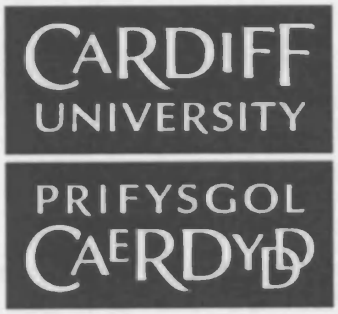
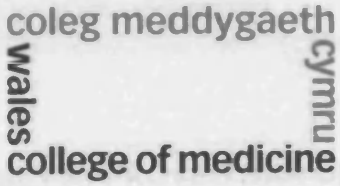
The Development of a Cell Based Chronic Wound Bioassay

Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy, Cardiff University

September 2008





Matthew Peter Caley
School of Dentistry,
Cardiff University,
Cardiff.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed  (candidate) Date 24/1/2011.....

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD

Signed  (candidate) Date 24/1/2011.....

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.
Other sources are acknowledged by explicit references.

Signed  (candidate) Date 24/1/2011.....

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed  (candidate) Date 24/1/2011.....

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed  (candidate) Date 24/1/2011

Acknowledgements

Firstly I would like to thank my project supervisors Professor Phil Stephens and Professor Dave Thomas for their constant support, advice and encouragement throughout the course of this study. I would also like to thank Professor David Kipling and Dr Llewelyn Roderick for their scientific advice, and help.

I would to thank Dr Peter Giles, Miss Megan Musson and Dr Amanda Redfern of the CBS microarray service for all their hard work and assistance in microarray analysis. This project would not have been possible without them. I would also like to thank the staff of the West Midlands Regional Genetics Laboratory for the karyotyping they carried out for me.

As part of this project I visited and worked at the Babraham Institute, I would like to thank all the members of the Molecular Signalling group for making my visit not only possible but productive and thoroughly enjoyable.

I am extremely grateful to my colleagues Dr Matthew Peake and Dr Ivan Wall for their advice, suggestions and encouragement throughout this study.

I would to thank all my colleagues on the 4th and 5th floors of the Dental School. I have enjoyed working with them and their help and support over the entire period of my study and the writing of my Thesis have helped keep me sane.

Finally I would like to thank my family, friends and especially my partner Anita for their belief and support throughout my PhD. Thank you all for making this possible.

Table of Contents

ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
C	Celcius
CAT	Chloramphenical Acetyltransferase
CEL	cell intensity file
cm	centimeter
CVI	chronic venous insufficiency
CWF	chronic wound fibroblast
CXCL	C-X-C chemokine ligand
DBTSS	Database of Transcriptional Start Sites
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EBV	Epstein Bar Virus
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EtOH	Ethanol
FAM	carboxyfluorescein
FCS	foetal calf serum
FDR	false discovery rate
FGF	fibroblast growth factor
FPCL	fibroblast-populated collagen lattice
FRET	Förster Resonance Energy Transfer
F-SCM	fibroblast – serum-containing medium
g	gram
g	gravitational constant
GAG	glycosaminoglycans
GFP	green fluorescent protein
GO	gene ontology
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hours
HA	hyaluronan or hyaluronic acid
HAS	hyaluronan synthase
HGF	Hepatocyte growth factor
HPV	human papiloma virus
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IL	interleukin
KEGG	Kyoto encyclopaedia of genes and genomes
KGF	keratinocyte growth factor
KO	knock out
L	litre
LASAF	Leica application suite, advanced fluorescence
LUX	Light Upon Extension system
M	molar
MAS 5	microarray analysis suite 5

MADRAS	microarray analysis and data review annotation system
mg	milligram
min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NF	normal fibroblast
ng	nanogram
nm	nanometer
nM	nanomolar
4-NPP	*p*-nitophenylphopshate
NTP	nucleotide triphosphate
ORF	open reading frame
P	passage
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	population doubling
PDGF	platelet-derived growth factor
PDL	population doubling level
PMNL	polymorphonuclear leukocyte
QRT-PCR	quantitative real time polymerase chain reaction
rma	robust multiarray analysis
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SA β-gal	senescence-associated β-galactosidase
SDS	sodium dodecyl sulphate
SNPs	single nucleotide polymorphisms
SV40	simian virus 40
TERC	rna component of telomerase reverse transcriptase
TERT	telomerase reverse transcriptase
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TLR	Toll like receptors
TNF	tumour necrosis factor
TRAP	telomeric repeat amplification protocol
TSS	transcriptional start sites
μg	microgram
μL	microlitre
μm	micrometer
μM	micromolar
UV	ultraviolet
v	volume
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
w	weight
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

Abstract

Impaired wound healing affects 3% of the population over 60 and costs over £1 billion annually in the UK. Chronic wounds are characterised by prolonged inflammation, impaired re-epithelialisation and defective extracellular matrix (ECM) remodelling. Fibroblasts play an important role in the closure of skin wounds, they replace and remodel the lost tissue and also influence both re-epithelialisation and angiogenesis. Numerous *in vivo* chronic wound animal models have been reported however, they fail to accurately model human chronic wounds.

This investigation aims to develop an *in vitro* chronic wound bioassay.Chronic wound fibroblast (CWF) and patient matched normal fibroblast (NF) cell strains (n=3) were retrovirally infected with a hTERT/puromycin construct. Population doubling levels (PDLs) were determined over an extended time in culture. RNA at defined time points was extracted and analysed using *Affymetrix*™ U133A microarrays. A cohort of gene expression changes were confirmed by QRT-PCR and the promoter regions of these genes used to construct fluorescent reporter human NF and CWF cell lines.

Population doubling levels indicated that, compared to primary (non-immortalised) CWF and NF, the hTERT infected fibroblasts had escaped replicative senescence and formed an immortalised cell line. *Affymetrix*™ microarray analysis of the CWF-hTERT with the NF-hTERT identified 247 genes that were significantly up- or down-regulated in CWF (validated by QRT-PCR). Upstream promoter regions of 13 genes of interest and a housekeeping gene have been identified by database searches/sequence analysis, amplified and cloned into the promoterless reporter vector pZsGreen1-DR. Reporter constructs have been transiently transfected into human NF and CWF cell lines and are currently being tested for efficacy.

CWF and NF cell lines have been created which demonstrated distinct gene expression profiles. The disease marker genes will form the basis of a low cost, highly reproducible fluorescent reporter cell-based bioassay which will be utilised in the discovery of novel therapeutics which have the potential to alter the chronic wound healing and, in turn, reduce unnecessary animal experimentation.

CHAPTER ONE

INTRODUCTION

1.1 The skin

The skin is, by volume, the largest single organ of the human body. It has multiple functions as diverse as temperature regulation and microbial protection. The skins primary function is as a barrier: it acts as the first line of defence against pathogens attempting to enter the body and as a water proof barrier preventing the loss of water from the body by evaporation. The skin protects underlying muscle and organs from mechanical, chemical and ultraviolet damage. The skin aids in the regulation of body temperature through the vasodilatation/vasoconstriction of blood vessels close to the skin, sweat glands and hair follicles. The skin also acts as a sensory system through nerve endings within the dermis allowing the detection of heat, cold, pressure and importantly tissue injury. The skin is also responsible for the body's production of vitamin D.

The skin is not composed of a single cell type but is the result of multiple cell types working together. The skin can be separated into three discrete layers, the epidermis, dermis and the hypodermis. The epidermis and dermis are separated by the basement membrane (a collagen rich membrane allowing communication between the dermis and epidermis and anchoring the cells of the epidermis).

1.1.1 The Epidermis

The epidermis is the outermost layer of skin. It is this layer that forms the waterproof barrier protecting the body from water loss. The epidermis is composed of many layers of cells forming a stratified epithelium with an underlying basement membrane. The epidermis contains four main cell types, keratinocytes, melanocytes, Merkel cells and Langerhans cells. The epidermis consists of five strata or sublayers, The outer stratum corneum is thickened in areas of the body with increased mechanical load

such as the ball of the foot. Below the stratum corneum are, in order, the stratum lucidum granulosum, spinosum and finally the stratum basale. The epidermis does not contain any blood vessels. Instead nutrients are supplied to the cells of the stratum basale by diffusion from the underlying dermis.

1.1.1.1 Keratinocytes

Keratinocytes are the most abundant cell of the epidermis and are responsible for the synthesis of keratin the main structural protein of the epidermis (Eckert et al., 1997). The type of keratin produced changes as the keratinocytes differentiate during keratinisation, with basal cells synthesising keratins 5 and 14 and the surface keratinocytes containing keratins 1 and 10 (Larjava et al., 1993). As well as keratin, keratinocytes are able to synthesise the components of the basement membrane such as collagen types IV and VII, laminin and fibronectin. This is important in wound healing as the keratinocytes are able to synthesize fresh basement membrane as they migrate during re-epithelisation (Romero-Graillet et al., 1996) .

1.1.1.2 Melanocytes

Melanocytes are neural crest derived cells, which through melanogenesis produce the pigment melanin. Melanin acts to protect the skin against ultra violet radiation damage and is synthesised in specialist organelles called melanosomes. Melanogenesis occurs at basal levels in the skin leading to differences in skin tone, and is upregulated by exposure of the cells to UVB radiation leading to an increase in skin pigmentation (Kim and Holbrook, 2001, Iggo and Muir, 1969).

1.1.1.3 Merkel Cells

Merkel cells are neural crest derived oval shaped cells found within the stratum basale of the epidermis. The exact function of Merkel cells is disputed, however they appear to function as mechanoreceptors (Ginhoux et al., 2006).

1.1.1.4 Langerhans cells

Langerhans cells are found in the stratum spinosum of the epidermis and are the only dendritic cell type found within the epidermis. Langerhans cells are derived from monocytes (Eckert, 1989) and form the immune systems first barrier to invading pathogens. Langerhans cells are mobile; they ingest antigens present in the skin, migrate to the lymph node and present the antigens to T-lymphocytes.

1.1.1.5 Keratinisation

Mitosis occurs in the stratum basale. Keratinocytes formed in this layer move up through the strata changing shape and cellular composition (McMillan et al., 2003). The cells becoming progressively flatter as they move up through the strata, dying as they are separated from the diffused nutrients from the dermis. The cells synthesise more of the protein keratin, a fibrous protein which replaces the cytoplasm within the cells, allowing them to become waterproof. Over this period these cells lose their nuclei and die. This gradual process of cornification is called keratinisation and normally takes four weeks. The cells that reach the outer stratum corneum are dead and because of the high keratin content are consequently waterproof. These cells are subsequently constantly sloughed off by friction.

1.1.2 The Basememt Membrane

The basement membrane zone is the junction between the epidermis and the dermis. The basement membrane is composed of a variety of different proteins including collagen type IV and type VII, perlecan and laminin (Metcalfe et al., 1997). The basement membrane allows the attachment of the epidermis to the underlying dermis.

1.1.3 The Dermis

The dermis is composed of a dense matrix of connective tissue known as the extracellular matrix (ECM). The ECM is predominantly made up of collagens however, it is also rich in proteoglycans, glycosaminoglycans and signalling molecules. The principle cell type of the dermis is the fibroblast. Fibroblasts are responsible for the synthesis and the remodelling of the ECM. In addition to fibroblasts, the dermis is also composed of macrophages and mast cells. The dermis contains an extensive network of blood capillaries supplying nutrients and growth factors to both the dermis and the stratum basale of the epidermis. The dermis contains many of the skins nerve endings and mechanoreceptors providing sensory information about touch and temperature in addition to lymphatic vessels, hair follicles and sweat glands.

1.1.3.1 Fibroblasts

Fibroblasts are mesenchyme-derived cells; they have a bipolar, spindle shaped morphology *in vivo* and play a vial role in the skins response to injury. In normal uninjured skin, fibroblasts are in the G0 phase of the cell cycle (quiescence), they do not actively proliferate, however they do still regulate the composition of the ECM.

Quiescent fibroblasts are still able to secrete ECM components as well as matrix metalloproteinases (MMPs), which are able to modify the ECM and tissue inhibitors of metalloproteinases (TIMPs) responsible for the control MMP activity. Upon wounding fibroblasts are activated by exposure to growth factors, re-enter the cell cycle and proliferate. Activated fibroblasts have an increased rough endoplasmic reticulum compared to quiescent fibroblasts and secrete a number of growth factors, cytokines and ECM protein components.

1.1.3.2 Macrophages

Macrophages are part of the body's innate immune system, giving a non specific response to pathogens. They are derived from monocytes, drawn into the dermis through the endothelium of the dermal blood vessels by chemotaxis. Monocytes are attracted to sites of tissue damage by damaged cells, pathogens and signals released by mast cells. When in the dermis they differentiate into macrophages. Macrophages are phagocytes (as are the monocytes from which they are derived) and engulf cellular debris and pathogens. Macrophages are antigen presenting cells; they activate the specific immune response via T-helper cells generating antibodies to the antigen and aiding further macrophage phagocytosis. A further function of macrophages within the dermis is as a source of growth factors and cytokines, aiding in the recruitment of cells to the wound environment.

1.1.3.3 Mast Cells

Mast cells are bone marrow derived tissue based inflammatory cells acting as part of both the innate and the acquired immune response. Mast cells are characterised by the presence of cytoplasmic granules rich in histamine and heparin (Prussin and Metcalfe,

2003). When activated through an IgE-dependent mechanism, mast cells release histamine and a number of cytokines (Watson et al., 2001, Smith et al., 1986). Mast cells play an important role in the wound response and in the defence against pathogens.

1.1.3.4 Extracellular Matrix

The ECM of the dermis like all ECM contains three classes of molecule, structural proteins, proteoglycans and adhesive glycoproteins all forming an interlocking mesh. The dermal ECM is generated and modified by dermal fibroblasts; it provides an anchorage for cells and a repository for growth factors.

1.1.3.4.1 Structural proteins of the ECM

The most abundant of the structural proteins in the ECM are the collagens,. Type I collagen is the most abundant of these structural ECM proteins, however collagen types III, V and VI are also present (Greenberg et al., 1991). Collagen fibres are made up of multiple collagen fibrils which are in turn made up of individual collagen molecules. Structurally collagen molecules are triple helices formed of three intertwined polypeptide chains. It is this multilayer quaternary structure, that gives ECM collagen fibres great mechanical strength. Collagen fibres are cross linked by a family of proteins known as transglutaminases. The transglutaminases are a family of acyl-transferases that form bonds between glutamine residues and primary amines (Kielty et al., 2002, Kielty and Shuttleworth, 1997, Montes, 1996). With collagens, transglutaminases form bonds between glutamine and lysine residues of adjacent fibres forming a strong meshwork of collagen fibres. The collagen fibres impart great strength to the ECM however, the fibres are rigid and do not impart flexibility into the

ECM. The skin must be able to flex and change shape to allow for movement of the body. This elasticity is provided by elastic fibres consisting of elastin and fibrillin rich microfibrils (Waller and Maibach, 2006, Lavker et al., 1986). Elastin molecules are cross linked by lysine-lysine covalent bonds, with the elastin network able to assume either an extended or compact configuration. This ability allows the elastin fibres to stretch giving the ECM the required flexibility. IN aged skin elastin fibres demonstrate signs of degradation and misfolding, thereby reducing the elasticity of the skin (Bernstein and Uitto, 1996).

1.1.3.4.2 Glycosaminoglycans, proteoglycans and glycoproteins of the ECM

Glycosaminoglycans (GAGs) are long unbranched polysaccharides consisting of repeating disaccharide units. The number and order of the different disaccharide units is unique to each GAG. In the dermis GAGs are important for the retention of water and the hydration of the skin. GAGs are able to bind many times their own volume of water (Choi et al., 1989). Glycosaminoglycans that are found in the dermis include hyaluronic acid (HA) and the chondroitin sulfate glycosaminoglycans. Glycosaminoglycans with a protein core are referred to as proteoglycans; this family includes the chondroitin sulphate glycosaminoglycans decorin and versican. One of the most abundant proteoglycan in the dermis is decorin, a member of the small leucine-rich proteoglycan family. Decorin is made up of a core protein with a single GAG chain containing either chondroitin sulfate or dermatan sulphate. In skin decorin GAG chain contains dermatan sulphate (Scott et al., 2003). Decorin is thought to be a dimer (Fleischmajer et al., 1991) that binds to type I collagen fibrils (Danielson et al., 1997, Corsi et al., 2002) and is thought to be involved in regulating the assembly of the collagen matrix. Decorin has been shown to inhibit collagen

fibrillogenesis (Lochner et al., 2007). The expression of decorin has been shown to decrease both with age and with UV-irradiation, linking decorin levels with structural changes seen in the skin with increased age (Zimmermann et al., 1994b). Versican is a proteoglycan expressed by both fibroblasts and keratinocytes. It is a member of a family of proteoglycans including aggrecan which is found in cartilage (du Cros et al., 1995, Soma et al., 2005). Versican has many different roles within the ECM. It is involved in matrix assembly as well as playing a structural role in the ECM. Versican present in the dermis is linked with hair follicle morphogenesis and cycling (Sorrell et al., 1999). The percentage of the ECM made up of versican changes, with fetal skin containing higher levels of versican than adult skin (Zimmermann et al., 1994a). Within the dermis, versican is associated with elastin microfibrils (Hasegawa et al., 2007). Versican has been shown to interact with other components of the ECM such as hyaluronan (HA) and elastin. Versican has a HA binding region at its N- terminus and a fibrillin binding domain at its C-terminus, which allows it to act as a connection between elastin microfibrils and HA (Sakai et al., 2000).

Hyaluronan (also referred to as hyaluronic acid or HA) is a major component of the extracellular matrix found in a variety of tissues within the body including the skin. HA is a polymer made up of disaccharides varying in size from 5KDa to 20,000KDa. The disaccharides that make up HA are composed of D-glucuronic acid and D-N-acetylglucosamine, linked by glycosidic bonds with up to 25,000 disaccharide repeats in each HA molecule. HA in humans is synthesised by the hyaluronan synthases HAS1, HAS2 and HAS3. These membrane bound proteins synthesise HA by repeatedly adding D-glucuronic acid and D-N-acetylglucosamine as the HA molecule is extruded from the cell membrane. HA is degraded by another family of enzymes the hyaluronidases. HA is found throughout the skin and has been identified in the

stratum corneum of the epidermis. The majority of the HA found in skin is, however, in the extracellular matrix of the dermis (Meran et al., 2007). HA has many roles within the dermis, with HA thought to play a role in the control of fibroblast adhesion, migration and differentiation. Studies have identified differences in HA synthesis between cells able to be stimulated by TGFβ-1 and those that are resistant to stimulation. HA synthesis appears to be linked with activation of fibroblasts (Meran et al., 2008) and with fibroblast proliferation (Podolnikova et al., 2003).

1.1.4 The Hypodermis

The hypodermis is the lowest layer of the skin lying beneath the dermis and is often referred to as subcutaneous tissue. Multiple cell types are found within the hypodermis including fibroblasts, macrophages and adipocytes, with adipocytes being the most numerous. The primary function of the hypodermis is as a fat store; providing an energy reserve for the body as well as insulation against heat loss.

1.2 Wound Healing

1.2.1 Acute Wound Healing

Wound healing is a complex multi cellular process involving fibroblasts, keratinocytes and endothelial cells as well as inflammatory cells. The healing process follows an orderly sequence of events incorporating three distinct, yet overlapping phases: the inflammatory phase (I), the proliferation phase (II) and the remodelling phase (III). The phases of wound healing are regulated by crosstalk between at least three different groups of molecules growth factors, integrins and metalloproteases. Secreted growth factors such as IGF-1, EGF, TGF-β and PDGF are involved in recruitment of cells to the wound area as well as controlling proliferation and

migration rates. Integrins allow for cells to interact with a wide variety of ECM proteins present in the wound bed, as well as at the wound margins. Differences in ECM makeup alter the way cells behave. MMPs promote degradation and modification of extracellular matrix proteins at the wound site, facilitating cell migration.

1.2.1.1 Inflammation

Inflammation is the first stage of the wound healing process. During the inflammatory phase of wound healing the aim is to prevent blood loss, infection and to remove dead or dying tissue. Several different events are grouped within the inflammatory response including haemostasis, cellular recruitment and cellular differentiation.

1.2.1.1.1 Haemostasis (immediate)

Haemostasis is the process where blood loss is halted at the site of injury. Initially vasoconstriction of the local blood vessels takes place, reducing blood flow and therefore blood loss. Vasoconstriction is followed by vasodilation caused by histamine released from proximal mast cells, as well as components of the complement cascade. Vasodilation allows the influx of platelets to the wound site, at which point they come into contact with exposed collagen that activates them. Activated platelets adhere to each other and to the blood vessel walls forming a clot. They release clotting factors leading to the deposition of fibrin at the site of injury. The fibrin clot acts as a provisional matrix, providing a site for platelet (Gailit et al., 1997, Farrell and Al-Mondhiry, 1997) and fibroblast binding (Kubo et al., 2001), but not keratinocyte binding (Laurens et al., 2006). Fibrin may provide a potential novel wound sealant to be used in poorly healing wounds (Heldin and Westermark, 1999).

Platelets also release cytokines that are responsible for the recruitment of cells to the wound area and effectively initiate the wound healing response. Platelets release multiple different cytokines and growth factors, among those platelet derived growth factor (PDGF) and transforming growth factor beta (TGFβ). PDGF is responsible for the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts to the wound area (Kim et al., 2008). TGFβ is also involved in the chemotaxis of macrophages and enhances the chemotaxis of smooth muscle cells and fibroblasts. In addition, TGFβ plays a role in the stimulation of collagen synthesis and the deposition of new ECM.

1.2.1.1.2 Early inflammatory phase (24 - 48 hours)

The activation of the complement cascade and the release of cytokines from the activated platelets at the wound site, leads to the infiltration of the wound area by neutrophils (also referred to as polymorphonuclear leukocytes or PMNLs). The recruitment of Neutrophils to the wound site occurs rapidly after wounding, with neutrophils numbers increasing by five times in the wound area within 18 hours of wounding (Dovi et al., 2003). Neutrophils remove bacteria, infiltrated foreign material, dead cells and damaged ECM from the wound site by phagocytosis and the release of lysozymes and oxygen free radicals. Though they play an important role in the prevention of wound infection neutrophils are not required for successful wound healing and have been shown to actually delay wound closure in animal models (Gillitzer and Goebeler, 2001, DiPietro et al., 1998). The neutrophils response lasts for 24 hours, at which time macrophages and lymphocytes have been successfully recruited to the wound site.

1.2.1.1.3 Late inflammatory phase (48-72 hours)

Around 48 hours after wounding, circulating monocytes move into the wound area, become activated and undergo a phenotypic change, becoming macrophages. Monocytes are attracted to the wound area by a number of chemoattractants. These include complement proteins, degraded ECM products, immunoglobulin G fragments, cytokines and by changes in the tissue oxygen tension (Leibovich and Ross, 1975). Macrophages decontaminate the wound site, working with leukocytes to remove cellular debris, bacteria filled neutrophils, dead cells and bacteria, thereby preparing the wound site for the next stage of the wound healing process. Reduction of macrophage number from the wound site has been shown to delay wound healing, identifying them as an important cells within the wound healing process (Martin, 1997). The continual recruitment of monocytes and their activation into macrophages is maintained by the production of pro-inflammatory cytokines at the wound site. These pro-inflammatory cytokines include interferon – γ (IFNγ), tumour necrosis factor (TNF) and interleukins 1, 2 and 8 (IL1, IL2 and IL8). Macrophages release MMPs which allow them to degrade damaged ECM components such as collagen and elastin. The degraded ECM components are then removed by phagocytosis. When the wound debridement is completed, macrophages release PDGF and TGFβ, which attract fibroblasts and smooth muscle cells to the wound site thereby beginning the proliferative phase of wound healing.

1.2.1.2 Proliferation (72 hours – 14 days)

The proliferative phase of wound healing begins approximately three days after the initial injury and continues for between two and four weeks. The predominant cell in the wound site during the proliferative phase is the fibroblast, which is responsible for

the deposition of new ECM and the formation of granulation tissue. During the proliferative phase of wound healing a new network of blood vessels is formed within the wound area (neovascularisation). Towards the end of the proliferative phase of wound healing keratinocyte proliferation leads to epithelisation of the wound and the restoration of the epidermis (Drew et al., 2001).

1.2.1.2.1 Fibroblasts in the wound

Fibroblasts migrate into the wound area: once within the wound they proliferate and begin to express ECM proteins. Within the wound the provisional fibrin matrix generated during the first stage of wound healing acts as a binding site for fibroblasts. The fibrin matrix is not required for successful wound closure, however, in it's absence cellular migration and ECM organisation is impaired (McClain et al., 1996). Once attached to the fibrin scaffold fibroblasts begin the production of collagen with fibroblast activation being the limiting step in the production of granulation tissue (Hakkinen et al., 1996, O'Kane and Ferguson, 1997). The provisional ECM laid down by the fibroblasts is predominantly composed of collagen (type III and type I), fibronectin and hyaluronan. The provisional ECM is laid down from the wound edges and base and, along with the new vasculature, (see section 1.2.1.2.2) forms what is known as granulation tissue. The progression of granulation is controlled by a number of growth factors; one of the most important is TGF-β. TGF-β modulates the activity of the fibroblasts within the granulation tissue, with fibroblast phenotype being dependent on TGF-β levels. At low levels TGF-β acts as a chemoattractant inducing fibroblast migration and proliferation. At higher concentrations fibroblast proliferation and migration is inhibited (Hughes, 2008). This feedback mechanism controls cell

numbers within the granulation tissue, as the more fibroblasts present in the tissue the more TGF-β produced.

1.2.1.2.2 Neovascularisation

Angiogenesis occurs within the new connective tissue. Endothelial cells are attracted to the wound site by the pro-angiogenic growth factors, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). This leads to the formation of new capillaries and their in-growth into the wound. It is the large number of blood capillaries that gives the granulation tissue its distinctive pink colouration. The infiltration of capillaries into the provisional ECM requires the interaction between fibroblasts and endothelial cells (Whitby et al., 1991).

1.2.1.2.3 Epithelisation

The surface of the wound is closed by re-epithelialisation with proliferating keratinocytes migrating over the granulation tissue as a sheet. Keratinocytes reform the stratified layers of the epidermis and generate a new basement membrane (Zeitz et al., 1978). Epithelialisation is controlled by growth factors and cell-cell interactions. Growth factors such as epithelial growth factor (EGF) and keratinocyte growth factor (KGF), as well as bFGF, stimulate keratinocyte proliferation and migration.

1.2.1.3 Remodelling and Scar maturation (week one onwards)

This is the longest phase of wound healing and can last for several years. The ECM laid down during the proliferative phase of wound healing is replaced or reorganised, the wound is contracted and the final scar is formed. During the remodelling phase of

wound healing there is a decrease in the level of type III collagen and HA produced. At the same time the production of type I collagen and proteoglycans increases. During the proliferative phase of wound healing the ECM laid down by fibroblasts mostly consists of collagen type III, this collagen is gradually degraded as new collagen type I is produced to replace it (Stamenkovic, 2003, Fini et al., 1992). Collagen fibres are proteolytically degraded by proteases belonging to the MMP family (Tomasek et al., 2002). The collagen fibres within the granulation tissue at the end of the proliferative phase of wound healing are disordered. As the wound remodelling continues the fibres become more ordered, with fresh collagen fibre deposition perpendicular to the wound edge. As the wound matures it begins to contract and fibroblasts within the wound undergo a phenotypic change into myofibroblasts (Mirastschijski et al., 2004). Myofibroblasts are similar to smooth muscle cells in that they have an actin rich cytoskeleton similar to that seen in smooth muscle cells and are responsible for the wound contraction (Racine-Samson et al., 1997). Myofibroblasts attach to the matrix via cell surface receptors, which allows the myofibroblasts to contract the ECM, further closing the wound and partially restoring tissue integrity. Myofibroblasts use the integrin $\alpha 1\beta 1$ to attach to collagen (Levenson et al., 1965). After wound contraction the resulting scar does not contain any hair follicles or melanocytes. The scar tissue has a lower tensile strength compared to the surrounding skin, with a 20% reduction in strength even after several years (Falanga, 1993).

1.2.2 Chronic Wounds

Wounds do not always follow the phases described in section 1.2.1. Those wounds that fail to heal are classified as chronic wounds. Chronic wounds fall into three

principal catagories: venous ulcers, pressure ulcers and diabetic foot ulcers (Menke et al., 2007). Though the underlying causes for chronic wounds varies, they are thought to have failed to resolve the inflammatory phase of the wound healing response (Graham et al., 2003, Moffatt et al., 2004).

Within the UK, the NHS has approximately 200,000 individual patients with chronic non-healing wounds (Gunnel and Hjelmgren, 2005). The annual cost to the NHS is estimated at between £2.3bn to £3.1bn per year, which is 3% of the annual NHS budget (Budgen, 2004, Iglesias et al., 2005, Palfreyman et al., 2007, van Korlaar et al., 2003). As well as being expensive to treat and manage, chronic wounds reduce the quality of life of those suffering from them (Margolis et al., 2002). The prevalence of chronic wounds increases with age, with 3% of the population over 65 suffering from a chronic wound (Boulton et al., 2004). The problem of chronic wounds is set to increase, as by 2020 nearly 20% of the UK population will be over 65.

Diabetic foot ulcers are seen in patients whose diabetes has lead to peripheral neuropathy. The neuropathy inhibits the perception of pain and pressure in the feet, which renders the foot susceptible to minor trauma and subsequent infection. Peripheral neuropathy coupled with a compromised vasculature and infection can lead to a non-healing wound (Ho and Bogie, 2007). Pressure ulcers occur in patients with conditions that inhibit their mobility. They are usually secondary to another disabling or restrictive condition and their effect on the patients quality of life is linked with the primary condition. Pressure ulcers are caused by a restriction of blood flow to a specific region of the body caused by sustained pressure. The pressure generates a region of ischemia with pressure in the tissue greater than that in the blood capillaries preventing blood flow and oxygenation (Mekkes et al., 2003, Bello and Phillips, 1998).

The most prevalent chronic wound, and the one this thesis will focus upon, is the venous leg ulcer. Between 15% and 25% of chronic wounds are linked with diabetes, 10% to 20% linked to pressure or arterial insufficiency and 45% to 60% linked to venous insufficiency (Berard et al., 2002, Grey et al., 2006). Unlike diabetic foot ulcers, (where diabetes and peripheral neuropathy) or pressure ulcers, (where lack of mobility) which have underlying causes there is no universally accepted theory for the cause of venous leg ulcers. The primary contributory factor in the development of a venous leg ulcers is chronic venous insufficiency in the lower limb leading to tissue ischemia and tissue damage caused by reperfusion (Mustoe, 2004). Three main theories for the formation of chronic venous leg ulcers are accepted. Whilst they each explain some of the observed pathology there is no one universally accepted theory. It is likely that multiple factors are involved in the formation of chronic venous leg ulcers, with different combinations of factors in each patient. Three factors that have been suggested as playing a role in most venous leg ulcers are cellular and systemic aging, repeated ischemia-reperfusion injury and bacterial colonization (Van de Scheur and Falanga, 1997, Burnand et al., 1982).

1.2.2.1 Theories explaining the formation of Chronic Wounds

1.2.2.1.1 The Fibrin Cuff Hypothesis

The fibrin cuff hypothesis suggests that the prolonged venous hypertension within the lower limb of patients is carried over into the capillary system within the skin. The increase in pressure within the capillaries leads to a stretching of the capillary walls, which in turn allows macromolecules to leave the capillaries and enter the tissue. Molecules such as fibrinogen would therefore build up around the capillaries in the perivascular space. Fibrinogen in the perivascular space would be converted to fibrin

in the same way as in the haemostasis phase of wound healing. The resulting fibrin cuff prevents the diffusion of oxygen and nutrients into the dermis around the blood capillary leading to cell death and the formation of a chronic wound (Falanga and Eaglstein, 1993). The fibrin cuff hypothesis explains the localised tissue ischemia and how this may be caused by venous hypertension.

1.2.2.1.2 The 'trap' hypothesis

A modified version of the fibrin cuff hypothesis was put forward that linked a build-up of perivascular macromolecules to the observed reduction in growth factors seen in chronic wounds. The 'trap' hypothesis like the fibrin cuff hypothesis suggests that venous hypertension could lead to the leaking of macromolecules into the perivascular space. In the 'trap' hypothesis the authors suggest that the macromolecules leaking into the dermis, trap growth factors and cytokines. While trapped these growth factors and cytokines are unable to stimulate or attract cells to the dermis (Thomas and Dormandy, 1988).

1.2.2.1.3 The leukocyte trapping hypothesis

Another theory for the formation of venous leg ulcers in patients with venous disease is the leukocyte trapping hypothesis. This hypothesis is based on observations made in patients with chronic venous insufficiency. Microscopic analysis of the skin of patients suffering from chronic venous insufficiency indicated a loss of functional capillary loops within the skin. Analysis of the circulation of leukocytes in patients with chronic venous insufficiency indicated a reduction in the leukocytes leaving the lower limbs compared to healthy volunteers (Coleridge Smith et al., 1988). This lead to the theory that the microvasculature of patients with chronic venous insufficiency

becomes blocked by an accumulation of leukocytes. These blockages lead to localised tissue ischemia and cell death, in turn leading to the formation of a venous leg ulcer (Weyl et al., 1996, Peschen et al., 1999, Smith et al., 1999). Further evidence and a demonstrated mechanism for the leukocyte trapping hypothesis has been shown in the years since its first proposal. The expression level of adhesion molecules by endothelial cells was shown to increase with the progression of chronic venous insufficiency. Specifically the levels of Inter-Cellular Adhesion Molecule, vascular cell adhesion molecule and endothelial leukocyte adhesion molecule were increased (Weyl et al., 1996, Peschen et al., 1999, Smith et al., 1999) A subset of leukocytes expressing leukocyte function-associated antigen-1 and very late activated antigen-4 were shown to adhere to vascular endothelium with increased expression of these adhesion molecules. This adherence was due to the affinity of the leukocyte expressed antigens to the endothelial expressed adhesion molecules. After adherence to the vascular endothelium the leukocytes move into the perivascular tissue (Stanley et al., 1997, Mendez et al., 1998b, Vande Berg et al., 1998, Loots et al., 1999) causing damage to both the endothelium and the surrounding tissue. The localisation of the increased adhesion molecule expression and perivascular leukocyte invasion to the chronic wound suggests a link with this observation and the initial formation of a chronic wound.

1.2.2.2 Cellular and Molecular changes observed in Chronic Wounds

Fibroblasts taken from chronic wounds (CWF) have been widely studied in an attempt to gain an insight into the failure of chronic wounds to heal. Fibroblasts play an important role in the wound healing process, expressing and remodelling ECM,

releasing growth factors and chemokines and as a source of myofibroblasts. Changes in fibroblast phenotype would therefore give an insight into chronic wounds.

1.2.2.2.1 Proliferation, Morphology and Senescence

The reduced proliferative life span and growth rate of CWF has been demonstrated in a number of studies when compared with fibroblasts from patient matched normal skin (NF) (Agren et al., 1999b). CWF have a reduced proliferative potential compared to fibroblasts taken from acute wounds (Agren et al., 1999b). It appears that it is the chronic wound environment rather than just a wound environment that reduces the proliferative potential of the cells.

Fibroblasts normally have a bipolar morphology, however CWF have been shown to display an altered morphology more similar to aged cultured fibroblasts. CWF appear polygonal with clear actin stress fibres, they also appear to be larger than normal fibroblasts (Yang and Hu, 2005).

Changes in morphology and reduced proliferative potential are potentially indications that CWF cells are closer to cellular senescence than cells taken from patient matched normal skin. A marker for senescence, that was until recently widely used, is senescence associated β-galactosidase activity (SA β-gal). This marker for senescence has recently been questioned. SA β-gal activity can be induced in non senescent cells (Mendez et al., 1998b, Stanley and Osler, 2001a). SA β-gal activity has been detected in a higher proportion of CWF compared to patient matched NF (Raffetto et al., 2001b, Lerman et al., 2003). The reduced proliferative potential and growth rate observed in CWF may be linked with cellular senescence. As well as reduced proliferation another marker for cellular senescence is a reduction in cellular migration. CWF have been shown to have a reduced migration rate compared to NF taken from the same patient (Hayflick and Moorhead, 1961). Cultured fibroblasts

have a limited replicative capacity, after a finite number of population they enter senescence (West et al., 1989, Millis et al., 1992, Wick et al., 1994). Many of the characteristics of chronic wound fibroblasts are similar to those of senescent cells. Senescent fibroblasts display altered morphological features such as cellular enlargement, polygonal shape and actin stress fibres throughout the cytoplasm. Senescent fibroblasts express higher levels of MMPs and lower levels of TIMPs than none senescent cells (Vasquez et al., 2004, Agren et al., 1999b, Loots et al., 1998, Lal et al., 2003).

1.2.2.2.2 Expression of Growth Factors and Cytokines

Growth factors and cytokines play a vital role in the coordination of the wound healing response. CWF have been shown to have a reduced response to various growth factors and cytokines compared to NF including TGF-β, EGF, bFGF, IGF and PDGF (Schmid et al., 1993): all known to be important in the wound healing response. The effect of growth factors and cytokines on cells taken from chronic wounds has been shown to be altered when compared to patient matched NF. This may explain the failure of chronic wounds to heal if the growth factors normally responsible for coordinating the healing process do not have an effect on cells within the wound. The levels of these signalling molecules within chronic wounds has also been studied and compared with acute wounds. For example TGFβ1 is expressed in acute wounds but not in chronic wounds (Lauer et al., 2000). The opposite trend is seen in the expression of VEGF with higher levels of mRNA detected within the cells of the chronic wounds compared to patient matched normal skin (Fivenson et al., 1997, Barone et al., 1998, Trengove et al., 2000, Li et al., 1998).

Cytokine levels have also been analysed in chronic and acute wounds, the pro-inflammatory cytokines IL-1α, IL1β, IL-6, IL10 and TNFα were shown to be increased in wound fluid taken from chronic wounds compared to acute wound fluid (Li et al., 1998, Loots et al., 1998). Therefore the high levels of pro-inflammatory cytokines within the chronic wounds may be responsible for their failure to resolve the inflammatory phase of wound healing.

The altered levels of growth factors and cytokines observed in chronic wounds may directly affect other cells involved in wound healing. Chronic wounds have been shown to contain significantly higher numbers of macrophages and B-lymphocytes than acute wounds (Loots et al., 1998). This increase is not seen in all types of inflammatory cell as T-lymphocytes are reduced in chronic wounds compared to acute wounds (Mendez et al., 1998b). The alterations in inflammatory cell numbers within chronic wounds may also play a role in the failure of chronic wounds to resolve the inflammatory phase. Too many inflammatory cells would increase the levels of pro-inflammatory cytokines within the wound environment, prolonging the inflammatory phase. Too few inflammatory cells would not be able to remove dead cells and bacteria from the wound site, preventing the proliferative phase of wound healing from beginning.

1.2.2.2.3 Changes to the Extracellular Matrix

As well as having altered growth factor and cytokine levels, chronic wounds display alterations to ECM components and to proteases that are important for the remodelling of the ECM. Three key components of the ECM have been studied in detail within the chronic wound environment: fibronectin (Herrick et al., 1992), fibrin (Herrick et al., 1996) and collagen (Mendez et al., 1998c). Fibronectin gene expression was shown to be up-regulated in CWF compared to patient matched NF

when cultured *in vitro* (Ongenae et al., 2000). In chronic wound tissue the same trend was observed with fibronectin mRNA up-regulated compared to acute wounds or normal unwounded skin (Herrick et al., 1992). Within the chronic wound there is no increase in fibronectin (Rao et al., 1995, Grinnell et al., 1992, Grinnell and Zhu, 1996). Though more fibronectin mRNA is produced within chronic wound tissue the increased fibronectin protein produced is degraded by increased proteases within the chronic wound fluid. Degraded fibronectin has been identified within wound fluid taken from chronic wounds, but not acute wounds, confirming that it is degraded by proteases within the wound fluid (Grinnell and Zhu, 1996, Rao et al., 1995). Analysis of wound fluid for proteases known to degrade fibronectin identified increased levels of elastase within chronic wound fluid (Brakman et al., 1992). Elastase is a serine protease that degrades fibronectin, the high levels of elastase found within chronic wound fluid account for the reduced levels of intact fibronectin in chronic wounds.

The fibrin cuff hypothesis explains a possible cause for the high levels of fibrin within chronic wounds; the leaking of fibrinogen into the perivascular space due to increased blood pressure. Normally fibrin can be broken down by the serine protease, plasmin, into fibrin degradation products. This process would break down the fibrin cuff preventing ischemia and tissue death. In chronic wounds an inhibitor to plasmin activity is present: plasminogen activator inhibitor 1 prevents the activation of plasmin and the breakdown of fibrin (Herrick et al., 1996).

The most abundant component of the ECM is collagen. Collagen is vital for successful wound healing. Collagen synthesis in CWF grown *in vitro* has been shown to be reduced compared to NF (Siddiqui et al., 1996). A reduction of collagen synthesis has also been seen in chronically hypoxic cells (Wysocki et al., 1993a, Weckroth et al., 1996, Rayment et al., 2008, Trengove et al., 1999, Saarialho-Kere,

1998) suggesting that the hypoxic environment within the chronic wound may be responsible for the alteration in collagen synthesis. The effect of the wound environment on any collagen or other ECM component that is synthesised has also been considered.

MMP activity within chronic wound fluid is higher than in acute wound fluid (Rayment et al., 2008). Proteases able to degrade various forms of collagen have been identified within chronic wounds. Expression of MMP9 is increased in chronic wounds (Han et al., 2001), MMP9 is a protease able to digest gellatin and type IV collagen with the level of MMP9 appearing to be an indicator of the severity of the wound. The increased levels of TNFα within chronic wounds have been identified as a factor leading to the increased expression on MMP2 (Nwomeh et al., 1999). MMP2 is also a gelatinase able to break down type IV collagen. Some other studies have identified MMP8 as the predominant collagenase within chronic wounds, with MMP1 also detected (Vaalamo et al., 1997). MMP8 is able to digest collagen type I, II and III, as well as collagen I, II and III. Furthermore, MMP1 has the ability to cleave collagen types VII and X. Both MMP1 and 8 are found in acute wounds however they are in an inactive form whereas they are both active in chronic wounds. MMP 13 has also been identified in chronic wounds but not in acute wounds. MMP13 is also a collagenase able to cleave collagen I, II and III with highest activity against collagen II (Trengove et al., 1999, Vaalamo et al., 1996). Potentially other proteases may also play a role in the chronic wound phenotype but have yet to be identified.

As well as protease levels is also important to consider the specific inhibitors of proteases within chronic wounds. Proteases are tightly controlled both by expression and by specific inhibitors. An increase in protease gene expression may have no effect if inhibitor gene expression increases to compensate. The expression levels of

members of a family of MMP inhibitors, the TIMPs have been studied in the chronic wound environment. In acute wounds TIMPs regulate collagenase activity, however in chronic wounds TIMP1 is either not expressed or its expression is severely reduced (Nwomeh et al., 1999). Levels of TIMP-1 protein in wound fluid have also been analysed and a reduction has been reported in chronic wound fluid compared to acute wound fluid (Vaalamo et al., 1999). The other members of the TIMP family have also been studied in chronic wounds, with TIMP-2,TIMP-3 and TIMP-4 all shown to be down regulated in chronic wounds (Frank et al., 2000, Stallmeyer et al., 1999, Yamasaki et al., 1998).

1.3 Models of Wound Healing

Wound healing is a complex process involving interactions between multiple cell types from the skin as well as inflammatory cells. The sequence of events that take place during wound healing is reasonably well understood, as macroscopic changes in cell numbers and tissue structure are easily observed. On a molecular level the wound healing process is less well understood. In order to better understand the wound healing process models of part, or all, of the wound healing process are used. Models of wound healing can be split into two groups *in vivo* models and *in vitro* models.

1.3.1 *In vivo* Models of wound healing

Many animal models for wound healing exist. At their most simple they involve wounding a laboratory animal and observing wound closure time. Various different species are used regularly for skin wounding experiments including rats, mice, rabbits and pigs. As well as these 'normal' laboratory animals, genetically modified animals

are also used to model human diseases such as diabetes and the effect this would have on wound healing.

1.3.1.1 Full Thickness Excision Wounds (Mouse Model)

This model allows the investigation of haemorrhage, granulation tissue formation, reepithelialisation and the formation of new blood vessels (Frank et al., 2000, Stallmeyer et al., 1999, Yamasaki et al., 1998). Wounds are generated by the surgical removal of all the layers of the skin (epidermis, dermis, subcutaneous fat and in the case of the mouse the subcutaneous smooth muscle layer) from the animals back. Multiple wounds may be generated on each animal allowing direct comparison between treatments. When mice are used with this wound healing model, wounds of up to 5mm are generally generated with six wounds per mouse. Wounds are generated under anaesthetic, hair is removed from the back of the mouse, the loose skin on the mousse back is lifted and cut with scissors generating a wound. Wound area may be recorded over time giving a wound healing rate. For histological analysis animals are sacrificed at desired time points after wounding and the wounded skin is removed for analysis. This model allows for not only the rate of wound healing to be recorded, but also histological analysis of the wounded skin at different time points after wounding (Goss and Grimes, 1975).The ability to generate multiple wounds on each animal increases the statistical value of this model. However, the wounds generated in this model of wound healing are not uniform as they are generated by hand. The depth and size of wounds, though similar, are not identical introducing an additional variable into the model.

1.3.1.2 Punch Wounds (Rabbit/Mouse)

Punch wounds generated in the ears of rabbits have been used as a model for full thickness wound healing for many years. Wounds are generated using biopsy punches cutting through the full thickness of the ear. The wound includes damage to the epidermis, dermis and cartilage that make up the ear. Rabbits have the ability to regenerate the tissue of the ear from the margin of the punch wound inwards (Patsy K. Williams-Boyce, 1980, Bos et al., 2001). The wounds heal over a period of weeks and can be taken at different time-points for histological analysis. As the wounds are straight through the ear the variation in depth possible in excision wounds on the back of animals is not seen in this model, and as the wounds are generated using a biopsy punch, the size of the wounds is reproducible. Multiple punch wounds can be generated in each ear allowing for experimental controls. As well as its use in rabbits (Colwell et al., 2006, Clark et al., 1998, Heber-Katz, 1999) this model of wound healing has also been used in mice (Schaffer et al., 1997). Modifications to this model of wound healing have been proposed as a chronic wound model (1.3.3.1)

1.3.1.3 Partial Thickness Excision Wounds

Unlike the full thickness and ear punch wounds described above, this model of wound healing aims to look at epidermal wound healing (Rommain et al., 1991, Nanchahal and Riches, 1982). Wounds are generated using a dermatome, using the same technique as is used to harvest skin for split thickness skin grafts. The dermatome is used to remove a set thickness of skin generating a partial thickness wound with the epidermis and some of the dermis removed. The depth of wound is somewhat reproducible if the dermatome is used correctly however, it is possible to generate an

uneven wound with the dermatome leaving patches of epidermis within the wound. Removal of the epidermis causes initial bleeding as the blood supply to the epidermis is disrupted. This model of wound healing generates a larger wound by area and is ideal for use on larger experimental animals, such as pigs. Multiple wounds may be generated on each animal allowing for experimental treatments and controls on the same animal. As with the full thickness model, differences in wound size and depth are problematic in this model. The dermatome is not a precise instrument and different users may generate different wound depths. Any variation in wound depth and size would alter wound healing rates.

1.3.1.4 Suction Blisters

This model of wound healing uses dry suction to separate the dermis and epidermis at the level of the basement membrane. Vacuum suction is applied to the skin. The suction slowly separates the epidermis from the dermis, with fluid filling the generated inter-dermal space. The wounds generated by this technique are of a uniform size (the size of the suction device) and of a uniform depth (down to the basement membrane). This method of wound generation has been used on experimental animal species (Leivo et al., 2000, Levy et al., 1995) and also in human volunteers (Cribbs et al., 1998, Brans et al., 1994). The blister formed can be deroofed leaving an exposed wound. As the dermis is not damaged this is a good model of re-epithelisation, with reproducible wounds and the potential to generate multiple wounds per animal.

1.3.1.5 Water Scald Burns

Wounds generated by scalding the skin of animals to generate blisters have been used in different species as a model of wound healing (Becic et al., 2003, von Bulow et al., 2005). Blisters are generated by exposing a fixed area of skin to heated water. The burning process is halted by the application of ice cold water to the site of injury. A blister forms at the site of injury and, as in the suction blister model, the blister may be deroofed to expose the wound. By keeping the temperature of the water and exposure time constant, repeatable wounds can be formed using this technique. With this model it is possible to control the depth of the partial thickness wound by altering exposure time. This model requires a water-tight seal to be generated around the desired wound area. In smaller animals it is harder to generate multiple wounds on a single animal with this technique. To determine the required exposure time to generate the desired depth of wound multiple trials must be used for each new species or strain of experimental animal.

1.3.1.6 Thermal Burns

Another method of generating wounds with thermal damage is through the direct application of heat to the skin (Brans et al., 1994). Partial thickness wounds can be reproducibly generated by applying heat to the skin for timed periods using a metal template. As with the suction blister and scald wound models, a blister is formed and can be deroofed to expose the dermis and leave an open wound. The depth of wound can be controlled by increase or decrease in either the time the heated template is applied to the skin or in the heat of the template. As in the water scald model, a series of trial experiments is required to determine the optimum conditions for generating a wound of a specified depth. Multiple wounds may be generated in each animal and

the size and shape of the wounds can be easily altered and is reproducible. This model of wound healing has been used in multiple different species of laboratory animal and is especially useful in the porcine model (Liang et al., 2007). The two thermally generated wound models both lead to denatured proteins in the wound environment due to the applied heat, these denatured proteins are not found in wounds generated by physical trauma and their influence on wound healing should be considered.

1.3.2 *In vitro* Models of wound healing

Animal models of wound healing generate complex wounds involving multiple cell types. In some circumstances the complexity of the wounding response can make it impossible to accurately measure the effect of a treatment or environmental change and difficult to determine which cells are specifically affected. Simpler models of wound healing are therefore useful for looking at specific parts of the wound healing response. Cells cultured either alone or in multi-cellular complex cultures have been used to study wound healing. As it is possible to use human cells in these models possible differences between wound responses in different species can be reduced. It is also possible to use cells from different human disease states or to genetically modify the cells to study the effect these changes may have on the wounding response.

1.3.2.1 Scratch wound

One of the simplest *in vitro* wound models is the monolayer scratch assay, allowing the quantification of cellular migration. When a confluent monolayer of cells is wounded the cells at the edge of the wound re orientate and migrate into the space closing the generated wound. By imaging the wound at the beginning of cell

migration, and at regular intervals during cell migration, it is possible to determine the rate of wound closure (Poujade et al., 2007). Differences in cellular migration can be observed using this method, keratinocytes migrate as a connected sheet (Kole et al., 2005) whereas fibroblasts migrate individually (Goldberg et al., 2008). Scratch wound models can be used by themselves to study specific responses to wounding, or in conjunction with animal models, with the scratch wounds allowing greater control of the wound environment (Kitano et al., 2007, Borensztajn et al., 2008, Diaz et al., 2008, Stevenson et al., 2008). By altering the environment, the cells or the media used to generate the scratch wound model it is possible to study the effect that different stimuli or disease states have on wound healing (Loryman and Mansbridge, 2008). Scratch wounds have been used to study chronic wounds either by the addition of compounds known to be found in chronic wounds such as bacterial lipopolysaccharide (Wall et al., 2008b) or by using cells isolated from chronic wounds in the scratch assay (Poujade et al., 2007).

Traditionally scratch wounds are generated by hand using mechanical trauma: this can generate wound of varying sizes and shapes. Automated scratch wounding devices are available, for example the Essen Woundmaker ™ (www.essen-instruments.com) which is capable of generating and analysing multiple scratch wounds. Another alternative recently suggested to improve the reproducibility of scratch wounds suggested the use of removable templates. Cells are grown within the templates, which when removed leaves uniform gaps between confluent strips of cells, cellular migration into the gaps can then be measured (Bell et al., 1979). However they are generated, scratch wounds are a low cost, simple and versatile model of wound healing.

1.3.2.2 Fibroblast Populated Collagen Lattice Model

Wound repair within the dermis requires the replacement and remodelling of extracellular matrix by the dermal fibroblasts. This process can be studied *in vitro* using a fibroblast populated collagen lattice (FPCL) (Paul Ehrlich et al., 2006b, O'Gorman et al., 2006, Mukhopadhyay et al., 2005). Fibroblasts are introduced into a solution of collagen collected from rat tail tendons. The mixture of cells, media and collagen is cast in a petri dish and allowed to set. To generate a free-floating collagen lattice the lattice is detached from the sides of the dish. As the fibroblasts remodel the collagen lattice, the area of the FPCL is reduced. This can be measured giving a value for the rate of fibroblast mediated collagen reorganisation. This wound model is more complex than simple monolayer scratch wounds and gives a insight into the ability of fibroblasts to remodel the ECM during wound healing. By altering the cells used to form the FPCL and the makeup of the media, the FPCL is cultured in different disease states and growth factors can be studied (Wall et al., 2008b, Cook et al., 2000b) including chronic wounds (Stark et al., 2004).

1.3.2.3 Organotypic Co-Culture Model

Single cell *in vitro* models do not allow any of the interactions between different cell types that are so important within the wound healing response. To study the interactions between dermal and epidermal cells without using animals, co-cultures of keratinocytes and fibroblasts can be used. Growing epidermal keratinocytes on a collagen matrix containing fibroblasts generates these epidermal equivalents. The keratinocytes are exposed to the air and form stratified layers as seen in the epidermis and are supplied with nutrients from the underlying matrix (Schneider et al., 2008, Kinsner et al., 2001, Wang et al., 2003, Maas-Szabowski et al., 2000, Stark et al.,

1999). Commercially available epidermal equivalents are available (www.skinethic.com): modelling full thickness skin, epidermis, and epithelium from different part of the body. Epidermal equivalents can be modified by using cells from different sources (Hoeller et al., 2001) or by the addition of hair follicles to the culture (Genever et al., 1993, O'Leary et al., 2002). Wound healing can be studied in the dermal equivalents by wounding the skin model and recording the cellular responses (Margolis et al., 2002). Organotypic co-culture models add a level of complexity to *in vitro* wound modelling, allowing the investigation of complex cell-cell interactions. The organotypic models described above do not involve vasculature or the immune response. Organotypic models therefore offer a simplified system to study some areas of wound healing such as dermal epidermal signalling and cellular migration.

1.3.3 Chronic Wound Models

In order to study wounds that fail to heal and develop potential treatments for them models of chronic wounds have been developed. These models are adaptations of the wound models described previously in this chapter. In humans, chronic wounds are more common among patients over 65 (Boulton et al., 2004) and are associated with diabetes (Ho and Bogie, 2007), immobility (Mekkes et al., 2003) or vascular disease (Engerman and Kramer, 1982, Zhang et al., 2005). Animal models of chronic wounds must first artificially generate the conditions associated with chronic wound formation and be utilised as reproducible wound model systems

1.3.3.1 Diabetic Wound Models

It is possible to artificially generate diabetes in animal models by the surgical removal of the pancreas (Howarth et al., 2008) or by chemical treatments to kill the β-cells of

the islets of Langerhans (Inoue et al., 1985). The models of induced diabetes demonstrate impaired wound healing compared to control animals (Inoue et al., 1985, Kwon et al., 2008, Lau et al., 2008, Morikawa et al., 2007, Rabelo et al., 2006, Velander et al., 2008). Induced diabetic animals have been used to study different wound treatments that may be useful in treating diabetic wounds (Chen et al., 1996). The surgical removal of the pancreas to induce diabetes is a stressful operation and generates other symptoms along with the diabetes. The use of alloxan and streptozotocin to chemically induce diabetes may also have other side effects associated with the use of chemotherapy agents potentially limiting the value of the model. Genetically diabetic animals are available for use in wounding studies, with one of the most widely used being the db/db diabetic mouse. This mouse lacks the receptor for leptin normally present within the hypothalamus (Tsuboi et al., 1992). Genetically diabetic mice have impaired wound healing (Scott et al., 2008, Trousdale et al., 2008, Michaels et al., 2007) and are therefore a useful model for the study of diabetic wounds (Quirinia et al., 1992, Quirinia and Viidik, 1992). Wounds in diabetic animals (induced or genetically diabetic) demonstrate a reduced ability to heal however, they do eventually heal. The diabetic wound models discussed here are models of impaired acute wounds rather than true non healing wounds.

1.3.3.2 Ischemic wound models

Both venous leg and pressure ulcers have an underlying tissue ischemia linked with their formation. Chronic wound models that can generate an ischemic environment have therefore been developed to model the human disease state. Two methods for generating ischemic wounds are widely used: a back and an ear model. The ischemic back wound (Buemi et al., 2004) is based on a skin flap generated by an H shaped

incision wound in the back of a rat. The generated wound shows reduced blood perfusion along the horizontal wound creating an ischemic environment. The vertical wounds do not have a reduced blood flow so can act as controls for wounding experiments. This is a relatively short-term wound model as the skin returns to normal levels of blood perfusion within 16 days. The model can be used to study the effect of potential treatments on chronic wound healing (Gould et al., 2005). Modifications to the basic skin flap ischemic wound model have also been reported, such as the insertion of a silicone sheet under the skin flap to reduce blood perfusion (Kloeters et al., 2007, Mogford et al., 2006, Mogford et al., 2004, Kamler et al., 1993, Chien, 2007). A second model of ischemic wounds involves the generation of an ischemic environment in a mouse, rat or rabbit ear and the subsequent wounding of the ear (Wu and Mustoe, 1995). The surgical division of the rostral and central arteries of the ear generates an ischemic environment in one ear of the animal. This has been shown to generate an ischemic environment in the ear for up to 28 days (Hayflick and Moorhead, 1961). In this model one ear is ischemic and the other acts as a control, when performed in the rabbit multiple punch wounds may be generated in each ear. While these models do generate impaired healing they do not create wounds that fail to heal. They provide useful tools for studying impaired wound healing but more accurately model delayed acute wound healing than true chronic wounds.

1.4 Cell Lines as Models of Disease

Normal human somatic cells have a finite lifespan in culture before they enter replicative senescence (Masters, 2002, Gey et al., 1952). Primary cells taken from a patient can therefore only be used for a limited period of time before fresh patient samples are required. To overcome this problem cell lines have been generated for

numerous different cell types. Initially cell lines were derived from spontaneously immortalised cells such as the HeLa cell line derived from a cervical tumour (Collins, 1996). An alternative approach for cell line generation has been the use of oncogenic viral proteins. The introduction of viral genes such as Simian virus 40 (SV40), T antigen, Epstein Barr virus (EBV), Adenovirus E1A and E1B, and human Papilomavirus (HPV) E6 and E7 can lead to the immortalisation of primary cells. The expansion of tumour biopsies into cell lines or the expression of viral oncogenes can generate useful immortalised cell lines, however, the cells are phenotypically different from the primary cells they were derived from. While this may be useful in the study of cancer biology, where the phenotypic changes in the cancer cell lines may relate to changes that occur in tumour formation, these cell lines are less useful for the study of wound healing.

Another method for generating cell lines from somatic cells is the overexpression of the enzyme telomerase. Telomerase is a DNA polymerase that specifically restores the ends of telomeres that are damaged at cell division. Telomerase adds repeats of TTAGGG onto the existing telomere ends

(Harley, 1991, Harley and Villeponteau, 1995, Bodnar et al., 1998). Telomerase is made up of two components: a protein component human telomerase reverse transcriptase (hTERT) and a RNA component human telomerase RNA component (HTERC or hTR). Most somatic cells do not express sufficient hTERT to maintain their telomeres at a constant length resulting in the shortening of their telomeres at each cell division (Kipling, 2001). This telomere shortening in some cells leads to growth arrest and senescence (Nakamura et al., 1997, Meyerson et al., 1997, Harrington et al., 1997). The gene for hTERT was isolated and cloned in 1997 (Jiang et al., 1999, Morales et al., 1999), allowing for the over-expression of hTERT in

human cells. The forced over-expression of hTERT by retroviral infection of somatic cells allows the generation of stable cell lines that maintain their previous phenotype (Bodnar et al., 1998, Yang et al., 1999, Kampinga et al., 2004). Forced over expression of hTERT has been used to generate immortalised cell lines in many different cell types. Skin fibroblasts, epithelial cells and endothelial cells have all been immortalised by the over expression of hTERT (Jiang et al., 1999, Morales et al., 1999). Importantly hTERT immortalised cells do not have a tumour like phenotype: they remain diploid, contact inhibited, anchorage dependent and differentiated (Wyllie et al., 2000).

Whilst hTERT immortalised cells have generally been shown to retain their pre immortalised phenotype in some cases this is not true. The over expression of telomerase in cells taken from patients with Werner syndrome reversed the accelerated aging of the cells normally seen *in vitro* (Thompson et al., 1993). However work carried out with fibroblasts isolated from chronic wounds indicates that the introduction of telomerase would not reverse the disease-specific phenotype of chronic wound fibroblasts (Stephens *et al.*, 2003). In order to gain a better understanding of chronic wounds an immortalised cell line retaining a chronic wound specific phenotype would provide a useful tool reducing the requirement for fresh patient samples. Such a cell line could be used in the *in vitro* models of wound healing described previously allowing for analysis of chronic wound healing without the need for animal models.

Before a cell line can be used it must be characterised to ensure that the over expression of hTERT during the immortalisation process has not altered the phenotype of the cells. Another potential drawback with retroviral infection and

insertion of hTERT is that the insertion may occur anywhere within the genome, potentially changing the cells by disrupting gene expression.

1.5 Reporter Constructs

To study the effect a treatment has on a model cell line some form of reporter assay is required. Changes in proliferation rate and cell motility may be studied using the models of described previously. While microarray and QRT-PCR analysis allow detailed investigation of gene expression changes they require the extraction and purification of RNA before they may be used. To study changes in gene expression without the need for RNA analysis promoter regions from genes of interest may be used to drive the expression of reporter molecules. These reporters may then be detected giving a rapid readout of changes in gene expression.

1.5.1 *In Vitro* Reporter Assays

To quantify this type of reporter assay cell or tissue lysates which contain the reporter protein or culture medium when the reporter protein is secreted are directly assayed. *In vitro* assays allow a quantitative and reproducible analysis of promoter and enhancer elements.

1.5.1.1 Chloramphenical Acetyltransferase (CAT)

This prokaryotic enzyme catalyses the transfer of acetyl groups from acetyl-coenzyme A to chloramphenicol. A high signal to noise ratio can be achieved because of the prokaryotic origin of the enzyme, in eukaryotic cells there is minimal competition for the enzyme creating a very accurate reporter. CAT is stable in mammalian cells with a half life of 50 hr (Balu and Adams, 2003) making it ideal for transient expression studies but unsuited for stable expression or some enhancer studies. Initially the CAT

assay was based on ^{14}C labelled chloramphenicol (Hruby et al., 1990). Cell lysates were incubated with the isotopically labelled compound, separated by chromatography and visualised on film. A non radioactive quantitative method for detecting chloramphenciol acetylation has been developed (Gendloff et al., 1990) and describes a fluorescently labelled chloramphenicol derivative capable of a single acetylation and is available commercially (www.invitrogen.com). An ELISA based assay for CAT activity was also developed (de Wet et al., 1987) allowing high throughput screening of samples (www.roche.com).

1.5.1.2 Firefly Luciferase

The *luc* gene found in the firefly *Photinus pyralis* was first cloned in 1987 (Bronstein et al., 1994) Luciferase bioluminescence requires not only the luciferase catalyst but also luciferin, ATP, Mg^{2+} and O_2. Cell lysate are mixed with these reageants generating a bioluminescent flash lasting less than 1 second. This can be detected by a luminometer or liquid scintillation counter, with total light emission being directly proportional to the amount of active luciferase. A modification of the original protocol with the addition of coenzyme A to the reaction (Li et al., 2007)gives a longer bioluminescent flash allowing a longer reading time, increases sensitivity and imporves reproducibility between samples. Because of its short halflife luciferase is useful for inducible systems, allowing detection of signal above basal expression levels (Fowler et al., 1970).

1.5.1.3 β-Galactosidase

β-Galactosidase is a bacterial protein found in the *Lac* operon of *Escherichia coli.* β-Galactosidase catalyses the hydrolysis of galactosides such as lactose into

monosaccharides. The sequence for β-Galactosidase was first published in 1970 (An et al., 1982). The β-Galactosidase gene can be expressed in mammalian cells with gene expression determined using an artificial substrate (MacGregor et al., 1987) . Currently 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-Gal) is routinely used to detect β-Galactosidase activity. β-Galactosidase can be used as a reporter of promoter activity in mammalian cells (Berger et al., 1988) however, X-Gal is toxic to the cells and the detection of β-Galactosidase expression is carried out at single timepoints in fixed cells.

1.5.1.4 Secreted Alkaline Phosphatase

Alkaline phosphatase is a hydrolase which removes phosphate groups from a wide range of molecules. The secreted form of alkaline phosphatase is a human placental variant of the enzyme (Shiraiwa et al., 2007), and on secretion from the cells can be easily quantified. A common method for quantification of alkaline phosphatase activity is the hydrolysis of *p*-nitophenylphopshate (4NPP), hydrolysis leads to an increase in absorbance at 405nm. Other substrates include 4-methylumbelliferyl phosphate which generates a fluorescent product upon alkaline phosphatase catalysed hydrolysis (Nolan et al., 1988).

1.5.2 *In Vivo* Reporter Assays

These reporter assays allow the detection of the reporter in live cells or tissue. The data obtained is less quantitiative than from *in vitro* reporter based systems however, analysis from living cells allows observation of dynamic changes in gene expression in real time. *In vivo* systems also allow for the same cells or tissue to be used in repeated experiments as there is no requirement for cell lysis.

1.5.2.1 β-Galactosidase

Through modifications of the substrate used to detect β-Galactosidase activity it is possible to use β-Galactosidase as a reporter in living cells. Compunds such fluorescein di-β-galactopyranoside (Arboleda-Velasquez et al., 2008, Yamazaki et al., 2007), which is cleaved by active β-Galactosidase into fluorescein a fluorescent molecule, have been used to determine gene expression over time in living cells. Coupled with FACS analysis this technique is still used to determine gene expression in living cells (Shimomura et al., 1962) however, the need to add a substrate before visualisation limits its use *in vivo*.

1.5.2.2 Fluorescent Proteins

Fluorescent proteins do not require any substrate for detection. Excitation of the proteins with specific wavelengths of light produces emissions of light at a shifted wavelength. Green fluorescent protein (GFP) from the luminescent jellyfish *Aequorea victoria* is one of the most widely used fluorescent proteins first extracted in the 1960s (Prendergast and Mann, 1978). The protein fluoresces in the green wavelength when excited with blue light (Prasher et al., 1992). The gene encoding GFP was succesfully cloned (Chalfie et al., 1994) and used as a reporter for gene expression (Heim et al., 1995). Various modifications to GFP have generating an enhanced (EGFP) mutant with increased fluorescence and stability (Matz et al., 1999). Other mutations to GFP and the discovery of new fluorescent proteins (Shaner et al., 2005) mean that fluorescence in far red, red, orange yellow, green and blue can be generated as a reporter of gene expression (Wall et al., 2008b, Stephens et al., 2003, Agren et al., 1999a, Raffetto et al., 2001c). Currently available fluorescent proteins

are destabilised, allowing the fluorescent proteins to be degraded within the cell (www.clontech.com). This destabilisation of fluorescent proteins is especially important if they are used as reporters of gene expression, as they can report a decrease in gene expression as well as an increase.

1.6 Aims of thesis

The overall aims of this thesis were to characterise hTERT immortalised chronic wound fibroblasts and patient matched normal fibroblasts over an extended period in culture. To study the phenotypes of immortalised chronic wound and patient matched normal fibroblasts using *in vitro* models of wound healing. To use Microarray analysis and QRT-PCR to identify disease specific genes altered between chronic wound and normal fibroblasts. To use promoter regions from these disease specific genes to generate reporter constructs and to test these constructs in both chronic wound and normal cells.

Specifically, it was hypothesised that:

- Fibroblasts will maintain their phenotype after hTERT immortalisation
- Chronic wound fibroblasts exhibit reduced growth rate and impaired wound healing *in vitro*
- Chronic wound fibroblasts have a distinct gene expression profile and this relates to the impaired wound healing *in vivo*
- Reporter constructs will allow the real time analysis of changes in gene expression allowing the study of potential treatments for Chronic wounds
- A characterised chronic wound cell line would be a real alternative to the use of animals in the study of impaired wound healing.

Chapter Two

Materials and Methods

2.1 Cell Culture

Recruitment of patients to the study was carried out at the out-patient clinics of the Wound Healing Research Unit, School of Medicine, Cardiff University.Informed consent was obtained from all subjects and local research ethical committee approval was obtained from the Cardiff and Vale NHS Trust and Bro Taf Health Authority

All cell culture carried out in this project has been conducted in sterile conditions in an Astec Microflow 2 cabinet. All flasks and plates were maintained in a Nuaire air-jacketed DH autoflow automatic CO_2 incubator at 37°C/5% CO_2.

2.1.1 Fibroblast Culture

The hTERT immortalised fibroblast cell lines used in this project were generated from chronic wound and patient matched normal fibroblasts donated by Ivan Wall (Wound Biology Group, Dept. Oral Surgery Medicine and Pathology, Cardiff University). Fibroblasts were transfected with the hTERT containing retroviral vector pBABE-hTERT. Positively transfected cells were selected by the addition of 1μg/ml puromycin to the growth medium (Fibroblast-Serum Containing Medium (F-SCM + Puro) consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (2mM), antibiotics (100U/mL penicillin G; 100μg/mL streptomycin sulphate; 0.25μg/mL amphotericin B), puromycin 1μg/ml and 10% (v/v) foetal calf serum (FCS) (all reagents supplied by Invitrogen, Paisley, UK). Upon reaching 80-90% confluence, fibroblast populations were passaged at a ratio of 1:3. The cells were washed twice with phosphate-buffered saline (PBS) and detached from the plastic by incubation with 1mL 0.05% trypsin/0.53mM ethylenediaminetetraacetic acid solution (Invitrogen) for approximately 5 minutes at 37°C/5% CO_2. Following incubation, the tissue culture vessel was washed with 9mL PBS to collect trypsinised cells. The solution was then transferred to a 15mL centrifuge tube (Greiner Bio-one, Gloucester,

UK) and spun at 1500rpm for 5 minutes in a Precision Durafuge 200. The supernatant was aspirated off and the resulting pellet was re-suspended in 1mL of F-SCM. Cells were counted using a Neubauer haemocytometer (Sigma-Aldrich, Poole, UK) and re-seeded as required.

2.1.2 Cryopreservation and retrieval of cells

For cryopreservation cells were re-suspended in a solution of 10% (v/v) dimethyl sulphoxide (DMSO) (Sigma-Aldrich) 90% (v/v) FCS, placed in cryogenic vials (Greiner) and stored at -80°C in an iso-propanol freezing container. After 24 hours cryovials were transferred for long term storage in liquid nitrogen (at -196°C). Frozen cells were retrieved from storage by rapid thawing at 37°C, washed in F-SCM and following centrifugation (1500 rpm) for 5 minutes the cells were re-suspended in F-SCM and cells counted using a haemocytometer. Cells were then seeded as required.

2.1.3 Calculation of population doubling levels

To determine and compare the proliferation of different fibroblast cell lines, cumulative population doubling levels were plotted against time. At each passage the total number of viable cells (determined by trypan blue exclusion) were counted using a haemocytometer and recorded. The number of cells subsequently reseeded was also recorded. Population doubling level (PDL) of the cell population was calculated using the following formula:

$$PDL = \frac{\log_{10}(\text{total cell count obtained}) - \log_{10}(\text{total cell count re-seeded})}{\log_{10}(2)}$$

2.1.4 Cell Size Analysis

Cells in monolayer culture were digitally imaged 24 hours after passage using a Nikon Coolpix 995 digital camera (Nikon, Kingston-upon-Thames, UK) attached to an Olympus CK2 microscope (x10 objective lens, F-Stop 3.5) (Olympus, Southall, UK).Images were taken at random from various regions of the flask. Digital images were viewed and cell area determined using ImageJ (http://rsb.info.nih.gov/ij/). Average cell size was calculated from no fewer than 100 individual cells.

2.1.5 Screening for Mycoplasma contamination

Cell lines were routinely screened for the presence of *Mycoplasma* contamination. Cells were cultured overnight ($1x10^4$ cells/well) under standard conditions in 24 well plates. Media was removed and the cells washed with 1mL of Hoechst 33258 stain (bisbenzimide; Aventis, Bad Soden, DE) at 1 μg mL^{-1} in methanol (Sigma-Aldrich). Fresh stain was applied and the cells incubated for 15 minutes at 37°C/5% CO_2. Staining solution was removed and the cells were washed 4 times with ddH_2O. Digital images of stained cells were acquired using a Zeiss Axiovert 200M microscope (with DAPI excitation 300-390nm, emmission 410-750nm) and a Hamamatsu digital camera and reviewed using Adobe Photoshop 6.

2.2 Microarray Analysis

2.2.1 RNA extraction

$1x10^6$ cells were plated out into a 14cm tissue culture plate (Greiner) and left for 24 hours to adhere. F-SCM + Puro was replaced with F-SCM + Puro containing 0.1% FCS and the cells serum starved for 48 hours. Cells were stimulated by the addition of F-SCM + Puro for 0, 1, 6 and 24 hours. After stimulation, cells were washed with PBS and incubated with 1.5ml TRIzol (Invitrogen) reagent (5min RT). The plates were scraped and 750μl of cell lysate placed into RNase free 1.5ml microcentrifuge

tubes (Greiner). 175µl chloroform (Sigma-Aldrich) was added to each tube and shaken. Chloroform/TRIzol mix was placed in 1.5ml heavy phase lock gels (Fisher Scientific, Loughborough, UK) and centrifuged (2min, 13000g, RT). The upper colourless aqueous layer was transferred to a RNase free 1.5ml microcentrifuge tube. RNA was precipitated by the addition of 500µl Isopropanol (Sigma-Aldrich), 1µl glycogen (Invitrogen) (10min, RT) the centrifuged (10min, 12000g, 4°C). The supernatant was removed and the pellet washed with 500µl 75% Ethanol (EtOH) (Sigma-Aldrich) and centrifuged (5min, 7500g, 4°C). The supernatant was removed and the pellet air-dried and re-suspended in 10µl of DEPC H_2O.

2.2.2 Ethanol Precipitation

RNA was precipitated (overnight -20°C) by the addition of $1/10^{th}$ volume NaOAc (Sigma-Aldrich), 2.5x volume 100% EtOH and 1µl glycogen. After overnight precipitation, the RNA was centrifuged (20min, 12000g, 4°C) the supernatant removed and washed twice 500µl 80% EtOH (Sigma-Aldrich) and centrifuged (10min, 12000g, 4°C). The supernatant was removed and the pellet air-dried and re-suspended in 10µl of DEPC H_2O. RNA samples were checked for purity (260/280 ratio >1.6) and concentration by nanodrop spectrometer. Analysis for integrity of RNA samples was carried out on an Agilent 2100 bioanalyser (Agilent Technologies, Stockport, UK). The ratio and integrity of 18S and 28S rRNA was analysed to determine if the RNA has been degraded.

2.2.3 Microarray Analysis

cDNA synthesis and Microarray analysis of mRNA was carried out by Megan Musson, Central Biotechnology Service, Department of Pathology, Cardiff University using *Affymetrix*™ Genechip U133A arrays (Affymetrix UK Ltd., Maidenhead, UK). Raw microarray expression data was initially processed using the microarray Analysis

Suite 5.0 (MAS5) provided by *Affymetrix*. The resulting data was stored in cell intensity (.CEL) files. Statistical analysis was carried out using Bioconductor (http://www.bioconductor.org) an open source R based software project for the analysis of genomic data. Data was also uploaded onto MADRAS Microarray Data Review and Annotation System (http://www.madras.uwcm.ac.uk).

2.3 Telomeric Repeat Amplification Protocol (TRAP ASSAY)

2.3.1 TRAP solutions

Unless otherwise stated all reagents used in this assay were from Sigma-Aldrich.

2.3.1.1 Wash Buffer per 1ml

100µl 0.1M Hepes-KOH

1.5µl 1M $MgCl_2$

1.0µl KCl

1µl 1M DTT (added before use)

DEPC water to 1ml

2.3.1.2 Lysis Buffer per 1ml

100µl Tris-HCL pH8.3

1.5µl 1M $MgCl_2$

10µl 0.1M EGTA

100µl Glycerol

100µl 5% CHAPS

2µl 0.5M PMSF (added before use)

3.5µl 10% β-mercaptoethanol (added before use)

DEPC water to 1ml

2.3.1.3 1x Reaction Buffer per reaction

20mM Tris-HCL, pH8.3

1.5mM $MgCl_2$

63mM KCL

0.005% TWEEN

1mM EGTA

50μM dNTP's (Promega, Southampton, UK)

0.1mg/ml BSA (Promega)

1μg T4 gene32 protein (Promega) (added before use)

100ng TS primer (Invitrogen Custom Primer) (added before use)

100ng CX primer (Invitrogen Custom Primer) (added after first step)

2.5U Taq polymerase (Promega) (added after first step)

2.3.1.4 TS Primer

5'-AATCCGTCGAGCAGAGTT-3'

2.3.1.5 CX Primer

5'-CCCTTACCCTTACCCTTACCCTAA-3'

2.3.2 Telomerase extraction

Cells ($5x10^5$) were re-suspended in 1 ml wash buffer (2.3.1.1) and spun at 15,000g for 2 min 4°C. The supernatant was removed and the cells re-suspended in 100μl lysis buffer (2.3.1.2). Cells were incubated on ice for 30 min. The lysate was then centrifuged at 20,000*g* for 30 min at 4°C and the supernatant collected and frozen on dry ice in 10μl aliquots.

2.3.3 TRAP assay

Reactions were set up in RNase free 0.5ml microtubes, each reaction containing 50μl of 1x reaction mix with 1μg T4 gene32 protein and 100ng TS primer added (2.3.1.3). Various volumes of protein extract (2.3.2) were added (1μl-5μl) to each reaction. Negative controls for each reaction were setup with heat denatured protein extracts.

Protein extracts were heat denatured for 10 minutes at 85°C. Reactions were incubated for 30 minutes at 30°C, the temperature was increased to 92°C and 100ng CX primer, 2.5U Taq polymerase was added to each reaction. TRAP products were amplified by 31 cycles (92°C for 30s, 50°C for 30s and 72°C for 90s). 10 µl of 6x loading dye (Promega) was added to each reaction. TRAP products were run on a 10% polyacrylamide (19:1) 20cm gel containing 0.5x TBE, 25µl of each reaction were added per well and run at 300V until bromophenol blue was approximately 3cm from the bottom of the gel. The gel was soaked in 1x Sybr Gold (Invitrogen) for 10 minutes and visualised on a Typhoon 9400 Variable Mode Imager (GE Healthcare, Little Chalfont, UK) using an excitation wavelength of 488nm and a 520 BP40 emission filter.

2.4 Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

2.4.1 cDNA generation

For QRT-PCR cDNA was generated from total RNA samples extracted as described in section 2.2. RNA concentration and integrity was quantified as described in section 2.2. For each reaction 1µg mRNA was added to 0.5µg random hexamer primers (Promega) in a RNase free 50µl PCR tube and made up to 13µl with DEPC treated ddH_2O. Reactions were heated to 70°C for 5min followed by quenching on ice for 5min. To each reaction the following reageants were added; 1µl RNAsin (Promega), 5µl MMLV 5x buffer, 5µl 10mM dNTP's (dATP, dCTP, dGTP and dTTP) (Promega) and 1µl M-MLV Reverse Transcriptase (Promega). Reactions were mixed and incubated for 10min 25°C, 60min 42°C and 5min 95°C. Reactions were stored at -80°C until required.

2.4.2 GAPDH PCR

2.4.2.1 GAPDH Primers

GAPDH 1 F (5' to 3') CAACTACATGGTTTACATGTTCCAA

GAPDH 1 R (5' to 3') TCAAGATCATCAGCAATGCCT

2.4.2.2 Confirmation of cDNA generation by Polymerase Chain Reaction

25 µl PCR reactions were setup with 10ng cDNA (1µl cDNA reactions after 1:4 dilution), 2.5µl 10x PCR buffer (Promega), 2µl 25mM $MgCl_2$, 2µl 10mM dNTP's (dATP, dCTP, dGTP and dTTP) (Promega),1µl 10µM GAPDH 1 F primer, 1µl 10µM GAPDH 1 R primer, 0.5µl Taq Polymerase (Promega) and 15µl DEPC treated ddH_2O. Reactions were denatured for 2 minutes at 95°C then run for 30 cycles of 94

°C for 20s, 55 °C for 30 s and 72 °C for 30s on a Hybaid thermal cycler (Thermo Electron).

2.4.2.3 Agarose Gel Electrophoresis of PCR products

A 1.5 % agarose gel containing 60 ml of TAE and 0.9 g of molecular grade agarose (Bioline, London, UK) was microwave-heated until the agarose was dissolved. After cooling to a temperature comfortable to touch, 0.6µl of ethidium bromide was added and the gel was poured into a 10 cm x 11.5 cm casting tray and allowed to set. The gel was then immersed in a tank containing 200 ml TAE and each well was loaded with 10 µl of PCR product containing 1x blue/orange loading dye (Promega). A 100 bp DNA ladder (Promega), also containing 1x blue-orange loading dye was loaded at a volume of 6 µl per well and the gel was run at 75 V for 60 min.

2.4.3 Light Upon Extension Quantitative Real Time PCR

2.4.3.1 Reference Control

To allow comparison between plates a reference control was used. Stratagene QPCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA) was supplied at s concentration of 1µg/µl in 0.1mMEDTA/RNase-free H_2O with a A_{260}/A_{280} ratio of 1.8. cDNA controls were generated from 4 pooled cDNA reactions each containing 5µg total RNA giving 100µl of 200ng/µl total cDNA.

2.4.3.2 Reference Gene

A predesigned certified FAM labelled LUX primer set for GAPDH (Invitrogen) was chosen as the housekeeping gene for all QRT-PCR primers. Primers were designed against GenBank Accession number NM_002046, human GAPDH mRNA and

amplify a region that spans the exon junction 4/5. Both labelled and unlabelled primers were supplied at 10μM concentration.

2.4.3.3 Primer Design

Primers pairs for each of my genes of interest were designed using the D-LUX™ Designer (https://orf.invitrogen.com/lux/). Genes were identified using their unique GenBank Accession number and the mRNA sequence inputed into the primer design program. Each sequence was compared by BLAST to other sequences in the Human database to identify unique regions for primer design. The following design parameters were defined, amplicon size (minimum 60bp, maximum 20bp, optimum 75bp), primer temperature (minimum 60, maximum 68, optimum 64) and primer length (minimum 20, maximum 24, optimum 22). Primers were selected within the translated region where possible and with at least a 4 star quality rating.

2.4.3.4 QRT-PCR

For each 96 well plate (StarLab, Milton Keynes, UK) a standard curve of total human cDNA (100ng, 10ng, 1ng and 100pg) was used in triplicate along with 4 GAPDH controls (10ng total human cDNA) to allow for comparison between plates. 25μl QRT-PCR reactions were setup for each sample in triplicate with one patients' samples per plate. 100ng cDNA (0.5μl cDNA reaction) was made up to 5μl with DEPC treated H_2O, to this 12.5μl Platinum qPCR SuperMix-UDG (Invitrogen) containing 3mM $MgCl_2$ and ROX reference dye, 0.5μl 10μM LUX labelled primer, 0.5μl 10μM unlabelled primer, 6.5μl DEPC treated H_2O. Plates were run on an ABI Prism 7000 (Applied BioSystems, Foster City, USA) with the following cycling

program; 2 min 50°C, 2 min 95°C followed by 50 cycles of 15 sec 95°C, 60 sec 60°C. Melting curve analysis was carried out for all reactions at 60°C.

2.4.3.5 Validation of Reference Gene

Variations in reference gene expression level were determined through analysis of ct values for a known concentration of cDNA. 10ng of reference cDNA was used and reactions set up with GAPDH primers as described in section 2.4.3.4 in total 106 reactions were run with positive results. ABI Prism 7000 SDS version 1.1 software was used to determine the ct value for each reaction. Data was statistically analysed in SPSS 14.02 and a 95% confidence interval for fold change determined.

2.4.3.6 Analysis of Expression Changes

ABI Prism 7000 SDS version 1.1 software was used to determine standard curves for each gene and these curves used to determine relative gene expression for each sample. The mean expression for the three replicates was taken and normalised by dividing by the relevant GAPDH reference gene value. These values were initially plotted on Excel spread sheets. Statistical analysis of data was carried out in SPSS 14.02.

2.5 Generation of Reporter Constructs

2.5.1 DNA extraction from CWF and NF

At sub-culture, cells were counted and $5x10^5$ cells were pelleted in a 1.5 ml RNase/ DNase free eppendorf tube (Greiner). Media was removed and cells were washed with PBS to remove residual culture medium. Samples were kept on ice and 300 µL lysis buffer (200 mM tris-HCl pH 7.5/250 mM NaCl/25 mM EDTA/0.5 % SDS in H_2O) along with 3 µl ribonuclease A (RNase A) was added to each pellet. 6 µL proteinase K (20 mg/ml) was added to each tube at room temperature before pulse spinning in a microcentrifuge (all reagents from Sigma-Aldrich). The solution was incubated overnight at 45 °C in a heat block. After incubation 300 µL phenol:chloroform:Iso-amyl alcohol (25:24:1; Sigma-Aldrich) was added to each tube and the tubes mixed on a revolving mixer for 30 min at room temperature. The solution was centrifuged at 13000 rpm for 5 min. The upper aqueous layer was carefully removed from the underlying phenol:chloroform:Iso-amyl alcohol layer and transferred to a new tube containing 300µl phenol:chloroform:Iso-amyl alcohol. The solution was mixed on a revolving mixer for 30 min at room temperature.The tubes were centrifuged at 13000 rpm for 5 min and the upper aqueous layer transferred to a new tube. DNA was precipitated by incubating with 300 µL NaOAc (3 M, pH 5.2; Sigma-Aldrich) and 900µl ice-cold ethanol (100 %) for 20 min at -20 °C. DNA was pelleted by centrifugation at 13000 rpm for 2 min, the supernatant removed and the pellet retained. The DNA pellet was washed with 500µl ice-cold ethanol (70 %) and centrifuged at 13000 rpm for 2 min. Supernatant was removed and the pellet air dried at room temperature for 2 h. DNA was re-suspended in 20µl DEPC treated ddH_2O and stored at -20°C until required.

2.5.2 Quantification of DNA

DNA concentration was calculated using the Fluorescent DNA quantitation kit (BioRad) and the FLUOROStar Optima (BMG LABTECH, Aylesbury, UK). Samples were thawed on ice and pulse centrifuged. The DNA quantification buffer (TEM), consisting of 100 mM Tris, 2 M NaCl and 10 mM EDTA pH 7.4 was prepared 1:10 in H_2O. Hoechst 33258 dye was added to the buffer at a concentration of 1 ng/ml. 1µl of each DNA sample was added to 200µl of TEM buffer containing Hoechst stain in a 96 well fluorometric plate. A standard curve was setup in triplicate with calf thymus DNA at the following concentrations 2000ng, 1000ng, 500ng, 100ng, 50ng and 20ng. A blank control was also setup with TEM buffer containing Hoechst stain but no DNA. DNA was quantified using a 355nm excitation filter and a 460 emission filter, 20 flashes per well. Sample DNA concentrations were calculated by comparison to the standard curve. DNA samples were then diluted to a final concentration of 10 ng/µl in 10mM Tris pH8.

2.5.3 Primer Design

Primers were designed using the online primer3 software (http://primer3.sourceforge.net/) using sequence information from the Database of Transcriptional Start Sites (http://dbtss.hgc.jp).

2.5.4 Long Range PCR

Long range PCR was carried out using genomic DNA extracted as described in section 2.5.1. The proof reading polymerase Expand HiFi (Roche, Welwyn Garden City, UK) following the manufacturers instructions. Two reaction mixes were prepared, and combined just prior to thermal cycling. Mix 1; 21.75 µl ddH_2O, 1 µl

10mM dNTP's (Promega), 1 µl 10mM 3'primer, 1 µl 10mM 5' primer, 100ng genomic DNA. Mix 2; 19.25 µl ddH_2O, 5 µl 10X Expand HiFi buffer (Roche), 0.75 µl Expand HiFi polymerase (Roche). Reactions were denatured for 5 minutes at 95°C and then run for 35 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 2 min with a final extension 72 °C for 7 min on a Hybaid thermal cycler (Thermo Electron). Products were run on a 0.8% TBE agarose gel as described previously.

2.5.5 Gel Extraction

Gel extraction was carried out using a Quiagen gel extraction kit (Quiagen, West Sussex, UK) following the manufactures instructions. Briefly bands were excised from the gel using a scalpel, weighed and three times the gel slice volume of QG buffer added. The gel slice was incubated with shaking at 50^{o+}C for 10 minutes. 800µl of gel/buffer was added to a QIAquick spin column and centrifuged for 1 min at 10,000 RPM with the flow through discarded. If any Gel/buffer solution remained it was added to the same column and the centrifugation repeated. 750µl PE buffer was added to the column, incubated at r/t for 5 min and then centrifuged for 1 min at 10,000 RPM and the flow through discarded. Remaining PE buffer was removed by a further centrifugation for 1 min at 13,000RPM and the column was placed in a clean 1.5ml tube. 30µl of EB buffer was added to the column membrane, incubated for 1 minute at r/t and centrifuged at 10,000RPM for 1 min. DNA concentration was determined by UV spectroscopy.

2.5.6 Ligation into Plasmids

Ligation reactions were setup either with pGEM-T recovery vector (Promega) or restriction digested pZsGreen reporter vector (Clontech, Saint-Germain-en-Laye,

France). Recovered DNA from gel extracts or restriction digested PCR products were used as insert. Ligation reactions were setup with 5 µl 2x T4 DNA ligase buffer (Promega), 1 µl Vector, 1 µl T4 DNA Ligase (Promega) 3 µl insert DNA. Reactions were mixed by gentle pipetting up and down and incubated at 16°C overnight.

2.5.7 Transformation into competent *E.coli*

Subcloning Efficiency ™ DH5α™ competent *E.coli* (Invitrogen) were used for all transformations following the manufacturers recommended protocol. Briefly DH5α™ cells were thawed on ice and mixed gently with a pipette tip. 50µl of cells were added to 2 µl of ligation reaction and mixed gently. Unused cells were flash frozen on dry ice/EtOH. Cells and DNA were incubated on ice for 30 min after which time they were heatshocked at 42°C for 30 seconds. After heat shocking cells were placed on ice for 2 min. 950 µl of warmed SOC media (Invitrogen) was added to the cells and the cells/media incubated at 37°C for 1 hour at 225rpm.100 µl of cells/media was spread onto agar plates with appropriate selection and incubated overnight at 37°C. Colonies were picked used to inoculate 5ml of LB media and grown overnight.

2.5.8 Plasmid MiniPrep

5ml of overnight bacterial culture was pelleted by centrifugation at 16,000 rpm for 5 minutes. Pelleted bacterial cells were resuspended in 250µl buffer P1 (Qiagen) and transferred to a fresh 1.5ml microcentrifuge tube. 250 µl of buffer P2 (Qiagen) was then added and mixed by inverting 4-6 times. Buffer N3 (Qiagen) was added and mixed by inverting 4-6 times. The lysed bacteria were centrifuged at 13,000 rpm for 10 minutes and the resulting supernatant added to a QIAprep spin column. The QIAprep column was centrifuged for 60 seconds at 13,000rpm and the flow through

discarded. The column was washed by the addition of 500 µl buffer PB (Qiagen) followed by centrifugation for 60 seconds at 13,000rpm and the flow through discarded. The column was washed by the addition of 750 µl of buffer PE (Qiagen) followed by centrifugation for 60 seconds at 13,000rpm and the flow through. A further centrifugation step of 60 seconds at 13,000rpm was used to remove left over PE buffer and the column was placed into a fresh 1.5ml centrifuge tube. DNA was eluted by the addition of 30 µl buffer EB (Qiagen), incubation for 1 minute at room temperature followed by centrifugation for 60 seconds at 13,000rpm. The resulting flowthrough was analysed for DNA concentration by absorbance at 260nm and stored at -20°C.

2.5.9 Restriction Digest

All restriction digests were carried out using Promeg restriction enzymes and preferred buffers. In the case of double digest the buffer with the highest percentage activity for both enzymes without star activity was used. 10 µl digests were setup with 1µl DNA, 1µl restriction enzyme(s), 1µl 10x buffer, 1µl 0.1M DTT, 6µl ddH_2O. Plasmid DNA from miniprep purifications or PCR product from gel extractions was used at a concentration no greater than 500ng/µl. Digests were incubated for 1 hour at 37°C and visualised on an agarose gel.

2.5.10 Transfection of Adherent Fibroblasts

Fibroblasts were plated out into a 6 well tissue culture plate (Greiner) at a density of $1x10^5$ cells per well. Cell were incubated overnight in FSCM at 37°C, 5% CO_2. After overnight incubation cells were between 50-80% confluent. For each well 100µl of serum free DMEM (Invitrogen) plus 3µl GeneJuice® transfection reagent (Merck

KGaA, Darmstadt, Germany) were prepared and incubated at room temperature for 5 minutes. For each well 1μg of plasmid DNA was added to the DMEM/GeneJuice mixture and incubated at room temperature for 15 minutes. The DNA/DMEM/GeneJuice mixture was added dropwise to the cells, with the drops evenly distributed across the well. Cells were incubated for 24 hours at 37°C, 5% CO_2 before media was changed.

2.5.11 Quantification of fluorescence

Fluorescence was quantified using the FLUOROStar Optima (BMG LABTECH). Transfected cells were serum starved for 48 hours prior to use, FSCM was added to the cells and fluorescence readings taken at 0, 1, 6 and 24 hours after stimulation. Untransfected cells were used as a negative control and all fluorescence was normalised to GAPDH.

2.5.12 Confocal Microscopy.

Transfected and untransfected cells were plated at a density of 2×10^4 cells per well into each well of an 8 well chamber slides (Greiner) and maintained for 24h under standard culture conditions. After discarding waste media, the cells were washed fixed in ice cold methanol and stained with 1 mL of Hoechst 33258 stain at 1 μg mL^{-1} in methanol (Sigma-Aldrich) for 15 minutes. Stain was removed and the slides mounted with Fluorosave reagent (Merck). Mounted slides were visualised using a Leica TCS SP5 confocal microscope(Leica, Milton Keynes, UK) running the Leica Application Suite, Advanced Fluorescence (LASAF) software.

CHAPTER THREE

Phenotypic Analysis

of

Immortalised Human Chronic wound and

Normal Fibroblasts Cell Lines

3.1 Introduction

In order to study chronic wounds without the use of animal models a cell based alternative is required. Fibroblasts can be isolated and cultured from chronic wounds, and they have been demonstrated to be phenotypically distinct from fibroblasts taken from the unwounded skin of the same patient, making them a potential tool for understanding chronic wound cell biology (Hayflick and Moorhead, 1961, Harley, 1991). These primary cells however have a finite replicative capacity before they enter senescence (Stanley and Osler, 2001b, Raffetto et al., 1999b, Mendez et al., 1998a). Therefore the potential usefulness of primary human fibroblasts isolated from chronic wounds as an experimental model is severely limited by their reduced replicative potential (Collins, 1996).

One of the mechanisms controlling replicative senescence is the erosion of telomeres. Telomere erosion can be halted by the addition of hTERT to the cells. Telomerase is a ribonucleoprotein DNA polymerase consisting of both protein and RNA components (Vaziri and Benchimol, 1998). Telomerase has the ability to add 6 base pair repeats to the end of the telomeres restoring their length after cell division. The addition of hTERT to somatic diploid fibroblasts through retroviral transfection has been shown to increase telomere length and extend cellular replicative life span (MacKenzie et al., 2000). The expression of hTERT allows the cells to escape telomere dependent senescence. After extended periods in culture hTERT infected cells may still enter a state of growth arrest in a telomere independent fashion (Milyavsky et al., 2003b) or take on a cancer like phenotype (Morales et al., 1999, Choi et al., 2001, Ouellette et al., 2000). Despite the possible drawbacks associated with long term growth of hTERT infected cell lines, the technology offers the potential to generate cell lines

from difficult to obtain primary cells without altering their phenotype (Wyllie et al., 2000, MacKenzie et al., 2000).

The aim of this chapter was to characterise hTERT infected chronic wound and patient matched normal skin fibroblasts over an extended time in culture. Before they can be used further it was important to confirm that the hTERT infected cells had escaped replicative senescence and were effectively immortalised. For the cells to be used as a model for chronic wounds it was also vital to confirm that the disease specific phenotype observed in the chronic wound derived cells was maintained, not only immediately after hTERT transfection but also after long term culture.

3.2 Results

3.2.1 Long term culture of hTERT infected fibroblasts

Work carried out previously within the Wound Biology Group in Cardiff involved the recruitment of three patients suffering with chronic venous leg ulcers. Biopsies were taken from both the ulcer bed and from the ipsilateral thigh for each patient. Fibroblasts were isolated from the biopsies giving 6 primary cell strains (Ivan Wall Thesis 2006). The cell strains were labelled H, I and K identifying the 3 separate patients, with 'w' indicating wound and 'n' indicating normal identifying the biopsy site. The primary cell strains were retrovirally infected with either an empty pBABE plasmid conferring puromycin resistance or with a pBABE plasmid expressing human telomerase (hTERT). The infected cells were treated with puromycin therby selecting infected cells. For each cell strain two cell lines were generated; a 'mock' cell line containing the empty vector and a hTERT cell line containing the telomerase expressing vector. The cell lines were maintained in continuous culture with accurate cell counts taken at each passage. Population doubling levels (PDL) were determined for each cell line and plotted against time in culture (Figs 3.1-3.3). The mock and hTERT cell lines for each patient and for wound and normal are shown on the same graph allowing comparison between growth rates he average growth rate for each cell line over the entire extended time in culture is shown in Table 3.1.

In patient H (Fig 3.1) the mock infected normal cells (nHm) underwent 55 population doublings (PD) over 340 days before reaching senescence. The mock infected wound cells (wHm) underwent 29 PD over 231 days before reaching senescence. The hTERT infected normal cell line (nHh) reached 55 PD in 175 days and 128 PD in 340 days with no sign of a reduction in cell proliferation. The hTERT infected wound cell line (wHh) underwent 29 PD in 119 days, 91 PD in 340 days and showed no sign of a

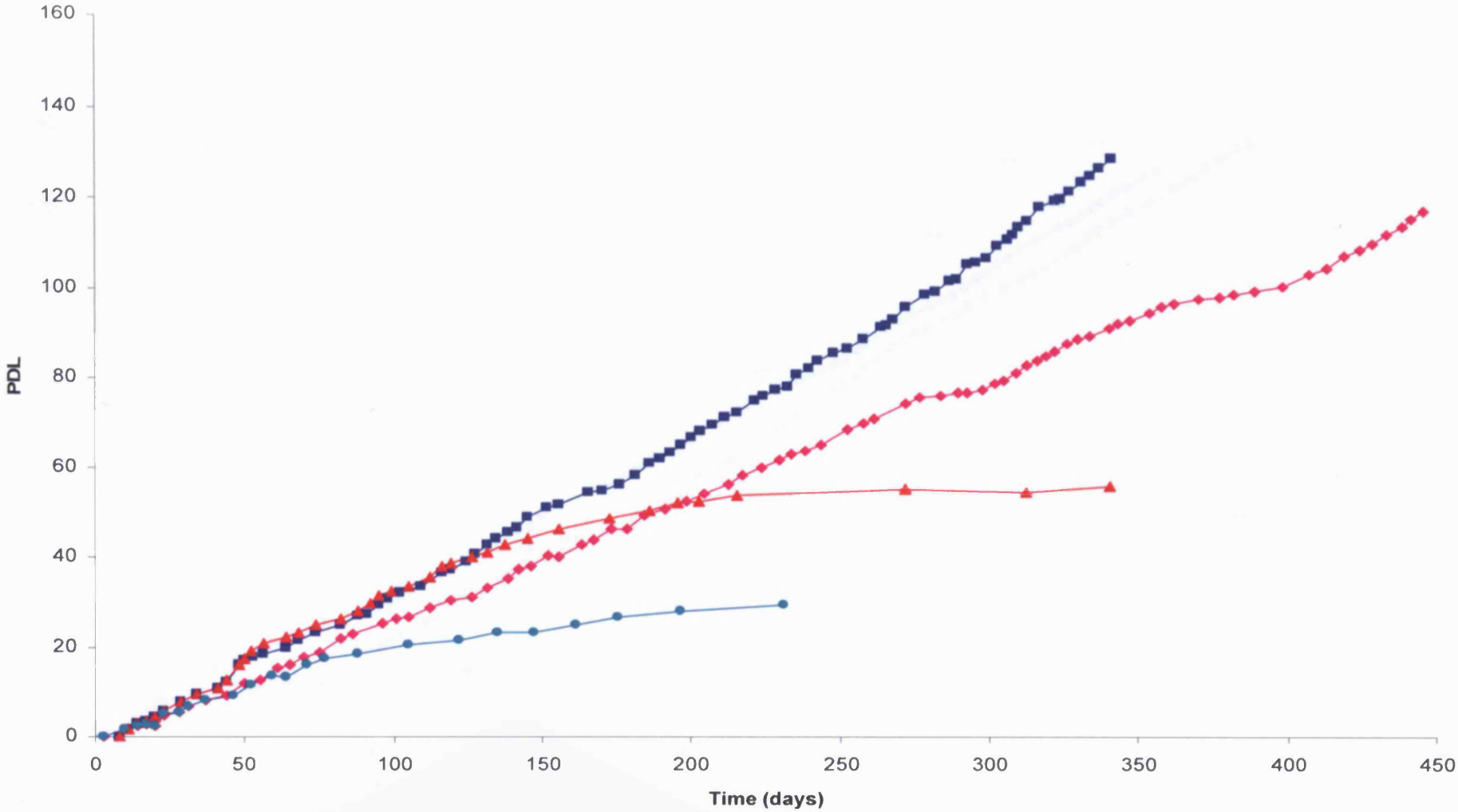


Figure 3.1: Relative PDL data for Patient H, mock infected cells CWF (—) and NF (—) and hTERT immortalised cell lines CWF (—) and NF (—). Mock infected CWF complete fewer PDs than NF with both cell strains senescing before 55 population doublings (senescence was determined when population doubling per week dropped below 0.5). The hTERT immortalised cell lines exceeded the maximum PD's seen in the mock immortalised cells completing more than 100 population doublings.

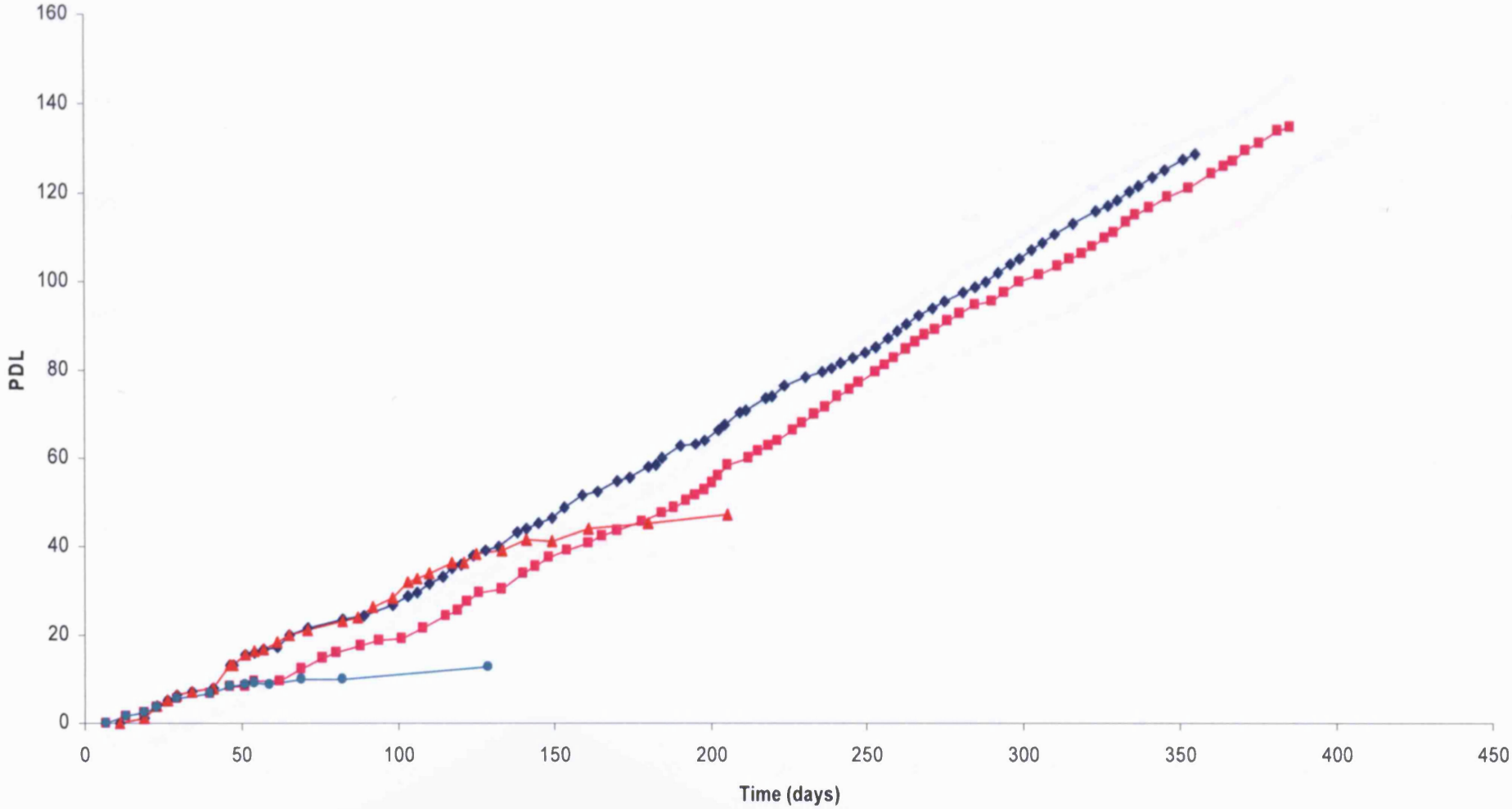


Figure 3.2: Relative PDL data for Patient I, mock infected cells CWF (—) and NF (—) and hTERT immortalised cell lines CWF (—) and NF (—). Mock infected CWF complete fewer PDs than NF with both cell strains senescing before 50 population doublings (senescence was determined when population doubling per week dropped below 0.5). The hTERT immortalised cell lines exceeded the maximum PD's seen in the mock immortalised cells completing more than 100 population doublings.

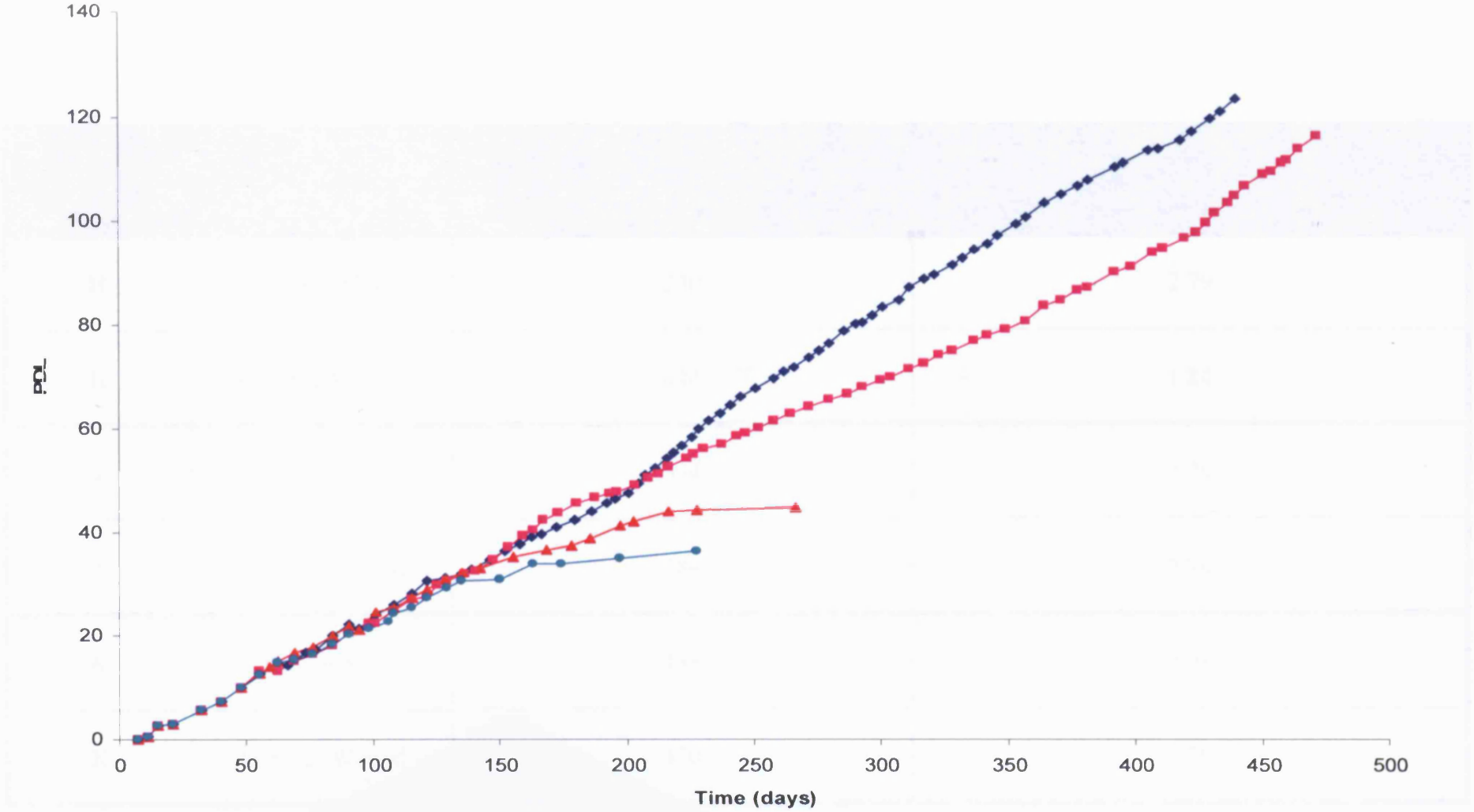


Figure 3.3: Relative PDL data for Patient K, mock infected cells CWF (—) and NF (—) and hTERT immortalised cell lines CWF (—) and NF (—). Mock infected CWF complete fewer PDs than NF with both cell strains senescing before 50 population doublings (senescence was determined when population doubling per week dropped below 0.5). The hTERT immortalised cell lines exceeded the maximum PD's seen in the mock immortalised cells completing more than 100 population doublings.

Patient	Type	Total Time in Culture (Days)	Average Growth Rate (Population Doubling/Week)
H	Normal Skin	340	2.79
H	Chronic Wound	444	1.84
I	Normal Skin	354	2.70
I	Chronic Wound	384	2.70
K	Normal Skin	438	2.03
K	Chronic Wound	470	1.78

Table 3.1: Table showing average growth rate for each of the hTERT immortalised cell lines

reduction in cell proliferation at 116 PD and 444 days in culture. The wHh cell line did however undergo a brief plateau in PDL at two points in culture between days 283-297 and between days 376-397. The PD/week dropped below 0.5 indicating senescence however, the wHh cell line recovered and PD/week returned to levels previously seen. Both the hTERT infected cell lines have escaped replicative senescence, undergoing more than 100 PD with no sign of a reduction in cell proliferation. Overall though the wHh cell line showed a reduced rate of proliferation compared to the nHh cell line (Table 3.1).

In patient I (Fig 3.2) the mock infected normal cells (nIm) underwent 47 population doublings (PD) over 205 days before reaching senescence. The mock infected wound cells (wIm) underwent 12 PD over 129 days before reaching senescence. The hTERT infected normal cell line (nIh) reached 47 PD in 149 days and 129 PD in 354 days with no sign of a reduction in cell proliferation. The hTERT infected wound cell line (wIh) underwent 12 PD in 69 days, 121 PD in 352 days and showed no sign of a reduction in cell proliferation at 134 PD and 384 days in culture. Compared to patient H the growth rates of the normal I and wound I hTERT infected cells were closer with no difference in average growth rates over the total time in culture (Table 3.1). At some points in culutre wIh did demonstrate a slightly reduced rate of proliferation compared to nIh with the greatest difference in proliferation rates observed between days 50 and 200. Both the hTERT infected cell lines escaped replicative senescence undergoing more than 125PD.

In patient K (Fig 3.3) the mock infected normal cells (nKm) underwent 45 population doublings (PD) over 266 days before reaching senescence. The mock infected wound cells (wKm) underwent 36 PD over 227 days before reaching senescence. The hTERT infected normal cell line (nKh) reached 45 PD in 192 days and 123 PD in 438 days

with no sign of a reduction in cell proliferation. The hTERT infected wound cell line (wKh) underwent 36 PD in 153 days, 104 PD in 438 days and 116 PD in 470 days with no sign of a reduction in cell proliferation. Both the hTERT infected cell lines demonstrated that they had escaped replicative senescence undergoing more than 110 PD. As in patient H there was a difference in proliferation rates between wKh and nKh with the wound derived cell line showing a reduced rate of cell proliferation (Table 3.1). Compared to the other patients the patient K hTERT cell lines showed generally slower rates of cell proliferation requiring a greater time in culture to reach 100PD.

For all three patients the wound derived mock infected cells senesced at a lower PDL than the patient matched mock infected normal cells. For all three patients the hTERT immortalised normal and wound derived cells escaped replicative senescence undergoing significantly more PD than the mock infected cells. In patients H and I the hTERT infected cells maintained a difference in cell proliferation rate between the normal skin and wound derived cell lines with the wound cells having a reduced proliferation rate (Table 3.1).

3.2.2 hTERT and hTR gene expression in CWF and NF cell lines

To be active, human telomerase requires a protein and RNA component hTERT and hTR respectively. The expression of these components was determined by RT-PCR with RNA extracted from each of the hTERT infected cell lines (Fig 3.4) Gene expression was observed in normal and wound derived cell lines for each of the three patients. However the presence of hTERT and hTR RNA does not confirm the presence of active telomerase in the hTERT infected cell lines.

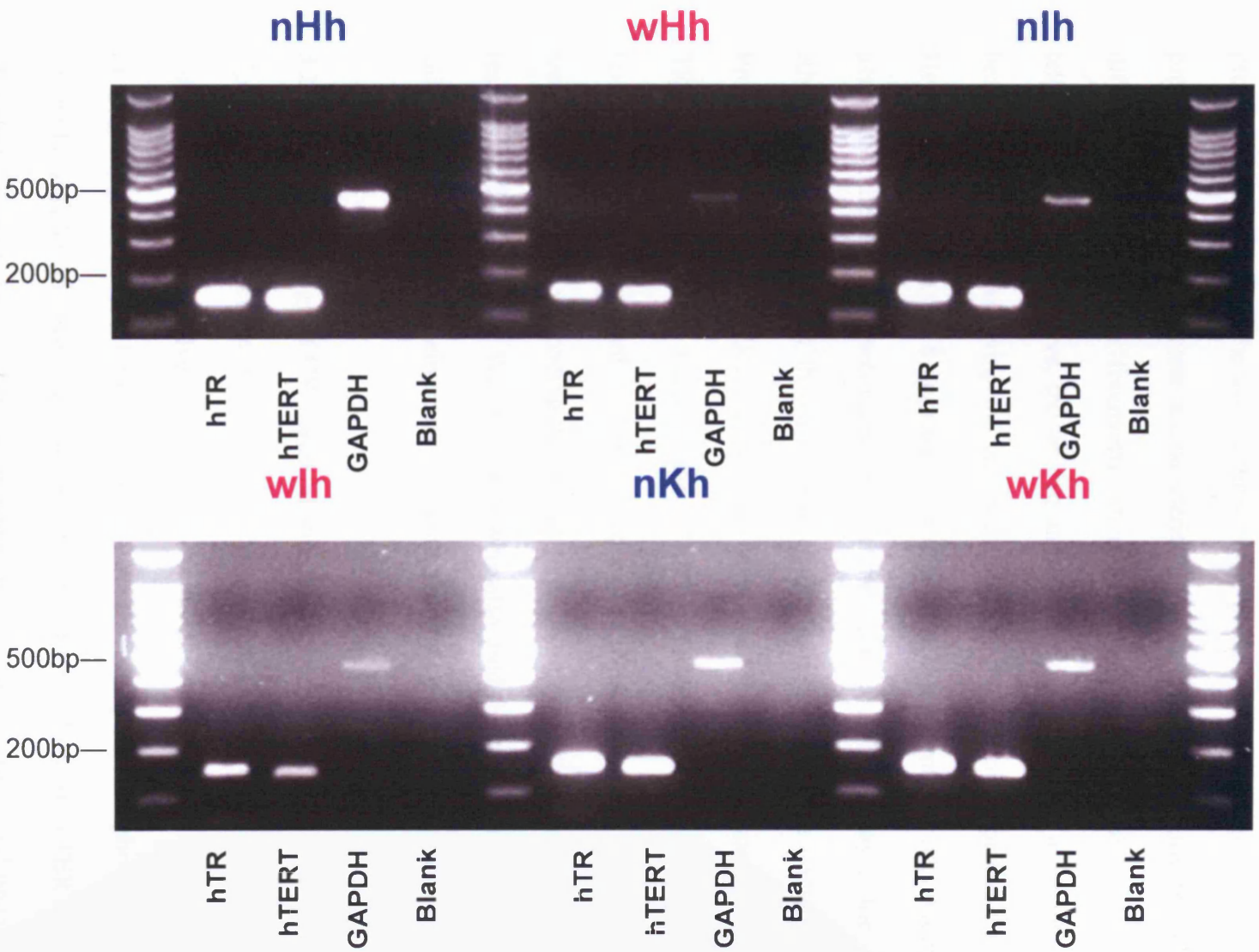


Figure 3.4: RT-PCR for hTR, hTERT and GAPDH expression. Expression of the RNA (hTR) and the protein (hTERT) components of Telomerase was confirmed in all 6 immortalised cell lines by RT-PCR Products of 170bp for hTR, 150bp for hTERT and 500bp for GAPDH were seen in each patient.

3.2.3 Telomerase activity in CWF and NF cell lines

To detect the presence of active telomerase the Telomere Repeat Amplification Protocol (TRAP) assay was utilised. Active telomerase will add 6bp repeats to the primer templates within the assay solution. The presence of active telomerase within protein extracts will generate a characteristic 'ladder' of DNA fragments with 6bp differences between the fragments when visualised by electrophoresis. Active telomerase is heat sensitive, the protein and RNA components will dissociate when heated above 80°C removing the ability to add 6bp repeats to the primer template. Heat inactivation acts as a negative control helping to confirm that it is telomerase generating the ladder, a second potential control for the TRAP assay is the use of RNase to digest the hTR this was not used due to fears of RNase contamination. Protein extracts from hTERT infected cells were tested for active telomerase using the TRAP assay (Fig 3.5). Each of the hTERT infected cell lines showed active telomerase as demonstrated by a DNA ladder. The DNA ladder was not observed in samples heat treated suggesting that a heat sensitive enzyme such as telomerase was responsible for the ladder. Water controls were also used to demonstrate that the DNA ladder was due to the protein extract and not an artefact of the TRAP assay.

3.2.4 Cell Morphology of CWF and NF cell lines

Upon reaching senescence fibroblast morphology changes dramatically, they become enlarged and take on a polygonal appearance with stress fibres visible within the cells. In contrast proliferating fibroblasts have a stretched bi-polar morphology and are generally smaller in size. To confirm that the morphology of hTERT infected fibroblasts did not change over an extended time in culture bright field images were taken of recently passaged cells at three timepoints in culture. As the different patient

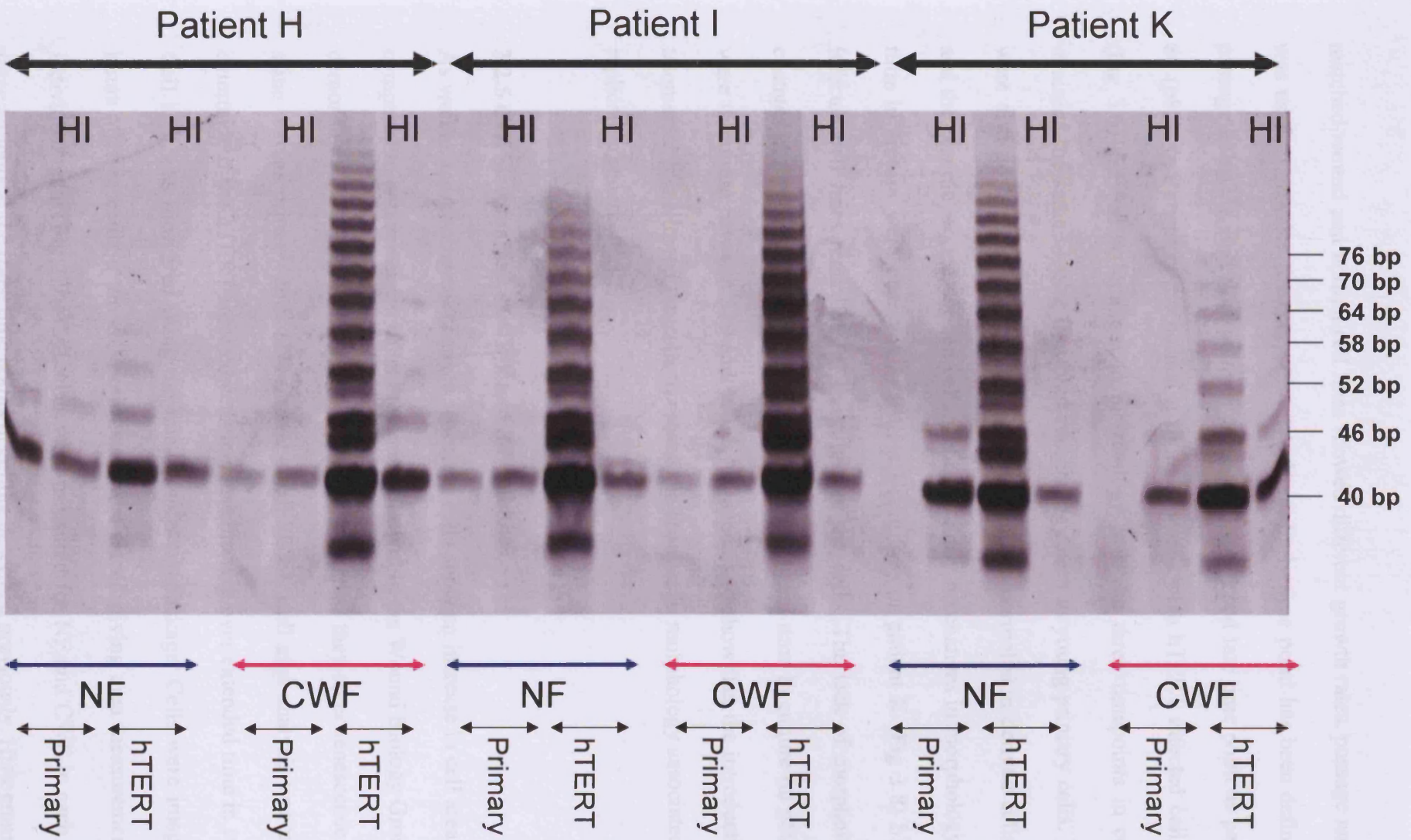
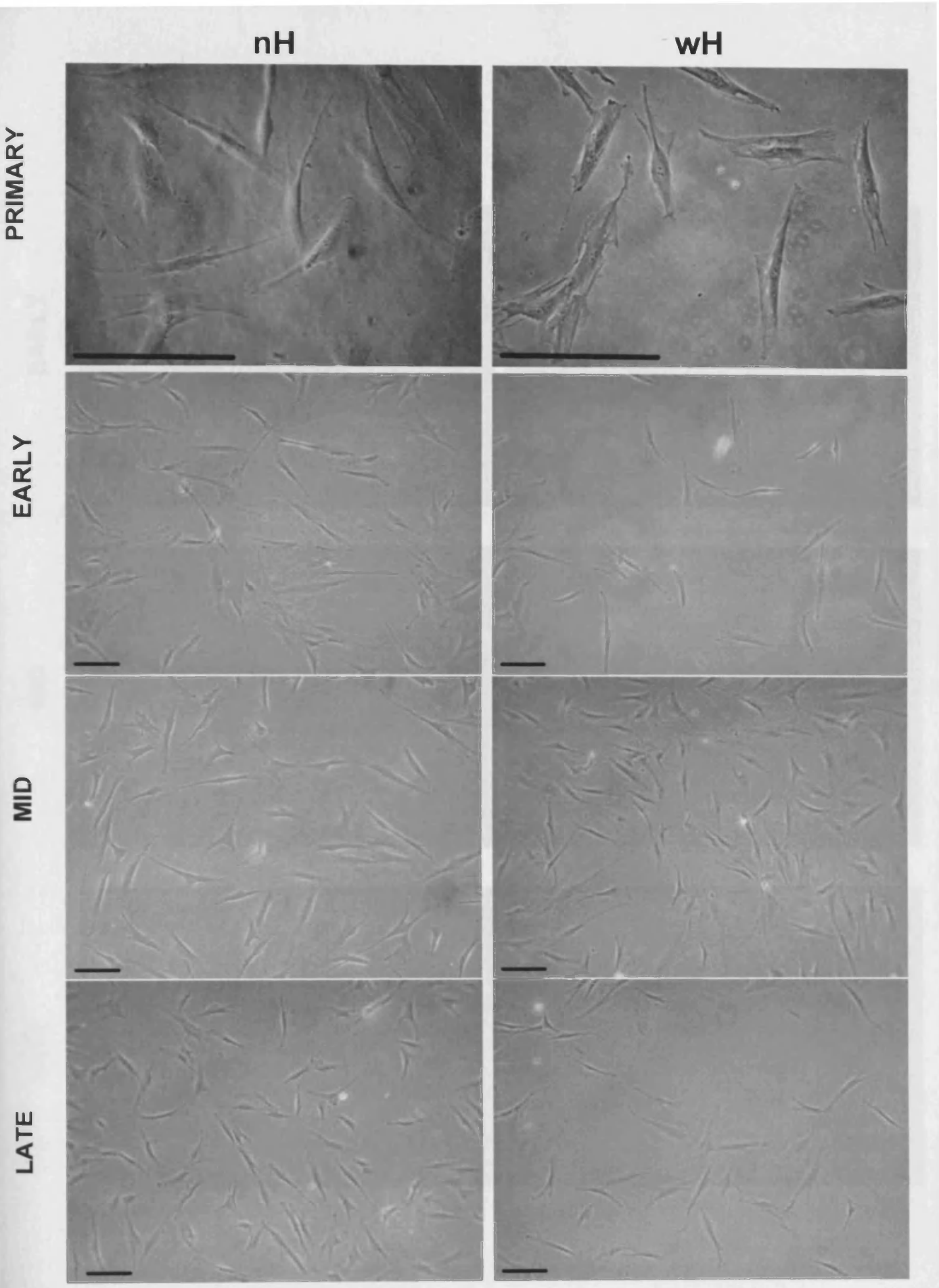


Figure 3.5: Telomerase Activity Assay (TRAP) comparing primary cells with hTERT immortalised cell lines. Active telomerase gives a 6bp incremental ladder which is not present after heat inactivation (HI).

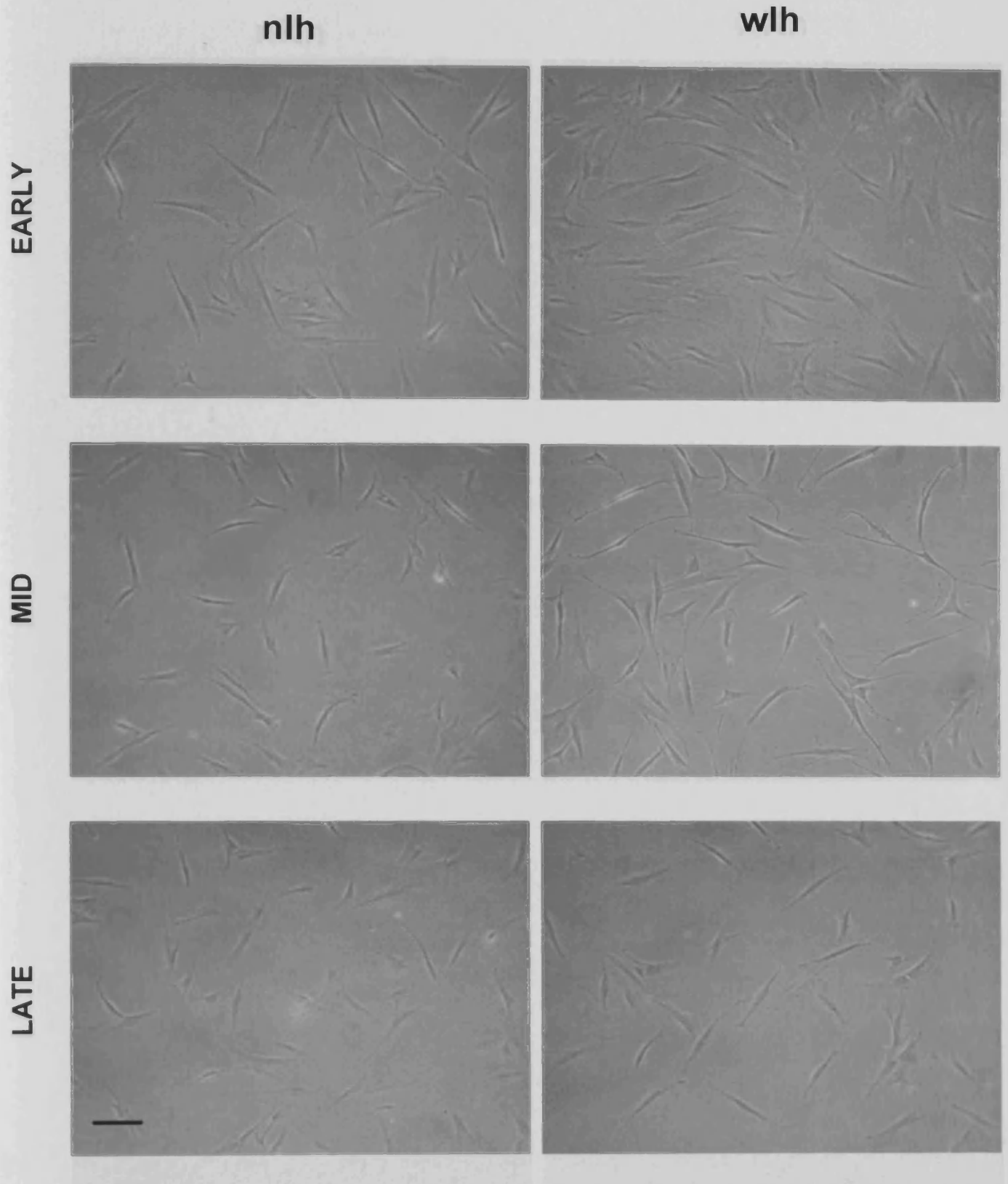
matched wound and normal cell lines showed different growth rates, passage number was used to describe the time point in culture. Early time point has been defined as passage 23 (p23), mid time point as passage 50 (p50) and late time point as passage 80 (p80). In Patient H the normal nHh and chronic wHh hTERT infected cell lines (Fig 3.6) showed no changes in morphology over the three timepoints in culture retaining the same bipolar fibroblast morphology seen in young primary cells. There were also no morphological differences between the normal skin derived cells nHh and the chronic wound derived cells wHh. Similarly no changes in morphology over time in culture were observed in patient I (Fig 3.7) or patient K (Fig 3.8) hTERT infected cell lines regardless of the origin of the cells. The lack of morphological changes in the hTERT infected cells even over extended time in culture (at p80 cells were in culture between 320 and 460 days), appears to show that the introduction of telomerase has allowed the cells to escape the changes in morphology associated with replicative senescence.

3.2.5 Cell Size analysis of CWF and NF cell lines

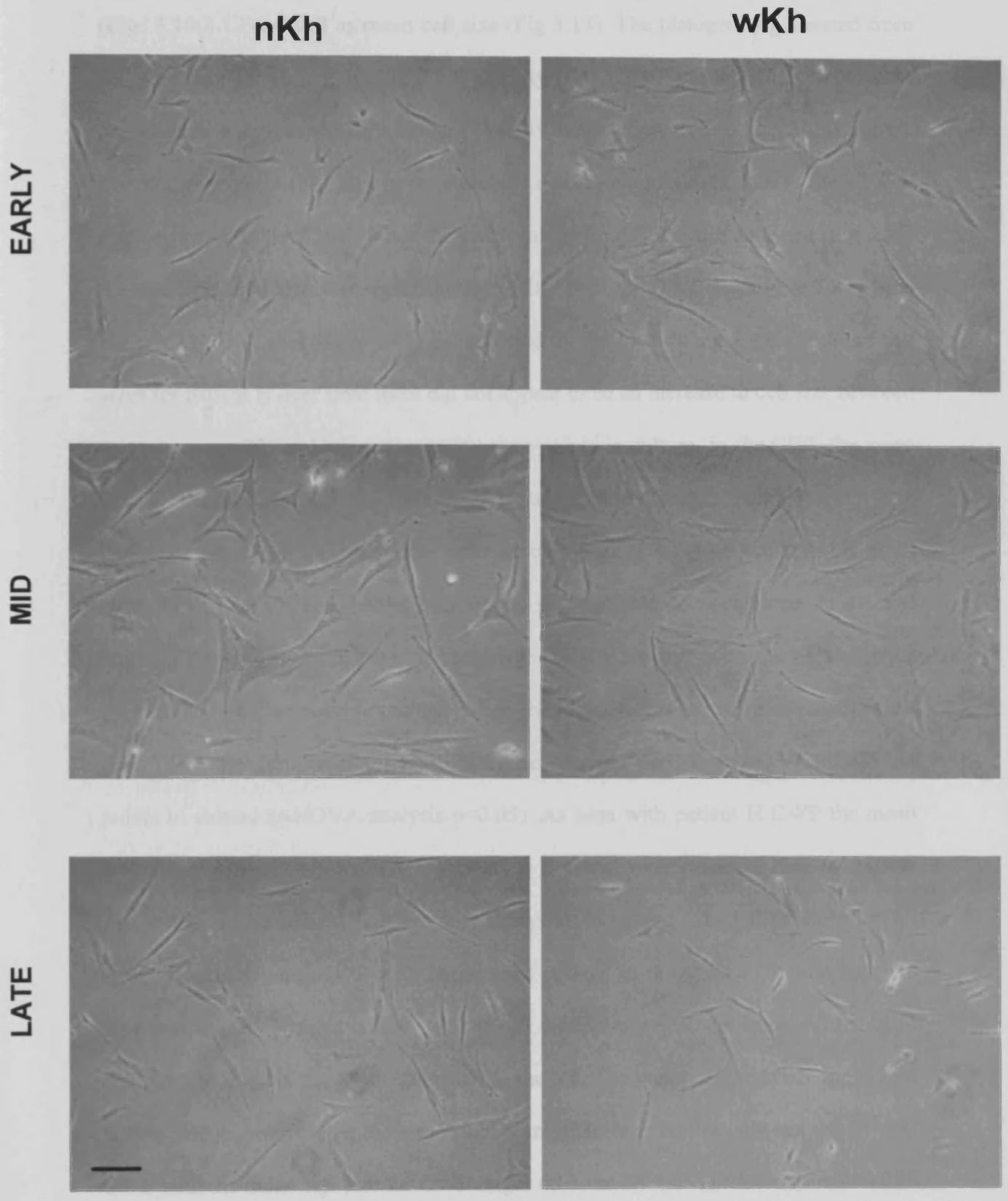
As well as morphological changes senescent cells show an increase in cell area when compared to non senescent cells. Previous work within the Wound Biology Group has demonstrated that mock infected cells increase in size as they enter senescence in the same way as primary cells (Thesis Ivan Wall 2006). Cell area analysis was used to determine if the hTERT infected cells increased in size over extended time in culture. Cell area was measured using the ImageJ software package. Cells were imaged 24 hours after reseeding, the cell perimeters were traced giving area measurements for individual cells (Fig 3.9). Cell areas were measured for NF and CWF in each of the three patients at the three time points in culture described previously. Histograms of



gure 3.6: Cellular Morphology Patient H at Early (p23), Mid (p50) and Late (p80) time points in culture for normal skin (NF) and chronic wound (CWF) derived hTERT infected fibroblasts. Also included are images of primary NF and CWF cells (Scale Bar = 100μm)



ure 3.7: Cellular Morphology Patient I at Early (p23), Mid (p50) and Late (p80) time points in culture for normal skin (NF) and chronic wound (CWF) derived hTERT infected fibroblasts. (Scale Bar = 100μm)



ure 3.8: Cellular Morphology Patient K at Early (p23), Mid (p50) and Late (p80) time points in culture for normal skin (NF) and chronic wound (CWF) derived hTERT infected fibroblasts. (Scale bar = 100μm)

cell size in mm^2 against percentage of cell population were generated for each patient (Figs 3.10-3.12) as well as mean cell size (Fig 3.13). The histograms generated from cell size data for patient H (Fig 3.10) at early (Fig 3.10a) and late (Fig 3.10c) time points show a shift to the right in the CWF histogram compared to the NF histogram. The mid time point (Fig 3.10 b) for patient H demonstrated a right shift in the NF cell size compared to the CWF. When the mean values for cell size are analysed (Fig 3.13) the mean NF cell size was significantly smaller than the CWF at early and late time points (ANOVA analysis $p<0.05$) as indicated by the histograms. Examining the cell sizes for patient H over time there did not appear to be an increase in cell size between early (passage 23) and late (passage 80) time points in culture. In the CWF the mean cell size appears to decrease over time in culture. The histogram showing cell size data for patient I at the early time point in culture (Fig 3.11) demonstrated a small right shift in the CWF cell sizes indicating a slight difference in cell size. At the mid and late time points in culture the same right shift is evident with the greatest shift seen in the late time point in culture. When the mean values for cell size are analysed (Fig 3.13) they show a significant difference between the NF and CWF at all time points in culture (ANOVA analysis $p<0.05$). As seen with patient H CWF the mean cell size in patient I NF and CWF appears to decrease over extended time in culture. The histogram for patient K at the early timepoint in culture (Fig 3.12) a spread of all sizes in both NF and CWF with larger cells present in the CWF. The mid and late time points demonstrated a more typical cell distribution with the same right shift in cell size observed in the CWF compared to the NF. The mean cell size data for patient K (Fig 3.13) demonstrated the same significant difference in size between the NF and CWF with the mean cell size for CWF larger than the NF (ANOVA analysis $p<0.05$). The same decrease in cell size over extended time in culture was

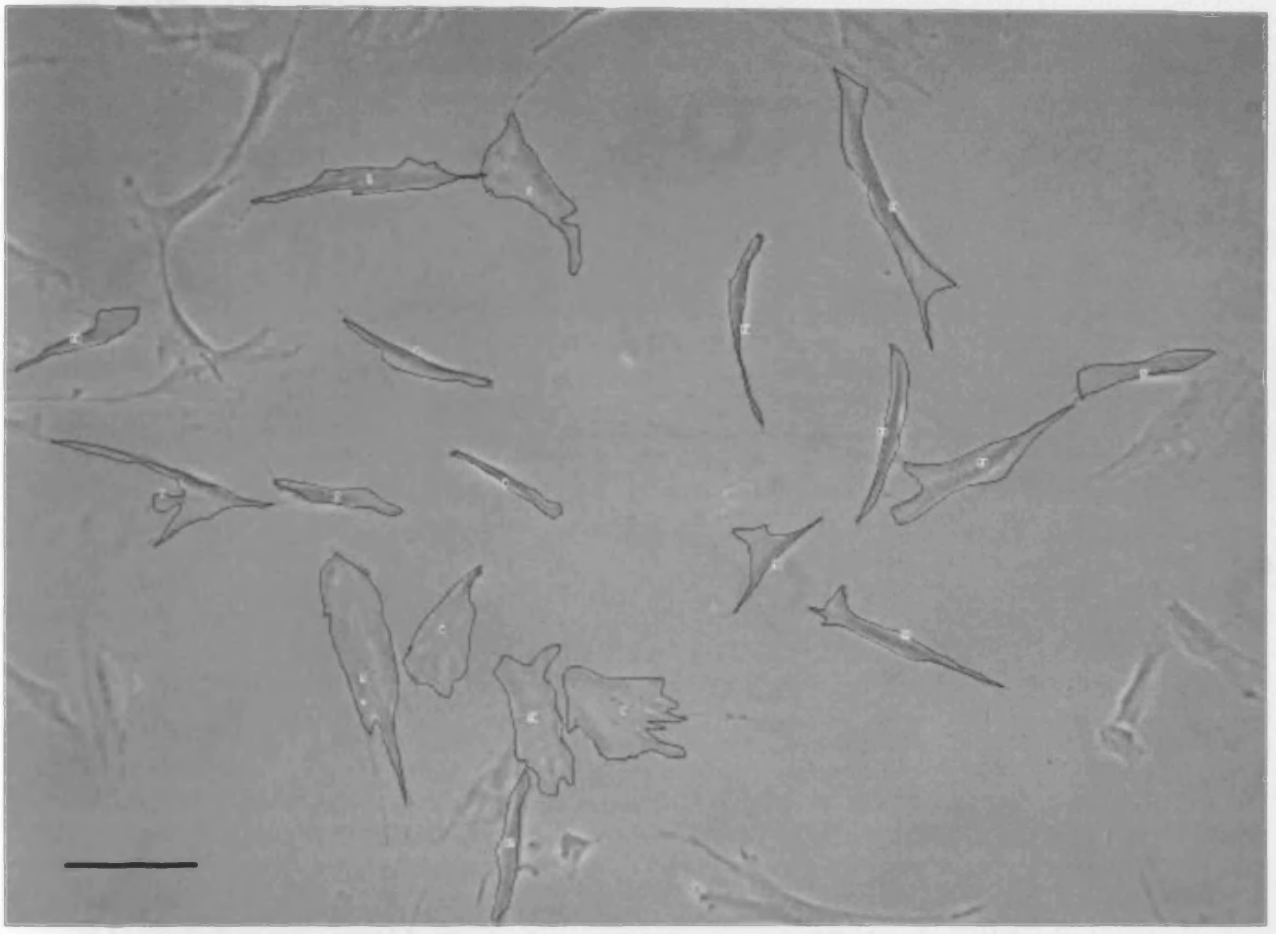


Figure 3.9: Cell size analysis was carried out on the ImageJ software package. Cell perimeter was traced and cell area was calculated based on scale calibration taken from a haemocytometer grid. Counted cells were marked and numbered to prevent repeated measurement. (Scale Bar 100μm)

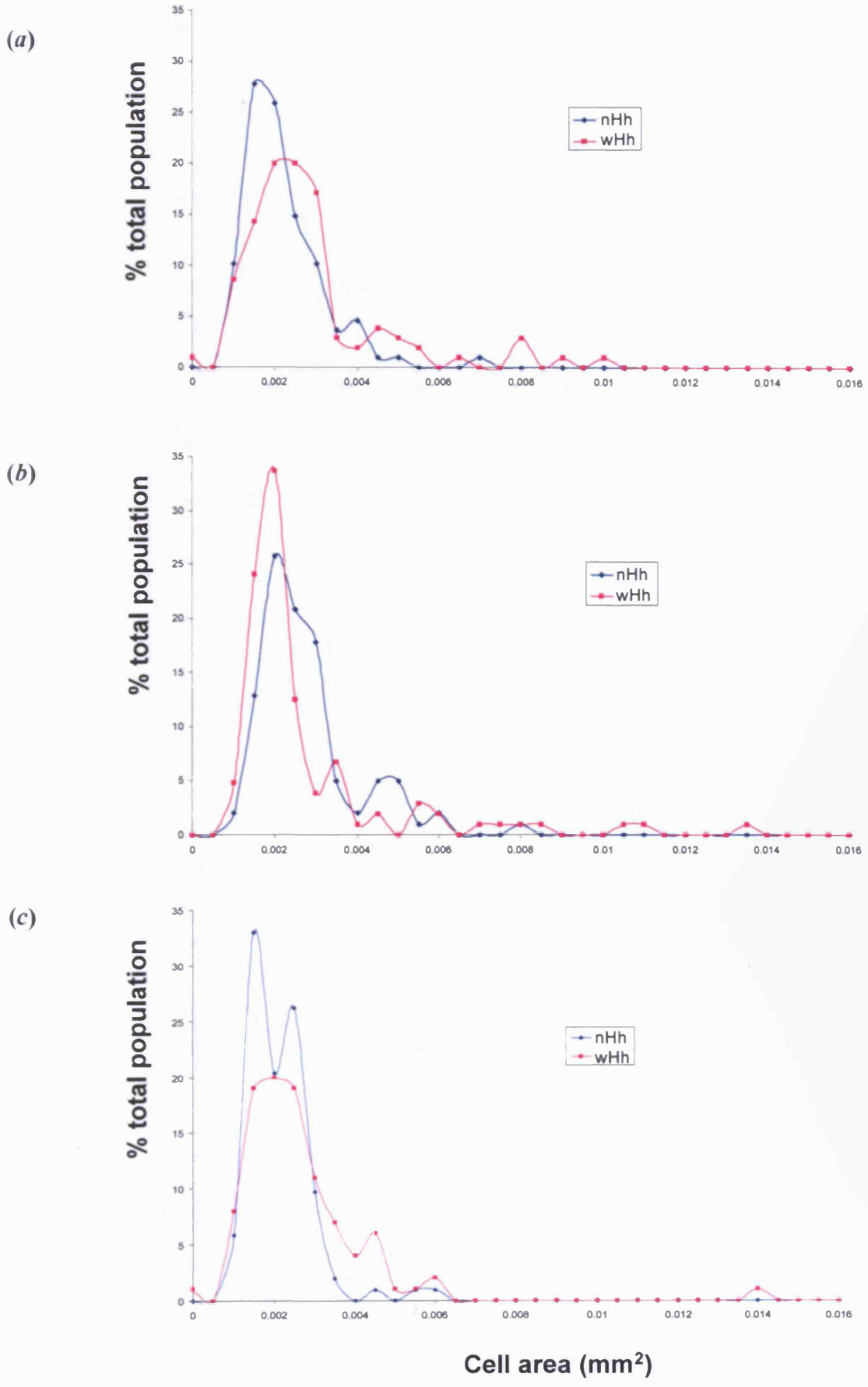


Figure 3.10: Histograms showing distribution of cell area in NF (-) and CWF (-) cell lines from patient H at (*a*) early, (*b*) mid and (*c*) late time point in culture.

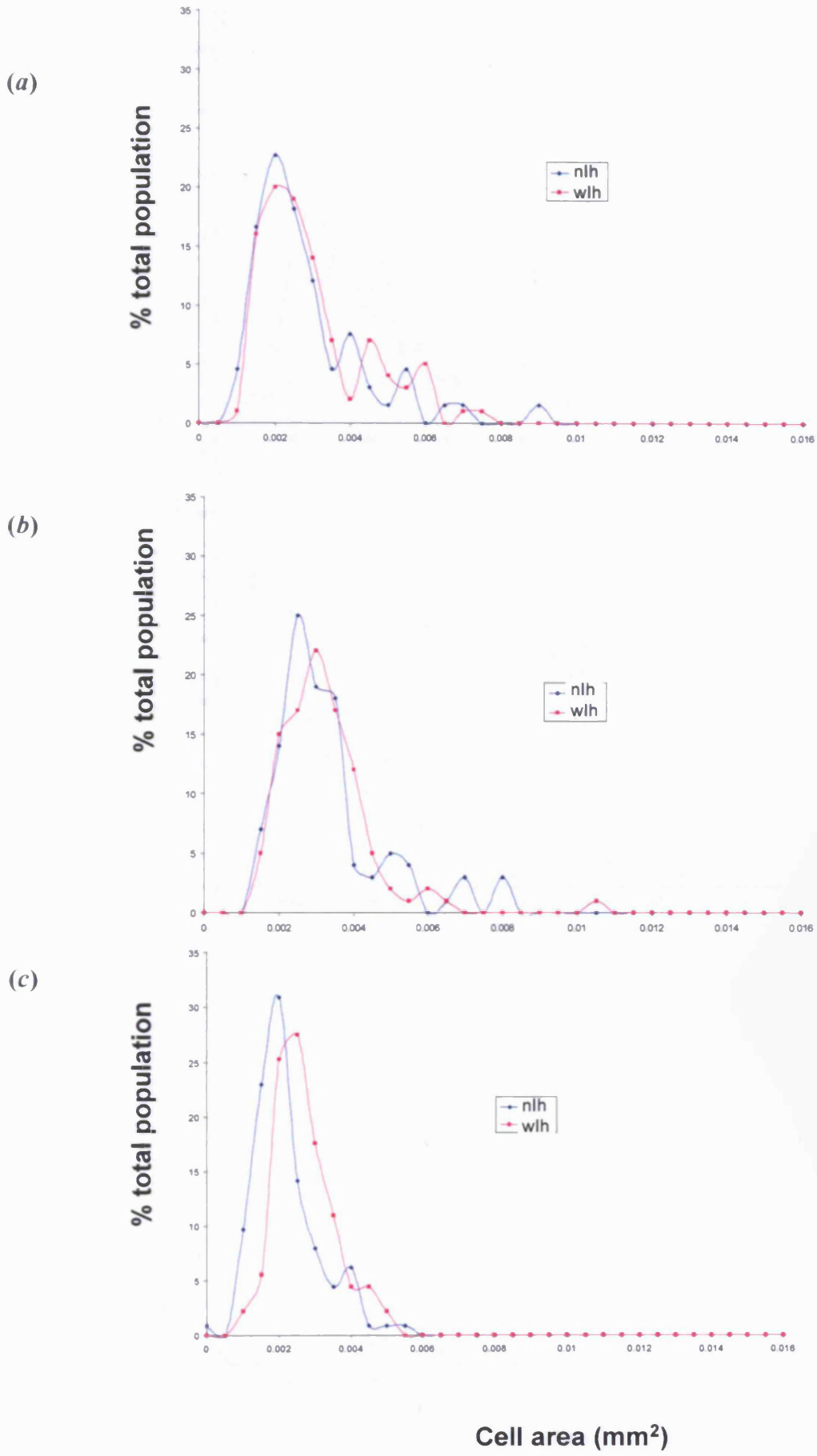


Figure 3.11: Histograms showing distribution of cell area in NF (-) and CWF (-) cell lines from patient I at (*a*) early, (*b*) mid and (*c*) late time point in culture.

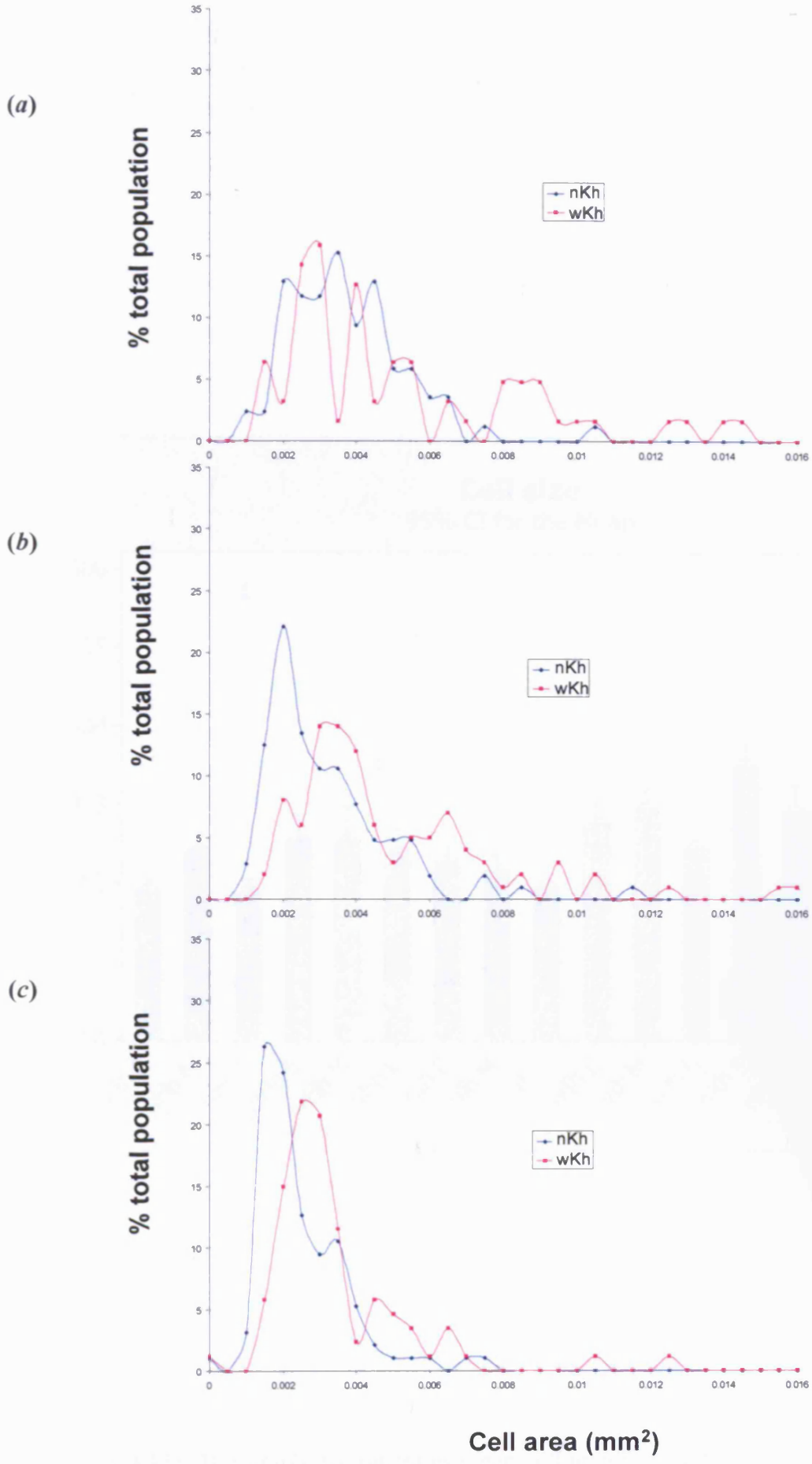


Figure 3.12: Histograms showing distribution of cell area in NF (-) and CWF (-) cell lines from patient K at (***a***) early, (***b***) mid and (***c***) late time point in culture.

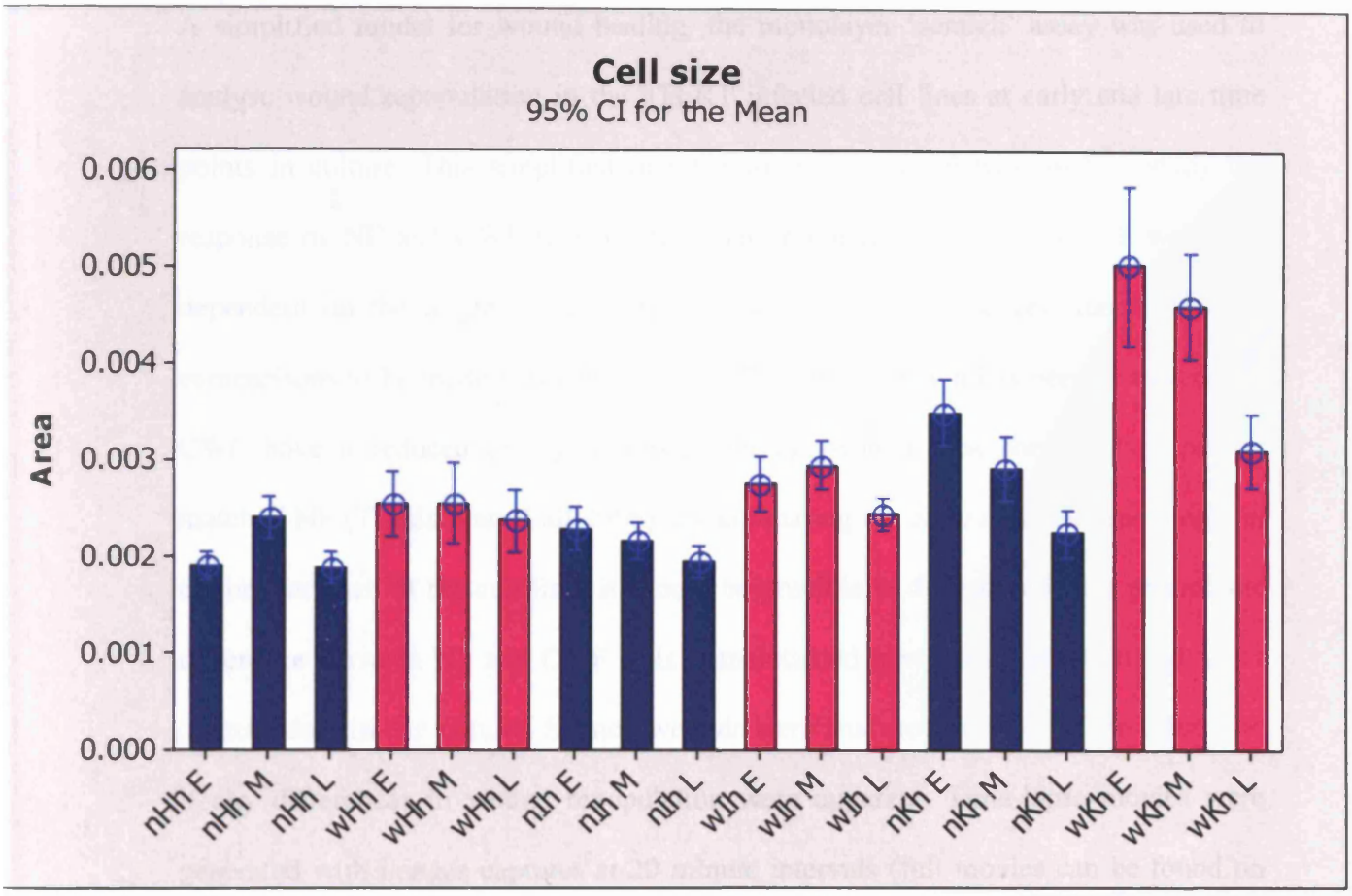


Figure 3.13: Bar graph demonstrates mean cell area in populations of NF and CWF from all three patients at three time point in culture. ANOVA analysis has shown a significant difference in size between NF and CWF.

observed in patient K in both NF and CWF. Cell size analysis showed that the hTERT infected cell lines for all three patients did not increase in size over extended time in culture. The mean cell sizes and histograms of cell size distribution demonstrated that the CWF cells were larger than the NF from the same patient and that this size difference was maintained over extended time in culture.

3.2.6 Repopulation of a monolayer scratch wound by CWF and NF cell lines

A simplified model for wound healing, the monolayer 'scratch' assay was used to analyse wound repopulation in the hTERT infected cell lines at early and late time points in culture. This simplified *in vitro* wounding model was used to study the response of NF and CWF to wounding. The repopulation of the scratch wound is dependent on the migratory and proliferative potential of the cells used allowing comparisons to be made between NF and CWF. Previously it has been observed that CWF have a reduced ability to repopulate the wound area compared to patient matched NF (Thesis Ivan Wall 2006). By comparing the early and late time points in culture for each of the cell lines it should be possible to determine if this phenotypic difference between NF and CWF cells is maintained post immortalisation and after an extended time in culture. Scratch wounds were analysed in two ways to determine if any differences in wound repopulation were apparent. Time-lapse movies were generated with images captures at 20 minute intervals (full movies can be found on the supplemental DVD); for comparison 6 time points were used to generate a montage for each wounding experiment. It should be noted however, that the wounds generated were not generally uniform with some differences in starting size altering the apparent rate of wound closure. The imageJ software package was used to determine wound area over the course of the experiment allowing for a comparison of wound repopulation rates over time.

Patient H NF hTERT infected cells had successfully repopulated the scratch wound between 18 hour and 24 hours post wounding at both the early (Fig 3.14) and late (3.15) time points in culture. In comparison the CWF hTERT infected cells failed to completely repopulate the scratch wound by 24 hours at the early time point in culture (Fig 3.14) and by 30 hours at the late timepoint in culture (Fig 3.15). The normalised wound repopulation data (Fig 3.16) demonstrated the differences between NF and CWF wound repopulation rates are maintained after an extended time in culture. At the early time point in culture patient I NF hTERT infected cells (Fig 3.17) repopulate the wound area between 18 and 24 hours at the late timepoint (Fig 3.18) they repopulate the wound area between 24 and 30 hours. The CWF hTERT infected cells repopulated the wound area by 30 hours at the early timepoint (Fig 3.17) and after 30 hours at the late timepoint (Fig 3.18). The normalised wound area data (Fig 3.19) demonstrated the same delayed wound repopulation by the CWF compared to the normal at the early and late time points in culture however, the difference between the NF and CWF cells appears to be less than was seen in patient H demonstrating some heterogeneity in patient response.

At the early time point in culture patient K NF hTERT infected cells repopulated the wound area between 18 and 24 hours (Fig 3.20) the CWF hTERT infected cells failed to repopulate the wound area within the 30 hours recorded in the montage taking almost 40 hours to repopulate the wound. The late time point in culture initially indicated a reversal of the wound repopulation phenotype with the CWF hTERT infected cells repopulating the wound area between 18 and 24 hours (Fig 3.21) and the NF hTERT infected cells repopulated the wound area between 24 and 30 hours. The normalised wound size data for the late time point in culture scratch wounds (Fig 3.22 b) demonstrated that when initial wound area is taken into account the CWF appear to

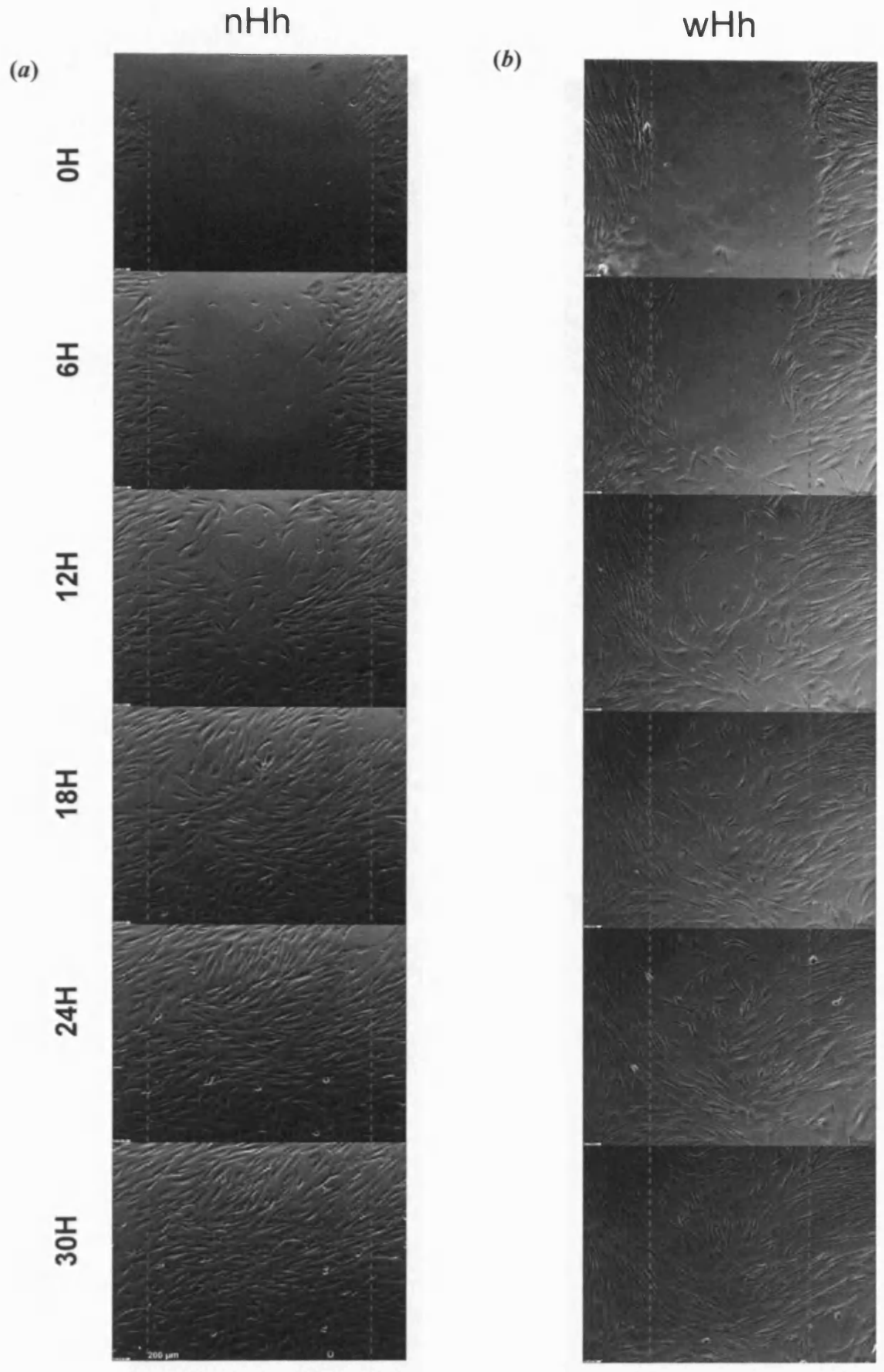


Figure 3.14: Scratch Wound montages for Patient H at Early time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies

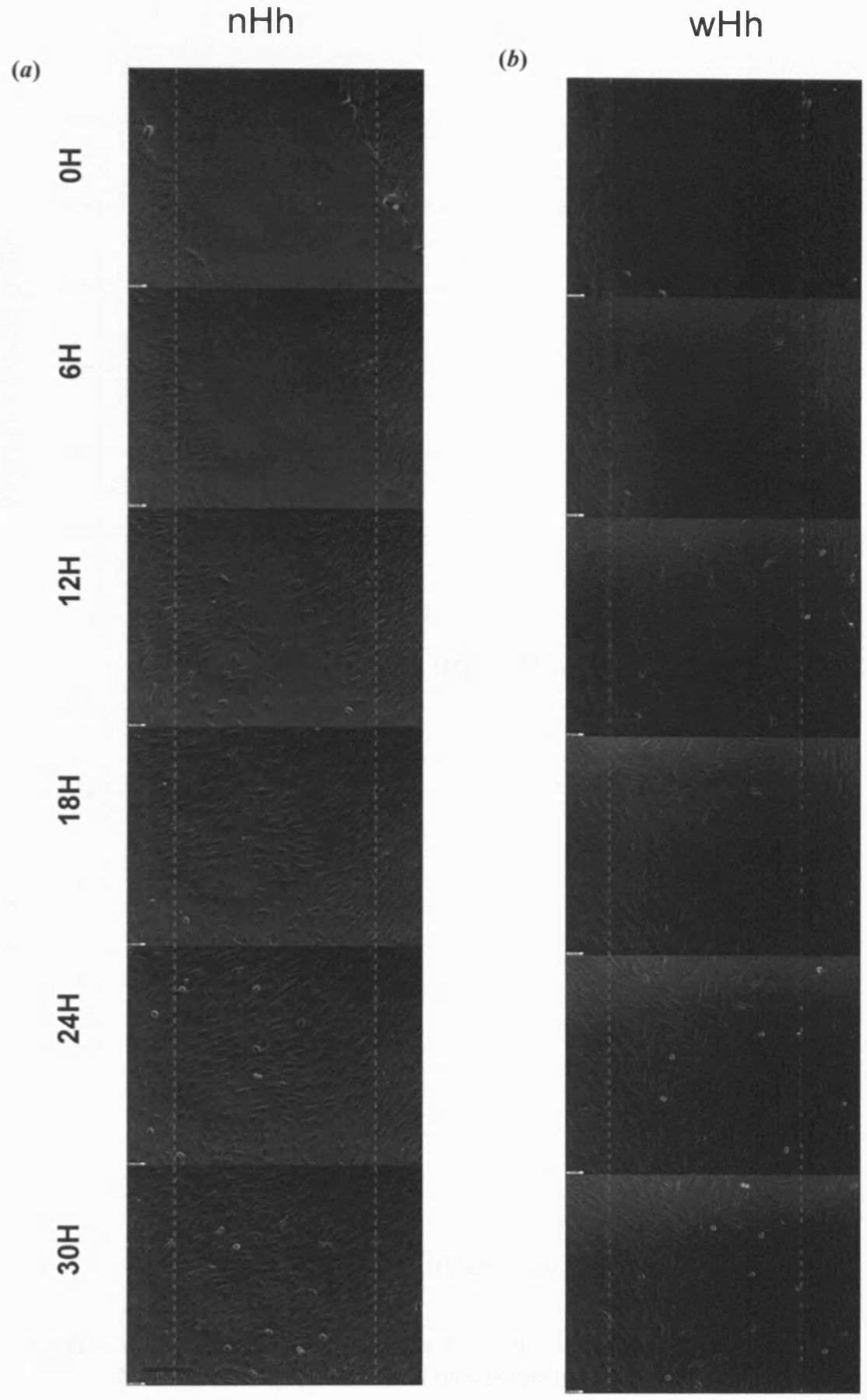


Figure 3.15: Scratch Wound montages for Patient H at Late time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies.

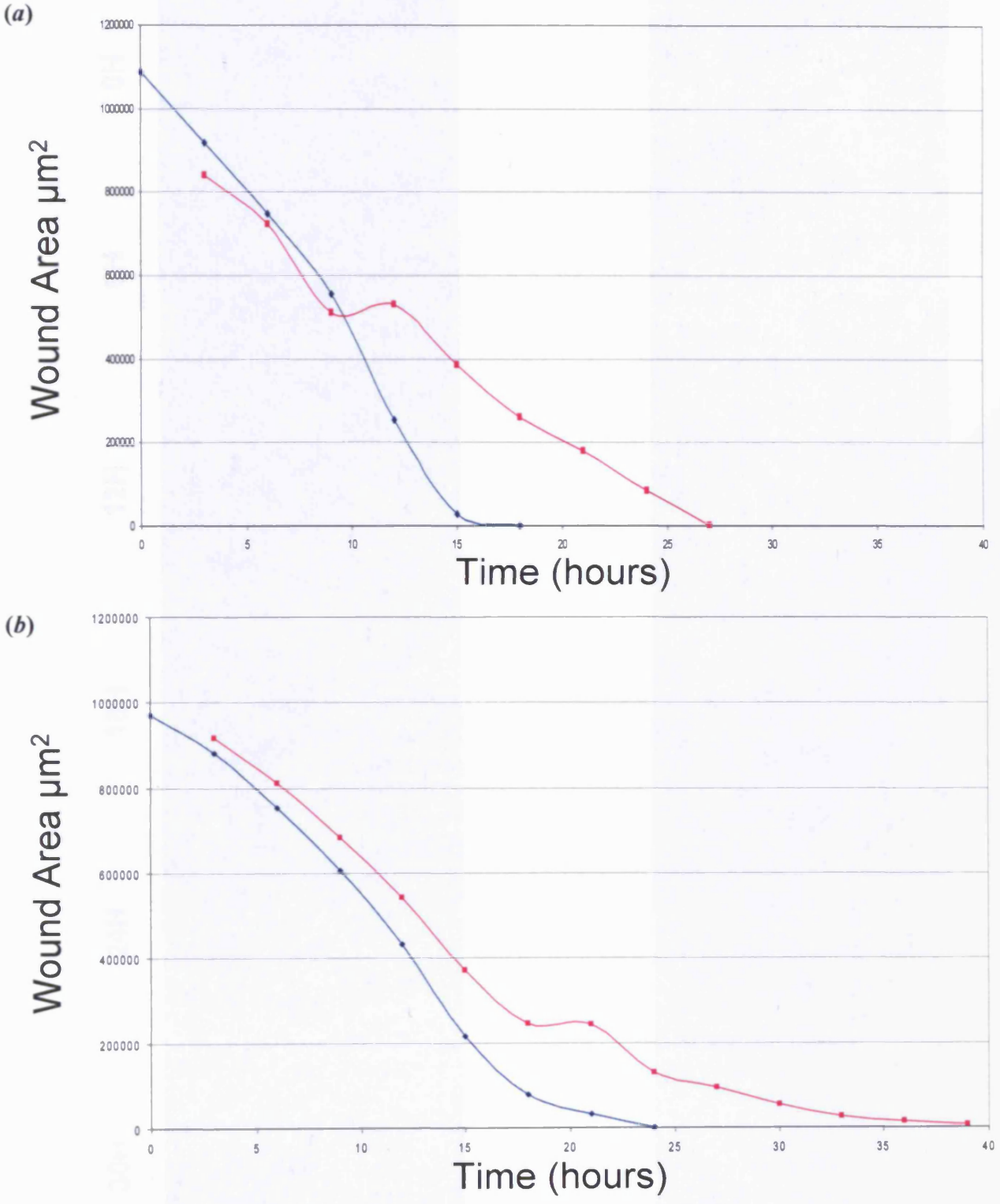


Figure 3.16: Wound Closure data for Patient H, hTERT immortalised cell lines CWF (---) and NF (---) at Early a) and Late b) time points in culture.

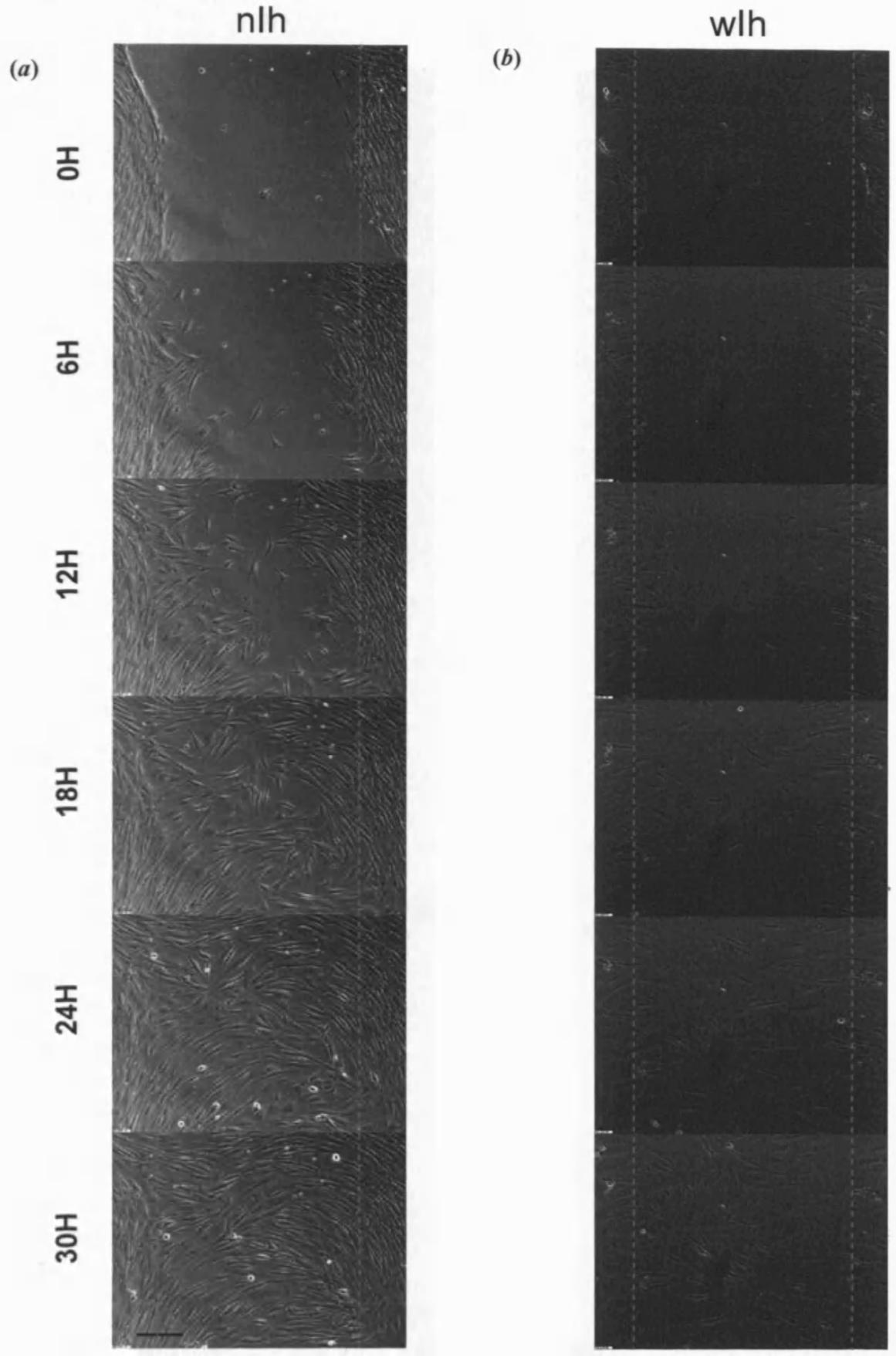


Figure 3.17: Scratch Wound montages for Patient I at Early time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies.

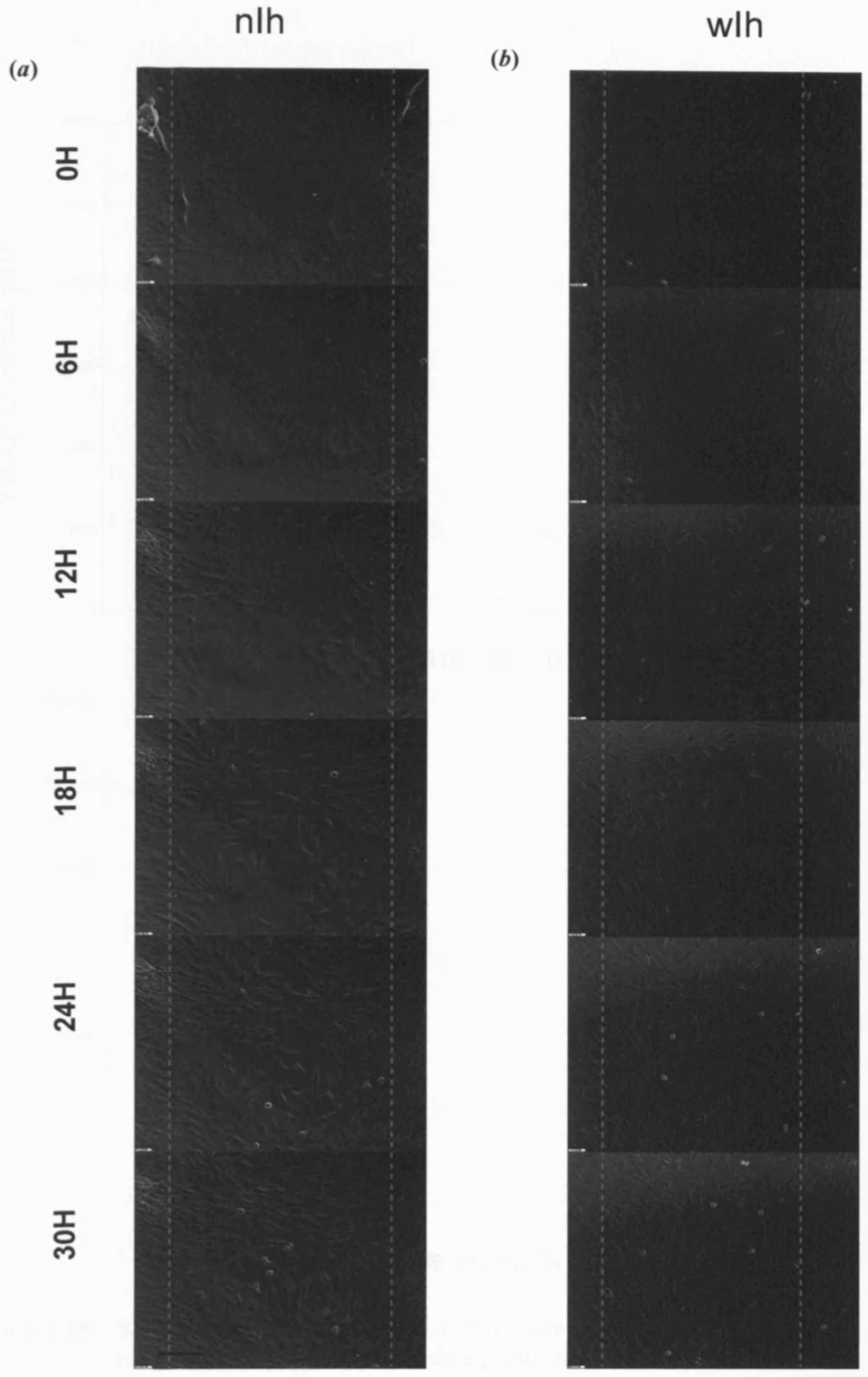


Figure 3.18: Scratch Wound montages for Patient I at Late time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies.

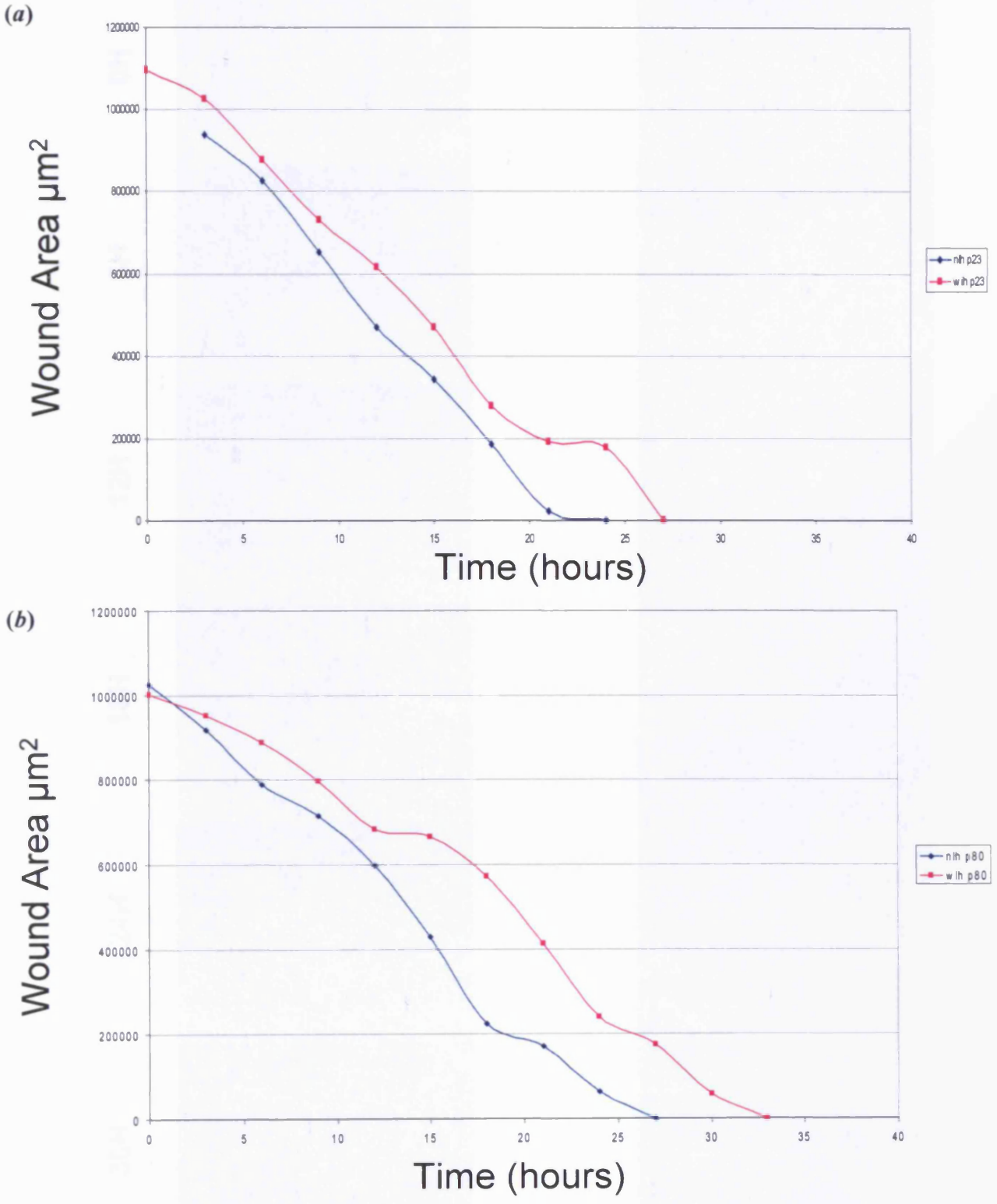


Figure 3.19: Wound Closure data for Patient I, hTERT immortalised cell lines CWF (---) and NF (---) at Early a) and Late b) time points in culture.

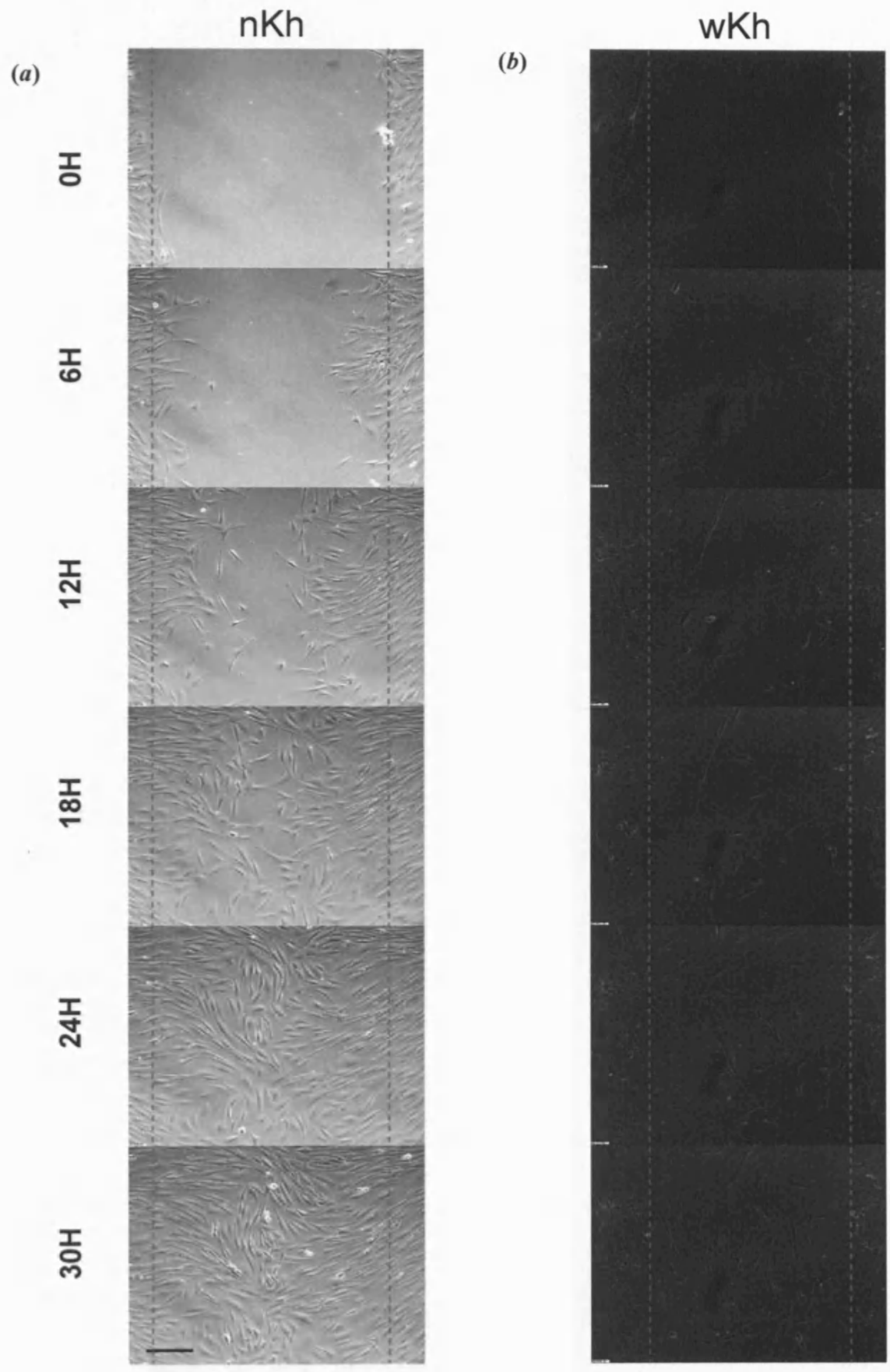


Figure 3.20: Scratch Wound montages for Patient K at Early time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies

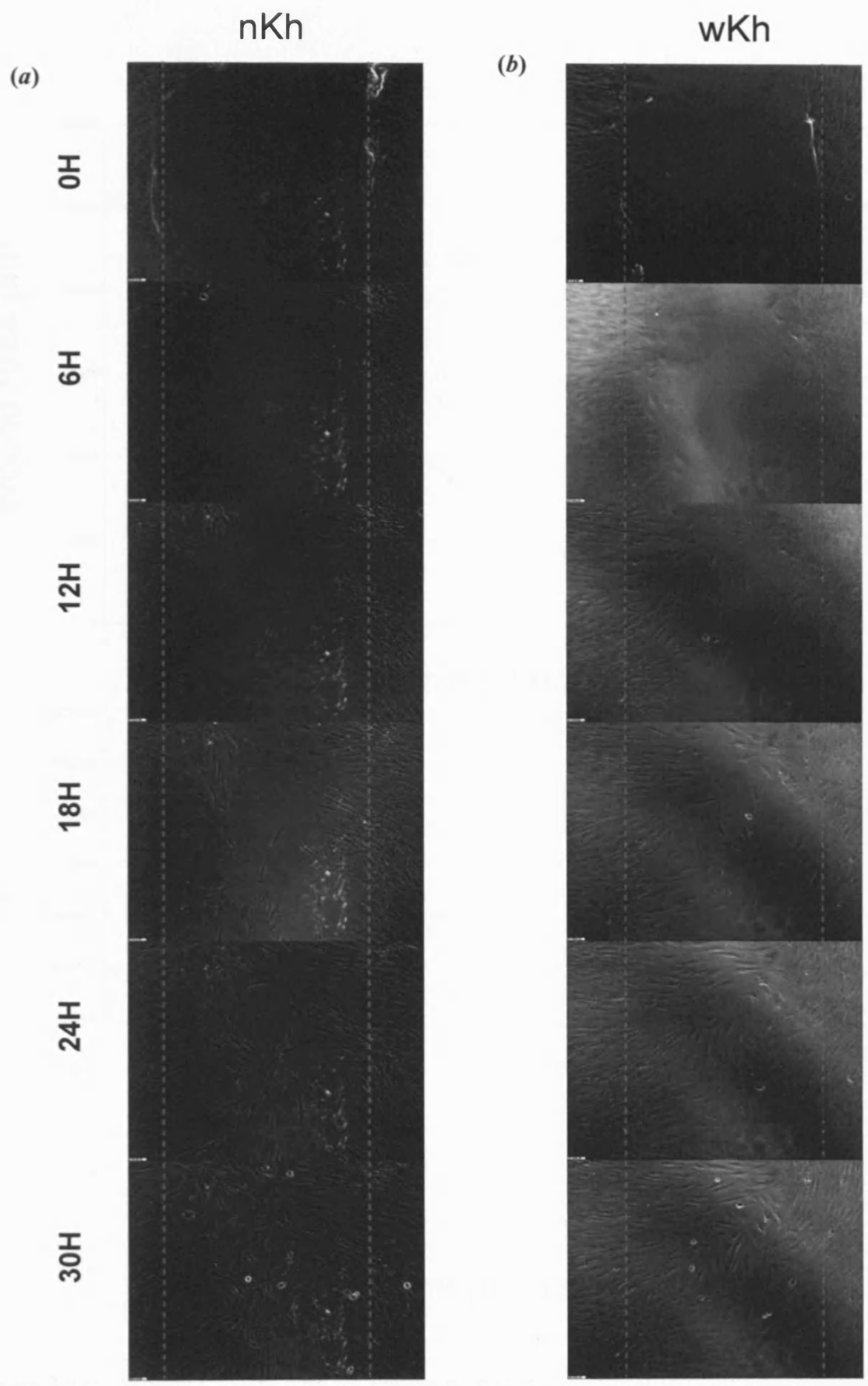


Figure 3.21: Scratch Wound montages for Patient K at Late time point in culture, a) NF b) CWF (Scale Bar = 200μm). Movies available on supplemental DVD under Timelapse Movies.

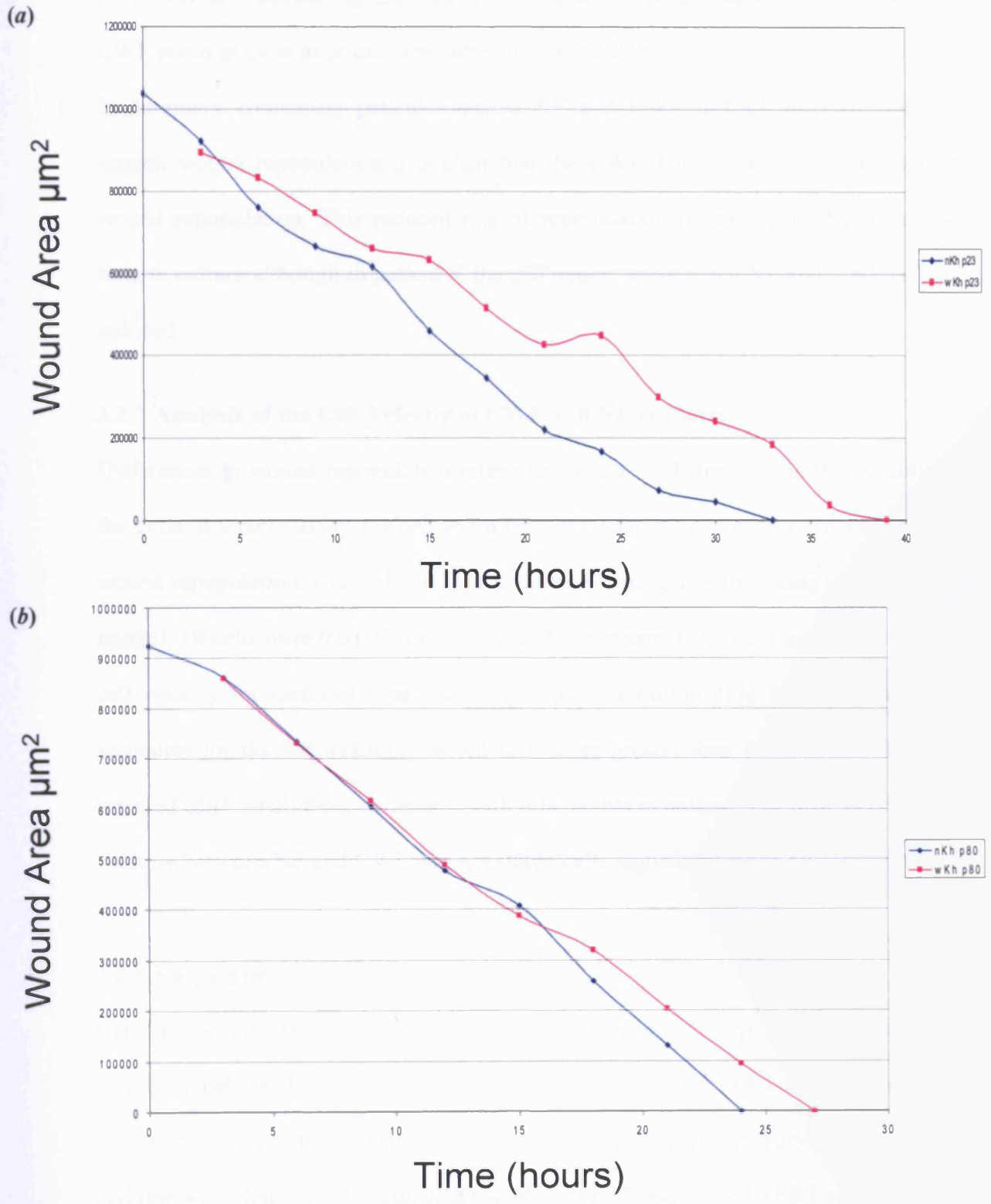


Figure 3.22: Wound Closure data for Patient K, hTERT immortalised cell lines CWF (---) and NF (---) at Early a) and Late b) time points in culture.

be slower at repopulating the wound area however the difference between NF and CWF is not as clear as at the early timepoint (Fig 3.22 a).

In summary comparing patient matched NF and CWF hTERT infected cells by scratch wound repopulation it is clear that the CWF demonstrate a reduced rate of wound repopulation. This reduced rate of repopulation is maintined after extended time in culture although in patient K the difference between the NF and CWF cells is reduced.

3.2.7 Analysis of the Cell Velocity of CWF and NF cell lines

Differences in wound repopulation rates may be due to differences in the motility of the cells, a simple assay for cell motility is the analysis of cellular velocity during wound repopulation. Cell velocity was determined using a cell tracking plugin within imageJ, 10 cells were tracked for each wound experiment (Fig 3.23) giving an average cell velocity for each cell type at each time point in culture (Fig 3.24). Average cell velocities for the NF hTERT infected cells were greater than for the CWF hTERT infected cells for all three patients at both time points in culture. The difference in cell velocity between NF and CWF was not statistically significant when taken together.

3.2.8 Karyotype

Cell lines in culture for extended periods of time have been reported to demonstrate chromosomal instability. To determine if the CWF and NF hTERT immortalised cell lines were chromosomally stable after extended periods in culture karyotypes for each cell line were determined. Cytogenetic analysis of NF and CWF hTERT infected cells was carried out at the West Midlands Regional Genetics Laboratory. Cells at passage 80 were analysed for gross chromosomal abnormalities, these cells had been continually cultured for more than a year and therefore the most likely to have

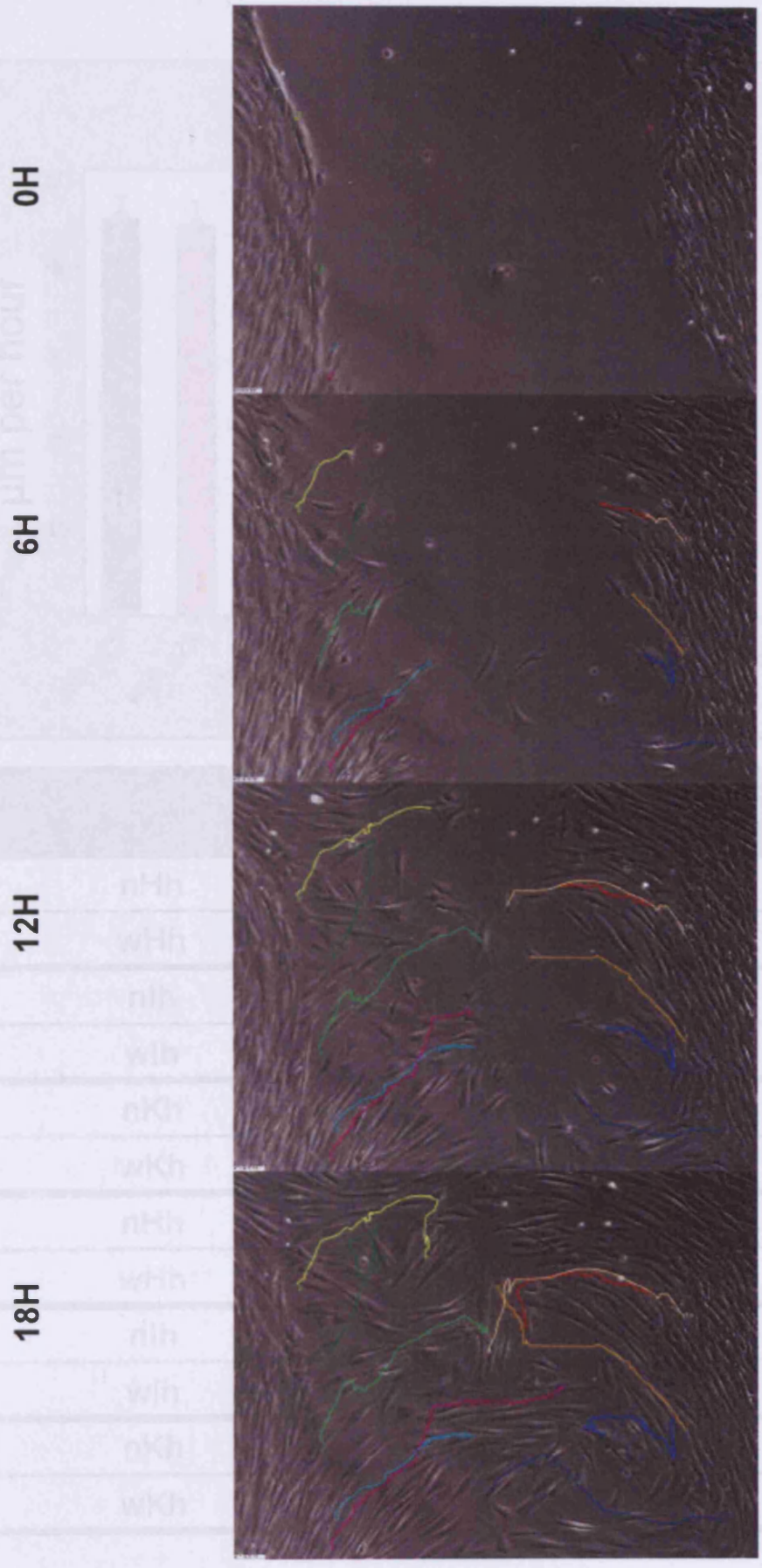
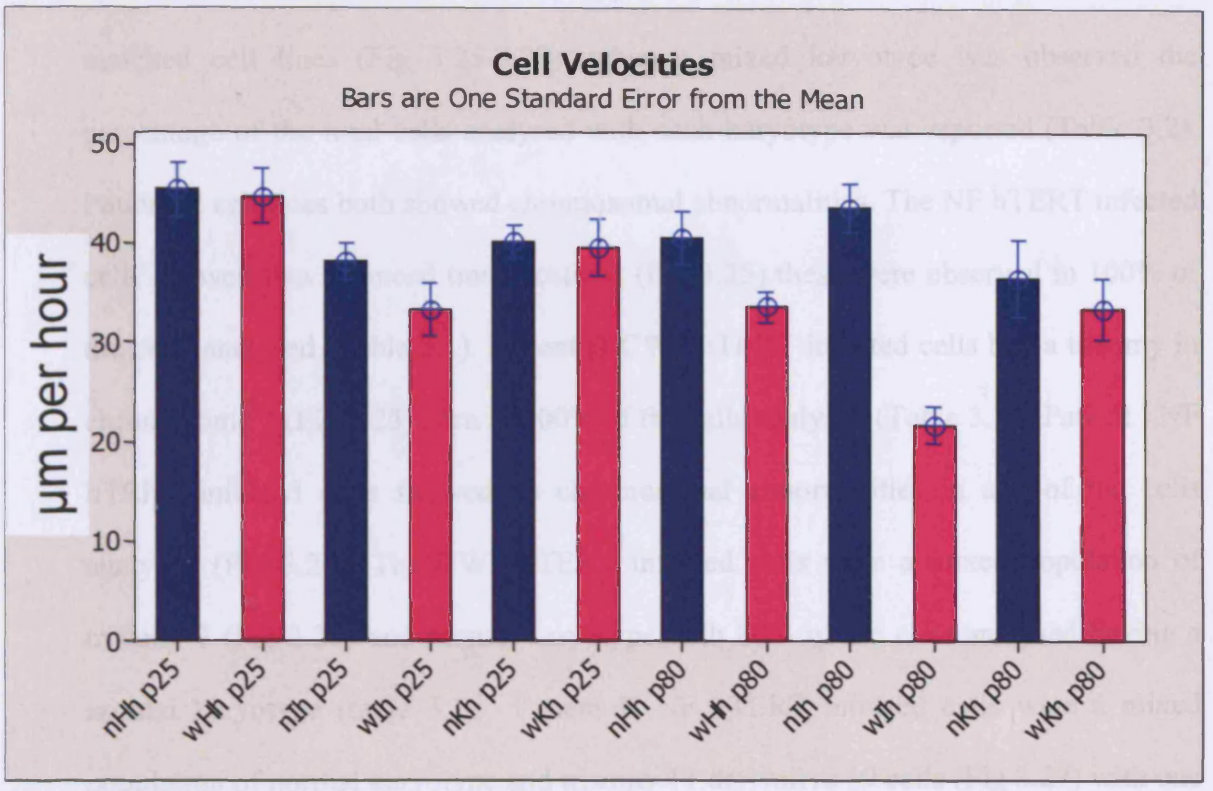


Figure 3.23: Scratch Wound montages showing cell tracking. 10 cells were tracked recording position and distance travelled allowing for calculations of cell velocity..



Cell Line	Passage	Average Cell Velocity µm/hour
nHh	P25	45.5
wHh	p25	44.9
nIh	p25	38.3
wIh	p25	33.4
nKh	p25	40.3
wKh	p25	39.7
nHh	p80	40.7
wHh	p80	33.7
nIh	p80	43.8
wIh	p80	21.7
nKh	p80	36.6
wKh	p80	33.6

Figure 3.24: Cell velocities for NF ■ and CWF ■ hTERT immortalised cells at Early (p25) and Late (p80) for all three patients.

chromosomal abnormalities. A karyotype was determined for each of the patient matched cell lines (Fig 3.25-3.27), when a mixed karyotype was observed the percentage of the total cells analysed with each karyotype was reported (Table 3.2). Patient H cell lines both showed chromosomal abnormalities. The NF hTERT infected cells showed two balanced translocations (Fig 3.25) these were observed in 100% of the cells analysed (Table 3.1). Patient H CWF hTERT infected cells had a trisomy in chromosome 7 (Fig 3.25) seen in 100% of the cells analysed (Table 3.1). Patient I NF hTERT infected cells showed no chromosomal abnormalities in any of the cells analysed (Fig 3.26). The CWF hTERT infected cells were a mixed population of trisomy 7 (Fig 3.26) and normal karyotype with 80% of the cells analysed having a normal karyotype (table 3.1). Patient K NF hTERT infected cells were a mixed population of normal karyotype and trisomy 11 derivative 19 cells (Fig 3.27) with one copy of chromosome 19 containing a large portion of the short arm of chromosome 7 in place of its own long arm making these cells partially trisomic for chromosome 7. 80% of the cells analysed had a normal karyotype (Table 3.1). The CWF hTERT infected cells all displayed a trisomy 7 karyotype (Table 3.1).

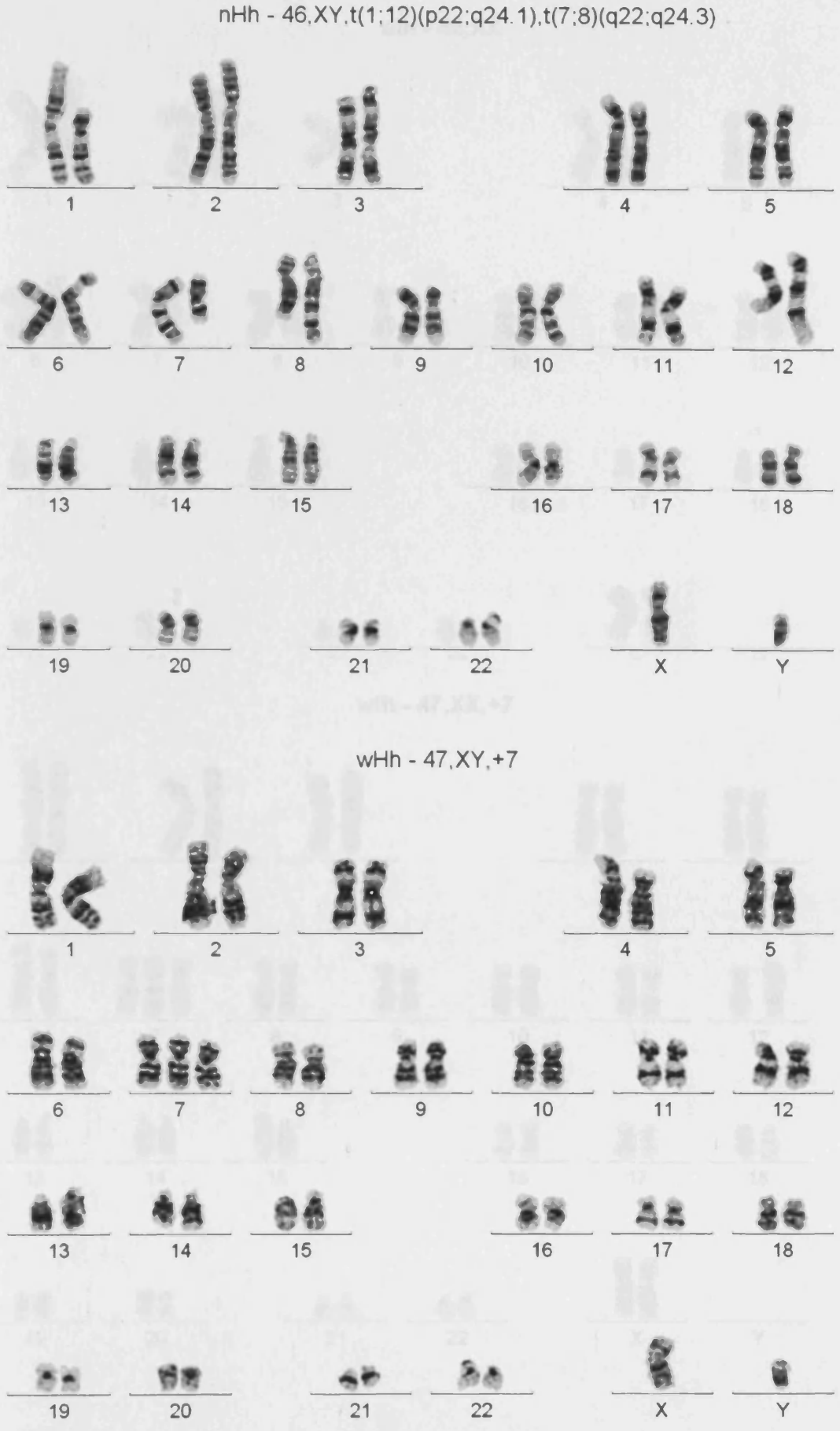


Figure 3.25: Karyotypes for Patient H showing examples of translocations in the NF cell line and trisomy 7 in the CWF cell line

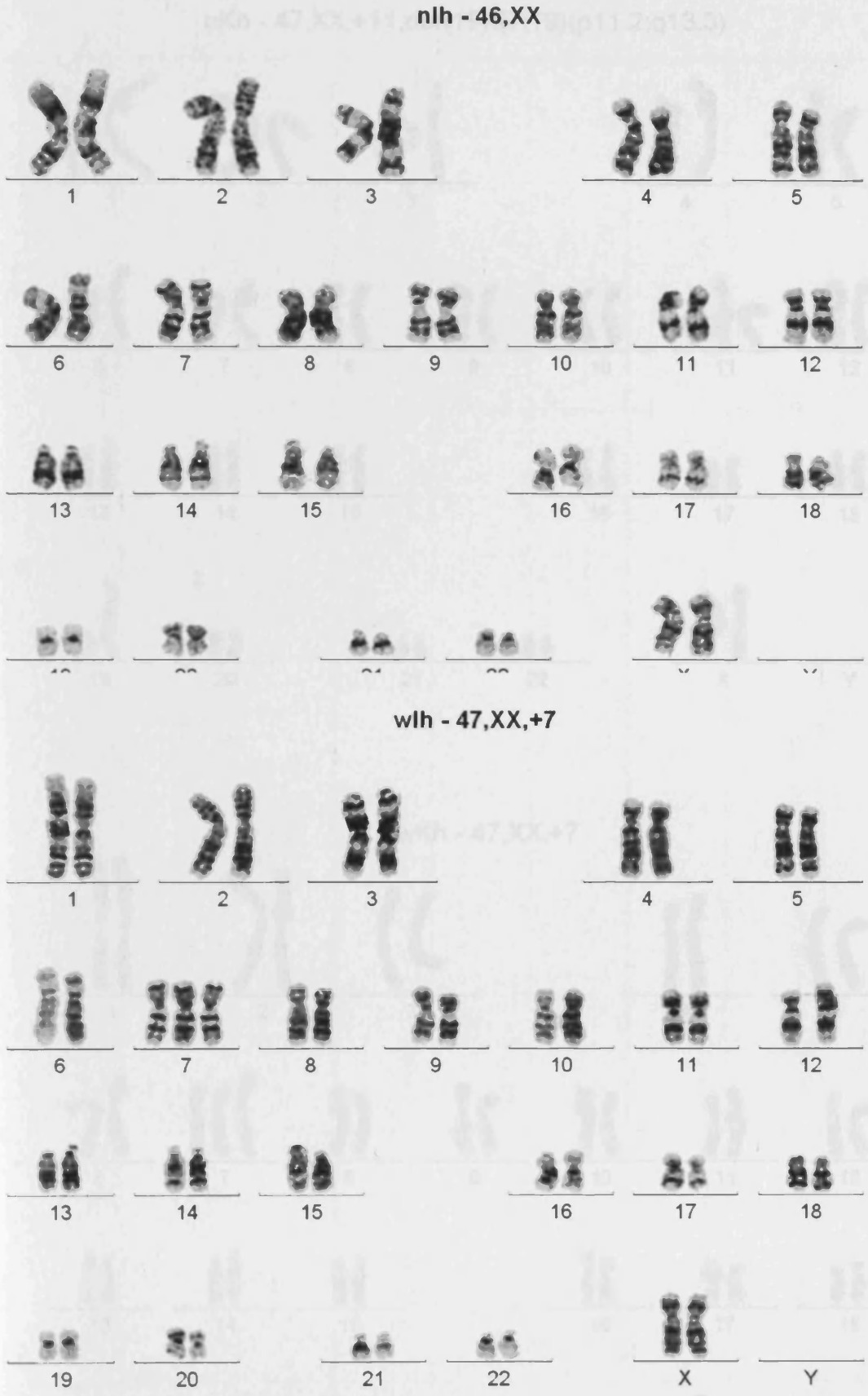


Figure 3.26: Karyotypes for Patient I showing normal chromosomes in the NF cell line and trisomy 7 in the CWF cell line

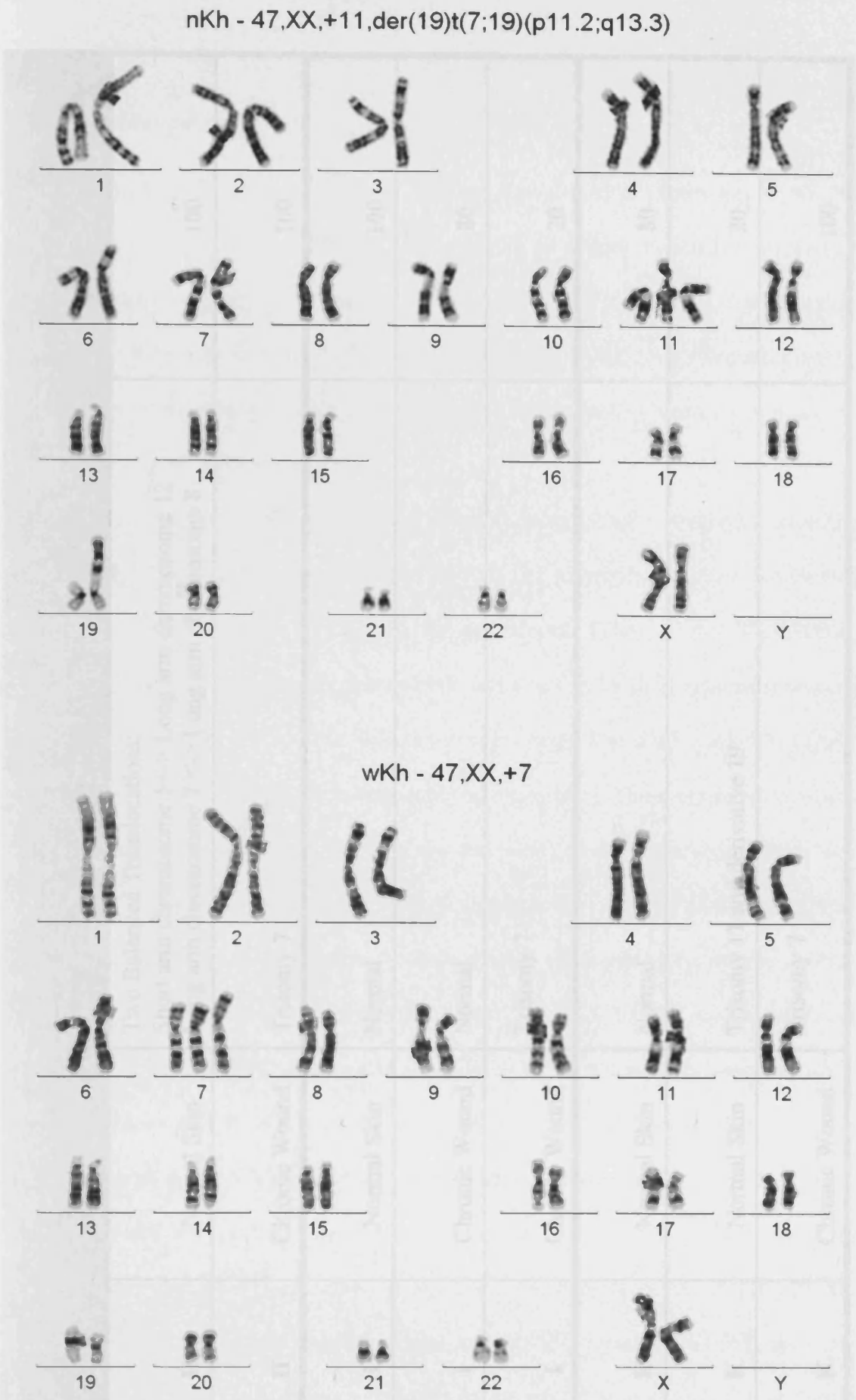


Figure 3.27: Karyotypes for Patient K showing trisomy 7 and derivative chromosome 19 in NF and trisomy 7 in CWF.

Patient	Type	Karyotype	Percentage of Cell Population
H	Normal Skin	Two Balanced Translocations: Short arm chromosome 1<-> Long arm chromosome 12 Long arm chromosome 7 <-> Long arm chromosome 8	100
H	Chronic Wound	Trisomy 7	100
I	Normal Skin	Normal	100
I	Chronic Wound	Normal	80
I	Chronic Wound	Trisomy 7	20
K	Normal Skin	Normal	80
K	Normal Skin	Trisomy 11 and derivative 19	20
K	Chronic Wound	Trisomy 7	100

Table 3.2: Table showing Karyotype of hTERT immortalised cell lines at p83

3.3 Discussion

The aims of this chapter were to confirm that the over expression of hTERT by retroviral infection permitted the CWF and NF to escape replicative senescence and investigate whether the phenotype of the CWF and NF remained distinct after long term culture. These were vital questions to answer if the cells were ultimately to be used as experimental models for the study of chronic wound healing and as a potential animal replacement system.

Patient matched CWF and NF were infected using either a retrovirus encoding the protein subunit of human telomerase (hTERT) or an empty retroviral vector (mock). Previous work within the Wound Biology Group (Thesis Ivan Wall 2006) had demonstrated that the mock infected NF and CWF cells both entered senescence at close to the same PD and non-infected primary cells. The CWF underwent fewer PD than the patient matched NF before entering senescence. These negative controls were important as they demonstrated that the retroviral 'mock' transfection alone was not sufficient for the cells to escape replicative senescence. The hTERT infected NF and CWF cells from each of the three patients studied all exceeded the number of PD seen in the primary and mock infected cells. All NF and CWF hTERT infected cells carried out more than 100 PD and can be considered to be successfully immortalised. The growth curves of the NF and CWF cell lines remained distinct after immortalisation in all three patients with the CWF showing a reduced rate of proliferation over the entire time in culture. Similar observations have been made in other hTERT immortalised fibroblast cell lines (Gorbunova et al., 2002, Gorbunova et al., 2003) with slower proliferation rates maintained after immortalisation. In patient H CWF cells showed two dips in their proliferation rate followed by a full recovery. hTERT immortalised fibroblasts have been shown to enter senescence after exposure to oxidative stress and

cultures of hTERT immortalised fibroblasts have been shown to contain subpopulations of senescent cells (Gorbunova et al., 2003). It is proposed that overexpression of hTERT can actually lead to cellular senescence in a similar manner to the overexpression of oncogenic Ras, Raf, and E2F1 (Swiggers et al., 2004). It is possible that in patient H a subset of the cells are expressing higher levels of telomerase and are therefore exiting the normal cell cycle and entering senescence. Further work on these cells would potentially confirm this hypothesis.

In order to confirm the hTERT infected cells were actually expressing telomerase and that the telomerase expressed was functionally active RT-PCR and TRAP experiments were carried out. The presence of RNA for hTERT confirmed by RT-PCR indicates that the retroviral transfection has been successful. The expression of RNA for hTR the RNA component of active telomerase in the infected cells demonstrates that the cells are able to generate active telomerase. However the presence of hTERT and hTR RNA has been shown to be insufficient evidence of hTERT activity and the succeaful immortalisation of the cells (Kim and Wu, 1997). The TRAP assay detects active telomerase in cell lysates (Hou et al., 2001), the presence of active telomerase is the best marker for successful transfection and subsequent immortalisation. All the hTERT infected cell lines were telomerase positive however, the technique used was not accurate enough to detect differences in levels of telomerase expression. A quantitative method for analysis of telomerase activity has been developed (Angello et al., 1989, Cristofalo and Pignolo, 1993, Greenberg et al., 1977) however, the presence or absence of telomerase activity was considered to be sufficient information for this study.

The final confirmation that the hTERT infected cells had escaped replicative senescence was the analysis of cell morphology and cell size. As fibroblasts age they

get larger (Gorbunova et al., 2003, Stephens et al., 2003, Stanley and Osler, 2001b) their morphology also shifts from the bipolar spindle shape associated with fibroblasts to a more flattened multi sided morphology. A further test for senescence associated with the increase in cell size and changes in morphology is the presence of SA β-galactosidase activity in the cells. Senescence associated β-galactosidase activity has been a widely used tool to quantify senescence within cell populations (Yang and Hu, 2005). However recent work has shown that it is possible to turn β-galactosidase activity on and off by simply controlling the environment the cells are exposed to (Gorbunova et al., 2003). Although under the right conditions β-galactosidase activity may still be used as a marker for senescence it is not definitive and for this reason was not used in this Thesis. Cell morphology did not appear to change over time in culture. At the late time point passage 80 the hTERT infected NF and CWF cells from all three patients were still small bipolar and spindle shaped. Cellular morphology did not appear to be visibly different between the NF and CWF cell lines however, the CWF cells did appear larger and therefore this observation lead to the analysis of cell size using the imageJ software package. Detailed cell size analysis demonstrated maintenance or in the case of patient K reduction in cell size over extended time in culture. However, the average cell size for Patient K NF and CWF cells over extended time in culture decreased bringing the average cell size closer to the average cell size seen in the other patients. This may indicate the presence of two populations of cells in cultures of in patient K NF and CWF cells one with larger and one with smaller cells. The histogram showing cell size for patient K (Fig 3.12) does appear to show multiple peaks at all time points in culture. The multiple peaks are reduced at the late timepoint in culture with the largest peak shifted to the left. This may be another effect of variable expression of hTERT leading to cells leaving the cell cycle (Wall et

al., 2008b). Indeed none of the hTERT infected cell lines were clonally selected and so they are therefore the result of expansion of a mixed population. As hTERT insertion into the genome is random it is possible that different levels of telomerase expression are linked with position of the insertion site.

Fibroblasts isolated from chronic wounds display a reduced ability to repopulate monolayer scratch wounds compared to patient matched normal fibroblasts (Liang et al., 2007). The monolayer scratch wound is a well utilised method for the study of wound repopulation *in vitro* (Raffetto et al., 2001c). Fibroblasts repopulate the scratch wound area through both proliferation and cellular migration mimicking their *in vivo* response to wounding. At the early time point after immortalisation CWF showed a reduced ability to repopulate the scratch wound compared to patient matched NF. This phenotype was retained at the late time point in culture across all three patients. The apparent reduction in the ability to repopulate a monolyer scratch wound may relate to the failure of chronic wounds to heal. For a wound to successfully heal fibroblasts must move into the wound where they are able to generate and remodel ECM. Without the fibroblast derived ECM re-epithelisation cannot occur and the wound will not heal.

Studies comparing the motility of CWF compared to NF have shown reduced motility in CWF (Stephens et al., 2004). To analyse cell motility cell velocity was also analysed, no significant differences were seen between the NF and CWF cells however, the CWF were generally slower than the patient matched NF cells. The observed reduced motility is not sufficient to explain the differences in wound repopulation rates. A possible explanation for the similarity in cell motility between NF and CWF but the difference in wound healing ability could be cell directionality. If the CWF cell movement was random rather than directed towards the wound area

they would show a reduction in the rate of wound repopulation. This effect has been seen in monolayer scratch wounds when extracellular matrix proteins were investigated (Jiang et al., 1999, Mondello et al., 2003).

The final analysis undertaken on the hTERT infected NF and CWF was an analyis of their karyotype. Chromosomal abnormalities are oftern seen in hTERT immortalised cell lines (Mondello et al., 1999). It was considered important to analyse the chromosomal stability of the hTERT infected cells as the intention was to continue to use these cells as a model for chronic wounds. Cells were karyotyped at passage 83 with many of the cell lines showing either a sub population or the entire population with chromosomal abnormalities. It has not been possible at this time to analyse the primary cells to determine if the abnormalities are due to the hTERT immortalisation or are seen in the original primary cells. The mixed nature of many of the cells karyotypes with a small population of the cells demonstrating an altered chromosome number or translocations suggests that the chromosomal alterations have been picked up over time in culture rather than being present at the point of transfection. All the CWF populations analysed showed some levels of trisomy 7, previously trisomy 7 has been observed in cultured cells taken from aged patients (Broberg et al., 2001). A further link between trisomy 7 and aging was demonstrated by the increase in trisomy 7 with age in certain tumours (Raimund W. Kinne et al., 2003). Trisomy 7 has also been observed in cells taken from patients with rheumatoid arthritis (Dahlén et al., 2001, Broberg et al., 1998) and osteoarthritis (Stanley and Osler, 2001b, Raffetto et al., 1999a, Mendez et al., 1998c, Fivenson et al., 1997) indicating a potential link with inflammation. Chronic wounds are sites of chronic inflammation and cells taken from them have the characteristics of aged cells (Stephens et al., 2003). The trisomy 7 seen

in the hTERT immortalised CWF could be due to the site from which the cells were taken rather than as a result of the hTERT transfection.

The data presented in this chapter demonstrates that the addition of hTERT to NF and CWF allows them to escape replicative senescence. The infected cells underwent more than 100PD, the cells express active telomerase and show no change in cell size or morphology with increased time in culture. Previous work within the Wound Biology Group suggested that hTERT immortalisation would not be sufficient to reverse the CWF phenotype (Ouellette et al., 2000) and the data from this chapter agrees with this suggestion. As observed in other disease states hTERT infected cells maintained their phenotype after transfection (Mogford et al., 2006). The CWF hTERT infected cells retained the reduced ability to proliferate and repopulate scratch wounds that was seen in the primary cell strains (Thesis Ivan Wall 2006). The addition of telomerase has been suggested as a potential treatment for chronic wounds (Brown and Botstein, 1999) based on data from animal models of ischemic wounds. The data presented in this chapter suggests that telomerase is not a potential treatment for chronic wounds however it can be used to generate cell lines to allow further study of the condition. Patient matched NF and CWF from three patients have been successfully immortalised through the addition of hTERT to the cells. The CWF have maintained some of their disease specific phenotypes after immortalisation, a reduced proliferation rate and a reduced ability to repopulate scratch wounds was observed in two of the three patients. These cell lines may be used to study chronic wound healing as an adjunct to the use of animal models. While simple wound assays cannot completely replace the use of animal wound models they may be useful for the high throughput screening of potential wound healing agents.

Chapter 4

Identification
of
Disease Marker Genes

4.1 Introduction

Microarray technology has lead to a paradigm shift in experimental design, allowing the coordinated analysis of expression levels for thousands of genes (Southern, 1975). Traditionally experiments focused on a single gene or protein, identifying and characterising it and linking it to a biological function. With the introduction of whole genome and proteome projects the identification of thousands of previously unknown genes and proteins and their functions is now possible.

Microarray technology is based on the original techniques described by Southern in the mid 1970s (Schena et al., 1995) where specific nucleotide fragments could be detected by probing with labelled nucleotide sequences. A number of microarray platforms are now available with the most common variants being printed cDNA microarrays (Lockhart et al., 1996) and photolithographically synthesised oligonucleotide probe microarrays (Weinstein, 1998). Printed cDNA arrays are generally made using an in house robotic spotter and can be tailored to meet the specific needs of the researcher, they are cheaper to produce but have limited quality control. Oligonucleotide arrays (Affymetrix™ GeneChip®) are commercially available, mass produced with stringent quality control at the time of production making them highly reproducible. GeneChip® arrays are composed of several hundred thousand synthesised 25mer oligos generated by photolithography. The HG-U133A arrays used in this investigation contains 22,283 probe sets representing 13,387 human genes. Each probe set is made up of between 11 and 20 complementary oligos specific to different regions of the same gene. To control for non-specific hybridisation mismatch probes are also included. These probes contain a single non-

specific base, binding to these non-specific probes give a background hybridisation reading for each probe set.

The use of microarray technology in academic research has been likened to a fishing expedition (Brown and Botstein, 1999) with no way of knowing which genes will be identified if any in your experiment. However a different viewpoint is that this can be seen as a good starting point for more in-depth analysis of identified genes of interest (Pusztai, 2006). Microarrays have been used to gain an understanding of complex diseases (Mogford et al., 2004, Charles et al., 2008, Wall et al., 2008a) including chronic wounds (Smith et al., 2008). In order to gain a better understanding of the molecular pathways involved in wound healing microarrays have been used extensively. Analysis of cell derived from keloid scars, identified a number of genes potentially linked with the disorder (Colwell et al., 2008, Hardman and Ashcroft, 2008). Genes involved in fibrosis were identified and offer a greater understanding of the underlying molecular pathways involved in the disease. As well as investigating disease specific changes in gene expression, microarrays have been used to study the effect of age on wound healing (Cooper et al., 2005, Roy et al., 2008, Theilgaard-Monch et al., 2004), the effect of inflammation (Swamy et al., 2004, Zhu et al., 2005) and the effect of different wound treatments on gene expression (Iyer et al., 1999). Microarray analysis of gene expression is a useful tool for the study of wound healing giving insight into the genes involved. Genes that have previously not been linked to wound healing may also be identified by microarray analysis.

In this chapter the NF and CWF at 3 time points after immortalisation were analysed using the serum starvation/stimulation model described by Iyer (Wall et al., 2008b) and compared to data obtained for the primary cell strains by Dr Ivan Wall (Iyer et al.,

1999). Originally, attempting to use microarrays to map the temporal response of fibroblasts to serum stimulation cells were serum starved for 48 hours. This synchronised the cells in G0 of the cell cycle. Addition of serum containing media stimulated the cells re-entry into the cell cycle. RNA was then extracted from the cells at numerous time points and analysed on printed cDNA microarrays. Analysis of the temporal gene expression profiles identified numerous genes relating to wound repair whose expression was controlled by serum stimulation (Chang et al., 2002). Whilst this is an understandably simplified model of *in vivo* wound healing it has been used to study gene expression in numerous fibroblast populations taken from varied anatomical sites (Cooper et al., 2004). Comparisons between gene list from serum starvation/re-stimulation experiments and *in* vivo wound models show a marked similarity between the early wound response *in* vivo and the serum stimulation model (Wall et al., 2008b).

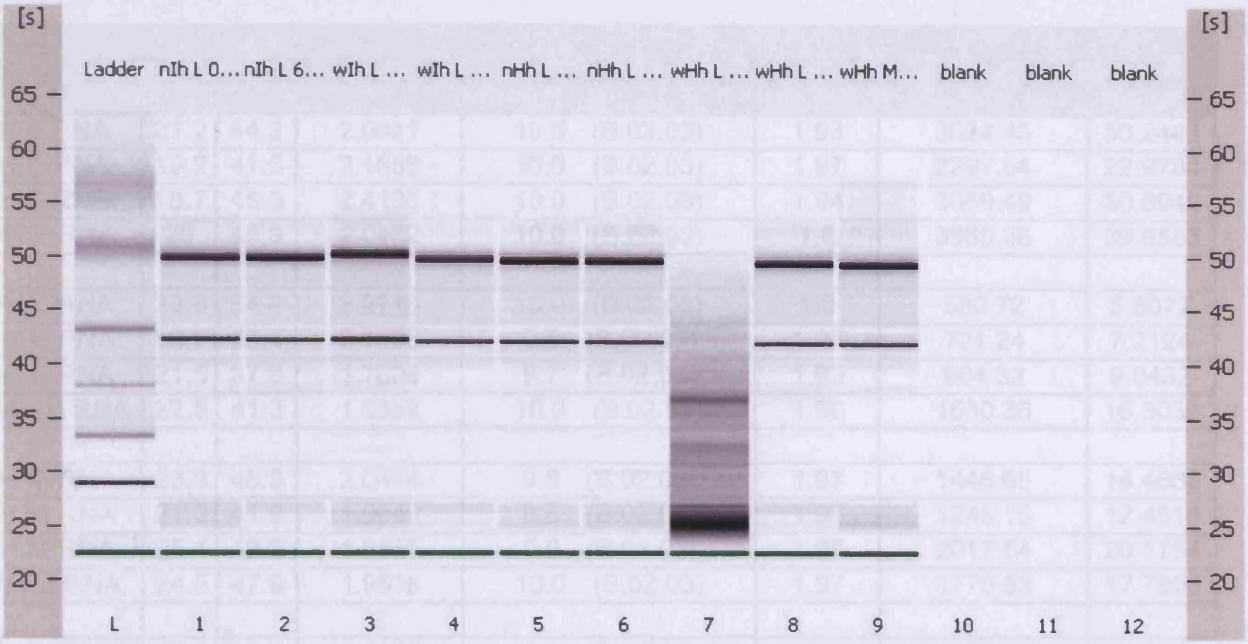
The overall aims of this Thesis were to characterise hTERT immortalised CWF and patient matched NF over an extended period in culture and to generate disease reporter constructs for use in these cells. In this Chapter microarray technology was used to compare gene expression between NF and CWF in a simplified model of wounding. Gene expression was analysed over extended time in culture. The gene expression of immortalised cells was also compared with primary cells to investigate the effect immortalisation had on the cells. A set of 'Disease Genes of Interest' were to be identified that were differentially regulated between NF and CWF and may be used for the generation of reporter constructs. Finally over-representation analysis was used to identify further sets of genes that may play a role in chronic wounds.

4.2 Results

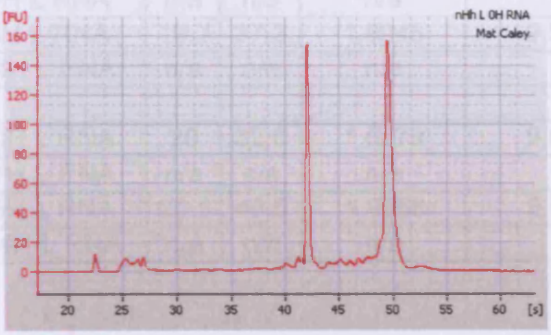
4.2.1 Microarray Data Analysis

Gene expression profiles of CWF and patient-matched NF (patients H, I and K) from quiescent (0 hour) and serum stimulated (6 hour) cells were characterised using *Affymetrix*™ GeneChip® technology. Data was previously collected from primary CWF and NF (Wall et al., 2008b) and this was compared to data collected from the hTERT-infected immortalised CWF and NF. Data was collected for the immortalised cell lines at three time points in culture, early (passage 23), mid (passage 50) and late (passage 80). RNA extracted from cells was checked for degradation using an Agilent 2100 bioanalyzer and RNA LabChip® technology The ratio and integrity of 18S and 28S rRNA analysed to determine if the RNA has been degraded (Fig 4.1). Although mRNA is not present in sufficient quantity for direct analysis, the more abundant ribosomal RNA can be used to give a value of RNA degradation. Any degraded RNA was identified by a lack of clear 26s and 18s bands on the virtual gel (Fig 4.1a) and on the detailed fluorescence profile (Fig 4.1c). 260/280 ratios were analysed on a NanoDrop® ND-1000 Spectrophotometer to detect protein or aromatic contamination and to accurately determine concentration and quantity of RNA. A 260/280 greater than 1.6 is considered acceptable, below this level protein or aromatic contamination maybe present which could interfere with the cDNA generation process or degrade the RNA. Data for RNA analysis is shown (Tables 4.1-4.3) with data points for 0, 1, 6 and 24 hours after serum stimulation.

(*a*)



(*b*)



(*c*)

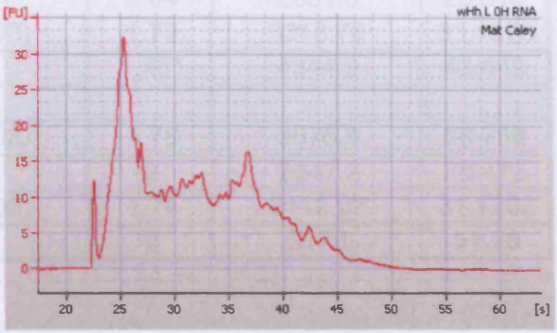


Figure 4.1: RNA integrity analysis carried out on the Agilent 2100 bioanalyser. RNA was analysed for both mass and size by a combination of microfluidics, capillary electrophoresis and fluorescence.

(*a*) A typical virtual gel generated by the Agilent 2100 bioanalyser labchip showing ribosomal RNA (rRNA) and the time at which it is detected. The larger the fragment the longer it will take to migrate through the chip. A DNA ladder is shown allowing calibration of the chip. Lanes 1-6 and 8-9 show weak ribosomal RNA bands at 26s 5S rRNA, 43s 18S rRNA and 50s 28S rRNA. Lane 7 shows degraded RNA with no detectable 18S or 28S rRNA bands.

(*b*) Typical bioanalyser profile for RNA showing Fluorescence plotted against time with clear peaks at 43s 18S rRNA and 50s 28S rRNA the ratio of the peak volumes (28S:18S) should ideally be 2.7:1 though a ratio of 2:1 is considered acceptable

(*c*) Bioanalyser profile of degraded RNA no peaks are visible for 18S or 28S rRNA.

Sample Name	18S	28S	rRNA Ratio [28S / 18S]:	RNA Integrity Number (RIN):	260:280 ratio	Concentration ng/µl	Quantity µg
nHh 0H E RNA	21.2	44.3	2.0841	10.0 (B.02.03)	1.93	3024.43	30.2443
nHh 1H E RNA	19.2	41.3	2.1558	10.0 (B.02.03)	1.97	2297.84	22.9784
nHh 6H E RNA	18.7	45.3	2.4186	10.0 (B.02.03)	1.94	3069.49	30.6949
nHh 24H E RNA	20	40.9	2.0452	10.0 (B.02.03)	1.8	3965.38	39.6538
wHh 0H E RNA	23.8	54.2	2.2716	10.0 (B.02.03)	1.9	580.72	5.8072
wHh 1H E RNA	16.7	35.4	2.1238	9.5 (B.02.03)	1.95	721.24	7.2124
wHh 6H E RNA	21.5	37.8	1.7608	9.7 (B.02.03)	1.93	904.32	9.0432
wHh 24H E RNA	22.5	41.3	1.8352	10.0 (B.02.03)	1.96	1630.36	16.3036
nHh 0H M RNA	23.6	48.3	2.0474	9.8 (B.02.03)	1.97	1446.65	14.4665
nHh 1H M RNA	21.3	41.9	1.9649	9.5 (B.02.03)	1.97	1245.15	12.4515
nHh 6H M RNA	25.1	42.2	1.6855	9.9 (B.02.03)	1.98	2017.54	20.1754
nHh 24H M RNA	24.5	47.9	1.9538	10.0 (B.02.03)	1.97	1778.53	17.7853
wHh 0H M RNA	17.8	30.9	1.7405	9.4 (B.02.03)	1.95	1474.77	14.7477
wHh 1H M RNA	21	37	1.7575	9.4 (B.02.03)	1.89	484.56	4.8456
wHh 6H M RNA	21	39.2	1.8672	10.0 (B.02.03)	1.85	358.59	3.5859
wHh 24H M RNA	21.7	37.7	1.7372	10.0 (B.02.03)	1.9	638.2	6.382
nHh 0H L RNA	19.8	37.7	1.9072	9.4 (B.02.03)	1.88	3459.28	34.5928
nHh 1H L RNA	n/a	n/a	n/a	n/a	2.04	2551.6	25.516
nHh 6H L RNA	19.7	37.3	1.8945	9.5 (B.02.03)	1.79	3318.78	33.1878
nHh 24H L RNA	n/a	n/a	n/a	n/a	1.48*	4344.9	43.449
wHh 0H L RNA	20	32.6	1.6279	9.5 (B.02.03)	1.78	3875.8	38.758
wHh 1H L RNA	n/a	n/a	n/a	n/a	2	2847.2	28.472
wHh 6H L RNA	20.3	40.5	1.9958	9.5 (B.02.03)	1.79	3112.02	31.1202
wHh 24H L RNA	n/a	n/a	n/a	n/a	2	3178	31.78

Table 4.1: RNA analysis Patient H. RNA integrity determined by analysis on the Agilent 2100 bioanalyser is displayed as rRNA Ratio 28S/18S. RNA purity is displayed as a ratio of absorbance determined by NanoDrop analysis. A 260:280 ratio of greater than 1.6 is considered acceptable. (* indicates an RNA sample with particularly high concentration which has masked the true 260:280 ratio).

Sample Name	18S	28S	rRNA Ratio [28S / 18S]:	RNA Integrity Number (RIN):	260:280 ratio	Concentration ng/µl	Quantity µg
nlh 0H E RNA	20.2	37.6	1.8625	9.4 (B.02.03)	1.93	1140.83	11.4083
nlh 1H E RNA	19.3	33.2	1.7155	9.1 (B.02.03)	1.96	1083.72	10.8372
nlh 6H E RNA	21.4	37.3	1.7439	9.6 (B.02.03)	1.94	1420.37	14.2037
nlh 24H E RNA	20.9	38.7	1.8482	9.8 (B.02.03)	1.95	1826.8	18.268
wlh 0H E RNA	20.4	35.2	1.7238	9.3 (B.02.03)	1.99	1578.16	15.7816
wlh 1H E RNA	20.2	32.3	1.5999	9.2 (B.02.03)	1.94	1018.13	10.1813
wlh 6H E RNA	19.5	34.1	1.7428	9.4 (B.02.03)	1.96	1613.14	16.1314
wlh 24H E RNA	26	30.9	1.19	9.6 (B.02.03)	1.96	2909.64	29.0964
nlh 0H M RNA	24.8	47.8	1.9311	10.0 (B.02.03)	1.98	2782.42	27.8242
nlh 1H M RNA	24.7	43.6	1.7651	9.8 (B.02.03)	1.98	1688.86	16.8886
nlh 6H M RNA	22.6	43.4	1.9197	10.0 (B.02.03)	1.99	1651.33	16.5133
nlh 24H M RNA	27.4	48.8	1.7812	10.0 (B.02.03)	1.99	2231.45	22.3145
wlh 0H M RNA	22.7	49.2	2.1659	10.0 (B.02.03)	1.98	2042.68	20.4268
wlh 1H M RNA	23.2	51.7	2.2342	10.0 (B.02.03)	1.99	2097.13	20.9713
wlh 6H M RNA	21.8	45.2	2.0777	10.0 (B.02.03)	1.97	2419.8	24.198
wlh 24H M RNA	23.1	44.3	1.9205	10.0 (B.02.03)	1.93	3314.78	33.1478
nlh 0H L RNA	18.9	32.9	1.7425	9.3 (B.02.03)	1.94	2234.37	22.3437
nlh 1H L RNA	n/a	n/a	n/a	n/a	2.05	935.82	9.3582
nlh 6H L RNA	20.3	39.4	1.9393	9.3 (B.02.03)	2.02	2321.04	23.2104
nlh 24H L RNA	n/a	n/a	n/a	n/a	2.04	2068.5	20.685
wlh 0H L RNA	21.1	33.4	1.5843	9.5 (B.02.03)	1.6	865.17	8.6517
wlh 1H L RNA	n/a	n/a	n/a	n/a	2.01	1390.1	13.901
wlh 6H L RNA	20.8	39.4	1.8912	9.5 (B.02.03)	1.97	1137.33	11.3733
wlh 24H L RNA	n/a	n/a	n/a	n/a	2.03	2194	21.94

Table 4.2: RNA analysis Patient I. RNA integrity determined by analysis on the Agilent 2100 bioanalyser is displayed as rRNA Ratio 28S/18S. RNA purity is displayed as a ratio of absorbance determined by NanoDrop analysis. A 260:280 ratio of greater than 1.6 is considered acceptable.

Sample Name	18S	28S	rRNA Ratio [28S / 18S]:	RNA Integrity Number (RIN):	260:280 ratio	Concentration ng/µl	Quantity µg
nKh 0H E RNA	22.9	46.5	2.0288	10.0 (B.02.03)	1.91	959.23	9.5923
nKh 1H E RNA	22.5	49.9	2.2179	10.0 (B.02.03)	1.94	711.24	7.1124
nKh 6H E RNA	26.9	44.5	1.6522	10.0 (B.02.03)	1.94	934.87	9.3487
nKh 24H E RNA	23.4	45	1.9248	10.0 (B.02.03)	1.92	3121.48	31.2148
wKh 0H E RNA	22.2	46.9	2.1152	10.0 (B.02.03)	1.96	2405.91	24.0591
wKh 1H E RNA	21.9	45.6	2.0845	10.0 (B.02.03)	1.96	2082.93	20.8293
wKh 6H E RNA	21.2	45.3	2.1333	10.0 (B.02.03)	1.91	3186.27	31.8627
wKh 24H E RNA	21.1	43.5	2.0602	9.9 (B.02.03)	1.95	2759.5	27.595
nKh 0H M RNA	20	34.1	1.7039	9.4 (B.02.03)	1.96	2679.96	26.7996
nKh 1H M RNA	19.1	34.2	1.7935	9.3 (B.02.03)	1.96	2643.06	26.4306
nKh 6H M RNA	20	37.3	1.8655	9.6 (B.02.03)	1.89	3485.43	34.8543
nKh 24H M RNA	23.6	46.6	1.9739	10.0 (B.02.03)	1.46*	4399.45	43.9945
wKh 0H M RNA	23.3	41.5	1.7841	9.7 (B.02.03)	1.99	2058.51	20.5851
wKh 1H M RNA	22.7	43.2	1.9001	9.7 (B.02.03)	1.98	2099.95	20.9995
wKh 6H M RNA	23.1	45.5	1.973	9.7 (B.02.03)	1.98	2388.49	23.8849
wKh 24H M RNA	28.8	28.6	0.9933	9.2 (B.02.03)	1.98	2455.07	24.5507
nKh 0H L RNA	19.5	42.3	2.16923077	9.8 (B.02.03)	1.75	4173.65	41.7365
nKh 1H L RNA	17.5	47.1	2.69142857	9.9 (B.02.03)	2.13	2034.04	20.3404
nKh 6H L RNA	19.9	40	2.01005025	10.0 (B.02.03)	1.47*	4345.91	43.4591
nKh 24H L RNA	19.2	45.4	2.36458333	10.0 (B.02.03)	1.61*	4304.51	43.0451
wKh 0H L RNA	18.6	47.1	2.53225806	9.9 (B.02.03)	2.02	3314.79	33.1479
wKh 1H L RNA	19.4	40.8	2.10309278	10.0 (B.02.03)	2.09	2616.63	26.1663
wKh 6H L RNA	15.8	37.4	2.36708861	9.8 (B.02.03)	2.07	2948.2	29.482
wKh 24H L RNA	19.1	41.6	2.17801047	10.0 (B.02.03)	1.97	3604.87	36.0487

Table 4.3: RNA analysis Patient K. RNA integrity determined by analysis on the Agilent 2100 bioanalyser is displayed as rRNA Ratio 28S/18S. RNA purity is displayed as a ratio of absorbance determined by NanoDrop analysis. A 260:280 ratio of greater than 1.6 is considered acceptable. (* indicates an RNA sample with particularly high concentration which has masked the true 260:280 ratio).

Raw microarray expression data was initially processed using the microarray Analysis Suite 5.0 (MAS5) provided by *Affymetrix*^{TM}. MAS 5 normalises probe intensities, combines the different probes for each gene to give a gene expression value and calculates the presence/absence call and the p-values of calls. This processing allows different chips to be compared as the signal intensities on each chip will vary dramatically depending on the amount of labelled cDNA hybridised to the chip. The normalisation process scales the average expression intensity for each chip to a single constant value. However, if the scaling factor is too great the data will be rejected; anything greater than a 3 fold range will be rejected. The resulting data was stored in cell intensity (.CEL) files. Data analysis was carried out using Bioconductor (http://www.bioconductor.org) an open source software project for the analysis of genomic data by Dr Peter Giles in the Department of Pathology, Cardiff University. Before analysis, quality control was carried out on each of the chips, identifying not only aberrations in the normalisation but also identifying variation between chips caused by faults in the fluidics system. Quality control looks at sets of housekeeping genes on each chip comparing their expression levels. If theses levels and the scaling factor are found to be within acceptable limits the data can be used (Fig 4.2). 2 of the 48 chips run were found to have abnormalities in the signal intensity though they did not fail MAS5 processing. These abnormalities are seen as areas of high signal intensity on the scanned chip images (Fig 4.3a) particularly obvious when compared to a normal chip image (Fig 4.3b) and are thought to be due to a non-critical fault in the scanner. The data obtained was within the limits set by Affymetrix^{TM} however, it was decided that a further normalisation step would be used to reduce the effect of any background noise caused by these chips. All chips were therefore further

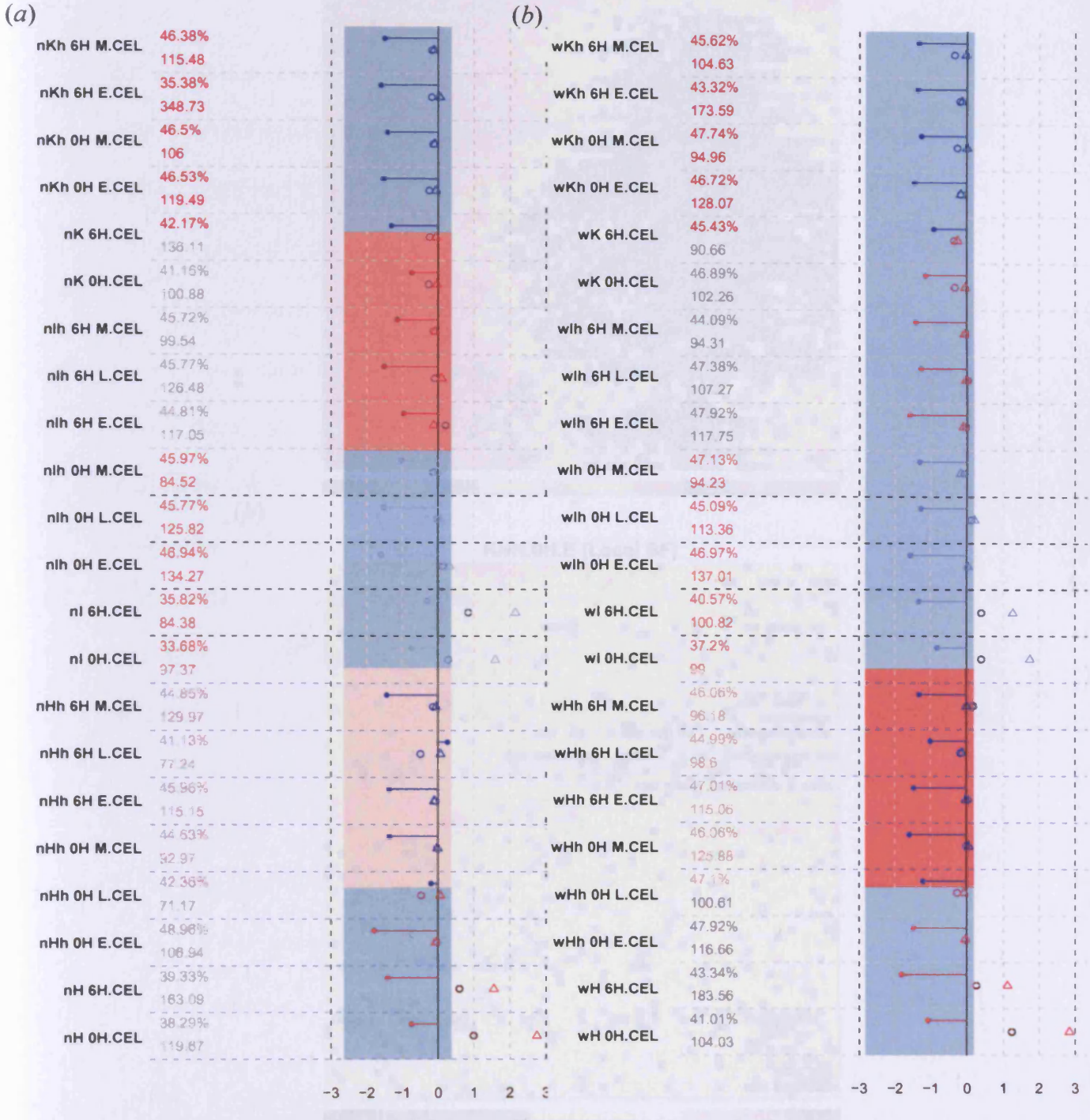
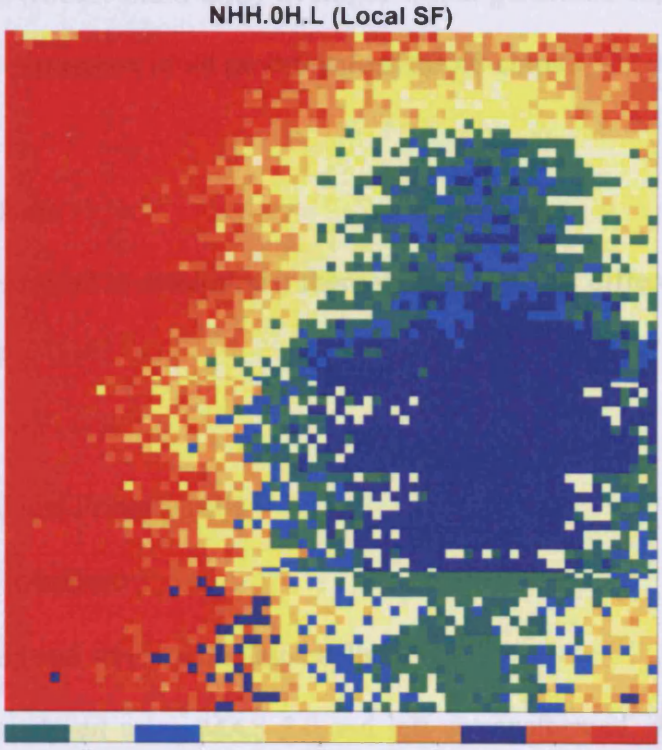


Figure 4.2: Quality control of MAS 5.0 data generated from Affymetrix™ GeneChip® experiments. Scaling factors should fall within a 3-fold range of each other as indicated in (***a***) for NF samples and (b) for CWF samples. The 3':5' ratios shown as blue lines all fall within the blue shaded region. Two built-in controls house keeping gene controls: ß-actin (△) and GAPDH (○) are also used and must fall within ± 3 for the data to be acceptable.

(a)



(b)

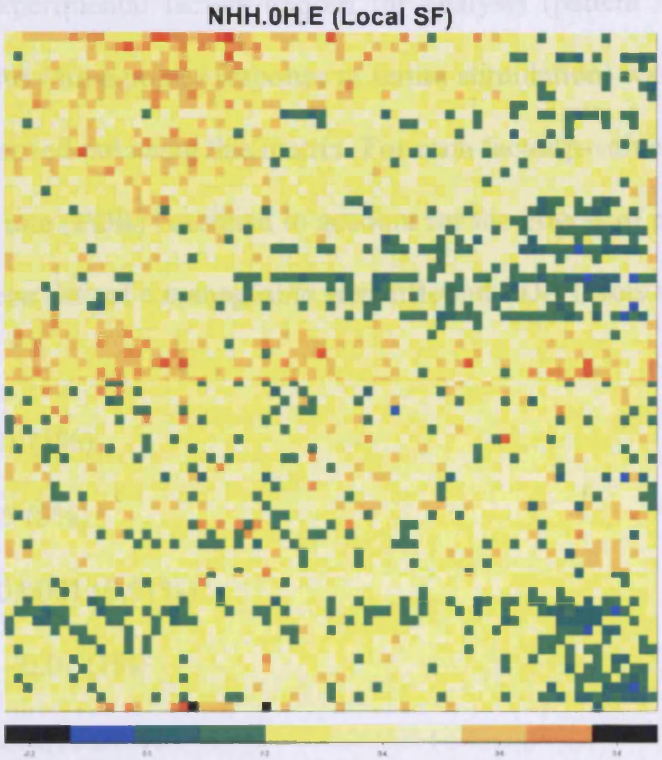


Figure 4.3: Scanned chip images of Affymetrix™ U133A microarrays. Analysis of scanned images identified high intensity expression in 2/48 chips used in this experiment (a) when compared to normal chip images (b) the high intensity of signal seen in these chips is easily identifiable.

processed using rma (robust multi-array average) which generated expression values relative to the mean expression of all probes across the 48 chips.

4.2.2 ANOVA Analysis

ANOVA analysis was used to determine if there were any global differences between:

- CWF and NF
- Patients
- Immortalised and Primary cells
- Time points in culture
- Serum starved and serum stimulated cells

CEL file data was analysed using MAS 5.0 and left untransformed. A design matrix containing the 5 experimental factors chosen for analysis (patient / NF v CWF / immortalisation status / time point / response to serum stimulation) was generated and ANOVA analysis carried out using this matrix. For each factor p-values were returned and false discovery rate (FDR) corrected to account for the effects of multiple testing. In order to create gene lists of a manageable size different FDR levels were chosen for each analysis

- Patient – 0.01 (1%)
- N/CW – 0.05 (5%)
- Immortal – 0.0001 (0.01%)
- Time Point – 0.01 (1%)
- Serum – 0.0001 (0.01%)

Heatmaps for the genes extracted for each factor were then produced, plotting both the whole dataset, and split to show genes moving in different directions (e.g. up in one plot, down in another plot). Changes in gene expression were determined in

comparison to the average expression level of the selected dataset. Changes in gene expression compared to this average were indicated by intensity of colour with red indicating up regulation and green indicating down regulation (Fig 4.4-4.7). For easy access to the data all gene lists were uploaded onto the microarray data review and annotation system (MADRAS; www.madras.uwcm.ac.uk). On Affymetrix™ microarrays multiple probe sets may represent a single gene. 110 probe sets representing 95 were identified as being up-regulated by hTERT immortalisation (Fig 4.4), while 206 probe sets representing 168 genes were identified as being down-regulated by hTERT immortalisation (Fig 4.5). A total of 263 genes were identified as altered by hTERT immortalisation representing 1.96% of the 13,387 genes represented on the microarray. When CWF and NF gene expression was compared 144 probe sets representing 123 genes were identified as being down-regulated in CWF compared to NF (Fig 4.6) and 102 probe sets representing 88 genes identified as being up-regulated in CWF compared to NF (Fig 4.7). 211 genes were therefore identified as being differentially regulated between NF and CWF.

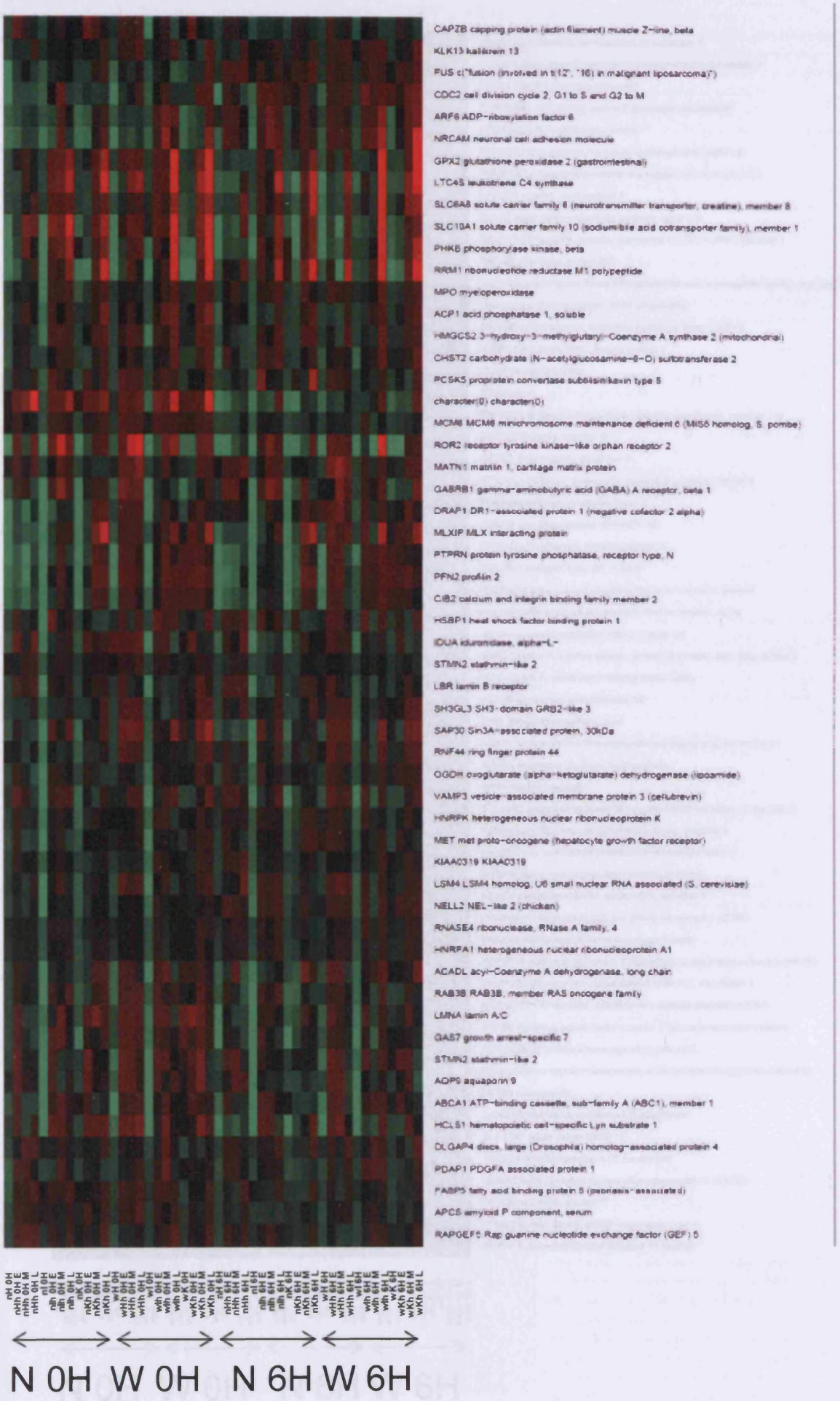


Figure 4.4a: Genes up-regulated by hTERT immortalisation. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.4b: Genes up-regulated by hTERT immortalisation. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change


CORO2B coronin, actin binding protein, 2B
TNFRSF10B tumor necrosis factor receptor superfamily, member 10b
ABCA1 ATP-binding cassette, sub-family A (ABC1), member 1
MCM6 MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe)
PTGDS prostaglandin D2 synthase 21kDa (brain)
PTGDS prostaglandin D2 synthase 21kDa (brain)
SLC22A2 solute carrier family 22 (organic cation transporter), member 2
RNASE4 ribonuclease, RNase A family, 4
HCLS1 hematopoietic cell-specific Lyn substrate 1
SLC17A7 solute carrier family 17
PAPPA pregnancy-associated plasma protein A, pappalysin 1
RANGAP1 Ran GTPase activating protein 1
ACP1 acid phosphatase 1, soluble
HSBP1 heat shock factor binding protein 1
COX10 COX10 homolog
MET met proto-oncogene (hepatocyte growth factor receptor)
GADD45G growth arrest and DNA-damage-inducible, gamma
character(0) character(0)
SULF1 sulfatase 1
HLA-DPA1 major histocompatibility complex, class II, DP alpha 1
TUBB tubulin, beta
CMKOR1 chemokine orphan receptor 1
CAP2 CAP, adenylate cyclase-associated protein, 2 (yeast)
COL5A1 collagen, type V, alpha 1
CKS2 CDC28 protein kinase regulatory subunit 2
RASSF1 Ras association (RalGDS/AF-6) domain family 1
MBP myelin basic protein
SULF1 sulfatase 1
CHST2 carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2
HLA-B major histocompatibility complex, class I, B
IGFBP6 insulin-like growth factor binding protein 6
NRCAM neuronal cell adhesion molecule
BUB3 BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)
PSMD14 proteasome (prosome, macropain) 26S subunit, non-ATPase, 14
FGF5 fibroblast growth factor 5
VAMP3 vesicle-associated membrane protein 3 (cellubrevin)
DRAP1 DR1-associated protein 1 (negative cofactor 2 alpha)
ADCY1 adenylate cyclase 1 (brain)
FGFR2 fibroblast growth factor receptor 2
SPON1 spondin 1, extracellular matrix protein
ADCY2 adenylate cyclase 2 (brain)
RAB3B RAB3B, member RAS oncogene family
H2AFV H2A histone family, member V
character(0) character(0)
SAP30 Sin3A-associated protein, 30kDa
GAS7 growth arrest-specific 7
PRKG2 protein kinase, cGMP-dependent, type II
IL17 interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)
PFN2 profilin 2
BTN2A3 butyrophilin, subfamily 2, member A3
CGA glycoprotein hormones, alpha polypeptide
NELL2 NEL-like 2 (chicken)
SULF1 sulfatase 1
RNASE4 ribonuclease, RNase A family, 4
TDRD7 tudor domain containing 7
FCGR2A Fc fragment of IgG, low affinity IIa, receptor (CD32)
TSPYL5 TSPY-like 5
LSM4 LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)
IDUA iduronidase, alpha-L-
FGF5 fibroblast growth factor 5
PEX3 peroxisomal biogenesis factor 3
THY1 Thy-1 cell surface antigen
STMN2 stathmin-like 2
CDC2 cell division cycle 2, G1 to S and G2 to M
NRG2 neuregulin 2
BTG2 BTG family, member 2
PDGFRA platelet-derived growth factor receptor, alpha polypeptide
TNFRSF11B tumor necrosis factor receptor superfamily, member 11b
HLA-C major histocompatibility complex, class I, C
MEFV Mediterranean fever
PCMT1 protein-L-isoaspartate (D-aspartate) O-methyltransferase
SLC6A8 solute carrier family 6 (neurotransmitter transporter, creatine), member 8
PCSK5 proprotein convertase subtilisin/kexin type 5
CD24 CD24 antigen (small cell lung carcinoma cluster 4 antigen)
N 0H
W 0H
N 6H
W 6H

Figure 4.5a

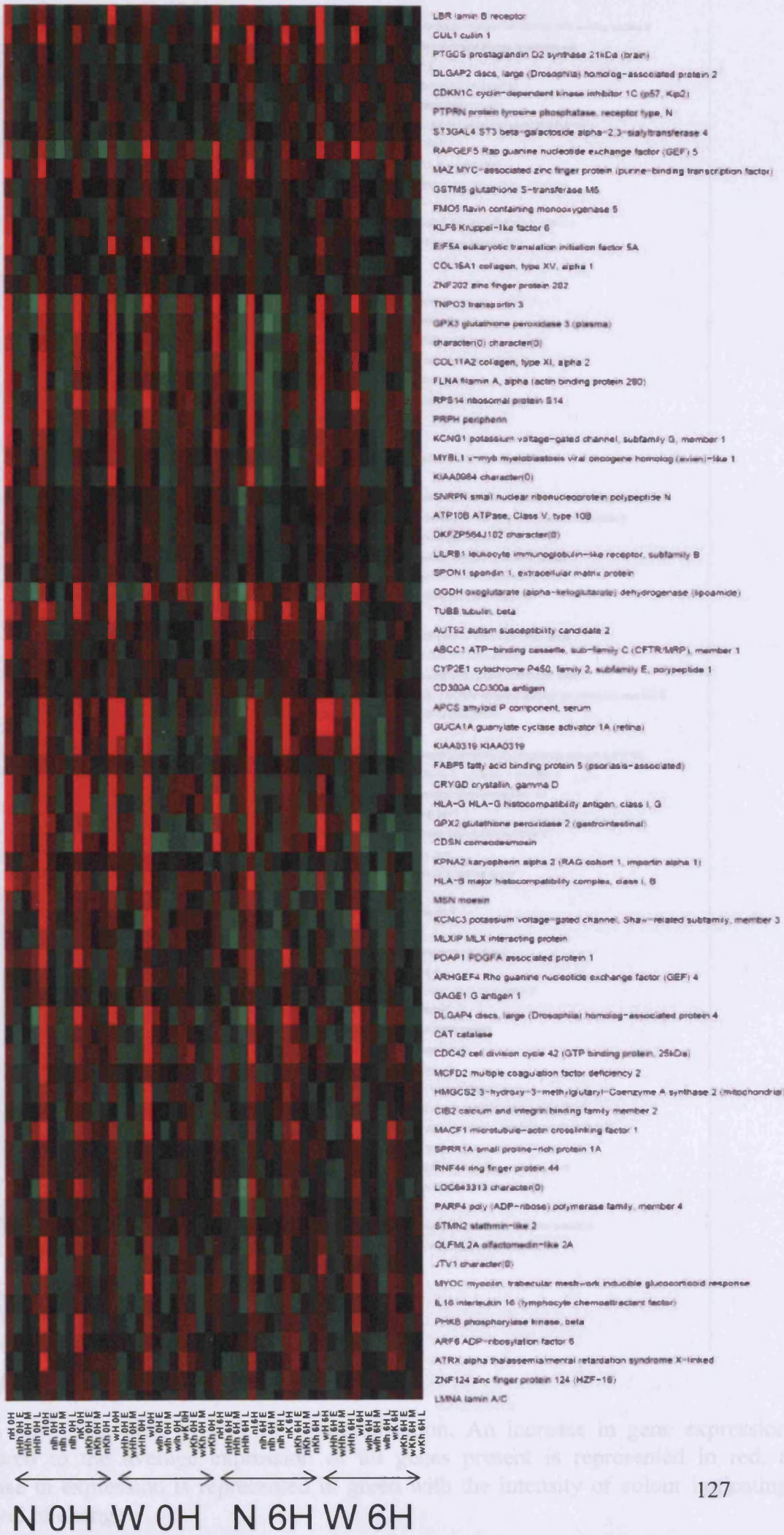

LBR lamin B receptor
CUL1 cullin 1
PTGDS prostaglandin D2 synthase 21kDa (brain)
DLGAP2 discs, large (Drosophila) homolog-associated protein 2
CDKN1C cyclin-dependent kinase inhibitor 1C (p57, Kip2)
PTPRN protein tyrosine phosphatase, receptor type, N
ST3GAL4 ST3 beta-galactoside alpha-2,3-sialyltransferase 4
RAPGEF5 Rap guanine nucleotide exchange factor (GEF) 5
MAZ MYC-associated zinc finger protein (purine-binding transcription factor)
GSTM5 glutathione S-transferase M5
FMO5 flavin containing monooxygenase 5
KLF6 Kruppel-like factor 6
EIF5A eukaryotic translation initiation factor 5A
COL16A1 collagen, type XV, alpha 1
ZNF202 zinc finger protein 202
TNPO3 transportin 3
GPX3 glutathione peroxidase 3 (plasma)
character(0) character(0)
COL11A2 collagen, type XI, alpha 2
FLNA filamin A, alpha (actin binding protein 280)
RPS14 ribosomal protein S14
PRPH peripherin
KCNG1 potassium voltage-gated channel, subfamily G, member 1
MYBL1 v-myb myeloblastosis viral oncogene homolog (avian)-like 1
KIAA0984 character(0)
SNRPN small nuclear ribonucleoprotein polypeptide N
ATP10B ATPase, Class V, type 10B
DKFZP564J102 character(0)
LILRB1 leukocyte immunoglobulin-like receptor, subfamily B
SPON1 spondin 1, extracellular matrix protein
OGDH oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)
TUBB tubulin, beta
AUTS2 autism susceptibility candidate 2
ABCC1 ATP-binding cassette, sub-family C (CFTR/MRP), member 1
CYP2E1 cytochrome P450, family 2, subfamily E, polypeptide 1
CD300A CD300a antigen
APCS amyloid P component, serum
GUCA1A guanylate cyclase activator 1A (retina)
KIAA0319 KIAA0319
FABP5 fatty acid binding protein 5 (psoriasis-associated)
CRYGD crystallin, gamma D
HLA-G HLA-G histocompatibility antigen, class I, G
GPX2 glutathione peroxidase 2 (gastrointestinal)
CDSN corneodesmosin
KPNA2 karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
HLA-B major histocompatibility complex, class I, B
MSN moesin
KCNC3 potassium voltage-gated channel, Shaw-related subfamily, member 3
MLXIP MLX interacting protein
PDAP1 PDGFA associated protein 1
ARHGEF4 Rho guanine nucleotide exchange factor (GEF) 4
GAGE1 G antigen 1
DLGAP4 discs, large (Drosophila) homolog-associated protein 4
CAT catalase
CDC42 cell division cycle 42 (GTP binding protein, 25kDa)
MCFD2 multiple coagulation factor deficiency 2
HMGCS2 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)
CIB2 calcium and integrin binding family member 2
MACF1 microtubule-actin crosslinking factor 1
SPRR1A small proline-rich protein 1A
RNF44 ring finger protein 44
LOC643313 character(0)
PARP4 poly (ADP-ribose) polymerase family, member 4
STMN2 stathmin-like 2
OLFML2A olfactomedin-like 2A
JTV1 character(0)
MYOC myocilin, trabecular meshwork inducible glucocorticoid response
IL16 interleukin 16 (lymphocyte chemoattractant factor)
PHKB phosphorylase kinase, beta
ARF6 ADP-ribosylation factor 6
ATRX alpha thalassemia/mental retardation syndrome X-linked
ZNF124 zinc finger protein 124 (HZF-16)
LMNA lamin A/C
N 0H W 0H N 6H W 6H

Figure 4.5b

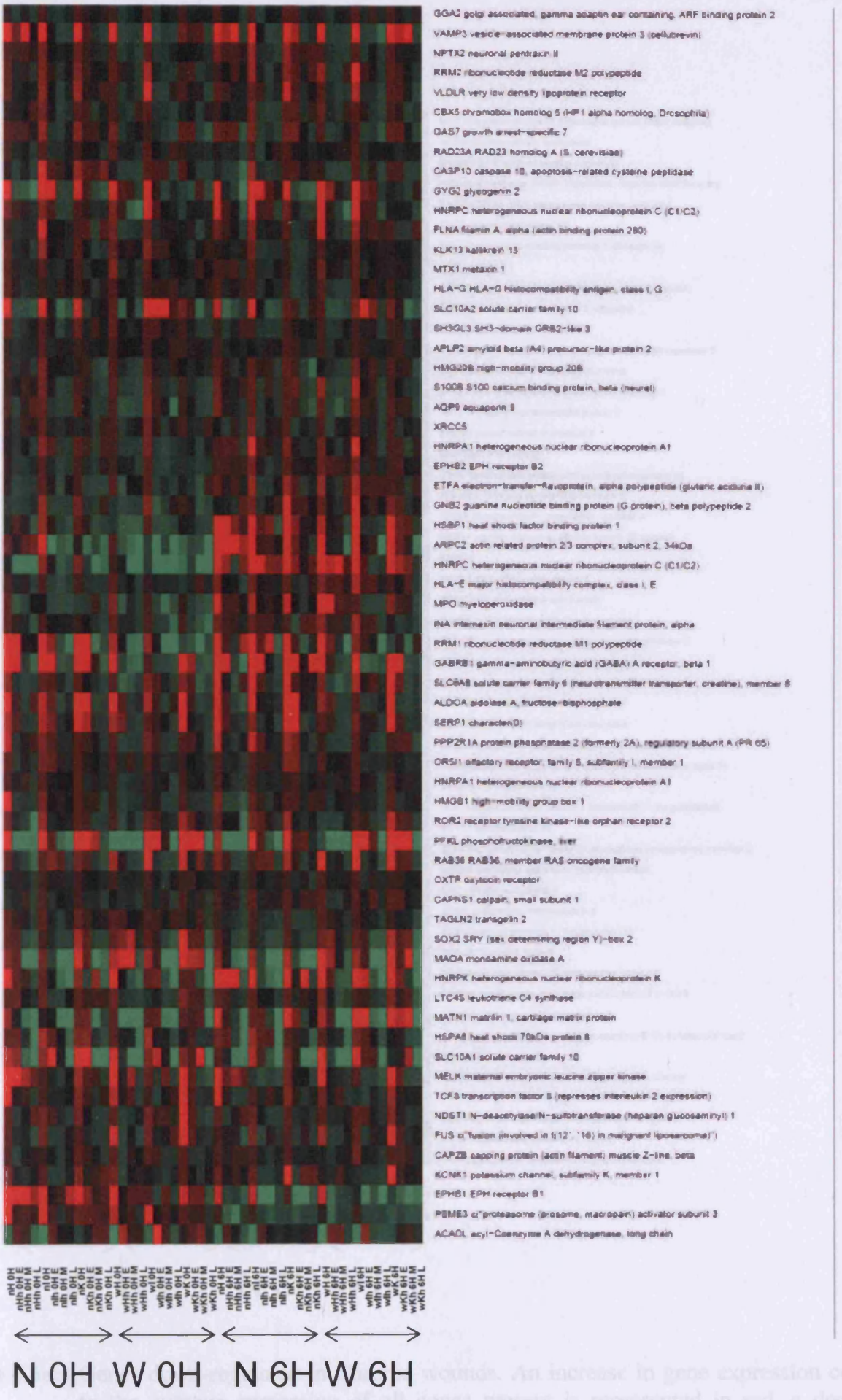


Figure 4.5c: Genes down-regulated by hTERT immortalisation. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.6a: Genes down-regulated in Chronic wounds. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

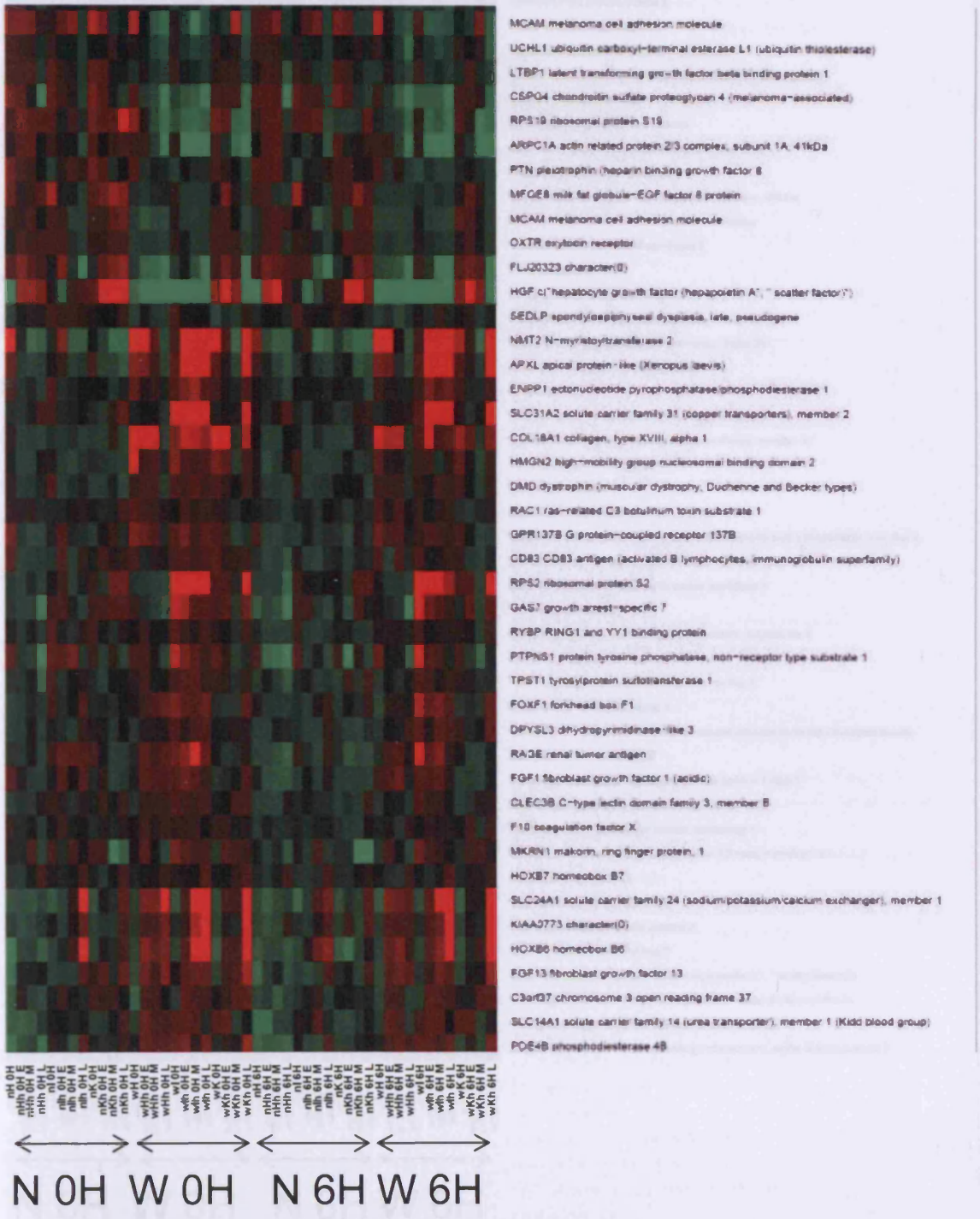


Figure 4.6b: Genes down-regulated in Chronic wounds. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

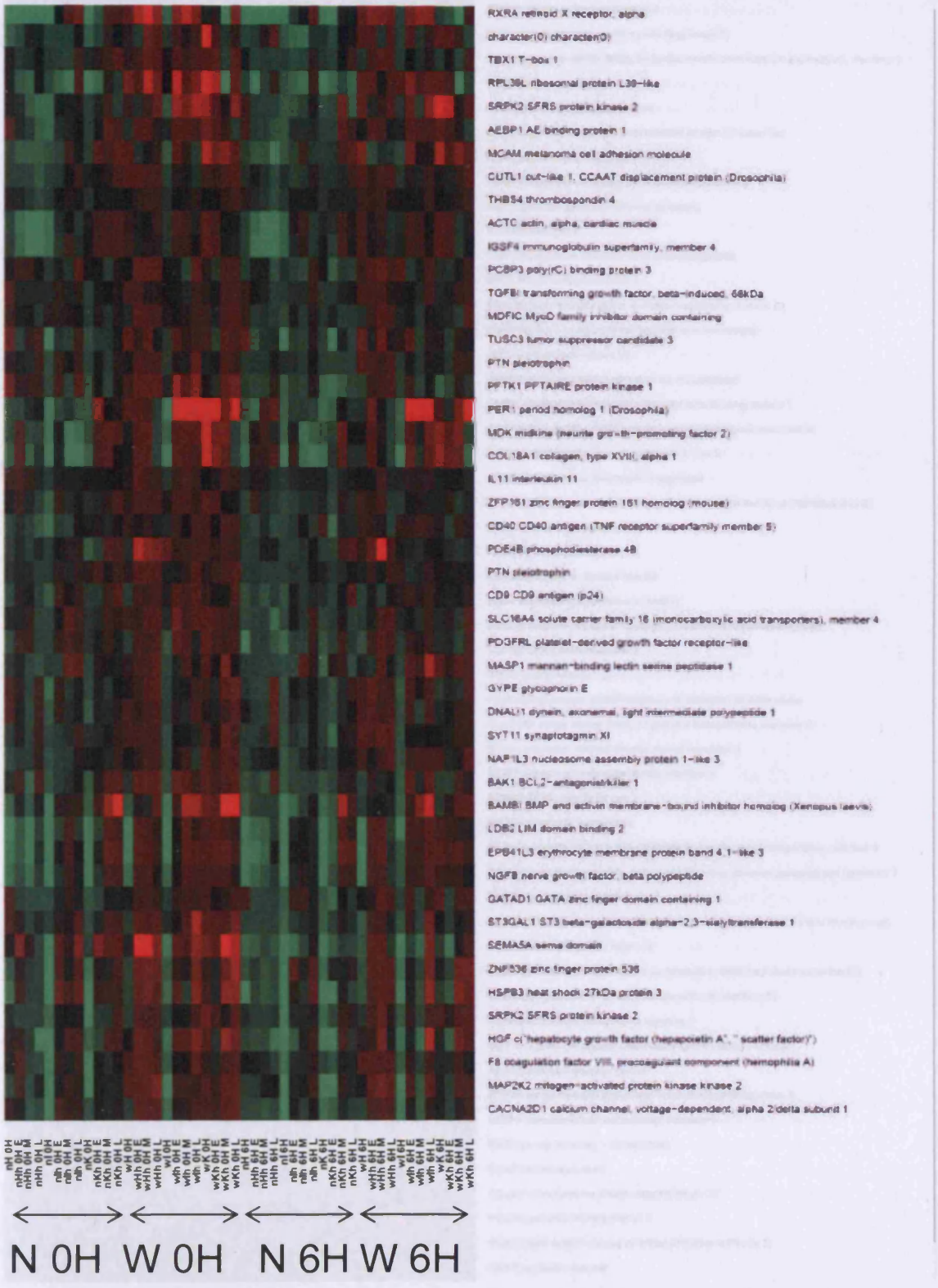


Figure 4.6c: Genes down-regulated in Chronic wounds. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

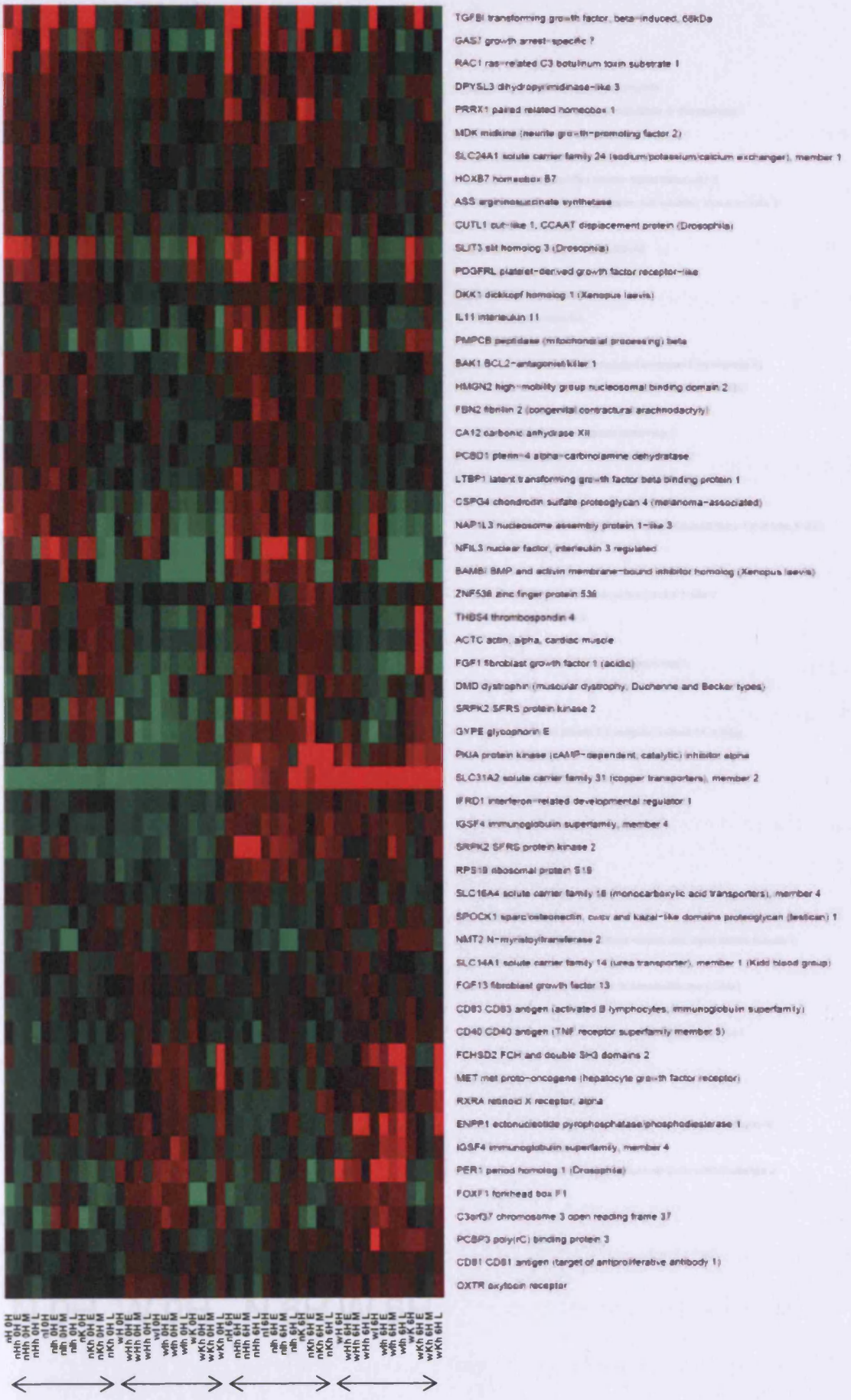


Figure 4.7a: Genes up-regulated in Chronic wounds. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

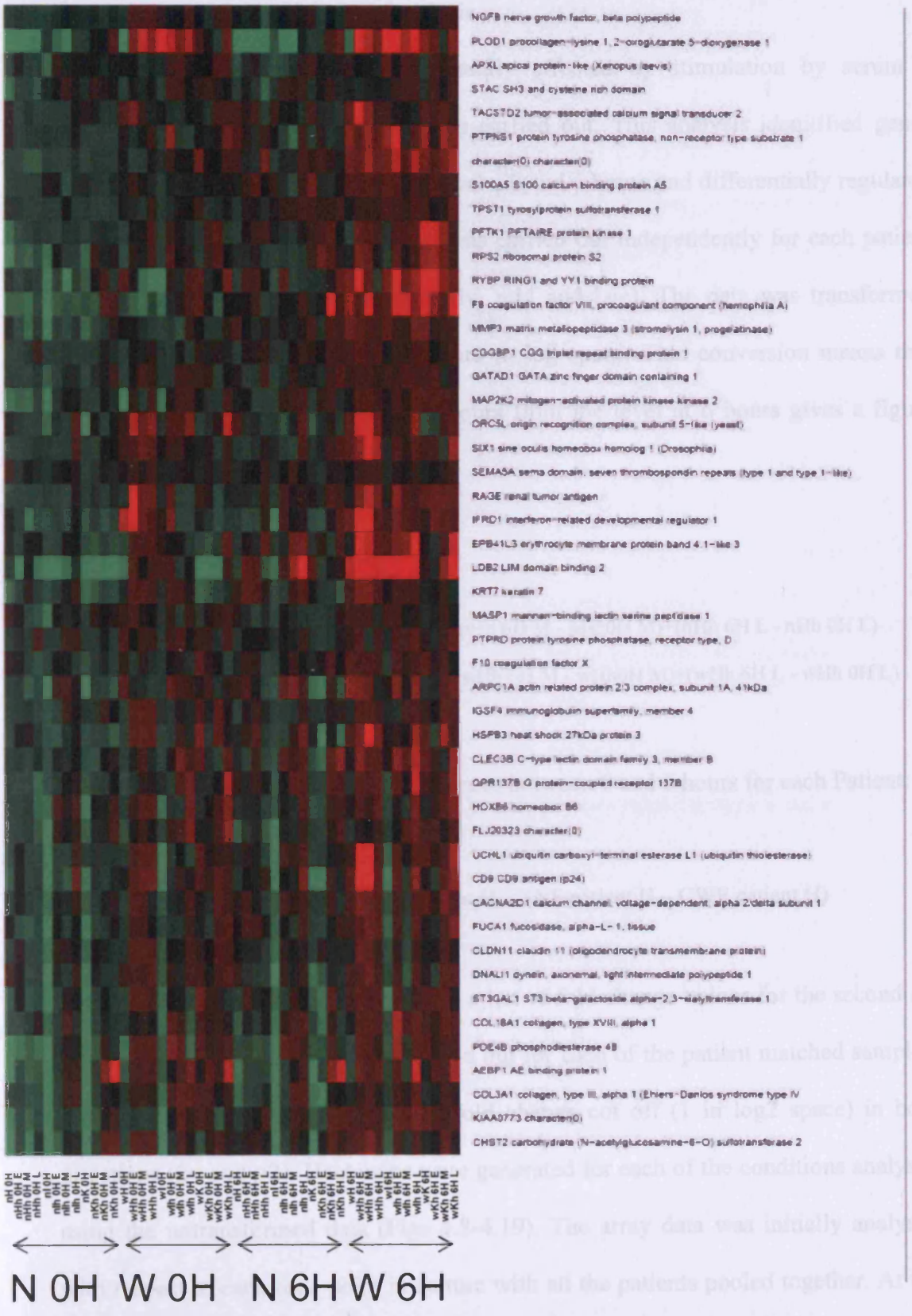


Figure 4.7b: Genes up-regulated in Chronic wounds. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

4.2.3 Difference of Differences Analysis

To identify genes that were differentially effected by stimulation by serum a 'difference of differences' analysis was carried out. This analysis idenitified genes which were differentially regulated between 0 and 6 hours and differentially regulated between NF and CWF. This analysis was carried out independently for each patient and for each timepoint in culture (early, mid and late). The data was transformed using RMA analysis converting the data to log space. This conversion means that subtraction of expression levels at 0 hours from the level at 6 hours gives a figure equivalent to fold change.

For each probe set:

NF Patient H (nHh 6H E - nHh 0H E)+(nHh 6H M - nHh0H M)+(nHh 6H L - nHh 0H L)

CWF Patient H (wHh 6H E - wHh 0H E)+(wHh 6H M - wHh0H M)+(wHh 6H L - wHh 0H L)

This gave two new sets with the differences between 0 and 6 hours for each Patient.

Difference of differences Patient H (NF patient H – CWF patient H)

The resultant data is then equivalent to a list of fold change values for the second set of differences. This process was carried out for each of the patient matched samples. The data was then filtered with a 2 fold change cut off (1 in log2 space) in both directions (2< and >2). Heat maps were generated for each of the conditions analysed using the untransformed data (Figs 4.8-4.19). The array data was initially analysed with respect to each time point in culture with all the patients pooled together. At the early time point in culture 17 genes were identified as being down regulate by the addition of serum in CWF compared to NF (Fig 4.8) and 60 genes as being up

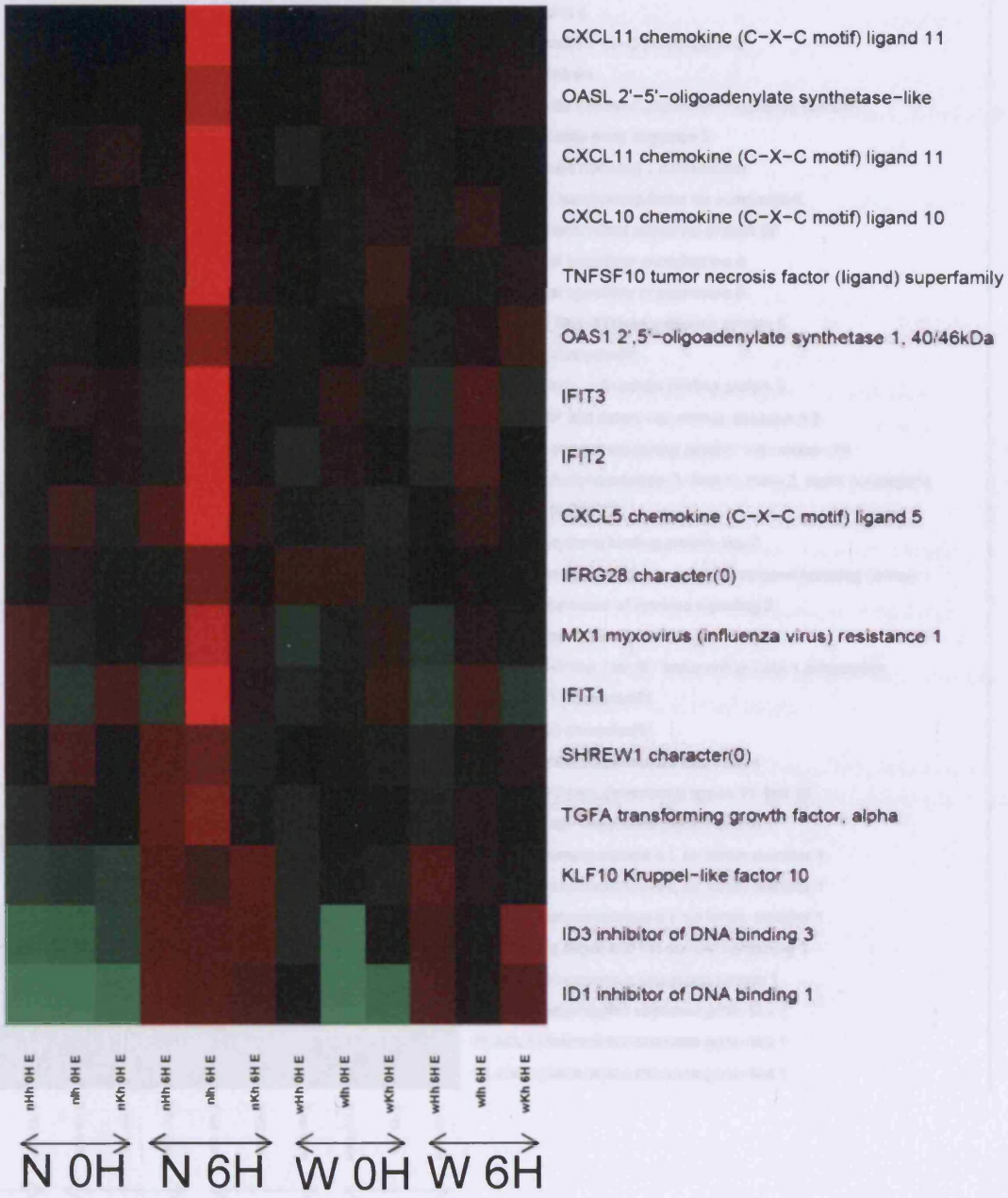


Figure 4.8: Difference of Differences analysis Early Time Point, genes down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

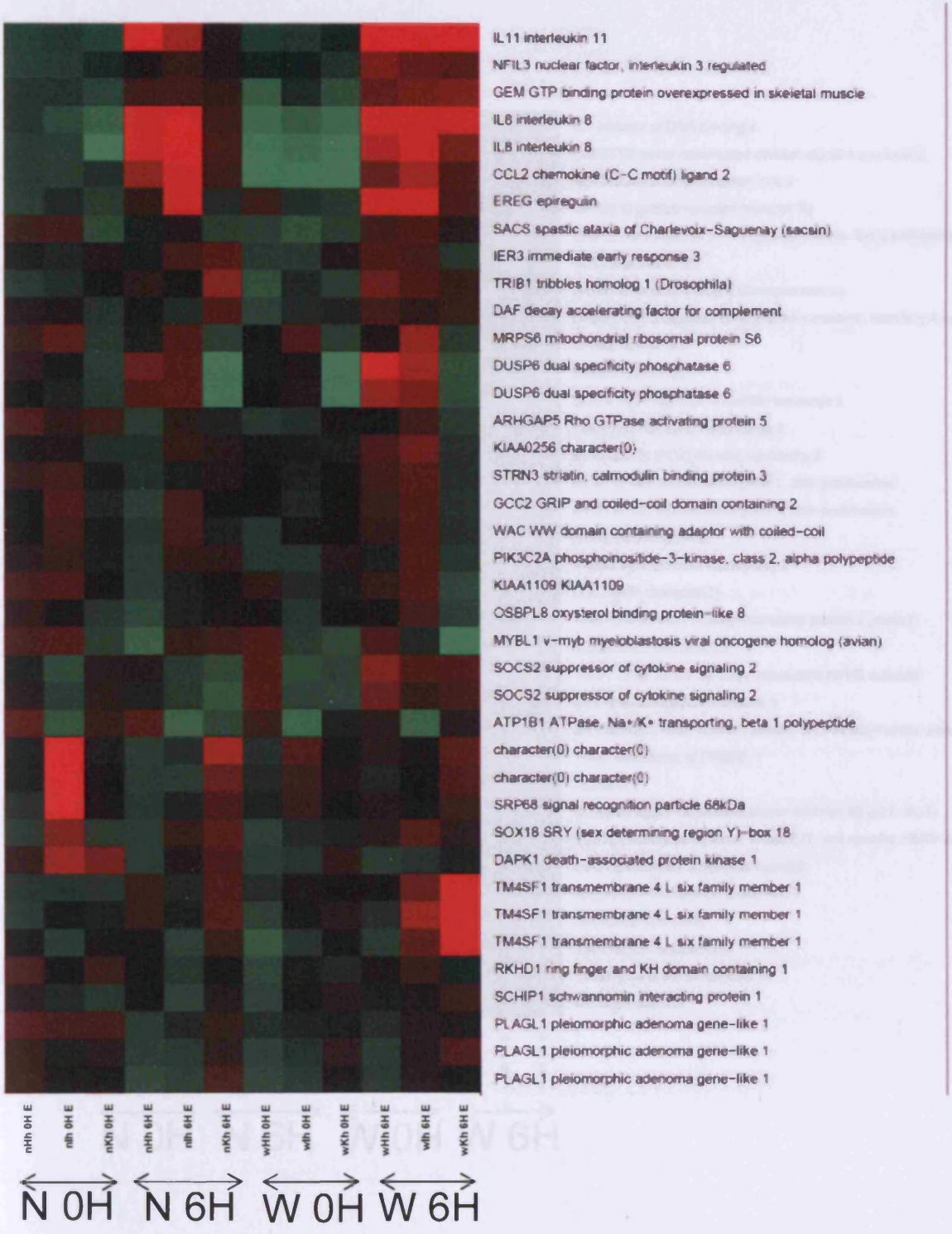


Figure 4.9a: Difference of Differences analysis Early Time Point, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

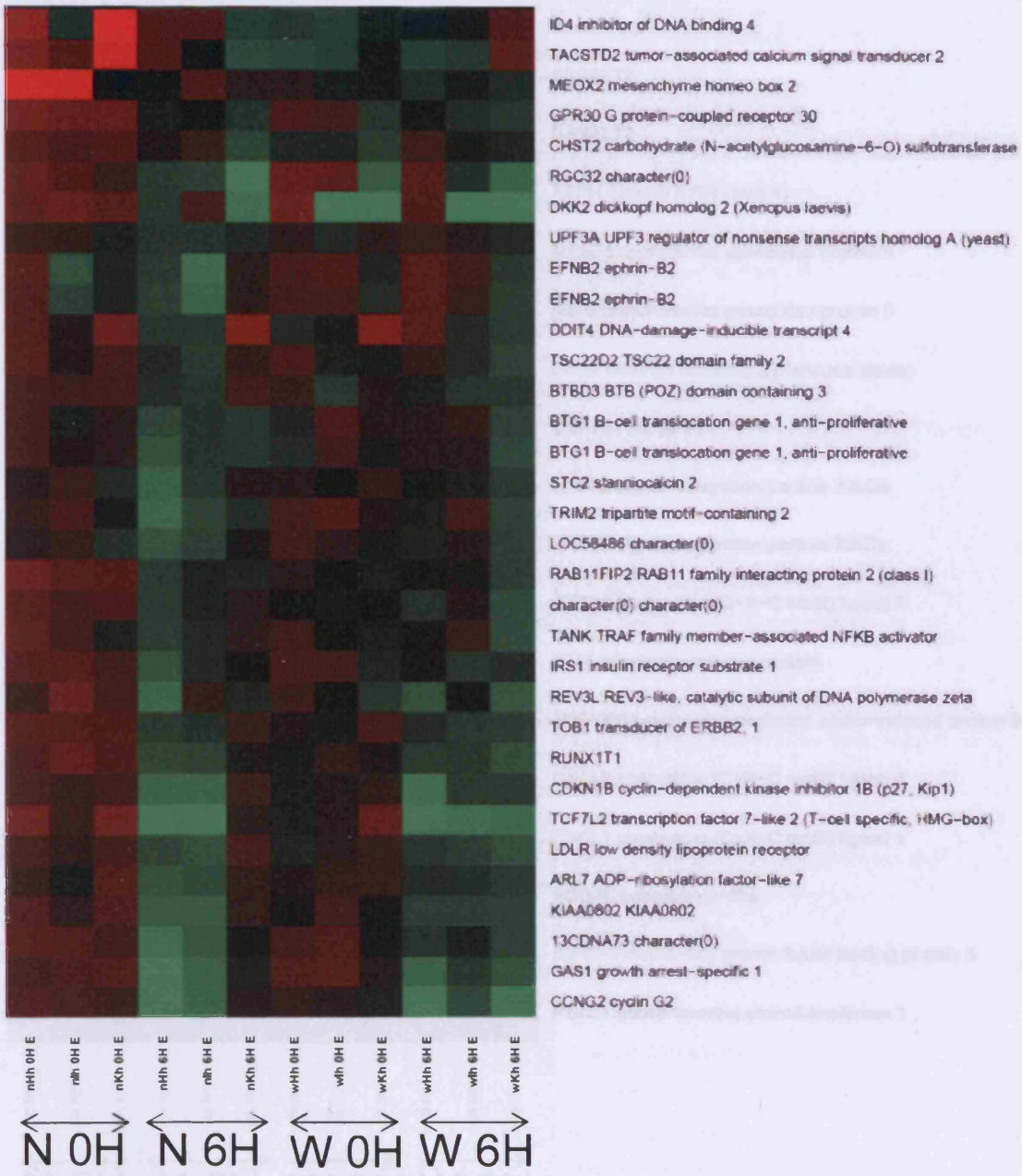


Figure 4.9b: Difference of Differences analysis Early Time Point, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

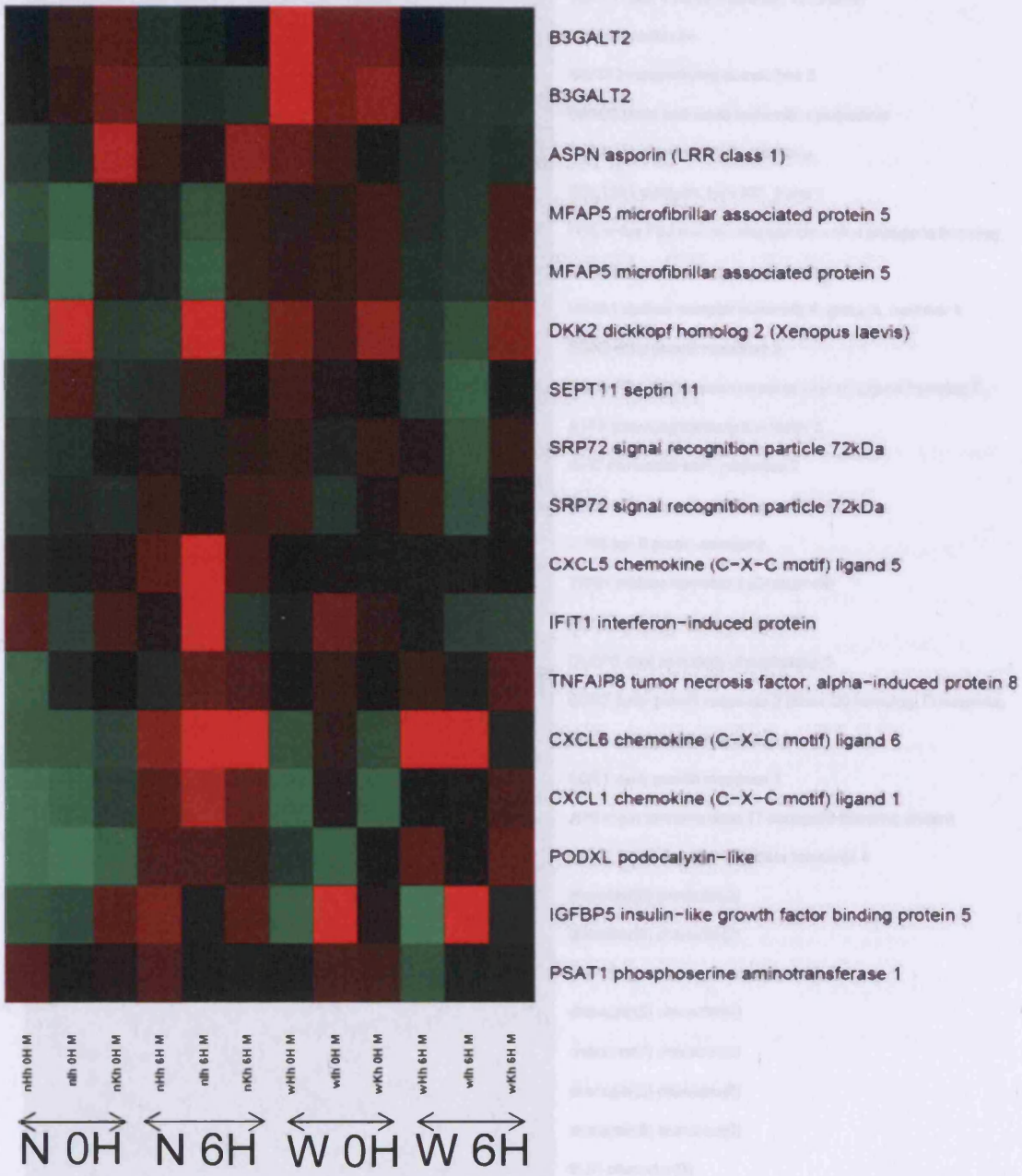


Figure 4.10: Difference of Differences analysis Mid Time Point, genes down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.11: Difference of Differences analysis Mid Time Point, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

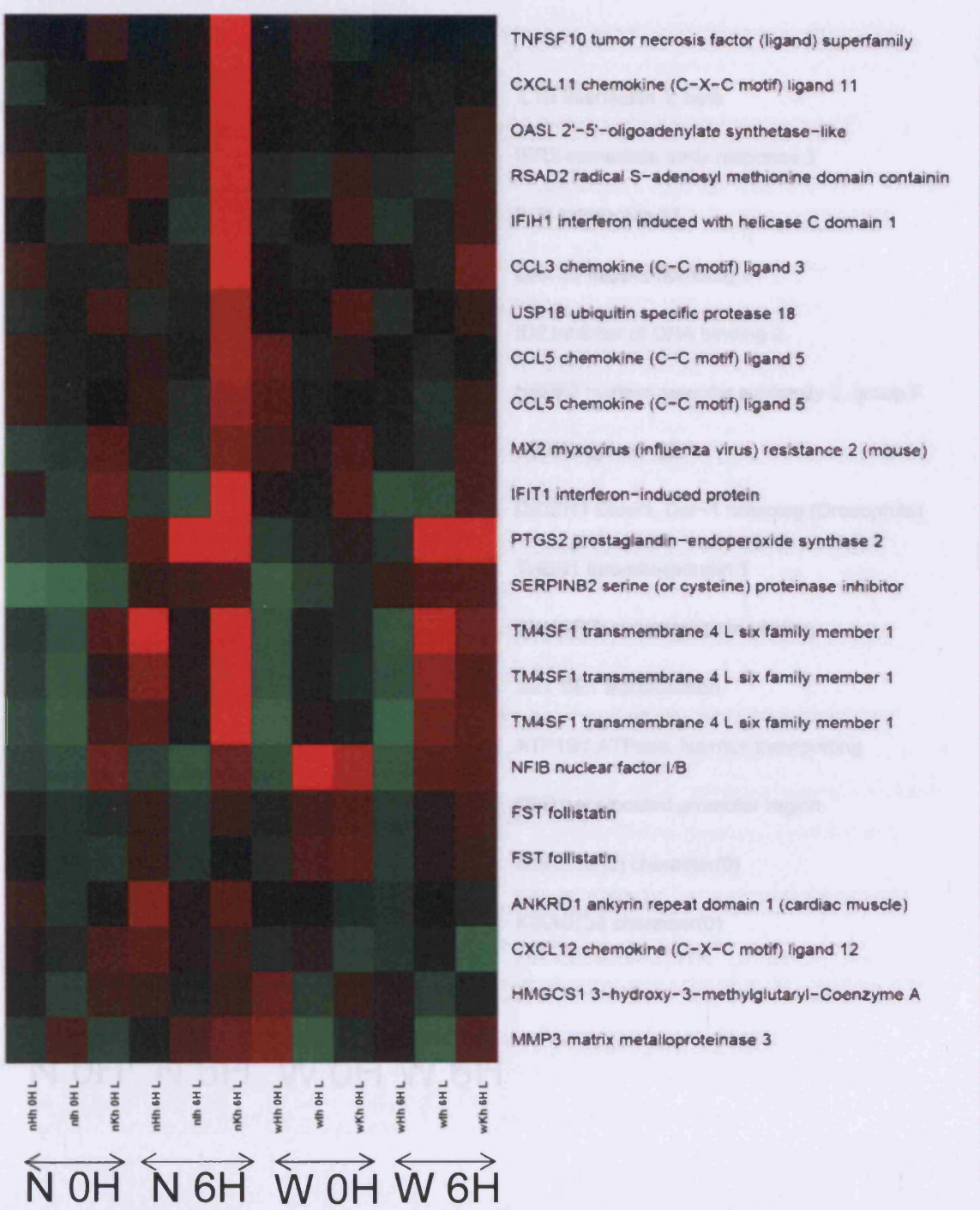


Figure 4.12 Difference of Differences analysis Late Time Point, genes down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

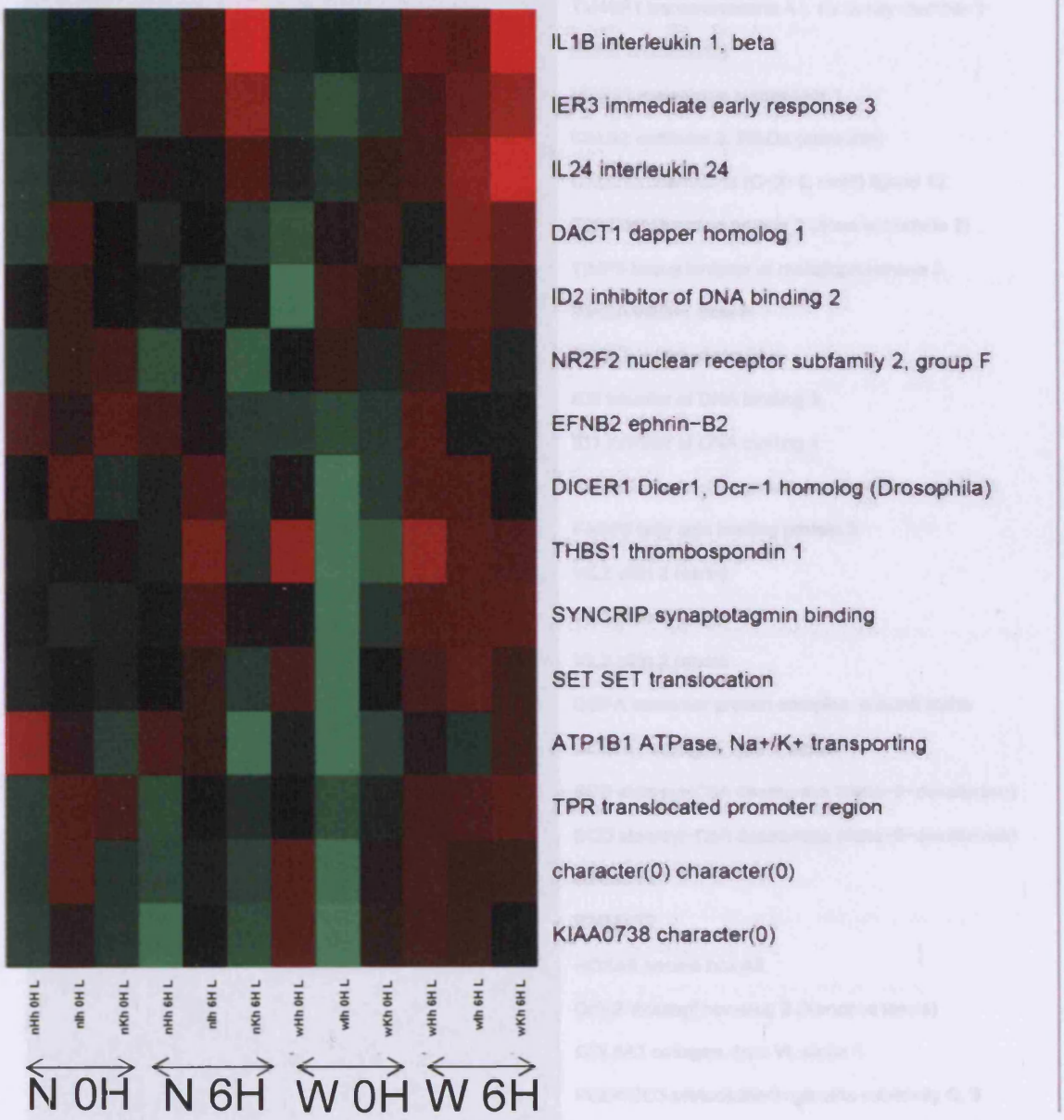


Figure 4.13 Difference of Differences analysis Late Time Point, genes Up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

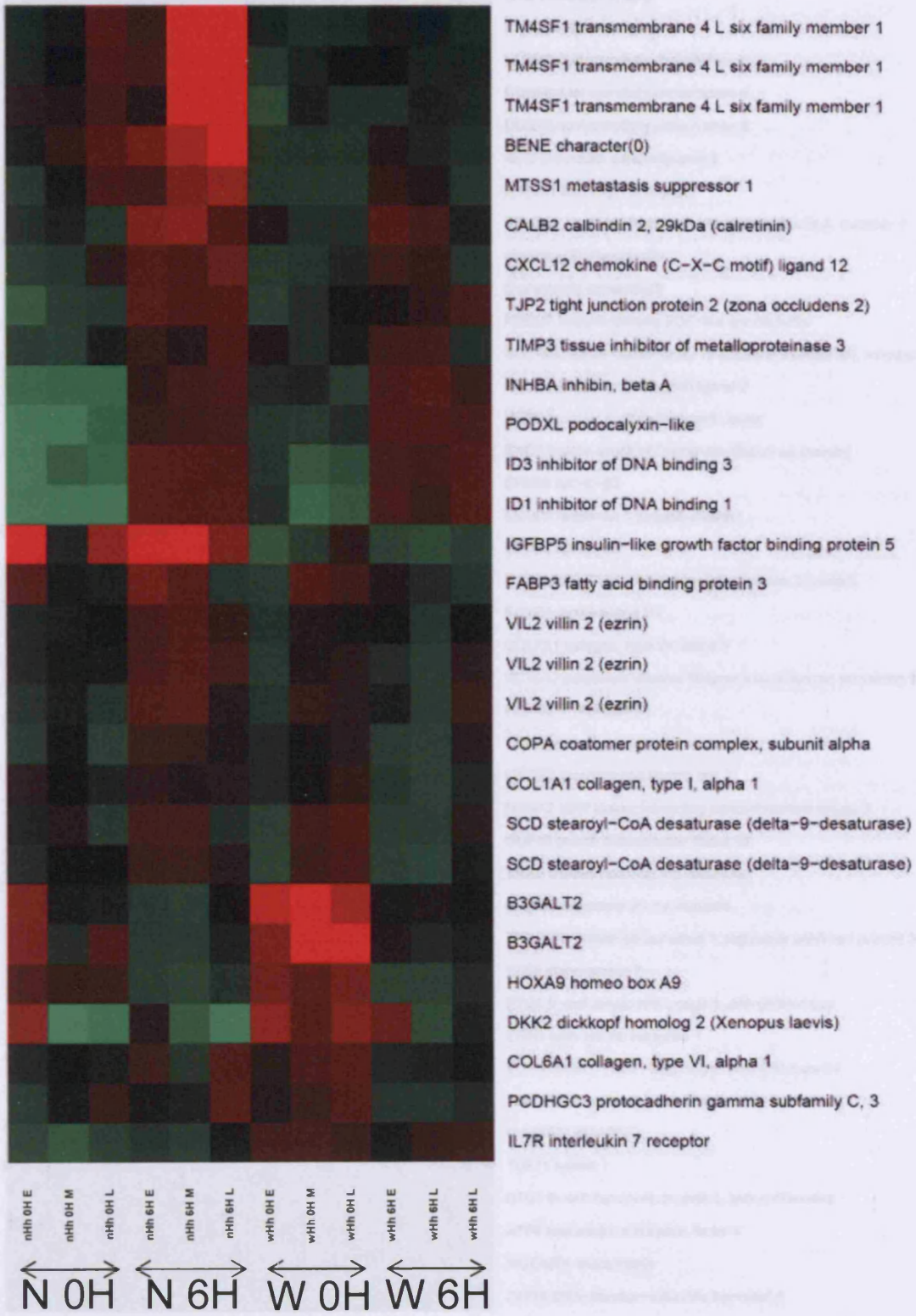


Figure 4.14 Difference of Differences analysis Patient H, genes Down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.15 Difference of Differences analysis Patient H, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.16 Difference of Differences analysis Patient I, genes down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

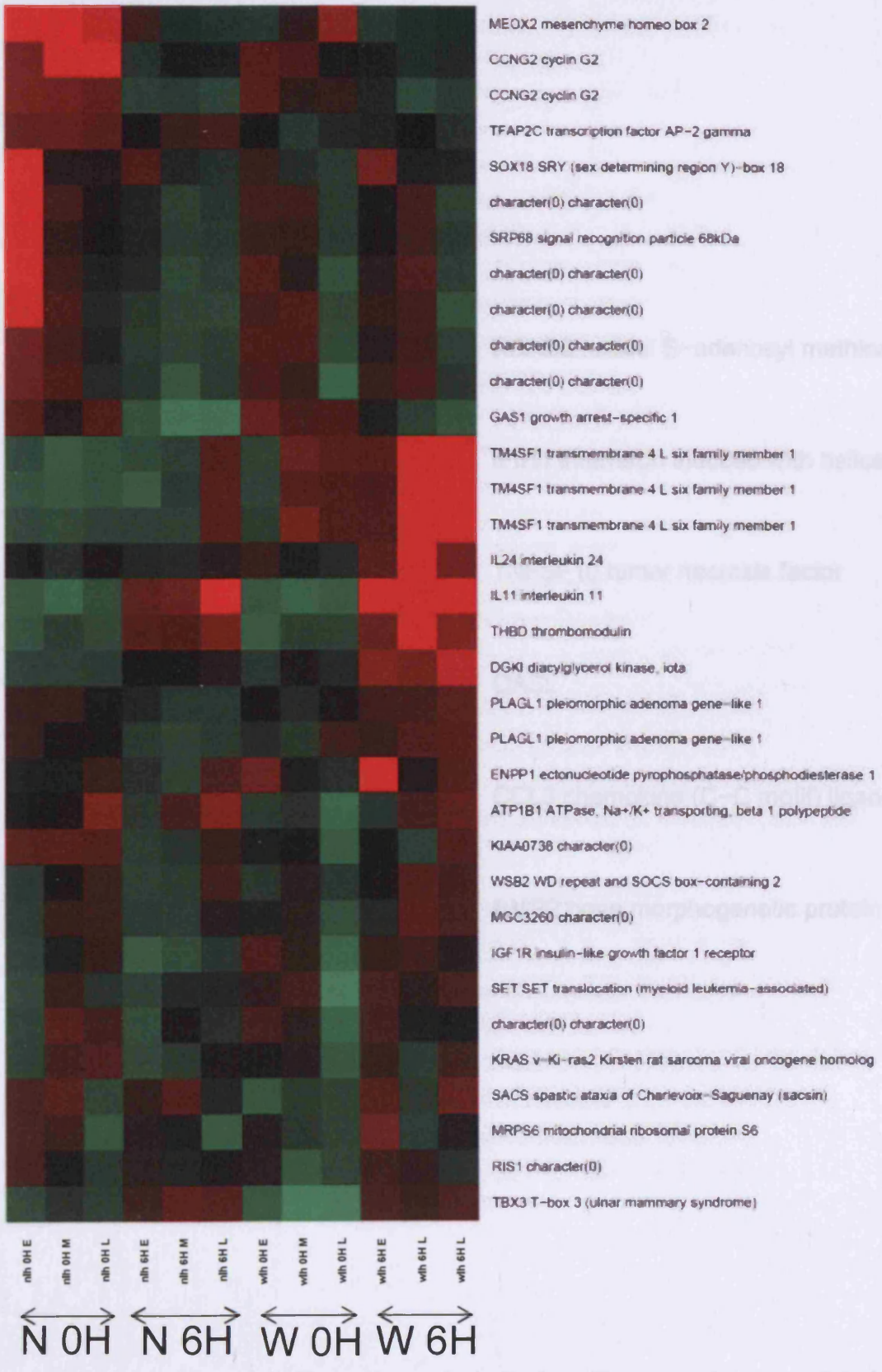


Figure 4.17 Difference of Differences analysis Patient I, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

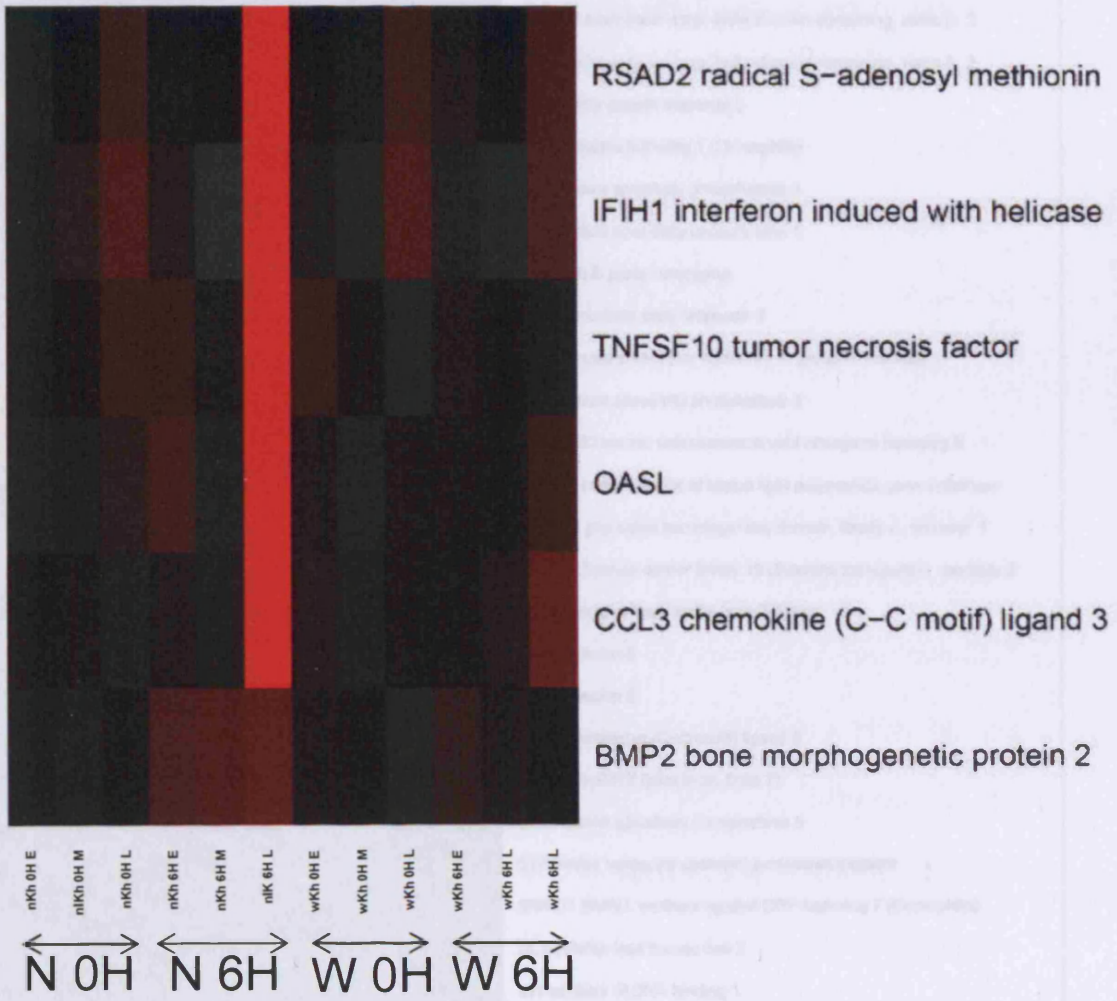


Figure 4.18 Difference of Differences analysis Patient K, genes down-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

Figure 4.19 Difference of Differences analysis Patient K, genes up-regulated by addition of serum. An increase in gene expression compared to the average expression of all genes present is represented in red, a decrease in expression is represented in green with the intensity of colour indicating the level of change

regulated by the addition of serum in CWF compared to NF (Fig 4.9). At the mid time point in culture 14 genes were identified as being down regulate by the addition of serum in CWF compared to NF (Fig 4.10) and 28 genes as being up regulated by the addition of serum in CWF compared to NF (Fig 4.11). At the late time point in culture 19 genes were identified as being down regulate by the addition of serum in CWF compared to NF (Fig 4.12) and 14 genes as being up regulated by the addition of serum in CWF compared to NF (Fig 4.13). Finally the data was analysed with respect to the individual patients with the different time points in culture pooled together. For patient H 23 genes were identified as being down regulated in CWF compared to patient matched NF by the addition of serum (Fig 4.14) and 39 genes were identified as being up regulated in CWF compared to patient matched NF (Fig 4.15). In patient I 51 genes were identified as being down regulated in CWF compared to patient matched NF by the addition of serum (Fig 4.16) and 24 genes were identified as being up regulated in CWF compared to patient matched NF (Fig 4.17). Finally in patient K 6 genes were identified as being down regulated in CWF compared to patient matched NF by the addition of serum (Fig 4.18) and 33 genes were identified as being up-regulated in CWF compared to patient matched NF (Fig 4.19). The difference of the differences analysis identified genes which were not seen in the ANOVA analysis. Difference of differences analysis focussed on changes in the response to serum between NF and CWF. Differences in the response to serum by CWF identified using this technique may relate to differences in wound healing potential.

4.2.4 Over representation analysis

Gene lists generated by ANOVA analysis and from pooled Difference of Differences analysis were first uploaded onto MADRAS. This system allows for the graphical representation of gene expression levels for all experiments as well as containing information about genes represented on Affymetrix™ GeneChip®. Importantly MADRAS allows for comparisons between gene lists. This function was used to remove genes altered by immortalisation and genes altered between patients from both the ANOVA and Difference of Differences generated gene lists (Fig 4.20). Genes altered by the addition of hTERT to the CWF and NF cells were identified by ANOVA analysis (Figs 4.4 & 4.5). They represent differences between the primary and immortalised cells; one of the aims of this thesis was to use immortalised cells as a model of chronic wounds. Genes altered by immortalisation were therefore excluded from further analysis as they do not reflect gene expression levels in the primary cells. Inter-patient variability also represented a problem, genes identified as variable between the three patients were also excluded from further analysis. Genes identified as being differentially regulated between CWF and NF were initially screened for changes due to immortalisation and over time in culture (Fig 4.20a). Genes unchanged by immortalisation of time in culture were then screened against genes known to show inter-patient variability (Fig 4.20b). Corrected gene lists were analysed by the Functional Annotation Tool from the DAVID Bioinformatics Resources web site (http://david.abcc.ncifcrf.gov/). Briefly this tool suite provides annotation and Gene Ontology (GO) term enrichment analysis to highlight the most relevant GO terms associated with the inputted gene list. Gene Ontology (www.geneontology.org) is a defined method for describing a gene by molecular function, biological processes and the cellular location of the gene product. The DAVID software covers over 40

Term	Count	% of Genes in List	P value	Genes
protein binding	37	37.00%	0.028766425	NUP205, MFGE8, RAC1, ACTR3B, PIK3R1, CNOT4, PTPRD, DKK1, IL11, HMG20A, DKK13, SNX13, FGF1, CD9, ORC5L, RABGEF1, HGF, EIF2AK1, DZIP3, GNAI1, MDK, ZFP161, NFIL3, TIAM1, CD58, MKRN1, MAGI2, PTN, ARPC1A, PDLIM5, PRRX1, DNMBP, SPON2, TSPYL2, DMD, SIX1, LDB2,
development	23	23.00%	0.004389058	MDK, SEMA5a, FBN2, RAC1, SPOCK, THUMPD2, IL11, SRPK2, DKK1, GPR65, PTN, FGF1, DPYSL3, CUTL1, PDLIM5, PRRX1, CD9, SPON2, TSPYL2, DMD, SIX1, IFRD1, LDB2,
extracellular space	9	9.00%	0.010360235	MDK, MFAP5, PTN, FGF1, SPOCK, SPON2, ECM1, IL11, F8,
developmental protein	8	8.00%	0.029130787	DKK13, MDK, SEMA5A, FGF1, CUTL1, PRRX1, SIX1, IFRD1,
sequence-specific DNA binding	7	7.00%	0.044487279	NFIL3, CUTL1, PRRX1, ORC5L, TSPYL2, SIX1, DKK1,
growth factor activity	6	6.00%	0.004908495	HGF, DKK1, MDK, PTN, FGF1, IL11,
extracellular matrix (sensu Metazoa)	6	6.00%	0.025645193	MFAP5, FBN2, SPOCK, SPON2, ECM1, DMD,
extracellular matrix	6	6.00%	0.026934459	MFAP5, FBN2, SPOCK, SPON2, ECM1, DMD,
protein complex assembly	6	6.00%	0.034382133	SRPK2, NAP1L3, H2AFV, CD9, LPXN, TSPYL2,
growth factor	5	5.00%	0.009505331	HGF, MDK, PTN, FGF1, IL11,
extracellular matrix	5	5.00%	0.021750277	MFAP5, FBN2, SPOCK, SPON2, ECM1,
REGULATION OF ACTIN CYTOSKELETON	5	5.00%	0.023041083	TIAM1, RAC1, FGF1, PIK3R1, ARPC1A,
blood coagulation	4	4.00%	0.026681393	HGF, CD9, IL11, F8,
coagulation	4	4.00%	0.02750818	HGF, CD9, IL11, F8,
hemostasis	4	4.00%	0.030951296	HGF, CD9, IL11, F8,
wound healing	4	4.00%	0.031845972	HGF, CD9, IL11, F8,
Cell division and chromosome partitioning	4	4.00%	0.039843129	DZIP3, 55841, CUTL1, PIK3R1,
regulation of body fluids	4	4.00%	0.041530846	HGF, CD9, IL11, F8,
heparin binding	3	3.00%	0.012005315	MDK, PTN, FGF1,
mitogen	3	3.00%	0.028131291	MDK, PTN, FGF1,
SF001949:pleiotrophin	2	2.00%	0.012269823	MDK, PTN,
L-carnitine dehydratase/bile acid-inducible protein F	2	2.00%	0.015381727	AMACR, C7orf10,
PTN/MK heparin-binding protein	2	2.00%	0.015381727	MDK, PTN,
PTN	2	2.00%	0.015599888	MDK, PTN,
domain:F5/8 type C 2	2	2.00%	0.035344181	MFGE8, F8,
domain:F5/8 type C 1	2	2.00%	0.035344181	MFGE8, F8,
transmembrane receptor protein tyrosine phosphatase signaling pathway	2	2.00%	0.045255371	PTN, PTPRD,

Table 4.4: Over-representation analysis of genes up-regulated in CWF compared to NF, genes altered by immortalisation and patient differences were removed. Genes were grouped according to over-represented term and listed according to P value with a cut off of 0.05.

Term	Count	% of Genes in List	P value	Genes
glycoprotein	22	30.99%	0.019850983	GYPE, F10, CD83, MMP3, PLOD1, CD40, ADRA2A, SLIT3, AMBP, C3, NGFB, CYBB, CLEC3B, BAMBI, THBS4, MASP1, NCAM1, CSPG4, TRPC6, MCAM, TRPV2, ST3GAL1,
glycosylation site:N-linked (GlcNAc...)	21	29.58%	0.035566759	GYPE, F10, CD83, MMP3, PLOD1, CD40, ADRA2A, SLIT3, AMBP, C3, NGFB, CYBB, BAMBI, THBS4, MASP1, NCAM1, CSPG4, TRPC6, MCAM, TRPV2, ST3GAL1,
development	19	26.76%	0.002967538	RPS19, RAPGEFL1, HOXB7, FGF13, HOXB6, PLOD1, CD40, DUSP22, S100A5, CECR1, SLIT3, NGFB, CLEC3B, TBX1, NCAM1, CSPG4, NEUROD2, MCAM, PPARD,
signal	18	25.35%	0.044	GYPE, F10, CD83, MMP3, PLOD1, CD40, CECR1, SLIT3, AMBP, C3, NGFB, CLEC3B, BAMBI, THBS4, MASP1, NCAM1, CSPG4, MCAM,
disulfide bond	17	23.94%	0.023534038	F10, CD83, MMP3, CD40, CD81, ADRA2A, SLIT3, AMBP, C3, NGFB, CLEC3B, THBS4, MASP1, NCAM1, CSPG4, MCAM, ST3GAL1,
direct protein sequencing	17	23.94%	0.043527695	GYPE, RPS19, F10, CD83, RPS2, MMP3, PLOD1, CD40, Sep-06, ADRA2A, C3, AMBP, CYBB, CLEC3B, BAMBI, CA2, MCAM,
structural molecule activity	12	16.90%	7.62E-04	SLIT3, LOC148430, RPS19, ACTC, RPL31, EPB41L3, THBS4, CLDN11, RPS2, RPL39L, KRT7, S100A5,
extracellular region	11	15.49%	0.04030241	MAP2K2, SLIT3, AMBP, C3, CLEC3B, F10, FGF13, THBS4, MASP1, MMP3, CSPG4,
EGF-like	6	8.45%	0.013497331	SLIT3, DKFZP586H2123, F10, THBS4, MASP1, CD40,
EGF-like region	6	8.45%	0.014409642	SLIT3, DKFZP586H2123, F10, THBS4, MASP1, CD40,
EGF-like calcium-binding	5	7.04%	0.003270514	SLIT3, DKFZP586H2123, F10, THBS4, MASP1,
humoral immune response	5	7.04%	0.012314652	C3, CYBB, MASP1, CD83, CD40,
EGF-like, type 3	5	7.04%	0.021191091	SLIT3, DKFZP586H2123, F10, THBS4, MASP1,
structural constituent of ribosome	5	7.04%	0.025082134	LOC148430, RPS19, RPL31, RPS2, RPL39L,
ribosome	5	7.04%	0.026042652	LOC148430, RPS19, RPL31, RPS2, RPL39L,
ribonucleoprotein	5	7.04%	0.027818444	RPS19, 54039, RPL31, RPS2, RPL39L,
glycosylation site:O-linked (GalNAc...)	4	5.63%	0.004519097	AMBP, CLEC3B, GYPE, F10,
MAPKKK cascade	4	5.63%	0.013625548	ADRA2A, AMBP, DUSP22, CD81,
SM00179:EGF_CA	4	5.63%	0.017201109	SLIT3, F10, THBS4, MASP1,
plasma	4	5.63%	0.023969735	AMBP, C3, CLEC3B, F10,
humoral defense mechanism (sensu Vertebrata)	4	5.63%	0.027541298	C3, CYBB, MASP1, CD40,
serine-type endopeptidase activity	4	5.63%	0.029904848	C3, DKFZP586H2123, F10, MASP1,
serine-type peptidase activity	4	5.63%	0.038791578	C3, DKFZP586H2123, F10, MASP1,
SM00042:CUB	3	4.23%	0.0189042	DKFZP586H2123, MASP1, PDGFD,
small ribosomal subunit	3	4.23%	0.019450491	LOC148430, RPS19, RPS2,
cytosolic ribosome (sensu Eukaryota)	3	4.23%	0.024685562	RPS19, RPS2, RPL39L,
IPR000859:CUB	3	4.23%	0.0253641	DKFZP586H2123, MASP1, PDGFD,
regulation of MAPK activity	3	4.23%	0.035179861	ADRA2A, DUSP22, CD81,
serine proteinase	3	4.23%	0.042107423	C3, F10, MASP1,
glycosylation site:O-linked (Xyl...) (chondroitin sulfate)	2	2.82%	0.023385439	AMBP, CSPG4,
Basic mechanism of action of PPARa	2	2.82%	0.032184646	RXRA, PPARD,
sialoglycoprotein	2	2.82%	0.03274671	GYPE, NCAM1,
alpha-crystallin-related small heat shock protein	2	2.82%	0.034537155	HSPB3, HSPB7,
Alpha crystallin	2	2.82%	0.046240368	HSPB3, HSPB7,
beta-hydroxyaspartic acid	2	2.82%	0.048722306	F10, THBS4,

Table 4.5: Over-representation analysis of genes down-regulated in CWF compared to NF, genes altered by immortalisation and patient differences were removed. Genes are grouped according to overrepresented term and listed according to P value with a cut off of 0.05.

Term	Count	% of Genes in List	P value	Genes
nuclear protein	31	36.05%	4.83E-04	JUNB, TFAP2C, MEOX2, FOS, SMAD7, JUN, VDR, FOSB, EGR1, SOX18, PHLDA1, PLAGL1, PLAGL1, TGIF, ATF4, DUSP5, EGR3, DGKI, BHLHB2, BHLHB2, ID1, FOXD1, OSR2, DUSP2, EGR2, TBX3, ZFP36, NR4A1, LOC389168, ZBTB38, TRIB3, DLX2, ZNF281,
dna-binding	22	25.58%	2.05E-05	ATF4, JUNB, TFAP2C, MEOX2, EGR3, BHLHB2, BHLHB2, FOXD1, FOS, EGR2, TBX3, ZFP36, JUN, NR4A1, VDR, FOSB, EGR1, SOX18, ZBTB38, DLX2, PLAGL1, PLAGL1, ZNF281, TGIF,
transcription regulation	21	24.42%	6.09E-05	ATF4, JUNB, TFAP2C, MEOX2, EGR3, BHLHB2, BHLHB2, FOXD1, SMAD7, EGR2, TBX3, JUN, NR4A1, VDR, EGR1, SOX18, ZBTB38, TRIB3, DLX2, PLAGL1, PLAGL1, ZNF281, TGIF,
transcription	19	22.09%	6.95E-04	ATF4, JUNB, TFAP2C, EGR3, BHLHB2, BHLHB2, FOXD1, SMAD7, EGR2, TBX3, JUN, NR4A1, VDR, EGR1, SOX18, ZBTB38, TRIB3, PLAGL1, PLAGL1, ZNF281, TGIF,
MAPK SIGNALING PATHWAY	11	12.79%	6.71E-05	ATF4, DUSP5, IL1B, IL1B, JUN, NR4A1, KRAS, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, MKNK2, FOS, DUSP2,
DNA binding	11	12.79%	7.56E-05	ATF4, JUNB, MEOX2, ZFP36, NR4A1, VDR, EGR3, EGR1, SOX18, DLX2, FOS,
developmental protein	9	10.47%	0.00653687	TBX3, MEOX2, EREG, EFNB2, EFNB2, ID1, VEGF, VEGF, DKK2, DLX2, JAG1,
phosphoprotein	9	10.47%	0.01062178	IGF1R, IL6, NFKBIA, ENPP1, DUSP5, DUSP1, DUSP1, LOC389168, NEF3, DUSP2,
CANCER	9	10.47%	0.02209049	IL6, IL1B, IL1B, GAS1, VDR, KRAS, IL8, IL8, IL24, VEGF, VEGF, IL8, IL8,
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	8	9.30%	0.00564969	IL6, CCL2, IL1B, IL1B, IL8, IL8, IL24, VEGF, VEGF, IL8, IL8, IL11,
:TOLL-LIKE RECEPTOR SIGNALING PATHWAY	7	8.14%	1.69E-04	IL6, NFKBIA, IL1B, IL1B, JUN, IL8, IL8, IL8, IL8, FOS,
cytokine	7	8.14%	5.41E-04	IL6, CCL2, IL1B, IL1B, GDF15, IL8, IL8, IL24, IL11,
activator	7	8.14%	0.02176692	EGR2, ATF4, TFAP2C, EGR1, ZBTB38, SOX18, PLAGL1, PLAGL1,
growth factor	6	6.98%	0.00106309	IL6, GDF15, EREG, HBEGF, VEGF, VEGF, IL11,
Basic-leucine zipper (bZIP) transcription factor	5	5.81%	2.64E-04	ATF4, JUNB, JUN, FOSB, FOS,
LUPUS	5	5.81%	2.65E-04	IL6, IL1B, IL1B, VDR, IL8, IL8, IL8, IL8,
BRLZ	5	5.81%	6.18E-04	ATF4, JUNB, JUN, FOSB, FOS,
EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	5	5.81%	7.91E-04	NFKBIA, JUN, IL8, IL8, HBEGF, IL8, IL8,
NFkB activation by Nontypeable Hemophilus influenzae	5	5.81%	0.0016108	NFKBIA, IL1B, IL1B, DUSP1, DUSP1, IL8, IL8, IL8, IL8,
INFECTION	5	5.81%	0.01328811	IL6, IL1B, IL1B, VDR, IL8, IL8, IL8, IL8,
zinc finger	5	5.81%	0.01792951	NR4A1, VDR, EGR3, EGR1, ZNF281,
nucleus	5	5.81%	0.03438949	MEOX2, NR4A1, LOC389168, DLX2, DUSP2,
proto-oncogene	5	5.81%	0.0497628	JUN, KRAS, LOC389168, BTG1, BTG1, FOS,
dual specificity protein phosphatase (MAP kinase phosphatase)	4	4.65%	1.40E-05	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
MAP kinase phosphatase	4	4.65%	2.34E-05	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
Rhodanese-like	4	4.65%	3.01E-04	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
RHOD	4	4.65%	5.34E-04	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
Dual specificity protein phosphatase	4	4.65%	6.67E-04	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
DSPc	4	4.65%	7.24E-04	DUSP5, DUSP1, DUSP1, DUSP6, DUSP6, DUSP6, DUSP2,
bZIP transcription factor, bZIP_1	4	4.65%	8.32E-04	ATF4, JUNB, JUN, FOS,
Genomic_reformatting_Brain_Ischemia	4	4.65%	0.0017469	JUNB, IL6, JUN, EGR1,
Cadmium induces DNA synthesis and proliferation in macrophages	4	4.65%	0.00306722	NFKBIA, JUN, KRAS, FOS,
Regulation of hematopoiesis by cytokines	4	4.65%	0.0037228	IL6, IL8, IL8, IL8, IL8, IL11,
IGF-1 Signaling Pathway	4	4.65%	0.00617828	IGF1R, JUN, KRAS, FOS,
IL 6 signaling pathway	4	4.65%	0.00716817	IL6, JUN, KRAS, FOS,

Table 4.6: Over-representation analysis of genes up-regulated in NF compared to CWF across all patients. Genes were identified by difference of the difference analysis. Genes altered by immortalisation and patient differences were removed. Genes were grouped according to over-represented term and listed according to P value with a cut off of 0.01.

		In List		
signal	28	35.44%	2.28E-04	PCDHGA12, PODXL, PCDHGA12, IGFBP5, CXCL1, PCDHGA12, TNFAIP6, TNFAIP6, CXCL3, COL6A1, CXCL5, CCL3L3, TIMP3, PCDHGA12, PCDHGA12, CXCL12, DKK2, PCDHGA12, , JAG1, JAG1, ICAM1, IL7R, BMP2, CCL3L3, CXCL10, CXCL2, INHBA, CXCL11, CXCL11, HBEGF,
direct protein sequencing	21	26.58%	0.02269497	G1P2, VIL2, VIL2, VIL2, IGFBP5, MX1, CXCL1, CCL3L3, COPA, CXCL10, TNFAIP6, TNFAIP6, CXCL3, COL6A1, CXCL2, CXCL5, CCL3L3, TIMP3, CXCL11, CXCL11, FABP3, HBEGF, PLSCR1, PLSCR1, , ICAM1,
interferon induction	16	20.25%	3.54E-24	IFITM1, IFITM1, G1P2, MX1, DDX58, CXCL10, IFIT2, IFIT5, OAS2, OAS1, OAS1, IFIT1, IFI44, GBP1, GBP1, IFI16, IFI16, OASL, OASL, MX2, IFIT3,
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	12	15.19%	5.54E-07	TNFSF10, CXCL2, CCL3L3, CXCL5, IL7R, INHBA, CXCL11, CXCL11, BMP2, CXCL12, CXCL1, CXCL10, CXCL3,
cytokine	11	13.92%	6.39E-08	TNFSF10, CXCL2, CCL3L3, CXCL5, CXCL11, CXCL11, BMP2, CXCL12, CXCL1, CCL3L3, CXCL10, CXCL3,
Small chemokine, interleukin-8-like	9	11.39%	1.41E-10	CXCL2, CCL3L3, CXCL5, CXCL11, CXCL11, CXCL12, CXCL1, CCL3L3, CXCL10, CXCL3,
SM00199:SCY	9	11.39%	5.58E-10	CXCL2, CCL3L3, CXCL5, CXCL11, CXCL11, CXCL12, CXCL1, CCL3L3, CXCL10, CXCL3,
cell adhesion	9	11.39%	0.00129348	COL6A1, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, TNFAIP6, TNFAIP6, ICAM1,
calcium	9	11.39%	0.032281709	PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, CALB2, PLSCR1, PLSCR1, JAG1, JAG1,
sensory transduction	8	10.13%	5.86E-04	CXCL2, CCL3L3, TIMP3, CXCL11, CXCL11, CXCL12, CCL3L3, CXCL10, CXCL3,
Small chemokine, C-X-C	7	8.86%	2.50E-10	CXCL2, CXCL5, CXCL11, CXCL11, CXCL12, CXCL1, CXCL10, CXCL3,
chemotaxis	7	8.86%	1.83E-06	CXCL2, CCL3L3, CXCL11, CXCL11, CXCL12, CCL3L3, CXCL10, CXCL3,
beta-thromboglobulin	6	7.59%	1.72E-08	CXCL2, CXCL5, CXCL12, CXCL1, CXCL10, CXCL3,
Cadherin-like	6	7.59%	1.66E-05	PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12,
inflammatory response	6	7.59%	3.14E-05	CXCL2, CCL3L3, CXCL11, CXCL11, CXCL1, CXCL10, CXCL3,
Cadherin	6	7.59%	3.46E-04	PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12,
SM00112:CA	6	7.59%	8.48E-04	PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12, PCDHGA12,
Small chemokine, C-X-C/Interleukin 8	5	6.33%	1.92E-06	CXCL2, CXCL5, CXCL12, CXCL1, CXCL3,
antiviral defense	5	6.33%	4.79E-05	IFIH1, MX1, DDX58, MX2, PLSCR1, PLSCR1,
growth factor	5	6.33%	0.007575534	INHBA, BMP2, CXCL12, CXCL1, HBEGF,
Tetratricopeptide-like helical	5	6.33%	0.016352099	IFIT1, SAMD9, IFIT5, IFIT2, IFIT3,
interferon-induced 56K protein	4	5.06%	2.43E-06	IFIT1, IFIT5, IFIT2, IFIT3,
Tetratricopeptide TPR_2	4	5.06%	0.002507113	IFIT1, IFIT5, IFIT2, IFIT3,
Chemokine_families	4	5.06%	0.009279094	CCL3L3, CXCL11, CXCL11, CXCL12, CXCL10,
tpr repeat	4	5.06%	0.022129383	IFIT1, IFIT5, IFIT2, IFIT3,
TGF-BETA SIGNALING PATHWAY	4	5.06%	0.022317208	INHBA, BMP2, ID1, ID3,
Tetratricopeptide TPR_1	4	5.06%	0.022879146	IFIT1, IFIT5, IFIT2, IFIT3,
2-5-oligoadenylate synthetase	3	3.80%	2.35E-04	OAS1, OAS1, OAS2, OASL, OASL,
:2-5 oligoadenylate synthetase, ubiquitin-like region	3	3.80%	3.91E-04	OAS1, OAS1, OAS2, OASL, OASL,
PAP/25A core	3	3.80%	0.002505496	OAS1, OAS1, OAS2, OASL, OASL,
Caspase Recruitment	3	3.80%	0.008417984	IFIH1, RIPK2, DDX58,
DNA replication, recombination, and repair	3	3.80%	0.047002226	IFIT1, IFIH1, DDX58,
dynamin-related protein VPS1	2	2.53%	0.025877093	MX1, MX2,
Antiviral defense	2	2.53%	0.027019297	G1P2, IRF7,
Dynamin GTPase effector	2	2.53%	0.037405996	MX1, MX2,
transcription repressor Id-2	2	2.53%	0.042764316	ID1, ID3,

Table 4.7: Over-representation analysis of genes down-regulated in NF compared to CWF across all patients. Genes were identified by difference of the difference analysis. Genes altered by immortalisation and patient differences were removed. Genes were grouped according to over-represented term and listed according to P value with a cut off of 0.05.

Term	Count	% of Genes in List	P value	Genes
nuclear protein	34	35.42%	7.18E-05	JUNB, CDKN1B, MEOX2, ID2, TPR, NR2F2, RKHD1, RUNX1T1, FOS, JUN, FOSB, EGR1, SOX18, PIK3C2A, ATF3, DACT1, SYNCRIP, PLAGL1, UPF3A, ARL7, MYBL1, DUSP5, EGR3, TCF7L2, KLF4, DUSP2, NFIL3, EGR2, TBX3, REV3L, ID4, NR4A1, LOC389168, ZBTB38,
dna-binding	21	21.88%	1.26E-04	JUNB, MYBL1, MEOX2, EGR3, TCF7L2, KLF4, NR2F2, FOS, RUNX1T1, NFIL3, EGR2, TBX3, REV3L, JUN, NR4A1, FOSB, EGR1, SOX18, ZBTB38, ATF3, PLAGL1,
transcription regulation	18	18.75%	0.00252764	JUNB, MYBL1, MEOX2, EGR3, TCF7L2, KLF4, NR2F2, RUNX1T1, NFIL3, EGR2, TBX3, JUN, NR4A1, EGR1, SOX18, ZBTB38, ATF3, PLAGL1,
transcription	16	16.67%	0.01638153	JUNB, MYBL1, EGR3, TCF7L2, KLF4, NR2F2, RUNX1T1, EGR2, TBX3, JUN, NR4A1, EGR1, SOX18, ZBTB38, ATF3, PLAGL1,
zinc-finger	13	13.54%	0.03779863	EGR3, TRIM2, KLF4, NR2F2, RKHD1, RUNX1T1, EGR2, TANK, REV3L, NR4A1, EGR1, EGR1, ZBTB38, PLAGL1,
DNA binding	9	9.38%	0.00228779	JUNB, MYBL1, MEOX2, NR4A1, EGR3, EGR1, EGR1, SOX18, NR2F2, FOS,
MAPK SIGNALING PATHWAY	8	8.33%	0.00368293	DUSP5, IL1B, JUN, NR4A1, DUSP1, DUSP6, DUSP6, FOS, DUSP2,
activator	8	8.33%	0.0074968	EGR2, MYBL1, TCF7L2, EGR1, EGR1, ZBTB38, SOX18, KLF4, PLAGL1, PLAGL1, PLAGL1,
Basic-leucine zipper (bZIP) transcription factor	6	6.25%	2.33E-05	NFIL3, JUNB, JUN, FOSB, ATF3, FOS,
BRLZ	6	6.25%	4.54E-05	NFIL3, JUNB, JUN, FOSB, ATF3, FOS,
proto-oncogene	6	6.25%	0.01456013	JUN, LOC389168, TPR, BTG1, BTG1, FOS, RUNX1T1,
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	6	6.25%	0.04293483	CCL2, IL1B, IL8, IL8, IL24, IL8, IL8, IL11,
TOLL-LIKE RECEPTOR SIGNALING PATHWAY	5	5.21%	0.00628661	IL1B, JUN, IL8, IL8, IL8, IL8, FOS,
cytokine	5	5.21%	0.0218373	CCL2, IL1B, IL8, IL8, IL24, IL11,
nucleus	5	5.21%	0.03894905	MYBL1, MEOX2, NR4A1, LOC389168, DUSP2,
dual specificity protein phosphatase (MAP kinase phosphatase)	4	4.17%	2.25E-05	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
MAP kinase phosphatase	4	4.17%	3.15E-05	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
Basic leucine zipper	4	4.17%	2.02E-04	NFIL3, FOSB, ATF3, FOS,
Rhodanese-like	4	4.17%	4.03E-04	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
RHOD	4	4.17%	5.63E-04	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
DSPc	4	4.17%	7.64E-04	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
Dual specificity protein phosphatase	4	4.17%	8.90E-04	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
inflammatory response	4	4.17%	0.00658322	CCL2, IL1B, IL8, IL8, CHST2,
NFkB activation by Nontypeable Hemophilus influenzae	4	4.17%	0.00866786	IL1B, DUSP1, IL8, IL8, IL8, IL8,
transcription factor	4	4.17%	0.01555411	JUNB, EGR3, ID2, NR2F2,
Tyrosine specific protein phosphatase and dual specificity protein phosphatase	4	4.17%	0.02115542	DUSP5, DUSP1, DUSP6, DUSP6, DUSP2,
fos transforming protein	3	3.12%	7.62E-04	FOSB, ATF3, FOS,
TSP-1 Induced Apoptosis in Microvascular Endothelial Cell	3	3.12%	0.0055225	JUN, THBS1, FOS,
Regulation of MAP Kinase Pathways Through Dual Specificity Phosphatases	3	3.12%	0.00927263	DUSP1, DUSP6, DUSP6, DUSP2,
inflammation	3	3.12%	0.01143063	CCL2, IL1B, IL8, IL8,
bZIP transcription factor, bZIP_1	3	3.12%	0.01793706	JUNB, JUN, FOS,
114.Genomic_reformatting_Brain_Ischemia	3	3.12%	0.02855055	JUNB, JUN, EGR1, EGR1,
Regulation of hematopoiesis by cytokines	3	3.12%	0.02875015	IL8, IL8, IL8, IL8, IL11,
LUPUS	3	3.12%	0.03759547	IL1B, IL8, IL8, IL8, IL8,
IGF-1 Signaling Pathway	3	3.12%	0.03972245	JUN, 204686_at, FOS,
Pertussis toxin-insensitive CCR5 Signaling in Macrophage	3	3.12%	0.04368458	CCL2, JUN, FOS,
Insulin Signaling Pathway	3	3.12%	0.04368458	JUN, IRS-1, FOS,
Transcription factor Jun	2	2.08%	0.02917256	JUNB, JUN,

Table 4.8: Over-representation analysis of genes up-regulated in NF compared to CWF across all time points in culture. Genes were identified by difference of the difference analysis. Genes altered by immortalisation and patient differences were removed. Genes were grouped according to over-represented term and listed according to P value with a cut off of 0.03.

Term	Count	% of Genes in List	P value	Genes
signal	19	43.18%	1.77E-04	PTGS2, PODXL, IGFBP5, CXCL6, SERPINB2, ASPN, CXCL1, CCL3L3, MMP3, TGFA, CXCL10, CCL5, CCL5, MFAP5, MFAP5, CCL3L3, CXCL5, CXCL11, CXCL12, FST, FST, DKK2,
direct protein sequencing	15	34.09%	0.006598163	IGFBP5, CXCL6, SERPINB2, ASPN, MX1, CXCL1, MMP3, CCL3L3, CXCL10, CCL5, CCL5, Sep-11, CCL3L3, CXCL5, CXCL11, FST,
cytokine	10	22.73%	3.50E-09	TNFSF10, CXCL6, CXCL5, CCL3L3, CXCL11, CXCL11, CXCL12, CXCL1, CCL3L3, CXCL10, CCL5,
CYTOKINE-CYTOKINE RECEPTOR INTERACTION	9	20.45%	9.68E-06	TNFSF10, CXCL6, CXCL5, CCL3L3, CXCL11, CXCL11, CXCL12, CXCL1, CXCL10, CCL5,
SCY	9	20.45%	3.01E-12	CXCL6, CXCL5, CCL3L3, CXCL11, CXCL11, CXCL12, CXCL1, CCL3L3, CXCL10, CCL5, CCL5,
:Small chemokine, interleukin-8-like	9	20.45%	1.34E-12	CXCL6, CXCL5, CCL3L3, CXCL11, CXCL11, CXCL12, CXCL1, CCL3L3, CXCL10, CCL5,
interferon induction	8	18.18%	4.96E-11	OAS1, IFIT1, MX1, OASL, MX2, CXCL10, IFIT2, IFIT3,
sensory transduction	7	15.91%	1.32E-04	CXCL6, CCL3L3, CXCL11, CXCL11, CXCL12, CCL3L3, CXCL10, CCL5, CCL5,
chemotaxis	7	15.91%	5.04E-08	CXCL6, CCL3L3, CXCL11, CXCL11, CXCL12, CCL3L3, CXCL10, CCL5, CCL5,
Chemokine_families	6	13.64%	2.34E-05	CXCL6, CCL3L3, CXCL11, CXCL11, CXCL12, CXCL10, CCL5, CCL5,
Small chemokine, C-X-C	6	13.64%	1.47E-09	CXCL6, CXCL5, CXCL11, CXCL11, CXCL12, CXCL1, CXCL10,
inflammatory response	5	11.36%	4.85E-05	CCL3L3, CXCL11, CXCL11, CXCL1, CXCL10, CCL5, CCL5,
Tetratricopeptide-like helical	4	9.09%	0.018346279	IFIT1, SRP72, SRP72, IFIT2, IFIT3,
TOLL-LIKE RECEPTOR SIGNALING PATHWAY	4	9.09%	0.010600023	CCL3L3, CXCL11, CXCL11, CXCL10, CCL5, CCL5,
TPR	4	9.09%	0.005235576	IFIT1, SRP72, SRP72, IFIT2, IFIT3,
Tetratricopeptide region	4	9.09%	0.005227872	IFIT1, SRP72, SRP72, IFIT2, IFIT3,
Tetratricopeptide TPR_1	4	9.09%	0.005078947	IFIT1, SRP72, SRP72, IFIT2, IFIT3,
tpr repeat	4	9.09%	0.004405128	IFIT1, SRP72, SRP72, IFIT2, IFIT3,
Small chemokine, C-X-C/Interleukin 8	4	9.09%	1.98E-05	CXCL6, CXCL5, CXCL12, CXCL1,
SF002522:beta-thromboglobulin	4	9.09%	1.86E-05	CXCL5, CXCL12, CXCL1, CXCL10,
transcription factor	3	6.82%	0.031733059	ID1, KLF10, ID3,
Tetratricopeptide TPR_2	3	6.82%	0.0106462	IFIT1, IFIT2, IFIT3,
antiviral defense	3	6.82%	0.005672997	IFIH1, MX1, MX2,
Chemokines_in_EAE	3	6.82%	0.003321686	CCL3L3, CXCL10, CCL5, CCL5,
GProt-coupled_Rec_T_Cell_med_Inflam m	3	6.82%	0.003321686	CCL3L3, CXCL10, CCL5, CCL5,
Small chemokine, C-C	3	6.82%	0.003123246	CCL3L3, CCL3L3, CCL5, CCL5,
macrophage inflammatory protein	3	6.82%	8.76E-04	CCL3L3, CCL3L3, CCL5, CCL5,
interferon-induced 56K protein	3	6.82%	1.48E-04	IFIT1, IFIT2, IFIT3,
Dynamin	2	4.55%	0.046364914	MX1, MX2,
PAP/25A core	2	4.55%	0.042874181	OAS1, OASL,
DYNc	2	4.55%	0.030917256	MX1, MX2,
GED	2	4.55%	0.026557629	MX1, MX2,
transcription repressor Id-2	2	4.55%	0.025214085	ID1t, ID3,
Dynamin central region	2	4.55%	0.021666336	MX1, MX2,
Dynamin GTPase effector	2	4.55%	0.021666336	MX1, MX2,

Table 4.9: Over-representation analysis of genes down-regulated in NF compared to CWF across all time points in culture. Genes were identified by difference of the difference analysis. Genes altered by immortalisation and patient differences were removed. Genes were grouped according to over-represented term and listed according to P value with a cut off of 0.02.

annotation categories these include; GO terms, protein-protein interactions, protein functional domains, disease associations, bio-pathways, sequence general features, homologies, gene functional summaries and gene tissue expressions. Genes are grouped and given an EASE score, which is a modified Fisher Exact p-value. The Fisher Exact is adopted to measure the gene-enrichment compared to the human genome background (30,000 gene total). Groups of genes with a p-value of <0.05 were selected for genes up regulated in CWF compared to NF (Table 4.4) and genes down regulated in CWF compared to NF (Table 4.5). Genes identified by difference of differences analysis as having altered responses to serum stimulation were also analysed. They were sub grouped into differences of differences observed when each patients data was analysed separately (Tables 4.6-4.7) and when each time points data was analysed separately (Tables 4.8-4.9). The over representation analysis included all the GO terms. This included GO terms such as 'protein binding' which was identified as the most relevant group of genes up regulated in CWF compared to NF (Table 4.4). The inclusion of potentially irrelevant data in the generated gene lists is a drawback to over representation analysis using this type of software.

4.2.5 Identification of Genes of Interest

Microarray analysis was carried out to identify potential disease marker genes which could then be used for the generation of chronic wound reporter constructs. For this project it was decided that a clear difference in gene expression between CWF and NF was required for a potential reporter gene. Therefore a list of Genes of Interest differentially regulated between NF and CWF was generated, including serum independent and serum stimulated genes (Table 4.10). This list was assembled from genes identified by ANOVA analysis and Difference of Differences analysis which

were not altered by immortalisation or between patients. Gene lists were manually sorted using MADRAS. Genes demonstrating stability across all three patients and ideally greater than 2 fold alterations in gene expression between NF and CWF were selected. Over representation analysis was carried out independently of the selection of genes of interest however the genes selected (Table 4.10) were also present in one or more important pathway or functional group as determined by DAVID over representation analysis. The selected genes are potential disease marker genes.

4.2.6 Pathway Architect Relevance Networks

The list of Genes of Interest (Table 4.10) was entered into Pathway Architect a pathway discovery tool. Pathway Architect uses natural language processing to search PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) abstracts from this data it constructs pathways involving the genes entered. The pathways generated are interaction networks demonstrating direct interactions, shortest path, putative targets and putative regulators. Pathway Architect will also suggest genes that though not originally entered have have high relevance and known interactions with the genes under analysis. Pathway Architect was used to generate relevance networks showing known interactions between the genes of interest (Fig 4.21). While this information is based solely on text mining the PubMed database and is not based on experimental interactions it did suggest that this list of genes shared some common factors.

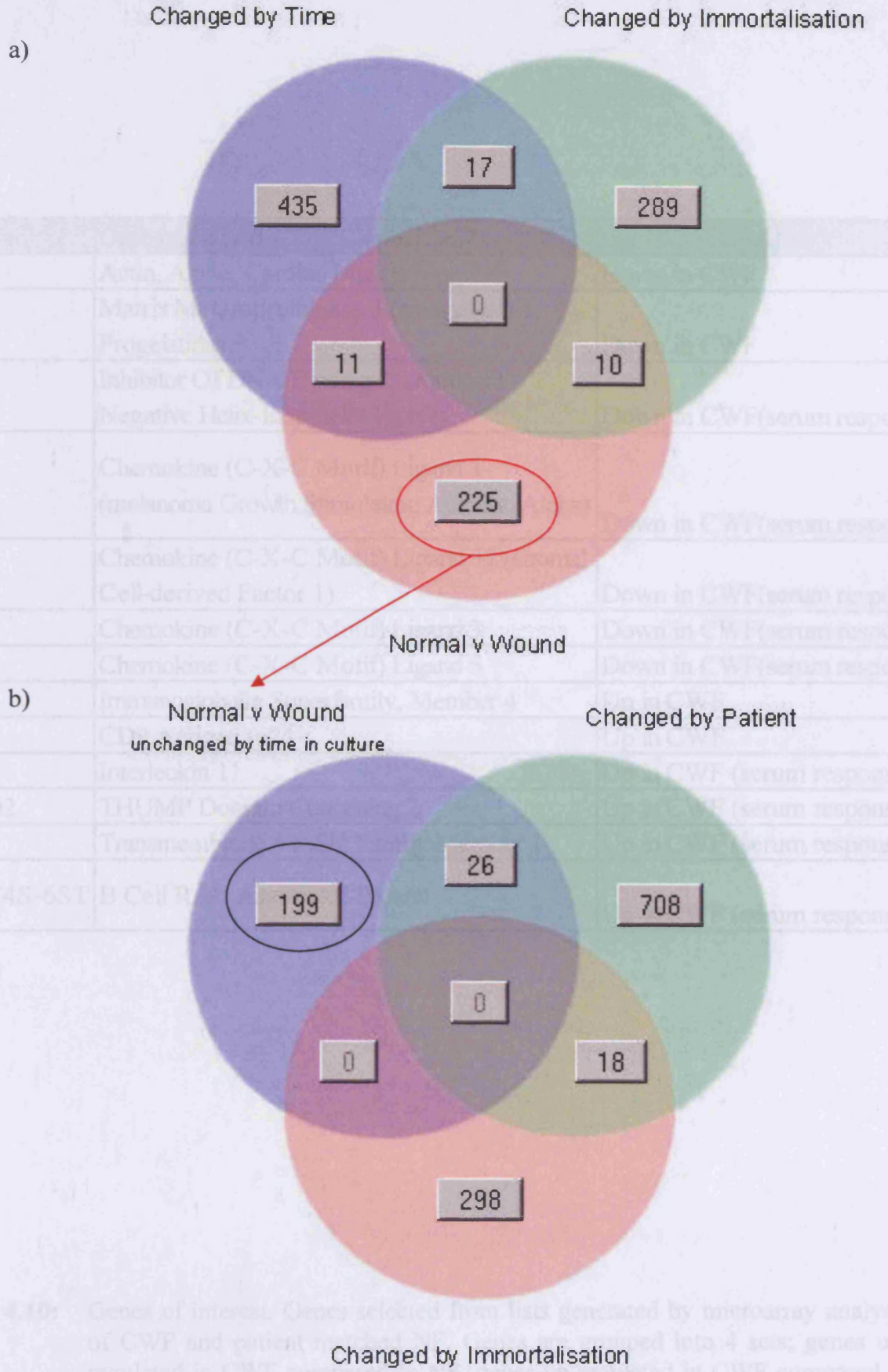


Figure 4.20: Venn diagrams showing gene lists from anova analysis. Genes were selected that were not altered by time in culture, by the process of immortalisation but were differentially expressed between NF and CWF a) These genes (ringed in red) were then controlled for inter patient variability b). This gave a final gene list of 199 genes (ringed in black).

Gene Symbol	Gene name	
ACTC	Actin, Alpha, Cardiac Muscle	Down in CWF
MMP3	Matrix Metalloproteinase 3 (stromelysin 1, Progelatinase)	Down in CWF
ID1	Inhibitor Of DNA Binding 1, Dominant Negative Helix-loop-helix Protein	Down in CWF(serum response)
CXCL1	Chemokine (C-X-C Motif) Ligand 1 (melanoma Growth Stimulating Activity, Alpha)	Down in CWF(serum response)
CXCL12	Chemokine (C-X-C Motif) Ligand 12 (stromal Cell-derived Factor 1)	Down in CWF(serum response)
CXCL3	Chemokine (C-X-C Motif) Ligand 3	Down in CWF(serum response)
CXCL5	Chemokine (C-X-C Motif) Ligand 5	Down in CWF(serum response)
IGSF4	Immunoglobulin Superfamily, Member 4	Up in CWF
CD9	CD9 Antigen (p24)	Up in CWF
IL11	Interleukin 11	Up in CWF (serum response)
THUMPD2	THUMP Domain Containing 2	Up in CWF (serum response)
TM4SF1	Transmembrane 4 L Six Family Member 1	Up in CWF (serum response)
GALNAC4S-6ST	B Cell RAG Associated Protein	Up in CWF (serum response)

Table 4.10: Genes of interest. Genes selected from lists generated by microarray analysis of CWF and patient matched NF. Genes are grouped into 4 sets; genes up-regulated in CWF compared to NF, genes up-regulated in CWF compared to NF in response to serum, genes down-regulated in CWF compared to NF and genes down-regulated in CWF compared to NF in response to serum

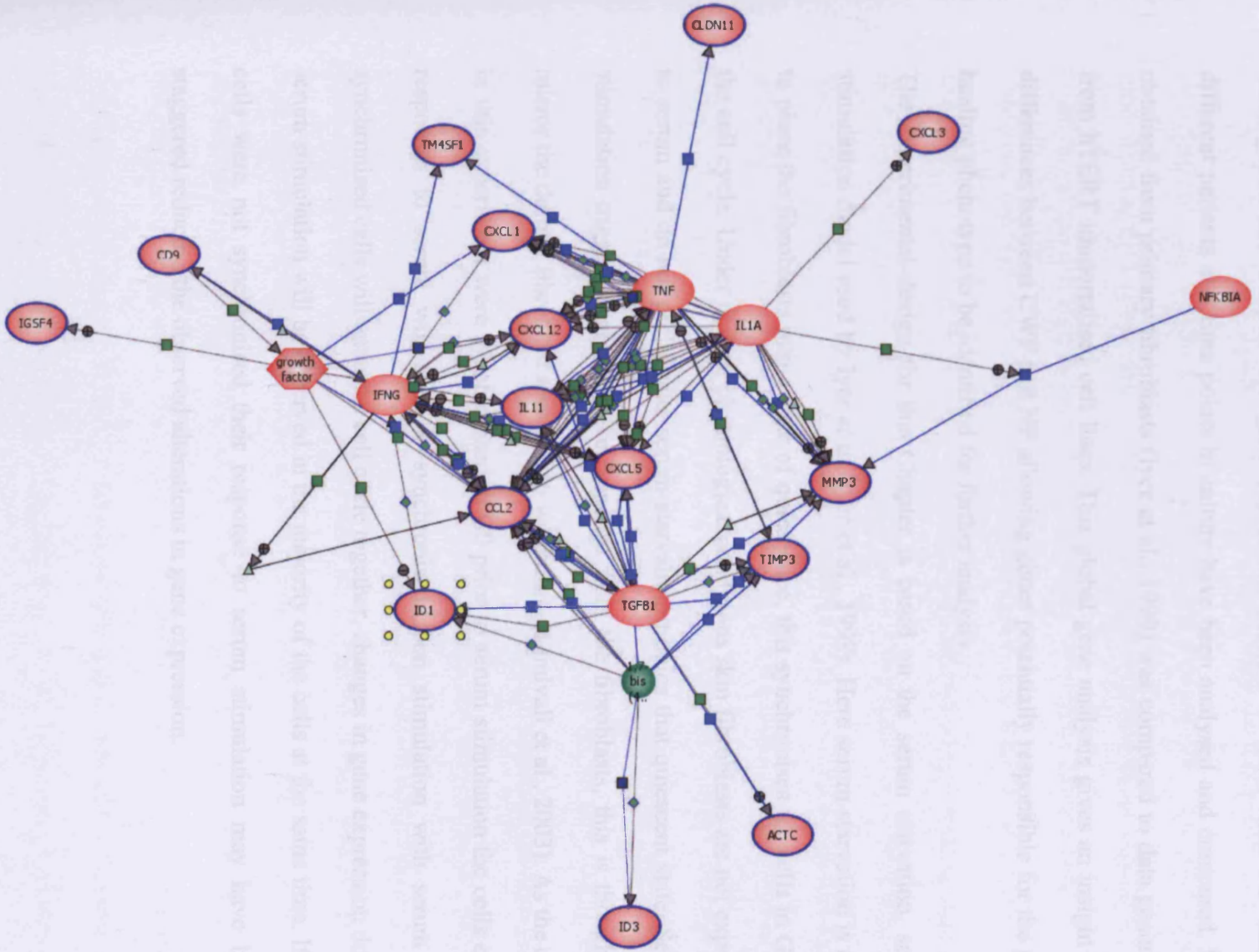


Figure 4.21: Pathway Architect representation of all known interaction between genes of interest identified by Microarray analysis. Lines shown in the diagram represent pubmed citations, the shorter the line the greater that number of citations. Genes not originally entered into Pathway Architect have no blue ring.

4.3 Discussion

Throughout this Chapter the gene expression profiles of CWF and NF from 3 different patients at 4 time points in culture have been analysed and compared. Data obtained from primary fibroblasts (Iyer et al., 1999) was compared to data generated from hTERT immortalised cell lines. This global gene analysis gives an insight into differences between CWF and NF allowing genes potentially responsible for the non-healing phenotype to be identified for further analysis.

The experimental design for this Chapter is based on the serum starvation, serum stimulation model used by Iyer *et al* (Iyer et al., 1999). Here serum starvation is used to place the fibroblasts into a state of quiescence, this synchronises the cells in G0 of the cell cycle. Under normal physiological conditions skin fibroblasts are not exposed to serum and do not proliferate; serum starvation mimics that quiescent state. Serum stimulation triggers a proliferative response from the fibroblasts, this is thought to mirror the dermal fibroblast response to wounding (Lindvall et al., 2003). As the cells in this experiment were synchronised in G0 prior to serum stimulation the cells early responses to serum will also be synchronised.Upon stimulation with serum the synchronised cells will enter the cell cycle together, changes in gene expression due to serum stimulation will be observed in the majority of the cells at the same time. If the cells were not synchronised their response to serum stimulation may have been staggered reducing the observed alterations in gene expression.

4.3.1 Analysis of changes due to immortalisation

Initial analysis was carried out comparing gene expression in primary cells with the hTERT immortalised cell lines. These comparisons were made between each primary cell strain and the matching immortalised cell line irrespective of the patient or cell type. In Chapter 3 it was demonstrated that hTERT immortalisation had allowed the cells to escape replicative senescence and form cell lines, demonstrated by their continued proliferation. Phenotypic studies demonstrated that the immortalised NF and CWF retained differences in proliferation rates and wound healing responses typical of primary cells. As discussed in Chapter 3 this retention of phenotype after immortalisation makes the NF and patient matched CWF cell lines a potential tool for the study of chronic wounds. Previous studies on hTERT immortalised fibroblast cell lines disagree as to the effect hTERT has on the cellular phenotype. Lindvall *et al* (2003) describe significant differences in gene expression in their hTERT immortalised cells including DNA repair genes and growth factors (Wang et al., 2000). Wang *et al* (2000) showed hTERT immortalisation activated the oncogene c-MYC (Farwell et al., 2000, Noble et al., 2004), the repression of p16 and chromosomal aberration after hTERT immortalisation as well as repression of p14 has been reported in some cell lines (Pirzio et al., 2004). Other studies however, contradict these findings. Pirzio *et al* (2004) showed low frequencies of chromosomal aberrations in hTERT immortalised fibroblasts suggesting a role in genome stability for telomerase (Milyavsky et al., 2003a). Milyavsky *et al* (2003) showed an up-regulation of p16 in their hTERT immortalised cells and the retention of chromosome stability and the ability to enter G0 (Lindvall et al., 2003). However, Milyavsky *et al* (2003) did demonstrate effects of hTERT immortalisation described in other papers after prolonged culture of the cells. They examined their cell lines at 500 PDLs after

telomerase immortalisation and demonstrated overexpression of c-MYC and Bmi-1 oncogenes and loss of p14 expression in later time points. In this Thesis gene expression analysis of the immortalised cell lines compared to the primary cell strains identified 263 genes altered significantly by immortalisation totalling only 1.96% of the genes analysed. None of the gene expression alterations described previously were seen in any of the patient matched NF or CWF cell lines. In other studies the oncogenes RAB36 and MAFB and the protooncogene B MET were all down regulated in immortalized cells. Over representation analysis of genes up regulated by immortalisation identified pathways relating to the cell cycle and glycolysis as well as genes linked with protein sequencing. These findings correlate with the increased proliferative ability of the immortalised cells. The hTERT immortalisations lead to more decreases in gene expression when compared to the primary cells than increases. However, fewer pathways were identified by overrepresentation analysis in the down regulated genes. Alterations to pathways associated with GAP junction signaling, genes linked with control of the cell cycle and growth factor binding were identified. The alteration in growth factor and cell cycle genes mirrors Lindvall *et al* (2003) findings (Wysocki, 1996, Fivenson et al., 1997, Barone et al., 1998, Schmid et al., 1993). In this Thesis the number of genes altered by immortalisation was small and few known pathways appear to have been effected. Therefore genotypically as well as phenotypically the hTERT immortalised cell lines appear to be a good model of the primary cell strains from which they were generated.

4.3.2 Analysis of Inter-patient variability

The three different patient matched samples demonstrated differences in phenotype in Chapter 3, with differences in proliferation rate, rate of wound repopulation and cell

size observed between the different patients. Taken separately each of the patient matched samples demonstrated the same differences between NF and CWF however, when taken together the inter-patient differences are often greater then those seen between the NF and CWF cell lines. Genotypic analysis also identified differences between the patient matched cells with 633 genes showing statistically significant differences across the three patients. Analysis of these genes identified no significantly altered pathways or GO terms. Despite the differences between the patients it was possible to identify genes altered between CWF and NF across all patients independent of the serum response with 246 genes identified. Though this gene list is relatively small the alterations in gene expression are relevant as they have been seen in all three patients before and after immortalisation irrespective of the time point in culture.

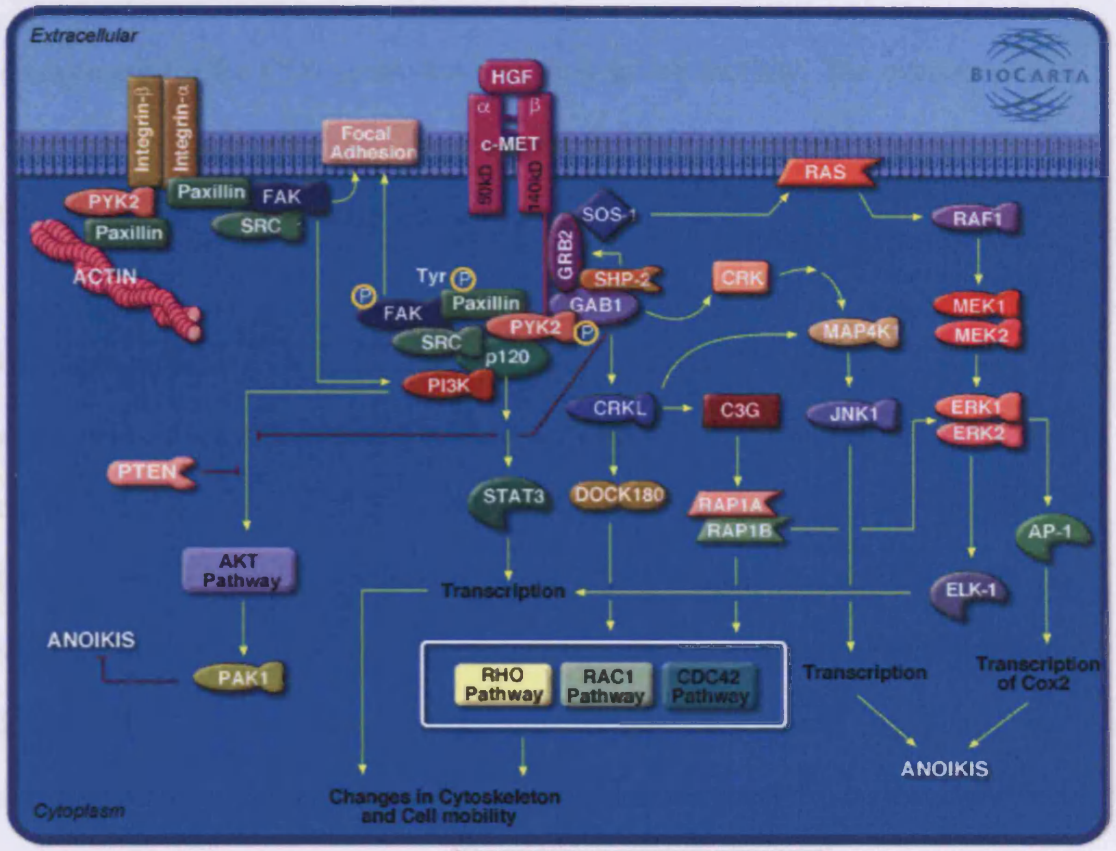
4.3.3 Gene expression and CWF phenotype

Chronic wounds are characterised by their failure to escape the inflammatory phase of wound healing. Chronic wounds have been shown to demonstrate alterations in levels of cytokines in the wound environment, the reduction of growth factors in the wound environment and aberrant extracellular matrix deposition (Agren et al., 1999a, Cook et al., 2000b, Raffetto et al., 2001a, Stephens et al., 2003, Wall et al., 2008b). Fibroblasts from chronic wounds display reduced proliferative ability, reduced mobility and altered extracellular matrix reorganisation (Sheterline, 1993, May et al., 1999). Over representation analysis of the gene lists generated by microarray comparison between NF and CWF has identified a number of groups of genes up- or down-regulated in CWF relating to these observed phenotypes.

4.3.3.1 Cell Migration

Cell migration requires the controlled polymerisation of actin molecules into filaments within laminapodia (Huang et al., 2006) and this process is controlled through a series of growth factor controlled signalling pathways. Genes involved in the regulation of the actin cytoskeleton were identified as being up regulated in CWF as well as growth factors known to influence actin filament formation (Table 4.4). CWF demonstrated a reduced ability to repopulate a scratch wound in Chapter 3 suggesting that CWF have a reduced migratory ability. Chronic wounds fail to resolve the inflammatory phase of wound healing, this may be due to the inability of fibroblasts to migrate into the wound.Hepatocyte growth factor (HGF) identified as a growth factor up-regulated in CWF regulates cell growth and motility through a tyrosine kinase signalling cascade. HGF binds to the c-MET receptor (which is also upregulated in CWF). The signalling cascade feeds into Ras/Rac pathway which in turn controls the Arp2/3 complex responsible for the formation of actin filament growth. The upregualation of RAC, HGF, ARPC1A and MET should increase the ability of the CWF to migrate. However the tetraspanin CD9 a cell surface signal transduction protein has been linked to regulation of the actin cytoskeleton (Bowler and Davies, 1999). Huang *et al* demonstrated that overexpression of CD9 reduced the motility of cells by affecting the formation of actin filaments. Huang *et al* identified WAVE2 and WASF2 both part of the Ras/Rac pathway as being regulated by CD9 (Fig 4.22). WASF2 forms a multiprotein complex that links the MET receptor and actin. The multiprotein complex serves to tranduce signals that involve changes in cell shape and motility. Over expression of CD9 and its subsequent effect on the formation of actin filaments could explain the upregulation of other cytoskeleton related genes. This upregulation may be an attempt

(a



(b

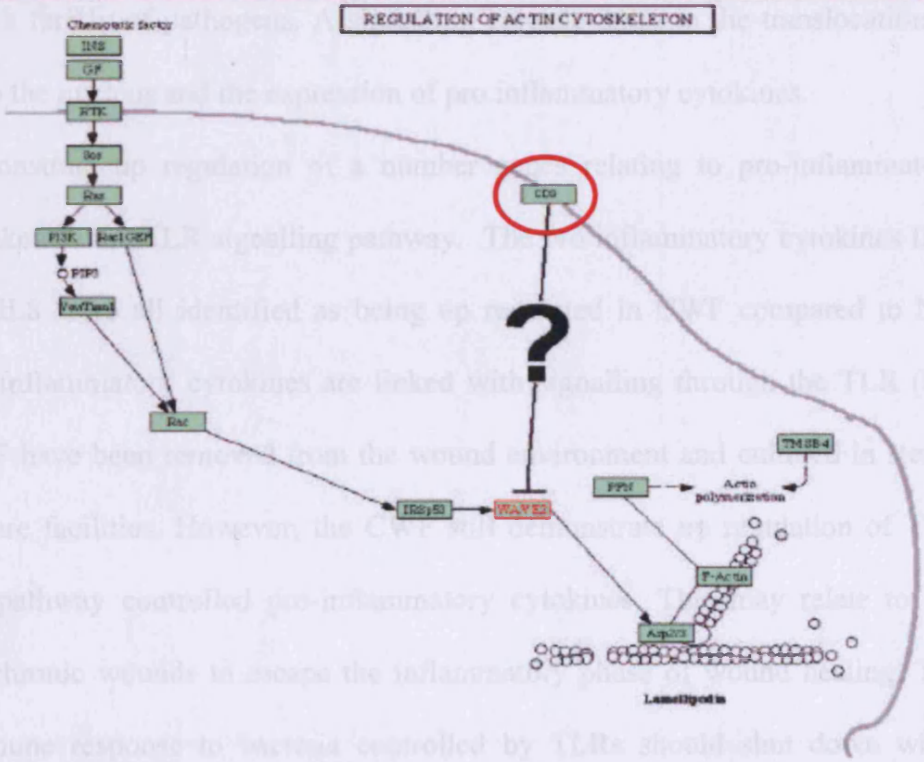


Figure 4.22: Pathways responsible for Actin filament formation. a) HGF signals through the MET receptor instigating a tyrosine kinase signalling cascade. This cascade activates the Ras/Rac pathway (b) however CD9 negatively regulates the Ras/Rac pathway through Wave 2 (Huang *et al* 2006) reducing actin filament formation. Images modified from biocarta (www.biocarta.com) (a) and KEGG (www.genome.jp/kegg) (b).

to compensate for the CD9 controlled reduction in cell motility. The overexpression of CD9 may explain the reduced motility observed in CWF compared to NF and the reduced ability to repopulate scratch wounds.

4.3.3.2 Inflammation

Chronic wounds are characterised by their high incidence of bacterial infection, with bacterial infection thought to be among the contributing factors to the non-healing nature of chronic wounds (Scanzello et al., 2008, Sabroe et al., 2008). Toll like receptors (TLR) play a vital role in the innate immune response to bacterial infection, linking the bodies innate and adaptive immune systems (Cole et al., 1998). TLRs found on the surface of cells within the wound space recognise different ligands, each specific to a family of pathogens. A signalling cascade leads to the translocation of NF-kB into the nucleus and the expression of pro inflammatory cytokines.

CWF demonstrate up regulation of a number genes relating to pro-inflammatory proteins linked to the TLR signalling pathway. The pro inflammatory cytokines IL6, IL1B and IL8 were all identified as being up regulated in CWF compared to NF. These pro inflammatory cytokines are linked with signalling through the TLR (Fig 4.23). CWF have been removed from the wound environment and cultured in sterile tissue culture facilities. However, the CWF still demonstrate up regulation of TLR signalling pathway controlled pro-inflammatory cytokines. This may relate to the failure of chronic wounds to escape the inflammatory phase of wound healing. The innate immune response to bacteria controlled by TLRs should shut down when bacteria are no longer present, if this is not the case as seen in CWF isolated from the wound site then the inflammatory phase of wound healing will not be resolved.

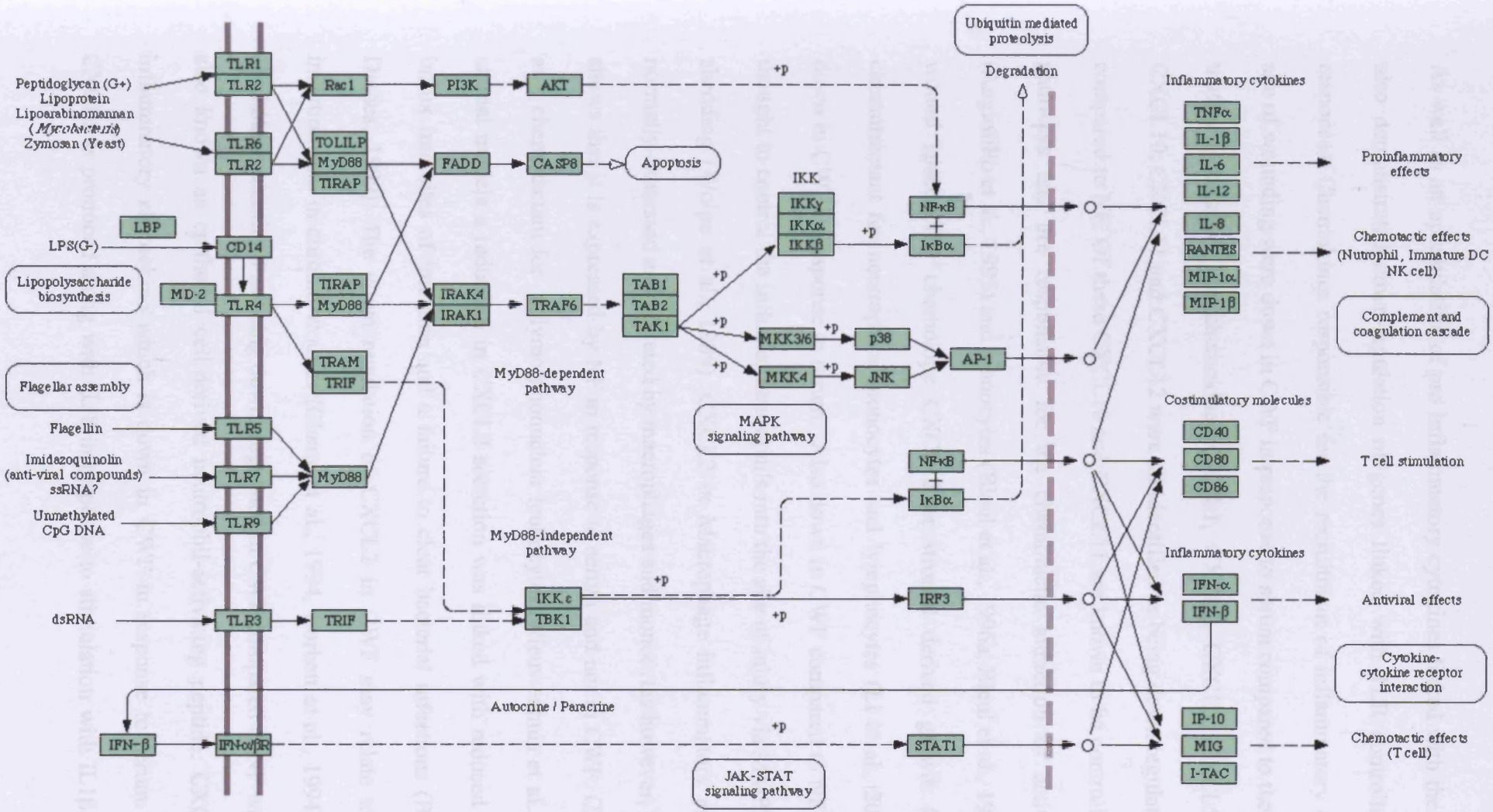


Figure 4.23: Toll like Receptor Signalling Pathway. Toll-like receptors (TLR) play a pivotal role in the regulation of innate immune responses; linking the innate and adaptive immune responses. Altered gene expression levels seen in CWF appear to be linked with the TLR pathway and could play a role in the continued inflammation observed in chronic wounds. Pathway taken from KEGG (www.genome.jp/kegg)

As well as an upregulation of pro inflammatory cytokines linked with the TLR CWF also demonstrated down-regulation of genes linked with TLR controlled immune responses. Chemokines responsible for the recruitment of inflammatory cells to the site of wounding were down in CWF in response to serum compared to the NF patient matched controls. The chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL10, CXCL11 and CXCL12 were all identified as being down regulated in CWF compared to NF. Of these CXCL10 and CXCL11 are known to be controlled by TLR pathways and are responsible for the chemotactic attraction of active T-Cells (Angiolillo et al., 1995) and monocytes (Bleul et al., 1996a, Bleul et al., 1996b) to the wound space. The chemokine CXCL12 or stromal derived growth factor is a chemotractant for neutrophils, monocytes and lymphocytes (Li et al., 2002) and is down in CWF in response to serum. Also down in CWF compared to NF CXCL1 is thought to control the influx of neutrophils into the site of injury via MMP7-mediated shedding (Wolpe et al., 1989). CXCL2 or Macrophage inflammatory protein 2 is normally expressed and secreted by macrophages and monocytes however, this Thesis shows that it is expressed by NF in response to serum and not in CWF. CXCL2 acts as a chemotactant for polymorphonuclear leukocytes (Nieuwenhuis et al., 2002). In animal models a reduction in CXCL2 secretion was linked with reduced neutrophil influx into sites of infection and a failure to clear bacterial infections (Bowler and Davies, 1999). The down regulation of CXCL2 in CWF may relate to the high infection rate in chronic wounds (Chang et al., 1994, Corbett et al., 1994). Another chemokine identified as being downr regulated in CWF compared to NF was CXCL5 also known as epithelial cell-derived neutrophil-activating peptide. CXCL5 is an inflammatory chemokine which is down in CWF in response to serum. Normally CXCL5 is produced along with IL18 in response to stimulation with IL1β or TNFα.

CXCL5 is responsible for the activation of neutrophils and their recruitment to the site of injury (Rovai et al., 1997, Wuyts et al., 1997). CXCL6 or granulocyte chemotactic protein 2 is a potent inflammatory mediator attracting neutrophilic granulocytes to the wound site, signalling through receptors CXCR1 and CXCR2 (Cole et al., 1998, Luo et al., 1998) Another chemokine down regulated in CWF was CXCL11 a chemokine known to induced a chemotactic response in activated T cells and monocytes and to be induced by IFNG (Hembruff et al., 2005, Mimmack et al., 2004).

Chemokines are clearly important in the normal wound healing response by the timely recruitment of inflammatory cells to the wound site. The observed down regulation of chemokines in CWF would relate to a failure to attract neutrophils, monocytes and lymphocytes to chronic wounds. In this model, serum stimulation was intended to mimic the change in cellular environment seen in wounding of the skin. The chemokines identified were all regulated by serum stimulation in NF. CWF display an impaired response to the pro-inflammatory cytokines found in serum. *In-vivo* a failure to produce the chemokines required for inflammatory cell recruitment could lead to the prolonged inflammation and bacterial colonisation seen in chronic wounds.

4.3.4 Potential CWF Disease Marker Genes

Genes from the pathways identified as well as genes that did not fall into any specific pathway but whose altered expression levels between NF and CWF were of interest were selected (Table 4.10). The genes selected include genes up in CWF compared to NF and genes down in CWF compared to NF independent of serum stimulation. The list also included genes up regulated in CWF compared to NF by the addition of serum and genes down regulated in CWF compared to NF in response to serum stimulation. These genes were identified despite the large inter-patient variability

which further validates them as genes important to the chronic wound phenotype. When analysed for known interactions by Pathway Architect multiple PubMed citations were found linking the genes of interest to each other. As well as these interactions a number of other genes were identified as relevant to the CWF disease marker genes. TNF, TGF-β1 and IFN-γ were all identified as interacting with the identified CWF disease marker genes (Fig 4.21).

This Chapter has presented data obtained by microarray analysis of primary and hTERT immortalised fibroblasts taken from chronic wounds and their patient matched normal controls. Analysis using over-representation and interactive networking has identified individual genes and whole pathways that are potentially interesting with respect to the chronic wound phenotype. One of the aims of this thesis was to validate hTERT immortalised CWF and NF cell lines as potential models for use in studying chronic wounds. Gene expression changes between NF and CWF were maintained after immortalisation. These changes relate to the observed chronic wound phenotype and may be important in the future design of chronic wound treatments. Importantly the data has shown the requirement for patient matched controls within these experiments as inter-patient variability accounted for the largest lists of identified gene alterations. A second aim of this thesis was to identify potential disease marker genes for use as reporter genes. A number of potentially interesting genes were identified (Table 4.10). These genes demonstrated significant changes in gene expression between NF and CWF as identified by ANOVA or difference of differences analysis. To confirm that the gene changes observed in the genes of interest selected are 'real' and to allow further time points in the serum response to be

analysed the genes of interest need next to be validated using quantitative realtime PCR.

Chapter Five

Confirmation of Disease Reporter Genes by Quantitative Real-Time PCR

5.1 Introduction

Microarray analysis is a invaluable tool for investigating genome wide alterations in gene expression levels as the technology allows all genes present on the array to be analysed at the same time. Though Affymetrix^{TM} gene chip technology allows for many levels of quality control it is generally considered necessary to validate observed expression changes by another method (Bermejo-Rodriguez et al., 2006, Sato et al., 2003). Two methods of validation are widely used: western blot analysis and quantitative real time PCR (QRT-PCR). These methods are often used in conjunction with one another (Langmann et al., 2005, Finn et al., 2007, Iyer et al., 1999). Western blotting allows for the quantitative analysis of protein concentration which confirms that gene expression changes observed in microarray analysis are actually having an effect at the protein levels. As the aim of this project was to specifically identify genes differently expressed between NF and CWF and generate promoter based reporter constructs for the disease marker genes identified, the alterations in gene transcription, not translation are of paramount concern. Thus an assay that investigates at the gene expression levels in more detail is more appropriate to validate the array data for this project.

Quantification of PCR reactions requires data collection at the exponential phase of PCR amplification, where the amount of PCR product present is doubled after each cycle. For end point PCR, this requires running reactions for varying numbers of cycles to identify the exponential phase for each reaction. Rather than altering cycle numbers to identify the exponential phase, QRT-PCR uses fluorescent dyes to take accurate readings for each sample at each cycle. This allows identification of the exponential and linear phases of the PCR reaction based on alterations in fluorescence levels.

There are three different methods for generating a fluorescent signal used by QRT-PCR systems, all taking advantage of the specific chemistries of fluorescent molecules: Förster Resonance Energy Transfer (FRET) used in TaqMan and Scorpion probes, SYBR Green dye and Light Upon Extension (LUX).

TaqMan (Applied BioSystems) utilises FRET, with a fluorescent reporter molecule attached to the 5' and quencher moiety attached to the 3' ends of the same oligonucleotide probe. The release of the reporter and quencher molecules is dependant on the 5'- nuclease activity of DNA polymerase used for PCR. The probe is designed to hybridise to the region between the forward and reverse primers. When the probe is free in solution the proximity of the quencher molecule to the fluorophore prevents any fluorescent signal through FRET. When PCR amplification takes place the 5'-nuclease activity of the polymerase digests the probe decoupling the fluorophore from the quencher and releasing it into solution increasing fluorescence. Fluorescence will therefore increase with each cycle as more probe is cleaved. TaqMan probes are able to be multiplexed as different fluorophores can be attached to each probe. However each target mRNA requires a new TaqMan probe which are expensive to synthesise. TaqMan based QRT-PCR has been used to validate microarray analysis (Hembruff et al., 2005).

SYBR Green based qRT-PCR allows the detection and quantification of PCR products through the fluorescence of the SYBR green dye. SYBR green is a DNA binding dye which when bound to DNA fluoresces upon excitation with 485nm light. As PCR product accumulates after repeated cycles the level of fluorescence increases. Unlike TaqMan based QRT-PCR any double-stranded DNA will cause an increase in fluorescence: this includes primer dimers and non-specific products. This can lead to an overestimation of the target cDNA concentration. SYBR green QRT-PCR is

inexpensive and with well designed primers SYBR green has been found to be as accurate as TaqMan (Mimmack et al., 2004). SYBR green will work with any primers without the need to design new probes which makes it an ideal choice for looking at multiple genes (Lowe et al., 2003, Chen et al., 2004, Nazarenko et al., 2002a, Nazarenko et al., 2002b, Rekhviashvili et al., 2007, Nordgren et al., 2007, Antal et al., 2007, Rekhviashvili et al., 2006). However, it is not possible to multiplex with SYBR green.

The system used in this Thesis was the Light Upon Extension system (LUX). LUX QRT-PCR is based on one normal and one labelled primer. The labelled primer has a fluorescent molecule attached to the 3' end within a hairpin structure. The hairpin configuration quenches the fluorophore removing the need for a separate quenching moiety. When free in solution the LUX primers have very low fluorescence. When incorporated into double stranded DNA the fluorophore is unquenched and thereby fluorescence increases (Nazarenko et al. 2002a; Nazarenko et al. 2002b). After each cycle the number of LUX primers incorporated into double stranded PCR product increases allowing the quantification of starting cDNA. LUX probes can be made with a range of fluorophores attached allowing multiplex QRT-PCR and as no quencher moieties are required the cost of each LUX primer is significantly less than a TaqMan probe. Any product which incorporates the primers will lead to an increase in fluorescence so good primer design is essential as is confirmation of primer specificity. The LUX system has been used for a number of different applications including viral identification (Ikeyama et al., 1993) and offers a cost effective QRT-PCR option.

The aims of this chapter were to analyse gene expression for disease reporter genes of interest identified by microarray analysis (Chapter 4), to confirm changes in gene expression identified by microarray and to gain a better understanding of the time course of gene expression. Gene expression analysis was carried out using QRT-PCR on cDNA generated from total RNA extracted from both CWF and NF at various experimental timepoints. For genes of interest to be taken forward for use in reporter constructs detailed expression data was required.

5.2 Results

5.2.2 Generation of cDNA

RNA was extracted from serum starved and serum stimulated NF and CWF (n=3) at 0H (serum starved) 1H, 6H and 24H after stimulation with 10%(w/v) FCS. The RNA was quantified and checked for quality as described in Chapter 4 with final RNA concentration, RNA quality and RNA integrity listed in Tables 4.1-3. For cDNA generation 1μg of RNA was taken for each time point/cell type/patient giving a total of 72 samples. To confirm successful cDNA generation non-quantitative PCR was carried out using primers for GAPDH designed to generate a product spanning an intron/exon boundary. A positive band would indicate successful cDNA synthesis. Agarose gel electrophoresis (Fig 5.1) demonstrates good amplification and single bands generated from Patients H, I and K for all timepoints. Variation in cDNA concentration was controlled for by reference to GAPDH in QRT-PCR reactions. To allow a reproducible standard curve to be generated for each of the genes of interest and for standards to allow comparison between plates Total Human RNA (Stratagene) was used. Microarray analysis data provided by Stratagene made it possible to confirm that transcripts from each of the genes under investigation were present in the pooled RNA. Total Human cDNA was generated as described previously with identical random hexamer primers and under the same conditions that were used for generating cDNA from each of the experimental RNA samples. To obtain sufficient cDNA to generate standard curves, 5μg of RNA was used per cDNA reaction, with 4 reactions carried out. To confirm successful cDNA generation non-quantitative PCR was carried out using primers specific for GAPDH. Agarose gel analysis of the products (Fig 5.1) demonstrated positive bands for each of the 4 cDNA generation reactions. The 4 reactions were pooled and used to generate a dilution series.

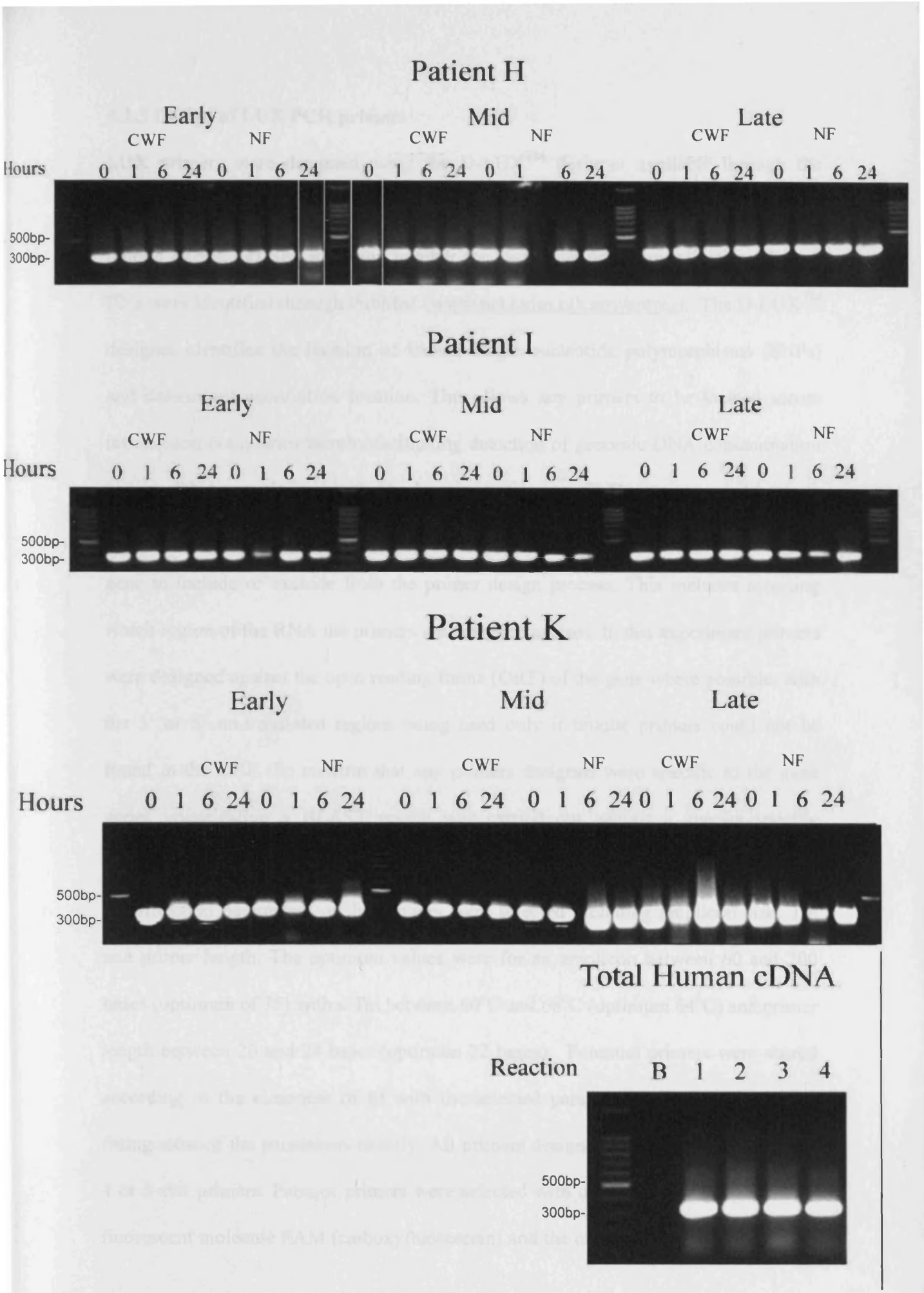


Figure 5.1: Confirmation of cDNA by PCR. The presence of GAPDH was confirmed by PCR with a band of approximately 300bp observed in each cDNA reaction.

5.2.3 Design of LUX PCR primers

LUX primers were designed using the D-LUX™ designer available through the Invitrogen website (www.invitrogen.com). The primer design software requires the unique Entrez Ref Seq accession number for each gene under investigation. Entrez ID's were identified through PubMed (www.ncbi.nlm.nih.gov/entrez). The D-LUX™ designer identifies the location of known single nucleotide polymorphisms (SNPs) and determines exon/intron location. This allows any primers to be located across intron/exon boundaries thereby facilitating detection of genomic DNA contamination in any cDNA sample analysed. The locations of known SNPs are important because they reduce the efficiency of primer binding. It was possible to select regions of the gene to include or exclude from the primer design process. This includes selecting which region of the RNA the primers are designed against. In this experiment primers were designed against the open reading frame (ORF) of the gene where possible, with the 3' or 5' un-translated regions being used only if unique primers could not be found in the ORF. To confirm that any primers designed were specific to the gene under investigation a BLAST search was carried out against a species specific database selecting primers against unique regions of the gene. Finally the amplification parameters of the primers were selected including amplicon size, Tm and primer length. The optimum values were for an amplicon between 60 and 200 bases (optimum of 75) with a Tm between 60°C and 68°C (optimum 64°C) and primer length between 20 and 24 bases (optimum 22 bases). Potential primers were scored according to the closeness of fit with the selected parameters with 5 star primers fitting most of the parameters exactly. All primers designed for this experiment were 4 or 5 star primers. Pairs of primers were selected with one primer labelled with the fluorescent molecule FAM (carboxyfluorescein) and the other primer unlabelled. The

exception to this was the primer pair for MMP3 where the fluorophore selected was JOE.

A housekeeping gene was selected from pre-designed LUX primers available from Invitrogen. GAPDH with a FAM label was selected. Athough the sequence of the primers was not available the primer pair was known to amplify the coding region spanning exon 4/5 of GAPDH.

5.2.4 Confirmation of primers

Each primer pair designed for this experiment was novel and had not been tested prior to this experiment therefore, each pair was tested to ensure that a single product was produced and that the product was of the size indicated by the D-LUX^{TM} designer. Reactions were carried out using an ABI Prism 7000 following the Invitrogen recommended protocol for this machine with total human cDNA. Successful amplification was detected for each primer pair by the ABI Prism through an increase in fluorescence. The products from each amplification were run on a 1.5% agarose gel (Fig 5.2). Each primer pair successfully generated a single product of the expected size.

5.2.5 Standard Curves

Due to the number of genes under investigation and the number of patient samples it was not possible to run all reactions on the same plate. To determine expression levels for each of the genes under investigation and to allow comparisons between plates a standard curve of reference cDNA was used on each plate. Comparison with the standard curve (Fig 5.3) gave expression levels for each sample. A separate plate was

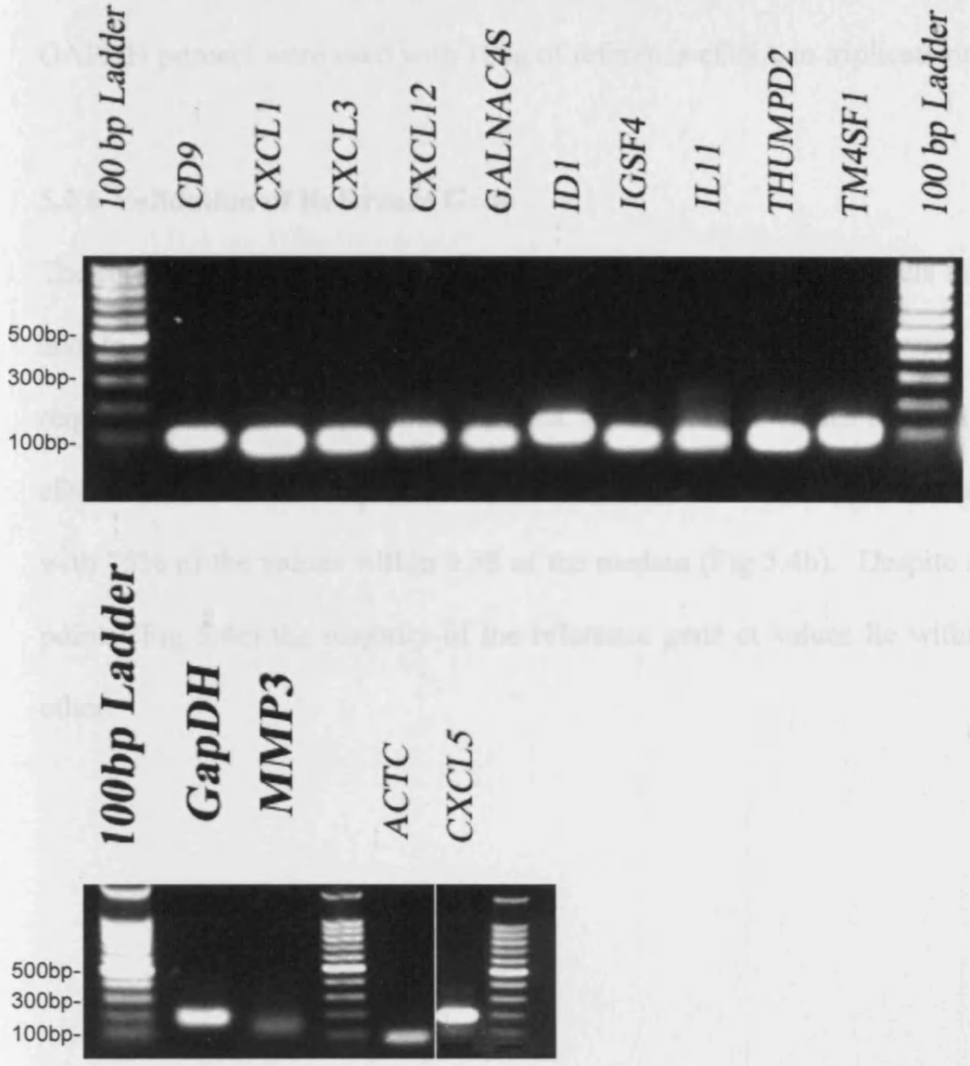


Figure 5.2: Confirmation of primers by PCR with cDNA generated from total human RNA supplied by Stratagene. Composite gels demonstrated the specificity of all LUX primers, only one band was observed from each primer pair with a DNA fragment of the appropriate size.

run for the housekeeping gene GAPDH. To allow comparisons between plates, GAPDH primers were used with 10ng of reference cDNA in triplicate on each plate.

5.2.6 Validation of Reference Gene

The reference gene GAPDH was used to normalise expression levels for each sample and to normalise variation between plates. Any variation between plates would require further normalisation of the data. Analysis of ct values for all 10ng reference cDNA with GAPDH (Fig 5.4a) shows the median level of expression at a ct of 18.3 with 75% of the values within 0.58 of the median (Fig 5.4b). Despite some outlying points (Fig 5.4c) the majority of the reference gene ct values lie within 1ct of each other.

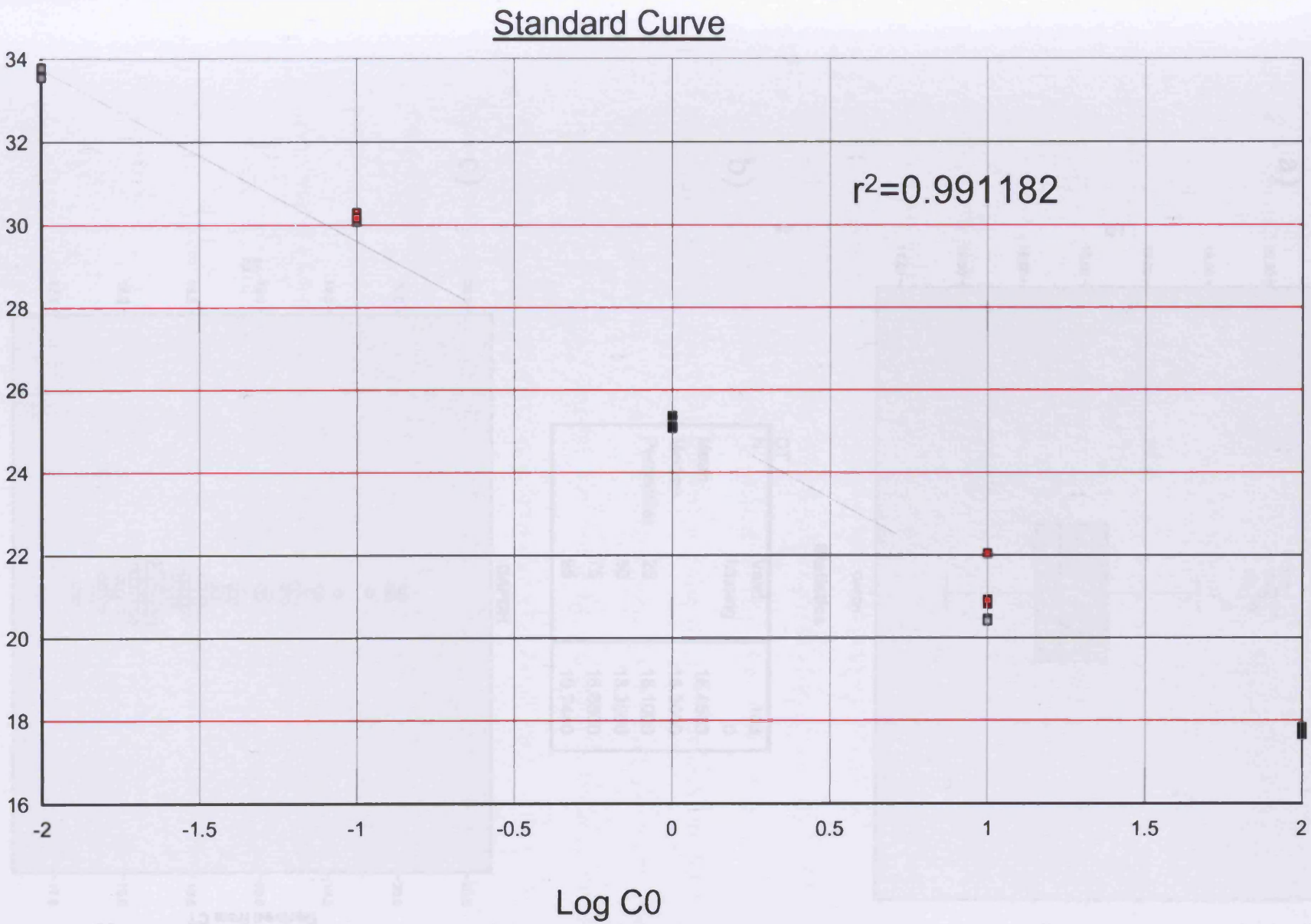
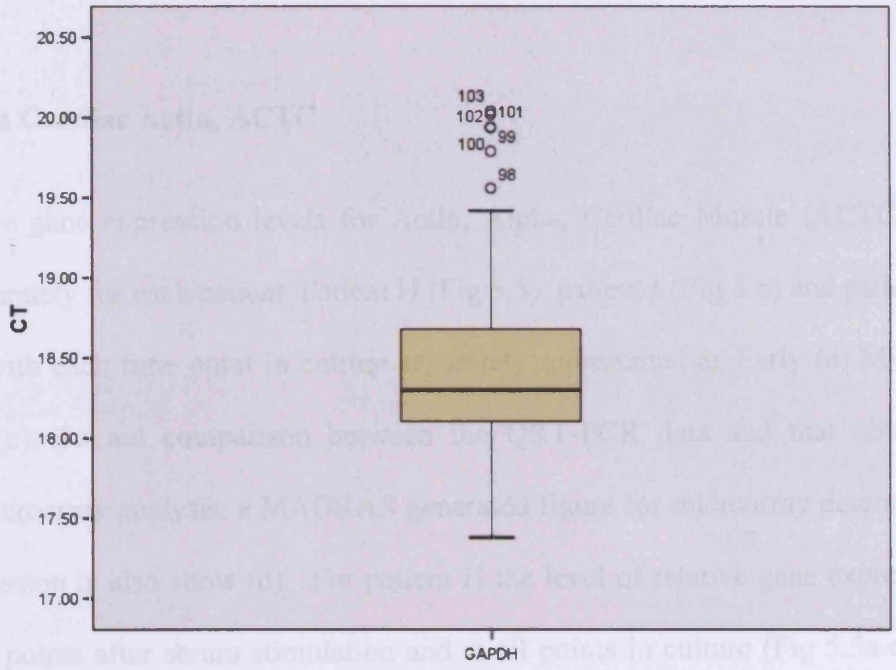


Figure 5.3: Graphs showing standard curve for GAPDH generated by serial dilution of reference cDNA. Standard curves were generated for each of the genes under investigation with at least 4 reference points per curve and r^2 vale greater than 0.97.

a)



b)

Statistics

CT

N	Valid	103
	Missing	0
Mean		18.4503
Median		18.3000
Percentiles	25	18.1000
	50	18.3000
	75	18.6800
	95	19.7440

c)

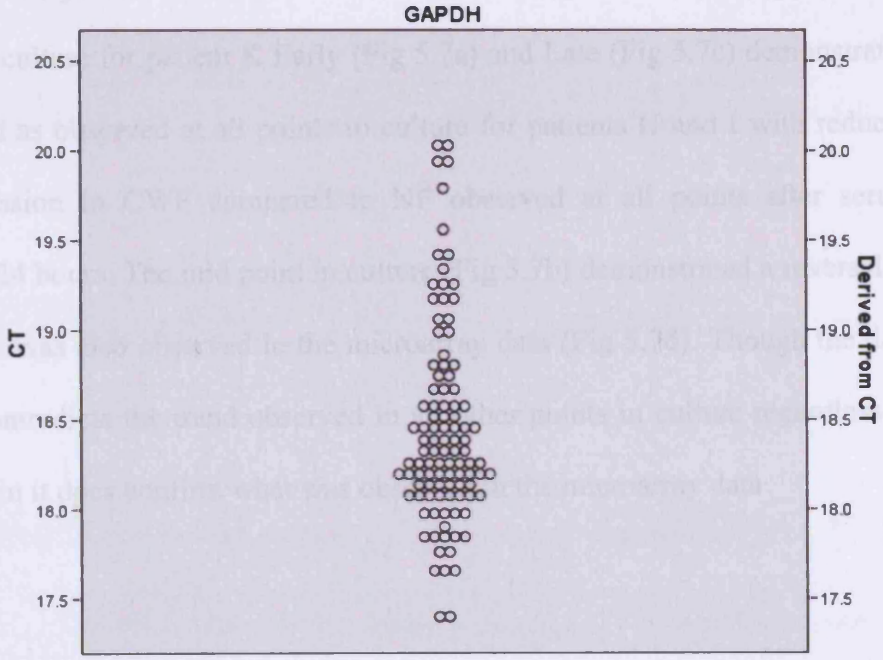


Figure 5.4: Validation of Reference Gene GAPDH. a) Box plot showing distribution of ct values for GAPDH with mean, upper and lower quartile and 95 percentile shown. b) Table showing mean and percentiles for GAPDH ct values. c) Symmetrical dot plot showing distribution of ct values for GAPDH.

5.2.7 Alpha Cardiac Actin, ACTC

The relative gene expression levels for Actin, Alpha, Cardiac Muscle (ACTC) are shown separately for each patient. Patient H (Fig 5.5), patient I (Fig 5.6) and patient K (Fig 5.7) with each time point in culture separately represented as Early (a) Mid (b) and Late (c). To aid comparison between the QRT-PCR data and that obtained through microarray analysis, a MADRAS generated figure for microarray determined gene expression is also show (d). For patient H the level of relative gene expression at all time points after serum stimulation and at all points in culture (Fig 5.5a-c) are lower in CWF compared to NF. This was also observed in the microarray data (Fig 5.5d). As microarray analysis investigated only two time points after serum stimulation (0 and 6 hours) the apparent increase of gene expression reported at 24 hours was not observed. The same trend was observed in patient I with lower relative expression levels in CWF than NF observed at all time points in culture (early, mid and late) and at all points after serum stimulation (1,6 and 24 hours). Two of the three time points in culture for patient K Early (Fig 5.7a) and Late (Fig 5.7c) demonstrated the same trend as observed at all points in culture for patients H and I with reduced relative expression in CWF compared to NF observed at all points after serum stimulation 0-24 hours. The mid point in culture (Fig 5.7b) demonstrated a reversal of the trend. This was also observed in the microarray data (Fig 5.7d). Though the data in Fig 5.7b contradicts the trend observed in all other points in culture regardless of patient of origin it does confirm what was observed in the microarray data

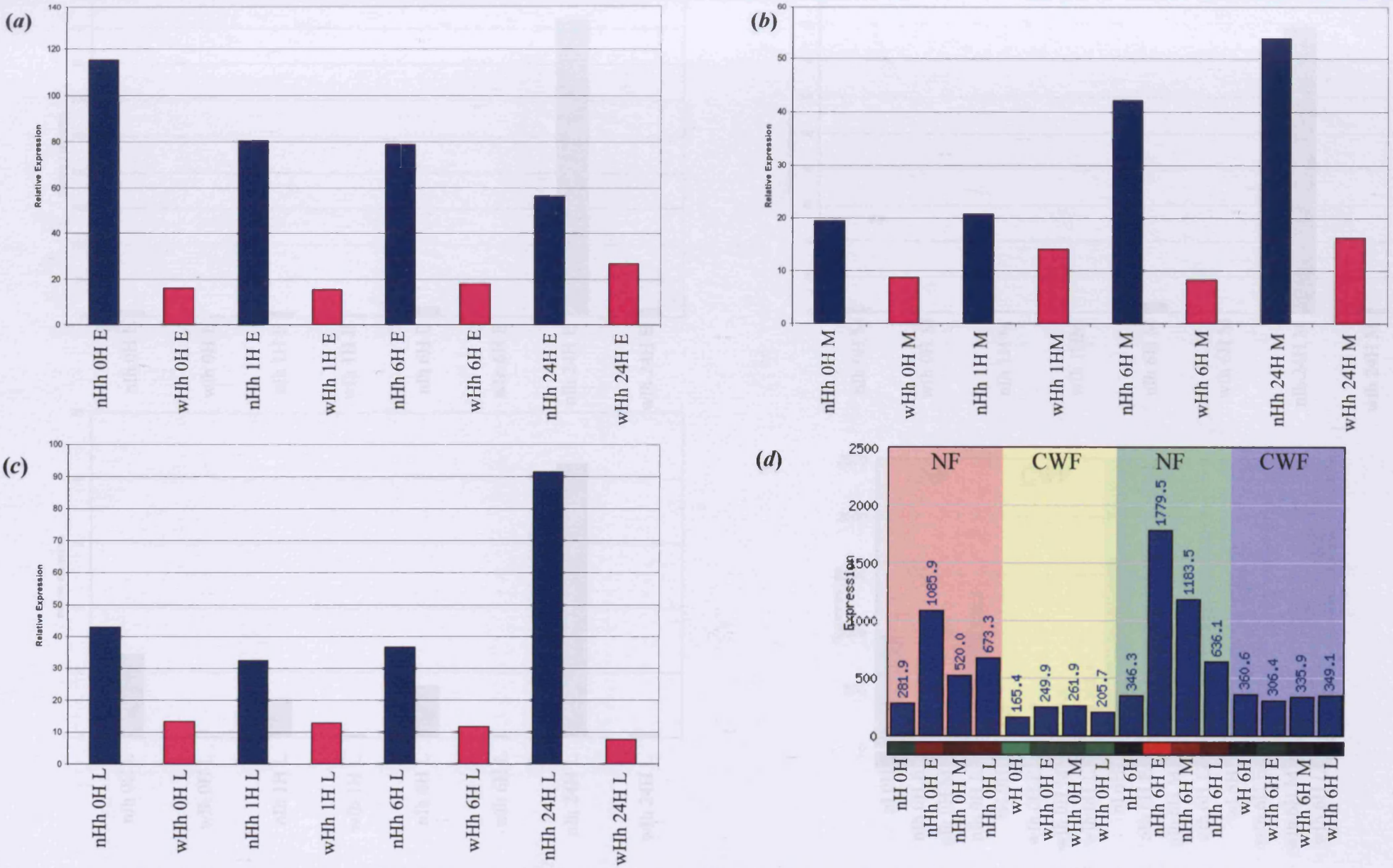


Figure 5.5: Graphs showing relative gene expression levels for ACTC in Patient H at Early (a) Mid (b) and Late (c) time points in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

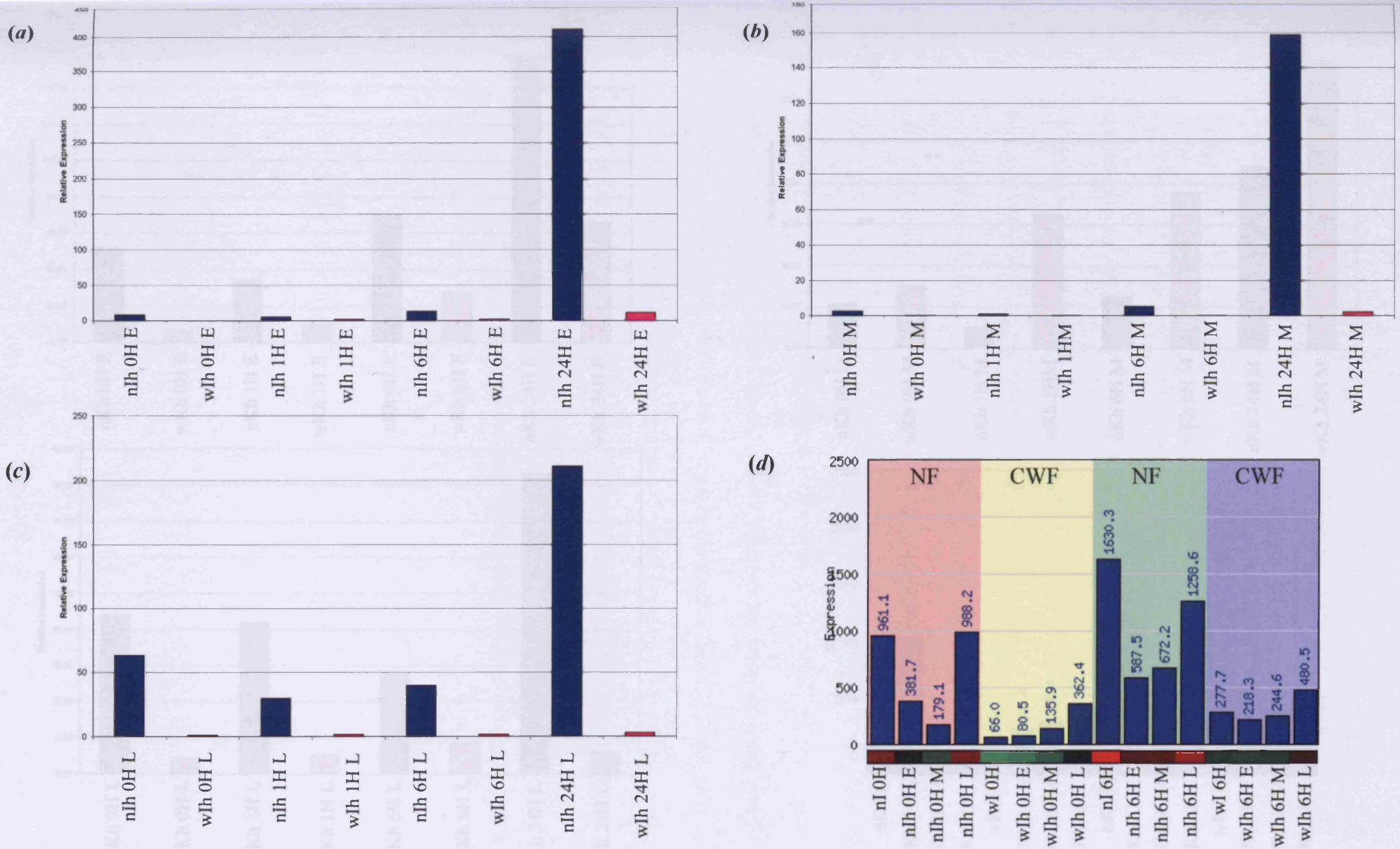


Figure 5.6: Graphs showing relative gene expression levels for ACTC in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

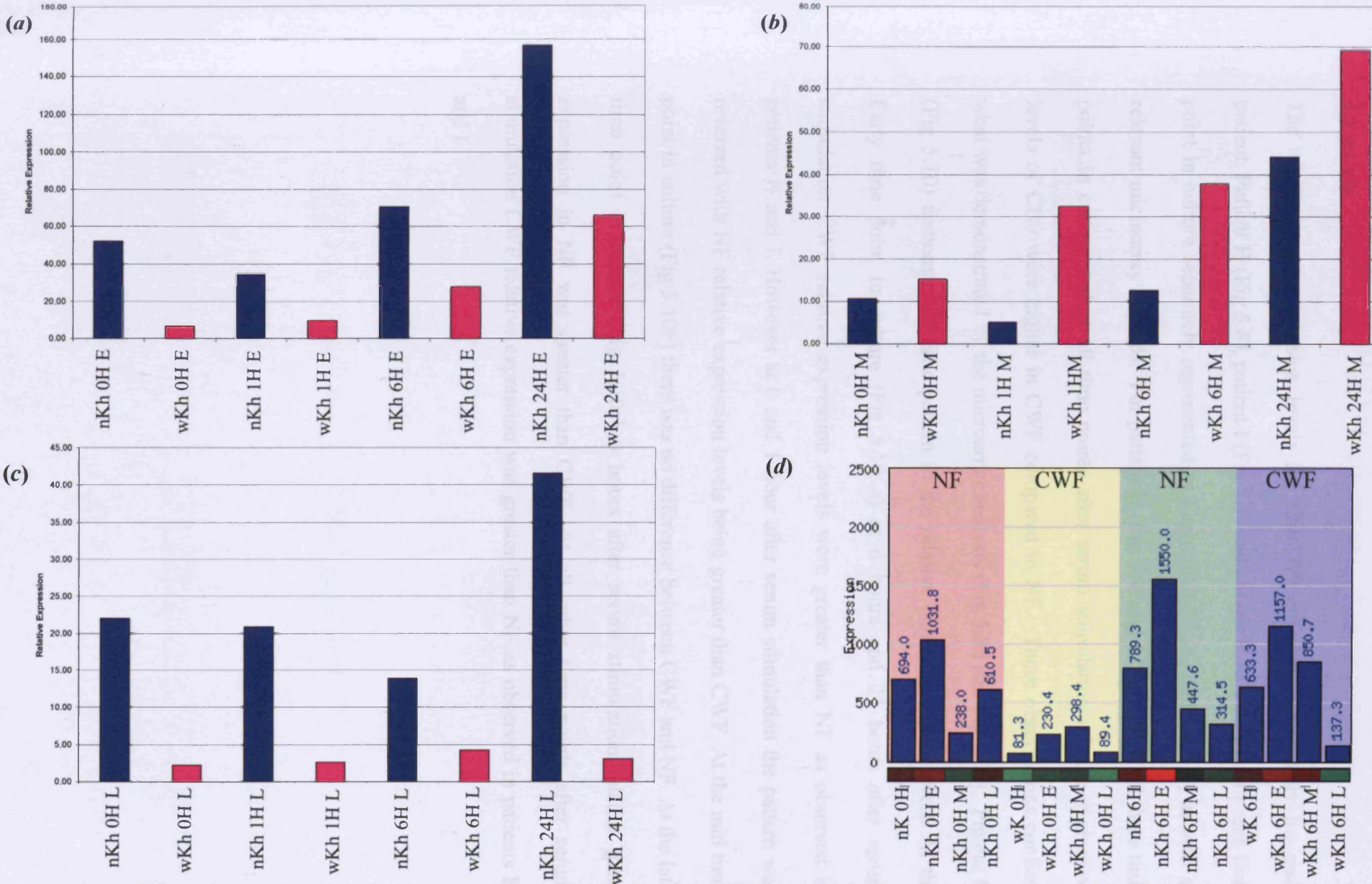


Figure 5.7: Graphs showing relative gene expression levels for ACTC in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d).

5.2.8 CD9

The relative gene expression levels for p24/CD9 are shown separately for each patient. Patient H (Fig 5.8), patient I (Fig 5.9) and patient K (Fig 5.10) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). For patient H (Fig 5.8) and I (Fig 5.9) at all three time points in culture and at all time points after serum stimulation relative expression levels of CD9 were higher in CWF compared to NF. These observations confirm what was demonstrated by the microarray analysis (Fig 5.8d and Fig 5.9d). Patient K (Fig 5.10) demonstrated little pattern in the relative gene expression levels. At the Early time point in culture (Fig 5.10 a) at 6 hours and 24 hours after serum stimulation CWF relative expression levels were greater than NF as observed in patients H and I. However at 0 and 1 hour after serum stimulation the pattern was reversed with NF relative expression levels being greater than CWF. At the mid time point in culture (Fig 5.10b) there was no difference between CWF and NF. At the late time point in culture (Fig 5.10c) 0 hours after serum stimulation relative gene expression in NF was greater than CWF. At all other time points after serum stimulation CWF relative expression was greater than NF as observed in patients H and I.

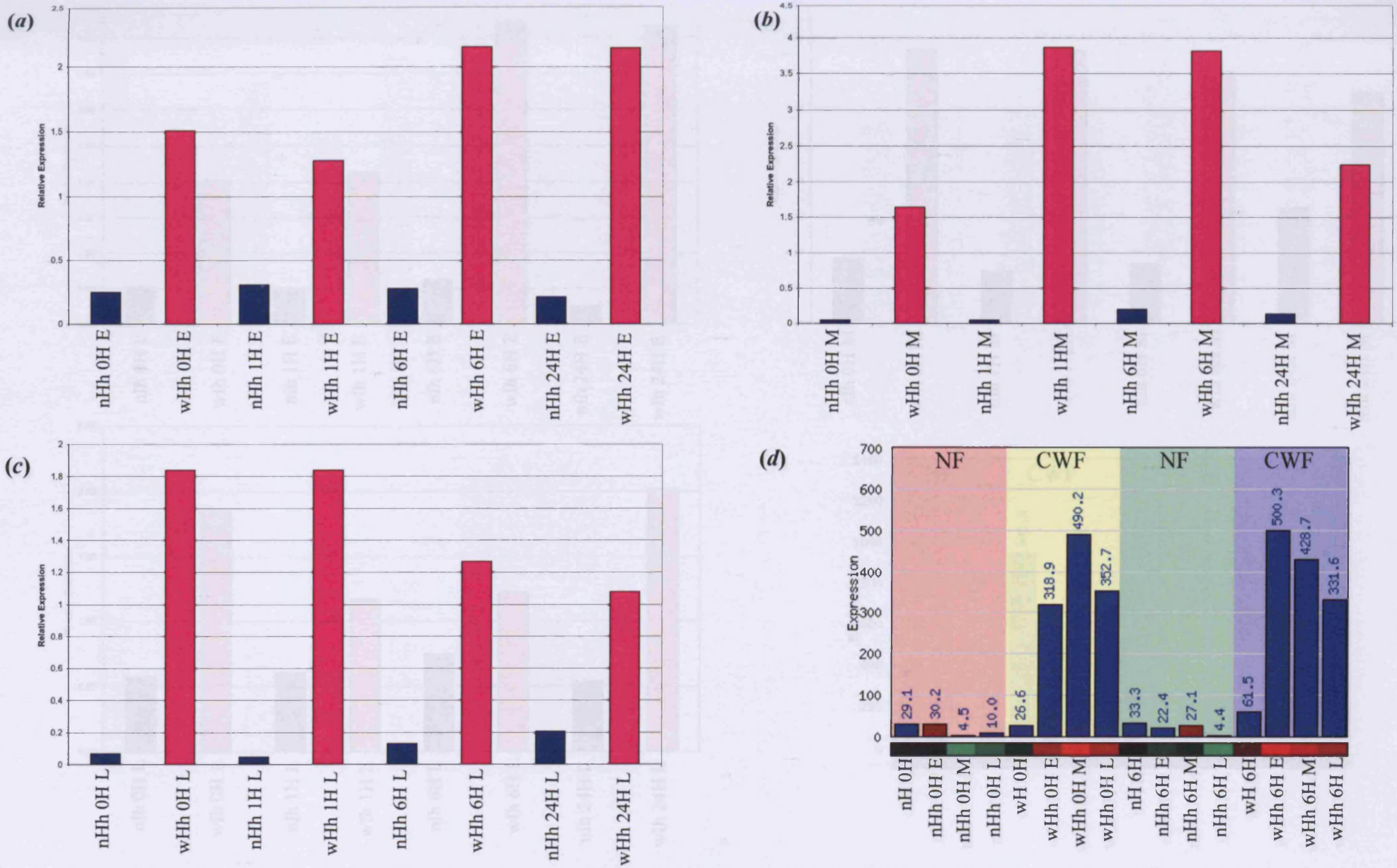


Figure 5.8: Graphs showing relative gene expression levels for CD9 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

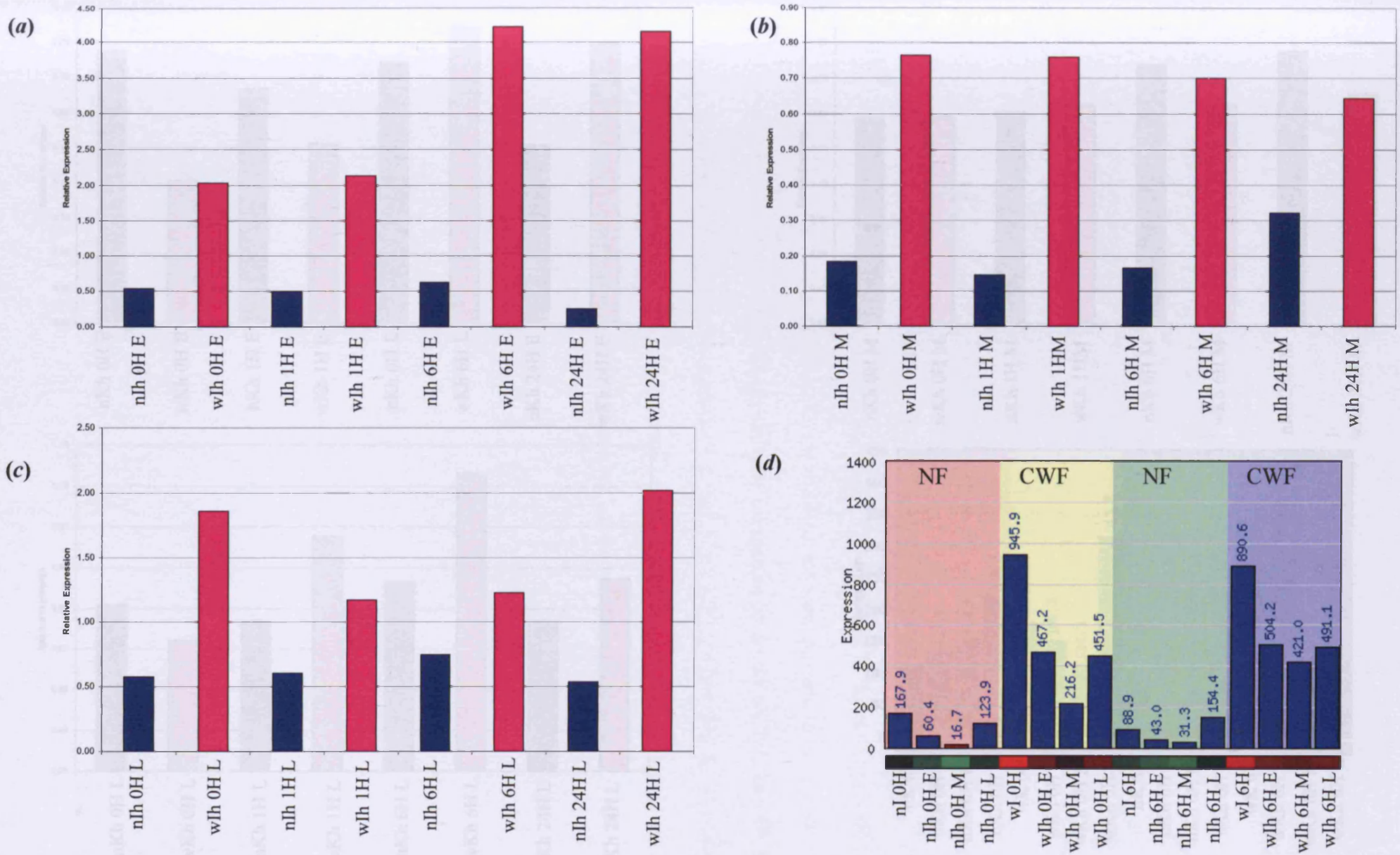


Figure 5.9: Graphs showing relative gene expression levels for CD9 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

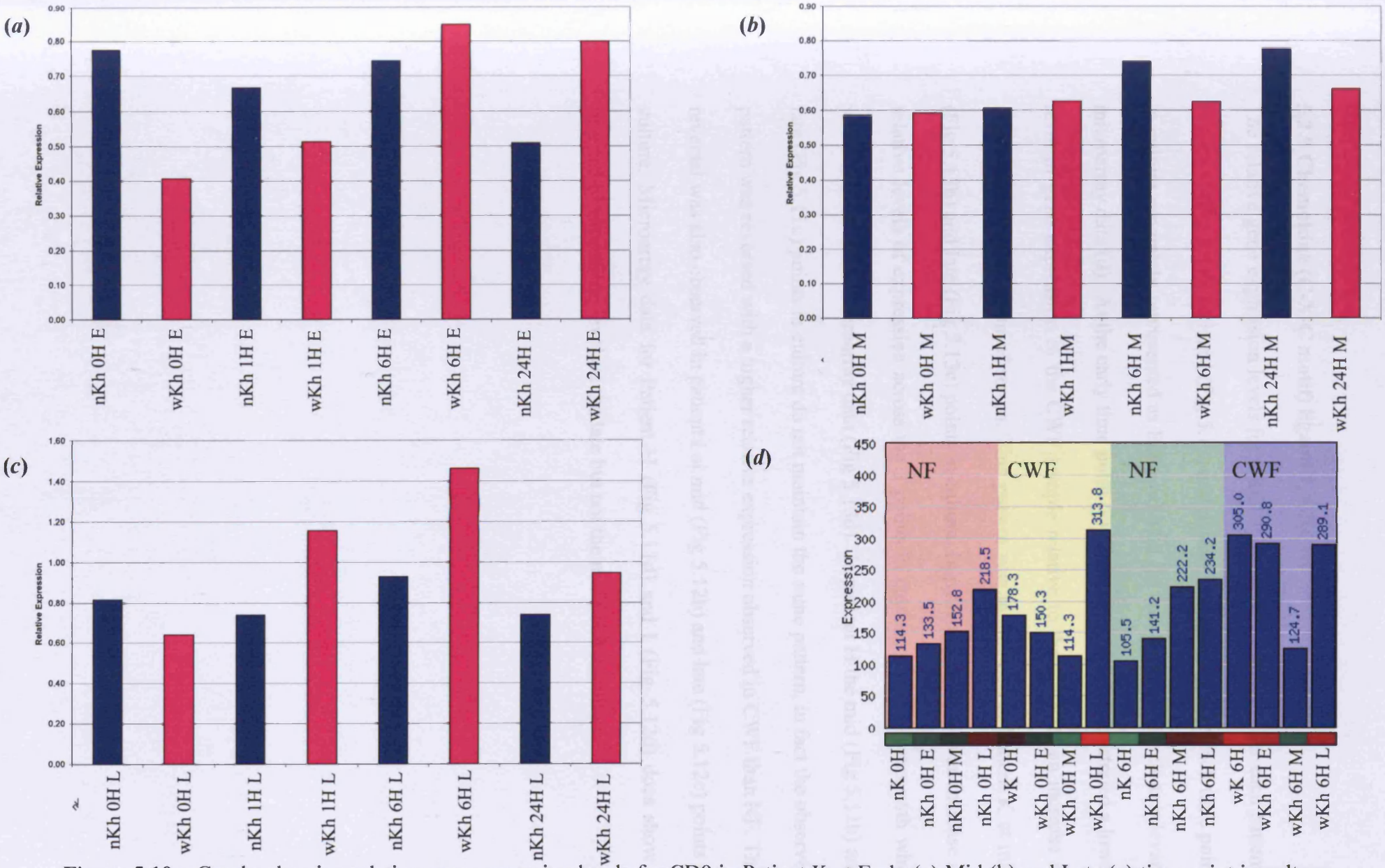


Figure 5.10: Graphs showing relative gene expression levels for CD9 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d).

5.2.9 Chemokine (C-X-C motif) ligand 1, CXCL1

The relative gene expression levels for CXCL1 are shown separately for each patient. Patient H (Fig 5.11), patient I (Fig 5.12) and patient K (Fig 5.13) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). At the early time point (a) all three patients demonstrated a lower level of gene expression in the CWF sample relative to the NF with an increase in expression upon addition of serum. This pattern was maintained in Patient K at mid (Fig 5.13b) and late (Fig 5.13c) points in culture. In patient K there was an increase in relative levels of expression across the 3 points in culture, this correlates with what was observed in the microarray data (Fig 5.13d). In patient H the mid (Fig 5.11b) and late (Fig 5.11c) points in culture do not maintain the same pattern, in fact the observed pattern was reversed with a higher relative expression observed in CWF than NF. This reversal was also observed in patient I at mid (Fig 5.12b) and late (Fig 5.12c) points in culture. Microarray data for Patient H (Fig 5.11d) and I (Fig 5.12d) does show a reversal of expression levels at the late but not the mid point in culture.

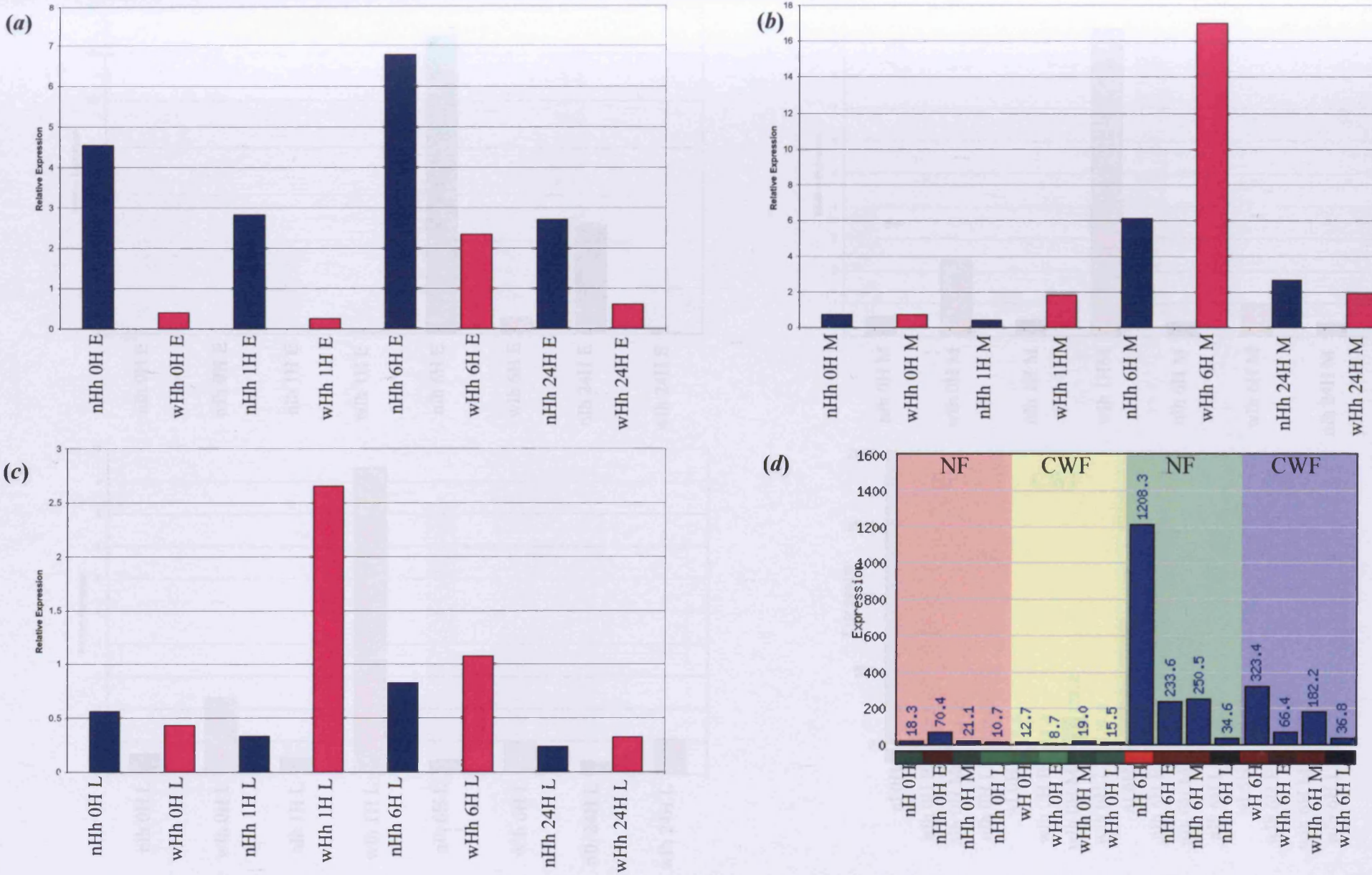


Figure 5.11: Graphs showing relative gene expression levels for CXCL1 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

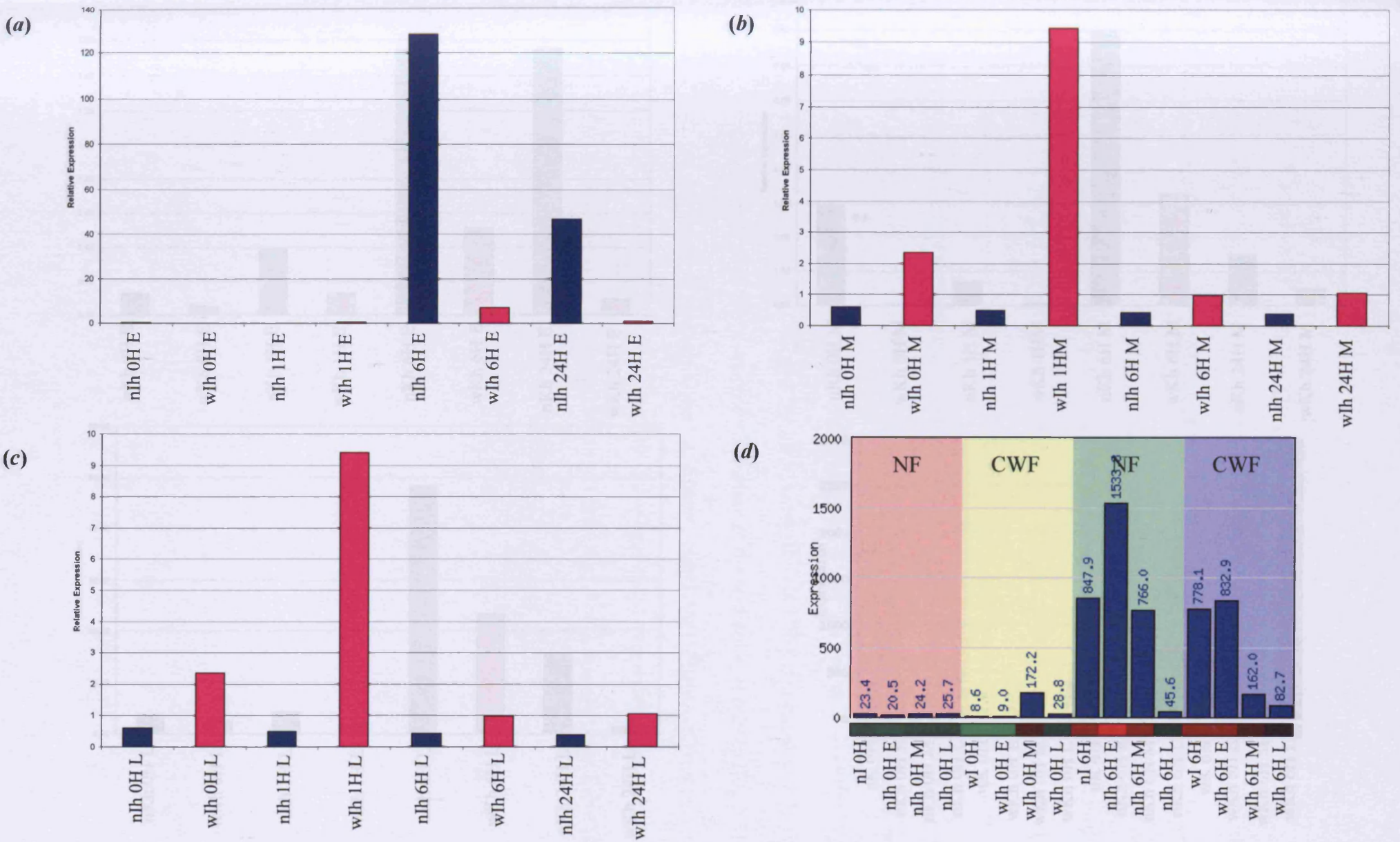


Figure 5.12: Graphs showing relative gene expression levels for CXCL1 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

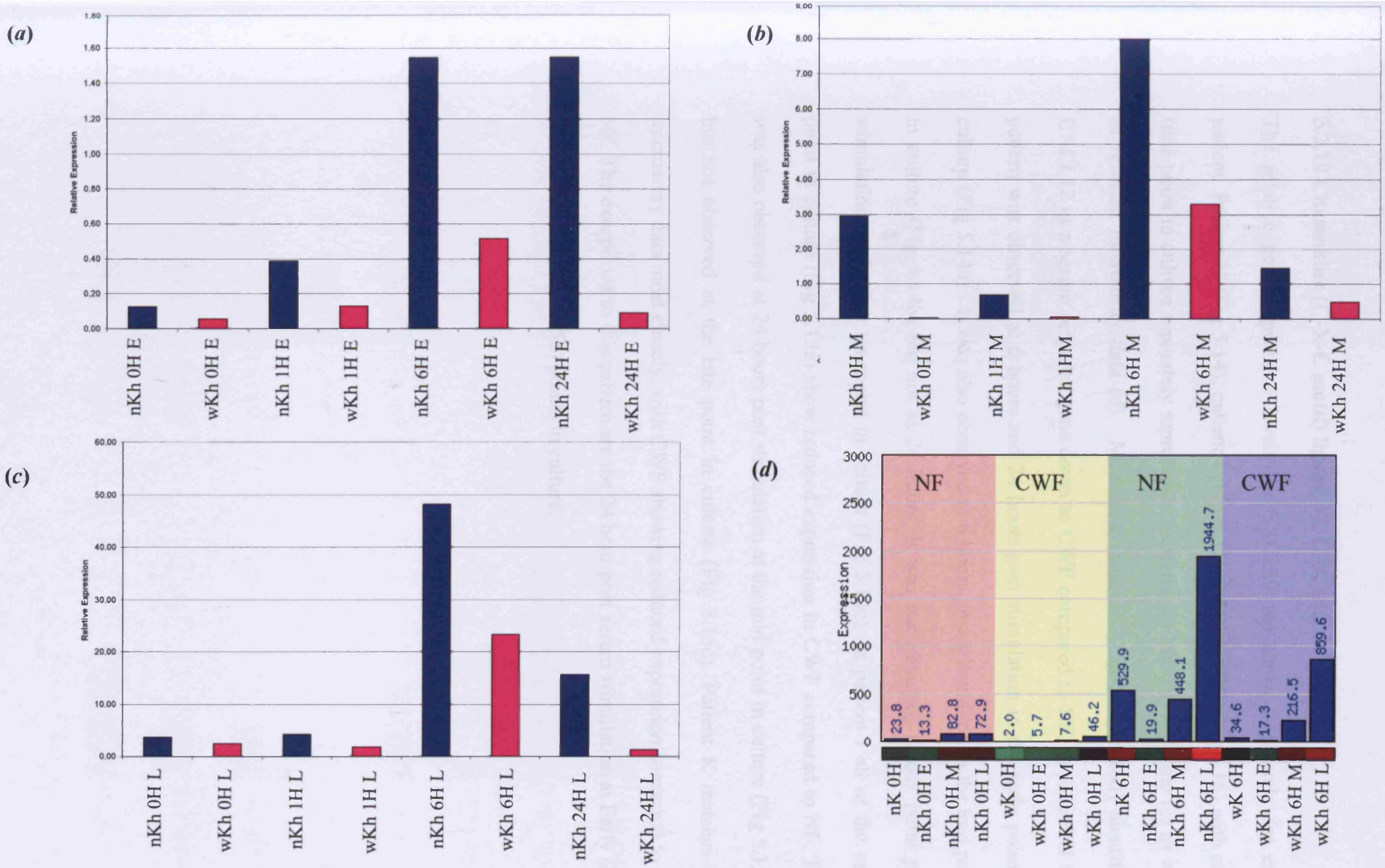


Figure 5.13: Graphs showing relative gene expression levels for CXCL1 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d).

5.2.10 Chemokine (C-X-C motif) ligand 12, CXCL12

The relative gene expression levels for CXCL12 are shown separately for each patient. Patient H (Fig 5.14), patient I (Fig 5.15) and patient K (Fig 5.16) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). Microarray analysis (Fig 5.14d-5.16d) identified CXCL12 as a serum response gene down in CWF compared to NF, in patient H this pattern was observed at 6 hours and 24 hours post stimulation in the early point in culture (Fig 5.14a) . It was also observed at 6 hours post stimulation in the mid point in culture (Fig 5.14b) but not at 24 hours. It was not observed at any point post stimulation at the late timepoint in culture (Fig 5.14c). In patient I all of the early point in culture (Fig 5.15a) show reduced expression in CWF compared to NF. This was also observed at 24 hours post stimulation at the mid point in culture (Fig 5.15b) but not observed at the late point in culture (Fig 5.15c). Patient K matches the microarray data most closely, with CWF showing reduced expression compared to the NF. The exceptions to this pattern are the 24 hour post serum stimulation at Early (Fig 5.16a) and mid (Fig 5.15b) points in culture.

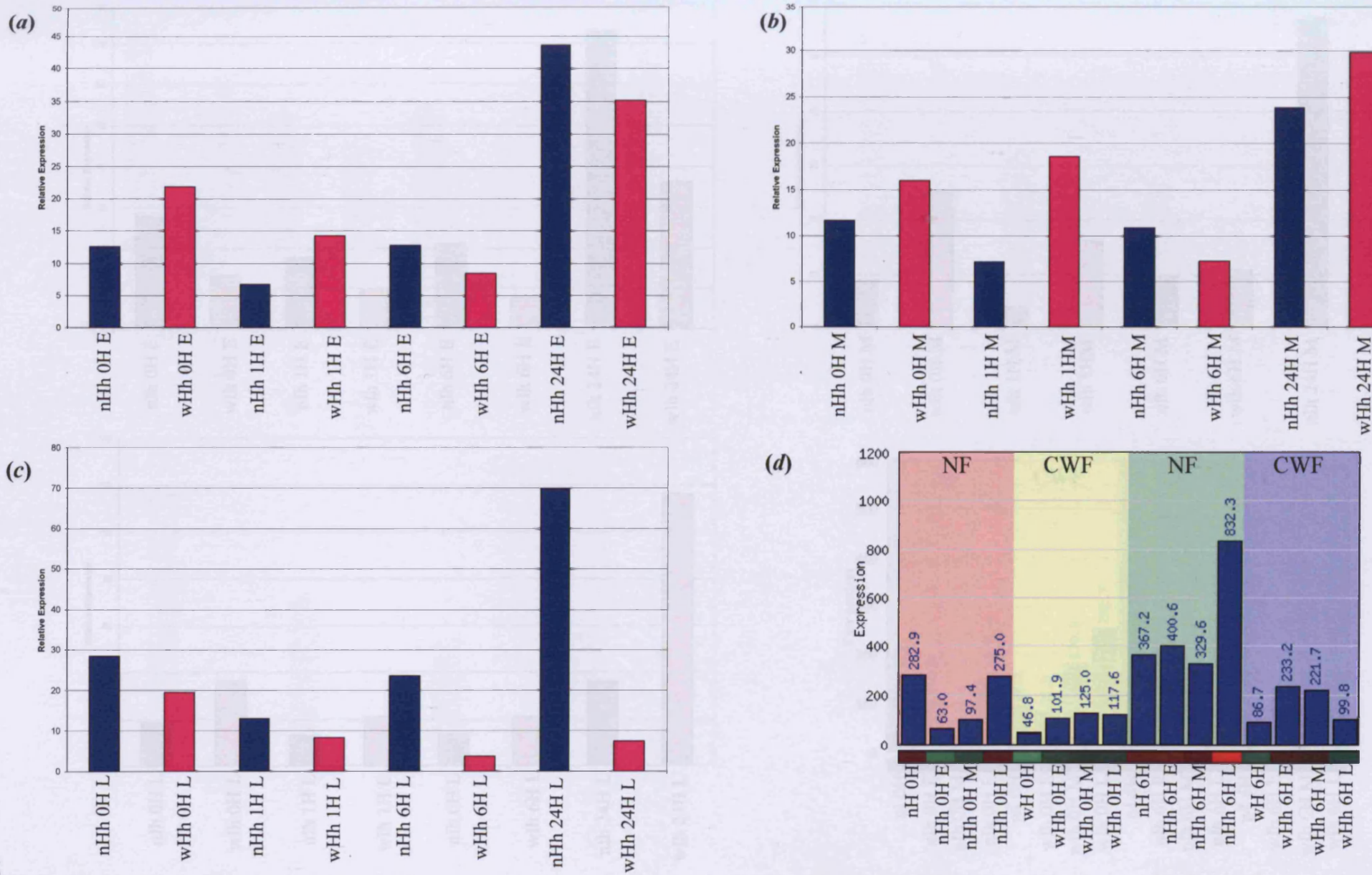


Figure 5.14: Graphs showing relative gene expression levels for CXCL12 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

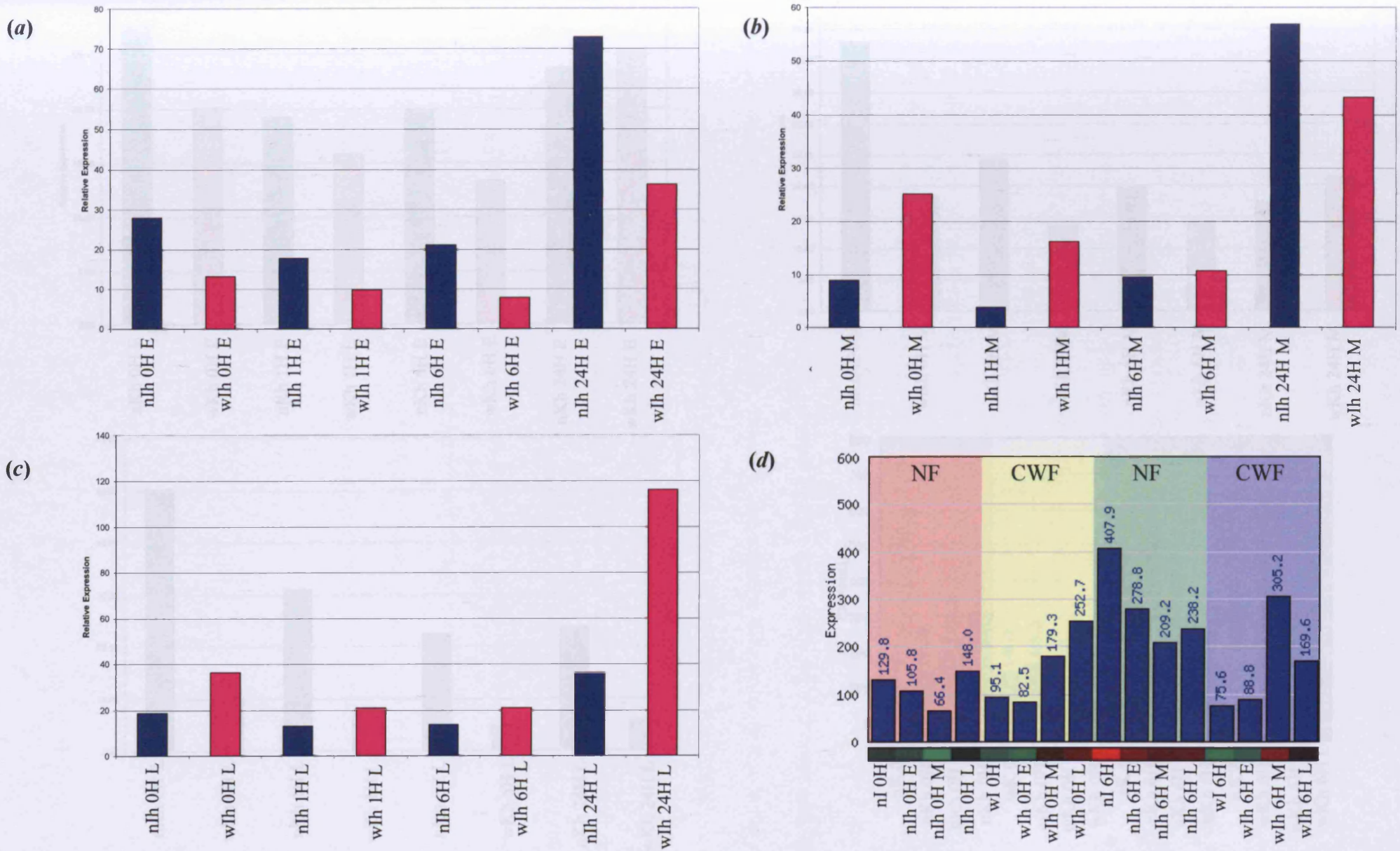


Figure 5.15: Graphs showing relative gene expression levels for CXCL12 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

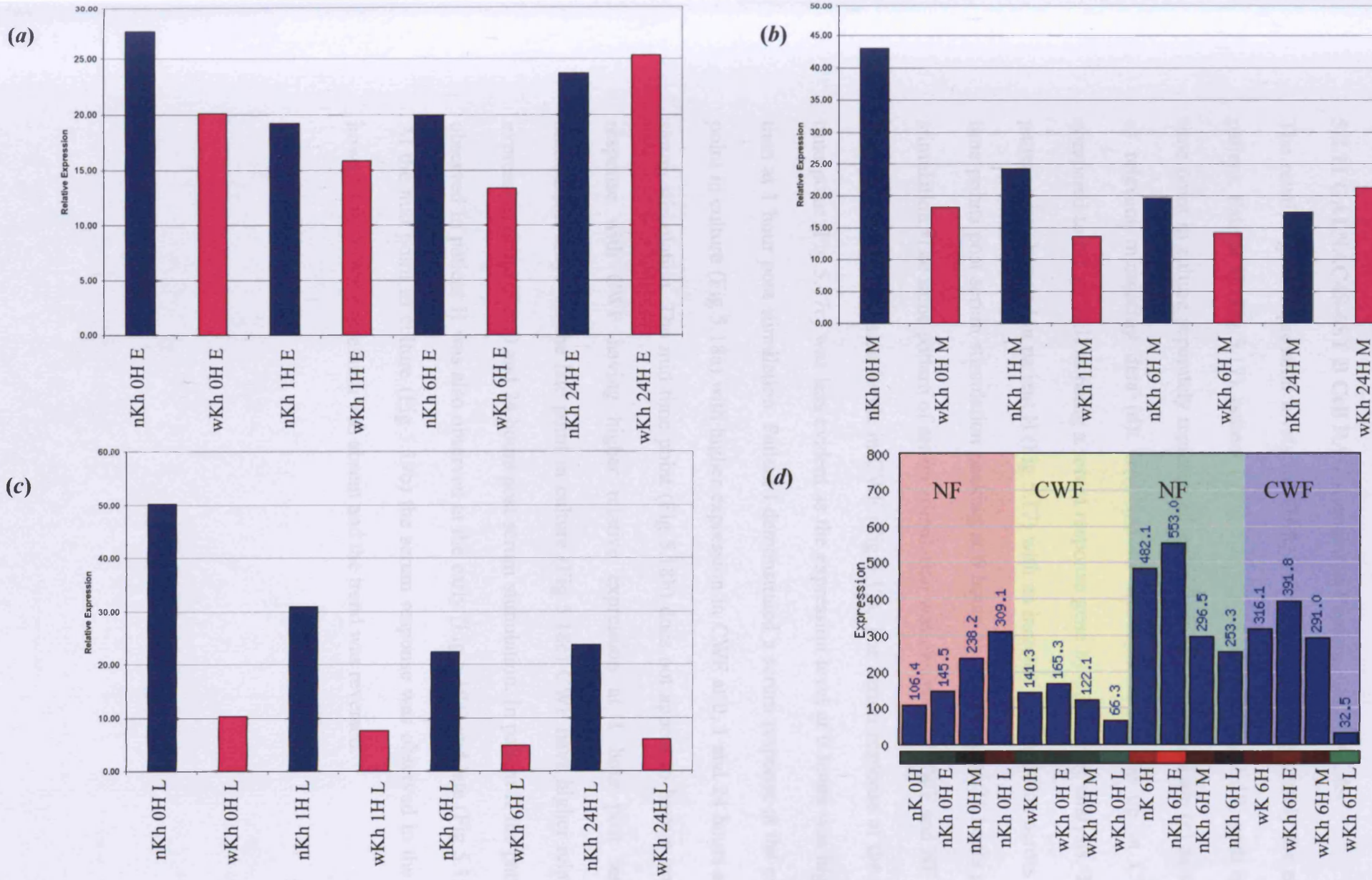


Figure 5.16: Graphs showing relative gene expression levels for CXCL12 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.11 GALNAC4S-6ST B Cell RAG Associated Protein, GALNAC4S

The relative gene expression levels for GALNAC4S are shown separately for each patient. Patient H (Fig 5.17), patient I (Fig 5.18) and patient K (Fig 5.19) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). GALNAC4S was observed to be up in CWF compared to NF as well as being a serum response gene by microarray analysis. This pattern was observed in patient H (Fig 5.17) with an increase in expression across the time points post serum stimulation peaking at 6 hours and reducing at 24 hours post stimulation. The same pattern of serum stimulation was observed in CWF and NF but with increased expression levels in CWF (Fig 5.17). The serum response at the late time point (Fig 5.17c) was less evident as the expression level at 0 hours was higher than at 1 hour post stimulation. Patient I demonstrated a serum response at the early point in culture (Fig 5.18a) with higher expression in CWF at 0, 1 and 24 hours after serum stimulation. The mid time point (Fig 5.18b) does not appear to have a serum response with CWF having higher relative expression at 1 hour post serum stimulation only. At the late point in culture (Fig 5.18c) CWF have higher relative expression than NF at 0 and 24 hours post serum stimulation. In patient K the pattern observed in patient H was also observed at the early (Fig 5.19a) and late (Fig 5.19c). At the mid point in culture (Fig 5.19b) the serum response was observed in the NF however the CWF expression was absent and the trend was reversed.

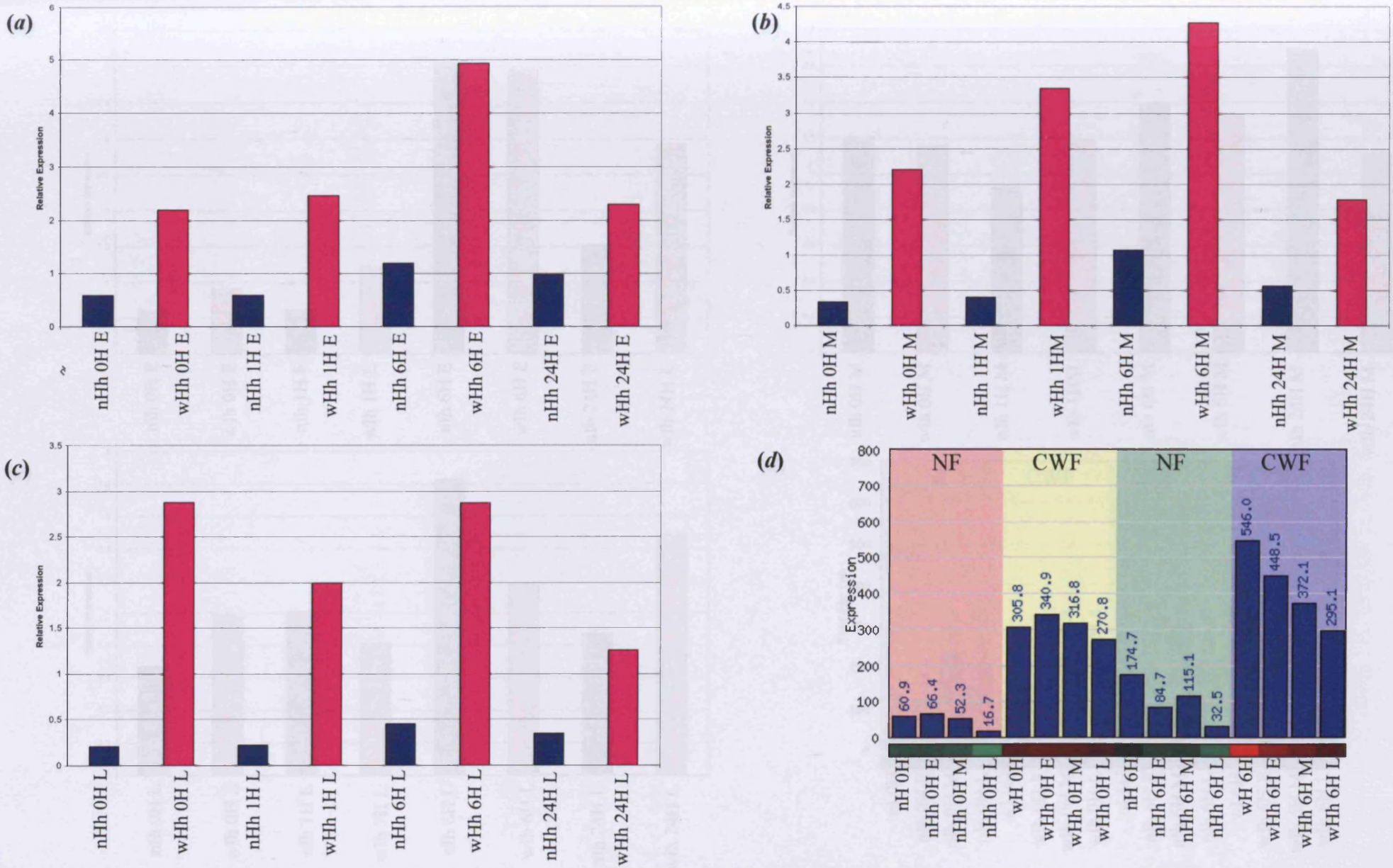


Figure 5.17: Graphs showing relative gene expression levels for GALNAC4S in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

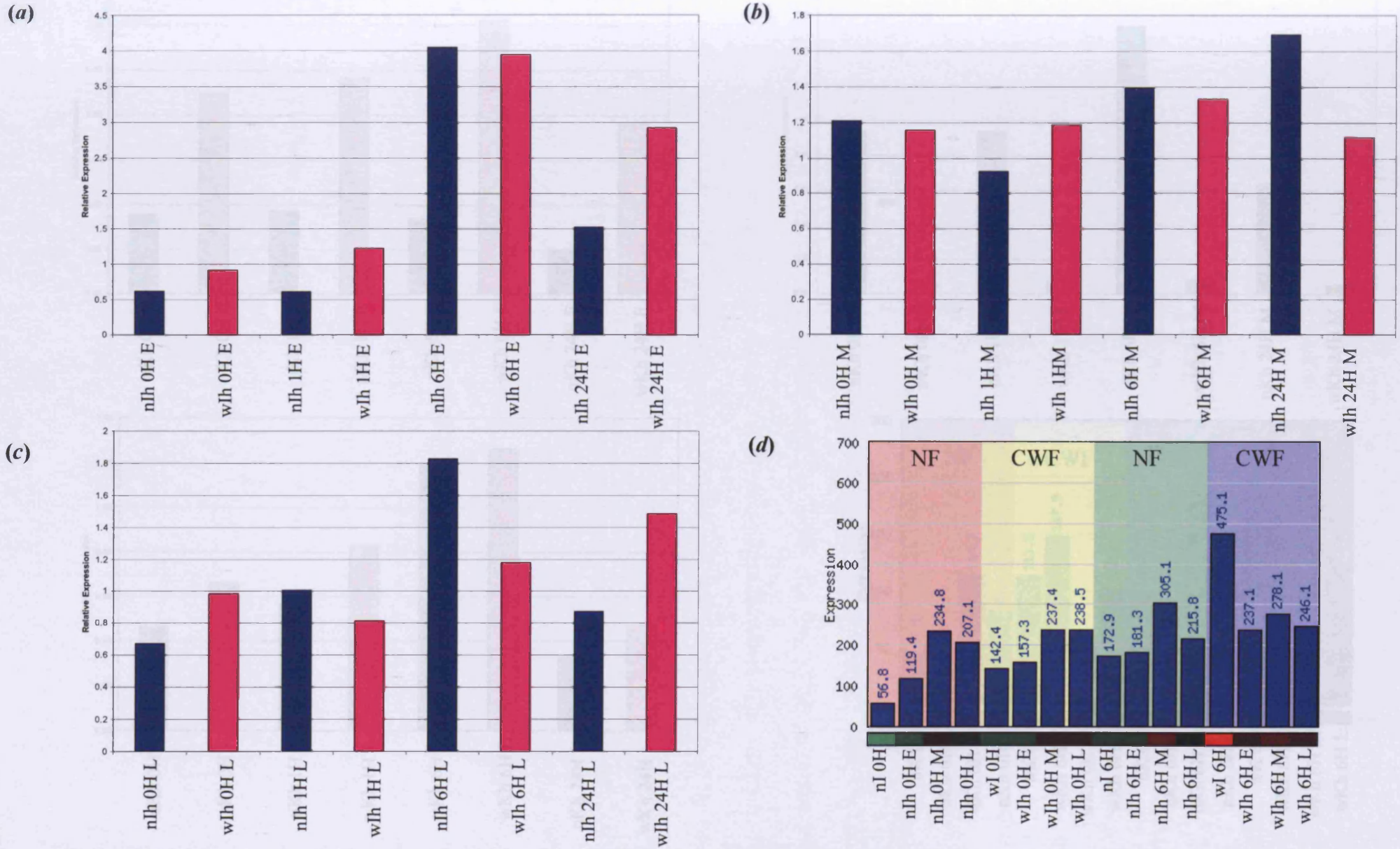


Figure 5.18: Graphs showing relative gene expression levels for GALNAC4S in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

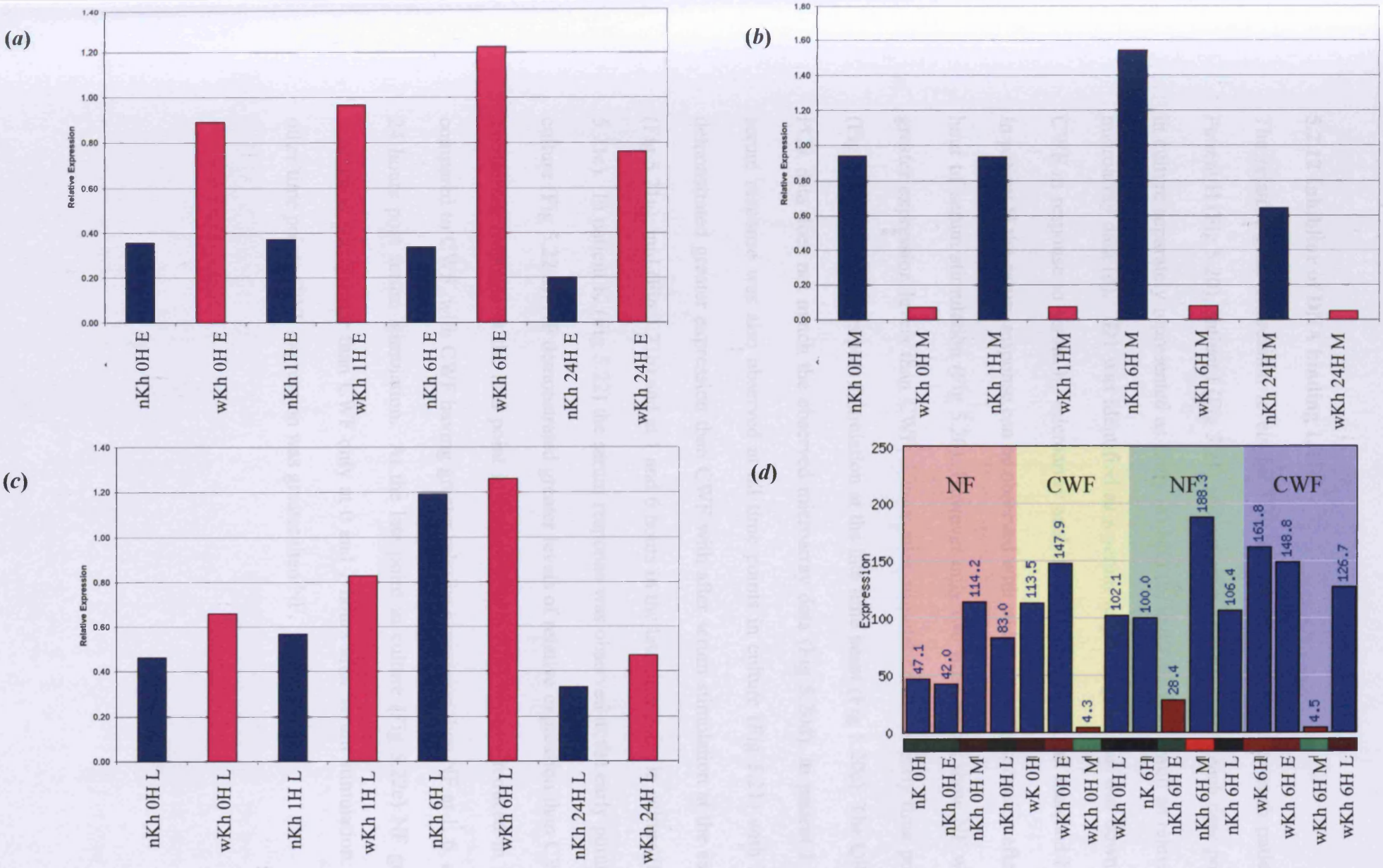


Figure 5.19: Graphs showing relative gene expression levels for GALNAC4S in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.12 Inhibitor of DNA binding 1, ID1

The relative gene expression levels for ID1 are shown separately for each patient. Patient H (Fig 5.20), patient I (Fig 5.21) and patient K (Fig 5.22) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). ID1 was identified as a serum response gene that was down in CWF in response to serum by microarray analysis compared to patient matched NF. In patient H the serum response can be observed with an increase of expression after 1 hour of serum stimulation (Fig 5.20). However only two time points show NF with greater expression levels than CWF; 1 hour after stimulation at the early time point (Fig 5.20a) and 6 hours post stimulation at the late time point (Fig 5.20c). The QRT-PCR data does not match the observed microarray data (Fig 5.20d). In patient I the serum response was also observed at all time points in culture (Fig 5.21) with NF demonstrated greater expression than CWF with after serum stimulation at the early (Fig 5.21a), mid (Fig 5.21b) and at 1 and 6 hours in the late time point in culture (Fig 5.21c). In patient K (Fig 5.22) the serum response was observed at the early point in culture (Fig 5.22 a), NF demonstrated greater levels of relative expression than CWF. The serum response at the mid point in culture (Fig 5.22b) appears reduced in NF compared to CWF, with CWF having greater relative expression than NF at 1, 6, and 24 hours post serum stimulation. At the late point in culture (Fig 5.22c) NF gene expression was greater than CWF only at 0 and 1 hours after serum stimulation. At other time points CWF expression was greater than NF.

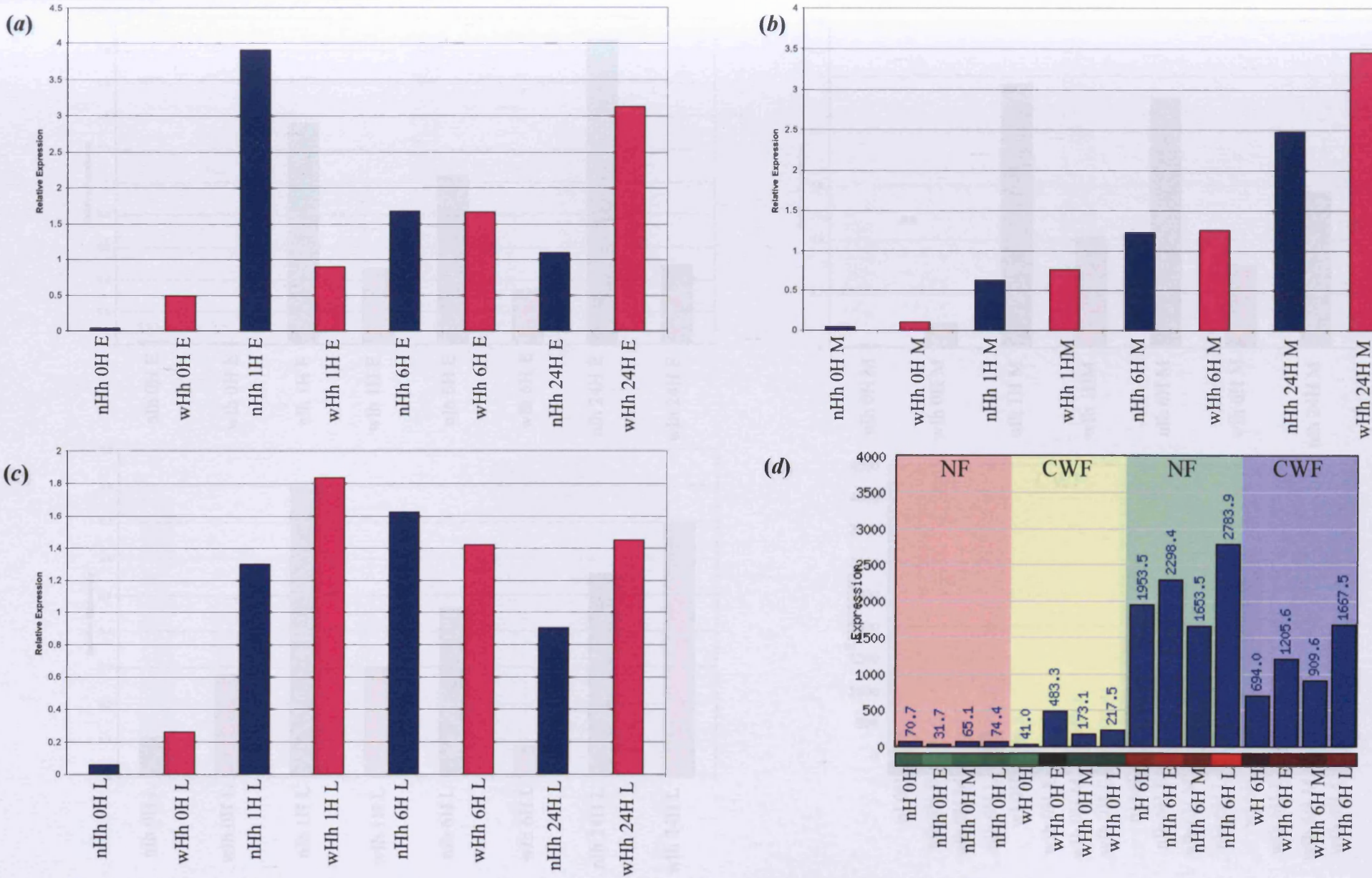


Figure 5.20: Graphs showing relative gene expression levels for ID1 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

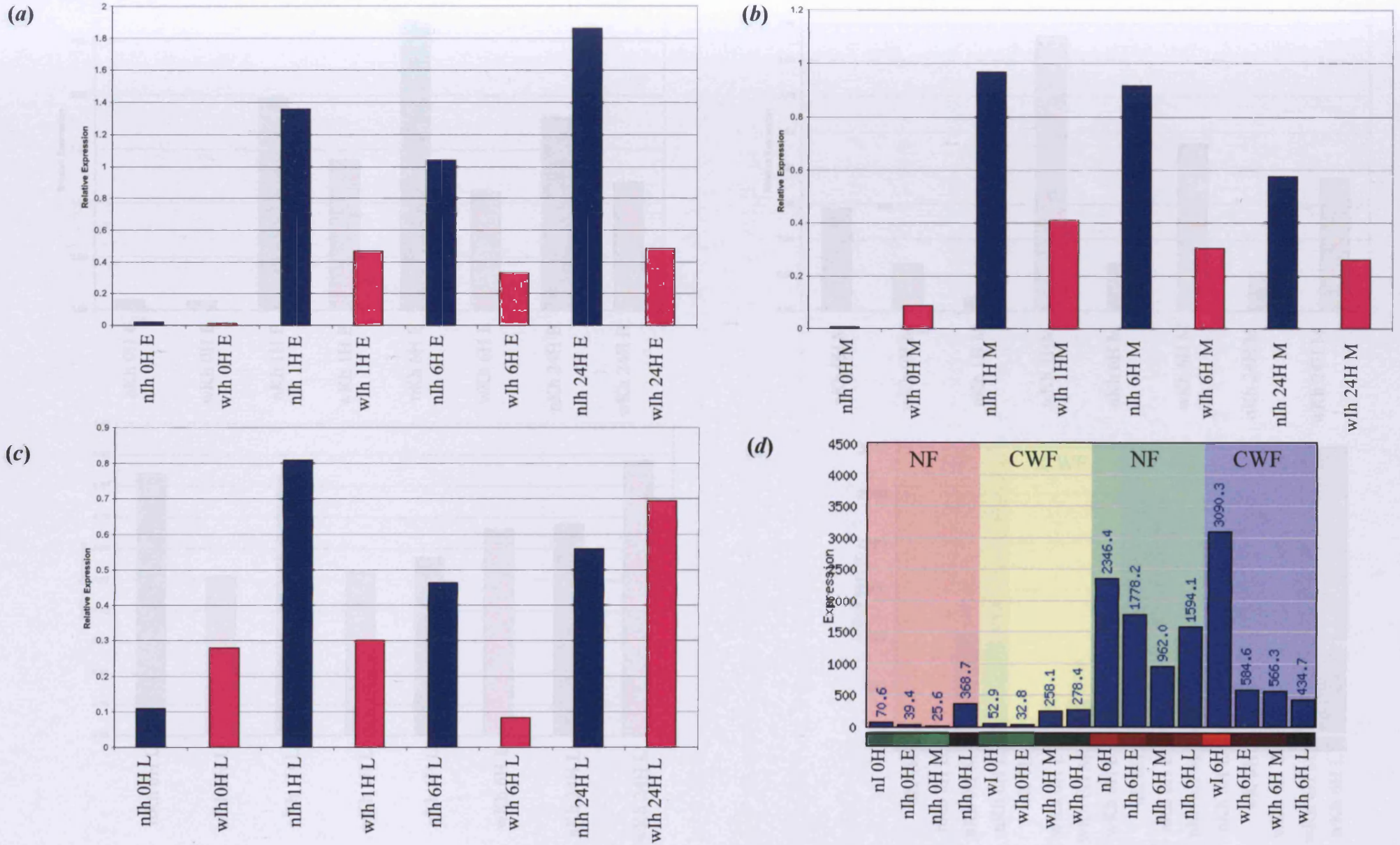


Figure 5.21: Graphs showing relative gene expression levels for ID1 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

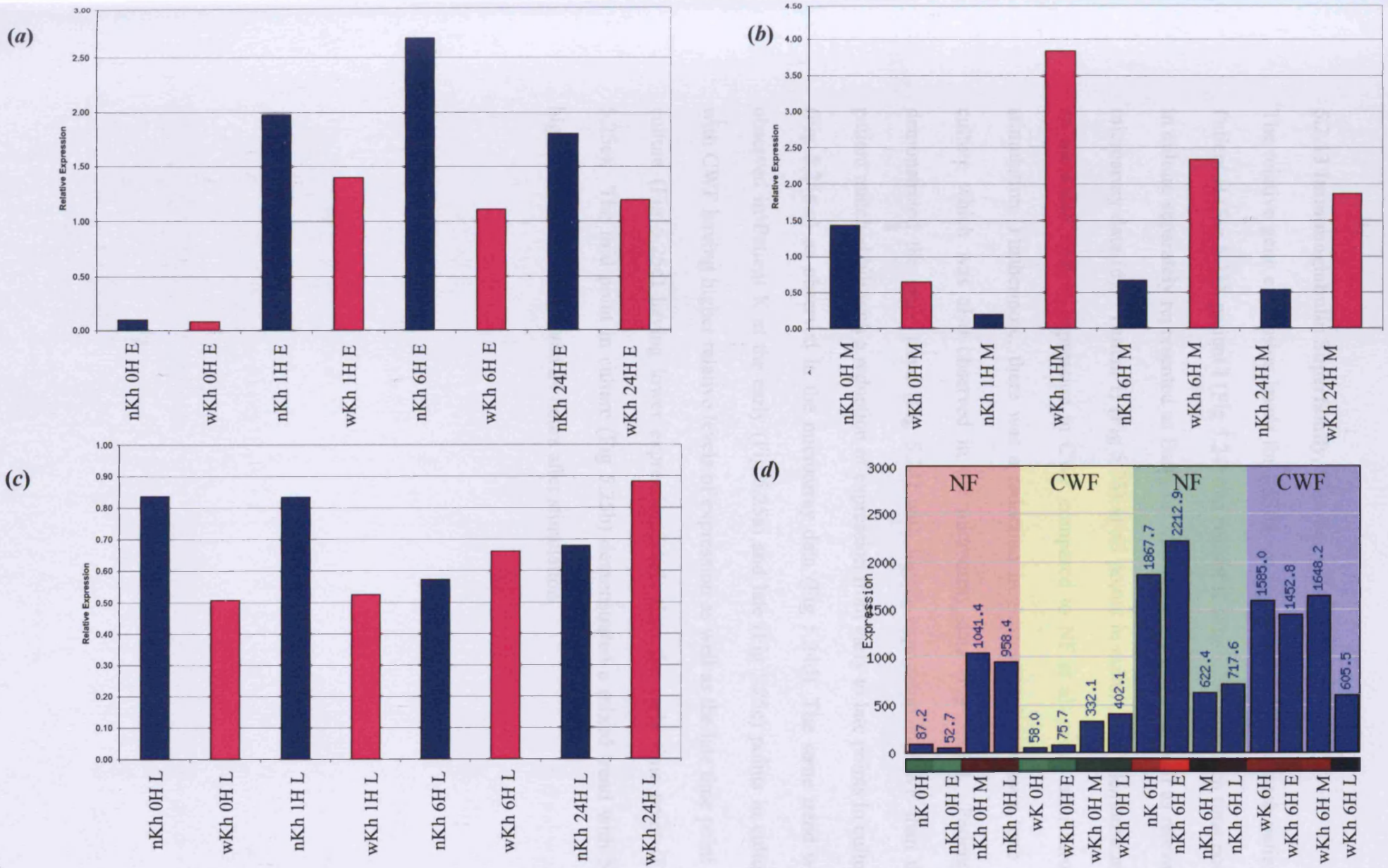


Figure 5.22: Graphs showing relative gene expression levels for ID1 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.13 Immunoglobulin Superfamily, Member 4, IGSF4

The relative gene expression levels for IGSF4 are shown separately for each patient. Patient H (Fig 5.23), patient I (Fig 5.24) and patient K (Fig 5.25) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). Patient H (Fig 5.23) at all points in culture (a-c) demonstrated an increased relative expression in CWF compared to NF at all points after serum stimulation. Furthermore, there was a reduction in expression levels over time in culture which was also observed in the microarray data (Fig 5.23d). Patient I demonstrated the same trend (Fig 5.24) with higher expression in CWF than the patient matched NF with a reduction of expression from early to late points in culture (Fig 5.24a-c) as observed in the microarray data (Fig 5.24d). The same trend was observed in Patient K at the early (Fig 5.25a) and late (Fig 5.25c) points in culture with CWF having higher relative levels of expression as well as the late time point in culture (Fig 5.25c) having lower expression levels than the early time point (Fig 5.25c). The mid point in culture (Fig 5.25b) demonstrated a mixed trend with NF higher than CWF at 0, 6 and 24 hours after stimulation.

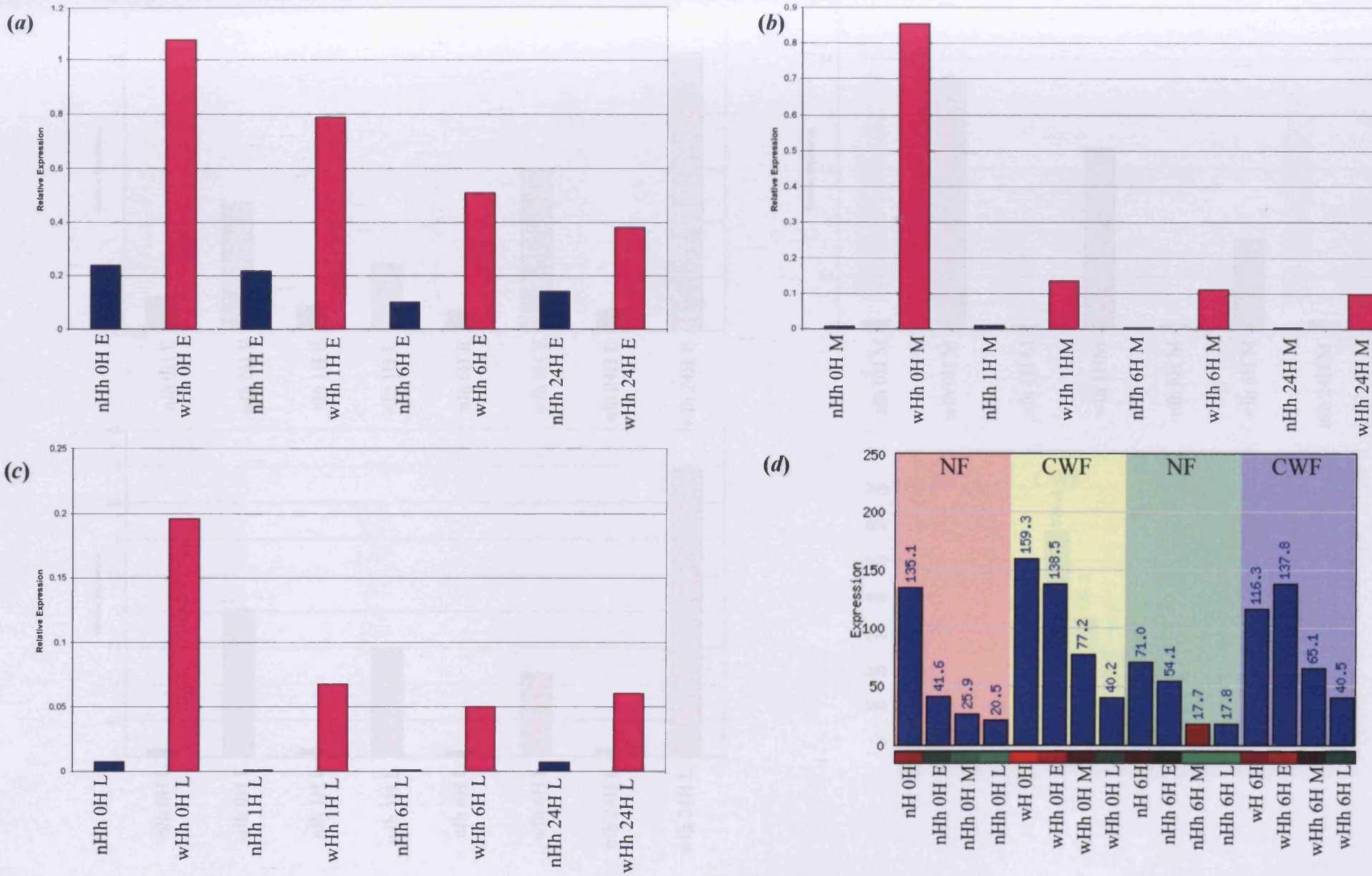


Figure 5.23: Graphs showing relative gene expression levels for IGSF4 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

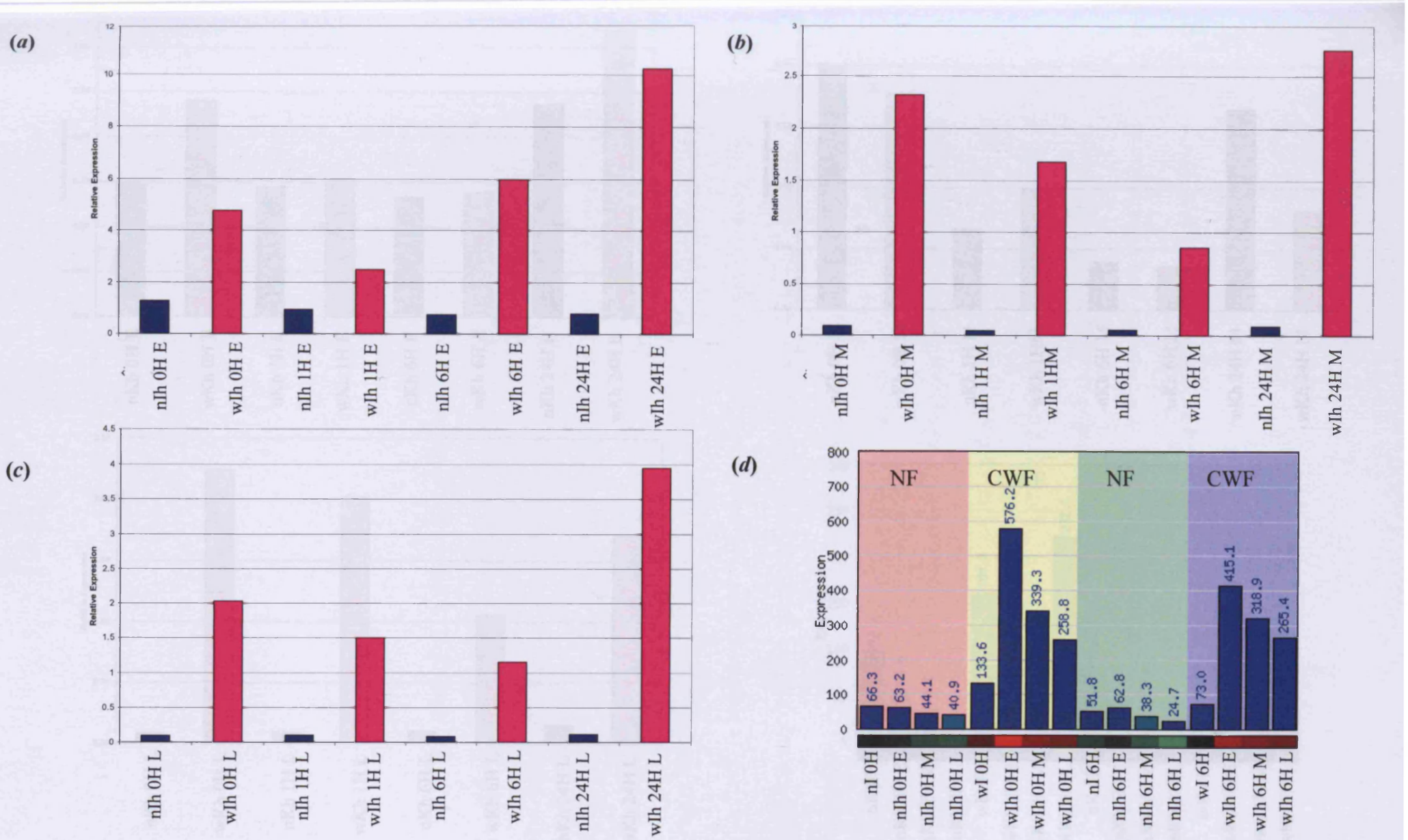


Figure 5.24: Graphs showing relative gene expression levels for IGSF4 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

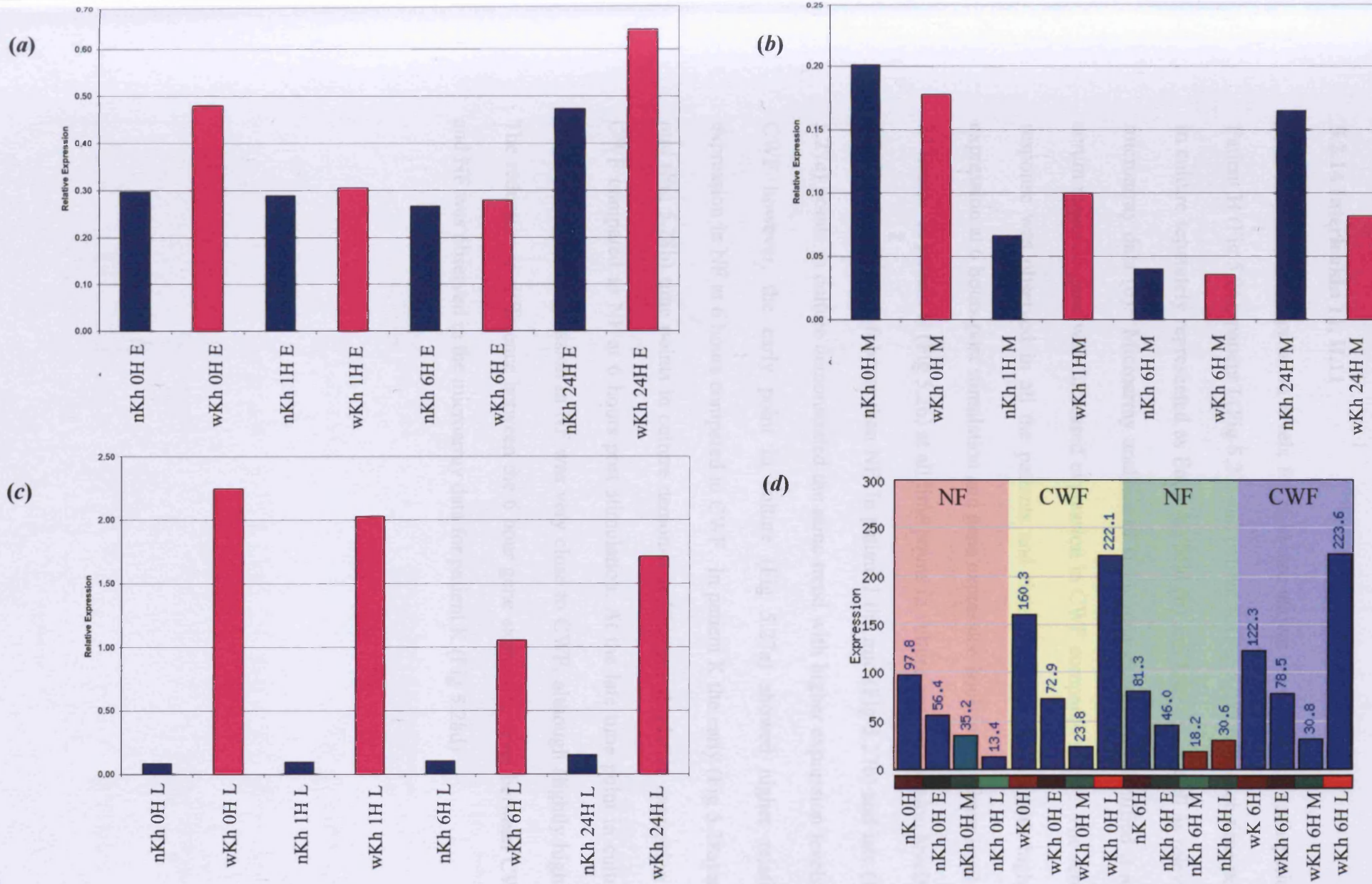


Figure 5.25. Graphs showing relative gene expression levels for IGSF4 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.14 Interleukin 11, IL11

The relative gene expression levels for IL11 are shown separately for each patient. Patient H (Fig 5.26), patient I (Fig 5.27) and patient K (Fig 5.28) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). Microarray analysis of IL11 gene expression identified it as a serum response gene with increased expression in CWF compared to NF. The serum response was observed in all the patients and time points in culture with highest expression at 6 hours post stimulation and gene expression back to the 0 hour level by 24 hours. In patient H (Fig 5.26) at all time points in culture gene expression levels in CWF were greater at 6 hours than NF. In patient I the mid (Fig 5.27b) and late (Fig 5.27c) points in culture demonstrated the same trend with higher expression levels in CWF however, the early point in culture (Fig 5.27a) showed higher relative expression in NF at 6 hours compared to CWF. In patient K the early (Fig 5.28a) and mid (Fig 5.28b) time points in culture demonstrated increased relative expression in CWF compared to NF at 6 hours post stimulation. At the late time point in culture (Fig 5.28c) gene expression in NF was very close to CWF, although slightly higher. The reduction in difference between the 6 hour gene expression level between CWF and NF was observed in the microarray data for patient K (Fig 5.28d)

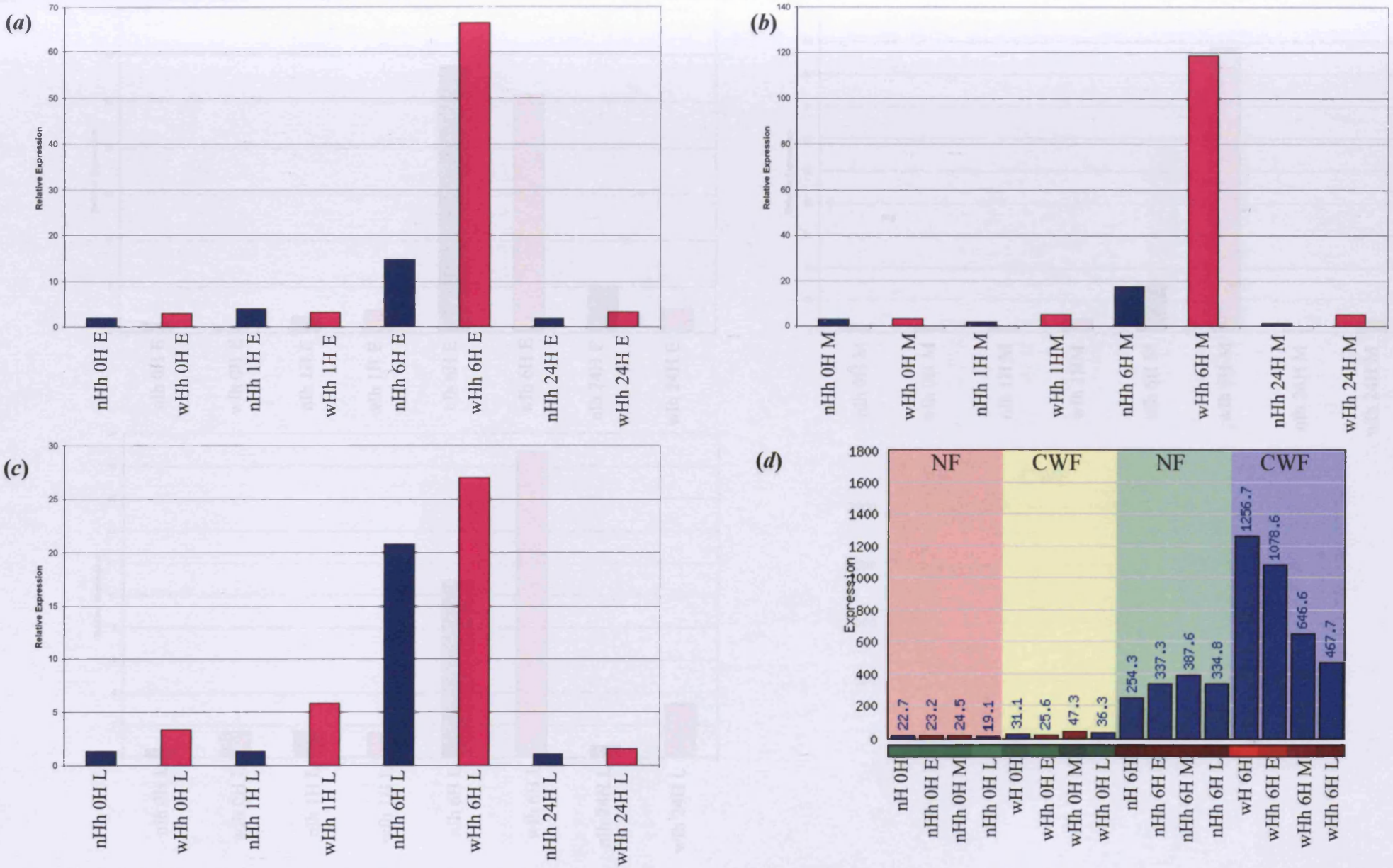


Figure 5.26: Graphs showing relative gene expression levels for IL11 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

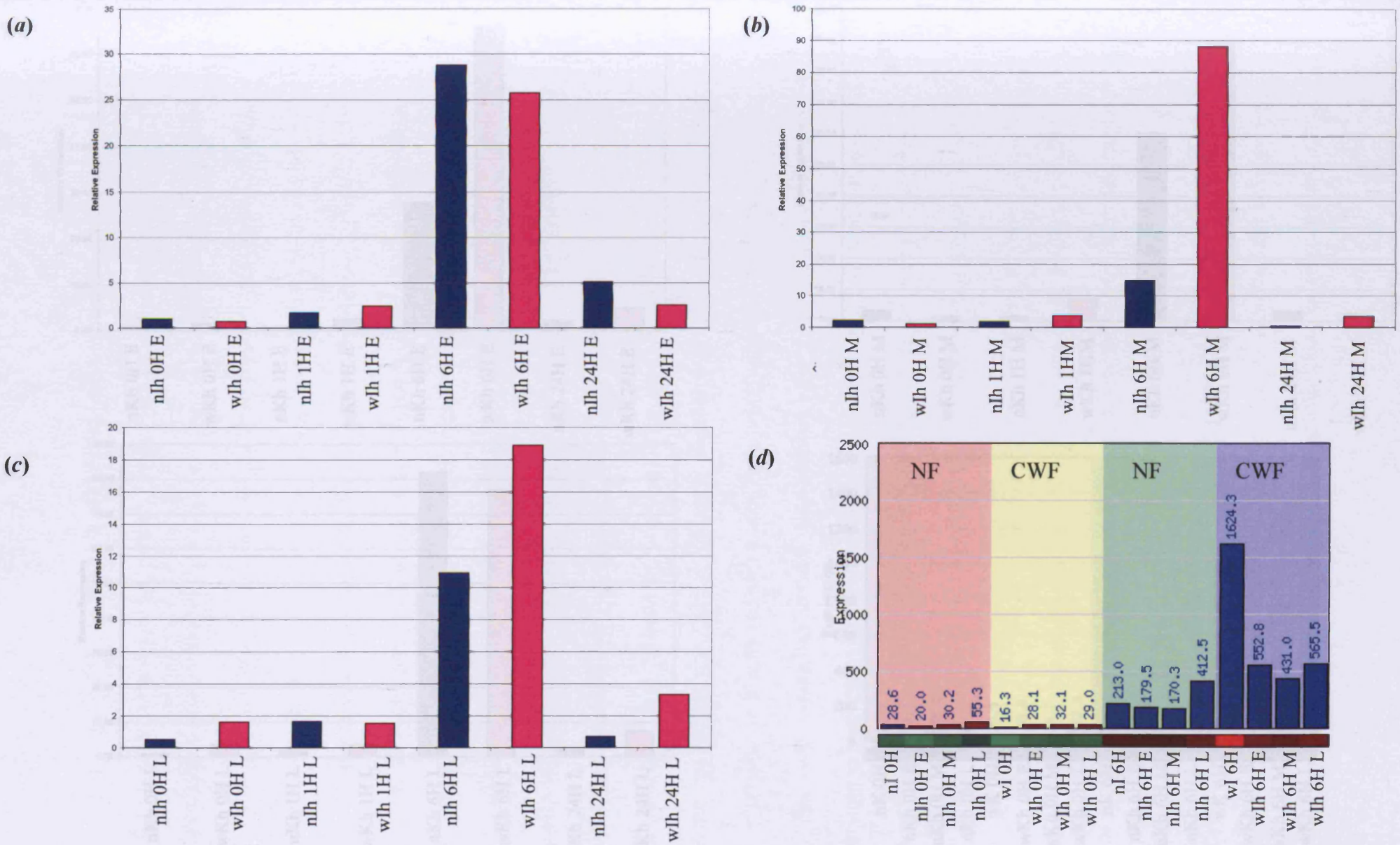


Figure 5.27: Graphs showing relative gene expression levels for IL11 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

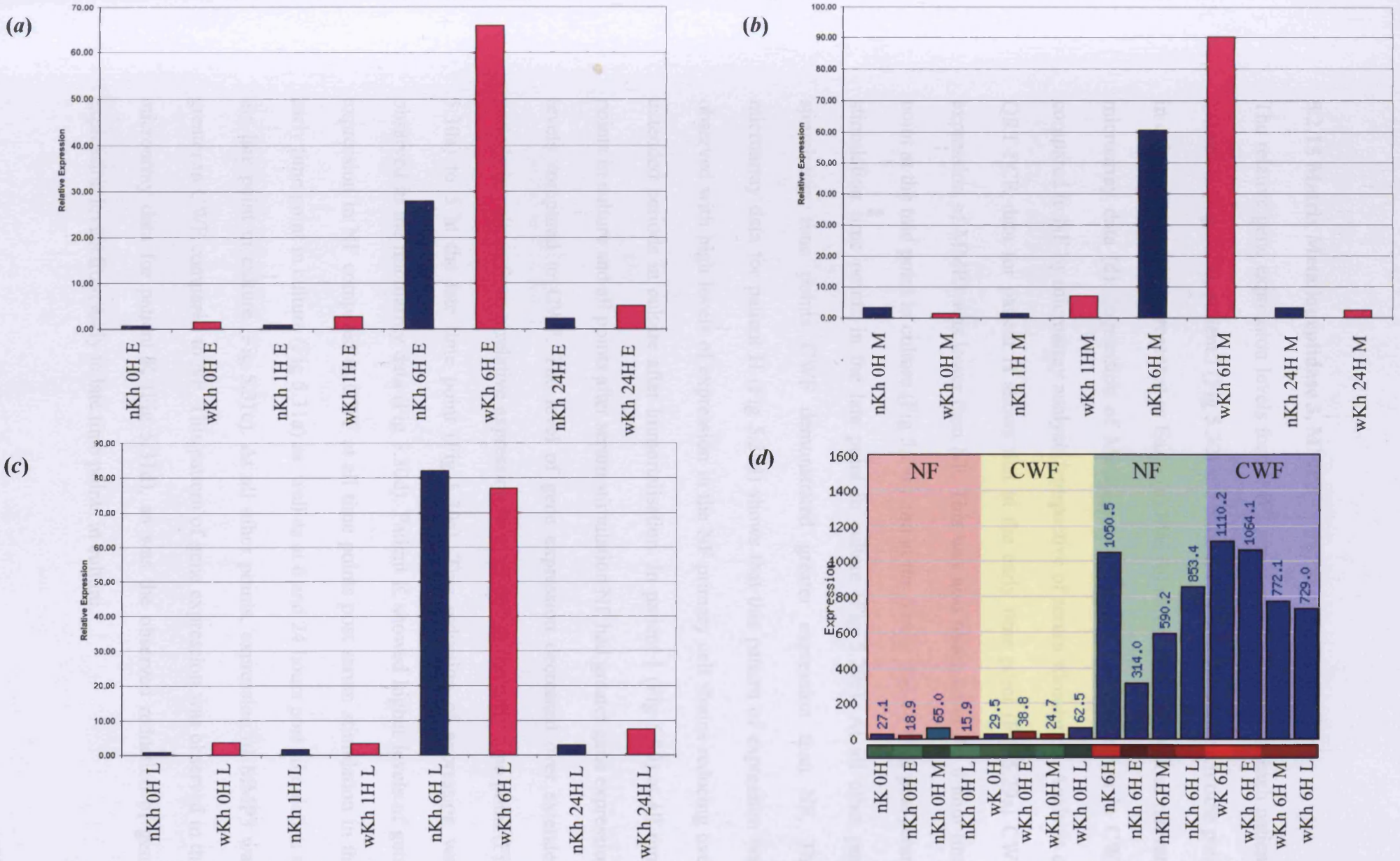


Figure 5.28: Graphs showing relative gene expression levels for IL11 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.15 Matrix Metallopeptidase 3, MMP3

The relative gene expression levels for MMP3 are shown separately for each patient. Patient H (Fig 5.29), patient I (Fig 5.30) and patient K (Fig 5.31) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). Expression of MMP3 was identified as being down in CWF compared to NF by microarray analysis irrespective of serum stimulation. Analysis of QRT-PCR data for patient H shows that at the early time point (Fig 5.29a) CWF expression of MMP3 was lower than NF. This was also observed at the 0 hour time point at the mid point in culture (Fig 5.29b) and at the 6 hour and 24 hour post serum stimulation time points in the late point in culture (Fig 5.29c). At all other post stimulation time points CWF demonstrated greater expression than NF. The microarray data for patient H (Fig 5.29d) shows that this pattern of expression was observed with high levels of expression in the NF primary cell strains reducing over extended periods in culture after immortalisation. In patient I (Fig 5.30) at all time points in culture and all points after serum stimulation NF had greater gene expression levels compared to CWF. The level of gene expression decreased over extended periods in culture from a relative expression level of 400 at the early time point (Fig 5.30a) to 5 at the late time point (Fig 5.30c). This reduction of expression was observed in the microarray data (Fig 5.30d). Patient K showed higher levels of gene expression in NF compared to CWF at all time points post serum stimulation in the early time point in culture (Fig 5.31a) as well as at 6 and 24 hours post stimulation at the late point in culture (Fig 5.31c). At all other points, expression of MMP3 was greater in CWF compared to NF. This pattern of gene expression was observed in the microarray data for patient K (Fig 5.31d), as was the observed reduction of gene expression levels from early to late time points in culture.

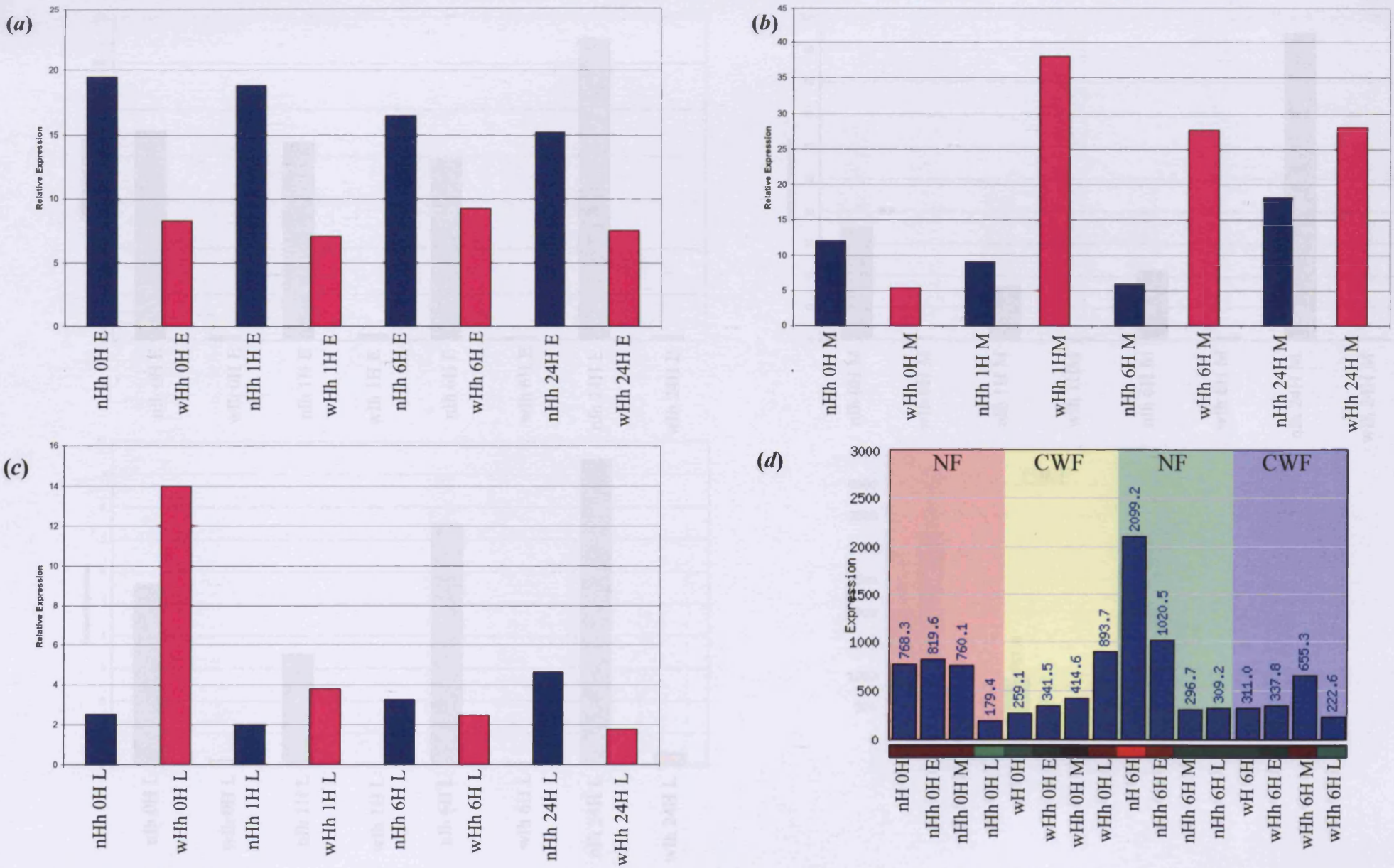


Figure 5.29: Graphs showing relative gene expression levels for MMP3 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

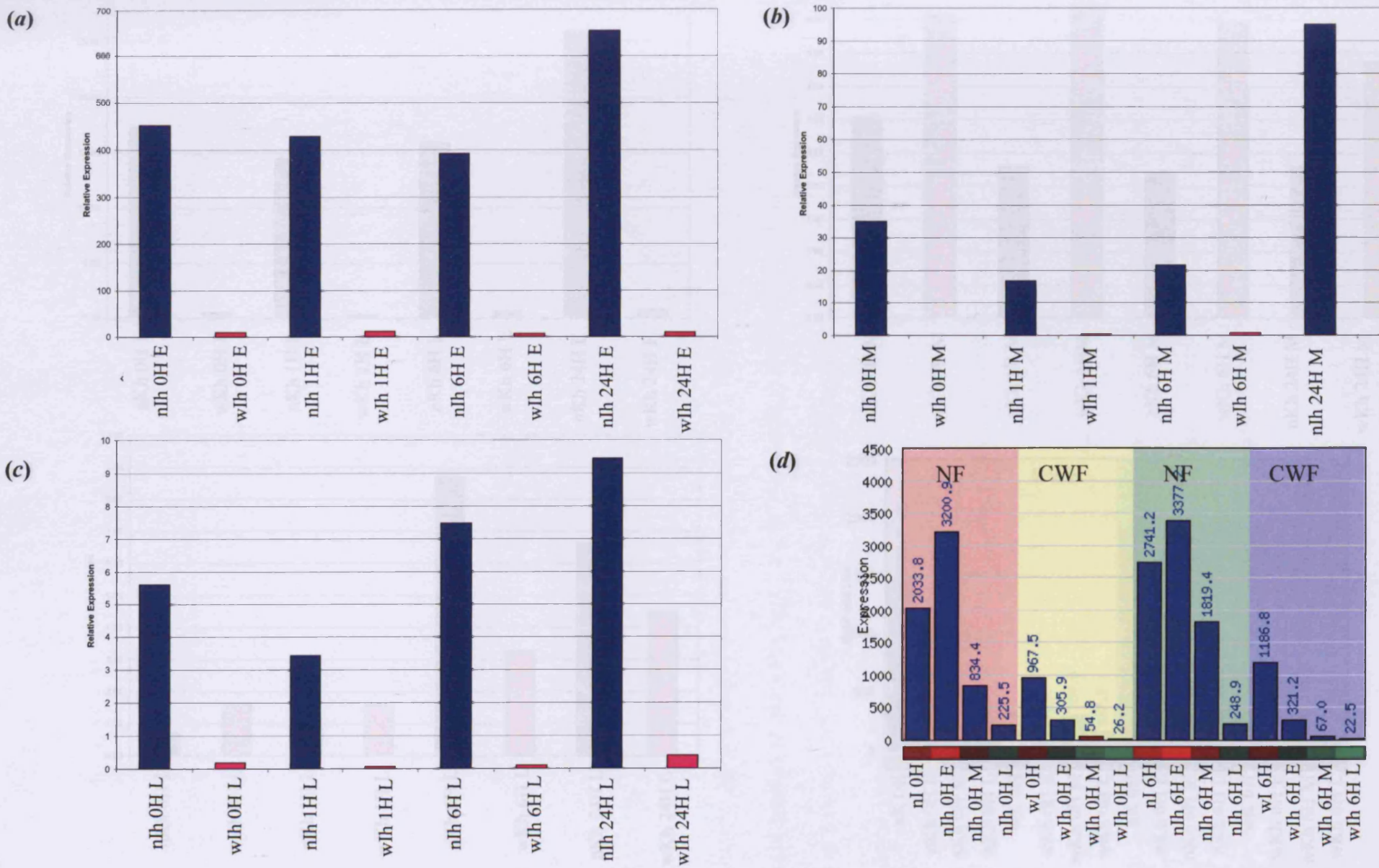


Figure 5.30: Graphs showing relative gene expression levels for MMP3 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

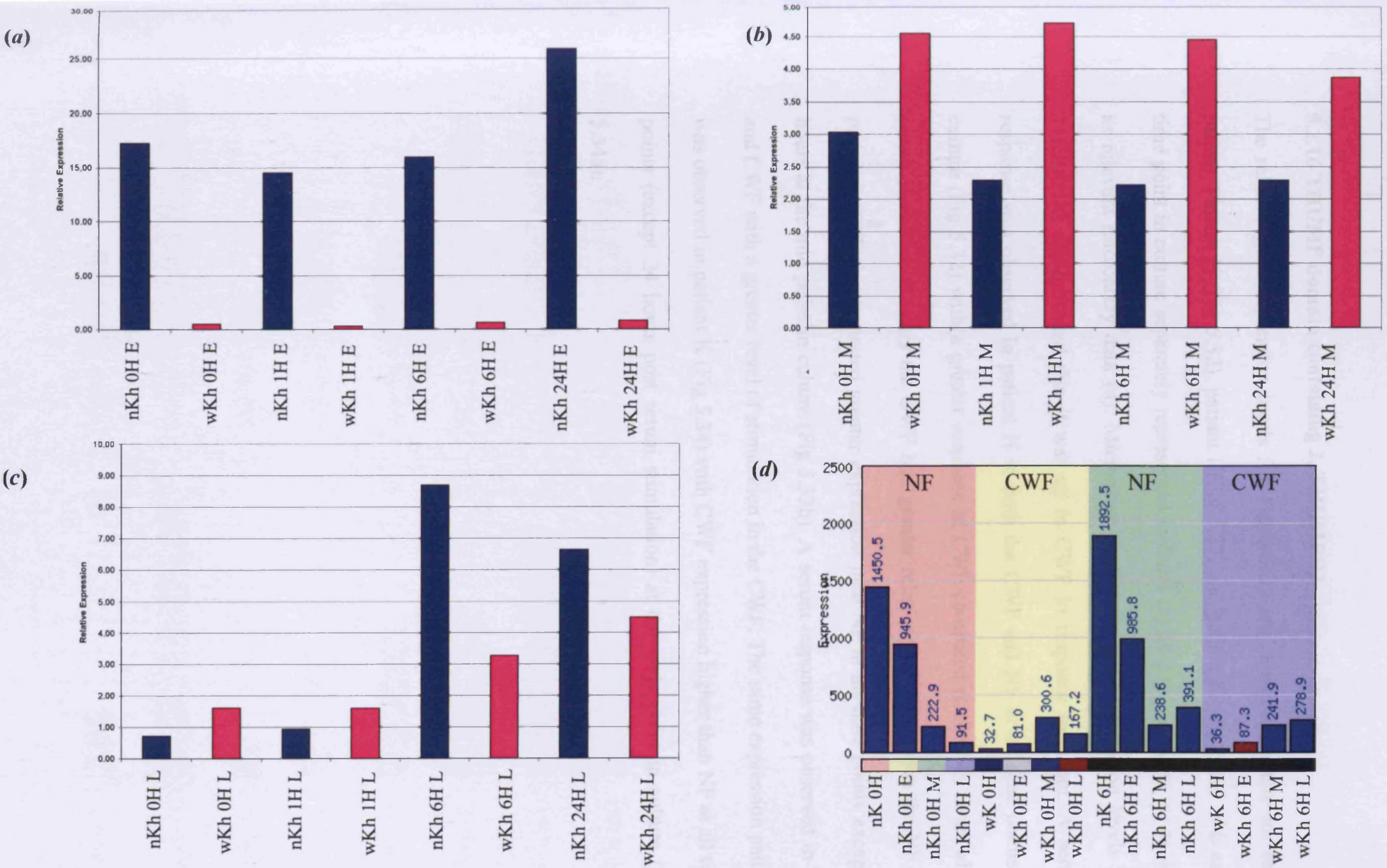


Figure 5.31: Graphs showing relative gene expression levels for MMP3 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.16 THUMP domain containing 2, THUMPD2

The relative gene expression levels for THUMPD2 are shown separately for each patient. Patient H (Fig 5.32), patient I (Fig 5.33) and patient K (Fig 5.34) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). Microarray analysis of gene expression levels for THUMPD2 demonstrated that it was up in CWF in response to serum. A serum response was observed in patient H in both the CWF and NF at all time points in culture (Fig 5.32) with a greater response in CWF compared to the NF. Even when serum starved at 0 hours the CWF had greater relative expression than the NF. In patient I CWF demonstrated greater expression than NF at all time points except 0 hours at the mid point in culture (Fig 5.33b). A serum response was observed in NF and CWF with a greater level of stimulation in the CWF. The same expression pattern was observed in patient K (Fig 5.34) with CWF expression higher than NF at all time points (except 24 hours post serum stimulation) at the early point in culture (Fig 5.34a).

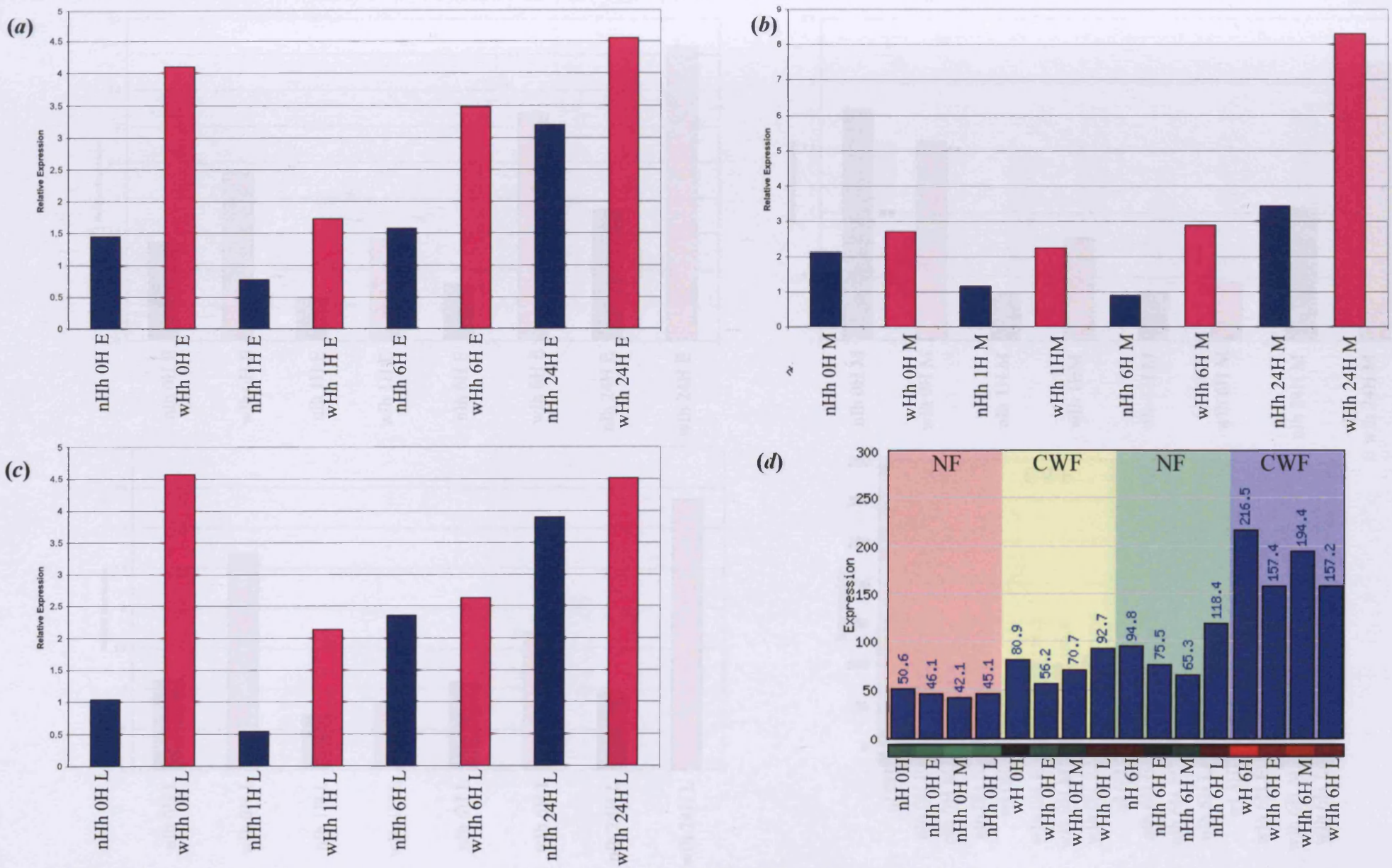


Figure 5.32: Graphs showing relative gene expression levels for THUMPD2 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

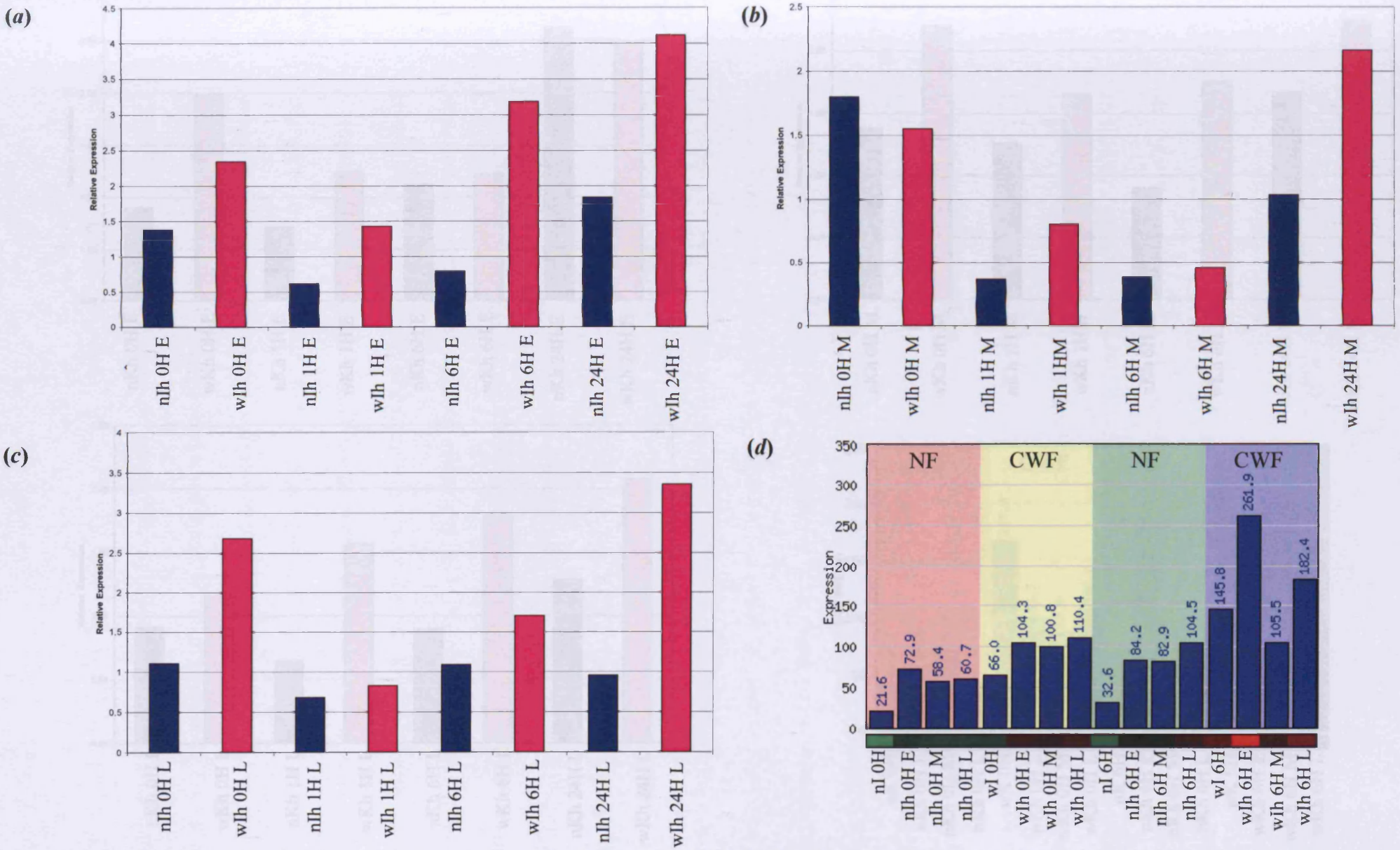


Figure 5.33: Graphs showing relative gene expression levels for THUMPD2 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

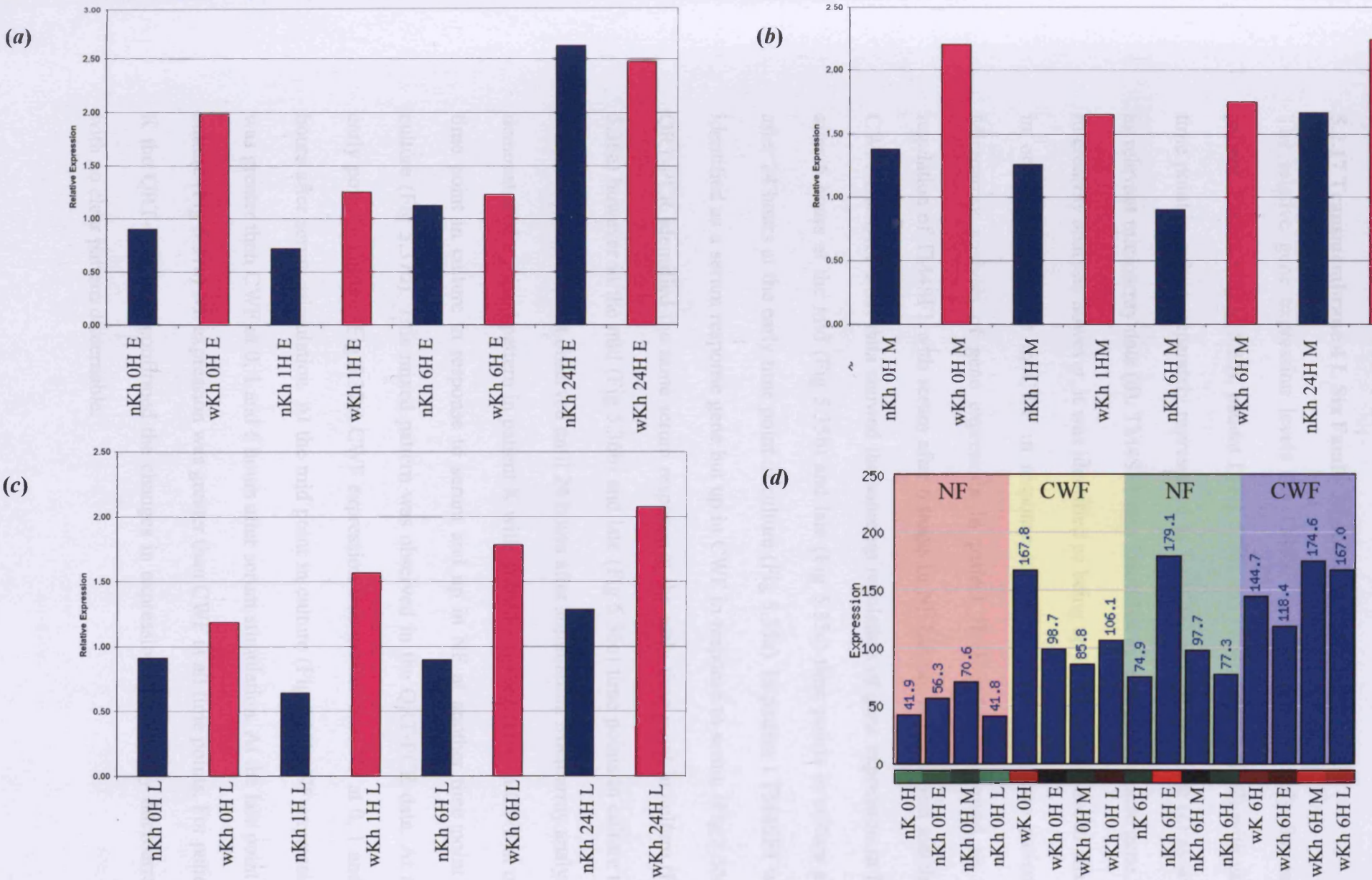


Figure 5.34: Graphs showing relative gene expression levels for THUMPD2 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.17 Transmembrane 4 L Six Family Member 1, TM4SF1

The relative gene expression levels for TM4SF1 are shown separately for each patient. Patient H (Fig 5.35), patient I (Fig 5.36) and patient K (Fig 5.37) with each time point in culture separately represented as Early (a) Mid (b) and Late (c) as well as relevant microarray data (d). TM4SF1 was identified as a serum response gene by microarray analysis however, it was identified as being up in NF in response to serum in one patient and up in CWF in response to serum in the other two patients. Microarray analysis of gene expression in patient H (Fig 5.35d) showed an up regulation of TM4SF1 with serum after 6 hours in NF but not in the patient matched CWF. The QRT-PCR data showed the same up regulation of gene expression in NF after 6 hours at the mid (Fig 5.35b) and late (Fig 5.35c) time points in culture and after 24 hours at the early time point in culture (Fig 5.35a). In patient I TM4SF1 was identified as a serum response gene but up in CWF in response to serum (Fig 5.36d). QRT-PCR identified the same serum response at the early time point in culture (Fig 5.36a) however in the mid (Fig 5.36b) and late (Fig 5.36c) time points in culture the serum response was not observed until 24 hours after stimulation. Microarray analysis demonstrated a mixed pattern in patient K with TM4SF1 up-regulated in CWF at one time point in culture in response to serum and up in NF at another time point in culture (Fig 5.37d). This mixed pattern was observed in the QRT-PCR data. At the early point in culture (Fig 5.37a) CWF expression was greater than NF at 0, 1 and 6 hours after serum stimulation. At the mid point in culture (Fig 5.37b) NF expression was greater than CWF at 0, 1 and 6 hours after serum stimulation. At the late point in culture (Fig 5.37c) NF expression was greater than CWF at all time points. For patient K the QRT-PCR data confirmed the changes in expression observed by microarray, with no clear pattern discernable.

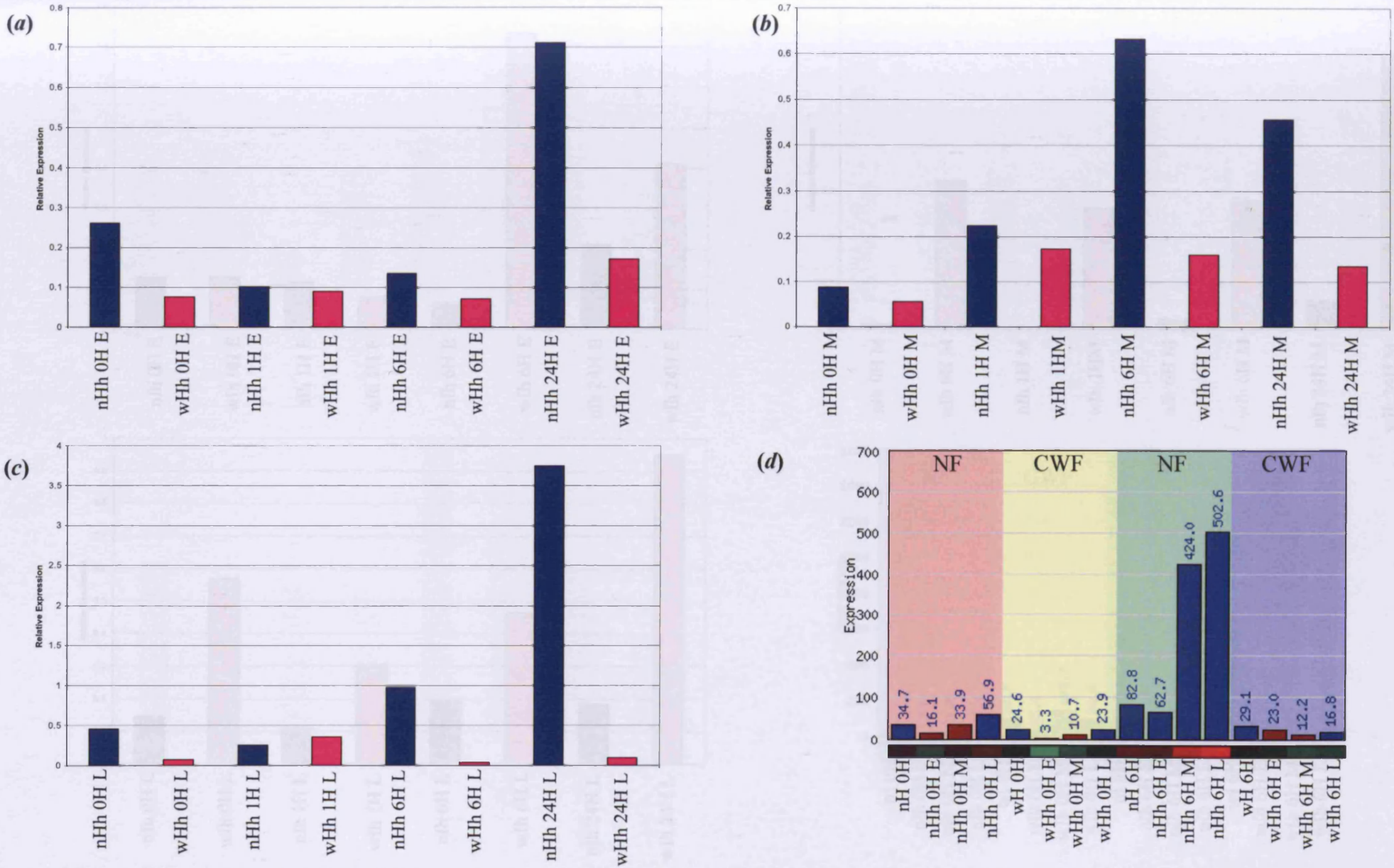


Figure 5.35: Graphs showing relative gene expression levels for TM4SF1 in Patient H at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

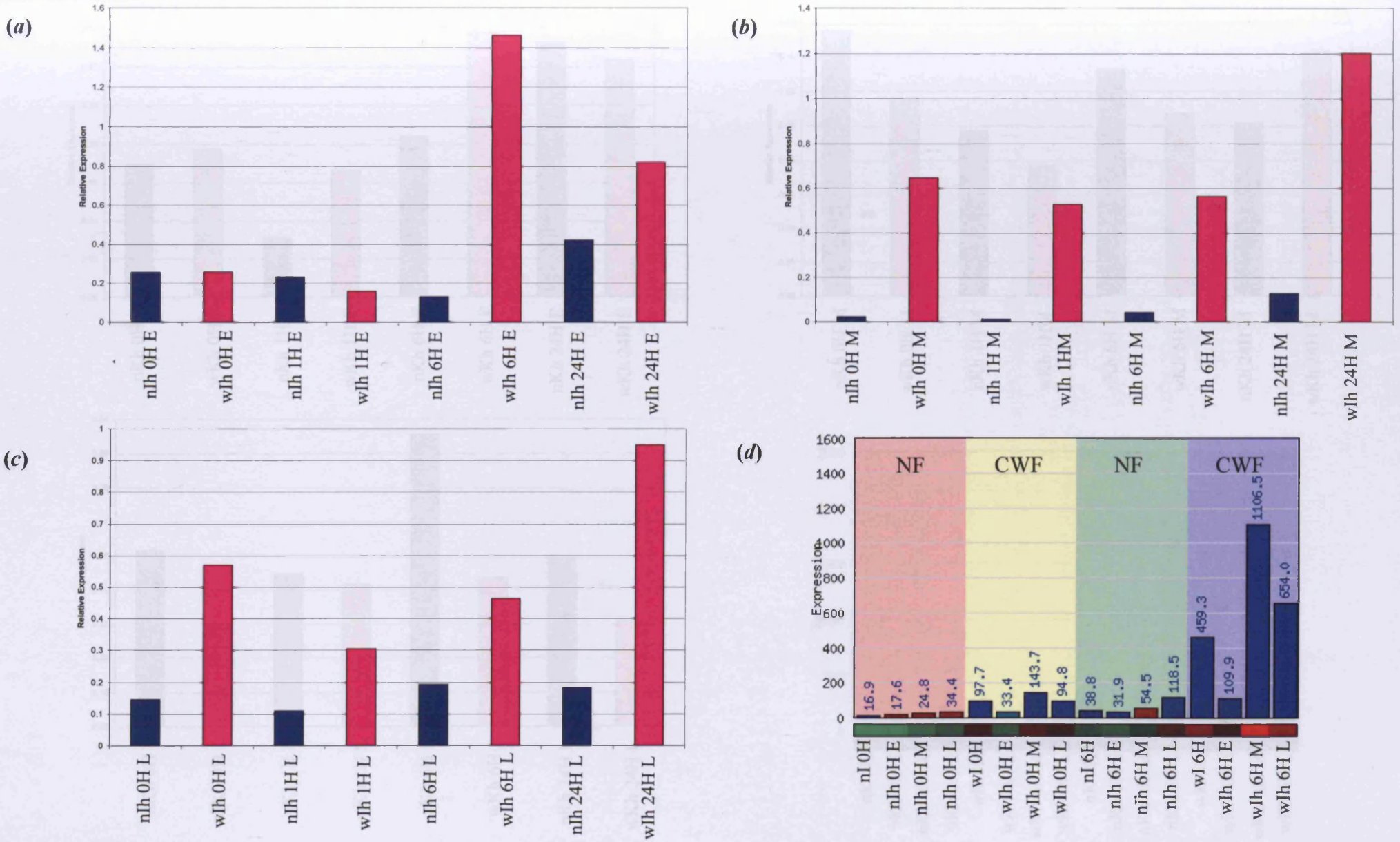


Figure 5.36: Graphs showing relative gene expression levels for TM4SF1 in Patient I at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

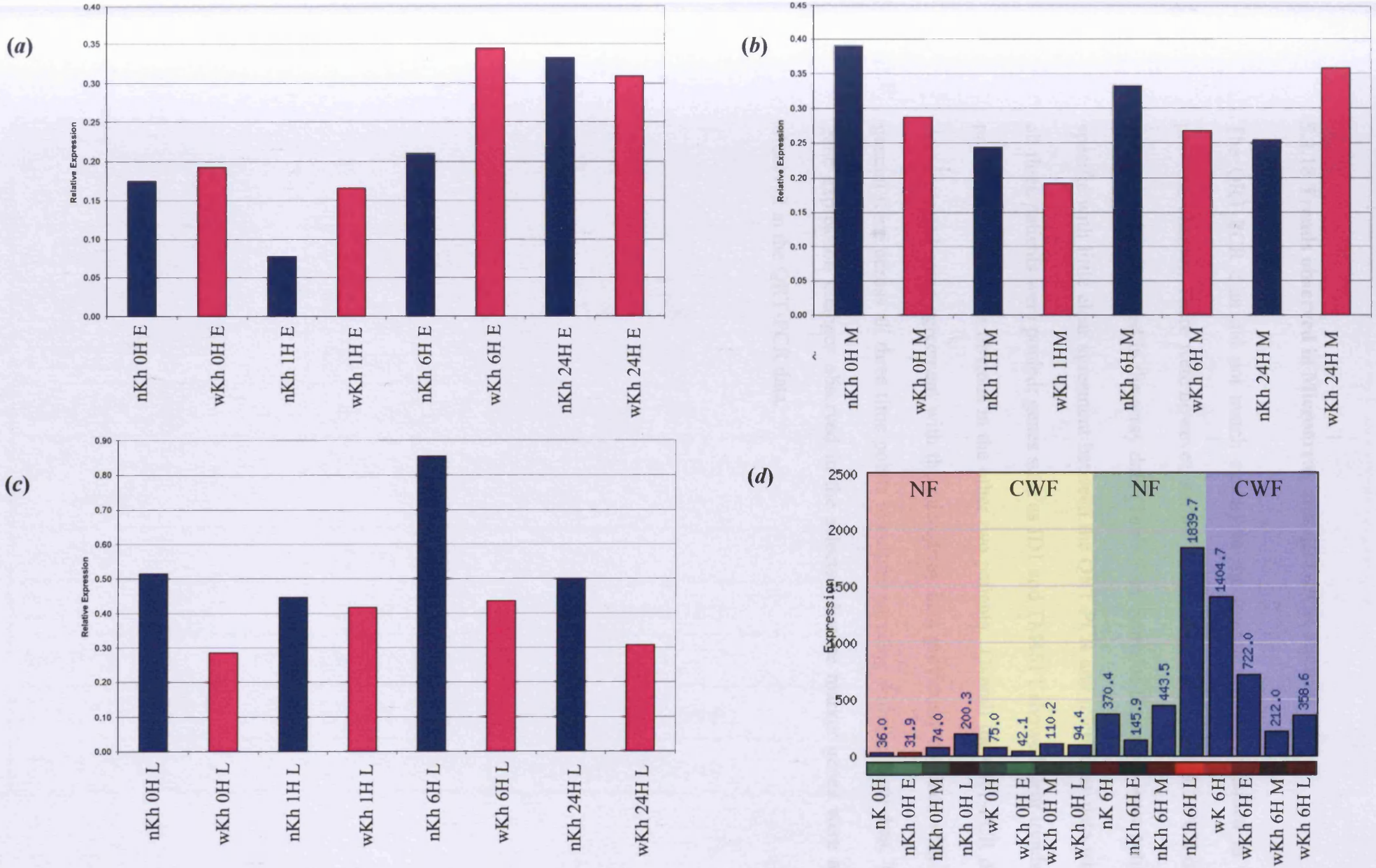


Figure 5.37: Graphs showing relative gene expression levels for TM4SF1 in Patient K at Early (a) Mid (b) and Late (c) time point in culture in NF (■) and CWF (■) at 0, 1, 6 and 24 hours after serum stimulation. Microarray data for each time point at 0 and 6 hours after stimulation is shown for comparison (d)

5.2.18 Trends observed in Microarray and qRT-PCR analysis

The QRT-PCR data did not match exactly to the microarray data obtained in the previous chapter. There were however, similar trends observed in the QRT-PCR data that were also observed in the array data (Table 5.1). Some of the trends were patient specific with little clear agreement between the QRT-PCR and microarray data when all three patients were pooled; genes such as ID1 and TM4SF1 showed clear trends in patient I but were less obvious in the other two patients. Overall the QRT-PCR data demonstrated some agreement with the microarray data previously obtained with the general trend across all three time points in culture agreeing with the array data. The gene expression changes observed in the selected disease marker genes were also observed in the QRT-PCR data.

Gene Name	Microarray Data	qRT-PCR
ACTC	Down in CWF	Down in CWF
CD9	Up in CWF	Up in CWF
CXCL1	Down in CWF (serum response)	Down in CWF (serum response)
CXCL12	Down in CWF (serum response)	Down in CWF (serum response)
GALNAC4S	Up in CWF (serum response)	Up in CWF (serum response)
ID1	Down in CWF (serum response)	Patient dependant, down in CWF (serum response)
IGSF4	Up in CWF	Up in CWF
IL11	Up in CWF (serum response)	Up in CWF (serum response)
MMP3	Down in CWF	Down in CWF
THUMPD2	Up in CWF (serum response)	Up in CWF (serum response)
TM4SF1	Up in CWF (serum response)	Patient dependant

Table 5.1: Table showing gene expression trends identified by microarray analysis and qRT-PCR with percentage agreement between the two techniques.

5.3 Discussion

In this Chapter the gene expression profiles for a set of genes identified by microarray analysis as being differentially regulated between CWF and NF (Chapter 4) were further analysed by qRT-PCR. This was done firstly to validate the microarray analysis carried out and secondly to generate a more detailed serum stimulation profile for each gene of interest. If the gene expression changes observed by microarray analysis can be validated then the potential role these genes play in chronic wounds can be explored.

Variations were observed between the three patients with respect to gene expression for each gene under investigation. During the microarray analysis the patients were analysed separately, allowing the identification of gene expression changes between CWF and NF unique to that patient. The patients were also pooled giving an overall view of the changes in gene expression levels between CWF and NF. In a similar way the different time points in culture did not always show the same trends in gene expression often with a reduction of gene expression over extended periods in culture, although without an obvious change in the pattern of expression. The reduction of gene expression was not uniform across all genes identified. MMP3 in patient I showed dramatic reductions in relative expression levels over time in culture but other genes did not. No references to this phenomenon have been identified through literature searches.

The genes studied in this chapter were not selected specifically for their known links to wound healing or chronic wounds. They were selected through manual curation of microarray derived gene lists, developed MADRAS thus allowing the expression levels for each gene to be observed. The genes selected were chosen because of the large changes of expression between NF and CWF and the maintenance of these

expression changes over time in culture as well as between patients. The large changes in expression level for the genes selected suggest a potential use as part of a reporter assay. Interactions between the genes selected have been identified (Fig 4.21) as well as interactions with known wound healing genes such as TNFα. Most of the genes selected have been previously characterised and potential links with wound healing and the chronic wound phenotype are discussed below.

5.3.1 Genes validated as being up in CWF by QRT-PCR

Two genes were identified as having an increased expression in CWF compared to the patient matched NF independent of serum stimulation. These genes were always over-expressed in CWF.

CD9 is also known as: BA2, BTCC-1, DRAP-27, GIG2, MIC3, MRP-1, P24 and TSPAN29. CD9 is a member of the transmembrane-4 superfamily of cell surface molecules and is thought to be involved in cell migration and adhesion. CD9 expression is higher in CWF compared to NF. CD9 has been over-expressed in a range of cell types with all of the cells showed suppressed cell motility (Miyake et al., 1995). In melanoma BL6 cells CD9 expression suppresses metastatic potential (Zheng et al., 2005). CD9 expression in OS3-R5 cells caused suppression of the liver metastasis (Huang et al., 2006) this effect was thought to be due to inhibition of cell proliferation and motility. CD9 overexpression effects cell motility, a possible mechanism for this is via the Wnt-independent signal pathway (Watabe et al., 2003). CD9 regulates the actin cytoskeleton through a down-regualation of WAVE2. In relation to thisCWF cells have been shown to have a reduced proliferation rate compared to patient matched NF as well as reduced motility (Chapter 3). This

reduction in proliferation and migration could be partially caused by the increased expression of CD9 observed in the CWF.

IGSF4 Immunoglobulin Superfamily, Member 4 is also known as BL2, DKFZp686F1789, IGSF4A, NECL2, ST17, SYNCAM, TSLC1 and sTSLC-1. Many roles for IGSF4 have been identified (Biederer et al., 2002). IGSF4 was identified as a cell adhesion molecule found at the synapse and thought to be brain specific (Ito and Oonuma, 2006). IGSF4 was shown to be the adhesion molecule involved in mast cell interactions with nerve cells, IGSF4 was shown to bind homophilically with both mast cells and neurons expression the protein (Trabucchi et al., 1988, Martin and Leibovich, 2005). Mast cells play an important role in wound healing possibly contributing to the early response to wounding and play a role in the inflammatory reaction as well as angiogenesis (Kuramochi et al., 2001, Masuda et al., 2005). IGSF4 has been identified as a tumor suppressor gene in non small cell lung cancer with high levels of IGSF4 expression leading to tumor suppression (Laurent K. Verkoczy, 1998). IGSF4 may therefore play a number of roles within the chronic wound environment, its over-expression may be responsible for the inhibition of cell proliferation as observed in tumor cells. Over-expression of IGSF4 may also maintain the mast cell population in the wound longer than normal adding to the prolonged inflammatory phase.

5.3.2 Genes validated as being up in CWF (Serum response) by QRT-PCR

Four genes were identified as having an increased response to serum stimulation in CWF compared to patient matched NF. In the model for wound healing used in this experiment these genes were over-expressed altering the CWF wounding response.

GALNAC4S-6ST B Cell RAG Associated Protein is also known as BRAG, N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase, DKFZp781H1369, KIAA0598 and MGC34346. GALNAC4S-6ST is a membrane-integrated glycoprotein expressed in B cells (Ohtake et al., 2001). GALNAC4S-6ST has been shown to have sulfotransferase activity (Ito and Habuchi, 2000) GALNAC and to transfer sulfate to chondroitin sulfate A and dermatan sulfate (Paul et al., 1990). The sulfation of chondroitin sulfate alters the biological function of the glycosaminoglycan. No role for GALNAC in the skin has been identified however, glycosaminoglycans are important in the ECM of the skin and GALNAC may play a role in the modification of the ECM generated by the dermal fibroblasts. The increases expression of GALNAC in CWF in response to serum may lead to modifications of the ECM not observed in normal skin.

IL11 is a member of the gp130 family of cytokines characterized by containing transmembrane signalling receptor IL6ST (gp130). IL11 has been shown to play a role in the development of B cells, specifically the development of immunoglobulin-producing B cells (Schinkel et al., 2005). IL11 has been identified as being up-regulated after trauma in patients (Du et al., 1997) and has been shown to have a protective effect in animal models of ischemic bowel necrosis (Du et al., 1994) and after combination radiation/chemotherapy (Takeuchi et al., 2002). IL11 has also been identified as having a role in bone formation, enhancing the effect of bone morphogenetic protein (Ameglio et al., 1997). Under normal conditions IL11 appears to have a positive effect on bone and on wound healing. Aberrant expression of IL11 has been observed in psoriatic lesions of the skin and after total hip replacement surgery (Ameglio et al., 1997). In the psoriatic lesions of the skin IL11 was found to be present at significantly higher levels than non lesion skin (Xu et al., 1998) possibly

playing a role in the disease. In total hip replacements a significantly higher number IL11 expressing cells were found compared to control tissue. IL11 expression was hypothesized to play a role in periprosthetic osteolysis the loss of bone in the vicinity of the implant (Marchler-Bauer et al., 2007). IL11 over-expression has been observed at sites of inflammation, in the skin and bone. The increased expression of IL11 observed in CWF may play a role in the extended inflammatory phase observed in chronic wounds.

THUMPD2 is also known C2orf8 and MGC2454. THUMPD2 is named after the THUMP domain which it contains. The THUMP domain is predicted to be a RNA-binding domain with a structure similar to the C-terminal domain of translation initiation factor 3 and ribosomal protein S8. No actual function has been determined for THUMPD2 however, the THUMP domain is thought to aid in the modification of RNA through delivery of RNA modifying enzymes (Zhang et al., 2001). THUMPD2 was identified as a methyltransferase gene linked to hereditary gingival fibromatosis with (Farrer-Brown et al., 1972, Bozzo et al., 1994, Araujo et al., 2003). Gingival fibromatosis can be characterized by gingival enlargement, because of an increase in the amount of connective tissue. The enlargement is due to fibrosis with densely arranged collagen bundles poorly vascularised connective tissue and a increase in the fibroblast population. In some cases mucosal ulceration and inflammation have been observed (Maecker et al., 1997). If gingival fibrosis is caused by aberrant expression of THUMPD2 as suggested by Zhand et al then the increased expression observed in CWF could be having an effect on the ECM deposition in chronic wounds.

Transmembrane 4 L Six Family Member 1 is also known as TM4SF1, H-L6, L6, M3S1 and TAAL6. TM4SF1 is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. This family of proteins are cell surface proteins

known to play a role in the regulation of cell development, activation, growth and motility (Marken et al., 1992). The TM4SF1 protein is a cell surface antigen and is highly expressed in different carcinomas including lung, breast, colonic and ovarian carcinomas (Kao et al., 2003). Over-expression of TM4SF1 in lung carcinoma cells increases their invasiveness (Storim et al., 2001). In human epidermal keratinocytes TM4SF1 is expressed after proteolytic detachment of the cells. The expression of TM4SF1 is increased after wounding in an *in vitro* keratinocyte wound model. Inhibition of TM4SF1 by antibodies reduces cell motility (Wysocki et al., 1993b). The increased expression of TM4SF1 has been linked to carcinoma invasiveness as well as keratinocyte motility and wound responses. The increased expression of TM4SF1 in CWF observed in a number of patients could be linked with the increased levels of MMPs observed in chronic wounds (Halbersztadt et al., 2006, Deryugina and Quigley, 2006). Increased MMP level is linked to invasiveness in carcinomas (Mogensen et al., 1999). TM4SF1 may play a role in generating the altered wound environment observed in chronic wounds and leading to the delay in the generation of extracellular matrix.

5.3.3 Genes validated as being down in CWF by QRT-PCR

Two of the genes studied in this chapter were observed to have a reduced expression in CWF, this was independent of serum stimulation suggesting that the genes are permanently down-regulated in CWF.

ACTC is a member of the actin family of proteins, actins are a major constituent of the microfilaments of the cytoskeleton of eukaryotic cells. Alpha actins are normally observed in muscle tissues, in these tissues they are a major constituent of the contractile apparatus. Defects in ACTC have been linked with familial hypertrophic

cardiomyopathy (Hinz et al., 2003). Another isoform of apha actin, alpha smooth muscle actin is known to be important in wound healing, (Chaponnier and Gabbiani, 2004, Hinz et al., 2001) specifically in myofibroblast contraction. ACTC does not show the same contractile ability as alpha smooth muscle actin (Cooper et al., 2004) however, ACTC was identified as an inflammation independent 'activation' gene activated by wounding in mouse skin (Chin et al., 1985, Wilhelm et al., 1987). While this mouse wounding experiment suggests a role for ACTC in wound healing it does not describe how ACTC is involved. ACTC gene expression was down-regulated in CWF compared to patient matched NF, as chronic wounds have impaired wound healing this may be further evidence for the importance of ACTC in the wound healing response.

MMP3 is also known as, Stromelysin 1, SL-1, STMY, STMY1, STR1 and TRANSIN. MMP3 is a proteoglycanase expressed in human fibroblasts, it is closely related to collagenase (MMP1) and is able to act on a number of different substrates. MMP3 is able to degrade proteoglycan, fibronectin, laminin, and type IV collagen (Madlener, 1998). MMP3 is normally expressed in acute wounds within 24 hours of wounding (Li et al., 2004). Studies of wound healing in the intestinal mucosa in a MMP3 KO mouse showed that tissue remodeling and wound healing were possible without the presence of MMP3. The mice did however, exhibit a delayed clearance of bacteria from the wound site (Bullard et al., 1999a). Skin wounds in MMP3 KO mice showed a failure of wound contraction in excisional wounds as well as delayed healing compared to wild type controls (Bullard et al., 1999b). Collagen gel reorganisation in MMP3 KO fibroblasts is significantly reduced compared to wild type controls (Saarialho-Kere et al., 1994). MMP3 expression was observed in keratinocytes at the edge of chronic wounds as well as in the dermis immediately

beneath and to the sides of the wound (Iyer et al., 1999). Initially the reduced expression of MMP3 in CWF reported in this Chapter does not agree with the observed MMP3 expression in the dermis of chronic wounds. However, the paper describing the expression of MMP3 in the dermis of chronic wounds does not quantify the levels of expression. The paper does not demonstrate that MMP3 is not expressed in the dermis of acute wounds. The QRT-PCR data shown in this Chapter and the microarray data shown in Chapter 4 demonstrate expression of MMP3 in both NF and CWF cells. The level of expression in CWF is however, reduced in comparison to patient matched NF. The MMP3 KO mouse model suggests that if CWF fibroblasts have reduced MMP3 expression as indicated by this data they would demonstrate a reduced ability to remodel and contract the wound. The data presented in this Chapter suggests that the role of MMP3 in chronic wound healing may warrant further investigation.

5.3.4 Genes validated as being down in CWF (serum response) by QRT-PCR

Three of the genes investigated in this Chapter were serum response genes whose induction by serum stimulation was decreased in CWF. The serum response in this experiment attempts to model wounding of the skin (Devalaraja et al., 2000). These genes potentially represent a loss of function in the CWF in response to wounding.

CXCL1 is also known as: GRO1, GROa, MGSA, MGSA alpha, MGSA-a, NAP-3 and SCYB1. CXCL1 is a member of the CXC family of chemokines. CXCL1 signals through the receptor CXCR2, this receptor has been knocked out in mice (Nanney et al., 1995) and wounding experiments carried out. Delayed wound healing was observed in the KO mice compared to controls with delayed epithelialisation and decreased neovascularisation. Analysis of the wounds for inflammatory cells

identified defective neutrophil recruitment in the KO mice as well as an altered temporal pattern of monocyte recruitment. CXCL1 expression has been observed in human burn wounds with a potential role as an important cytokine involved in the inflammatory and proliferative phases of cutaneous wound repair. (Engelhardt et al., 1998). In cutaneous wound healing the expression of CXCL1 is directly linked to neutrophil infiltration (Smith et al., 2005). CXCL1 also has a role in monocyte recruitment (Bleul et al., 1996b) mediating monocyte arrest, one of the earliest steps in monocyte recruitment. CXCL1 plays an important role in the recruitment of inflammatory cells to the wound area in normal wound healing. The reduced expression of CXCL1 observed in CWF compared to NF in this Chapter may play a role in the failure of chronic wounds to escape the inflammatory phase of wound healing.

CXCL12 is also known as: PBSF, SCYB12, SDF-1a, SDF-1b, SDF1, SDF1A, SDF1B, TLSF-a, TLSF-b and TPAR1. CXCL12 is a small cytokine, a member of the intercrine family of cytokines. These cytokines are known to activate leukocytes after induction by pro-inflammatory stimuli. CXCL12 has chemotactic properties, acting on lymphocytes and monocytes but not neutrophils (Florin et al., 2005). In the skin CXCL12 from skin fibroblasts has been shown to promote keratinocyte proliferation (Norton, 2000). In this chapter CXCL12 gene expression was shown to be down in CWF compared to patient matched NF. CXCL12 is able to recruit lymphocytes and monocytes to the wound site and promote keratinocyte proliferation; the reduced expression in CWF could explain the failure of chronic wounds to escape the inflammatory phase of wound healing and their failure to reepithelialise.

ID1 is a member of the ID family of helix-loop-helix motif containing protein known to regulate tissue-specific transcription through the binding of basic helix-loop-helix

containing transcription factors. ID proteins have roles in cell cycle progression, cell proliferation and are thought to have a role in tumour growth and metastasis (Hara et al., 1994). ID1 is expressed transiently in human fibroblasts with expression highest G phase of the cell cycle, quiescent cells do not express ID1. Upon serum stimulation early passage fibroblast show an induction of ID1 expression, this induction of expression is reduced in senescent cells (Nickoloff et al., 2000). ID1 has the ability to delay senescence in keratinocytes (Alani et al., 2001). The observed inhibition of senescence has been shown to be through direct repression of the tumor-suppressor protein $p16^{Ink4a}$ (Ding et al., 2006). In bladder cancer ID1 was shown to be up-regulated, ID1 was suggested as a therapeutic target as a reduction of expression appeared to inhibit the invasive phenotype (Volpert et al., 2002). ID1 null mice have been generated, they have defects in angiogenesis (Thompson et al., 1993). ID1 expression has been shown to be lower in senescent cells in response to serum stimulation. The expression of ID1 expression is lower in CWF in response to serum than patient matched NF, this may indicate that CWF even after immortalisation have characteristics of senescent cells. The impaired angiogenesis observed in ID1 null mice could also be important, since angiogenesis is a crucial step in wound healing.

In conclusion, this Chapter has attempted to show the data obtained from a qRT-PCR study of gene expression in CWF compared with patient-matched NF. This data has validated the changes in gene expression observed by microarray analysis. Importantly the data has added to the microarray data obtained previously with the extra data points giving a more complete picture of gene expression changes between CWF and NF. Further study of this reduced set of disease marker genes has identified potential roles for the genes within the chronic wound. Trends in gene expression

have been validated for each of the genes identified by microarray analysis. Therefore all the genes validated in this chapter will be taken on for the generation of reporter constructs. From the 3 patients studied Patient I showed the most consistent maintenance of gene expression profile across the different time points in culture. Cells from patient I will therefore be used in the generation of reporter cell lines.

Chapter Six

Generation and Testing of Chronic Wound Reporter Constructs

6.1 Introduction

To measure changes in gene expression in real time it is possible to link cis-acting sequences from genes of interest to the coding sequence of a reporter gene. A reporter gene can be defined as any gene whose transcribed protein may be detected. There are a number of different commonly used reporter genes some of which are described below. There are two main categories of reporter constructs giving different levels of information about the expression of the gene under examination. Transcriptional reporters are made from the promoter region of the gene under investigation driving the expression of the reporter gene. The promoter region normally consists of approximately 2Kb of DNA upstream of the transcriptional start site, this region contains enough gene specific regulatory sequences to give a reliable expression pattern for the gene of interest. Translational reporters are gene fusions between the reporter and the gene of interest. A translational reporter should contain the entire genomic locus of the gene of interest, this includes 5'upstream regions, introns, exons and 3' untranslated regions. The reporter is inserted anywhere in the open reading frame where it will not disrupt the function of the protein.

To make a good reporter, the protein should not be normally present in the host, or easily distinguishable from host proteins. The detection of the reporter should be both rapid and sensitive so small short lived changes in gene expression can be detected. Ideally the assay for detecting the reporter should cover a wide range of reporter levels so both small and large changes can be detected with the same system. Most importantly the presence and expression of the reporter gene must not alter the phenotype of the host. Reporter constructs fall into two groups; *in vitro* assays such as CAT (de Wet et al., 1987), Luciferase (Fowler et al., 1970), β-Galactosidase (Berger et al., 1988) and Secreted Alkaline Phosphatase (Nolan et al., 1988) and *in*

vivo assays such as β-Galactosidase (Shimomura et al., 1962) and fluorescent proteins (Arhondakis et al., 2008). *In vitro* reporter assays require the detection of the reporter in cell / tissue lysates or culture medium when the reporter protein is secreted. *In vitro* assays are quantitative and reproducible but are not able to give results in real time. *In vivo* Reporter Assays allow the detection of the reporter in live cells or tissue usually through fluorescence. *In vivo* Reporter Assays are less quantitiative than *in vitro* reporter based systems however, analysis from living cells allows observation of dynamic changes in gene expression in real time. As well as giving real time data on gene expression *in vivo* systems allow for the same cells or tissue to be used in repeated experiments as there is no requirement for cell lysis.

For this Chapter the aim was to generate reporter cell lines using both NF and CWF able to quantifiably determine real time changes in gene expression over extended time in culture. For this to work the cells must not be lysed as this would only give a fixed timepoint reading of gene expression. A fluorescence based reporter was chosen as this should allow the quantification of gene expression in living cells.

6.2 Results

6.2.1 Identification of Promoter regions

Promoter regions for each of the genes of interest were identified by bioinformations at the Babraham Institute using the Database of Transcriptional Start Sites (DBTSS http://dbtss.hgc.jp/). The DBTSS is a comprehensive database containing sequence information for most of the genes identified in the human genome, this data includes transcriptional start sites (TSS) and the regions upstream of the TSS. Information about known promoter regions identified experimentally is available as well as hypothetical transcription factor binding sites and enhancer regions. A region of 2Kb upstream of the TSS for each gene was identified as the most likely promoter region. Full sequences can be found in Appendix 1.

6.2.2 Design of promoter amplification primers

For each gene of interest a segment that extended from 2450bp upstream to 300bp downstream of the TSS was identified from the DBTSS and added to the primer3 primer design software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_results.cgi). The selected region was masked for human repeats and a region (between 500bp and 2000bp upstream of the TSS) was delimited within which primers were not selected as this contained the desired promoter region. The product range was set to 2000-2300bp and a primer length of between 28 and 30. The Tm was set to 66-70 with a maximum difference of 1 degree. Primers were identified for each gene of interest (Table 6.1).

Gene Name		Primer start position	Product Length	Sequence
ACTC	Left primer	457	2237	AATCAACCTGATTCTTTAAAAGGCAGAGC
ACTC	Right primer	-1780	2237	CCCAGGACAAAACAAGTTAGAACCAAATC
CD9	Left primer	452	2153	GCCTTCATTCATTCTTCTCTCACCTGAAC
CD9	Right primer	-1701	2153	GTTAAATCCGAACAGCAGGTATTTGATGC
CXCL1	Left primer	440	2288	TCAGGAAGATGTGAAAAGAGAAATAATTGA
CXCL1	Right primer	-1848	2288	CCTAGGGAAGAAGAGACTCACTGACTG
CXCL12	Left primer	473	2261	CAGCGGCCAGGCCTGAATCCCTGGTT
CXCL12	Right primer	-1791	2261	TAAGCGAGCTGCCTCCACCCCCACTGTGT
GALNAC4S-6ST	Left primer	448	2144	GTATAAATACCTTTGGAAAGTCCCTGGTG
GALNAC4S-6ST	Right primer	-1969	2144	ACCTAACTTGTACCTGAGAAGCCTGAGTC
ID1	Left primer	298	2252	GTCGTGTGGAGCCTCAGTTTCCTTTGTAT
ID1	Right primer	-1954	2252	GATTCTTGGCGACTGGCTGAAACAGAAT
IGSF4	Left primer	304	2492	AGGATTCTTGCCATATAGGACCTGCCTTT
IGSF4	Right primer	-2188	2492	CTCAGCCCTGAGTTTCCTCTCCACACT
IL11	Left primer	111	2494	GAGAGAGAGCTCTTACCTGAGGCGATG
IL11	Right primer	-2383	2494	AACCAACTTACAGTTCATGTCCCCACAG
MMP3	Left primer	389	2298	AAAATAGAGTAGCAGAGGCAGGTACAAGG
MMP3	Right primer	-1909	2298	AAGACACACAGAATGGTTTCAGCTTACTC
THUMPD2	Left primer	342	2219	AAACAAGAGTAGCCCCGACCTTGCTGACC
THUMPD2	Right primer	-1877	2219	CACCTCTCGCATTACGAACGGCTCCAG
TM4SF1	Left primer	1	1868	GTAGTTTAAGCATCACTTTCCCCCATTT
TM4SF1	Right primer	-1868	1868	CTTCTCTTTGTCTTCAGCTCAGTGATACC
GAPDH	Left primer	391	2347	ACTCGTGTTCCCTAATCCCATCTCCAAAG
GAPDH	Right primer	-1956	2347	GGAGGGAGAGAACAGTGAGCGCCTAGT

Table 6.1: Table showing primer sequences, primer position relative to the transcriptional start site of each gene and the product size.

6.2.3 Amplification of Promoter regions

Genomic DNA from Patient I CWF was used a template for PCR amplification of the promoter regions of each gene of interest, with 100ng used in each reaction. Polymerase Expand HiFi was chosen for its proof reading ability as well as the single A overhang left at the 3' end of the product removing the need for a second A-tailing reaction. Products were visualized on a 0.8% Agarose BE gel with the Invitrogen 1Kb ladder used for sizing (Fig 6.1). Bands of approximately 2400bp (see Table 6.1 for sizes) were observed for ACTC, CXCL12, ID1, THUMPD2, GAPDH, GALNAC4S and TM4SF1 (Fig 6.1). Single bands were not seen in these reactions as the amplification from genomic DNA required sub optimum annealing temperatures and long extension times. Despite repeated attempts and alteration to the PCR conditions strong bands of the correct size were not observed for CD9, CXCL1, IGSF4, IL11 and MMP3 (Fig 6.1) and so these genes were not taken forward for further analysis. Bands of the correct size were excised from the gel and recovered using a gel extraction kit eluting in ddH_2O.

6.2.4 Recovery into pGEM-T

The amplified promoter regions were recovered into pGEM-T allowing amplification and analysis of the fragments. The pGEM-T vector is shipped linearised after restriction digestion with *Eco*R V, the linearisation leaves a single 3' thymine overhang preventing re-circularisation of the plasmid (Fig 6.2a). The enzyme used to amplify the promoter regions was a proofreading polymerase but unlike most proofreading polymerases it leaves a single 3' adenine overhang at each end of the product (Fig 6.2c). The PCR product and linearised pGEM-T vector were incubated together with a DNA ligase and the complementary 3' overhanging bases allowed

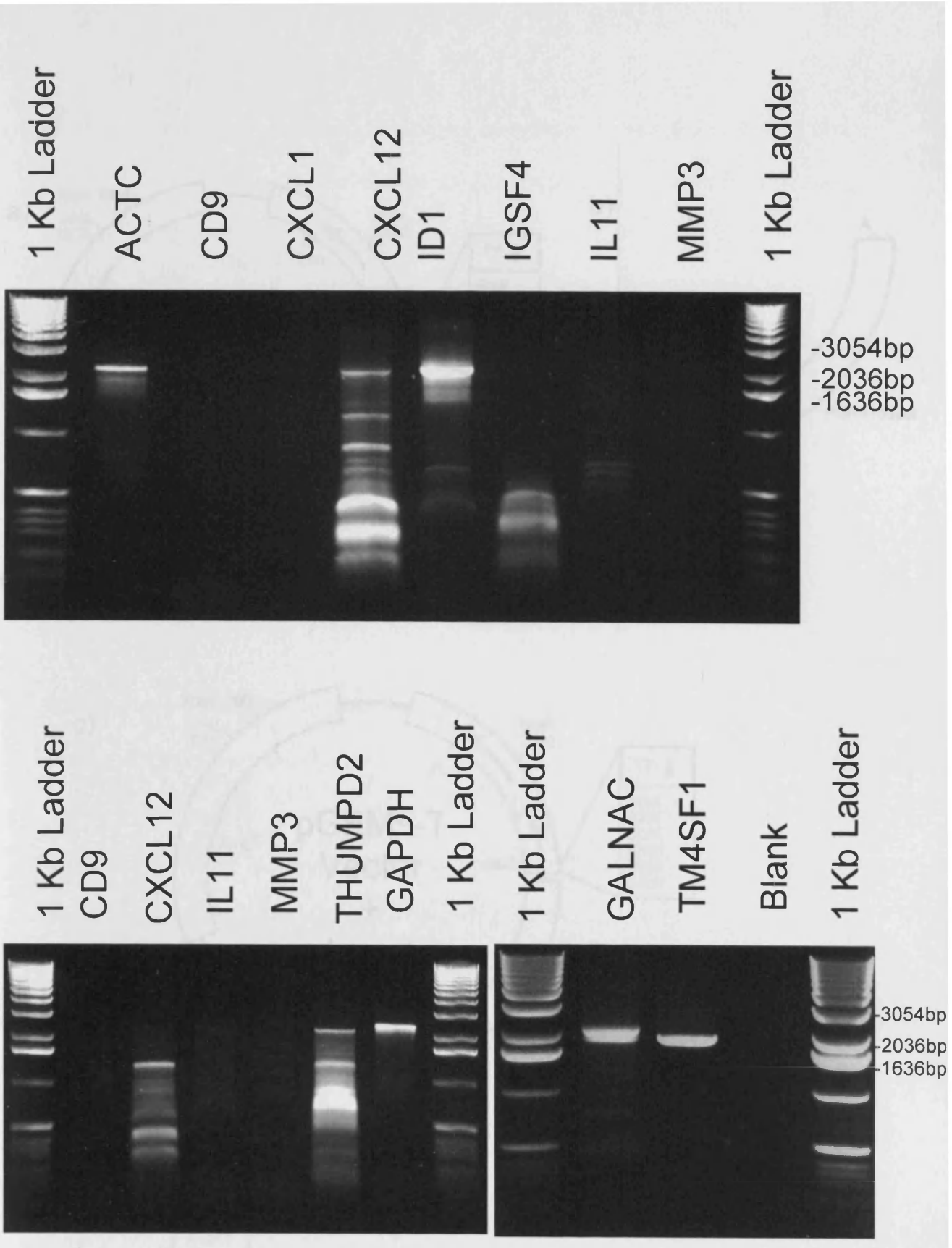


Figure 6.1: Long range PCR amplification of 11 genes of interest and GAPDH from genomic DNA isolated from Patient I CWF.

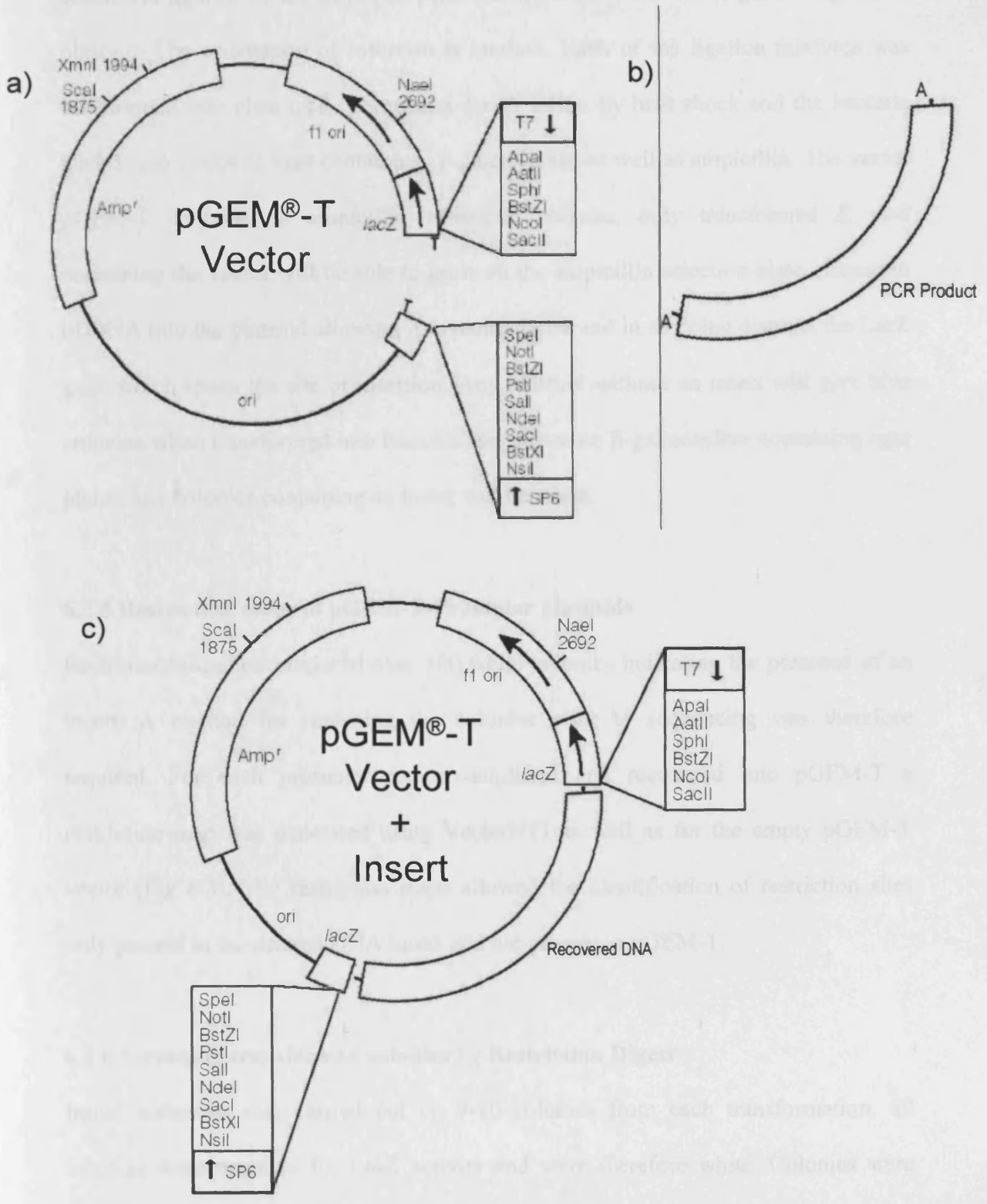


Figure 6.2: Schematic representation of the recovery of PCR products into pGEM-t plasmid. a) pGEM-T plasmid restriction digested gives a single 3' T overhangs preventing re-circularisation of the plasmid, the *lacZ* gene is disrupted by the digest. b) PCR product has single 3' A overhangs at each end. c)T4 DNA Ligase is used to ligate the PCR product and the digested plasmid generating a complete plasmid, insertion of the PCR product disrupts the *lacZ* gene. (Images adapted from www.promega.com/tbs/)

successful ligation of the amplified promoter region into the vector generating a new plasmid. The orientation of insertion is random. Each of the ligation mixtures was transformed into chemically competent *E.coli* DH5α by heat shock and the bacteria plated onto selective agar containing β-galctosidase as well as ampicillin. The vector pGEM-T contains an ampicillin resistance cassette, only transformed *E. coli* containing the vector will be able to grow on the ampicillin selection plate. Insertion of DNA into the plasmid allowing it to recircularise and in so doing disrupts the LacZ gene which spans the site of insertion. Any plasmid without an insert will give blue colonies when transformed into bacteria and grown on β-galctosidase containing agar plates, any colonies containing an insert will be white.

6.2.5 Restriction maps of pGEM-T-Promoter plasmids

Each transformation produced over 100 white colonies indicating the presence of an insert. A method for screening the colonies prior to sequencing was therefore required. For each promoter region amplified and recovered into pGEM-T a restriction map was generated using VectorNTI as well as for the empty pGEM-T vector (Fig 6.3). The restriction maps allowed the identification of restriction sites only present in the desired DNA insert and not present in pGEM-T.

6.2.6 Screening transformed colonies by Restriction Digest

Initial screening was carried out on 9-10 colonies from each transformation, all colonies were negative for LacZ activity and were therefore white. Colonies were selected and grown overnight in LB media containing ampicillin. Plasmid preps were carried out and plasmid DNA analysed by UV spectroscophy to determine DNA concentration. Restriction digests were carried out on each colony with specific

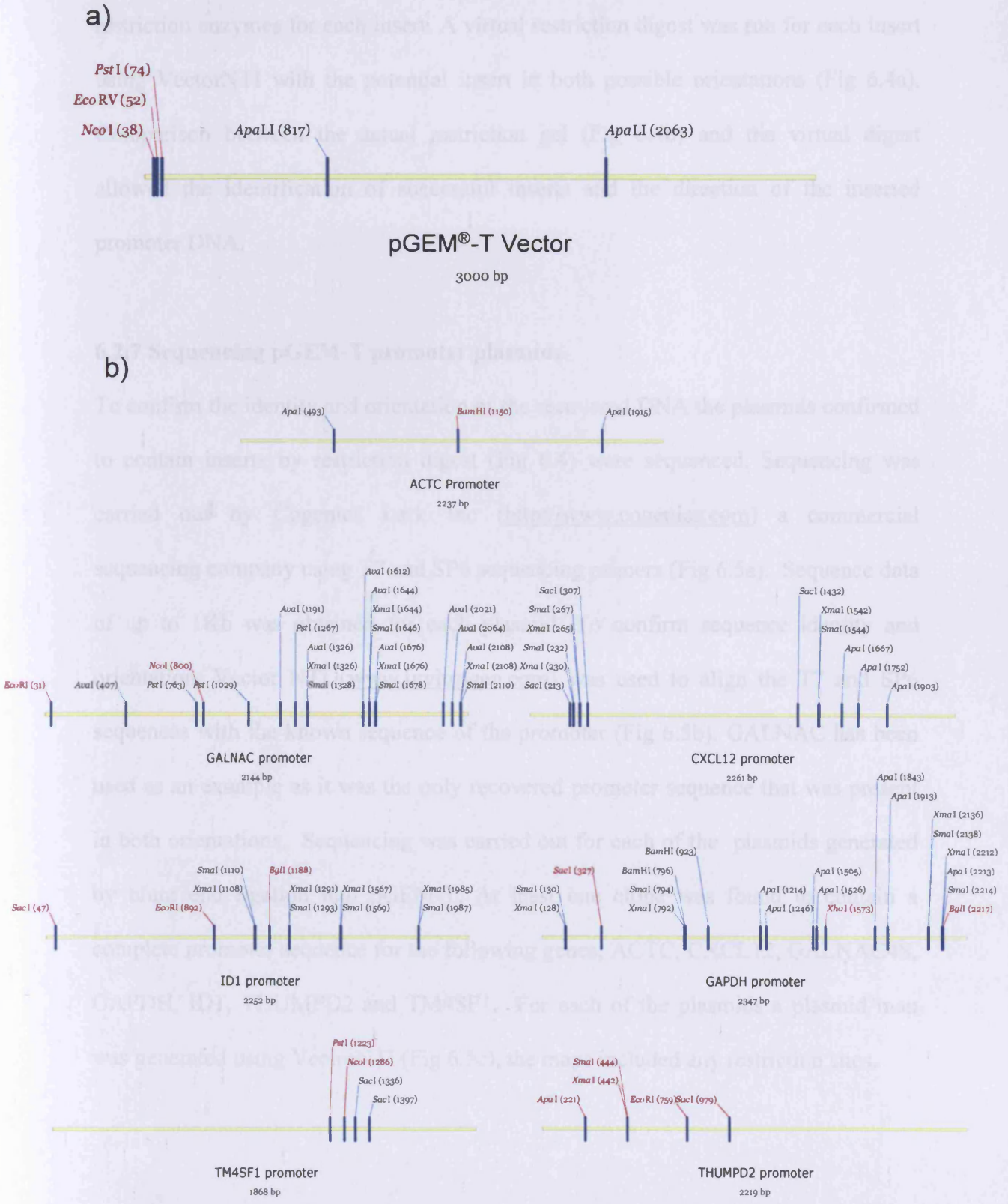


Figure 6.3: Restriction maps for pGEM-T (a) and all succesfully amplified promoter regions (b). Restriction sites for ApaLI, AvaI, BamHI, ClaI, EcoRI, HindIII, NcoI, PstI, SacI, SmaI and XmaI are shown. Generated in Vector NTI (Invitrogen)

restriction enzymes for each insert. A virtual restriction digest was run for each insert using VectorNTI with the potential insert in both possible orientations (Fig 6.4a). Comparison between the actual restriction gel (Fig 6.4b) and the virtual digest allowed the identification of successful inserts and the direction of the inserted promoter DNA.

6.2.7 Sequencing pGEM-T promoter plasmids

To confirm the identity and orientation of the recovered DNA the plasmids confirmed to contain inserts by restriction digest (Fig 6.4) were sequenced. Sequencing was carried out by Cogenics Lark Inc (http://www.cogenics.com) a commercial sequencing company using T7 and SP6 sequencing primers (Fig 6.5a). Sequence data of up to 1Kb was obtained for each plasmid. To confirm sequence identity and orientation, Vector NTI (www.invitrogen.com) was used to align the T7 and SP6 sequences with the known sequence of the promoter (Fig 6.5b). GALNAC has been used as an example as it was the only recovered promoter sequence that was present in both orientations. Sequencing was carried out for each of the plasmids generated by blunt end ligation into pGEM-T. At least one clone was found to contain a complete promoter sequence for the following genes; ACTC, CXCL12, GALNAC4S, GAPDH, ID1, THUMPD2 and TM4SF1. For each of the plasmids a plasmid map was generated using VectorNTI (Fig 6.5c), the maps included any restriction sites.

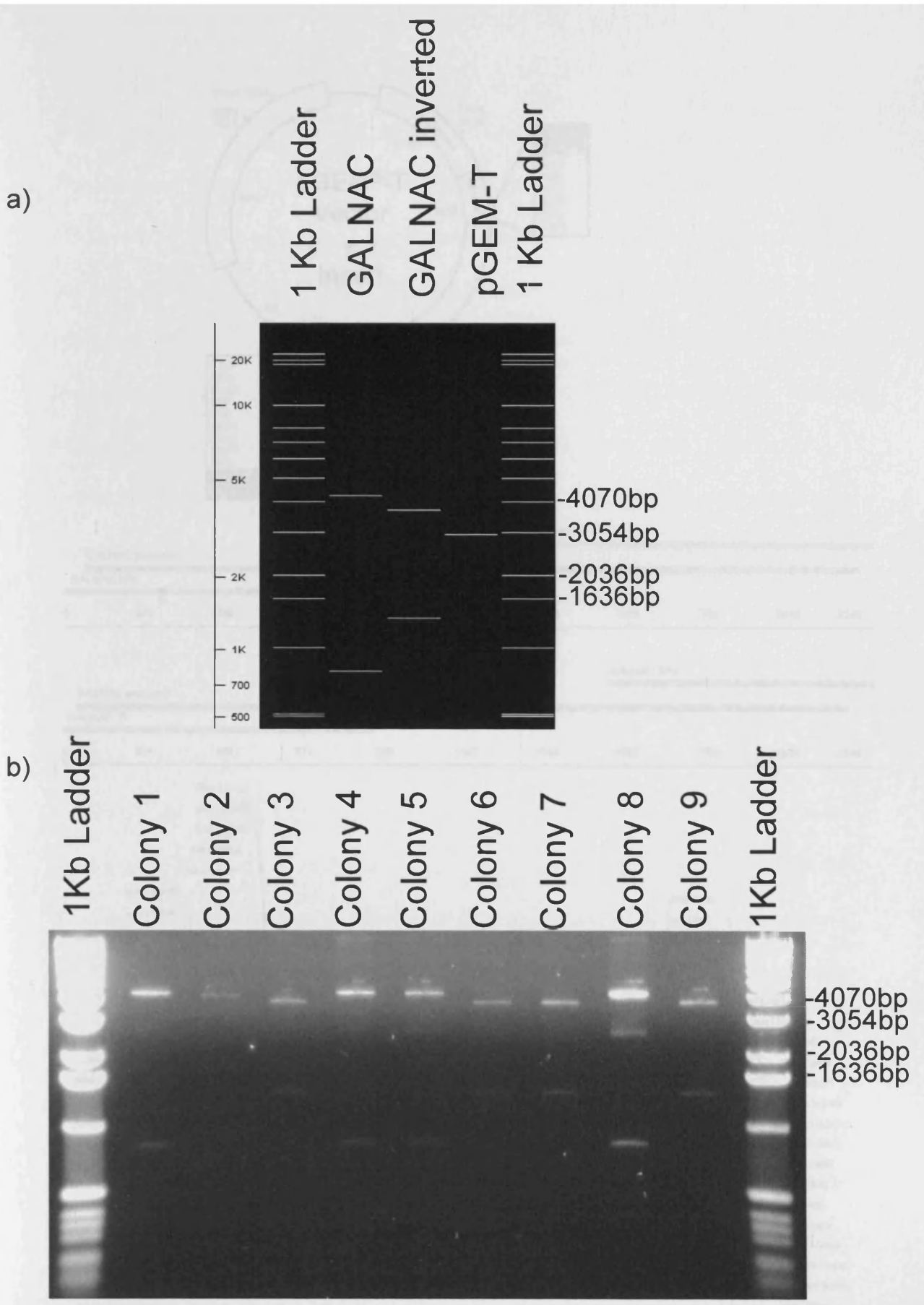
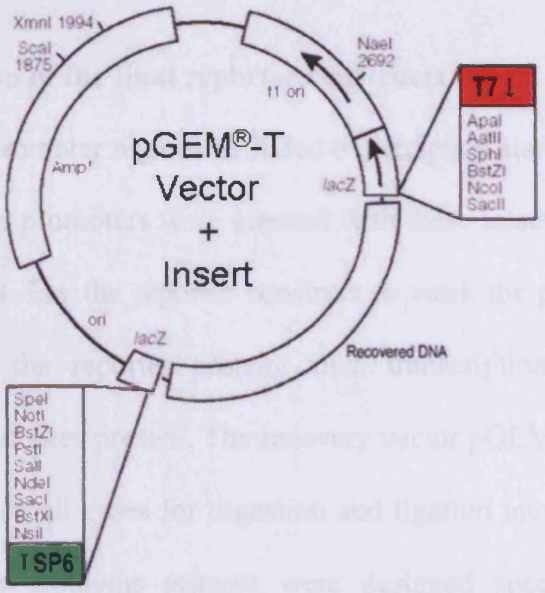
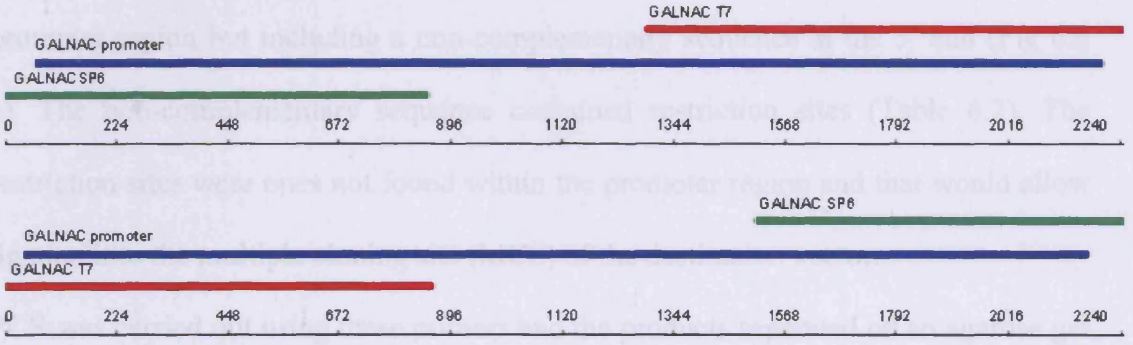


Figure 6.4: Restriction Digest of pGEM-T plasmid containing recovered PCR product. a) Virtual restriction digest carried out in Vector NTI for both possible orientations of GALNAC promoter region and empty pGEM-T. b) Restriction digest gel of 9 selected colonies, colonies 1, 4 and 5 contain GALNAC in one orientation and colonies 3, 6, 7 and 9 in the inverted orientation. Colony 8 appears to contain a mix of empty pGEM-T and GALNAC containing vector.

a)



b)



c)

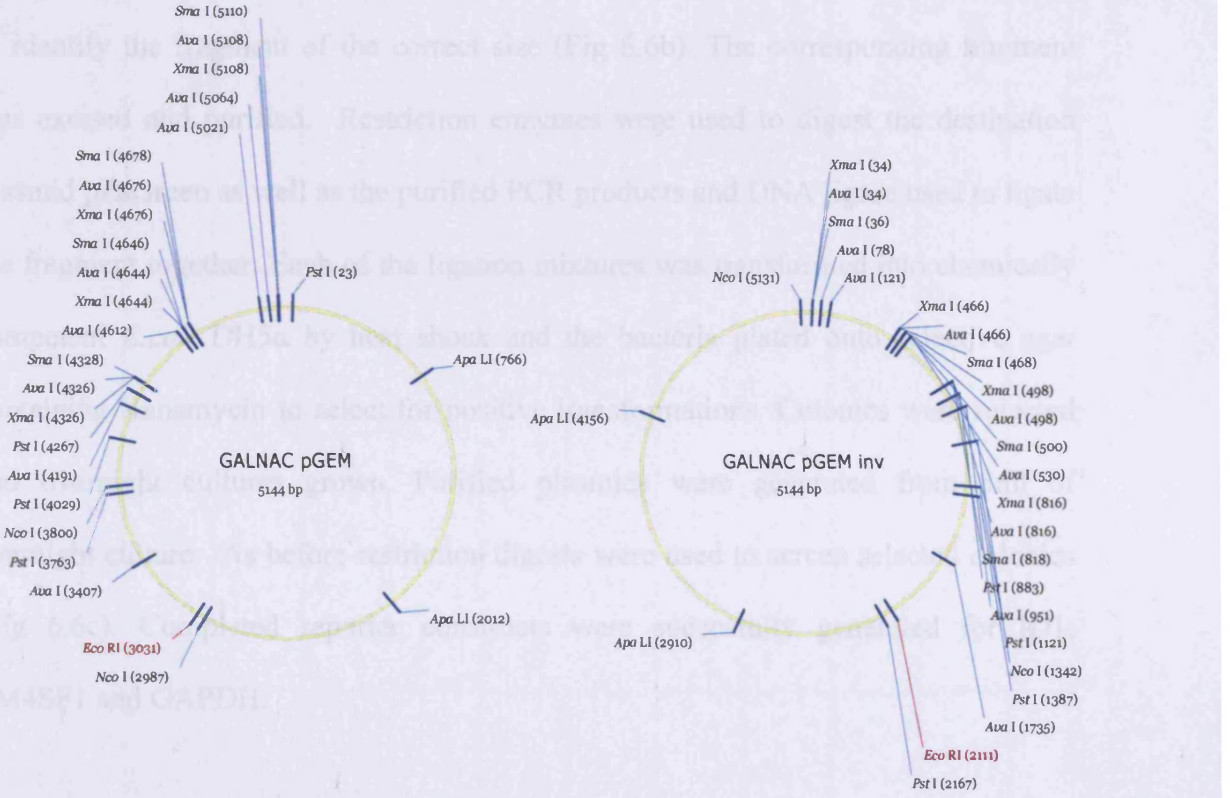


Figure 6.5: Confirmation of insert identity by sequencing a) Sequence analysis using T7 ■ and SP6 ■ primers allows sequencing of the recovered DNA. b) sequences were aligned with the known promoter sequence and contigs generated using Vector NTI. The two different orientations of GALNAC promoter are shown here. c) Plasmid maps for each sequenced plasmid were generated using Vector NTI.

6.2.8 Generation of the final reporter constructs

The recovered promoter regions included transcription start sites for the original genes of interest. If the promoters were inserted with these intact the reporter protein would not be produced. For the reporter construct to work the promoter sequence must be upstream from the reporter proteins own transcription start site with no other transcription start sites present. The recovery vector pGEM-T did not have the correct restriction sites in all cases for digestion and ligation into the destination vector. To overcome these problems primers were designed specificaly to each recovered promoter region but including a non-complementary sequence at the 5' end (Fig 6.6 a). The non-complementary sequence contained restriction sites (Table 6.2). The restriction sites were ones not found within the promoter region and that would allow ligation into the multiple cloning site (MCS) of the destination vector.

PCR was carried out using these primers and the products seperated on an agarose gel to identify the fragment of the correct size (Fig 6.6b). The corresponding fragment was excised and purified. Restriction enzymes were used to digest the destination plasmid pZsGreen as well as the purified PCR products and DNA ligase used to ligate the fragment together. Each of the ligation mixtures was transformed into chemically competent *E.coli* DH5α by heat shock and the bacteria plated onto selective agar containing Kanamycin to select for positive transformations. Colonies were selected and overnight cultures grown. Purified plasmids were generated from 5ml of overnight culture. As before restriction digests were used to screen selected colonies (Fig 6.6c). Completed reporter constructs were succesfully generated for ID1, TM4SF1 and GAPDH.

Gene Name		Restriction Enzyme	Seq
ACTC	3'	SP6 primer/ SacI	ATTTAGGTGACACTATAG
ACTC	5'	KpnI	CTAGccatggGCCTTGGCTGAATGC
CXCL12	3'	EcoRI	GATCgaattcAGGCCTGAATCCCTGGTTCG
CXCL12	5'	BamHI	CTAGggatccGCAATGCGGCTGAC
ID1	3'	XhoI	GATCctcgagGTCGTGTGGAGCCTCAG
ID1	5'	BamHI	CTAGggatccACGTGGAATGAGAGTGC
THUMPD2	3'	XhoI	GATCctcgagCAAGAGTAGCCCCGACCTTG
THUMPD2	5'	BamHI	CTAGggatccACCCGCAGTGCAGAAGAATC
TM4SF1	3'	EcoRI	GATCgaattcATAGATCAGCAGACAGGAG
TM4SF1	5'	BamHI	CTAGggatccATTAGATGAAAGTGTGC
GAPDH	3'	EcoRI	GATCgaattcGAAGTTCAGATCATG
GAPDH	5'	KpnI	CTAGccatggCGTCTTCACCTGGCGACGC

Table 6.2: Table showing primer sequences for introduction of restriction sites and the restriction site to be included in the product. Restriction sites are shown in red, linker DNA in blue. ACTC 3' primer is for the SP6 sequencing primer as the SacI restriction site lies between the SP6 primer and the promoter region.

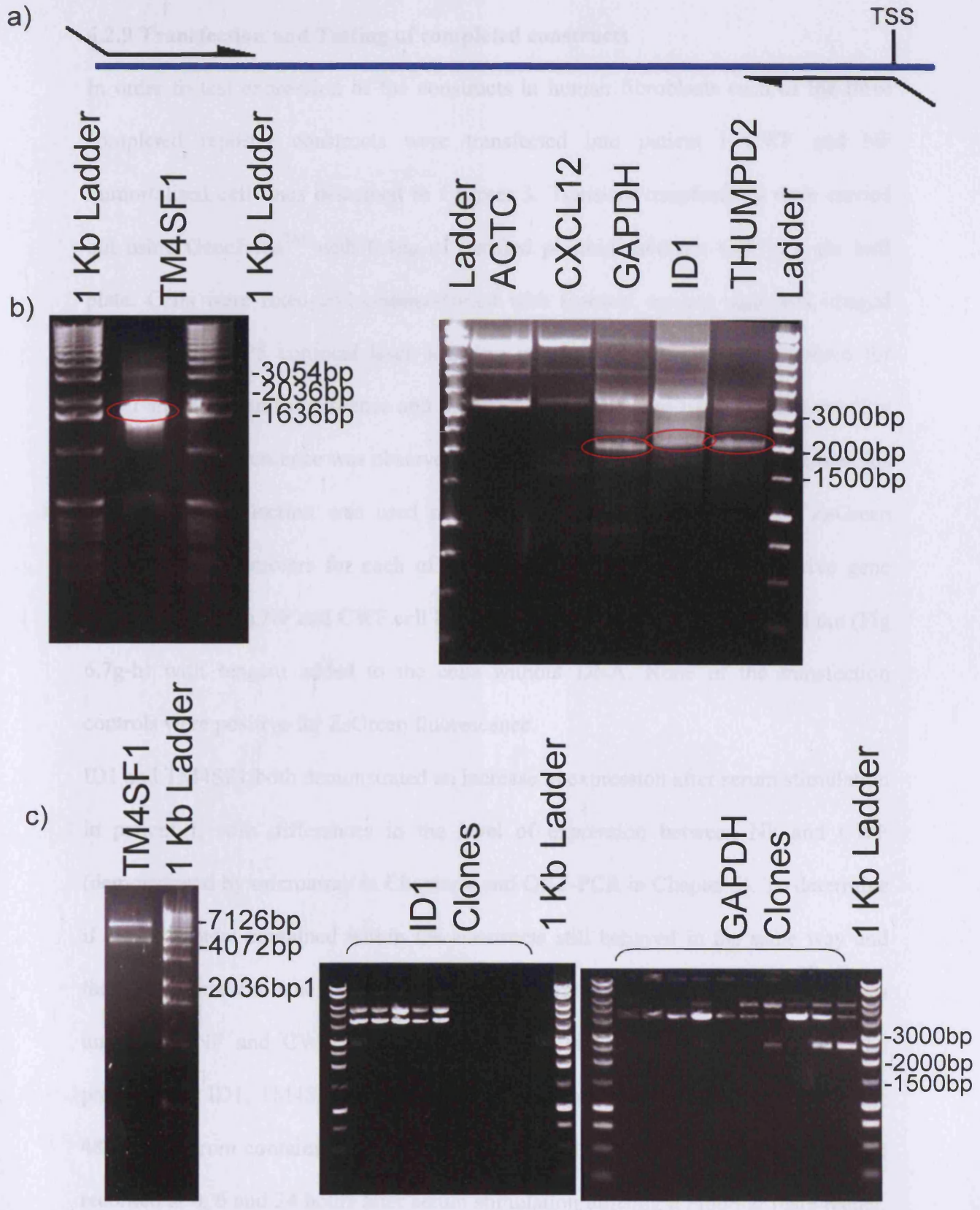


Figure 6.6: a) Primers designed to amplify promoter regions have non-complementary sequences at the tail encoding restriction sites. b) PCR amplification of promoter regions from pGEM-T recovery vector introducing restriction sites at either end. Successful products were seen for TM4SF1, GAPDH, ID1 and THUMPD2 (ringed). c)Restriction digests to screen potential vectors for the correct size inserts identified positive colonies for TM4SF1, ID1 and GAPDH.

6.2.9 Transfection and Testing of completed constructs

In order to test expression of the constructs in human fibroblasts each of the three completed reporter constructs were transfected into patient I CWF and NF immortalised cell lines described in Chapter 3. Transient transfections were carried out using GeneJuice™ with 0.4μg of purified plasmid used per well in a six well plate. Cells were fixed and counterstained with Hoescht nuclear stain and imaged using a Leica SP5 confocal laser scanning microscope. Images were captured for DAPI and ZsGreen fluorescence and the images merged using LAS AF software (Fig 6.7). Green fluorescence was observed in NF and CWF for each of the three plamids tested. As no selection was used not all cells present were expressing ZsGreen however, the promoters for each of the genes of interest were able to drive gene expression in both NF and CWF cell lines. Transfection controls were carried out (Fig 6.7g-h) with reagent added to the cells without DNA. None of the transfection controls were positive for ZsGreen fluorescence.

ID1 and TM4SF1 both demonstrated an increase in expression after serum stimulation in patient I, with differences in the level of expression between NF and CWF (demonstrated by microarray in Chapter 4 and QRT-PCR in Chapter 5). To determine if the promoters contained within the constructs still behaved in the same way and therefore represent true reporters a serum starvation/stimulation experiment was undetaken. NF and CWF cells transfected with either a construct containing the promoter for ID1, TM4SF1 or GAPDH or with reagent alone were serum starved for 48 hours. Serum containing medium was then added and fluorescence/reporter levels recorded at 0, 6 and 24 hours after serum stimulation utilising a FluoStar plate reader. Background levels of fluorescence were recorded from the transfection control cells and used as blank readings. To control for changes in global gene expression the

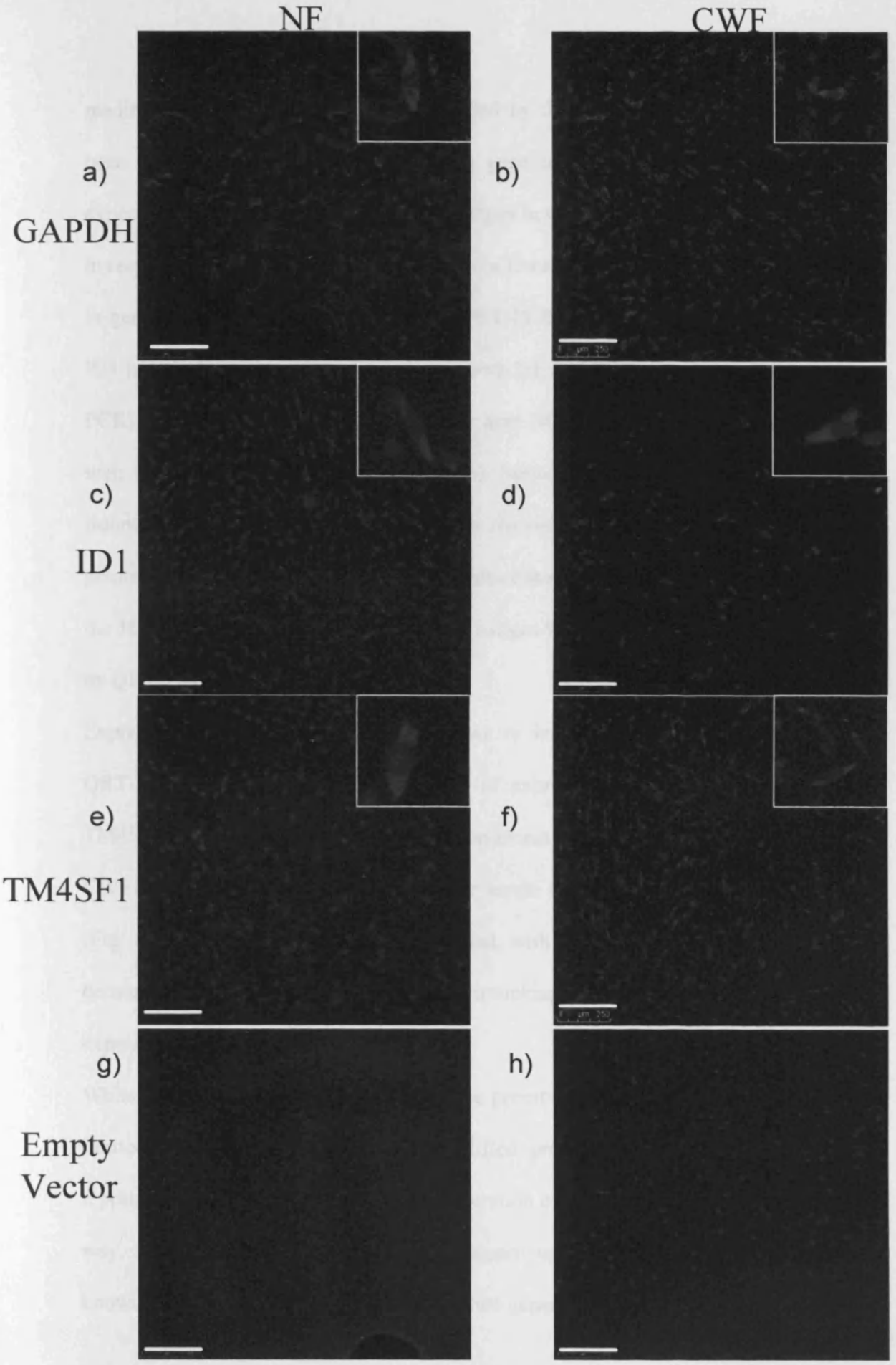


Figure 6.7: Confocal images of patient I NF and CWF transiently transfected with reporter constructs; reporter expression is seen in green with hoechst stained nuclei shown in blue . Images a and b show expression of GAPDH promoter linked ZsGreen expression. Images c and d show ID1 promoter linked ZsGreen expression. Images e and f show TM4SF1 promoter linked ZsGreen expression. Images g and h show untransfected cells (scale bar = 250μm).

readings for ID1 and TM4SF1 were divided by the GAPDH readings. GAPDH has been previously used as a housekeeping gene in both microarray and QRT-PCR experiments. In the fibroblasts used no changes in GAPDH gene expression were seen in response to serum stimulation. Changes in fluorescence were compared to changes in gene expression level determined by QRT-PCR (Chapter 5). Expression levels of ID1 increased upon serum stimulation in both NF and CWF (Demonstrated by QRT-PCR) but this increase was lower in CWF than NF (Fig 6.8a). The same pattern was seen with the reporter construct (Fig 6.8b). Serum stimulation led to an increase in fluorescence in both NF and CWF but the fluorescence levels observed in NF were greater than in CWF. The changes in fluorescence observed in cells transfected with the ID1 reporter construct mimicked the changes in ID1 gene expression determined by QRT-PCR.

Expression levels of TM4SF1 were shown to increase with serum stimulation by QRT-PCR (Fig 6.8c) with higher levels of expression in CWF in patient I. The TM4SF1 reporter construct demonstrated an increase in fluorescence for both NF and CWF after serum stimulation with higher levels of fluorescence observed in CWF (Fig 6.8d). As with ID1 cells transfected with the TM4SF1 reporter construct demonstrated changes in fluorescence mimicking the changes in TM4SF1 gene expression observed by QRT-PCR.

Whilst these initial experiments testing the promoter based reporter constructs were limited, the data suggests that the amplified promoter regions are sufficient for reporter gene expression and therefore generation of fluorescence in a disease specific way. Importantly changes in this fluorescence upon serum stimulation match the known changes in gene expression for the two genes of interest.

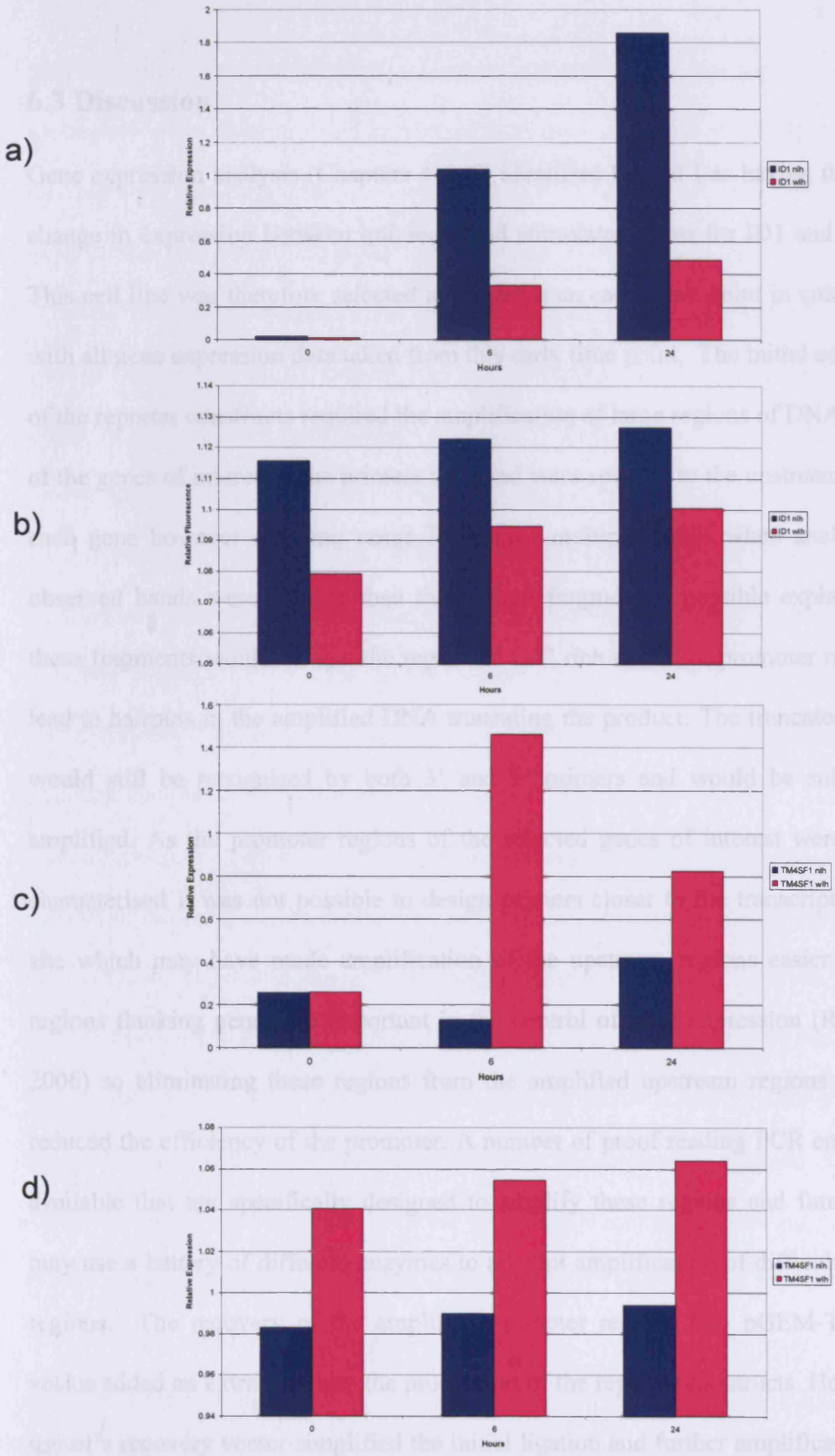


Figure 6.8: Graphs showing relative expression of genes of interest in response to serum. a) ID1 expression determined by qRT-PCR (Chapter 5). b) ID1 expression determined by reporter fluorescence. c) TM4SF1 expression determined by qRT-PCT (Chapter 5). d) TM4SF1 expression determined by reporter fluorescence.

6.3 Discussion

Gene expression analysis (Chapters 4 & 5) identified Patient I as having the greatest change in expression between quiescent and stimulated states for ID1 and TM4SF1. This cell line was therefore selected and used at an early time point in culture (p23), with all gene expression data taken from this early time point. The initial construction of the reporter constructs required the amplification of large regions of DNA upstream of the genes of interest. The primers designed were specific to the upstream region of each gene however the long range PCR gave multiple bands when analysed. The observed bands were smaller than the desired fragment, a possible explanation for these fragments would be that the repetitive G/C rich nature of promoter regions has lead to hairpins in the amplified DNA truncating the product. The truncated products would still be recognised by both 3' and 5' primers and would be subsequently amplified. As the promoter regions of the selected genes of interest were not well characterised it was not possible to design primers closer to the transcriptional start site which may have made amplification of the upstream regions easier. G/C rich regions flanking genes are important in the control of gene expression (Remington, 2006) so eliminating these regions from the amplified upstream regions may have reduced the efficiency of the promoter. A number of proof reading PCR enzymes are available that are specifically designed to amplify these regions and future studies may use a battery of different enzymes to attempt amplification of difficult upstream regions. The recovery of the amplified promoter regions into pGEM-T recovery vector added an extra step into the production of the reporter constructs. However the use of a recovery vector simplified the initial ligation and further amplification of the promoter regions. The recovery vector required only that the PCR products contained a single nucleotide overhang for successful insertion making ligation easier. With the

amplified region contained within a plasmid further PCR was not required to amplify the region as the plasmid could be transformed into *E. coli*. After transformation with the plasmid the competent *E. coli* would replicate the plasmid allowing large quantities of the amplified promoter regions to be generated. The chosen plasmid pGEM-T allowed screening of the bacteria for the presence of the plasmid in three ways. The antibiotic resistance cassette incorporated into the plasmid allowed selection of any bacteria containing a pGEM-T plamid. The lacZ gene into which any recovered DNA was incorporated allowed blue/white screening of colonies using XGal identifying plasmids with added DNA. Finally the plasmids themselves were purified and restriction digests used to identify the specific amplified and recovered promoter regions. The final advantage of using recovery vectors was for the incorporation of specific restriction sites into the amplified promoter regions to allow insertion into the promoter less reporter vector.

The introduction of specific restriction sites into each promoter region by PCR had a number of advantages. Originally the transcriptional start site of each gene of interest was amplified along with the upstream region. The transcriptional start site if retained would interfere with the transcriptional start site of the reporter so had to be removed. The introduction of a restriction site just upstream of the transcriptional start site of the gene of interest ensured that the upstream promoter regions were as close to the reporter proteins transcriptional start site as possible. The introduced restriction sites allowed the direction of the promoter regions insertion into the final reporter vector to be determined with only one possible orientation.

The transfection of the completed reporter constructs for GAPDH, ID1 and TM4SF1 into immortalised NF and CWF was carried out using FuGene transfection reagent. The reporter construct contained a resistance cassette imparting resistance to the

antibiotic G418 to transfected cells. This allowed selection of transfected cells, however after 4 weeks of selection the cells appeared to loose G418 resistance and died. It was decided that for these preliminary experiments transient transfections without selection would be used.

The comparison between gene expression levels determined by QRT-PCR and the changes in fluorescence in the reporter cells identified a potential problem with the fluorescent protein based reporter system. Though the pattern of fluorescent levels between NF and CWF did match the differences seen by QRT-PCR analysis the time course data was less convincing. Fluorescence did increase over time after stimulation with FCS however not to the extent expected from the QRT-PCR data. The lag time between changes in gene expression and changes in fluorescence from reporter proteins has been identified as a problem with this system with a delay of between 2 and 4 hours observed in GFP (Cubitt et al., 1995) (Matz et al., 1999). To fluoresce ZsGreen must first assemble itself into a tetramer, this occurs auto-catalytically however as in GFP this process takes a number of hours (Matz et al., 1999). The supplier Clontech (www.clontech.com) suggest an initial delay of between 8-12 hours after transfection before fluorescence is detectable but do not give specific details on maturation time of the fluorescent protein.

To control/normalise for cell division over the time of the experiment a way of determining cell number in real time was required. Commercial systems to label and track cells are available. Cell tracker dyes such as the green dye 5-chloromethylfluorescein diacetate (CMFDA) (www.invitrogen.com) label living cells giving up to 72 hours of trackable labelling. Nanocrystals can also be used to label cells over a period of time in culture the Qdot® nanocrystal system (www.invitrogen.com) offers 7 different colours of crystal with labelling covering 6

generations of cells. These commercially available systems would not allow for a determination of cell number in real time as the dye or nanocrystals remain constant and are equally divided between daughter cells. Methods for quantifying cell numbers such as the Cell Titre Glo system (www.promega.com) require cell lysis before cell number can be determined. To allow for non-lethal determination of cell number the GAPDH promoter driven reporter construct was constructed. GAPDH has been shown to be stably expressed in CWF and NF by microarray analysis (Chapter 4) and was used as a housekeeping gene in all QRT-PCR reactions carried for Chapter 5. The GAPDH gene expression was not altered between NF and CWF, and was not altered by the presence or absence of serum. Fluorescence from the GAPDH reporter construct should therefore increase with cell number allowing a normalisation of fluorescence. In these experiments the GAPDH reporter was expressed in separate cells to the ID1 and TM4SF1 reporters. The GAPDH reporter cells were treated in the same way as the ID1 and TM4SF1 cells in order for them to act as a control. The choice for separate reporter cell lines was determined by antibiotic resistance cassettes available in the Clontech promoter-less reporter plasmids. Although Red Green and Yellow fluorescent proteins are available in promoter-less reporter plasmids they all contain the same resistance cassettes. This would make selection of cells containing 2 different reporter constructs impossible. Future work with this system should focus on replacing the G418 resistance cassette with one for Zeomycin or Hygromycin allowing co-transfection of a housekeeping reporter construct and the reporter construct for the gene of interest.

These proof of principle experiment demonstrated that the reporter constructs work, and that with further refinement they may become useful tools forn studying chronic wounds. These reporter constructs demonstrate the potential for studying changes in

gene expression in real time. While the constructs developed in this Chapter contained only one reporter it may be possible to place multiple reporters in a single construct. This would allow the transfection of reporters for both the gene of interest as well as a housekeeping control at the same time. With the diverse range of fluorescent proteins now available (Trzaska et al., 2005) multiple fluorophores may be detected in the same cell without cross talk. With a larger panel of reporter constructs covering the genes of interest identified in previous Chapters new treatments for chronic wounds could be screened. High throughput assays for new therapeutics based on plates containing CWF with various reporter constructs would allow the rapid screening of potential therapeutics. Indeed, fluorescent reporter constructs have previously been developed to screen chemicals. An immunotoxicity test based on multiple cell types derived from lymphocytes has been developed, with these cells transfected with GFP based cytokine reporter gene constructs (Jones et al., 2007). The assay was proposed as a simple way to screen chemicals for immunotoxic effect. In order to study latent HIV in memory T cells a dual fluorescence reporter cell line has been developed. The assay uses green fluorescence as a marker for HIV activity and red fluorescence as a marker of cell viability. The reporter cells have been used in a high throughput screen for potential HIV therapeutics (Cahill et al., 2004). The Yeast based 'GreenScreen® assay', a DNA repair reporter assay based on detection of changes in the expression of the *S.cerevisiae RAD54* through a GFP reporter has also been described (Cahill et al., 2004). DNA damage leads to an increase in fluorescence however, other stresses such as oxidative stress does not lead to an increase in fluorescence. The GreenScreen assay was developed as a high throughput screen for genotoxic compounds (Cahill et al., 2004, Knight et al., 2007, Van Gompel et al., 2005). The GreenScreen® assay in commercially available from Gentronix (www.gentronix.co.uk) and has been assessed

by pharmaceutical companies as a potentially useful drug screen (Hastwell et al., 2006, Billinton et al., 2008). A human cell version of the GreenScreen® genotoxicity assay has been developed, based on a *GADD45a-GFP* reporter (Graham et al., 2003, Moffatt et al., 2004). The assay is described as being useful for detecting mutagens, clastogens, aneugens, topoisomerase and polymerase inhibitors in a high throughput 96 well microplate format. Reporter cell lines already play a role in biomedical research, immunotoxicity and DNA damage testing and could play a role in the study of chronic wounds. While cell based screening assays cannot replace the use of animal models for validation of novel therapeutic effects they can greatly reduce the number of animals used. A cell based real time reporter assay may be used to reduce the number of potential therapeutics prior to animal trails. This would not only speed up the screening process and reduce the number of animals required but it would also reduce the cost.

In conclusion, reporter constructs for a housekeeping gene and two genes of interest were generated. Previous work indicates that these genes may be important in the chronic wound phenotype. The reporter constructs were successfully transfected into CWF and NF fibroblasts with fluorescence observed with fluorescence microscopy. The generated reporter constructs were shown to be stimulated through addition of serum to the transfected cells. The scientific and commercial interest in cell based fluorescent reporters as useful tools in screening potentially harmful or useful chemicals has been demonstrated in the literature. The constructs described in this Chapter have the potential to be developed into a high throughput screen for the identification of novel therapeutic for the treatment of chronic venous leg ulcers.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 General discussion and future work

In this Thesis, the characterisation of hTERT immortalised dermal fibroblast cell lines was described. These cell lines were based on primary cells taken from both chronic wounds and patient matched normal skin. Chronic wounds are a burden on the patients that suffer from them and on the health services that treat them. Within the UK, the NHS has approximately 200,000 individual patients with chronic non-healing wounds (Gunnel and Hjelmgren, 2005), with a treatment cost between £2.3bn and £3.1bn per year (Menke et al., 2007). Though the underlying causes for chronic wounds varies, they are thought to have failed to resolve the inflammatory phase of the wound healing response (Agren et al., 1999a, Cook et al., 2000a, Raffetto et al., 2001c, Stephens et al., 2003).

In order to study chronic wounds within controllable laboratory conditions several models of impaired wound healing have been developed. These models aim to recreate the chronic wound environment in laboratory animals either through a specific genetic background (diabetic mouse model) or through surgically generated tissue ischemia. These models do demonstrate impaired wound healing taking longer to heal than none ischemic wounds in the same animal however, they do eventually heal unlike chronic wounds in human patients. A second consideration when considering the use of the impaired wound healing models is the increased suffering of the experimental animals. The work in this thesis was funded by the Dr Hadwen Trust for Humane Research (http://www.drhadwentrust.org.uk) to develop a human cell based alternative to the animal models of impaired wounding. Whilst primary human cell strains taken from chronic wounds have been used in the past (Jiang et al., 1999, Mondello et al., 2003) these cells have a limited life span and reproducibility in the laboratory and therefore new patient samples are required to continue work.

Therefore, the first aim of this thesis was to phenotypically characterise and compare hTERT immortalised fibroblast cell lines from chronic wound and patient matched normal with the primary cells from which they were derived. For the cells to be a useful research tool they must be able to escape replicative senescence, they must also retain their phenotypic differences both directly after immortalisation and after extended time in culture.

In Chapter 3 the growth potential of the CWF an NF cell lines was investigated along with their size, morphology, ability to repopulate a scratch wound and karyotype. It was demonstrated that all 6 hTERT infected cell lines had escaped replicative senescence, undergoing more than 100 population doublings with no marked reduction in proliferation rate. In two of the patients (H and K) there was a clear difference in proliferation rate between the CWF and NF. The CWF underwent fewer population doublings per week than the patient matched NF over the entire time in culture. In the third patient (I) there was no difference in the average population doubling rate between the CWF and NF cell lines. The hTERT infected cell lines were imaged over their extended time in culture to compare cellular morphology not only between NF and CWF cell lines but also between early and late passage cells. Both the morphology of the cells and their cell area were analysed looking for indications of senescence. No changes in cellular morphology were observed between the NF and CWF cell lines or between early and late passage cells within the same cell line. Differences in cell size were observed with CWF cells generally larger than patient matched NF however, no increase in cell size was observed with increased time in culture. While cellular proliferation, morphology and size by themselves are not

sufficient to confirm that cells have escaped replicative senescence when taken together they are more convincing.

To confirm that the hTERT infected cell lines were expressing active telomerase RT-PCR was used to identify transcripts for hTERT and hTR in all six cell lines. Active telomerase was identified using the TRAP assay with telomerase activity identified in all six cell lines. This data suggest that the hTERT infected cells had successfully escaped replicative senescence through the introduction of active telomerase. Additionally in Chapter 3, a simplified wound model was used to study the ability of the hTERT infected cell lines to repopulate a wound. The repopulation of a wound by fibroblasts is an essential part of the wound healing process. In this model the CWF demonstrated a reduced ability to repopulate monolayer scratch wounds in all three patients compared to the patient matched NF. This phenotype was observed at both the early and late time point in culture. Whilst time-lapse microscopy allowed the automated documentation of the wound repopulation, variability between the size of the initial wound and limitations in access to the time-lapse microscope restricted the data collected. Scratch wounding machines such as the Essen Woundmaker^{TM} offer the potential to generate reproducible wounds and to analyse multiple plates at a time. In this assay wound repopulation is through cellular migration and proliferation however, it may be useful to study wound repopulation in these cell through migration alone. This can be achieved by the use of an anti proliferative agent such as mitomycin c.

To address potential cross talk between CWF and NF a co-culture model using the fluorescent cell tracker dyes (www.invitrogen.com) CMFDA (green) and CMTPX (red) was designed. The CWF were labelled with CMFDA and the NF with CMTPX, an equal number of cells were used to generate a confluent monolayer which was then

wounded. Through the use of fluorescent time-lapse microscopy the cells repopulating the wound area could be identified and counted. Results from this model were initially inconclusive but with further more in-depth work it may provide an insight into the importance of cell-cell communication in chronic wounds. The final phenotypic analysis carried out on the cell lines was to determine their karyotype. Since chromosomal abnormalities are often seen in hTERT immortalised cell lines (Mondello et al., 1999, Broberg et al., 2001, Broberg et al., 1998, Dahlén et al., 2001). NF and CWF cell lines were karyotyped after more than a year in continuous culture. Many of the cell lines had either a sub population or the entire population with chromosomal abnormalities. The mixed nature of many of the cells karyotypes, with a small population of the cells demonstrating an altered chromosome number or translocations suggests that the chromosomal alterations have been picked up over time in culture rather than being present at the point of transfection. Interestingly all the CWF populations analysed showed some levels of trisomy 7, which has previously been seen in aged patient samples or in cells taken from areas of prolonged inflammation (Scott et al., 1998, Bell et al., 1979). Comparison between the karyotypes seen in the late passage immortalised cells and primary cell strains at very early passage would indicate if the chromosomal abnormalities have been picked up during culture. If detected in the primary cells the trisomy 7 seen in all the CWF cell lines may prove to play a role in the failure of chronic wounds to heal.

Overall the analysis carried out in Chapter 3 indicated that the hTERT infection of CWF and patient matched NF had allowed them to escape replicative senescence. Importantly the immortalised cell lines retained phenotypic differences in growth rate and wound repopulation rate after immortalisation. An experiment to assesses the response of the fibroblasts in 3D culture were not carried out on the hTERT

immortalised cell lines due to understandable restrictions set in place by the funding charity with respect to the use of rat tail collagen. Free floating fibroblast populated collagen lattices generated from rat tail type I collagen determine the ability of fibroblasts to remodel extracellular matrix. Lattice reorganisation is caused by the rearrangement of collagen fibrils by the fibroblasts within the lattice (Paul Ehrlich et al., 2006a). Tethered collagen lattices may also be used to study the ability of fibroblasts to generate tension as may be observed within a wound (Wall et al., 2008b). These experiments would give important phenotypic information about the ability of the cell lines to remodel the wound environment which cannot be obtained by 2D assays such as the scratch wound model.

The second aim of this Thesis was to characterise genotypic responses of CWF and NF cell lines to wounding and to compare this response to that of the primary cells. In Chapter 4, a microarray analysis of cellular responses to serum stimulation was conducted with responses compared with those of primary cells (Iyer et al., 1999). Cells were serum starved for 48 hours synchronising them in G_0 of the cell cycle (Quiescence), this mimics their state in the undamaged dermis (Wall et al., 2008b). The addition of serum back onto the cells stimulates cellular proliferation, mimicking the exposure of fibroblasts within the dermis to serum from damaged blood vessels which occurs upon wounding. As well as mimicking the *in vivo* wound response the synchronisation of all the cells within the culture makes the identification of up or down regulated gene expression simpler. A limitation set on the microarray experiment carried out was the need to compare the data generated with the primary cells. The same Affymetrix™ U133A 2.0 GeneChip® technology that had been used previously (Romagnani et al., 2004, Smith et al., 2005, Gillitzer and Goebeler, 2001)

was therefore used to measure gene expression at 0 and 6 hours after serum stimulation. Comparisons between the immortalised and primary cells identified a number of genes that had been altered by hTERT infection. Overrepresentation analysis of these genes identified pathways relating to the cell cycle and glycolysis up regulated in immortalised cells as well as genes linked with protein sequencing. These findings correlate with the increased proliferative ability the immortalised cells show. The number of genes altered by immortalisation was small and few known pathways appear to have been effected, genotypically the hTERT immortalised cell lines appear to be a good model of the primary cell strains from which they were generated. Comparisons between the CWF and NF identified a number of individual genes and pathways that were differentially expressed between CWF and NF. Among these genes were a number of members of the CXC chemokine family whose expression was down in CWF. This family of chemokines is responsible for the recruitment of neutrophils to the wound area controlling the inflammatory response seen in acute wounds (Huang et al., 2006). As previously mentioned chronic wounds fail to resolve the inflammatory phase of wound healing, the reduced expression of these chemokines may in part be responsible for this.

Microarray analysis identified pro-inflammatory components of the toll like receptor pathway to be up regulated in CWF compared to NF. The cytokines IL6, IL1B and IL8 were up regulated in CWF. In a wound toll like receptors detect the presence of bacterial and viral infection and instigate an inflammatory response, however, CWF are cultured in aseptic conditions free from bacterial and viral stimuli. The up regulated cytokines are downstream, of the TLR pathway and may indicate miss-signalling by the TLR. As with the CXC family of cytokines the over expression of pro inflammatory cytokines by CWF may play a role in the failure of chronic wounds

to heal. Further studies in this area looking at the ability of CWF and NF to induce the migration of inflammatory cells would be interesting. As the TLR pathway is well understood specific inhibitors could be used to confirm that this is in fact the cause of the altered gene expression seen in the CWF. Genes linked with cell motility were also identified as being differentially expressed between CWF and NF. Components of signalling pathways linked to the actin cytoskeleton were identified and may play a role in the reduced wound repopulation rates seen in the CWF. Genes such as CD9 which inhibits signalling through the WAVE2 pathway (Jones et al., 2007) and whose expression is increased in CWF may be the target for gene silencing using siRNA. The knock down of specific genes through the introduction of siRNA into cells is a useful tool for studying the role of individual genes within a cell. In the case of CWF it may be possible to reverse some of the chronic wound phenotype described in chapter 3 by the specific inhibition of genes identified by microarray analysis discussed in chapter 4.

Microarray data from this thesis has been archived in MADRAS (http://madras.uwcm.ac.uk) where it is fully searchable. This data adds value to the CWF and NF cell lines and increases their usefulness for future experiments. Gene expression in the cell lines over extended time in culture was demonstrated to be stable. The data also demonstrates that they retain not only phenotypic but also genotypic differences after hTERT infection.

The third aim of this Thesis was to generate promoter based reporter constructs using genes whose expression was identified as being altered in CWF. From the lists of genes differentially expressed between the CWF and NF a subset of genes of interest were manually selected. These genes demonstrated differences in expression between

CWF and NF across all three patients. The differences in expression between NF and CWF were thought to be large enough for detection with a promoter based reporter system. To allow for testing of the reporter constructs a set of genes which were not only differentially expressed between CWF and NF but were also induced or repressed by the addition of serum to the cells were selected.

For this subset of genes of interest quantitative real time PCR was used to confirm the microarray results. As well as confirming expression changes seen in the microarray data this allowed for the analysis of more time points after serum stimulation (1h and 24h) giving a broader picture of gene expression after stimulation. The cDNA used in the confirmation of the microarray data was generated from the same RNA that was used for microarray analysis. With more time repeats of the serum starvation/stimulation experiments and the generation of multiple RNA samples for each cell line would have been carried out. These experimental repeats would give more data about the reliability of the changes in gene expression observed in chapter 5 and allow statistical analysis to be carried out on the data. As with the phenotypic analysis, variations were seen between the three patients in gene expression for each gene under investigation. The QRT-PCR data demonstrated the same changes in gene expression observed in the microarray data. From the 3 patients studied Patient I showed the most consistent maintenance of gene expression profile across the different time points in culture. Further validation of the changes in gene expression by analysis of protein levels were not carried out in this thesis. The reporter constructs rely only on the promoter driven mRNA transcription of their targeted genes. Therefore changes in mRNA level detected by both microarray and QRT-PCR are sufficient to generate changes in reporter expression. With more time the study of changes in protein expression on an individual gene level by western blotting, or at a

proteome level by 2D gel electrophoresis would have been carried out. This data would confirm that changes in gene expression identified by microarray and QRT-PCR translate to changes in protein level. As the proteins expressed by CWF play an important part in the altered phenotype this information would be useful in targeted chronic wound treatments.

The generation of reporter constructs initially required the cloning of the promoter regions for the selected genes of interest. Whilst this was successfully carried out for the majority of the genes of interest not all promoter regions were successfully cloned. With more time to redesign primers and optimise the long range PCR reactions the remaining promoters may be successfully cloned. After cloning restriction sites were introduced at the ends of the promoter regions and used to insert the promoter regions into a promoterless reporter vector. As with the initial cloning it was not possible to introduce restriction sites into each of the promoter regions by PCR. Further optimisation or redesign of the primers used would allow this to be achieved in the future. Another approach could include the use of commercially available promoter driven reporter constructs such as the GENOME-WIDE PROMOTER LIBRARY ™ (www.switchgeargenomics.com). This system uses approximately 1Kb of the upstream region of genes of interest to drive expression of a luciferase reporter.

The completed reporter constructs were successfully transfected into NF and CWF cell lines from patient I, as this patient displayed the most consistent maintenance of gene expression profile across the different time points in culture. The cells were tested and expression of the green fluorescent reporter protein was detected by microscopy and its controlled induction by a fluorescent plate reader. These reporter cell lines were however, generated by transient transfection. For the reporter cell lines to be a useful tool in the investigation of chronic wounds the creation of stable

reporter cell lines is required. Unlike the transient reporter lines a stable line would be a consistent platform for the screening of therapeutic agents. Stable reporter cell lines may be generated through viral transduction, an efficient and widely used technique. Using a system such as the ViraPower™ Promoterless Lentiviral Gateway Vector Expression system (www.invitrogen.com) stable reporter cell lines could be generated. This technology would also allow for the introduction of more than one reporter per cell. This would allow a housekeeping gene reporter construct to be in the same cell as the reporter construct for the gene of interest. The presence of both reporters in the same cell controls for changes in global gene expression. If the chosen housekeeping gene is constitutively expressed it may also act as a control for cell number. Other controls looking at cross talk between the different fluorphores used, oxidative stress levels within the cells and cellular phototoxicity caused by exposure of the cells to light must also be carried out.

From the proof of principle work carried out in this Thesis the potential of a chronic wound reporter cell line is clear. With further development, screening of potential therapeutic agents could be carried out using such a cell line (Wall, 2006, Colwell et al., 2006), with the cells in a 96 well plate format. While such a cell line would not entirely replace the use of animal models in the testing of novel wound healing therapeutics it may play a part in reducing their use in large scale drug screening.

In this Thesis an attempt was made to establish hTERT immortalised cell lines generated from chronic wounds, as well as patient matched normal skin as a potential replacement for animals in chronic wound research. The cell lines were shown to be immortalised through retroviral infection by hTERT and to be expressing active telomerase. The CWF cell were shown to retain phenotypic and genotypic difference after immortalisation compared to patient matched NF. The phenotypic and genotypic

changes seen in CWF relate to changes seen in chronic wounds. Cell lines with detailed gene expression data are now available for use in future studies of chronic wound healing and are currently being used in a number of other research projects. Reporter constructs were generated for two disease genes and a housekeeping gene, these reporter constructs were successfully transfected into NF and CWF cell lines and tested. Work on the generation of stable reporter cell lines is ongoing, using immortalised fibroblasts taken from diabetic foot ulcers as well as the chronic wound fibroblasts discussed in this Thesis. It is envisaged that these cell lines may eventually offer a real alternative to the use of animal models in chronic wound research.

References

AGREN, M. S., STEENFOS, H. H., DABELSTEEN, S., HANSEN, J. B. & DABELSTEEN, E. (1999a) Proliferation and Mitogenic Response to PDGF-BB of Fibroblasts Isolated from Chronic Venous Leg Ulcers is Ulcer-Age Dependent. *J Invest Dermatol,* 112, 463-469.

AGREN, M. S., STEENFOS, H. H., DABELSTEEN, S., HANSEN, J. B. & DABELSTEEN, E. (1999b) Proliferation and mitogenic response to PDGF-BB of fibroblasts isolated from chronic venous leg ulcers is ulcer-age dependent. *J Invest Dermatol,* 112, 463-469.

ALANI, R. M., YOUNG, A. Z. & SHIFFLETT, C. B. (2001) Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. *Proceedings of the National Academy of Sciences,* 98, 7812-7816.

AMEGLIO, F., BONIFATI, C., FAZIO, M., MUSSI, A., TRENTO, E., CORDIAL FEI, P., DONATI, P., PIMPINELLI, F., D'AURIA, L. & CARDUCCI, M. (1997) Interleukin-11 production is increased in organ cultures of lesional skin of patients with active plaque-type psoriasis as compared with nonlesional and normal skin. Similarity to interleukin-1 beta, interleukin-6 and interleukin-8. *Arch Dermatol Res,* 289, 399-403.

AN, G., HIDAKA, K. & SIMINOVITCH, L. (1982) Expression of Bacterial {beta}-Galactosidase in Animal Cells. *Mol. Cell. Biol.,* 2, 1628-1632.

ANGELLO, J. C., PENDERGRASS, W. R., NORWOOD, T. H. & PROTHERO, J. (1989) Cell enlargement: one possible mechanism underlying cellular senescence. *J Cell Physiol,* 140, 288-94.

ANGIOLILLO, A. L., SGADARI, C., TAUB, D. D., LIAO, F., FARBER, J. M., MAHESHWARI, S., KLEINMAN, H. K., REAMAN, G. H. & TOSATO, G. (1995) Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J Exp Med,* 182, 155-62.

ANTAL, M., FARKAS, T., GERMAN, P., BELAK, S. & KISS, I. (2007) Real-time reverse transcription-polymerase chain reaction detection of Newcastle disease virus using light upon extension fluorogenic primers. *J Vet Diagn Invest,* 19, 400-4.

ARAUJO, C. S., GRANER, E., ALMEIDA, O. P., SAUK, J. J. & COLETTA, R. D. (2003) Histomorphometric characteristics and expression of epidermal growth factor and its receptor by epithelial cells of normal gingiva and hereditary gingival fibromatosis. *J Periodontal Res,* 38, 237-41.

ARBOLEDA-VELASQUEZ, J. F., ZHOU, Z., SHIN, H. K., LOUVI, A., KIM, H.-H., SAVITZ, S. I., LIAO, J. K., SALOMONE, S., AYATA, C., MOSKOWITZ, M. A. & ARTAVANIS-TSAKONAS, S. (2008) Linking Notch signaling to ischemic stroke. *Proceedings of the National Academy of Sciences,* 105, 4856-4861.

ARHONDAKIS, S., CLAY, O. & BERNARDI, G. (2008) GC level and expression of human coding sequences. *Biochemical and Biophysical Research Communications,* 367, 542-545.

BALU, B. & ADAMS, J. H. (2003) Fluorescent chloramphenicol as a substitute for radioactive [14C]-chloramphenicol for CAT reporter assays in Plasmodium falciparum. *Mol Biochem Parasitol,* 126, 285-6.

BARONE, E. J., YAGER, D. R., POZEZ, A. L., OLUTOYE, O. O., CROSSLAND, M. C., DIEGELMANN, R. F. & COHEN, I. K. (1998) Interleukin-1alpha and collagenase activity are elevated in chronic wounds. *Plast Reconstr Surg,* 102, 1023-7; discussion 1028-9.

BECIC, F., MORNJAKOVIC, Z., ZULIC, I., PRASOVIC, S., MULABEGOVIC, N., KAPIC, E. & MIJANOVIC, M. (2003) Animal model of thermal injuries. *Bosn J Basic Med Sci,* 3, 41-6.

BELL, E., IVARSSON, B. & MERRILL, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci U S A,* 76, 1274-8.

BELLO, Y. M. & PHILLIPS, T. J. (1998) Management of venous ulcers. *J Cutan Med Surg,* 3 Suppl 1, S1-6-12.

BERARD, A., ABENHAIM, L., PLATT, R., KAHN, S. R. & STEINMETZ, O. (2002) Risk factors for the first-time development of venous ulcers of the lower limbs: the influence of heredity and physical activity. *Angiology,* 53, 647-57.

BERGER, J., HAUBER, J., HAUBER, R., GEIGER, R. & CULLEN, B. R. (1988) Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene,* 66, 1-10.

BERMEJO-RODRIGUEZ, CAMINO PEREZ-CARO, M., PEREZ-MANCERA, P. A., SANCHEZ-BEATO, M., PIRIS, M. A. & SANCHEZ-GARCIA, I. (2006) Mouse cDNA microarray analysis uncovers Slug targets in mouse embryonic fibroblasts. *Genomics,* 87, 113-118.

BERNSTEIN, E. F. & UITTO, J. (1996) The effect of photodamage on dermal extracellular matrix. *Clin Dermatol,* 14, 143-51.

BIEDERER, T., SARA, Y., MOZHAYEVA, M., ATASOY, D., LIU, X., KAVALALI, E. T. & SUDHOF, T. C. (2002) SynCAM, a Synaptic Adhesion Molecule That Drives Synapse Assembly. *Science,* 297, 1525-1531.

BILLINTON, N., HASTWELL, P. W., BEERENS, D., BIRRELL, L., ELLIS, P., MASKELL, S., WEBSTER, T. W., WINDEBANK, S., WOESTENBORGHS, F., LYNCH, A. M., SCOTT, A. D., TWEATS, D. J., VAN GOMPEL, J., REES, R. W. & WALMSLEY, R. M. (2008) Interlaboratory assessment of the GreenScreen HC GADD45a-GFP genotoxicity screening assay: An enabling study for independent validation as an alternative method. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 653, 23-33.

BLEUL, C. C., FARZAN, M., CHOE, H., PAROLIN, C., CLARK-LEWIS, I., SODROSKI, J. & SPRINGER, T. A. (1996a) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature,* 382, 829-33.

BLEUL, C. C., FUHLBRIGGE, R. C., CASASNOVAS, J. M., AIUTI, A. & SPRINGER, T. A. (1996b) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med,* 184, 1101-9.

BODNAR, A. G., OUELLETTE, M., FROLKIS, M., HOLT, S. E., CHIU, C. P., MORIN, G. B., HARLEY, C. B., SHAY, J. W., LICHTSTEINER, S. & WRIGHT, W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science,* 279, 349-352.

BORENSZTAJN, K., STIEKEMA, J., NIJMEIJER, S., REITSMA, P. H., PEPPELENBOSCH, M. P. & SPEK, C. A. (2008) Factor Xa stimulates proinflammatory and profibrotic responses in fibroblasts via protease-activated receptor-2 activation. *Am J Pathol,* 172, 309-20.

BOS, P. K., VAN OSCH, G. J. V. M., FRENZ, D. A., VERHAAR, J. A. N. & VERWOERD-VERHOEF, H. L. (2001) Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model. *Osteoarthritis and Cartilage,* 9, 382-389.

BOULTON, A. J. M., KIRSNER, R. S. & VILEIKYTE, L. (2004) Neuropathic Diabetic Foot Ulcers. *N Engl J Med,* 351, 48-55.

BOWLER, P. G. & DAVIES, B. J. (1999) The microbiology of infected and noninfected leg ulcers. *Int J Dermatol,* 38, 573-8.

BOZZO, L., DE ALMEDIA, O. P., SCULLY, C. & ALDRED, M. J. (1994) Hereditary gingival fibromatosis. Report of an extensive four-generation pedigree. *Oral Surg Oral Med Oral Pathol,* 78, 452-4.

BRAKMAN, M., FABER, W. R., KERCKHAERT, J. A., KRAAIJENHAGEN, R. J. & HART, H. C. (1992) Immunofluorescence studies of atrophie blanche with antibodies against fibrinogen, fibrin, plasminogen activator inhibitor, factor VIII-related antigen, and collagen type IV. *Vasa,* 21, 143-8.

BRANS, T. A., DUTRIEUX, R. P., HOEKSTRA, M. J., KREIS, R. W. & DU PONT, J. S. (1994) Histopathological evaluation of scalds and contact burns in the pig model. *Burns,* 20 Suppl 1, S48-51.

BROBERG, K., HÖGLUND, M., LINDSTRAND, A., TOKSVIG-LARSEN, S., MANDAHL, N. & MERTENS, F. (1998) Polyclonal expansion of cells with trisomy 7 in synovia from patients with osteoarthritis.

BROBERG, K., TOKSVIG-LARSEN, S., LINDSTRAND, A. & MERTENS, F. (2001) Trisomy 7 accumulates with age in solid tumors and non-neoplastic synovia. *Genes, Chromosomes and Cancer,* 30, 310-315.

BRONSTEIN, I., FORTIN, J., STANLEY, P. E., STEWART, G. S. & KRICKA, L. J. (1994) Chemiluminescent and bioluminescent reporter gene assays. *Anal Biochem,* 219, 169-81.

BROWN, P. O. & BOTSTEIN, D. (1999) Exploring the new world of the genome with DNA microarrays. *Nat Genet,* 21, 33-37.

BUDGEN, V. (2004) Evaluating the impact on patients of living with a leg ulcer. *Nurs Times,* 100, 30-1.

BUEMI, M., GALEANO, M., STURIALE, A., IENTILE, R., CRISAFULLI, C., PARISI, A., CATANIA, M., CALAPAI, G., IMPALA, P., ALOISI, C., SQUADRITO, F., ALTAVILLA, D., BITTO, A., TUCCARI, G. & FRISINA, N. (2004) Recombinant human erythropoietin stimulates angiogenesis and healing of ischemic skin wounds. *Shock,* 22, 169-73.

BULLARD, K. M., LUND, L., MUDGETT, J. S., MELLIN, T. N., HUNT, T. K., MURPHY, B., RONAN, J., WERB, Z. & BANDA, M. J. (1999a) Impaired wound contraction in stromelysin-1-deficient mice. *Ann Surg,* 230, 260-5.

BULLARD, K. M., MUDGETT, J., SCHEUENSTUHL, H., HUNT, T. K. & BANDA, M. J. (1999b) Stromelysin-1-deficient fibroblasts display impaired contraction in vitro. *J Surg Res,* 84, 31-4.

BURNAND, K. G., WHIMSTER, I., NAIDOO, A. & BROWSE, N. L. (1982) Pericapillary fibrin in the ulcer-bearing skin of the leg: the cause of lipodermatosclerosis and venous ulceration. *Br Med J (Clin Res Ed),* 285, 1071-2.

CAHILL, P. A., KNIGHT, A. W., BILLINTON, N., BARKER, M. G., WALSH, L., KEENAN, P. O., WILLIAMS, C. V., TWEATS, D. J. & WALMSLEY, R. M. (2004) The GreenScreen(R) genotoxicity assay: a screening validation programme. *Mutagenesis,* 19, 105-119.

CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W. W. & PRASHER, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science,* 263, 802-5.

CHANG, H. Y., CHI, J.-T., DUDOIT, S., BONDRE, C., VAN DE RIJN, M., BOTSTEIN, D. & BROWN, P. O. (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. *PNAS,* 99, 12877-12882.

CHANG, M. S., MCNINCH, J., BASU, R. & SIMONET, S. (1994) Cloning and characterization of the human neutrophil-activating peptide (ENA-78) gene. *J Biol Chem,* 269, 25277-82.

CHAPONNIER, C. & GABBIANI, G. (2004) Pathological situations characterized by altered actin isoform expression. *J Pathol,* 204, 386-95.

CHARLES, C. A., TOMIC-CANIC, M., VINCEK, V., NASSIRI, M., STOJADINOVIC, O., EAGLSTEIN, W. H. & KIRSNER, R. S. (2008) A gene signature of nonhealing venous ulcers: Potential diagnostic markers. *J Am Acad Dermatol.*

CHEN, H., CHARLAT, O., TARTAGLIA, L. A., WOOLF, E. A., WENG, X., ELLIS, S. J., LAKEY, N. D., CULPEPPER, J., MORE, K. J., BREITBART, R. E., DUYK, G. M., TEPPER, R. I. & MORGENSTERN, J. P. (1996) Evidence That the Diabetes Gene Encodes the Leptin Receptor: Identification of a Mutation in the Leptin Receptor Gene in db/db Mice. *Cell,* 84, 491-495.

CHEN, R., HUANG, W., LIN, Z., ZHOU, Z., YU, H. & ZHU, D. (2004) Development of a novel real-time RT-PCR assay with LUX primer for the

detection of swine transmissible gastroenteritis virus. *Journal of Virological Methods,* 122, 57-61.

CHIEN, S. (2007) Ischemic rabbit ear model created by minimally invasive surgery. *Wound Repair Regen,* 15, 928-35.

CHIN, J. R., MURPHY, G. & WERB, Z. (1985) Stromelysin, a connective tissue-degrading metalloendopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase. Biosynthesis, isolation, characterization, and substrates. *J. Biol. Chem.,* 260, 12367-12376.

CHOI, D., WHITTIER, P. S., OSHIMA, J. & FUNK, W. D. (2001) Telomerase expression prevents replicative senescence but does not fully reset mRNA expression patterns in Werner syndrome cell strains. *FASEB J.,* 15, 1014-1020.

CHOI, H. U., JOHNSON, T. L., PAL, S., TANG, L. H., ROSENBERG, L. & NEAME, P. J. (1989) Characterization of the dermatan sulfate proteoglycans, DS-PGI and DS- PGII, from bovine articular cartilage and skin isolated by octyl- sepharose chromatography. *J. Biol. Chem.,* 264, 2876-2884.

CLARK, L. D., CLARK, R. K. & HEBER-KATZ, E. (1998) A New Murine Model for Mammalian Wound Repair and Regeneration. *Clinical Immunology and Immunopathology,* 88, 35-45.

COLE, K. E., STRICK, C. A., PARADIS, T. J., OGBORNE, K. T., LOETSCHER, M., GLADUE, R. P., LIN, W., BOYD, J. G., MOSER, B., WOOD, D. E., SAHAGAN, B. G. & NEOTE, K. (1998) Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med,* 187, 2009-21.

COLERIDGE SMITH, P. D., THOMAS, P., SCURR, J. H. & DORMANDY, J. A. (1988) Causes of venous ulceration: a new hypothesis. *Br Med J (Clin Res Ed),* 296, 1726-7.

COLLINS, K. (1996) Structure and function of telomerase. *Curr Opin Cell Biol,* 8, 374-80.

COLWELL, A. S., LONGAKER, M. T. & PETER LORENZ, H. (2008) Identification of differentially regulated genes in fetal wounds during regenerative repair. *Wound Repair Regen,* 16, 450-9.

COLWELL, A. S., THOMAS M. KRUMMEL, T. M., KONG, W., LONGAKER, M. T. H. & LORENZ, P. (2006) Skin wounds in the MRL/MPJ mouse heal with scar. *Wound Repair and Regeneration,* 14, 81-90.

COOK, H., DAVIES, K. J., HARDING, K. G. & THOMAS, D. W. (2000a) Defective extracellular matrix reorganization by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. *J Invest Dermatol,* 115, 225-33.

COOK, H., STEPHENS, P., DAVIES, K. J., HARDING, K. G. & THOMAS, D. W. (2000b) Defective Extracellular Matrix Reorganization by Chronic Wound Fibroblasts is Associated with Alterations in TIMP-1, TIMP-2, and MMP-2 Activity. *J Invest Dermatol,* 115, 225-233.

COOPER, L., JOHNSON, C., BURSLEM, F. & MARTIN, P. (2004) Wound healing and inflammation genes revealed by array analysis of 'macrophageless' PU.1 null mice. *Genome Biology,* 6, R5.

COOPER, L., JOHNSON, C., BURSLEM, F. & MARTIN, P. (2005) Wound healing and inflammation genes revealed by array analysis of 'macrophageless' PU.1 null mice. *Genome Biol,* 6, R5.

CORBETT, M. S., SCHMITT, I., RIESS, O. & WALZ, A. (1994) Characterization of the gene for human neutrophil-activating peptide 78 (ENA-78). *Biochem Biophys Res Commun,* 205, 612-7.

CORSI, A., XU, T., CHEN, X. D., BOYDE, A., LIANG, J., MANKANI, M., SOMMER, B., IOZZO, R. V., EICHSTETTER, I., ROBEY, P. G., BIANCO, P. & YOUNG, M. F. (2002) Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. *J Bone Miner Res,* 17, 1180-9.

CRIBBS, R. K., LUQUETTE, M. H. & BESNER, G. E. (1998) A standardized model of partial thickness scald burns in mice. *J Surg Res,* 80, 69-74.

CRISTOFALO, V. J. & PIGNOLO, R. J. (1993) Replicative senescence of human fibroblast-like cells in culture. *Physiol Rev,* 73, 617-38.

CUBITT, A. B., HEIM, R., ADAMS, S. R., BOYD, A. E., GROSS, L. A. & TSIEN, R. Y. (1995) Understanding, improving and using green fluorescent proteins. *Trends in Biochemical Sciences,* 20, 448-455.

DAHLÉN, A., BROBERG, K., DOMANSKI, H. A., TOKSVIG-LARSEN, S., LINDSTRAND, A., MANDAHL, N. & MERTENS, F. (2001) Analysis of the distribution and frequency of trisomy 7 in vivo in synovia from patients with osteoarthritis and pigmented villonodular synovitis. *Cancer Genetics and Cytogenetics,* 131, 19-24.

DANIELSON, K. G., BARIBAULT, H., HOLMES, D. F., GRAHAM, H., KADLER, K. E. & IOZZO, R. V. (1997) Targeted Disruption of Decorin Leads to Abnormal Collagen Fibril Morphology and Skin Fragility. *J. Cell Biol.,* 136, 729-743.

DE WET, J. R., WOOD, K. V., DELUCA, M., HELINSKI, D. R. & SUBRAMANI, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol,* 7, 725-37.

DERYUGINA, E. I. & QUIGLEY, J. P. (2006) Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev,* 25, 9-34.

DEVALARAJA, R. M., NANNEY, L. B., DU, J., QIAN, Q., YU, Y., DEVALARAJA, M. N. & RICHMOND, A. (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol,* 115, 234-44.

DIAZ, G., BATETTA, B., SANNA, F., UDA, S., REALI, C., ANGIUS, F., MELIS, M. & FALCHI, A. M. (2008) Lipid droplet changes in proliferating and quiescent 3T3 fibroblasts. *Histochem Cell Biol,* 129, 611-21.

DING, Y., WANG, G., LING, M. T., WONG, Y. C., LI, X., NA, Y., ZHANG, X., CHUA, C. W., WANG, X. & XIN, D. (2006) Significance of Id-1 up-regulation and its association with EGFR in bladder cancer cell invasion. *Int J Oncol,* 28, 847-54.

DIPIETRO, L. A., BURDICK, M., LOW, Q. E., KUNKEL, S. L. & STRIETER, R. M. (1998) MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. *J Clin Invest,* 101, 1693-8.

DOVI, J. V., HE, L.-K. & DIPIETRO, L. A. (2003) Accelerated wound closure in neutrophil-depleted mice. *J Leukoc Biol,* 73, 448-455.

DREW, A. F., LIU, H., DAVIDSON, J. M., DAUGHERTY, C. C. & DEGEN, J. L. (2001) Wound-healing defects in mice lacking fibrinogen. *Blood,* 97, 3691-3698.

DU CROS, D. L., LEBARON, R. G. & COUCHMAN, J. R. (1995) Association of versican with dermal matrices and its potential role in hair follicle development and cycling. *J Invest Dermatol,* 105, 426-31.

DU, X., LIU, Q., YANG, Z., ORAZI, A., RESCORLA, F. J., GROSFELD, J. L. & WILLIAMS, D. A. (1997) Protective effects of interleukin-11 in a murine model of ischemic bowel necrosis. *Am J Physiol Gastrointest Liver Physiol,* 272, G545-552.

DU, X. X., DOERSCHUK, C. M., ORAZI, A. & WILLIAMS, D. A. (1994) A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy. *Blood,* 83, 33-37.

ECKERT, R. L. (1989) Structure, function, and differentiation of the keratinocyte. *Physiol. Rev.,* 69, 1316-1346.

ECKERT, R. L., CRISH, J. F. & ROBINSON, N. A. (1997) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol. Rev.,* 77, 397-424.

ENGELHARDT, E., TOKSOY, A., GOEBELER, M., DEBUS, S., BROCKER, E.-B. & GILLITZER, R. (1998) Chemokines IL-8, GRO{alpha}, MCP-1, IP-10, and Mig Are Sequentially and Differentially Expressed During Phase-Specific Infiltration of Leukocyte Subsets in Human Wound Healing. *Am J Pathol,* 153, 1849-1860.

ENGERMAN, R. L. & KRAMER, J. W. (1982) Dogs with induced or spontaneous diabetes as models for the study of human diabetes mellitus. *Diabetes,* 31, 26-9.

FALANGA, V. (1993) Chronic Wounds: Pathophysiologic and Experimental Considerations. *J Investig Dermatol,* 100, 721-725.

FALANGA, V. & EAGLSTEIN, W. H. (1993) The "trap" hypothesis of venous ulceration. *Lancet,* 341, 1006-8.

FARRELL, D. H. & AL-MONDHIRY, H. A. (1997) Human Fibroblast Adhesion to Fibrinogen. *Biochemistry,* 36, 1123-1128.

FARRER-BROWN, G., LUCAS, R. B. & WINSTOCK, D. (1972) Familial gingival fibromatosis: an unusual pathology. *J Oral Pathol,* 1, 76-83.

FARWELL, D. G., SHERA, K. A., KOOP, J. I., BONNET, G. A., MATTHEWS, C. P., REUTHER, G. W., COLTRERA, M. D., MCDOUGALL, J. K. & KLINGELHUTZ, A. J. (2000) Genetic and epigenetic changes in human epithelial cells immortalized by telomerase. *Am J Pathol,* 156, 1537-47.

FINI, M. E., GIRARD, M. T. & MATSUBARA, M. (1992) Collagenolytic/gelatinolytic enzymes in corneal wound healing. *Acta Ophthalmol Suppl,* 26-33.

FINN, S. P., SMYTH, P., CAHILL, S., STRECK, C., O'REGAN, E. M., FLAVIN, R., SHERLOCK, J., HOWELLS, D., HENFREY, R., CULLEN, M., TONER, M., TIMON, C., O'LEARY, J. J. & SHEILS, O. M. (2007) Expression microarray analysis of papillary thyroid carcinoma and benign thyroid tissue: emphasis on the follicular variant and potential markers of malignancy. *Virchows Arch,* 450, 249-60.

FIVENSON, D. P., FARIA, D. T., NICKOLOFF, B. J., POVERINI, P. J., KUNKEL, S., BURDICK, M. & STRIETER, R. M. (1997) Chemokine and inflammatory cytokine changes during chronic wound healing. *Wound Repair Regen,* 5, 310-22.

FLEISCHMAJER, R., FISHER, L. W., MACDONALD, E. D., JACOBS, L., JR., PERLISH, J. S. & TERMINE, J. D. (1991) Decorin interacts with fibrillar collagen of embryonic and adult human skin. *J Struct Biol,* 106, 82-90.

FLORIN, L., MAAS-SZABOWSKI, N., WERNER, S., SZABOWSKI, A. & ANGEL, P. (2005) Increased keratinocyte proliferation by JUN-dependent expression of PTN and SDF-1 in fibroblasts. *J Cell Sci,* 118, 1981-1989.

FOWLER, A. V., ZABIN, I. & WITH THE TECHNICAL ASSISTANCE OF KARIN, P. (1970) The Amino Acid Sequence of {beta}-Galactosidase. I. ISOLATION AND COMPOSITION OF TRYPTIC PEPTIDES. *J. Biol. Chem.,* 245, 5032-5041.

FRANK, S., STALLMEYER, B., KAMPFER, H., KOLB, N. & PFEILSCHIFTER, J. (2000) Leptin enhances wound re-epithelialization and constitutes a direct function of leptin in skin repair. *J Clin Invest,* 106, 501-9.

GAILIT, J., CLARKE, C., NEWMAN, D., TONNESEN, M. G., MOSESSON, M. W. & CLARK, R. A. F. (1997) Human Fibroblasts Bind Directly to Fibrinogen at RGD Sites through Integrin [alpha]v[beta]3. *Experimental Cell Research,* 232, 118-126.

GENDLOFF, E. H., BOWEN, B. & BUCHHOLZ, W. G. (1990) Quantitation of chloramphenicol acetyl transferase in transgenic tobacco plants by ELISA and correlation with gene copy number. *Plant Mol Biol,* 14, 575-83.

GENEVER, P. G., WOOD, E. J. & CUNLIFFE, W. J. (1993) The wounded dermal equivalent offers a simplified model for studying wound repair in vitro. *Experimental Dermatology,* 2, 266-273.

GEY, G. O., COFFMAN, W. D. & KUBICEK, M. T. (1952) Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.,* 12, 264-265.

GILLITZER, R. & GOEBELER, M. (2001) Chemokines in cutaneous wound healing. *J Leukoc Biol,* 69, 513-21.

GINHOUX, F., TACKE, F., ANGELI, V., BOGUNOVIC, M., LOUBEAU, M., DAI, X.-M., STANLEY, E. R., RANDOLPH, G. J. & MERAD, M. (2006) Langerhans cells arise from monocytes in vivo. *Nat Immunol,* 7, 265-273.

GOLDBERG, S. R., QUIRK, G. L., SYKES, V. W., MCKINSTRY, R. P., KORDULA, T. & LANNING, D. A. (2008) Differential use of Erk1/2 and transforming growth factor beta pathways by mid- and late-gestational murine fibroblasts. *J Pediatr Surg,* 43, 971-6.

GORBUNOVA, V., SELUANOV, A. & PEREIRA-SMITH, O. M. (2002) Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J Biol Chem,* 277, 38540-9.

GORBUNOVA, V., SELUANOV, A. & PEREIRA-SMITH, O. M. (2003) Evidence that high telomerase activity may induce a senescent-like growth arrest in human fibroblasts. *Journal of Biological Chemistry,* 278, 7692-8.

GOSS, R. J. & GRIMES, L. N. (1975) Epidermal downgrowths in regenerating rabbit ear holes. *Journal of Morphology,* 146, 533-542.

GOULD, L. J., LEONG, M., SONSTEIN, J. & WILSON, S. (2005) Optimization and validation of an ischemic wound model. *Wound Repair and Regeneration,* 13, 576-582.

GRAHAM, I. D., HARRISON, M. B., NELSON, E. A., LORIMER, K. & FISHER, A. (2003) Prevalence of lower-limb ulceration: a systematic review of prevalence studies. *Adv Skin Wound Care,* 16, 305-16.

GREENBERG, C. S., BIRCKBICHLER, P. J. & RICE, R. H. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.,* 5, 3071-3077.

GREENBERG, S. B., GROVE, G. L. & CRISTOFALO, V. J. (1977) Cell size in aging monolayer cultures. *In Vitro,* 13, 297-300.

GREY, J. E., HARDING, K. G. & ENOCH, S. (2006) Venous and arterial leg ulcers. *Bmj,* 332, 347-50.

GRINNELL, F., HO, C.-H. & WYSOCKI, A. (1992) Degradation of Fibronectin and Vitronectin and Vitronectin in Chronic Wound Fluid: Analysis by Cell Blotting, Immunoblotting, and Cell Adhesion Assays. *J Invest Dermatol,* 98, 410-416.

GRINNELL, F. & ZHU, M. (1996) Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor, and alpha2-macroglobulin. *J Invest Dermatol,* 106, 335-41.

GUNNEL, R. T. & HJELMGREN, J. (2005) Annual costs of treatment for venous leg ulcers in Sweden and the United Kingdom. *Wound Repair and Regeneration,* 13, 13-18.

HAKKINEN, L., WESTERMARCK, J., KAHARI, V. M. & LARJAVA, H. (1996) Human granulation-tissue fibroblasts show enhanced proteoglycan gene expression and altered response to TGF-beta 1. *J Dent Res,* 75, 1767-78.

HALBERSZTADT, A., HALON, A., PAJAK, J., ROBACZYNSKI, J., RABCZYNSKI, J. & ST GABRYS, M. (2006) [The role of matrix metalloproteinases in tumor invasion and metastasis]. *Ginekol Pol,* 77, 63-71.

HAN, Y. P., TUAN, T. L., WU, H., HUGHES, M. & GARNER, W. L. (2001) TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. *J Cell Sci,* 114, 131-139.

HARA, E., YAMAGUCHI, T., NOJIMA, H., IDE, T., CAMPISI, J., OKAYAMA, H. & ODA, K. (1994) Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. *J. Biol. Chem.,* 269, 2139-2145.

HARDMAN, M. J. & ASHCROFT, G. S. (2008) Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly. *Genome Biol,* 9, R80.

HARLEY, C. B. (1991) Telomere loss: mitotic clock or genetic time bomb? *Mutation Research/DNAging,* 256, 271-282.

HARLEY, C. B. & VILLEPONTEAU, B. (1995) Telomeres and telomerase in aging and cancer. *Current Opinion in Genetics & Development,* 5, 249-255.

HARRINGTON, L., ZHOU, W., MCPHAIL, T., OULTON, R., YEUNG, D. S., MAR, V., BASS, M. B. & ROBINSON, M. O. (1997) Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev,* 11, 3109-15.

HASEGAWA, K., YONEDA, M., KUWABARA, H., MIYAISHI, O., ITANO, N., OHNO, A., ZAKO, M. & ISOGAI, Z. (2007) Versican, a major hyaluronan-binding component in the dermis, loses its hyaluronan-binding ability in solar elastosis. *J Invest Dermatol,* 127, 1657-63.

HASTWELL, P. W., CHAI, L.-L., ROBERTS, K. J., WEBSTER, T. W., HARVEY, J. S., REES, R. W. & WALMSLEY, R. M. (2006) High-specificity and high-sensitivity genotoxicity assessment in a human cell line: Validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 607, 160-175.

HAYFLICK, L. & MOORHEAD, P. S. (1961) The serial cultivation of human diploid cell strains. *Experimental Cell Research,* 25, 585-621.

HEBER-KATZ, E. (1999) The regenerating mouse ear. *Seminars in Cell and Developmental Biology,* 10, 415-419.

HEIM, R., CUBITT, A. B. & TSIEN, R. Y. (1995) Improved green fluorescence. *Nature,* 373, 663-664.

HELDIN, C.-H. & WESTERMARK, B. (1999) Mechanism of Action and In Vivo Role of Platelet-Derived Growth Factor. *Physiol. Rev.,* 79, 1283-1316.

HEMBRUFF, S. L., VILLENEUVE, D. J. & PARISSENTI, A. M. (2005) The optimization of quantitative reverse transcription PCR for verification of cDNA microarray data. *Analytical Biochemistry,* 345, 237-249.

HERRICK, S. E., IRELAND, G. W., SIMON, D., MCCOLLUM, C. N. & FERGUSON, M. W. (1996) Venous ulcer fibroblasts compared with normal fibroblasts show differences in collagen but not fibronectin production under both normal and hypoxic conditions. *J Invest Dermatol,* 106, 187-193.

HERRICK, S. E., SLOAN, P., MCGURK, M., FREAK, L., MCCOLLUM, C. N. & FERGUSON, M. W. (1992) Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. *Am J Pathol,* 141, 1085-95.

HINZ, B., CELETTA, G., TOMASEK, J. J., GABBIANI, G. & CHAPONNIER, C. (2001) Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell,* 12, 2730-41.

HINZ, B., DUGINA, V., BALLESTREM, C., WEHRLE-HALLER, B. & CHAPONNIER, C. (2003) Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. *Mol Biol Cell,* 14, 2508-19.

HO, C. H. & BOGIE, K. (2007) The prevention and treatment of pressure ulcers. *Phys Med Rehabil Clin N Am,* 18, 235-53.

HOELLER, D., HUPPERTZ, B., ROOS, T. C., GUTIÉRREZ, P. P., MERK, H. F., FRANK, J. & JUGERT, F. K. (2001) An improved and rapid method to construct skin equivalents from human hair follicles and fibroblasts. *Experimental Dermatology,* 10, 264-271.

HOU, M., XU, D., BJORKHOLM, M. & GRUBER, A. (2001) Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. *Clin Chem,* 47, 519-24.

HOWARTH, F. C., JACOBSON, M., SHAFIULLAH, M. & ADEGHATE, E. (2008) Long-term effects of type 2 diabetes mellitus on heart rhythm in the Goto-Kakizaki rat. *Exp Physiol,* 93, 362-9.

HRUBY, D. E., BRINKLEY, J. M., KANG, H. C., HAUGLAND, R. P., YOUNG, S. L. & MELNER, M. H. (1990) Use of a fluorescent chloramphenicol derivative as a substrate for CAT assays. *Biotechniques,* 8, 170-1.

HUANG, C. L., UENO, M., LIU, D., MASUYA, D., NAKANO, J., YOKOMISE, H., NAKAGAWA, T. & MIYAKE, M. (2006) MRP-1//CD9 gene transduction regulates the actin cytoskeleton through the downregulation of WAVE2. *Oncogene,* 25, 6480-6488.

HUGHES, C. C. (2008) Endothelial-stromal interactions in angiogenesis. *Curr Opin Hematol,* 15, 204-9.

IGGO, A. & MUIR, A. R. (1969) The structure and function of a slowly adapting touch corpuscle in hairy skin. *J Physiol,* 200, 763-96.

IGLESIAS, C. P., BIRKS, Y., NELSON, E. A., SCANLON, E. & CULLUM, N. A. (2005) Quality of life of people with venous leg ulcers: a comparison of the discriminative and responsive characteristics of two generic and a disease specific instruments. *Qual Life Res,* 14, 1705-18.

IKEYAMA, S., KOYAMA, M., YAMAOKO, M., SASADA, R. & MIYAKE, M. (1993) Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. *J Exp Med,* 177, 1231-7.

INOUE, N., NISHIKATA, S., FURUYA, E., TAKITA, H., KAWAMURA, M. & NISHIKAZE, O. (1985) Streptozotocin diabetes: prolonged inflammatory response with delay in granuloma formation. *Int J Tissue React,* 7, 27-33.

ITO, A. & OONUMA, J. (2006) Direct interaction between nerves and mast cells mediated by the SgIGSF/SynCAM adhesion molecule. *J Pharmacol Sci,* 102, 1-5.

ITO, Y. & HABUCHI, O. (2000) Purification and Characterization of N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase from the Squid Cartilage. *J. Biol. Chem.,* 275, 34728-34736.

IYER, V. R., EISEN, M. B., ROSS, D. T., SCHULER, G., MOORE, T., LEE, J. C., NBSP, F, TRENT, J. M., STAUDT, L. M., HUDSON, J., JR., BOGUSKI, M. S., LASHKARI, D., SHALON, D., BOTSTEIN, D. & BROWN, P. O. (1999) The Transcriptional Program in the Response of Human Fibroblasts to Serum. *Science,* 283, 83-87.

JIANG, X.-R., JIMENEZ, G., CHANG, E., FROLKIS, M., KUSLER, B., SAGE, M., BEECHE, M., BODNAR, A. G., WAHL, G. M., TLSTY, T. D. & CHIU, C.-P. (1999) Telomerase expression in human somatic cells does not induce changes associated with a transformed phenotype. *Nat Genet,* 21, 111-114.

JONES, J., RODGERS, J., HEIL, M., MAY, J., WHITE, L., MADDRY, J. A., FLETCHER, T. M., 3RD, SHAW, G. M., HARTMAN, J. L. T. & KUTSCH, O. (2007) High throughput drug screening for human immunodeficiency virus type 1 reactivating compounds. *Assay Drug Dev Technol,* 5, 181-89.

KAMLER, M., LEHR, H. A., BARKER, J. H., SAETZLER, R. K., GALLA, T. J. & MESSMER, K. (1993) Impact of ischemia on tissue oxygenation and wound healing: intravital microscopic studies on the hairless mouse ear model. *Eur Surg Res,* 25, 30-7.

KAMPINGA, H. H., VAN WAARDE-VERHAGEN, M. A., VAN ASSEN-BOLT, A. J., NIEUWENHUIS, B., RODEMANN, H. P., PROWSE, K. R. & LINSKENS, M. H. (2004) Reconstitution of active telomerase in primary human foreskin fibroblasts: effects on proliferative characteristics and response to ionizing radiation. *International Journal of Radiation Biology,* 80, 377-88.

KAO, Y.-R., SHIH, J.-Y., WEN, W.-C., KO, Y.-P., CHEN, B.-M., CHAN, Y.-L., CHU, Y.-W., YANG, P.-C., WU, C.-W. & ROFFLER, S. R. (2003) Tumor-associated Antigen L6 and the Invasion of Human Lung Cancer Cells. *Clin Cancer Res,* 9, 2807-2816.

KIELTY, C. M., SHERRATT, M. J. & SHUTTLEWORTH, C. A. (2002) Elastic fibres. *J Cell Sci,* 115, 2817-2828.

KIELTY, C. M. & SHUTTLEWORTH, C. A. (1997) Microfibrillar elements of the dermal matrix. *Microsc Res Tech,* 38, 413-27.

KIM, D. K. & HOLBROOK, K. A. (2001) The nerve-dependency of Merkel cell proliferation in cultured human fetal glabrous skin. *Yonsei Med J,* 42, 311-5.

KIM, M.-H., LIU, W., BORJESSON, D. L., CURRY, F.-R. E., MILLER, L. S., CHEUNG, A. L., LIU, F.-T., ISSEROFF, R. R. & SIMON, S. I. (2008) Dynamics of Neutrophil Infiltration during Cutaneous Wound Healing and Infection Using Fluorescence Imaging. *J Invest Dermatol,* 128, 1812-1820.

KIM, N. & WU, F. (1997) Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucl. Acids Res.,* 25, 2595-2597.

KINSNER, A., LESIAK-CYGANOWSKA, E. & SLADOWSKI, D. (2001) In vitro reconstruction of full thickness human skin on a composite collagen material. *Cell Tissue Bank,* 2, 165-71.

KIPLING, D. (2001) Telomeres, replicative senescence and human ageing. *Maturitas,* 38, 25-37.

KITANO, A., SAIKA, S., YAMANAKA, O., IKEDA, K., OKADA, Y., SHIRAI, K. & REINACH, P. S. (2007) Emodin suppression of ocular surface inflammatory reaction. *Invest Ophthalmol Vis Sci,* 48, 5013-22.

KLOETERS, O., JIA, S.-X., ROY, N., SCHULTZ, G. S., LEINFELLNER, G. & MUSTOE, T. A. (2007) Alteration of Smad3 signaling in ischemic rabbit dermal ulcer wounds. *Wound Repair and Regeneration,* 15, 341-349.

KNIGHT, A. W., BILLINTON, N., CAHILL, P. A., SCOTT, A., HARVEY, J. S., ROBERTS, K. J., TWEATS, D. J., KEENAN, P. O. & WALMSLEY, R. M. (2007) An analysis of results from 305 compounds tested with the yeast RAD54-GFP genotoxicity assay (GreenScreen GC) including relative predictivity of regulatory tests and rodent carcinogenesis and performance with autofluorescent and coloured compounds. *Mutagenesis,* 22, 409-416.

KOLE, T. P., TSENG, Y., JIANG, I., KATZ, J. L. & WIRTZ, D. (2005) Intracellular mechanics of migrating fibroblasts. *Mol Biol Cell,* 16, 328-38.

KUBO, M., VAN DE WATER, L., PLANTEFABER, L. C., MOSESSON, M. W., SIMON, M., TONNESEN, M. G., TAICHMAN, L. & CLARK, R. A. F. (2001) Fibrinogen and Fibrin are Anti-Adhesive for Keratinocytes: A Mechanism for Fibrin Eschar Slough During Wound Repair. 117, 1369-1381.

KURAMOCHI, M., FUKUHARA, H., NOBUKUNI, T., KANBE, T., MARUYAMA, T., GHOSH, H. P., PLETCHER, M., ISOMURA, M., ONIZUKA, M., KITAMURA, T., SEKIYA, T., REEVES, R. H. & MURAKAMI, Y. (2001) TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. *Nat Genet,* 27, 427-430.

KWON, A. H., QIU, Z., HASHIMOTO, M., YAMAMOTO, K. & KIMURA, T. (2008) Effects of medicinal mushroom (Sparassis crispa) on wound healing in streptozotocin-induced diabetic rats. *Am J Surg.*

LAL, B., SAITO, S., PAPPAS, P. J., PADBERG, F. T., CERVEIRA, J. J., HOBSON, R. W. & DURÃ¡N, W. N. (2003) Altered proliferative responses of dermal fibroblasts to TGF-Î²1 may contribute to chronic venous stasis ulcer1 1 Competition of interest: none. *Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter,* 37, 1285-1293.

LANGMANN, T., LIEBISCH, G., MOEHLE, C., SCHIFFERER, R., DAYOUB, R., HEIDUCZEK, S., GRANDL, M., DADA, A. & SCHMITZ, G. (2005) Gene expression profiling identifies retinoids as potent inducers of macrophage lipid efflux. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1740, 155-161.

LARJAVA, H., SALO, T., HAAPASALMI, K., KRAMER, R. H. & HEINO, J. (1993) Expression of integrins and basement membrane components by wound keratinocytes. *J Clin Invest,* 92, 1425-35.

LAU, T. W., SAHOTA, D. S., LAU, C. H., CHAN, C. M., LAM, F. C., HO, Y. Y., FUNG, K. P., LAU, C. B. & LEUNG, P. C. (2008) An in vivo investigation on the wound-healing effect of two medicinal herbs using an animal model with foot ulcer. *Eur Surg Res,* 41, 15-23.

LAUER, G., SOLLBERG, S., COLE, M., FLAMME, I., STURZEBECHER, J., MANN, K., KRIEG, T. & EMING, S. A. (2000) Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. *J Invest Dermatol,* 115, 12-8.

LAURENS, N., KOOLWIJK, P. & DE MAAT, M. P. M. (2006) Fibrin structure and wound healing. *Journal of Thrombosis and Haemostasis,* 4, 932-939.

LAURENT K. VERKOCZY, P. A. M. N. L. B. (1998) hBRAG, a novel B cell lineage cDNA encoding a type II transmembrane glycoprotein potentially involved in the regulation of recombination activating gene 1 (RAG1). *European Journal of Immunology,* 28, 2839-2853.

LAVKER, R. M., ZHENG, P. S. & DONG, G. (1986) Morphology of aged skin. *Dermatol Clin,* 4, 379-89.

LEIBOVICH, S. J. & ROSS, R. (1975) The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol,* 78, 71-100.

LEIVO, T., KIISTALA, U., VESTERINEN, M., OWARIBE, K., BURGESON, R. E., VIRTANEN, I. & OIKARINEN, A. (2000) Re-epithelialization rate and protein expression in the suction-induced wound model: comparison between intact blisters, open wounds and calcipotriol-pretreated open wounds. *Br J Dermatol,* 142, 991-1002.

LERMAN, O. Z., GALIANO, R. D., ARMOUR, M., LEVINE, J. P. & GURTNER, G. C. (2003) Cellular Dysfunction in the Diabetic Fibroblast: Impairment in Migration, Vascular Endothelial Growth Factor Production, and Response to Hypoxia. *Am J Pathol,* 162, 303-312.

LEVENSON, S. M., GEEVER, E. F., CROWLEY, L. V., OATES, J. F., 3RD, BERARD, C. W. & ROSEN, H. (1965) The Healing of Rat Skin Wounds. *Ann Surg,* 161, 293-308.

LEVY, J. J., VON ROSEN, J., GASSMULLER, J., KLEINE KUHLMANN, R. & LANGE, L. (1995) Validation of an in vivo wound healing model for the quantification of pharmacological effects on epidermal regeneration. *Dermatology,* 190, 136-41.

LI, C. K. F., PENDER, S. L. F., PICKARD, K. M., CHANCE, V., HOLLOWAY, J. A., HUETT, A., GONCALVES, N. S., MUDGETT, J. S., DOUGAN, G., FRANKEL, G. & MACDONALD, T. T. (2004) Impaired Immunity to Intestinal Bacterial Infection in Stromelysin-1 (Matrix Metalloproteinase-3)-Deficient Mice. *J Immunol,* 173, 5171-5179.

LI, Q., PARK, P. W., WILSON, C. L. & PARKS, W. C. (2002) Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell,* 111, 635-46.

LI, X., SHEN, F., ZHANG, Y., ZHU, J., HUANG, L. & SHI, Q. (2007) Functional characterization of cell lines for high-throughput screening of human neuromedin U receptor subtype 2 specific agonists using a luciferase reporter gene assay. *European Journal of Pharmaceutics and Biopharmaceutics,* 67, 284-292.

LI, Y. Q., DOYLE, J. W., ROTH, T. P., DUNN, R. M. & LAWRENCE, W. T. (1998) IL-10 and GM-CSF expression and the presence of antigen-presenting cells in chronic venous ulcers. *J Surg Res,* 79, 128-35.

LIANG, C. C., PARK, A. Y. & GUAN, J. L. (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc,* 2, 329-33.

LINDVALL, C., HOU, M., KOMURASAKI, T., ZHENG, C., HENRIKSSON, M., SEDIVY, J. M., BJORKHOLM, M., TEH, B. T., NORDENSKJOLD, M. & XU, D. (2003) Molecular characterization of human telomerase reverse transcriptase-immortalized human fibroblasts by gene expression profiling: activation of the epiregulin gene. *Cancer Res,* 63, 1743-7.

LOCHNER, K., GAEMLICH, A., SUDEL, K. M., VENZKE, K., MOLL, I., KNOTT, A., STAB, F., WENCK, H., DORING, O., BOTTGER, M. & GALLINAT, S. (2007) Expression of decorin and collagens I and III in different layers of human skin in vivo: a laser capture microdissection study. *Biogerontology,* 8, 269-82.

LOCKHART, D. J., DONG, H., BYRNE, M. C., FOLLETTIE, M. T., GALLO, M. V., CHEE, M. S., MITTMANN, M., WANG, C., KOBAYASHI, M., NORTON, H. & BROWN, E. L. (1996) Expression monitoring by

hybridization to high-density oligonucleotide arrays. *Nat Biotech,* 14, 1675-1680.

LOOTS, M. A., LAMME, E. N., ZEEGELAAR, J., MEKKES, J. R., BOS, J. D. & MIDDELKOOP, E. (1998) Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol,* 111, 850-857.

LOOTS, M. A. M., LAMME, E. N., MEKKES, J. R., BOS, J. D. & MIDDELKOOP, E. (1999) Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. *Archives of Dermatological Research,* 291, 93-99.

LORYMAN, C. & MANSBRIDGE, J. (2008) Inhibition of keratinocyte migration by lipopolysaccharide. *Wound Repair Regen,* 16, 45-51.

LOWE, B., AVILA, H. A., BLOOM, F. R., GLEESON, M. & KUSSER, W. (2003) Quantitation of gene expression in neural precursors by reverse-transcription polymerase chain reaction using self-quenched, fluorogenic primers. *Analytical Biochemistry,* 315, 95-105.

LUO, Y., KIM, R., GABUZDA, D., MI, S., COLLINS-RACIE, L. A., LU, Z., JACOBS, K. A. & DORF, M. E. (1998) The CXC-chemokine, H174: expression in the central nervous system. *J Neurovirol,* 4, 575-85.

MAAS-SZABOWSKI, N., STARK, H. J. & FUSENIG, N. E. (2000) Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. *J Invest Dermatol,* 114, 1075-84.

MACGREGOR, G. R., MOGG, A. E., BURKE, J. F. & CASKEY, C. T. (1987) Histochemical staining of clonal mammalian cell lines expressing E. coli beta galactosidase indicates heterogeneous expression of the bacterial gene. *Somat Cell Mol Genet,* 13, 253-65.

MACKENZIE, K. L., FRANCO, S., MAY, C., SADELAIN, M. & MOORE, M. A. (2000) Mass cultured human fibroblasts overexpressing hTERT encounter a growth crisis following an extended period of proliferation. *Exp Cell Res,* 259, 336-50.

MADLENER, M. (1998) Differential expression of matrix metalloproteinases and their physiological inhibitors in acute murine skin wounds. *Arch Dermatol Res,* 290 Suppl, S24-9.

MAECKER, H. T., TODD, S. C. & LEVY, S. (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J.,* 11, 428-442.

MARCHLER-BAUER, A., ANDERSON, J. B., DERBYSHIRE, M. K., DEWEESE-SCOTT, C., GONZALES, N. R., GWADZ, M., HAO, L., HE, S., HURWITZ, D. I., JACKSON, J. D., KE, Z., KRYLOV, D., LANCZYCKI, C. J., LIEBERT, C. A., LIU, C., LU, F., LU, S., MARCHLER, G. H., MULLOKANDOV, M., SONG, J. S., THANKI, N., YAMASHITA, R. A., YIN, J. J., ZHANG, D. & BRYANT, S. H. (2007) CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res,* 35, D237-40.

MARGOLIS, D. J., BILKER, W., SANTANNA, J. & BAUMGARTEN, M. (2002) Venous leg ulcer: incidence and prevalence in the elderly. *J Am Acad Dermatol,* 46, 381-6.

MARKEN, J. S., SCHIEVEN, G. L., HELLSTROM, I., HELLSTROM, K. E. & ARUFFO, A. (1992) Cloning and Expression of the Tumor-Associated Antigen L6. *Proceedings of the National Academy of Sciences,* 89, 3503-3507.

MARTIN, P. (1997) Wound Healing--Aiming for Perfect Skin Regeneration. *Science,* 276, 75-81.

MARTIN, P. & LEIBOVICH, S. J. (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol,* 15, 599-607.

MASTERS, J. R. (2002) HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer,* 2, 315-319.

MASUDA, M., KIKUCHI, S., MARUYAMA, T., SAKURAI-YAGETA, M., WILLIAMS, Y. N., GHOSH, H. P. & MURAKAMI, Y. (2005) Tumor Suppressor in Lung Cancer (TSLC)1 Suppresses Epithelial Cell Scattering and Tubulogenesis. *J. Biol. Chem.,* 280, 42164-42171.

MATZ, M. V., FRADKOV, A. F., LABAS, Y. A., SAVITSKY, A. P., ZARAISKY, A. G., MARKELOV, M. L. & LUKYANOV, S. A. (1999) Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat Biotech,* 17, 969-973.

MAY, R. C., HALL, M. E., HIGGS, H. N., POLLARD, T. D., CHAKRABORTY, T., WEHLAND, J., MACHESKY, L. M. & SECHI, A. S. (1999) The Arp2/3 complex is essential for the actin-based motility of Listeria monocytogenes. *Curr Biol,* 9, 759-62.

MCCLAIN, S. A., SIMON, M., JONES, E., NANDI, A., GAILIT, J. O., TONNESEN, M. G., NEWMAN, D. & CLARK, R. A. (1996) Mesenchymal cell activation is the rate-limiting step of granulation tissue induction. *Am J Pathol,* 149, 1257-70.

MCMILLAN, J. R., AKIYAMA, M. & SHIMIZU, H. (2003) Epidermal basement membrane zone components: ultrastructural distribution and molecular interactions. *J Dermatol Sci,* 31, 169-77.

MEKKES, J. R., LOOTS, M. A., VAN DER WAL, A. C. & BOS, J. D. (2003) Causes, investigation and treatment of leg ulceration. *Br J Dermatol,* 148, 388-401.

MENDEZ, M., STANLEY, A., PARK, H.-Y., SHON, K., PHILLIPS, T. & MENZOIAN, J. (1998a) Fibroblasts cultured from venous ulcers display cellular characteristics of senescence. *Journal of Vascular Surgery,* 28, 876-883.

MENDEZ, M. V., STANLEY, A., PARK, H. Y., SHON, K., PHILLIPS, T. & MENZOIAN, J. O. (1998b) Fibroblasts cultured from venous ulcers display cellular characteristics of senescence. *J Vasc Surg,* 28, 876-883.

MENDEZ, M. V., STANLEY, A., PARK, H. Y., SHON, K., PHILLIPS, T. & MENZOIAN, J. O. (1998c) Fibroblasts cultured from venous ulcers display cellular characteristics of senescence. *J Vasc Surg,* 28, 876-83.

MENKE, N. B., WARD, K. R., WITTEN, T. M., BONCHEV, D. G. & DIEGELMANN, R. F. (2007) Impaired wound healing. *Clinics in Dermatology,* 25, 19-25.

MERAN, S., THOMAS, D., STEPHENS, P., MARTIN, J., BOWEN, T., PHILLIPS, A. & STEADMAN, R. (2007) Involvement of hyaluronan in regulation of fibroblast phenotype. *J Biol Chem,* 282, 25687-97.

MERAN, S., THOMAS, D. W., STEPHENS, P., ENOCH, S., MARTIN, J., STEADMAN, R. & PHILLIPS, A. O. (2008) Hyaluronan facilitates transforming growth factor-beta1-mediated fibroblast proliferation. *J Biol Chem,* 283, 6530-45.

METCALFE, D. D., BARAM, D. & MEKORI, Y. A. (1997) Mast cells. *Physiol Rev,* 77, 1033-79.

MEYERSON, M., COUNTER, C. M., EATON, E. N., ELLISEN, L. W., STEINER, P., CADDLE, S. D., ZIAUGRA, L., BEIJERSBERGEN, R. L., DAVIDOFF, M. J., LIU, Q., BACCHETTI, S., HABER, D. A. & WEINBERG, R. A. (1997) hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell,* 90, 785-95.

MICHAELS, J., CHURGIN, S. S., BLECHMAN, K. M., GREIVES, M. R., AARABI, S., GALIANO, R. D. & GURTNER, G. C. (2007) db/db mice exhibit severe wound-healing impairments compared with other murine diabetic strains in a silicone-splinted excisional wound model. *Wound Repair Regen,* 15, 665-70.

MILLIS, A. J., MCCUE, H. M., KUMAR, S. & BAGLIONI, C. (1992) Metalloproteinase and TIMP-1 gene expression during replicative senescence. *Exp Gerontol,* 27, 425-8.

MILYAVSKY, M., SHATS, I., EREZ, N., TANG, X., SENDEROVICH, S., MEERSON, A., TABACH, Y., GOLDFINGER, N., GINSBERG, D., HARRIS, C. C. & ROTTER, V. (2003a) Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype. *Cancer Research,* 63, 7147-57.

MILYAVSKY, M., SHATS, I., EREZ, N., TANG, X., SENDEROVICH, S., MEERSON, A., TABACH, Y., GOLDFINGER, N., GINSBERG, D., HARRIS, C. C. & ROTTER, V. (2003b) Prolonged culture of telomerase-immortalized human fibroblasts leads to a premalignant phenotype. *Cancer Res,* 63, 7147-57.

MIMMACK, M. L., BROOKING, J. & BAHN, S. (2004) Quantitative polymerase chain reaction: validation of microarray results from postmortem brain studies. *Biological Psychiatry,* 55, 337-345.

MIRASTSCHIJSKI, U., HAAKSMA, C. J., TOMASEK, J. J. & AGREN, M. S. (2004) Matrix metalloproteinase inhibitor GM 6001 attenuates keratinocyte migration, contraction and myofibroblast formation in skin wounds. *Exp Cell Res,* 299, 465-75.

MIYAKE, M., NAKANO, K., IEKI, Y., ADACHI, M., HUANG, C. L., ITOI, S., KOH, T. & TAKI, T. (1995) Motility related protein 1 (MRP-1/CD9) expression: inverse correlation with metastases in breast cancer. *Cancer Res,* 55, 4127-31.

MOFFATT, C. J., FRANKS, P. J., DOHERTY, D. C., MARTIN, R., BLEWETT, R. & ROSS, F. (2004) Prevalence of leg ulceration in a London population. *Qjm,* 97, 431-7.

MOGENSEN, J., KLAUSEN, I. C., PEDERSEN, A. K., EGEBLAD, H., BROSS, P., KRUSE, T. A., GREGERSEN, N., HANSEN, P. S., BAANDRUP, U. & BORGLUM, A. D. (1999) Alpha-cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy. *J Clin Invest,* 103, R39-43.

MOGFORD, J. E., LIU, W. R., REID, R., CHIU, C., SAID, H., CHEN, S., HARLEY, C. B. & MUSTOE, T. A. (2006) Adenoviral human telomerase reverse transcriptase dramatically improves ischemic wound healing without detrimental immune response in an aged rabbit model. *Hum Gene Ther,* 17, 651-60.

MOGFORD, J. E., SISCO, M., BONOMO, S. R., ROBINSON, A. M. & MUSTOE, T. A. (2004) Impact of aging on gene expression in a rat model of ischemic cutaneous wound healing1. *Journal of Surgical Research,* 118, 190-196.

MONDELLO, C., CHIESA, M., REBUZZINI, P., ZONGARO, S., VERRI, A., COLOMBO, T., GIULOTTO, E., D'INCALCI, M., FRANCESCHI, C. & NUZZO, F. (2003) Karyotype instability and anchorage-independent growth in telomerase-immortalized fibroblasts from two centenarian individuals. *Biochemical and Biophysical Research Communications,* 308, 914-921.

MONDELLO, C., MORALLI, D., FRANCESCHI, C. & NUZZO, F. (1999) Occurrence and expansion of trisomy 7 in a fibroblast strain from a centenarian individual[small star, filled]. *Experimental Gerontology,* 34, 715-719.

MONTES, G. S. (1996) STRUCTURAL BIOLOGY OF THE FIBRES OF THE COLLAGENOUS AND ELASTIC SYSTEMS. *Cell Biology International,* 20, 15-27.

MORALES, C. P., HOLT, S. E., OUELLETTE, M., KAUR, K. J., YAN, Y., WILSON, K. S., WHITE, M. A., WRIGHT, W. E. & SHAY, J. W. (1999) Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet,* 21, 115-8.

MORIKAWA, T., TOYAMA, T., KUDO, N. & KAWASHIMA, Y. (2007) Reducing effect of matrix metalloproteinase inhibitors on serum triacylglycerol in streptozotocin-induced diabetic rats and Zucker fa/fa rats. *Biol Pharm Bull,* 30, 1461-7.

MUKHOPADHYAY, A., TAN, E. K., KHOO, Y. T., CHAN, S. Y., LIM, I. J. & PHAN, T. T. (2005) Conditioned medium from keloid keratinocyte/keloid fibroblast coculture induces contraction of fibroblast-populated collagen lattices. *Br J Dermatol,* 152, 639-45.

MUSTOE, T. (2004) Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy. *The American Journal of Surgery,* 187, S65-S70.

NAKAMURA, T. M., MORIN, G. B., CHAPMAN, K. B., WEINRICH, S. L., ANDREWS, W. H., LINGNER, J., HARLEY, C. B. & CECH, T. R. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science,* 277, 955-9.

NANCHAHAL, J. & RICHES, D. J. (1982) The healing of suction blisters in pig skin. *J Cutan Pathol,* 9, 303-15.

NANNEY, L. B., MUELLER, S. G., BUENO, R., PEIPER, S. C. & RICHMOND, A. (1995) Distributions of melanoma growth stimulatory activity of growth-regulated gene and the interleukin-8 receptor B in human wound repair. *Am J Pathol,* 147, 1248-1260.

NAZARENKO, I., LOWE, B., DARFLER, M., IKONOMI, P., SCHUSTER, D. & RASHTCHIAN, A. (2002a) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res,* 30, e37.

NAZARENKO, I., PIRES, R., LOWE, B., OBAIDY, M. & RASHTCHIAN, A. (2002b) Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Res,* 30, 2089-195.

NICKOLOFF, B. J., CHATURVEDI, V., BACON, P., QIN, J.-Z., DENNING, M. F. & DIAZ, M. O. (2000) Id-1 Delays Senescence but Does Not Immortalize Keratinocytes. *J. Biol. Chem.,* 275, 27501-27504.

NIEUWENHUIS, E. E., MATSUMOTO, T., EXLEY, M., SCHLEIPMAN, R. A., GLICKMAN, J., BAILEY, D. T., CORAZZA, N., COLGAN, S. P., ONDERDONK, A. B. & BLUMBERG, R. S. (2002) CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med,* 8, 588-93.

NOBLE, J. R., ZHONG, Z. H., NEUMANN, A. A., MELKI, J. R., CLARK, S. J. & REDDEL, R. R. (2004) Alterations in the p16(INK4a) and p53 tumor

suppressor genes of hTERT-immortalized human fibroblasts. *Oncogene,* 23, 3116-21.

NOLAN, G. P., FIERING, S., NICOLAS, J.-F. & HERZENBERG, L. A. (1988) Fluorescence-Activated Cell Analysis and Sorting of Viable Mammalian Cells Based on {beta} -D-galactosidase Activity after Transduction of Escherichia coli lacZ. *Proceedings of the National Academy of Sciences,* 85, 2603-2607.

NORDGREN, J., BUCARDO, F., DIENUS, O., SVENSSON, L. & LINDGREN, P. E. (2007) Novel LUXTM Real-time PCR Assays for Detection and Quantification of Genogroups I and II Noroviruses in Clinical Specimens. *J Clin Microbiol.*

NORTON, J. D. (2000) ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci,* 113, 3897-3905.

NWOMEH, B. C., LIANG, H. X., COHEN, I. K. & YAGER, D. R. (1999) MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res,* 81, 189-95.

O'GORMAN, D. B., WU, Y., SENEY, S., ZHU, R. D. & GAN, B. S. (2006) Wnt expression is not correlated with beta-catenin dysregulation in Dupuytren's Disease. *J Negat Results Biomed,* 5, 13.

O'KANE, S. & FERGUSON, M. W. J. (1997) Transforming growth factor [beta]s and wound healing. *The International Journal of Biochemistry & Cell Biology,* 29, 63-78.

O'LEARY, R., ARROWSMITH, M. & WOOD, E. J. (2002) Characterization of the living skin equivalent as a model of cutaneous re-epithelialization. *Cell Biochem Funct,* 20, 129-41.

OHTAKE, S., ITO, Y., FUKUTA, M. & HABUCHI, O. (2001) Human N-Acetylgalactosamine 4-Sulfate 6-O-Sulfotransferase cDNA Is Related to Human B Cell Recombination Activating Gene-associated Gene. *J. Biol. Chem.,* 276, 43894-43900.

ONGENAE, K. C., PHILLIPS, T. J. & PARK, H. Y. (2000) Level of fibronectin mRNA is markedly increased in human chronic wounds. *Dermatol Surg,* 26, 447-51.

OUELLETTE, M. M., MCDANIEL, L. D., WRIGHT, W. E., SHAY, J. W. & SCHULTZ, R. A. (2000) The establishment of telomerase-immortalized cell

lines representing human chromosome instability syndromes. *Hum. Mol. Genet.*, 9, 403-411.

PALFREYMAN, S., MICHAELS, J. & BRAZIER, J. (2007) Development of a tool to examine the effect of venous ulcers on patients' quality of life. *Nurs Stand,* 21, 57-8, 60, 62 passim.

PATSY K. WILLIAMS-BOYCE, J. C. D., JR. (1980) Regeneration of rabbit ear tissue. *Journal of Experimental Zoology,* 212, 243-253.

PAUL EHRLICH, H., SUN, B., KAINTH, K. S. & KROMAH, F. (2006a) Elucidating the mechanism of wound contraction: Rapid versus sustained myosin ATPase activity in attached-delayed-released compared with free-floating fibroblast-populated collagen lattices. *Wound Repair and Regeneration,* 14, 625-632.

PAUL EHRLICH, H., SUN, B., KAINTH, K. S. & KROMAH, F. (2006b) Elucidating the mechanism of wound contraction: rapid versus sustained myosin ATPase activity in attached-delayed-released compared with free-floating fibroblast-populated collagen lattices. *Wound Repair Regen,* 14, 625-32.

PAUL, S. R., BENNETT, F., CALVETTI, J. A., KELLEHER, K., WOOD, C. R., O'HARA, R. M., JR., CLEARY, A. C., SIBLEY, B., CLARK, S. C., WILLIAMS, D. A. & YANG, Y. (1990) Molecular Cloning of a cDNA Encoding Interleukin 11, a Stromal Cell-Derived Lymphopoietic and Hematopoietic Cytokine. *Proceedings of the National Academy of Sciences,* 87, 7512-7516.

PESCHEN, M., LAHAYE, T., HENNIG, B., WEYL, A., SIMON, J. C. & VANSCHEIDT, W. (1999) Expression of the adhesion molecules ICAM-1, VCAM-1, LFA-1 and VLA-4 in the skin is modulated in progressing stages of chronic venous insufficiency. *Acta Derm Venereol,* 79, 27-32.

PIRZIO, L. M., FREULET-MARRIERE, M. A., BAI, Y., FOULADI, B., MURNANE, J. P., SABATIER, L. & DESMAZE, C. (2004) Human fibroblasts expressing hTERT show remarkable karyotype stability even after exposure to ionizing radiation. *Cytogenet Genome Res,* 104, 87-94.

PODOLNIKOVA, N. P., YAKUBENKO, V. P., VOLKOV, G. L., PLOW, E. F. & UGAROVA, T. P. (2003) Identification of a Novel Binding Site for Platelet Integrins {alpha}IIb{beta}3 (GPIIbIIIa) and {alpha}5{beta}1 in the {gamma}C-domain of Fibrinogen. *J. Biol. Chem.*, 278, 32251-32258.

POUJADE, M., GRASLAND-MONGRAIN, E., HERTZOG, A., JOUANNEAU, J., CHAVRIER, P., LADOUX, B., BUGUIN, A. & SILBERZAN, P. (2007) Collective migration of an epithelial monolayer in response to a model wound. *Proc Natl Acad Sci U S A,* 104, 15988-93.

PRASHER, D. C., ECKENRODE, V. K., WARD, W. W., PRENDERGAST, F. G. & CORMIER, M. J. (1992) Primary structure of the Aequorea victoria green-fluorescent protein. *Gene,* 111, 229-233.

PRENDERGAST, F. G. & MANN, K. G. (1978) Chemical and physical properties of aequorin and the green fluorescent protein isolated from Aequorea forskalea. *Biochemistry,* 17, 3448-3453.

PRUSSIN, C. & METCALFE, D. D. (2003) 4. IgE, mast cells, basophils, and eosinophils. *Journal of Allergy and Clinical Immunology,* 111, 486-494.

PUSZTAI, L. (2006) Chips to Bedside: Incorporation of Microarray Data into Clinical Practice. *Clin Cancer Res,* 12, 7209-7214.

QUIRINIA, A., JENSEN, F. T. & VIIDIK, A. (1992) Ischemia in wound healing. I: Design of a flap model--changes in blood flow. *Scand J Plast Reconstr Surg Hand Surg,* 26, 21-8.

QUIRINIA, A. & VIIDIK, A. (1992) Ischemia in wound healing. II: Design of a flap model--biomechanical properties. *Scand J Plast Reconstr Surg Hand Surg,* 26, 133-9.

RABELO, S. B., VILLAVERDE, A. B., NICOLAU, R., SALGADO, M. C., MELO MDA, S. & PACHECO, M. T. (2006) Comparison between wound healing in induced diabetic and nondiabetic rats after low-level laser therapy. *Photomed Laser Surg,* 24, 474-9.

RACINE-SAMSON, L., ROCKEY, D. C. & BISSELL, D. M. (1997) The Role of alpha 1beta 1 Integrin in Wound Contraction. A QUANTITATIVE ANALYSIS OF LIVER MYOFIBROBLASTS IN VIVO AND IN PRIMARY CULTURE. *J. Biol. Chem.,* 272, 30911-30917.

RAFFETTO, J. D., MENDEZ, M. V., MARIEN, B. J., BYERS, H. R., PHILLIPS, T. J., PARK, H.-Y. & MENZOIAN, J. O. (2001a) Changes in cellular motility and cytoskeletal actin in fibroblasts from patients with chronic venous insufficiency and in neonatal fibroblasts in the presence of chronic wound fluid. *Journal of Vascular Surgery,* 33, 1233-1241.

RAFFETTO, J. D., MENDEZ, M. V., MARIEN, B. J., BYERS, H. R., PHILLIPS, T. J. & PARK, H. Y. (2001b) Changes in cellular motility and cytoskeletal actin in fibroblasts from patients with chronic venous insufficiency and in neonatal fibroblasts in the presence of chronic wound fluid. *J Vasc Surg,* 33, 1233-1241.

RAFFETTO, J. D., MENDEZ, M. V., MARIEN, B. J., BYERS, H. R., PHILLIPS, T. J., PARK, H. Y. & MENZOIAN, J. O. (2001c) Changes in cellular motility and cytoskeletal actin in fibroblasts from patients with chronic venous insufficiency and in neonatal fibroblasts in the presence of chronic wound fluid. *J Vasc Surg,* 33, 1233-41.

RAFFETTO, J. D., MENDEZ, M. V., PHILLIPS, T. J., PARK, H.-Y. & MENZOIAN, J. O. (1999a) The effect of passage number on fibroblast cellular senescence in patients with chronic venous insufficiency with and without ulcer. *The American Journal of Surgery,* 178, 107-112.

RAFFETTO, J. D., MENDEZ, M. V., PHILLIPS, T. J., PARK, H. Y. & MENZOIAN, J. O. (1999b) The effect of passage number on fibroblast cellular senescence in patients with chronic venous insufficiency with and without ulcer. *Am J Surg,* 178, 107-12.

RAIMUND W. KINNE, KUNISCH, E., BEENSEN, V., ZIMMERMANN, T., EMMRICH, F., PETROW, P., LUNGERSHAUSEN, W., HEIN, G., BRAUN, R. K., FOERSTER, M., KROEGEL, C., WINTER, R., LIESAUS, E., FUHRMANN, R. A., ROTH, A., CLAUSSEN, U. & LIEHR, T. (2003) Synovial fibroblasts and synovial macrophages from patients with rheumatoid arthritis and other inflammatory joint diseases show chromosomal aberrations. *Genes, Chromosomes and Cancer,* 38, 53-67.

RAO, C. N., LADIN, D. A., LIU, Y. Y., CHILUKURI, K., HOU, Z. Z. & WOODLEY, D. T. (1995) Alpha 1-antitrypsin is degraded and non-functional in chronic wounds but intact and functional in acute wounds: the inhibitor protects fibronectin from degradation by chronic wound fluid enzymes. *J Invest Dermatol,* 105, 572-8.

RAYMENT, E. A., UPTON, Z. & SHOOTER, G. K. (2008) Increased matrix metalloproteinase-9 (MMP-9) activity observed in chronic wound fluid is related to the clinical severity of the ulcer. *Br J Dermatol,* 158, 951-61.

REKHVIASHVILI, N., STEVENS, G., SCOTT, L. & STEVENS, W. (2006) Fluorogenic LUX primer for quantitation of HIV-1 by real-time RT-PCR. *Mol Biotechnol,* 32, 101-10.

REKHVIASHVILI, N., STEVENS, W., MARINDA, E., GONIN, R., STEVENS, G., MCINTYRE, J. & WOOD, R. (2007) Clinical performance of an in-house real-time RT-PCR assay using a fluorogenic LUXtrade mark primer for quantitation of human immunodeficiency virus type-1 (HIV-1). *J Virol Methods.*

REMINGTON, S. J. (2006) Fluorescent proteins: maturation, photochemistry and photophysics. *Current Opinion in Structural Biology,* 16, 714-721.

ROMAGNANI, P., LASAGNI, L., ANNUNZIATO, F., SERIO, M. & ROMAGNANI, S. (2004) CXC chemokines: the regulatory link between inflammation and angiogenesis. *Trends in Immunology,* 25, 201-209.

ROMERO-GRAILLET, C., ABERDAM, E., BIAGOLI, N., MASSABNI, W., ORTONNE, J.-P. & BALLOTTI, R. (1996) Ultraviolet B Radiation Acts through the Nitric Oxide and cGMP Signal Transduction Pathway to Stimulate Melanogenesis in Human Melanocytes. *J. Biol. Chem.,* 271, 28052-28056.

ROMMAIN, M., BROSSARD, C., PIRON, M. A. & SMETS, P. (1991) A skin suction blister model in hairless rats: application to the study of anti-inflammatory and immunomodulatory drugs. *Int J Immunopharmacol,* 13, 379-84.

ROVAI, L. E., HERSCHMAN, H. R. & SMITH, J. B. (1997) Cloning and characterization of the human granulocyte chemotactic protein-2 gene. *J Immunol,* 158, 5257-66.

ROY, S., KHANNA, S., RINK, C., BISWAS, S. & SEN, C. K. (2008) Characterization of the acute temporal changes in excisional murine cutaneous wound inflammation by screening of the wound-edge transcriptome. *Physiol Genomics,* 34, 162-84.

SAARIALHO-KERE, U. K. (1998) Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. *Arch Dermatol Res,* 290 Suppl, S47-54.

SAARIALHO-KERE, U. K., PENTLAND, A. P., BIRKEDAL-HANSEN, H., PARKS, W. C. & WELGUS, H. G. (1994) Distinct populations of basal keratinocytes express stromelysin-1 and stromelysin-2 in chronic wounds. *J Clin Invest,* 94, 79-88.

SABROE, I., PARKER, L. C., DOWER, S. K. & WHYTE, M. K. (2008) The role of TLR activation in inflammation. *J Pathol,* 214, 126-35.

SAKAI, S., YASUDA, R., SAYO, T., ISHIKAWA, O. & INOUE, S. (2000) Hyaluronan exists in the normal stratum corneum. *J Invest Dermatol,* 114, 1184-7.

SATO, M., OHGURO, H., OHGURO, I., MAMIYA, K., TAKANO, Y., YAMAZAKI, H., METOKI, T., MIYAGAWA, Y., ISHIKAWA, F. & NAKAZAWA, M. (2003) Study of pharmacological effects of nilvadipine on RCS rat retinal degeneration by microarray analysis. *Biochemical and Biophysical Research Communications,* 306, 826-831.

SCANZELLO, C. R., PLAAS, A. & CROW, M. K. (2008) Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound? *Curr Opin Rheumatol,* 20, 565-72.

SCHAFFER, C. J., REINISCH, L., POLIS, S. L. & STRICKLIN, G. P. (1997) Comparisons of wound healing among excisional, laser-created, and standard thermal burns in porcine wounds of equal depth. *Wound Repair Regen,* 5, 52-61.

SCHENA, M., SHALON, D., DAVIS, R. W. & BROWN, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science,* 270, 467-70.

SCHINKEL, C., WICK, M., MUHR, G. & KOLLER, M. (2005) Analysis of systemic interleukin-11 after major trauma. *Shock,* 23, 30-4.

SCHMID, P., COX, D., BILBE, G., MCMASTER, G., MORRISON, C., STAHELIN, H., LUSCHER, N. & SEILER, W. (1993) TGF-beta s and TGF-beta type II receptor in human epidermis: differential expression in acute and chronic skin wounds. *J Pathol,* 171, 191-7.

SCHNEIDER, R. K., NEUSS, S., STAINFORTH, R., LADDACH, N., BOVI, M., KNUECHEL, R. & PEREZ-BOUZA, A. (2008) Three-dimensional epidermis-like growth of human mesenchymal stem cells on dermal equivalents: contribution to tissue organization by adaptation of myofibroblastic phenotype and function. *Differentiation,* 76, 156-67.

SCOTT, J. R., TAMURA, R. N., MUANGMAN, P., ISIK, F. F., XIE, C. & GIBRAN, N. S. (2008) Topical substance P increases inflammatory cell density in genetically diabetic murine wounds. *Wound Repair Regen,* 16, 529-33.

SCOTT, K. A., WOOD, E. J. & KARRAN, E. H. (1998) A matrix metalloproteinase inhibitor which prevents fibroblast-mediated collagen lattice contraction. *FEBS Letters,* 441, 137-140.

SCOTT, P. G., GROSSMANN, J. G., DODD, C. M., SHEEHAN, J. K. & BISHOP, P. N. (2003) Light and X-ray scattering show decorin to be a dimer in solution. *J Biol Chem,* 278, 18353-9.

SHANER, N. C., STEINBACH, P. A. & TSIEN, R. Y. (2005) A guide to choosing fluorescent proteins. *Nat Meth,* 2, 905-909.

SHETERLINE, P. (1993) Mechanisms of actin filament turnover in animal cells. *Symp Soc Exp Biol,* 47, 339-52.

SHIMOMURA, O., JOHNSON, F. H. & SAIGA, Y. (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J Cell Comp Physiol,* 59, 223-39.

SHIRAIWA, T., KANETO, H., MIYATSUKA, T., KATO, K., YAMAMOTO, K., KAWASHIMA, A., KAJIMOTO, Y., MATSUOKA, T.-A., MATSUHISA, M., YAMASAKI, Y. & FUJITANI, Y. (2007) Establishment of a non-invasive mouse reporter model for monitoring in vivo pdx-1 promoter activity. *Biochemical and Biophysical Research Communications,* 361, 739-744.

SIDDIQUI, A., GALIANO, R. D., CONNORS, D., GRUSKIN, E. & WU, L. (1996) Differential effects of oxygen on human dermal fibroblasts: acute versus chronic hypoxia. *Wound Repair and Regeneration,* 4, 211-218.

SMITH, A., QUARMBY, J. W., COLLINS, M., LOCKHART, S. M. & BURNAND, K. G. (1999) Changes in the levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. *Thromb Haemost,* 82, 1593-9.

SMITH, D. F., GALKINA, E., LEY, K. & HUO, Y. (2005) GRO family chemokines are specialized for monocyte arrest from flow. *Am J Physiol Heart Circ Physiol,* 289, H1976-1984.

SMITH, J. C., BOONE, B. E., OPALENIK, S. R., WILLIAMS, S. M. & RUSSELL, S. B. (2008) Gene profiling of keloid fibroblasts shows altered expression in multiple fibrosis-associated pathways. *J Invest Dermatol,* 128, 1298-310.

SMITH, L. T., HOLBROOK, K. A. & MADRI, J. A. (1986) Collagen types I, III, and V in human embryonic and fetal skin. *Am J Anat,* 175, 507-21.

SOMA, T., TAJIMA, M. & KISHIMOTO, J. (2005) Hair cycle-specific expression of versican in human hair follicles. *J Dermatol Sci,* 39, 147-54.

SORRELL, J. M., CARRINO, D. A., BABER, M. A. & CAPLAN, A. I. (1999) Versican in human fetal skin development. *Anat Embryol (Berl),* 199, 45-56.

SOUTHERN, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol,* 98, 503-17.

STALLMEYER, B., KAMPFER, H., KOLB, N., PFEILSCHIFTER, J. & FRANK, S. (1999) The function of nitric oxide in wound repair: inhibition of inducible nitric oxide-synthase severely impairs wound reepithelialization. *J Invest Dermatol,* 113, 1090-8.

STAMENKOVIC, I. (2003) Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol,* 200, 448-64.

STANLEY, A. & OSLER, T. (2001a) Senescence and the healing rates of venous ulcers. *J Vasc Surg,* 33, 1206-1211.

STANLEY, A. & OSLER, T. (2001b) Senescence and the healing rates of venous ulcers. *J Vasc Surg,* 33, 1206-11.

STANLEY, A. C., PARK, H. Y., PHILLIPS, T. J., RUSSAKOVSKY, V. & MENZOIAN, J. O. (1997) Reduced growth of dermal fibroblasts from chronic venous ulcers can be stimulated with growth factors. *J Vasc Surg,* 26, 994-999.

STARK, H. J., BAUR, M., BREITKREUTZ, D., MIRANCEA, N. & FUSENIG, N. E. (1999) Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. *J Invest Dermatol,* 112, 681-91.

STARK, H. J., SZABOWSKI, A., FUSENIG, N. E. & MAAS-SZABOWSKI, N. (2004) Organotypic cocultures as skin equivalents: A complex and sophisticated in vitro system. *Biol Proc Online,* 6, 55–60.

STEPHENS, P., COOK, H., HILTON, J., JONES, C. J., HAUGHTON, M. F., WYLLIE, F. S., SKINNER, J. W., HARDING, K. G., KIPLING, D. & THOMAS, D. W. (2003) An analysis of replicative senescence in dermal fibroblasts derived from chronic leg wounds predicts that telomerase therapy would fail to reverse their disease-specific cellular and proteolytic phenotype. *Experimental Cell Research,* 283, 22-35.

STEPHENS, P., GRENARD, P., AESCHLIMANN, P., LANGLEY, M., BLAIN, E., ERRINGTON, R., KIPLING, D., THOMAS, D. & AESCHLIMANN, D. (2004) Crosslinking and G-protein functions of transglutaminase 2 contribute differentially to fibroblast wound healing responses. *J Cell Sci,* 117, 3389-3403.

STEVENSON, S., TAYLOR, A. H., MESKIRI, A., SHARPE, D. T. & THORNTON, M. J. (2008) Differing responses of human follicular and nonfollicular scalp cells in an in vitro wound healing assay: effects of estrogen on vascular endothelial growth factor secretion. *Wound Repair Regen,* 16, 243-53.

STORIM, J., FRIEDL, P., SCHAEFER, B. M., BECHTEL, M., WALLICH, R., KRAMER, M. D. & REINARTZ, J. (2001) Molecular and Functional Characterization of the Four-Transmembrane Molecule L6 in Epidermal Keratinocytes. *Experimental Cell Research,* 267, 233-242.

SWAMY, S. M., TAN, P., ZHU, Y. Z., LU, J., ACHUTH, H. N. & MOOCHHALA, S. (2004) Role of phenytoin in wound healing: microarray analysis of early transcriptional responses in human dermal fibroblasts. *Biochem Biophys Res Commun,* 314, 661-6.

SWIGGERS, S. J. J., NIBBELING, H. A. J., ZEILEMAKER, A., KUIJPERS, M. A., MATTERN, K. A. & ZIJLMANS, J. M. J. M. (2004) Telomerase activity level, but not hTERT mRNA and hTR level, regulates telomere length in telomerase-reconstituted primary fibroblasts. *Experimental Cell Research,* 297, 434-443.

TAKEUCHI, Y., WATANABE, S., ISHII, G., TAKEDA, S., NAKAYAMA, K., FUKUMOTO, S., KANETA, Y., INOUE, D., MATSUMOTO, T., HARIGAYA, K. & FUJITA, T. (2002) Interleukin-11 as a Stimulatory Factor for Bone Formation Prevents Bone Loss with Advancing Age in Mice. *J. Biol. Chem.,* 277, 49011-49018.

THEILGAARD-MONCH, K., KNUDSEN, S., FOLLIN, P. & BORREGAARD, N. (2004) The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J Immunol,* 172, 7684-93.

THOMAS, P. R. & DORMANDY, J. A. (1988) White cell and platelet trapping in patients with chronic venous insufficiency. *Phlebologie,* 41, 771-6.

THOMPSON, T. A., GOULD, M. N., BURKHOLDER, J. K. & YANG, N. S. (1993) Transient promoter activity in primary rat mammary epithelial cells evaluated

using particle bombardment gene transfer. *In Vitro Cell Dev Biol,* 29A, 165-70.

TOMASEK, J. J., GABBIANI, G., HINZ, B., CHAPONNIER, C. & BROWN, R. A. (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol,* 3, 349-63.

TRABUCCHI, E., RADAELLI, E., MARAZZI, M., FOSCHI, D., MUSAZZI, M., VERONESI, A. M. & MONTORSI, W. (1988) The role of mast cells in wound healing. *Int J Tissue React,* 10, 367-72.

TRENGOVE, N. J., BIELEFELDT-OHMANN, H. & STACEY, M. C. (2000) Mitogenic activity and cytokine levels in non-healing and healing chronic leg ulcers. *Wound Repair Regen,* 8, 13-25.

TRENGOVE, N. J., STACEY, M. C., MACAULEY, S., BENNETT, N., GIBSON, J., BURSLEM, F., MURPHY, G. & SCHULTZ, G. (1999) Analysis of the acute and chronic wound environments: the role of proteases and their inhibitors. *Wound Repair Regen,* 7, 442-452.

TROUSDALE, R. K., JACOBS, S., SIMHAEE, D. A., WU, J. K. & LUSTBADER, J. W. (2008) Wound Closure and Metabolic Parameter Variability in a db/db Mouse Model for Diabetic Ulcers. *J Surg Res.*

TRZASKA, D., ZEMBEK, P., OLSZEWSKI, M., ADAMCZEWSKA, V., ULLERAS, E. & DASTYCH, J. (2005) "Fluorescent Cell Chip" for immunotoxicity testing: Development of the c-fos expression reporter cell lines. *Toxicology and Applied Pharmacology,* 207, 133-141.

TSUBOI, R., SHI, C. M., RIFKIN, D. B. & OGAWA, H. (1992) A wound healing model using healing-impaired diabetic mice. *J Dermatol,* 19, 673-5.

VAALAMO, M., LEIVO, T. & SAARIALHO-KERE, U. (1999) Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) in normal and aberrant wound healing. *Hum Pathol,* 30, 795-802.

VAALAMO, M., MATTILA, L., JOHANSSON, N., KARINIEMI, A. L., KARJALAINEN-LINDSBERG, M. L., KAHARI, V. M. & SAARIALHO-KERE, U. (1997) Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. *J Invest Dermatol,* 109, 96-101.

VAALAMO, M., WECKROTH, M., PUOLAKKAINEN, P., KERE, J., SAARINEN, P., LAUHARANTA, J. & SAARIALHO-KERE, U. K. (1996) Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. *British Journal of Dermatology,* 135, 52-59.

VAN DE SCHEUR, M. & FALANGA, V. (1997) Pericapillary fibrin cuffs in venous disease. A reappraisal. *Dermatol Surg,* 23, 955-9.

VAN GOMPEL, J., WOESTENBORGHS, F., BEERENS, D., MACKIE, C., CAHILL, P. A., KNIGHT, A. W., BILLINTON, N., TWEATS, D. J. & WALMSLEY, R. M. (2005) An assessment of the utility of the yeast GreenScreen assay in pharmaceutical screening. *Mutagenesis,* 20, 449-454.

VAN KORLAAR, I., VOSSEN, C., ROSENDAAL, F., CAMERON, L., BOVILL, E. & KAPTEIN, A. (2003) Quality of life in venous disease. *Thromb Haemost,* 90, 27-35.

VANDE BERG, J. S., RUDOLPH, R., HOLLAN, C. & HAYWOOD-REID, P. L. (1998) Fibroblast senescence in pressure ulcers. *Wound Repair Regen,* 6, 38-49.

VASQUEZ, R., MARIEN, B. J., GRAM, C., GOODWIN, D. G., MENZOIAN, J. O. & RAFFETTO, J. D. (2004) Proliferative capacity of venous ulcer wound fibroblasts in the presence of platelet-derived growth factor. *Vasc Endovascular Surg,* 38, 355-60.

VAZIRI, H. & BENCHIMOL, S. (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol,* 8, 279-82.

VELANDER, P., THEOPOLD, C., HIRSCH, T., BLEIZIFFER, O., ZUHAILI, B., FOSSUM, M., HOELLER, D., GHEERARDYN, R., CHEN, M., VISOVATTI, S., SVENSSON, H., YAO, F. & ERIKSSON, E. (2008) Impaired wound healing in an acute diabetic pig model and the effects of local hyperglycemia. *Wound Repair Regen,* 16, 288-93.

VOLPERT, O. V., PILI, R., SIKDER, H. A., NELIUS, T., ZAICHUK, T., MORRIS, C., SHIFLETT, C. B., DEVLIN, M. K., CONANT, K. & ALANI, R. M. (2002) Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. *Cancer Cell,* 2, 473-483.

VON BULOW, S., HARTMANN, T., FUCHS, P. C., SCHRIMPF, C. & PALLUA, N. (2005) Endothelial thrombomodulin (CD 141) in a rabbit burn model. *Burns,* 31, 459-64.

WALL, I. B. (2006) A cellular and molecular analysis of chronic wound and normal dermal fibroblasts. *Wound Biolgy Group.* Cardiff, Cardiff University.

WALL, I. B., MOSELEY, R., BAIRD, D. M., KIPLING, D., GILES, P., LAFFAFIAN, I., PRICE, P. E., THOMAS, D. W. & STEPHENS, P. (2008a) Fibroblast Dysfunction Is a Key Factor in the Non-Healing of Chronic Venous Leg Ulcers. *J Invest Dermatol,* 128, 2526-2540.

WALL, I. B., MOSELEY, R., BAIRD, D. M., KIPLING, D., GILES, P., LAFFAFIAN, I., PRICE, P. E., THOMAS, D. W. & STEPHENS, P. (2008b) Fibroblast Dysfunction Is a Key Factor in the Non-Healing of Chronic Venous Leg Ulcers. *J Invest Dermatol.*

WALLER, J. M. & MAIBACH, H. I. (2006) Age and skin structure and function, a quantitative approach (II): protein, glycosaminoglycan, water, and lipid content and structure. *Skin Res Technol,* 12, 145-54.

WANG, T. W., HUANG, Y. C., SUN, J. S. & LIN, F. H. (2003) Organotypic keratinocyte-fibroblast cocultures on a bilayer gelatin scaffold as a model of skin equivalent. *Biomed Sci Instrum,* 39, 523-8.

WANG, Z., YI, J., LI, H., DENG, L. F. & TANG, X. M. (2000) [Extension of life-span of normal human fibroblasts by reconstitution of telomerase activity]. *Shih Yen Sheng Wu Hsueh Pao: Journal of Experimental Biology,* 33, 129-40.

WATABE, K., ITO, A., KOMA, Y. I. & KITAMURA, Y. (2003) IGSF4: a new intercellular adhesion molecule that is called by three names, TSLC1, SgIGSF and SynCAM, by virtue of its diverse function. *Histol Histopathol,* 18, 1321-9.

WATSON, R. E. B., BALL, S. G., CRAVEN, N. M., BOORSMA, J., EAST, C. L., SHUTTLEWORTH, C. A., KIELTY, C. M. & GRIFFITHS, C. E. M. (2001) Distribution and expression of type VI collagen in photoaged skin. *British Journal of Dermatology,* 144, 751-759.

WECKROTH, M., VAHERI, A., LAUHARANTA, J., SORSA, T. & KONTTINEN, Y. T. (1996) Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol,* 106, 1119-24.

WEINSTEIN, J. N. (1998) Fishing Expeditions. *Science,* 282, 627g-.

WEST, M. D., PEREIRA-SMITH, O. M. & SMITH, J. R. (1989) Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity. *Exp Cell Res,* 184, 138-47.

WEYL, A., VANSCHEIDT, W., WEISS, J. M., PESCHEN, M., SCHOPF, E. & SIMON, J. (1996) Expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin and their ligands VLA-4 and LFA-1 in chronic venous leg ulcers. *J Am Acad Dermatol,* 34, 418-23.

WHITBY, D. J., LONGAKER, M. T., HARRISON, M. R., ADZICK, N. S. & FERGUSON, M. W. (1991) Rapid epithelialisation of fetal wounds is associated with the early deposition of tenascin. *J Cell Sci,* 99 (Pt 3), 583-6.

WICK, M., BURGER, C., BRUSSELBACH, S., LUCIBELLO, F. C. & MULLER, R. (1994) A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation, and senescence. *J Biol Chem,* 269, 18953-60.

WILHELM, S. M., COLLIER, I. E., KRONBERGER, A., EISEN, A. Z., MARMER, B. L., GRANT, G. A., BAUER, E. A. & GOLDBERG, G. I. (1987) Human Skin Fibroblast Stromelysin: Structure, Glycosylation, Substrate Specificity, and Differential Expression in Normal and Tumorigenic Cells. *Proceedings of the National Academy of Sciences,* 84, 6725-6729.

WOLPE, S. D., SHERRY, B., JUERS, D., DAVATELIS, G., YURT, R. W. & CERAMI, A. (1989) Identification and characterization of macrophage inflammatory protein 2. *Proc Natl Acad Sci U S A,* 86, 612-6.

WU, L. & MUSTOE, T. A. (1995) Effect of ischemia on growth factor enhancement of incisional wound healing. *Surgery,* 117, 570-6.

WUYTS, A., VAN OSSELAER, N., HAELENS, A., SAMSON, I., HERDEWIJN, P., BEN-BARUCH, A., OPPENHEIM, J. J., PROOST, P. & VAN DAMME, J. (1997) Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. *Biochemistry,* 36, 2716-23.

WYLLIE, F. S., JONES, C. J., SKINNER, J. W., HAUGHTON, M. F., WALLIS, C., WYNFORD-THOMAS, D., FARAGHER, R. G. A. & KIPLING, D. (2000) Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts. *Nat Genet,* 24, 16-17.

WYSOCKI, A. B. (1996) Wound fluids and the pathogenesis of chronic wounds. *Journal of WOCN,* 23, 283-290.

WYSOCKI, A. B., STAIANO-COICO, L. & GRINNELL, F. (1993a) Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol,* 101, 64-8.

WYSOCKI, A. B., STAIANO-COICO, L. & GRINNELL, F. (1993b) Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol,* 101, 64-68.

XU, J. W., LI, T. F., PARTSCH, G., CEPONIS, A., SANTAVIRTA, S. & KONTTINEN, Y. T. (1998) Interleukin-11 (IL-11) in aseptic loosening of total hip replacement (THR). *Scand J Rheumatol,* 27, 363-7.

YAMASAKI, K., EDINGTON, H. D., MCCLOSKY, C., TZENG, E., LIZONOVA, A., KOVESDI, I., STEED, D. L. & BILLIAR, T. R. (1998) Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J Clin Invest,* 101, 967-71.

YAMAZAKI, H., TSUNETO, M., YOSHINO, M., YAMAMURA, K.-I. & HAYASHI, S.-I. (2007) Potential of Dental Mesenchymal Cells in Developing Teeth. *Stem Cells,* 25, 78-87.

YANG, J., CHANG, E., CHERRY, A. M., BANGS, C. D., OEI, Y., BODNAR, A., BRONSTEIN, A., CHIU, C.-P. & HERRON, G. S. (1999) Human Endothelial Cell Life Extension by Telomerase Expression. *J. Biol. Chem.,* 274, 26141-26148.

YANG, N.-C. & HU, M.-L. (2005) The limitations and validities of senescence associated-[beta]-galactosidase activity as an aging marker for human foreskin fibroblast Hs68 cells. *Experimental Gerontology,* 40, 813-819.

ZEITZ, M., RUIZ-TORRES, A. & MERKER, H. J. (1978) Collagen metabolism in granulating wounds of rat skin. *Arch Dermatol Res,* 263, 207-14.

ZHANG, Y., GORRY, M. C., HART, P. S., PETTENATI, M. J., WANG, L., MARKS, J. J., LU, X. & HART, T. C. (2001) Localization, genomic organization, and alternative transcription of a novel human SAM-dependent methyltransferase gene on chromosome 2p22p21. *Cytogenetic and Genome Research,* 95, 146-152.

ZHANG, Z. D., HAN, F. H. & MENG, L. X. (2005) Establishment of a pig model with enteric and portal venous drainage of pancreatoduodenal transplantation. *World J Gastroenterol,* 11, 5475-9.

ZHENG, R., YANO, S., ZHANG, H., NAKATAKI, E., TACHIBANA, I., KAWASE, I., HAYASHI, S. & SONE, S. (2005) CD9 overexpression suppressed the liver metastasis and malignant ascites via inhibition of proliferation and motility of small-cell lung cancer cells in NK cell-depleted SCID mice. *Oncol Res,* 15, 365-72.

ZHU, M. J., KIM, C. D., KWON, Y. B., KYE, K. C., CHEN, Y. Y., LEE, W. H., LEE, S., LIM, J. S., SEO, Y. J., SUHR, K. B., PARK, J. K. & LEE, J. H. (2005) Induction of connective tissue growth factor expression by sphingosylphosphorylcholine in cultured human skin fibroblasts. *Exp Dermatol,* 14, 509-14.

ZIMMERMANN, D. R., DOURS-ZIMMERMANN, M. T., SCHUBERT, M. & BRUCKNER-TUDERMAN, L. (1994a) Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J Cell Biol,* 124, 817-25.

ZIMMERMANN, D. R., DOURS-ZIMMERMANN, M. T., SCHUBERT, M., BRUCKNER-TUDERMAN, L. & HEITZ, P. U. (1994b) [Expression of the extracellular matrix proteoglycan, versican, in human skin]. *Verh Dtsch Ges Pathol,* 78, 481-4.

Appendix 1

Promoter Sequences

ACTC Promoter

```
   1  AATCAACCTG ATTCTTTAAA AGGCAGAGCT ACTACTGCCT CCAAAAGTAT CATTATTACA
      TTAGTTGGAC TAAGAAATTT TCCGTCTCGA TGATGACGGA GGTTTTCATA GTAATAATGT

 101  TTGTTTCTAA CTATTACAAT ACACAATAAC TTCTGCAACA AGTGTTATGG TATCACTTTT
      AACAAAGATT GATAATGTTA TGTGTTATTG AAGACGTTGT TCACAATACC ATAGTGAAAA

 201  CTCAAGACCA AACAGATAGT TAGTAAGTGA TAAAGGCAGC ATTTGAGCCC AGTTCTGTCT
      GAGTTCTGGT TTGTCTATCA ATCATTCACT ATTTCCGTCG TAAACTCGGG TCAAGACAGA

 301  TCTATATTTT CACGAGACAA GGCAGTTCTA CTCTTGGCAT CCTGTCAATA AACATTTTCA
      AGATATAAAA GTGCTCTGTT CCGTCAAGAT GAGAACCGTA GGACAGTTAT TTGTAAAAGT

 401  AGGTCTCTTA CCCGACCCCA CCACACAGCT GCCCATCGCG TTTCTTCTCC AGACCAACCC
      TCCAGAGAAT GGGCTGGGGT GGTGTGTCGA CGGGTAGCGC AAAGAAGAGG TCTGGTTGGG

 501  CCCAGTCGGG ACAAGTATCT AATCAGCAGG CCTCCTCAGC ATATCAGCCC AAGACCACTC
      GGGTCAGCCC TGTTCATAGA TTAGTCGTCC GGAGGAGTCG TATAGTCGGG TTCTGGTGAG

 601  GTGTGAACTG GAGGAAAAAC ACAAGCATTA AAAATCAAAG CTCCAGTCAA GAACTGAGAA
      CACACTTGAC CTCCTTTTTG TGTTCGTAAT TTTTAGTTTC GAGGTCAGTT CTTGACTCTT

 701  TTACATCTCC CTCTTCCTTC TCCCTCTCCT CATTTCATGT CATTTTTCTT TTTCCTTCTC
      AATGTAGAGG GAGAAGGAAG AGGGAGAGGA GTAAAGTACA GTAAAAAGAA AAAGGAAGAG

 801  GATGATATCT GTCATTTATG CCAATGCTTC TCACACTTTA ATGTACATAC CAAGCAAATG
      CTACTATAGA CAGTAAATAC GGTTACGAAG AGTGTGAAAT TACATGTATG GTTCGTTTAC

 901  TGCATTTCTA GCTCATTTCC AGGTTATGAC AATACTGCTT GTCCATGGGC TGCACTTTGA
      ACGTAAAGAT CGAGTAAAGG TCCAATACTG TTATGACGAA CAGGTACCCG ACGTGAAACT

1001  TATGTTCTCC ATCAAAGTAT TTGCCTTTGC AGTCTTGTCT CCACCTGGAG TGAGGGCCTC
      ATACAAGAGG TAGTTTCATA AACGGAAACG TCAGAACAGA GGTGGACCTC ACTCCCGGAG

1101  GGGCAAGCTA CTTTTGGCAT TTGTTCCTTT ATTCACTTTG GCAGCAGGGG ATCCTTTTCT
      CCCGTTCGAT GAAAACCGTA AACAAGGAAA TAAGTGAAAC CGTCGTCCCC TAGGAAAAGA

1201  GCATGGATGA GGGCTACCCA ATAACTGGGC TGGGGTAAAC CTGGTCCCTT CTCCAGCAAG
      CGTACCTACT CCCGATGGGT TATTGACCCG ACCCCATTTG GACCAGGGAA GAGGTCGTTC

1301  CCTGGCCAGA CCCTGATCCA TGCATGGGTT GGAACTCGGG TGGCCTTGGT TAATCTCAGC
      GGACCGGTCT GGGACTAGGT ACGTACCCAA CCTTGAGCCC ACCGGAACCA ATTAGAGTCG

1401  AGCCATTCAG GCTGGCGGGG ACTAGACCAG AAGGGTGCCC TGTCCTGGGT GCCCCTTTTG
      TCGGTAAGTC CGACCGCCCC TGATCTGGTC TTCCCACGGG ACAGGACCCA CGGGGAAAAC

1501  TGGCAGCCCT GGAAGATGAG AAGCCGCTGT TGCTCCCCTG CTGCCCTGGC CCAGCTGTCA
      ACCGTCGGGA CCTTCTACTC TTCGGCGACA ACGAGGGGAC GACGGGACCG GGTCGACAGT

1601  GCATGTGGCT TATTGTCCCC AAGGCTGGCA GGGCTGCGGA GGACCGAATC CACAGACCAT
      CGTACACCGA ATAACAGGGG TTCCGACCGT CCCGACGCCT CCTGGCTTAG GTGTCTGGTA

1701  TGGCGTCACT TAGTCTTCCC CTGCCCCCTA CCCTTCAGCG CCTGCCCCTC CCCAGCTCCC
      ACCGCAGTGA ATCAGAAGGG GACGGGGGAT GGGAAGTCGC GGACGGGGAG GGGTCGAGGG

1801  ATGGTCTGGG GGCTCCCTGG CTGATCCTCT CCCCTGCCCT TGGCTCCATG AATGGCCTCG
      TACCAGACCC CCGAGGGACC GACTAGGAGA GGGGACGGGA ACCGAGGTAC TTACCGGAGC

1901  TGGCAGACCG GGCCCCCCAC CCCTGCCCCC GGCTGCTCCA ACTGACCCTG TCCATCAGCG
      ACCGTCTGGC CCGGGGGGTG GGGACGGGGG CCGACGAGGT TGACTGGGAC AGGTAGTCGC

2001  CGCTGCCGCC GGAGCCGAGC CGACCCGCCC CGCCGACGGT GAGTCAGCGC CCGGCCCTCC
      GCGACGGCGG CCTCGGCTCG GCTGGGCGGG GCGGCTGCCA CTCAGTCGCG GGCCGGGAGG

2101  CAGCCCCGCG CCGCCACCTG GCCAGCCCGG CCCGCATTCA GCCAAGGCCC CAGCTCCTGC
      GTCGGGGCGC GGCGGTGGAC CGGTCGGGCC GGGCGTAAGT CGGTTCCGGG GTCGAGGACG

2201  CACTTTTTGA TTTGGTTCTA ACTTGTTTTG TCCTGGG
      GTGAAAAACT AAACCAAGAT TGAACAAAAC AGGACCC
```

CD9 promoter

```
   1  GCCTTCATTC ATTCTTCTCT CACCTGAACT ACTCCACAGC CTCCTAGCAG ATTTGCACCT
      CGGAAGTAAG TAAGAAGAGA GTGGACTTGA TGAGGTGTCG GAGGATCGTC TAAACGTGGA

 101  ATTACCTGAT TGCGTCTCCC CAAATACAAC ATCTCCATGT TCCATGCAAT CCCGTAAAGC
      TAATGGACTA ACGCAGAGGG GTTTATGTTG TAGAGGTACA AGGTACGTTA GGGCATTTCG

 201  CTCAAGGGGC TTCCTACCTG CCCCTCCGGC CTTCCCCCAC CCAACTACCT AAAACACAGG
      GAGTTCCCCG AAGGATGGAC GGGGAGGCCG GAAGGGGGTG GGTTGATGGA TTTTGTGTCC

 301  TGCTTGAACC TTTCGGTGGC CCCTAATGCT CAGAGTGCAA AGTCCAGGAG CACAAGGCTC
      ACGAACTTGG AAAGCCACCG GGGATTACGA GTCTCACGTT TCAGGTCCTC GTGTTCCGAG

 401  CAGCCCAGAG CCCGGACAGC CCCTGCCCCA GCCTTTGCCC AGGATTCTCC CTTGGCGGCA
      GTCGGGTCTC GGGCCTGTCG GGGACGGGGT CGGAAACGGG TCCTAAGAGG GAACCGCCGT

 501  TCAGAGGCCT CTCCTTAGCC TCTTAGCCCC CAAGCCTCTC CCCGTAACTG CACCTATCAC
      AGTCTCCGGA GAGGAATCGG AGAATCGGGG GTTCGGAGAG GGGCATTGAC GTGGATAGTG

 601  GTCCCTGAGC AGAAACAAGG ACTAATCCAT GTTTTTCAGC TCCTACCCCT AATGAAATAC
      CAGGGACTCG TCTTTGTTCC TGATTAGGTA CAAAAAGTCG AGGATGGGGA TTACTTTATG

 701  AGTCTACTGA TAATAAACAG GCAAATAACA GCAATGTGCT AACAGTGGCT GTTTCTGGTG
      TCAGATGACT ATTATTTGTC CGTTTATTGT CGTTACACGA TTGTCACCGA CAAAGACCAC

 801  TCGGTTGTTT CTGTAATGAC TGTATCTTGC TTTTGTAATC ATAAAGGACA GTAAAGGAAA
      AGCCAACAAA GACATTACTG ACATAGAACG AAAACATTAG TATTTCCTGT CATTTCCTTT

 901  AAAGAAGGTG ACTCTCCTCG TCCACTCCAG AGAATCAGGA AAGGAGTGTT TAAAGACCCA
      TTTCTTCCAC TGAGAGGAGC AGGTGAGGTC TCTTAGTCCT TTCCTCACAA ATTTCTGGGT

1001  CAGGGCGCAT GCCGGCCAGC GAGTGGGGTG GGCTCTGGGT CCGGGAGAAG TCAGATCAGT
      GTCCCGCGTA CGGCCGGTCG CTCACCCCAC CCGAGACCCA GGCCCTCTTC AGTCTAGTCA

1101  CTTAGGTCCT TGAATCTGGA GAGCTACAGA AAGTTTGTGA GCTCAGGAGA AGCGCTCCGA
      GAATCCAGGA ACTTAGACCT CTCGATGTCT TTCAAACACT CGAGTCCTCT TCGCGAGGCT

1201  CCAAAGACGC AGGAGGGAGG GTGGGGTGGA GGAGTAGAGA GAAAACAGAA GCCGTCTACA
      GGTTTCTGCG TCCTCCCTCC CACCCCACCT CCTCATCTCT CTTTTGTCTT CGGCAGATGT

1301  GAAGCGCTTG GGGACTGCCC AGCCCTCAGC TGTGTTATTA TTCGGTGATA GGTATTTGCT
      CTTCGCGAAC CCCTGACGGG TCGGGAGTCG ACACAATAAT AAGCCACTAT CCATAAACGA

1401  CTGCGCGCTT CTAATTCCTC CTACCCCACA TGCTGTGCCC AATGAAAAGT ATGGTCAGCG
      GACGCGCGAA GATTAAGGAG GATGGGGTGT ACGACACGGG TTACTTTTCA TACCAGTCGC

1501  CCAGGCGGCT TCCCTTTAAA TCCTCGCAAA GCAGAAGGGC CCCTCACTCT GGCAGCAGGC
      GGTCCGCCGA AGGGAAATTT AGGAGCGTTT CGTCTTCCCG GGGAGTGAGA CCGTCGTCCG

1601  GTCTCCCAAA GCAGAACGCC CGGTCCGGCG CCCAGACCAA ACGCGGGGGA ACCGGAAGGG
      CAGAGGGTTT CGTCTTGCGG GCCAGGCCGC GGGTCTGGTT TGCGCCCCCT TGGCCTTCCC

1701  TGGCTGCCTG CAAAGACCCC AAGACGGCTT GAAGAAGGAG TGGGTGGCGG GTGGGGGTTA
      ACCGACGGAC GTTTCTGGGG TTCTGCCGAA CTTCTTCCTC ACCCACCGCC CACCCCCAAT

1801  CGGGGCAGCT GGGGCCGGGG CTCGCCGACC TGGGCGGGGG CGGGGGGCGG GGGGGGTGCG
      GCCCCGTCGA CCCCGGCCCC GAGCGGCTGG ACCCGCCCCC GCCCCCCGCC CCCCCCACGC

1901  GGGGAGTGGG GGCGGCTTTT CCCGGCACAT GCGCACCGCA GCGGGTCGCG CGCCCTAAGG
      CCCCTCACCC CCGCCGAAAA GGGCCGTGTA CGCGTGGCGT CGCCCAGCGC GCGGGATTCC

2001  GCCGCCTGCA TCTGTATCCA GCGCCAGGTC CCGCCAGTCC CAGCTGCGCG CGCCCCCCAG
      CGGCGGACGT AGACATAGGT CGCGGTCCAG GGCGGTCAGG GTCGACGCGC GCGGGGGGTC

2101  TGCCGGTCAA AGGAGGCACC AAGTGCATCA AATACCTGCT GTTCGGATTT AAC
      ACGGCCAGTT TCCTCCGTGG TTCACGTAGT TTATGGACGA CAAGCCTAAA TTG
```

CXCL1 promoter

```
   1  TCAGGAAGAT GTGAAAAGAG AAATAATTGA AAAAGACTGG AGTACATATA CTATCTACAG
      AGTCCTTCTA CACTTTTCTC TTTATTAACT TTTTCTGACC TCATGTATAT GATAGATGTC

 101  CCCTTGACTA AGATTACTGT AATGAGAGCA CCAGTACCCC TGAGTAACCG AAAGGGCATT
      GGGAACTGAT TCTAATGACA TTACTCTCGT GGTCATGGGG ACTCATTGGC TTTCCCGTAA

 201  TTCCAGGACC TGCCTTGATT TATTCAAGAC TCATACTGCT GTATATGGTG TTGTATACAT
      AAGGTCCTGG ACGGAACTAA ATAAGTTCTG AGTATGACGA CATATACCAC AACATATGTA

 301  AAATGTAAAA AACACTGGGG AGTGAACCTT TCCATTATAT ATATATATAT ATATATATAT
      TTTACATTTT TTGTGACCCC TCACTTGGAA AGGTAATATA TATATATATA TATATATATA

 401  ACATCAGGAT GAGTTTCTGT TTAGGCAATG TTGGAAAACG CTATTTCCAT TTTTTTTTTA
      TGTAGTCCTA CTCAAAGACA AATCCGTTAC AACCTTTTGC GATAAAGGTA AAAAAAAAAT

 501  TGCTGGCTCT TACAAATGTT GACTCATTTC TCATAACCAC CTTGGGGTAG AAACGGAGAG
      ACGACCGAGA ATGTTTACAA CTGAGTAAAG AGTATTGGTG GAACCCCATC TTTGCCTCTC

 601  GTGGCAGCCT TGCCTGTGTT CTCTGCTTGG CTAGGAACAC AGGTCTTACC TATTGAGCTG
      CACCGTCGGA ACGGACACAA GAGACGAACC GATCCTTGTG TCCAGAATGG ATAACTCGAC

 701  GCAGAAGCCT CTGCTTTTTC CCCCTCCTCC CATCTTACTC CATGTCTCAG AGAGCTCTGA
      CGTCTTCGGA GACGAAAAAG GGGGAGGAGG GTAGAATGAG GTACAGAGTC TCTCGAGACT

 801  AATGATGAAT AAAAATAAGT TCTCTAGAAC TTCTGGAGAA AAAAGTAATA AAGCTACCAG
      TTACTACTTA TTTTTATTCA AGAGATCTTG AAGACCTCTT TTTTCATTAT TTCGATGGTC

 901  TGTTTCTCTA GAAAGGGGGC CCAATACTGA ATACCAGGAA GTCCTATAGT AAATGGAATG
      ACAAAGAGAT CTTTCCCCCG GGTTATGACT TATGGTCCTT CAGGATATCA TTTACCTTAC

1001  GCATGTCTGC TGCTTCAGTG GGAAGGGTGC TTGCACACCA GGTACCCACT CCCTGGTGTC
      CGTACAGACG ACGAAGTCAC CCTTCCCACG AACGTGTGGT CCATGGGTGA GGGACCACAG

1101  CCCATGAGCC TGCTGGACCC AGCCCCTCCG AGGTCCGGAG TGACAACCAG TGCCGTATTT
      GGGTACTCGG ACGACCTGGG TCGGGGAGGC TCCAGGCCTC ACTGTTGGTC ACGGCATAAA

1201  CAGGGGATTT TGAAAGTTCC AACATTTTAC AGGGAAGAAG GAAGATAAGC AGGATATGAA
      GTCCCCTAAA ACTTTCAAGG TTGTAAAATG TCCCTTCTTC CTTCTATTCG TCCTATACTT

1301  TAACACTGGA TTCAGCTTTT GACACTGATA ATCTGTTGCC ACCAAATGGA AAACGTAAAC
      ATTGTGACCT AAGTCGAAAA CTGTGACTAT TAGACAACGG TGGTTTACCT TTTGCATTTG

1401  AACAAGCAGA ACATTCTTCT CTGGAATCTG ACATAATGGA CTGTACTTTC ACAGACAGCA
      TTGTTCGTCT TGTAAGAAGA GACCTTAGAC TGTATTACCT GACATGAAAG TGTCTGTCGT

1501  AGAAAGGCTG AGGCAGAGAG GATAATATAG CTCCAGCCTA TCTCCCAGCA CCTTGTTAAT
      TCTTTCCGAC TCCGTCTCTC CTATTATATC GAGGTCGGAT AGAGGGTCGT GGAACAATTA

1601  TTCCTCCAAA GAGGTCGCGC CTTCTCTGTG GTGGTTCTCA GGGATCCGCC CCAGCTCCTT
      AAGGAGGTTT CTCCAGCGCG GAAGAGACAC CACCAAGAGT CCCTAGGCGG GGTCGAGGAA

1701  AAGATCCCAC CTCTCAGGTG GTATCTTCAG CGCAGGCTGC CACTCAGCCC CCCTCCAGGG
      TTCTAGGGTG GAGAGTCCAC CATAGAAGTC GCGTCCGACG GTGAGTCGGG GGGAGGTCCC

1801  ACAGGAGTTA CTCTGAAGGG CGAGGCGCGG GCTGCATCAG TGGACCCCCA CACCCCACCC
      TGTCCTCAAT GAGACTTCCC GCTCCGCGCC CGACGTAGTC ACCTGGGGGT GTGGGGTGGG

1901  TCCTTCCGGA CTCGGGATCG ATCTGGAACT CCGGGAATTT CCCTGGCCCG GGGGCTCCGG
      AGGAAGGCCT GAGCCCTAGC TAGACCTTGA GGCCCTTAAA GGGACCGGGC CCCCGAGGCC

2001  CTCGGAGAGC CACAGAGCCC GGGCCGCAGG CACCTCCTCG CCAGCTCTTC CGCTCCTCTC
      GAGCCTCTCG GTGTCTCGGG CCCGGCGTCC GTGGAGGAGC GGTCGAGAAG GCGAGGAGAG

2101  TGCTCTCTCC GCCGCCCCCA GCAATCCCCG GCTCCTGCGA GTGGCACTGC TGCTCCTGCT
      ACGAGAGAGG CGGCGGGGGT CGTTAGGGGC CGAGGACGCT CACCGTGACG ACGAGGACGA

2201  CGCCCTGGGG TCCCCGGGCC GGACGCGGCT GGGGTAGGCA CCCAGCGCCG ACAGCCTCGC
      GCGGGACCCC AGGGGCCCGG CCTGCGCCGA CCCCATCCGT GGGTCGCGGC TGTCGGAGCG
```

CXCL12 promoter

```
   1  CAGCGGCCAG GCCTGAATCC CTGGTTCGCC CTCGACTCAG CCCACGCGGG CCTTTCAGGC
      GTCGCCGGTC CGGACTTAGG GACCAAGCGG GAGCTGAGTC GGGTGCGCCC GGAAAGTCCG

 101  GTGGCAAAGA AAAAAAGAAG AAGAAGAAGC GAGAGGCGAT GGCGTTTGCT TTGGCCAGAT
      CACCGTTTCT TTTTTTCTTC TTCTTCTTCG CTCTCCGCTA CCGCAAACGA AACCGGTCTA

 201  GATCTCCGAG CTCCAGGGCG CCCCTCCACC CGGGTGTAGA TTTCCCGCGG ACCCCTTCGC
      CTAGAGGCTC GAGGTCCCGC GGGGAGGTGG GCCCACATCT AAAGGGCGCC TGGGGAAGCG

 301  AGAGCTCTGG GAGCGGGGAG GGAGGCGCCG CCACCAGAGG GCGCCGGAGC CCCAGCGCTG
      TCTCGAGACC CTCGCCCCTC CCTCCGCGGC GGTGGTCTCC CGCGGCCTCG GGGTCGCGAC

 401  AGGCCTGGGG ACGCCCGACG GCTGCCTTCT TCACTGGACC TCACTGCCTT CAGTTCTTTA
      TCCGGACCCC TGCGGGCTGC CGACGGAAGA AGTGACCTGG AGTGACGGAA GTCAAGAAAT

 501  AGTGCCCAGG GTGGGTGGGT GGGTGGGTGG GTGGGTGGAT GGATGGATGG ATGGATGGAT
      TCACGGGTCC CACCCACCCA CCCACCCACC CACCCACCTA CCTACCTACC TACCTACCTA

 601  TGGAAAAAAA ATGCACAGAA GACACCTACT CCCACCAGCG GAGTTCCGGA GCCCTCGCAG
      ACCTTTTTTT TACGTGTCTT CTGTGGATGA GGGTGGTCGC CTCAAGGCCT CGGGAGCGTC

 701  CCCACTCCCC GACGGCCAGG ACTCCCCAGG GACAGGGACG TGTCCCCAGG GCAGGCCCCT
      GGGTGAGGGG CTGCCGGTCC TGAGGGGTCC CTGTCCCTGC ACAGGGGTCC CGTCCGGGGA

 801  GCTGGGAAGG ACACAGAGAG GTGGCTGGGG CAGCCTGCGG TCACAAAGCG AGGCCCAAAG
      CGACCCTTCC TGTGTCTCTC CACCGACCCC GTCGGACGCC AGTGTTTCGC TCCGGGTTTC

 901  GCACCCTCCC TTCGCCACCG GACTGGGGCC ATCTGGGATG TCTCGGGGGT ATCCGGAGGG
      CGTGGGAGGG AAGCGGTGGC CTGACCCCGG TAGACCCTAC AGAGCCCCCA TAGGCCTCCC

1001  TGGACCCAGA AGGCAGACGC CAGTAGTACT GTCCTAGGAG TCGGAGGTCG GGGTGGGGGA
      ACCTGGGTCT TCCGTCTGCG GTCATCATGA CAGGATCCTC AGCCTCCAGC CCCACCCCCT

1101  AGGCAGTAAG TACTTAGGTT TAAAGGACTT ACTTACAGCT ACCATTTATT GAGTACTGTC
      TCCGTCATTC ATGAATCCAA ATTTCCTGAA TGAATGTCGA TGGTAAATAA CTCATGACAG

1201  CGGGTCTCAT TGAATCTCCC GTCCCACTCC GCGGGGTGGG CCTGTGATTA GCTCATTTCA
      GCCCAGAGTA ACTTAGAGGG CAGGGTGAGG CGCCCCACCC GGACACTAAT CGAGTAAAGT

1301  ACTGAGAGCC GCCGGCGCCT TCTGCTTTGT TTGTACAGGC GAGGAAACTG AGGCTCGGCT
      TGACTCTCGG CGGCCGCGGA AGACGAAACA AACATGTCCG CTCCTTTGAC TCCGAGCCGA

1401  AAAGACGGGT CTCATTCCCG CAGATCGAGC TCTGCCGGCG GCTGCGCCGC AAGCCGGGCA
      TTTCTGCCCA GAGTAAGGGC GTCTAGCTCG AGACGGCCGC CGACGCGGCG TTCGGCCCGT

1501  CCTCGGCTGC CTTGGGCCGC CACCGCCAGC AGGCCCTCCG CCCGGGACTA ACTTGTTTGC
      GGAGCCGACG GAACCCGGCG GTGGCGGTCG TCCGGGAGGC GGGCCCTGAT TGAACAAACG

1601  CCGTCCCGCA GCTTTCCACG CGCGCCCCAC TTTACGCCTA AGGTCCTCAG TCTCTCCAGT
      GGCAGGGCGT CGAAAGGTGC GCGCGGGGTG AAATGCGGAT TCCAGGAGTC AGAGAGGTCA

1701  CGTCGCTCAG GCTGCGGACC TCACTGCAGA CCGGGCCAGC GGTGCGGGGC CCAGCGGAGC
      GCAGCGAGTC CGACGCCTGG AGTGACGTCT GGCCCGGTCG CCACGCCCCG GGTCGCCTCG

1801  CACAGAGGGA GCGGAGGAGG GGCGAAGGGG ATGGGTGGGG GGTGCCGCCG AGGGAGTCGC
      GTGTCTCCCT CGCCTCCTCC CCGCTTCCCC TACCCACCCC CCACGGCGGC TCCCTCAGCG

1901  CCCGCCCCTC ACCGCGCGCC CCGCCCCGCC CCGCCTGGCT CTCCCCTCTA AAGCGCCCGG
      GGGCGGGGAG TGGCGCGCGG GGCGGGGCGG GGCGGACCGA GAGGGGAGAT TTCGCGGGCC

2001  GCATTGCCCG CTCGGCGTCC GGCCCCCGAC CCGCGCTCGT CCGCCCGCCC GCCCGCCCGC
      CGTAACGGGC GAGCCGCAGG CCGGGGGCTG GGCGCGAGCA GGCGGGCGGG CGGGCGGGCG

2101  GTGCTGACCG CGCTCTGCCT CAGCGACGGT AAGTGCGCTC GGCGGGGAGG CCCTGGCGAG
      CACGACTGGC GCGAGACGGA GTCGCTGCCA TTCACGCGAG CCGCCCCTCC GGGACCGCTC

2201  CCTCTGCCTG CGCCCGCAGT TTGCGCCGCC CGACACAGTG GGGGTGGAGG CAGCTCGCTT
      GGAGACGGAC GCGGGCGTCA AACGCGGCGG GCTGTGTCAC CCCCACCTCC GTCGAGCGAA
```

CXCL3 promoter

```
   1  AATGACCTCC CATCTATGTG CATCTCCTGG TAAAGTACAG ATGTGTCTTC CTTTCTGAAA
      TTACTGGAGG GTAGATACAC GTAGAGGACC ATTTCATGTC TACACAGAAG GAAAGACTTT

 101  TAAATTCCCA ATTGAACTTT ATTTTTCATT TATAAGTTTG CAGCATTTAT TACAGCTTGA
      ATTTAAGGGT TAACTTGAAA TAAAAAGTAA ATATTCAAAC GTCGTAAATA ATGTCGAACT

 201  GGGTCAATGT TTTTTCTGTA TCTATCCTTT CTTTCAAGGA ATTTAAGTAG TCAGGGAGAT
      CCCAGTTACA AAAAAGACAT AGATAGGAAA GAAAGTTCCT TAAATTCATC AGTCCCTCTA

 301  TTACCTATAG TGTCTATTTT AAAGTAACAG GACATTCTAG CACACCTTTC TATCCCTTGT
      AATGGATATC ACAGATAAAA TTTCATTGTC CTGTAAGATC GTGTGGAAAG ATAGGGAACA

 401  CAGTAACTAC AAGACCATTT TGGAAACTGG GATTGTGGTG TTTATATGAA CATTCTTCTT
      GTCATTGATG TTCTGGTAAA ACCTTTGACC CTAACACCAC AAATATACTT GTAAGAAGAA

 501  TATATCAGTT AGAGACAGGG AGAGTTGGGT GGCAGTAAGG GATACAGAAA ATTTTAAATG
      ATATAGTCAA TCTCTGTCCC TCTCAACCCA CCGTCATTCC CTATGTCTTT TAAAATTTAC

 601  TTTTTTCCTT TACAGAATTC ATAGCAGGCT GAGTTTTTGT TCAGGCAATG TCGGGAAAGA
      AAAAAAGGAA ATGTCTTAAG TATCGTCCGA CTCAAAAACA AGTCCGTTAC AGCCCTTTCT

 701  TAACAACCAT TAATAAGGCA CTTAATCCAT GTTGTCTCTT ACAAATGTTA ACTCATTTCT
      ATTGTTGGTA ATTATTCCGT GAATTAGGTA CAACAGAGAA TGTTTACAAT TGAGTAAAGA

 801  TGCAAGTGAT ACTCTGCAAC TAAATACAGG CAGCACAGTT GCACGCTCTG TGTTTGGTTA
      ACGTTCACTA TGAGACGTTG ATTTATGTCC GTCGTGTCAA CGTGCGAGAC ACAAACCAAT

 901  CCCTGTGCTT CAGGCAGATG CCATTCCTTT CTCCTCTCCT CCATCCTACT CCACGTCTCA
      GGGACACGAA GTCCGTCTAC GGTAAGGAAA GAGGAGAGGA GGTAGGATGA GGTGCAGAGT

1001  TCTAAAACCT AATGACAAAT CAAAATACAT TATCTAAAAC TAGAAAAAGG GACAAAGGTG
      AGATTTTGGA TTACTGTTTA GTTTTATGTA ATAGATTTTG ATCTTTTTCC CTGTTTCCAC

1101  GCCCTGTTAA TGTTTCCAGA GAAAGATCCC CCAGTGCTGA ATACCAGAAA ATTCTATAGT
      CGGGACAATT ACAAAGGTCT CTTTCTAGGG GGTCACGACT TATGGTCTTT TAAGATATCA

1201  TCATCAGCAG GCAGCTGTTT GCTCCTTCAG TGGGAATGGT GTTTGCACAC CAGGTACCTA
      AGTAGTCGTC CGTCGACAAA CGAGGAAGTC ACCCTTACCA CAAACGTGTG GTCCATGGAT

1301  AGCCCATGAT CCTGCTGGAC CCAGCCCCTC CACAGCTTCA GAGTGACAGC CAGTGTCATA
      TCGGGTACTA GGACGACCTG GGTCGGGGAG GTGTCGAAGT CTCACTGTCG GTCACAGTAT

1401  CTCCAGGGGA TTTAGGAAGT TCCAACATTT CAGAGGGAGG AAGGACGTTA TGCAGGATAC
      GAGGTCCCCT AAATCCTTCA AGGTTGTAAA GTCTCCCTCC TTCCTGCAAT ACGTCCTATG

1501  CACTAACACT GAATTCAAAT TTTGACACTG ATAATCTGTT GCCACCAAAT GGAAAACGTA
      GTGATTGTGA CTTAAGTTTA AAACTGTGAC TATTAGACAA CGGTGGTTTA CCTTTTGCAT

1601  AGGAACAAGT AGAACATTCT TCTCTGGAAT CCGAGACGAT GGCTGTACTT TCACAGAGAG
      TCCTTGTTCA TCTTGTAAGA AGAGACCTTA GGCTCTGCTA CCGACATGAA AGTGTCTCTC

1701  TGAGAGAGGC AGAGACCGGG AGGTTAACAT AGAGGATAGA CTATATAGAG AGAGGATAGC
      ACTCTCTCCG TCTCTGGCCC TCCAATTGTA TCTCCTATCT GATATATCTC TCTCCTATCG

1801  GATAACTAAG GAGACAAAGC TCTCTTCCTC TAAAGAAGTC GTGCCTTTCC TGTCCTGGTT
      CTATTGATTC CTCTGTTTCG AGAGAAGGAG ATTTCTTCAG CACGGAAAGG ACAGGACCAA

1901  TACGCGGGGG GTCATCGGGG ACCTAAGGTC CCCCCTCACA GGCTGTATCT TCAGCGAGGT
      ATGCGCCCCC CAGTAGCCCC TGGATTCCAG GGGGGAGTGT CCGACATAGA AGTCGCTCCA

2001  TATCCCAAAG TCCCAGAGTG CACGGGGGTT ACTCTGGAGG GCGAGGCGTA GGCGTCACCA
      ATAGGGTTTC AGGGTCTCAC GTGCCCCCAA TGAGACCTCC CGCTCCGCAT CCGCAGTGGT

2101  GGGGCCGTCG CCTTCCTTCG GGACTCCGGA TCGATCTGGA GCTCCGGGAA TTTCCCTGGC
      CCCCGGCAGC GGAAGGAAGC CCTGAGGCCT AGCTAGACCT CGAGGCCCTT AAAGGGACCG

2201  AGGGTTCGCC GATCTTGGG
      TCCCAAGCGG CTAGAACCC
```

CXCL5 promoter

```
   1  CTTTTCTCAT GATCCATCTC AGATTTCACC ATTTGGCGCC AAAAACAAAT TCGTGTGTTC
      GAAAAGAGTA CTAGGTAGAG TCTAAAGTGG TAAACCGCGG TTTTTGTTTA AGCACACAAG

 101  CACACACACA CACACAGTTT AAAAGTCAAT AACCTATGTA ATTAAACTTC TTTTAAAAGA
      GTGTGTGTGT GTGTGTCAAA TTTTCAGTTA TTGGATACAT TAATTTGAAG AAAATTTTCT

 201  GAATTTAATT GCCCAAGATT TTTTGAAGTG TTATCACTTC ACCTAGGCTT AACTTTTGTC
      CTTAAATTAA CGGGTTCTAA AAAACTTCAC AATAGTGAAG TGGATCCGAA TTGAAAACAG

 301  TCTGCAGGCT GTTTTCTACA GATTACCACT CCCCAGAAAG ATGAAGAATT ACATTTTCTG
      AGACGTCCGA CAAAAGATGT CTAATGGTGA GGGGTCTTTC TACTTCTTAA TGTAAAAGAC

 401  CTTCTCATTT AGTTTCCCAC AGAGTAAGGC CCATACCACC AAACCTTACT CAGCCAGTGA
      GAAGAGTAAA TCAAAGGGTG TCTCATTCCG GGTATGGTGG TTTGGAATGA GTCGGTCACT

 501  TATGACATCT CTTTCTTGCT CACCAATGGA GGTGATGGAT GGGGGAGTTA ATAAAATTCC
      ATACTGTAGA GAAAGAACGA GTGGTTACCT CCACTACCTA CCCCCTCAAT TATTTTAAGG

 601  CTGGTTTCTC ATTGCCATCA TGTTTACTGA TTACCTTTGC TGGTCTGCTG TGAGGGCAAG
      GACCAAAGAG TAACGGTAGT ACAAATGACT AATGGAAACG ACCAGACGAC ACTCCCGTTC

 701  CATGAAAGAA AGCCTATGTG TCATTCCAAT ACAATAACCC ATTCCTGCCA TGGAGCAAGA
      GTACTTTCTT TCGGATACAC AGTAAGGTTA TGTTATTGGG TAAGGACGGT ACCTCGTTCT

 801  ACAGATAACA GAAGGAGAGA GGGAGAGGAA GCTGGAAACT ACTTCACCCT TCAATTTCAG
      TGTCTATTGT CTTCCTCTCT CCCTCTCCTT CGACCTTTGA TGAAGTGGGA AGTTAAAGTC

 901  GGAAACAGCG TTGGTCACCA GTAATCATGA AACTCGATGG TTCCAGAAAC CTGTGGGGAG
      CCTTTGTCGC AACCAGTGGT CATTAGTACT TTGAGCTACC AAGGTCTTTG GACACCCCTC

1001  GGGTGCTGTT GTGCTGCCAA ATTGATACAA ACCCCTACAA GAAGCTTTCA CATGAGCCCT
      CCCACGACAA CACGACGGTT TAACTATGTT TGGGGATGTT CTTCGAAAGT GTACTCGGGA

1101  AAATACATAT CTGGGGCTGG GAACAGAGCT GGGGCAGGCC TGGGATCCCT GGCAAGCTTT
      TTTATGTATA GACCCCGACC CTTGTCTCGA CCCCGTCCGG ACCCTAGGGA CCGTTCGAAA

1201  CAAAACCTCT TCCAATTCAA TAGCATAGCA ACTGCCAATA GCAAGGTATC TTCCATGGCA
      GTTTTGGAGA AGGTTAAGTT ATCGTATCGT TGACGGTTAT CGTTCCATAG AAGGTACCGT

1301  GGACACACGT ATACTTGCTA TATACTACAA GTACAGAATT CTCAGTAAGC GGACTTACCA
      CCTGTGTGCA TATGAACGAT ATATGATGTT CATGTCTTAA GAGTCATTCG CCTGAATGGT

1401  CCTTTCAGGC CACTGACTTC AAGTGGCAAG AGACAAGGGT CTCTTGTTAT CATGTTATCT
      GGAAAGTCCG GTGACTGAAG TTCACCGTTC TCTGTTCCCA GAGAACAATA GTACAATAGA

1501  CATTCAAGAA ACCTAGAATG ACCTGCCTGC AAGGAAGACA GGAAGGACTT TCAGTTTATA
      GTAAGTTCTT TGGATCTTAC TGGACGGACG TTCCTTCTGT CCTTCCTGAA AGTCAAATAT

1601  AATAATTAGA AAGGATTGAC TTTCAGAAAT TTTTCTCAAA TCAAGGCTCC TGTTACTTTG
      TTATTAATCT TTCCTAACTG AAAGTCTTTA AAAAGAGTTT AGTTCCGAGG ACAATGAAAC

1701  CCAGAGGGCT GCGTGCCTCA GAAAAGCCGG CATCCCTAGC CCGCTCTGGC ACAGGCCATG
      GGTCTCCCGA CGCACGGAGT CTTTTCGGCC GTAGGGATCG GGCGAGACCG TGTCCGGTAC

1801  CTGGAGCCAC AGCTCCCTCC ACCGCGGAGC AGGGTTACAA CGTCCCTCTC GGTAGAGGTG
      GACCTCGGTG TCGAGGGAGG TGGCGCCTCG TCCCAATGTT GCAGGGAGAG CCATCTCCAC

1901  TGTCTGGCCC CCCTCCCCCA ACCTCTTCTT TCCACACTGC CCCATGAGTT CAGGGAATTT
      ACAGACCGGG GGGAGGGGGT TGGAGAAGAA AGGTGTGACG GGGTACTCAA GTCCCTTAAA

2001  AGATGAGTGT AGATAAAAGG AGTGCAGAAG GCACGAGGAA GCCACAGTGC TCCGGATCCT
      TCTACTCACA TCTATTTTCC TCACGTCTTC CGTGCTCCTT CGGTGTCACG AGGCCTAGGA

2101  CAGGAACCCG CGACCGCTCG CAGCGCTCTC TTGACCACTA TGAGCCTCCT GT
      GTCCTTGGGC GCTGGCGAGC GTCGCGAGAG AACTGGTGAT ACTCGGAGGA CA
```

GALNAC promoter

```
   1  GTATAAATAC CTTTGGAAAG TCCCTGGTGG AATTCATATC AAAGCGGCAC CGTGATTTTT
      CATATTTATG GAAACCTTTC AGGGACCACC TTAAGTATAG TTTCGCCGTG GCACTAAAAA

 101  ACACCTGAAT TTGAAAGTGA TCTGGGTGGG CCCAGCGCCA TCTCCATAAC GGAGTTATGT
      TGTGGACTTA AACTTTCACT AGACCCACCC GGGTCGCGGT AGAGGTATTG CCTCAATACA

 201  CAGTATGTGT GGCCCTGGAA TAATGTTCCA CACACCCTTA CAGAAGGGGA TTTCCTCCTT
      GTCATACACA CCGGGACCTT ATTACAAGGT GTGTGGGAAT GTCTTCCCCT AAAGGAGGAA

 301  TTGCACACTG GTCTGACAGC TGCCTGGCCC AGGGCCTTCC TTGCAGACGC ATGTACAACC
      AACGTGTGAC CAGACTGTCG ACGGACCGGG TCCCGGAAGG AACGTCTGCG TACATGTTGG

 401  AATTACTCGA GTATATAAAC CTCACTGAAA GGCAGGCTTT TCTTGGGAGC TAATAAATAA
      TTAATGAGCT CATATATTTG GAGTGACTTT CCGTCCGAAA AGAACCCTCG ATTATTTATT

 501  ATCATCATCT ATTGGGAATT GTGAACCCTG CAAAAAGCAG CTCGTTGTAT TTTCAGAAAT
      TAGTAGTAGA TAACCCTTAA CACTTGGGAC GTTTTTCGTC GAGCAACATA AAAGTCTTTA

 601  AAGTGGAGTC TATATGGAAA AAGCAATCTA GTCTGAGATT CTAAGGCAAG TCTCTTTGCT
      TTCACCTCAG ATATACCTTT TTCGTTAGAT CAGACTCTAA GATTCCGTTC AGAGAAACGA

 701  TTTGCAGGAA AACAAGACAG GATTTTAAAG AACTCCCCTC TCATCCCGGT TTCCCATCTG
      AAACGTCCTT TTGTTCTGTC CTAAAATTTC TTGAGGGGAG AGTAGGGCCA AAGGGTAGAC

 801  ATGGGTGCTG AGCAGAAGTT TATGACCTCA GGGTGGACTG TAGCTTCAGG GGGGCGAGAT
      TACCCACGAC TCGTCTTCAA ATACTGGAGT CCCACCTGAC ATCGAAGTCC CCCCGCTCTA

 901  GTCAGGAATG ACCCTTCGGT GGCATGTAGT CTACAGTTAA CGGAGCACTT TCATTTCCAC
      CAGTCCTTAC TGGGAAGCCA CCGTACATCA GATGTCAATT GCCTCGTGAA AGTAAAGGTG

1001  CGGGGACACA GGCCGGCAAG AGACTGCAGA TTCTTTTTGT CCCTTGGACC TCAGTTTCCT
      GCCCCTGTGT CCGGCCGTTC TCTGACGTCT AAGAAAAACA GGGAACCTGG AGTCAAAGGA

1101  TCTGCCAGAA TTTCATGTGT CTTTTCTTTA ACTGATGGCA TCTTCAGCCC AGAGTCAGTG
      AGACGGTCTT AAAGTACACA GAAAAGAAAT TGACTACCGT AGAAGTCGGG TCTCAGTCAC

1201  CAGGTGGTTC CCTGGGGCAG CCGACTCCAG GCCACTGCGG CAGGCCGTGG CGATCGGGGT
      GTCCACCAAG GGACCCCGTC GGCTGAGGTC CGGTGACGCC GTCCGGCACC GCTAGCCCCA

1301  GGGCCAAAGA GCCGGGCCAG CCTTCCCGGG ATGCAAAAAT ACAACAGAGG GCGCCGAACT
      CCCGGTTTCT CGGCCCGGTC GGAAGGGCCC TACGTTTTTA TGTTGTCTCC CGCGGCTTGA

1401  TCCAGACCGC CAAGTTCCTT AAAGATGTCT CTGGCGGCCG GGCGCGGTGG CTTACGCCTG
      AGGTCTGGCG GTTCAAGGAA TTTCTACAGA GACCGCCGGC CCGCGCCACC GAATGCGGAC

1501  TGAGGTCGGG AGTTCGAGAC CAGCCTGACC AACATGGAGA AACCCCGTCT GTACTAAAAA
      ACTCCAGCCC TCAAGCTCTG GTCGGACTGG TTGTACCTCT TTGGGGCAGA CATGATTTTT

1601  ATCCCGGCTA CTCGGGAGGC TGAGGCAGGA GACTCTCTTG AACCCGGGAG GCTGAGGCAG
      TAGGGCCGAT GAGCCCTCCG ACTCCGTCCT CTGAGAGAAC TTGGGCCCTC CGACTCCGTC

1701  CGAGATCGCG CCATTGCATT CCAGCCTGGG CAACAAGAGC GAAACTCTGT CTTTAAAAAA
      GCTCTAGCGC GGTAACGTAA GGTCGGACCC GTTGTTCTCG CTTTGAGACA GAAATTTTTT

1801  TTTCGATGTG CTTTTATTTG GAATCACCGC AACACACAAG CAGTGTTGAC TCGGAGGGGG
      AAAGCTACAC GAAAATAAAC CTTAGTGGCG TTGTGTGTTC GTCACAACTG AGCCTCCCCC

1901  TGATCTCACA TTCCAGACTG GAGCTGATGT GTGTCAGCGC GACATGGGGC GGGGGGAGGG
      ACTAGAGTGT AAGGTCTGAC CTCGACTACA CACAGTCGCG CTGTACCCCG CCCCCCTCCC

2001  CAAACTTTTC CTCCCAGCGC TCGGGGAGTC AGGTTGACGC GCCCTGGATG CGGGGCTAGA
      GTTTGAAAAG GAGGGTCGCG AGCCCCTCAG TCCAACTGCG CGGGACCTAC GCCCCGATCT

2101  GAGGCTCCCG GGCTGGACTC AGGCTTCTCA GGTACAAGTT AGGT
      CTCCGAGGGC CCGACCTGAG TCCGAAGAGT CCATGTTCAA TCCA
```

GAPDH promoter

```
   1  ACTCGTGTTC CCTAATCCCA TCTCCAAAGG TTGGCAGCAG ACCGGCCCAG GCTCGTGGAA
      TGAGCACAAG GGATTAGGGT AGAGGTTTCC AACCGTCGTC TGGCCGGGTC CGAGCACCTT
 101  CCTGTCTCCC AGCATTCCTC ATTGTTCCCG GGTCTGCAGA GGGCGTGAGC TATGCTGCAG
      GGACAGAGGG TCGTAAGGAG TAACAAGGGC CCAGACGTCT CCCGCACTCG ATACGACGTC
 201  CTCAAGTCTT CTCTGCTGAG TCACCTTCGA ACCGGAGGCT GTGAGCTGGC TGTCGTGACC
      GAGTTCAGAA GAGACGACTC AGTGGAAGCT TGGCCTCCGA CACTCGACCG ACAGCACTGG
 301  CAGTAGTGCT CCCTGGGCAC TGAGCTCCCT CTTTGCGGGG AGAAGACAGT AATGAAAAAT
      GTCATCACGA GGGACCCGTG ACTCGAGGGA GAAACGCCCC TCTTCTGTCA TTACTTTTTA
 401  GCAGGAGCAT GGAGGTGCTC TTAATGCTCT CAATGAGAAA GGGTTAACGG TCCTGGTTGC
      CGTCCTCGTA CCTCCACGAG AATTACGAGA GTTACTCTTT CCCAATTGCC AGGACCAACG
 501  CACCCCACCC CTTTCACCAT TAGGGACCTT CTTGCCTTGC TCTTGCTACT CTGCTCTGGG
      GTGGGGTGGG GAAAGTGGTA ATCCCTGGAA GAACGGAACG AGAACGATGA GACGAGACCC
 601  GCCAGACGAG GACACAGCCT GGCTCTGGGT CCCAGCAGGA AAGGCAATCC CAGAAAGGCA
      CGGTCTGCTC CTGTGTCGGA CCGAGACCCA GGGTCGTCCT TTCCGTTAGG GTCTTTCCGT
 701  GATTATCACC AGGCAGGCTA AACTTAGCAA CCGGCTTTTA GCTAGAAGGG CAGGGGGCTG
      CTAATAGTGG TCCGTCCGAT TTGAATCGTT GGCCGAAAAT CGATCTTCCC GTCCCCCGAC
 801  CCCTCCCATG CACCTGCTGA TGGGCCAAGG CCACCCCACC CCACCCCCTT CCTTACAAGT
      GGGAGGGTAC GTGGACGACT ACCCGGTTCC GGTGGGGTGG GGTGGGGGAA GGAATGTTCA
 901  CTGCCCTCCT ACCAGAAGAA TGGATCCCCT GTGGGAGGGG GCAGGGGACC TGTTCCCACC
      GACGGGAGGA TGGTCTTCTT ACCTAGGGGA CACCCTCCCC CGTCCCCTGG ACAAGGGTGG
1001  GACTCACCCT GCCCTCAATA TCCCCCGGCG CAGCCAGTGA AAGGGAGTCC CTGGCTCCTG
      CTGAGTGGGA CGGGAGTTAT AGGGGGCCGC GTCGGTCACT TTCCCTCAGG GACCGAGGAC
1101  TCACGTCCCG CTCTTCGCCC CAGGCTGGAT GGAATGAAAG GCACACTGTC TCTCTCCCTA
      AGTGCAGGGC GAGAAGCGGG GTCCGACCTA CCTTACTTTC CGTGTGACAG AGAGAGGGAT
1201  AGGCCTCTGG GCCCCCACCA GCCATCCTGT CCTCCGCCTG GGGCCCCAGC CCGGAGAGAG
      TCCGGAGACC CGGGGGTGGT CGGTAGGACA GGAGGCGGAC CCCGGGGTCG GGCCTCTCTC
1301  TCAGGTCCAG GCTACAGGGC TGCAGGACAT CGTGACCTTC CGTGCAGAAA CCTCCCCCTC
      AGTCCAGGTC CGATGTCCCG ACGTCCTGTA GCACTGGAAG GCACGTCTTT GGAGGGGGAG
1401  CCCAGTGCCC AGTGCCCAGT GCCCAGCCCA GGCCTCGGTC CCAGAGATGC CAGGAGCCAG
      GGGTCACGGG TCACGGGTCA CGGGTCGGGT CCGGAGCCAG GGTCTCTACG GTCCTCGGTC
1501  GGCCCCCGCC CGAGGCCCAT GGGCCCCTCC TAGGCCTTTG CCTGAGCAGT CCGGTGTCAC
      CCGGGGGCGG GCTCCGGGTA CCCGGGGAGG ATCCGGAAAC GGACTCGTCA GGCCACAGTG
1601  CTCTCAGCCT TTGAAAGAAA GAAAGGGGAG GGGGCAGGCC GCGTGCAGCC GCGAGCGGTG
      GAGAGTCGGA AACTTTCTTT CTTTCCCCTC CCCCGTCCGG CGCACGTCGG CGCTCGCCAC
1701  AGTCCTCCTG TTTCATCCAA GCGTGTAAGG GTCCCCGTCC TTGACTCCCT AGTGTCCTGC
      TCAGGAGGAC AAAGTAGGTT CGCACATTCC CAGGGGCAGG AACTGAGGGA TCACAGGACG
1801  CCATCGGGCC AATCTCAGTC CCTTCCCCCC TACGTCGGGG CCCACACGCT CGGTGCGTGC
      GGTAGCCCGG TTAGAGTCAG GGAAGGGGGG ATGCAGCCCC GGGTGTGCGA GCCACGCACG
1901  GAAAGTAGGG CCCGGCTACT AGCGGTTTTA CGGGCGCACG TAGCTCAGGC CTCAAGACCT
      CTTTCATCCC GGGCCGATGA TCGCCAAAAT GCCCGCGTGC ATCGAGTCCG GAGTTCTGGA
2001  AGTCACCGCC TGCCGCCGCG CCCCCGGTTT CTATAAATTG AGCCCGCAGC CTCCCGCTTC
      TCAGTGGCGG ACGGCGGCGC GGGGGCCAAA GATATTTAAC TCGGGCGTCG GAGGGCGAAG
2101  TTTGCGTCGC CAGGTGAAGA CGGGCGGAGA GAAACCCGGG AGGCTAGGGA CGGCCTGAAG
      AAACGCAGCG GTCCACTTCT GCCCGCCTCT CTTTGGGCCC TCCGATCCCT GCCGGACTTC
2201  GCGGGGTGGG CCCGGGCGGC CTCCGCATTG CAGGGGCGGG CGGAGGACGT GATGCGGCGC
      CGCCCCACCC GGGCCCGCCG GAGGCGTAAC GTCCCCGCCC GCCTCCTGCA CTACGCCGCG
2301  CGTGTGTGTC GGCCGGGGCC ACTAGGCGCT CACTGTTCTC TCCCTCC
      GCACACACAG CCGGCCCCGG TGATCCGCGA GTGACAAGAG AGGGAGG
```

ID1 promoter

```
   1  GTCGTGTGGA GCCTCAGTTT CCTTTGTATA AAGAAACCAA AGAGCTCCTT TCTTTAGAGT
      CAGCACACCT CGGAGTCAAA GGAAACATAT TTCTTTGGTT TCTCGAGGAA AGAAATCTCA

 101  AGTAAGACCC TGTATCATTA TTATTTTATT TTGAAACAGA GTCCCACTCT GTCGCCCAGG
      TCATTCTGGG ACATAGTAAT AATAAAATAA AACTTTGTCT CAGGGTGAGA CAGCGGGTCC

 201  GCCTCCTGGG TTCCAGCGAG TCTCCTGCCT CAGCCTCCTG AGTAGCTGGG GTATTACAGG
      CGGAGGACCC AAGGTCGCTC AGAGGACGGA GTCGGAGGAC TCATCGACCC CATAATGTCC

 301  AGACGGGATT TCACCATGTT GGTCAGGCTG GTCTCGAACT CCTGACCTCG TGATCTGCCC
      TCTGCCCTAA AGTGGTACAA CCAGTCCGAC CAGAGCTTGA GGACTGGAGC ACTAGACGGG

 401  ACTGCGCCCG GCCCCCTGTA TCCTTTTTTT TTTTTTTTTT TTTGAGACGA GTCTCACTCT
      TGACGCGGGC CGGGGGACAT AGGAAAAAAA AAAAAAAAAA AAACTCTGCT CAGAGTGAGA

 501  GCAACCTCCG CCTCCCAGGT TCAAGCAATT CTCCTGTCTC AGCCTCCCGA GTAGCTGGGA
      CGTTGGAGGC GGAGGGTCCA AGTTCGTTAA GAGGACAGAG TCGGAGGGCT CATCGACCCT

 601  TTTAGTAGAG ATGGGGTTTC ACCATATTGG TCAGGCTGGT CTCGAACTCC TGACCTCAGG
      AAATCATCTC TACCCCAAAG TGGTATAACC AGTCCGACCA GAGCTTGAGG ACTGGAGTCC

 701  GGCGTGAGCC ACCGCGCAGC CCTTCATTTT TTAAAATAAG AAATAAATGA AAAAAGATGA
      CCGCACTCGG TGGCGCGTCG GGAAGTAAAA AATTTTATTC TTTATTTACT TTTTTCTACT

 801  AGAAATTAAT AATGGTCACG TTTGTGATTA TTATTTAAAC ACCAAGCAGA TATTAAATAT
      TCTTTAATTA TTACCAGTGC AAACACTAAT AATAAATTTG TGGTTCGTCT ATAATTTATA

 901  AGCTGCAGAG CTGGAAAGAG AACTCAGGCC TTTTTCCCCA CGCTGGAAGG GGTAGCTGGG
      TCGACGTCTC GACCTTTCTC TTGAGTCCGG AAAAAGGGGT GCGACCTTCC CCATCGACCC

1001  CCAAGCTGTG GGTCTGGGTT GGGAGACTCG CAGGTGTGGG GCGGGGAGGT AAGGTGACCC
      GGTTCGACAC CCAGACCCAA CCCTCTGAGC GTCCACACCC CGCCCCTCCA TTCCACTGGG

1101  CTCCCGCCCG GGGTCTGCAG GTGACGGGCT GGGGGGAGCA CGGGAACTAG CTAGACCAGT
      GAGGGCGGGC CCCAGACGTC CACTGCCCGA CCCCCCTCGT GCCCTTGATC GATCTGGTCA

1201  ACAGTCCGTC CGGGTTTTAT GAATGGGTGA CGTCACAGGC CTGGCGTCTA ACGGTCTGAG
      TGTCAGGCAG GCCCAAAATA CTTACCCACT GCAGTGTCCG GACCGCAGAT TGCCAGACTC

1301  AGGGGGGAGA CTGTAGCTCC GCAGCTGCCG CGCCGTGGGA GGGAGACCCT GCTCTGAGGT
      TCCCCCCTCT GACATCGAGG CGTCGACGGC GCGGCACCCT CCCTCTGGGA CGAGACTCCA

1401  AAACATTAAA AAATCACGAA CTGTTGCAGG TTCAGGAAAT TTTTGCAAGG AGCTGCAAAT
      TTTGTAATTT TTTAGTGCTT GACAACGTCC AAGTCCTTTA AAAACGTTCC TCGACGTTTA

1501  ATCTAGTTCA CTTCCCAGCC ACCCAGCCCC AAACTTACTA GACTTTCCCG AATTAATTGC
      TAGATCAAGT GAAGGGTCGG TGGGTCGGGG TTTGAATGAT CTGAAAGGGC TTAATTAACG

1601  GGAACACGAA CAGCAACATT ATTTAGGAAT TGAGAAAGCC CAGGGGAGCA GATGTAAAGG
      CCTTGTGCTT GTCGTTGTAA TAAATCCTTA ACTCTTTCGG GTCCCCTCGT CTACATTTCC

1701  GGGTCCGAGA AGCATCTTCC AAGAGGGTCC GGAAACCCAG GCTCTCTGGG CTATCCAGAG
      CCCAGGCTCT TCGTAGAAGG TTCTCCCAGG CCTTTGGGTC CGAGAGACCC GATAGGTCTC

1801  TGAAATGGAG CTGGAGAAGT GAGAAAGTGA ATATGGGAAA AACTGATTCC TGAGGTCTTT
      ACTTTACCTC GACCTCTTCA CTCTTTCACT TATACCCTTT TTGACTAAGG ACTCCAGAAA

1901  CCAGAGGAGC CCAGTGCGGC CCTCGCCTCT ACCCGCTGCC CTGCCCGGTG CCCTGCCACC
      GGTCTCCTCG GGTCACGCCG GGAGCGGAGA TGGGCGACGG GACGGGCCAC GGGACGGTGG

2001  CACTGCGAGC AGGCACTAGA CGAGCAGGAG GCTGGACCTA GGAGCGCGGG TCACGCCCCC
      GTGACGCTCG TCCGTGATCT GCTCGTCCTC CGACCTGGAT CCTCGCGCCC AGTGCGGGGG

2101  CCTCCGGGGG CCGTGGCGTG TTTATAAAAG ACAAGCTGTG GCTCCGCACT CTCATTCCAC
      GGAGGCCCCC GGCACCGCAC AAATATTTTC TGTTCGACAC CGAGGCGTGA GAGTAAGGTG

2201  CACCTCATTT TTTTCGCTTT GCCCATTCTG TTTCAGCCAG TCGCCAAGAA TC
      GTGGAGTAAA AAAAGCGAAA CGGGTAAGAC AAAGTCGGTC AGCGGTTCTT AG
```

IGSF4 promoter

```
   1  AGGATTCTTG CCATATAGGA CCTGCCTTTA TAGTTTACAT ATCACTTTCA GCATACATAT
      TCCTAAGAAC GGTATATCCT GGACGGAAAT ATCAAATGTA TAGTGAAAGT CGTATGTATA

 101  TCGCCCAGGC TGGAGTGCAA TGATGCGATC TCAGCTCACT GCAACCTCCG CCTCCTGGGT
      AGCGGGTCCG ACCTCACGTT ACTACGCTAG AGTCGAGTGA CGTTGGAGGC GGAGGACCCA

 201  TTACAGGTGT GTGTCATCAC GCCTGGCTAA TTTTTGTATT TTTATTAGAG ACGGAGTTTC
      AATGTCCACA CACAGTAGTG CGGACCGATT AAAAACATAA AAATAATCTC TGCCTCAAAG

 301  TGATCCATCT GCCTCAACCT CCCAAAGTGC TGGGATTACA GGCATCAGTC AGCGTGCCCG
      ACTAGGTAGA CGGAGTTGGA GGGTTTCACG ACCCTAATGT CCGTAGTCAG TCGCACGGGC

 401  GCTCTCTATT TGGTCCGACC CAGGTACCTC ACTGCCAGAG GTAGTAAGAA CGGGTAGGAC
      CGAGAGATAA ACCAGGCTGG GTCCATGGAG TGACGGTCTC CATCATTCTT GCCCATCCTG

 501  GTTTCCTGTC TCCTGTAGTG CTGTTCTCCT GTTGGAGCTT TCCCCACTTC TGAAGGACTG
      CAAAGGACAG AGGACATCAC GACAAGAGGA CAACCTCGAA AGGGGTGAAG ACTTCCTGAC

 601  ATATCCAGGG CTGTGAAGAC CGGGCAGTCT CGAAATTAGT TTATGCATAA TGGATAATAG
      TATAGGTCCC GACACTTCTG GCCCGTCAGA GCTTTAATCA AATACGTATT ACCTATTATC

 701  ATCATGACTT CCTTTTTGAT ATGTATTATT TATTTATTTT ATAGCAAGAT GAGGACAGAA
      TAGTACTGAA GGAAAAACTA TACATAATAA ATAAATAAAA TATCGTTCTA CTCCTGTCTT

 801  CTCAGACTTA GCAATTTACA TTCAGTAATC TCTTGGTCAT TTAGCCACAG ATAATTTGTC
      GAGTCTGAAT CGTTAAATGT AAGTCATTAG AGAACCAGTA AATCGGTGTC TATTAAACAG

 901  CAAATTGTAT TTCCTACATC TTATTGTTCT GCATTCTATG AATACTTAAA TTATTATAAA
      GTTTAACATA AAGGATGTAG AATAACAAGA CGTAAGATAC TTATGAATTT AATAATATTT

1001  GAGCAACAGA GGGTCAGAGC GCACACGATC TTCTAGAGGG GAAGAAAATA AGTAATTAAA
      CTCGTTGTCT CCCAGTCTCG CGTGTGCTAG AAGATCTCCC CTTCTTTTAT TCATTAATTT

1101  AAGCAAAGAG AATTTCGGGG AGGGAGAGTG AGTCCGGAGA TGCAAGTCGC TTTAGCAGGA
      TTCGTTTCTC TTAAAGCCCC TCCCTCTCAC TCAGGCCTCT ACGTTCAGCG AAATCGTCCT

1201  GGTCCCCAGC TTCCTTAGGA AGGGCTGGGG GCGCAGAGGA GGAGTGAGAG GGTTTGAGCC
      CCAGGGGTCG AAGGAATCCT TCCCGACCCC CGCGTCTCCT CCTCACTCTC CCAAACTCGG

1301  CCTCTCCCCA ACTCCCACCA CCCCCGCTCT TCACCTGAAG CCTTGACTAA TTTTTTCCGT
      GGAGAGGGGT TGAGGGTGGT GGGGGCGAGA AGTGGACTTC GGAACTGATT AAAAAAGGCA

1401  CACATATATT CAACACACAC CTATATATTA AAACCAGGGA GGAGACCCTC GACAAGCGGA
      GTGTATATAA GTTGTGTGTG GATATATAAT TTTGGTCCCT CCTCTGGGAG CTGTTCGCCT

1501  TTCTGCCGCA AAAAGACCGA CTGGAAAATC TCAGAACCCG ACTCTACGGC TGCCTTCTCC
      AAGACGGCGT TTTTCTGGCT GACCTTTTAG AGTCTTGGGC TGAGATGCCG ACGGAAGAGG

1601  TCCCGCTCCG CCGGACGTGC AAAGCACGCA TGCACTTCTC CCAGATTGTT TTGTCAATCC
      AGGGCGAGGC GGCCTGCACG TTTCGTGCGT ACGTGAAGAG GGTCTAACAA AACAGTTAGG

1701  GTTCCCAAAG ATCTATTCCT TCGGTGCAAG GTGAGTGACG GAAATTTGCA ACGTCTGGTT
      CAAGGGTTTC TAGATAAGGA AGCCACGTTC CACTCACTGC CTTTAAACGT TGCAGACCAA

1801  TCTTAAGGGA GATTCTCCAG TCGTCGGTCT GATACAGCGA TTGCTATAAA CATTCCTAAT
      AGAATTCCCT CTAAGAGGTC AGCAGCCAGA CTATGTCGCT AACGATATTT GTAAGGATTA

1901  GTCCTTGCAC GCCAGGCGCC CGGGAGAACA CTTTTTCCTT GATCCGGGGA AAGCAAAACC
      CAGGAACGTG CGGTCCGCGG GCCCTCTTGT GAAAAAGGAA CTAGGCCCCT TTCGTTTTGG

2001  TGGCCCCGCC CCTAGGTGGC GCGGGCGCGC CGCCGAACGC CAGCGCCAGG GGGCGGGGTG
      ACCGGGGCGG GGATCCACCG CGCCCGCGCG GCGGCTTGCG GTCGCGGTCC CCCGCCCCAC

2101  CGGCGCTGTG ATTGGTCTGC CCGGACTCCG CCTCCAGCGC ATGTCATTAG CATCTCATTA
      GCCGCGACAC TAACCAGACG GGCCTGAGGC GGAGGTCGCG TACAGTAATC GTAGAGTAAT

2201  CTGAGGCAGG TGCCCGACAT GGCGAGTGTA GTGCTGCCGA GCGGATCCCA GTGTGCGGCG
      GACTCCGTCC ACGGGCTGTA CCGCTCACAT CACGACGGCT CGCCTAGGGT CACACGCCGC

2301  TTCTGCTGTT GCTCTTCTCC GCCGCGGCAC TGATCCCCAC AGGTAGGTGT GGGCACCGAC
      AAGACGACAA CGAGAAGAGG CGGCGCCGTG ACTAGGGGTG TCCATCCACA CCCGTGGCTG

2401  GGCCACAGAG GGGGGAAACG TTTCCATGAC AACCTCCCCC CACTTCCTAC ATCCGAAGCG
      CCGGTGTCTC CCCCCTTTGC AAAGGTACTG TTGGAGGGGG GTGAAGGATG TAGGCTTCGC
```

```
   1  GAGAGAGAGC TCTTACCTGA GGCGATGAGG TCATGTCCCA CCGTGGGGGG GCTGTGACCT
      CTCTCTCTCG AGAATGGACT CCGCTACTCC AGTACAGGGT GGCACCCCCC CGACACTGGA

 101  CTGGATTGGG AAGGGGAAGC CTCCAACCAG CCCGGCAGTC TCGGAGGCTG ACACGAGGGG
      GACCTAACCC TTCCCCTTCG GAGGTTGGTC GGGCCGTCAG AGCCTCCGAC TGTGCTCCCC

 201  TCAGTCCCAT CCTGTCCTTC CCTGCCATCT AGGTCTGGGG GCTCTCGGGG ACATGGCTTG
      AGTCAGGGTA GGACAGGAAG GGACGGTAGA TCCAGACCCC CGAGAGCCCC TGTACCGAAC

 301  CTCCTCTGCC CCCATGCCTG GGGCTGGCCC ACTGTGAGCC TGGGTAGGCG TCGCTGCCTC
      GAGGAGACGG GGGTACGGAC CCCGACCGGG TGACACTCGG ACCCATCCGC AGCGACGGAG

 401  CTGCCTCCAC CTCTGTGCGG TGACGTCCAG GGCTCTGGCC CCGGTGGTTC TGGCCACTGT
      GACGGAGGTG GAGACACGCC ACTGCAGGTC CCGAGACCGG GGCCACCAAG ACCGGTGACA

 501  CTGCCCCATC TCTCTCGAAG GCGACATCTT TTTCTCTGTG TCCCTGATGT GTTCAGAAAA
      GACGGGGTAG AGAGAGCTTC CGCTGTAGAA AAAGAGACAC AGGGACTACA CAAGTCTTTT

 601  CTTCACGTGA ATGAGCTCGC TTTATCCTCA TCGTGGCATT TAGGCGGATG AGGGTGCATC
      GAAGTGCACT TACTCGAGCG AAATAGGAGT AGCACCGTAA ATCCGCCTAC TCCCACGTAG

 701  GGGGAGAGTC GCACATGGAG GTCTTGCCTC TATGGAGTGT CTCAGCGCCT CTTGGTCCTC
      CCCCTCTCAG CGTGTACCTC CAGAACGGAG ATACCTCACA GAGTCGCGGA GAACCAGGAG

 801  CTTTGAGAAA GGCTTTTCAA AAGGCCAGGC GTGGTGGTTC ACACCTGTAT TCCCACCACT
      GAAACTCTTT CCGAAAAGTT TTCCGGTCCG CACCACCAAG TGTGGACATA AGGGTGGTGA

 901  TTGAGACCAG CCTGGGCAAC ATGGCAAAAC CCTATCTCTA CTAAAAATAC AAAAAATAGC
      AACTCTGGTC GGACCCGTTG TACCGTTTTG GGATAGAGAT GATTTTTATG TTTTTTATCG

1001  AGGCTGAGGC ATGAGAATCA CTTGAACCTG GGAGGCGGAG GTTACAGTGA GCTGAGATCA
      TCCGACTCCG TACTCTTAGT GAACTTGGAC CCTCCGCCTC CAATGTCACT CGACTCTAGT

1101  TCTCAAAAAA ACCAAAAACG AGGCCAGGCA CGGTAGCTCA CACCTGTCAT CCCAGCACTT
      AGAGTTTTTT TGGTTTTTGC TCCGGTCCGT GCCATCGAGT GTGGACAGTA GGGTCGTGAA

1201  AGACCAGCCT GGCCAACATG GTAAGACCCC GTCTCTACTA AAAATACAAA ATTAGCCGGG
      TCTGGTCGGA CCGGTTGTAC CATTCTGGGG CAGAGATGAT TTTTATGTTT TAATCGGCCC

1301  TGAGGCAGGA GAATCGCTTG AACCCGGGAG GTGGAGGTTG CAGTGAGCTG AGATTGTGCC
      ACTCCGTCCT CTTAGCGAAC TTGGGCCCTC CACCTCCAAC GTCACTCGAC TCTAACACGG

1401  AGACTCAGTA CCAAAAAACA AACAAACAAA AAACAAACAA AAAATGAGAA AGGCTTTTAC
      TCTGAGTCAT GGTTTTTTGT TTGTTTGTTT TTTGTTTGTT TTTTACTCTT TCCGAAAATG

1501  CTGTCTTTCT AATATCTCTG TCTCCCCTTT TCTGTCCCTG GGGCCTCTCC GTCCCTGTCA
      GACAGAAAGA TTATAGAGAC AGAGGGGAAA AGACAGGGAC CCCGGAGAGG CAGGGACAGT

1601  CTGCGGCATC CTCTGTCTCA GAGTCTTGGT GTCTCTGTTC CTTTCCCCTC GGGGTCTCCC
      GACGCCGTAG GAGACAGAGT CTCAGAACCA CAGAGACAAG GAAAGGGGAG CCCCAGAGGG

1701  TCTGATCTCT GACTCCCAGA ACCTCTCCCT CTGTCTCCAG GGCTGCCCCT CTGATCCTCT
      AGACTAGAGA CTGAGGGTCT TGGAGAGGGA GACAGAGGTC CCGACGGGGA GACTAGGAGA

1801  TGGATCTCCA TCTCCCTGTC TCTGTCTCAG TCTGTCCTTC ACTCTGTGTG TGTGTGTGTC
      ACCTAGAGGT AGAGGGACAG AGACAGAGTC AGACAGGAAG TGAGACACAC ACACACACAG

1901  CTCCACCTCT CCAGGCCCCT GTCTTGTCCC TCCGTCCGGC CTTTCTCTGC CTTTCCGTCC
      GAGGTGGAGA GGTCCGGGGA CAGAACAGGG AGGCAGGCCG GAAAGAGACG GAAAGGCAGG

2001  GGACCCCCAC CCACAGTCGG GCCCCAGCGC TTGAGCCTGA GTGTCTGCTC CGGCCCGTGG
      CCTGGGGGTG GGTGTCAGCC CGGGGTCGCG AACTCGGACT CACAGACGAG GCCGGGCACC

2101  TCCGACCACC CCCCCCTTTC CCTTTTCAAC TTTTCCAACT TTTCCTTCCG TGCCCTCCTC
      AGGCTGGTGG GGGGGGAAAG GGAAAAGTTG AAAAGGTTGA AAAGGAAGGC ACGGGAGGAG

2201  CTGAATGGAA AAGGCAGGCA GGGAGGGTGA GTCAGGATGT GTCAGGCCGC CCTCCCCTGC
      GACTTACCTT TTCCGTCCGT CCCTCCCACT CAGTCCTACA CAGTCCGGCG GGAGGGGACG

2301  CCCCCAGGCG TCCACACTCC CTCACTGCCG CGGCCCTGCT GCTCAGGGCA CATGCCTCCC
      GGGGGTCCGC AGGTGTGAGG GAGTGACGGC GCCGGGACGA CGAGTCCCGT GTACGGAGGG

2401  GGCAGCGGAC AGGGAAGGGT TAAAGGCCCC CGGCTCCCTG CCCCCTGCCC TGGGGAACCC
      CCGTCGCCTG TCCCTTCCCA ATTTCCGGGG GCCGAGGGAC GGGGGACGGG ACCCCTTGGG
```

MMP3 promoter

```
   1  AAAATAGAGT AGCAGAGGCA GGTACAAGGT ACCATTGAAC AATGATTAAT GACTCTTCCT
      TTTTATCTCA TCGTCTCCGT CCATGTTCCA TGGTAACTTG TTACTAATTA CTGAGAAGGA

 101  TTTTAAAATA TAGCAAACTT TTGTATTTAG TTCAGGAACA GCATGGCCCA TTTTGCCAAT
      AAAATTTTAT ATCGTTTGAA AACATAAATC AAGTCCTTGT CGTACCGGGT AAAACGGTTA

 201  GGCTTTCCTC TAAACTTTAA ATATGTTTTA TAAGTTATAA CTCCAGAGAA AATTTACAAA
      CCGAAAGGAG ATTTGAAATT TATACAAAAT ATTCAATATT GAGGTCTCTT TTAAATGTTT

 301  TTCTACACTT TTAACCTCTC AATATTTTAT CTGTTGGGCT CCACTGTTTC TTCCTGGAAT
      AAGATGTGAA AATTGGAGAG TTATAAAATA GACAACCCGA GGTGACAAAG AAGGACCTTA

 401  CAATGTGGCC AAATATTTTC CCTGTATTTC AATCAGGACA AGACATGGTT TTTCCCCCCA
      GTTACACCGG TTTATAAAAG GGACATAAAG TTAGTCCTGT TCTGTACCAA AAAGGGGGGT

 501  TCTTTAGGCC AGGTGCCGTG ACCCATGTCT GTAATCCTAG CACTTTGAGA GGTTGAGGCA
      AGAAATCCGG TCCACGGCAC TGGGTACAGA CATTAGGATC GTGAAACTCT CCAACTCCGT

 601  AACATAGTGA AACCTCTGTC TCTATTTTTT AAATAAAATT TGAAAAAGTC TTTAGACATA
      TTGTATCACT TTGGAGACAG AGATAAAAAA TTTATTTTAA ACTTTTTCAG AAATCTGTAT

 701  CCCTGCCAAG TGGCTATCAA CTGTGTGGGC ATCTTCAGTC ATAGGGATCT TATTGCCACA
      GGGACGGTTC ACCGATAGTT GACACACCCG TAGAAGTCAG TATCCCTAGA ATAACGGTGT

 801  GTTTATTAAG AAACACACAA AAAATAAAGC AAAGAAGAAA ATGCAAAAGA GTTATAAATG
      CAAATAATTC TTTGTGTGTT TTTTATTTCG TTTCTTCTTT TACGTTTTCT CAATATTTAC

 901  GCACATTTGT ATCCATCATT CTACTGAGTT CTTACTCCCA AGATGTTCTT CCCTTTAGCA
      CGTGTAAACA TAGGTAGTAA GATGACTCAA GAATGAGGGT TCTACAAGAA GGGAAATCGT

1001  AATGTGGTGA TCAGGGAAGC ATTGAGGAGA TGGATGGTGG CAGGTGGCAA GAGGACTATA
      TTACACCACT AGTCCCTTCG TAACTCCTCT ACCTACCACC GTCCACCGTT CTCCTGATAT

1101  CTTGCATTCA AGCATTTATT ATACACCAAT AATGTGAGCA ACACTTTACT TGACAAAGAA
      GAACGTAAGT TCGTAAATAA TATGTGGTTA TTACACTCGT TGTGAAATGA ACTGTTTCTT

1201  GAGAAAATTT AGGATGGATT CTGTTCTTCA ACTTCAAAGC ATCTGCTAAT TTGAATTTAG
      CTCTTTTAAA TCCTACCTAA GACAAGAAGT TGAAGTTTCG TAGACGATTA AACTTAAATC

1301  GAAGACAAGG ACAGAGAGAA TTTCAGTCCG GTAAGCAATG TAATTCATTT CAATTCTACA
      CTTCTGTTCC TGTCTCTCTT AAAGTCAGGC CATTCGTTAC ATTAAGTAAA GTTAAGATGT

1401  TAAATTGGTT ACAGAATTAA AACCAACCCA AAGGGAATAT ACTTCCTTCT TTTTCACAGA
      ATTTAACCAA TGTCTTAATT TTGGTTGGGT TTCCCTTATA TGAAGGAAGA AAAAGTGTCT

1501  AGAACCAGCA AATCCAACGA CAGTCAATAG CAGGCATTAC AAATCAGATT CAGAAAAATA
      TCTTGGTCGT TTAGGTTGCT GTCAGTTATC GTCCGTAATG TTTAGTCTAA GTCTTTTTAT

1601  GTTTTGAGTA TAATTGTATA TAGTATAGAC TATAGCTATG TATGTACACT TTCCACTTAC
      CAAAACTCAT ATTAACATAT ATCATATCTG ATATCGATAC ATACATGTGA AAGGTGAATG

1701  CTGCTTTTAG AAGTTCTGCA CAATTCTGAT TTTTACCAAG TCAACCTACT TCTTCTCTCA
      GACGAAAATC TTCAAGACGT GTTAAGACTA AAAATGGTTC AGTTGGATGA AGAAGAGAGT

1801  TTTCCAGAAG AAAAAAAATG CTCCAGTTTT CTCCTCTACC AAGACAGGAA GCACTTCCTG
      AAAGGTCTTC TTTTTTTTAC GAGGTCAAAA GAGGAGATGG TTCTGTCCTT CGTGAAGGAC

1901  GAGAGAAATT AGTAAAGTAG GTTGTATCAT CCTACTTTGA ATTTGGAATG TTTGGAAATG
      CTCTCTTTAA TCATTTCATC CAACATAGTA GGATGAAACT TAAACCTTAC AAACCTTTAC

2001  TGCGGGTGAT CCAAACAAAC ACTGTCACTC TTTAAAAGCT GCGCTCCCGA GGTTGGACCT
      ACGCCCACTA GGTTTGTTTG TGACAGTGAG AAATTTTCGA CGCGAGGGCT CCAACCTGGA

2101  AGAGCTAAGT AAAGCCAGTG GAAATGAAGA GTCTTCCAAT CCTACTGTTG CTGTGCGTGG
      TCTCGATTCA TTTCGGTCAC CTTTACTTCT CAGAAGGTTA GGATGACAAC GACACGCACC

2201  TGAGGACACC AGCATGAACC TTGTTCAGGT AATTAACACT AACTGACCTG GCCAGGTGGG
      ACTCCTGTGG TCGTACTTGG AACAAGTCCA TTAATTGTGA TTGACTGGAC CGGTCCACCC
```

THUMPD2 promoter

```
   1  AAACAAGAGT AGCCCCGACC TTGCTGACCA AAGGCTCCTG GAGGGGAAAA GACATCAGAA
      TTTGTTCTCA TCGGGGCTGG AACGACTGGT TTCCGAGGAC CTCCCCTTTT CTGTAGTCTT

 101  AAACTGCAGC CTCAGCTCAG CTGTGGTTTC ACCCAGGCCA GCACACTGCT GGTCGGTGCT
      TTTGACGTCG GAGTCGAGTC GACACCAAAG TGGGTCCGGT CGTGTGACGA CCAGCCACGA

 201  TGTACTGACA TAACTGGGCC CCTACCCACC GCTCTACCGC ATCTCTATCT ACTCCACTCA
      ACATGACTGT ATTGACCCGG GGATGGGTGG CGAGATGGCG TAGAGATAGA TGAGGTGAGT

 301  TTTCCTGTTC CCCACCCCCC ACACGCCGTC TCATTTTCAT CTGCTGCTTC TCCCTCTTAC
      AAAGGACAAG GGGTGGGGGG TGTGCGGCAG AGTAAAAGTA GACGACGAAG AGGGAGAATG

 401  AGCCCTCCCT GCCTCCTTTT TGTCGGTGCT GTCATAGCTT CCCGGGTATG TCCTCAGTGA
      TCGGGAGGGA CGGAGGAAAA ACAGCCACGA CAGTATCGAA GGGCCCATAC AGGAGTCACT

 501  ACCCTTGCTT ACCCATCTTT GAATCCTCAA GACAGGGTGG TGACTGGCGG AGAATAGGTG
      TGGGAACGAA TGGGTAGAAA CTTAGGAGTT CTGTCCCACC ACTGACCGCC TCTTATCCAC

 601  ATACTTAACA TCTTGGGCAC AGAATGTAGA GAGAACTAAA GAGGGTTCCT GTCCTCCCCA
      TATGAATTGT AGAACCCGTG TCTTACATCT CTCTTGATTT CTCCCAAGGA CAGGAGGGGT

 701  AAAATGAACT ACGGCAGTAG CCCAGAAAAG GAAAACATGT GTCTGAGGGC AAGAAAAGAA
      TTTTACTTGA TGCCGTCATC GGGTCTTTTC CTTTTGTACA CAGACTCCCG TTCTTTTCTT

 801  TCCCAGCACT TTGGGAGGCC GAGGCAGGCG GATCGCTTGA TCTCAGGAGT TCGAGACCAG
      AGGGTCGTGA AACCCTCCGG CTCCGTCCGC CTAGCGAACT AGAGTCCTCA AGCTCTGGTC

 901  AAAAATTAGC CCGGTGTGGT GGGGCTTGCC TGTGGTCCCA GCTACTGGAC AGGCTGAGGT
      TTTTTAATCG GGCCACACCA CCCCGAACGG ACACCAGGGT CGATGACCTG TCCGACTCCA

1001  GCCAAGATCA CGTCACTGCG CTCCAGCCTG GGCGACAGAG GGAGACCCTG TCGCAAAAAA
      CGGTTCTAGT GCAGTGACGC GAGGTCGGAC CCGCTGTCTC CCTCTGGGAC AGCGTTTTTT

1101  ACCTTGGAAA AGCTCATGTT ATGTGACTCC ATGAAGAATT TTTACTGTAC GGCCCAGTAG
      TGGAACCTTT TCGAGTACAA TACACTGAGG TACTTCTTAA AAATGACATG CCGGGTCATC

1201  CGGGGGAGGT GAGGGAGTAT CAGTTCTTCA CCTTAACATC CCATTCTGAA GCACAGGACA
      GCCCCCTCCA CTCCCTCATA GTCAAGAAGT GGAATTGTAG GGTAAGACTT CGTGTCCTGT

1301  TAACTGACTC AATATACAGC TACAGAGCTA ATTTCACGCT GTTTATCTTG TAACATGGAT
      ATTGACTGAG TTATATGTCG ATGTCTCGAT TAAAGTGCGA CAAATAGAAC ATTGTACCTA

1401  AAAATTCAGA GTTTAGAAAA ATGCCTTCAA ATACCGTTAG GAGAACCATT CAAATCAGGT
      TTTTAAGTCT CAAATCTTTT TACGGAAGTT TATGGCAATC CTCTTGGTAA GTTTAGTCCA

1501  TGGTTAACTA AGTAGTGCAA GAAATATGAG CTGTTTCAGA ACGCAGACGT TTGTTAGAGA
      ACCAATTGAT TCATCACGTT CTTTATACTC GACAAAGTCT TGCGTCTGCA AACAATCTCT

1601  GGCCCGATTC TTTGTGAAAA CAGACCAGGG CTCCTTCTAT GAGAAGAGTA GAGGAGAGGA
      CCGGGCTAAG AAACACTTTT GTCTGGTCCC GAGGAAGATA CTCTTCTCAT CTCCTCTCCT

1701  AAAAGGCATT ATGTTAGATA GGGAGAAATC TAGGCTCCAG CCTCAGCTGT GCTGCTAACT
      TTTTCCGTAA TACAATCTAT CCCTCTTTAG ATCCGAGGTC GGAGTCGACA CGACGATTGA

1801  TACAAAATAA GGCAACTGGA CTGAATTATC AATTCTAACC CTTCCACCTG TGATTCTAAC
      ATGTTTTATT CCGTTGACCT GACTTAATAG TTAAGATTGG GAAGGTGGAC ACTAAGATTG

1901  CAGGAAGACC CATAGGGGCC ACAGAGGGCT GATGCGACCC TCCCAGGCTC TCCGTCTCTC
      GTCCTTCTGG GTATCCCCGG TGTCTCCCGA CTACGCTGGG AGGGTCCGAG AGGCAGAGAG

2001  CCTCTCCCTC TCCCTCTCCC CTTTGGCATC ACACGCCCCT GCGCGCCGCC TCCAGGAGCG
      GGAGAGGGAG AGGGAGAGGG GAAACCGTAG TGTGCGGGGA CGCGCGGCGG AGGTCCTCGC

2101  GGCGCGCCTG AGCCGCCATG TCGGAGGCGC GTGGAGAGCC AGGGTCCGGG CCTGAGGCTG
      CCGCGCGGAC TCGGCGGTAC AGCCTCCGCG CACCTCTCGG TCCCAGGCCC GGACTCCGAC

2201  GTTCGTAATG CGAGAGGTG
      CAAGCATTAC GCTCTCCAC
```

TM4SF1 promoter

```
   1  GTAGTTTAAG CATCACTTTC CCCCATTTTG GCCCCAGGTG GGATAGATCA GCAGACAGGA
      CATCAAATTC GTAGTGAAAG GGGGTAAAAC CGGGGTCCAC CCTATCTAGT CGTCTGTCCT

 101  CACCCATAGG CTCTGTCACC AGTGGAATCC TGTCTCTAAG CCATCGGGCC AAACACACGT
      GTGGGTATCC GAGACAGTGG TCACCTTAGG ACAGAGATTC GGTAGCCCGG TTTGTGTGCA

 201  TTATGAGGAT TTGCCTGCCT CCGCCTGAGC AGGTGGGTGG TTCCACGCTT CCCAACTCCA
      AATACTCCTA AACGGACGGA GGCGGACTCG TCCACCCACC AAGGTGCGAA GGGTTGAGGT

 301  CCCTGTTAGT GTGCTGAGGC CACACTTCTT GGGCCCTCCT CCGGTCCCAT GCCACATCAC
      GGGACAATCA CACGACTCCG GTGTGAAGAA CCCGGGAGGA GGCCAGGGTA CGGTGTAGTG

 401  CCTCTAGTTC CGGGCCAAAT CCTTCAGACT CTAGATTCGT GAACCTCATT CACCTTCTTC
      GGAGATCAAG GCCCGGTTTA GGAAGTCTGA GATCTAAGCA CTTGGAGTAA GTGGAAGAAG

 501  TCTGTACTCT CTATCTTGTC CTGGTGACAA GGCCCTCCAG GCCTGGCCCC ACCCGGCCAC
      AGACATGAGA GATAGAACAG GACCACTGTT CCGGGAGGTC CGGACCGGGG TGGGCCGGTG

 601  TGCTGTTCAA GGCATAAATT TATTCAGTGC CCAGCCCCTC CTTCTGCAAG GTAACCACCT
      ACGACAAGTT CCGTATTTAA ATAAGTCACG GGTCGGGGAG GAAGACGTTC CATTGGTGGA

 701  AGCCTGTGGA AGCTGGTCTC AGGATCTGGC TGGGTAAACC ATCTGTTTAC CTCTGTGTGG
      TCGGACACCT TCGACCAGAG TCCTAGACCG ACCCATTTGG TAGACAAATG GAGACACACC

 801  TTTCTCCACT CTGCTTGCCA AGTCCCTTTG TCTTATTTTT TTCTCCCTCT CCCTCCTTCC
      AAAGAGGTGA GACGAACGGT TCAGGGAAAC AGAATAAAAA AAGAGGGAGA GGGAGGAAGG

 901  TTCCTCCCTC CCTCCCTTCC TCCCTTTTTC CCTCTCTCCT TCTCTCCCTC CTTCTCTTCT
      AAGGAGGGAG GGAGGGAAGG AGGGAAAAAG GGAGAGAGGA AGAGAGGGAG GAAGAGAAGA

1001  TCCCATTCTC TTCCTCCCCC TCTCTTCTTC CTCTTTTTAT TTTTGTCCCA TGCTTTAGGC
      AGGGTAAGAG AAGGAGGGGG AGAGAAGAAG GAGAAAAATA AAAACAGGGT ACGAAATCCG

1101  CACTCAATAA TTTTTAAAAA GCACCTGAGC CCTTTGTGTT GACTGATGCC ACCTCTGTTG
      GTGAGTTATT AAAAATTTTT CGTGGACTCG GGAAACACAA CTGACTACGG TGGAGACAAC

1201  GAACACTGAA ATCCATCCTG CAGAGCTTGC TGTAGGCAGA GTAAAGAAGT TTGCTCTGTA
      CTTGTGACTT TAGGTAGGAC GTCTCGAACG ACATCCGTCT CATTTCTTCA AACGAGACAT

1301  AGACACCTTG TATTTAATAG ACAAGGAAAT GAGCTCAGGA GATTTGCCCC AGGTTGTAAA
      TCTGTGGAAC ATAAATTATC TGTTCCTTTA CTCGAGTCCT CTAAACGGGG TCCAACATTT

1401  GATTGGAAGC TGTCCAGACA GCTGCATGGA GCAGGGGCAC AGCTCACTGT CTTTCAAAGC
      CTAACCTTCG ACAGGTCTGT CGACGTACCT CGTCCCCGTG TCGAGTGACA GAAAGTTTCG

1501  TGGAAAGTCA TGTTAGGCTT TCCTGTTGAT TCTTTATCTT GTGGAGGAGT TGCACAGTCA
      ACCTTTCAGT ACAATCCGAA AGGACAACTA AGAAATAGAA CACCTCCTCA ACGTGTCAGT

1601  ACTGAGGCTG TTTCTCACAT CCTGCCCCTG GTTTTGCCTG TCCCAAGGAT TTCGTCTGAA
      TGACTCCGAC AAAGAGTGTA GGACGGGGAC CAAAACGGAC AGGGTTCCTA AAGCAGACTT

1701  GCCTGCCATT AGGACCAATG AAAGCAAAGT ACCTCATCCC CTCAGTGACT AAGAATCGCA
      CGGACGGTAA TCCTGGTTAC TTTCGTTTCA TGGAGTAGGG GAGTCACTGA TTCTTAGCGT

1801  GCTTTCTCCA CCAGAAGGGC ACACTTTCAT CTAATTTGGG GTATCACTGA GCTGAAGACA
      CGAAAGAGGT GGTCTTCCCG TGTGAAAGTA GATTAAACCC CATAGTGACT CGACTTCTGT
```