The molecular and cellular impact of EPLIN (Epithelial Protein Lost in Neoplasm) on the healing of human wounds

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Medical Doctorate (MD)

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Abstract

EPLIN (Epithelial Protein Lost In Neoplasm) is a cytoskeletal associated protein whose expression is often reduced in cancer cells. It may function as a tumour suppressor through its effects on cancer cell migration and invasion. To date, its role in wound healing has not been elucidated. We examine the impact of EPLIN on keratinocyte migration and its implications in wound healing. A mammalian expression construct containing the full EPLIN coding sequence was used to overexpress EPLIN in human keratinocyte cell (HaCaT). Following overexpression verification, the impact of EPLIN on HaCaT cell migration was assessed using a conventional scratch wounding assay and an electric cell-substrate impedance sensing (ECIS) system-based assay. Protein expression was examined using western blot, ICC and IFC analysis. Transfection of HaCaT cells with the EPLIN expression construct successfully resulted in enhanced HaCaT EPLIN expression. Enhanced EPLIN levels were seen to negatively impact on cell migration as determined by both the scratch wound assay and the ECIS model system with migration rates of HaCaT cells over-expressing EPLIN being substantially less than the control HaCaT cells. Overexpression of EPLIN was found to slow keratinocyte migration rates using two independent assays as well as show convincing association and interaction with two NWASP and E-Cadherin. These important findings suggest novel routes to positively manipulate the wound healing process and has significance in further translational research.

CONTENTS

Chapter 1. Literature review

CONTENT OF FIGURES AND TABLES

CONTENT OF TABLES

Abbreviations and Acronyms

Abbreviation or Acronym	Expanded Term
3'UTR	UnTranslated Region
AA	Amino Acid
ABC	Avidin-Biotin complex
AJ	Adherens Junction
AC	Alternating current
APS	Ammonium persulfate
ARP	Actin Related Protein
bFGF	Fibroblast growth factor b
BSS	Balanced Salt Solution
cDNA	Complimentary DNA
CAM	Cell Adhesion Molecule
CWIS	Cardiff Wound Impact Schedule
Da	Dalton (unit)
DC	Direct Current
DEPC	Diethylpyrocarbonate
DM	Diabetes mellitus
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
ECIS	Electrical Cell-Substrate Impedance Sensing
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinases
EPLIN	Eplithelial Protein Lost in Neoplasm
FAM	Fluorescent tag
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IL-1	Interleukin-1
IP	Immunoprecipitation
MMP	Matrix metalloproteinase
M_r	Relative Molecular Mass
mRNA	Messenger Ribonucleic acid
NHS	National Health Service
NPF	Nucleation promoting factor
NWASP	Neuronal Wiskott – Aldrich syndrome protein
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEF	Empty plasmid control cell line (name)
PF4	Platelet factor 4
PGs	Proteoglycans
PI	Propidium iodide
pi-PLC	Phosphoinositide specific-phospholipase C
PLCγ	Phospholipase Cγ
PMN	polymorphonuclear leukocytes
PTB	phosphothyrosine binding
PTP-BAS	Protein tyrosine phosphatise-basophil
PTPH	Protein tyrosine phosphatises
qRT-PCR	Quantative real-time PCR
rRNA	Ribosomal Ribonucleic acid
RT	Reverse Transcriptase
SCCs	Squamous Cell Carcinoma
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBE	Tris-Boric acid-EDTA
TBS	Tris Buffered Saline

TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor β
TNF	Tumour necrosis factor
TNM	Tumour, node, metastasis
TRITC	Tetramethylrhodamine-5-(and 6)-isothiocyanate
tRNA	Transfer Ribonucleic acid
UC	Universal container
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
WASP	Wiskott–Aldrich syndrome protein
WT	Wild Type
ZA	Zona Adherens

List of Presentations

Saravolac VM, Sanders AJ, Bosanquet DC, Jiang WG, Harding KG **Impact of epithelial protein lost in neoplasm (EPLIN) on HaCaT adhesion and migration rates: implications in wound healing**. Presented 1st–3rd February 2011, Wounds International. South Africa

Sanders AJ, **Saravolac VM**, Jiang DG, Harding KG, Jiang WG**, The impact of VEGI (Vascular Endothelial cell Growth Inhibitor) on keratinocyte migration is influenced by the Decoy Receptor DcR-3,** Presented 1st–3rd February 2011, Wounds International. South Africa

Sanders AJ, **Saravolac VM**, Jiang DG, Harding KG, Jiang WG**, Keratinocyte migration in response to DR3/VEGI may involve ROCK signalling.** Presented 1st–3rd February 2011, Wounds International. South Africa

Bosanquet DC, Ye L, **Saravolac VM,** Saunders A, Rangaraj A, Harding KG, Jiang WG, **The role of HuR in keratinocyte migration and wound Healing,** April 14-17th 2011 Symposium on advanced wound care, Dallas, Texas

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **Keratinocyte migration, wound healing and the role of the novel protein Ehm2,** April 14-17th 2011 Symposium on advanced wound care, Dallas, Texas

Bosanquet DC, Ye L, **Saravolac VM**, Rangaraj A, Jiang WG, Harding KG, **Identifying wounds prone to chronicity; the role of the protein Ehm2 and its place in a novel diagnostic test**, Presented 5-6th May 2011, Welsh surgeons summer meeting, Llantrisant

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **Keratinocyte migration, wound healing and the role of the novel protein Ehm2 (Abstract),** March 2011 European tissue regeneration and repair (**ETRS**) meeting Amsterdam

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **The role of HuR in keratinocyte migration and wound Healing (Abstract),** March 2011 European tissue regeneration and repair (**ETRS**) meeting Amsterdam

Saravolac VM, Sanders AJ, Bosanquet DC, Jiang WG, Harding KG, **EPLIN (Epithelial Protein Lost In Neoplasm): Implications in Wound Healing,** 30th November, BAPRAS (British association of Plastic Reconstructive and Aesthetic Surgeons) London England

Saravolac VM, Jiang DG, Harding KG, **Relationship of Extracellular signal Regulated Kinase (ERK) and Epithelial Protein Lost in Neoplasm (EPLIN) in HaCaT cell migration,** October 2012 European tissue regeneration and repair (**ETRS**) meeting Athens

Saravolac VM, Jiang DG, Harding KG, **Potential role for Focal Adhesion Kinase (FAK) in EPLIN regulated migration of HaCaT cells,** October 2012 European tissue regeneration and repair (**ETRS**) meeting Athens

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **EHM2 promotes wound healing; the involvement of FAK and NWASP**. October 2012 European tissue regeneration and repair (**ETRS**) meeting Athens

Saravolac VM, Bradbury S, Harding KG, **Differentiating between pressure and Ischaemic ulcers**, European Pressure Ulcer Advisory Panel (**EPAUP**) October 2012 Cardiff

List of Publications

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **Keratinocyte migration, wound healing and the role of the novel protein Ehm2,** Wound Repair and Regeneration (International), 2011 March; 19(2): 14-15

Bosanquet DC, Ye L, **Saravolac VM**, Saunders A, Rangaraj A, Harding KG, Jiang WG, **The role of HuR in keratinocyte migration and wound Healing,** Wound Repair and Regeneration (International), 2011 March; 19(2): 14-15

List of Book Chapters

ELECTRIC CELL-SUBSTRATE IMPEDANCE SENSING AND CANCER METASTASIS

ECIS as a Tool in the Study of Metastasis Suppressor Genes: Epithelial Protein Lost In Neoplasm (EPLIN)

Andrew J. Sanders, **Vladimir M. Saravolac**, Malcolm D. Mason and Wen G. Jiang

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CHAPTER 1 - INTRODUCTION

1. Introduction (Literature Review)

1.1 History of wound healing

Since the caveman, man has been tending to his wounds. The wound care itself evolved from the magical incantations, potions and ointments to a systematic text of wound care and surgery from the breakthroughs Hippocrates to Celcus, the advances initially becoming lost after the fall of the Roman empire with the regression of wound care through the dark ages and then the subsequent re-emergence of wound care when large armies using muskets and cannons re-kindled the importance and breakthroughs in wound care (Broughton, Janis et al. 2006). In contrast to the recent large numbers of technological innovations over the last 100 years, progress beyond ancient wound care practises is a relatively recent phenomenon. Thus it is essential to know the historical aspects of wound treatment (both the successes and the failures) in order to continue and embellish on this progress and provide future direction (Hirano, Kimoto et al. 1992).

Knowledge of wound biology and wound healing has proliferated through the ages, along with the developments and innovations of new wound care products. Perhaps the earliest evidence of man's attention to wound healing comes to us from the Sumerians, in cuneiform tablets believed to be older than 2000 BC. Translations have revealed descriptions of differing types of wound injury as well as modes of treatment which broadly fell into either spiritual management by incantations or physical applications of substances in the form of poultice (including dust, plants, milk, wine, beer and flour) (Crissey and Parish 1984).

1.1.1 Egyptians

The Egyptian civilisation was one of the oldest in history and was renowned for its scientific and artistic achievements, with medicine being no exception. The ancient Egyptians were masters in applying and arranging bandages, and they recognised the cardinal signs of infection and inflammation (Shapiro and Weis 2009).

Wound care management during the early Egyptian civilisation paradoxically resembles current approaches, with treatments consisting primarily of wound closure through suturing or open wound therapy in unhealthy wounds with subsequent debridement followed by antibacterial therapies, albeit not intentionally. Wounds were cleansed with wine, vinegar and hot water and following cleansing dressed with a combination of dry metal powders including zinc, copper and silver in order to prevent inflammation. Little known to the Egyptians was that the reaction of copper, white wine and vinegar lead to the formation of a strong antibacterial compound copper acetate. Copper was predominantly sourced from the island of Cyprus, the ease of its discovery arising from its blue colour, which according to ancient scriptures was used to paint the wounds (Hirano, Kimoto et al. 1992). Furthermore a case could also be made that the Egyptians were the first to unknowingly employ the moist wound healing principle something we know of today to be of paramount importance. They dressed their wounds in linen soaked in honey oil and lint, which prevented the dressing from sticking to the wound thus creating a non-adherent dressing. Lint has been recently linked in conjunction with wound packing to create an oxygen deprived environment thereby promoting angiogenesis, and honey can be beneficial as a wound dressing because it inhibits the growth of microorganisms through the production of glucuronic acid and hydrogen peroxide by enzyme glucose

oxidase, thus attracting an abundant secretion of leukocytes and antibodies. (Donnelly 1998) Honey was by far the most popular Egyptian drug, mentioned in 500 out of 900 remedies (Shapiro and Weis 2009). Other components of dressings such as wine, willow bark and *Matricaria chamomilla* also provided potent anti-microbial effects amongst others.

"When you examine a man with an irregular wound ... and that wound is inflamed ... (there is) a concentration of heat; it pours out of the opening of that wound against your hand; the lips of the wound are reddened and that man is hot in consequence; then you must say: a man with an irregular wound ... a sickness that I can treat. Then you must make a cooling substance for him to draw the heat out. . . leaves of the willow" (Breasted 1931).

This information confirms the recognition by ancient Egyptians of the cardinal signs of inflammation and infection, using a known antiseptic of the time in the form of bark and leaves of the willow tree to reduce infection. The antiseptic qualities of the willow bark are well known to modern science (Shapiro and Weis 2009).

The Egyptians also favoured the use of larval therapy observing that maggots would in fact clean a puss filled infected wound very efficiently. Larval therapy was routinely used until antibiotics were introduced. Maggots are chemical factories; they produce a powerful mixture of proteolytic enzymes that break down necrotic tissue and liquefy it. The maggots then ingest this liquid and in the process, ingest, and digest, the bacteria in the wounds. The resultant secretions increase the pH of the wound to around 8 to 8.5 by the production of ammonia, which in turn inhibits the growth of some bacteria. Maggots have also been shown to secrete antimicrobial

chemicals. Their presence in the wound stimulates granulation tissue formation and fibroblast development, thereby accelerating the healing process (Shapiro and Weis 2009).

1.1.2 Greeks and Romans

The Greek medical practice of wound care greatly resembled that of the Egyptians with a few notable key exceptions. One important distinction was the promotion of puss instead of the prevention of inflammation as they believed puss to be an active by-product of a healthy healing wound. Another key change in wound management introduced by *Hippocrates* 300- 350 BC who advocated the importance of dry wound therapy in order to promote healing by primary intention all the while observing the recuperative powers of nature and preserving the high standard of ethical conduct embodying the now famous Hippocratic oath (Hirano, Kimoto et al. 1992).

The first science based medical manuscript (*De Medicina*) was the by product of the Roman era with *Celsus* (25 BC- 50AC) describing the four cardinal signs constituting infection; rubor, calor, dolor and tumor still in use by medical practitioners to this day. *Celsus* also addressed the importance of rigorous wound cleansing.

"Clean the wound of the old blood because this can cause infection and change into puss, which inhibits wound healing" (Hirano, Kimoto et al. 1992).

Despite these insightful observations by a select few such as *Celsus*, the principles of dry dressings and wound healing environment along with the promotion of puss were set to continue well into the 19th century.

A dominant figure in Greek medicine related to wound healing was *Galen of Pergamum* (120–201 A.D.). Galen gained specific experience with wound management when he was appointed the "game doctor" to the Roman gladiators in Pergamum. Gladiatorial combat generated many wounds for Galen to treat and he experimented with a variety of topical treatments (Ovington 2002). Galen recognised just as the Egyptians had before him, that wounds healed optimally in a continuously moist environment, in his case providing this environment through the use of a damp cotton cloth and sponge (Cohen 2007).

Our knowledge of the pharmacopoeia of ancient Greece is clearly inadequate to support many of the sweeping generalizations about the use and effectiveness of the recorded drug therapy regimens. Thus drawing actual conclusions concerning the efficacy of the pharmacological treatments of the time is difficult for a number of reasons including incomplete pharmacopeia translations, lack of information as to how often a therapy was used and difficulty in the certainty of definition of conditions being treated. However we can conclude that the Greek drug therapy had evolved from empirical observations and a system rooted in magic and superstition (Majno 1975).

At that time the Jews Arabs and early Christian church held surgery in low regard. The sick were considered unclean and no educated person would consider touching a patient. Thus surgery became the profession practiced by itinerant barbers, cutters and others, while physicians came from better educated backgrounds. Lack of systematized surgical teaching caused a number of important observations in wound

management to be overlooked and forgotten (Forrest 1982).

1.1.3 Middle Ages to 18th century

Inflexible adherence to texts, in particular those of Galen and Celsus and rigidity of thought dominated through the Middle Ages with limitations to surgical techniques to the extent that wound care actually declined and took a step back during this period. The practice of copying manuscripts by successive generations of scribes led to the embodiment of errors that enshrined the misconceptions in the texts (Forrest 1982).

Wound debridement made a return in the 16th century, especially following the introduction of gun powder and a dramatic shift in warfare techniques. Battlefield wound were treated with red hot iron pokes, cleaned with boiling oil and covered in suppuration provoking substances as observed by one Abroise Pare (1509- 1590) (Hirano, Kimoto et al. 1992).

In 1536 an inexperienced barber-surgeon Pare joined in his first military campaign as a field cutter and spent two years in Italy. He published his experiences in 1545 in his 'La Methode de Traiter les playes Faites per Harquebutes et Autres Batons de Feu' in which he tells the now famous story of how he was forced to dress wounds of a large number of soldiers with egg yolk, rose oil and turpentine after his boiling oil ran out. Following a particularly bloody battle he came to notice that those treated with the oil to have faired much worse than the others who were in less pain and afebrile (Forrest 1982). Abroise Pare exemplified well the predicament of the physician in the 16th century. He is best remembered for the phrase which immortalized him

"I dressed the wound; GOD healed it."

His insightful observations of battlefield wound and experiences eventually changed the wound care and management of the time, at the expense of his reputation at the time (Cohen 2007). Pare realized and worried that failure to follow to ascribed methods would lead to his rejection by his peers and superiors by challenging the practiced methods. He demonstrated courage strength and conviction, qualities needed to move the world forward to a more creative world in science and medicine (Hirano, Kimoto et al. 1992).

1.1.4 19th Century to present

Nineteen centuries later (Gilje 1948) and (Winter 1962) would scientifically prove the healing benefit of a moist environment and spark an explosive burst in the evolution of materials used as wound dressings that would address this difficulty of maintaining a moist wound environment (Gilje 1948; Winter 1962).

Wound treatment methods evolved to incorporate cleansing, removal of foreign debris, approximation of wound edges, and dressing with a bandage or poultice; however, the materials used for these dressings did not change significantly during this time (Ovington 2002).

The above reflection of wound therapies and care through time reinforces that the more things change perhaps paradoxically the more they stay the same. As we analysed the ways in which the ancient Egyptians and Greeks cared for wounds, the realisation that becomes apparent is that our own practice is directly impacted by colleagues that have preceded us (Cohen 2007).

One of the greatest advancements in wound healing during the 19th century is in the context of antisepsis treatment. During the same period a Hungarian Dr Ignaz Phillip Semmelweis (1818–1865), and the American physician Dr Oliver Wendell Holmes (1809–1894), postulated that patients suffering with post partum infective complications were happening as a result of inadequate hand washing by the doctors and nurses treating them. Indeed following the introduction of a hospital wide policy of mandatory hand washing introduced by Semmelweis the maternal mortality was markedly reduced. However this novel approach was met with substantial criticism leading to Semmelweis being dismissed from his duties by the higher authorities within the institution who remained unconvinced by his novel ideas (Brown 1992). It was not until years later that Louis Pasteur (1822 – 1895) and Joseph Lister (1827 – 1912) proved that germs were responsible for such outbreaks leading to the subsequent development of antiseptics. Further improvements in hygiene subsequently continued, with William Stewart Halsted (1852 –1922) actively advocating the use of rubber gloves in surgery and Ernst von Bergman (1836 – 1907) developing heat sterilisation that could be used on surgical instruments (Ovington 2002).

The time period that followed led to a number of important milestones in the contemporary knowledge and understanding of wound healing biology. A French surgeon, Alexis Carrell (1873 –1944), first described a method for measuring wounds in dogs, and as a result of his observations, was possibly the first to postulate on the contractile nature of granulation tissue. He also recognised that there were four stages of wound healing as early as 1910 (quiescent period, granulomatous reaction, epidermisation and a cicatricle period), though these are not the same as the four

stages we know of today (Crissey and Parish 1984). Another of his notable achievements is his work with Henry Drysdale Dakin to develop the Carrel-Dakin method of treating wounds, utilising a hypochlorite solution that led to the contemporary investigation of the cellular mechanisms underpinning wound healing, subsequently resulting in the award 'Légion d'honneur' for his contributions to science. Around the same time a German doctor Rudolph Virchow (1821-1902) made his most widely known academic contribution "*Cellular pathology as based on physiological and pathological history*", that built on the work from Theodor Schwann. In this work he presented his ideas about regeneration, hypothesising that cellular regeneration is dependant on cellular proliferation and showing that the origin of cells was based on the division of pre existing cells. This in turn cascaded a series of works investigating cellular mechanisms underpinning wound healing as we know it today (Broughton, Janis et al. 2006).

Notably however the arrival of antibiotics changed the approach to the management of infection and was the most significant advance from the 20th century. Sulphonamides were introduced in the mid 1930s and were applied to wounds in powder form for treatment of local infection (Forrest 1982). The discovery of penicillin by the Scottish scientist and Nobel Laureate Sir Alexander Flemming in 1928 showed that, if *Penicillium notatum* were grown in the appropriate substrate, it would exude a substance with antibiotic properties, which he dubbed penicillin. (Cohen 2007) This serendipitous observation began the modern era of antibiotic discovery.

Other notable discoveries include that of Corticosteroids which were isolated in the late 1940s with steroid creams soon becoming available for the treatment of

inflammatory skin conditions (Forrest 1982). First known use in 1944 according to Tadeusz Reichstein together with Edward Calvin Kendall and Philip Showalter Hench were awarded the Nobel Prize for Physiology and Medicine in 1950 for their work on hormones of the adrenal cortex, which culminated in the isolation of cortisone (Slocumb 1965).

1.2 Biology of wound healing

Human beings must repair wounds rapidly in order to restore the skin's critical protective function and at the same time attempt to preserve normal sensation, pliability and cosmesis. Wound healing is the process of tissue repair and regeneration occurring following injury. It is a complex process, consisting of an intrinsically regulated sequence of cellular and biochemical events. The response can be subdivided into four distinct but overlapping phases Haemostasis, Inflammation, Proliferation and Maturation or Remodelling (Schilling 1976). The stimulation and precise regulation by growth factor and matrix signals, of relatively sedentary cell lineages at the wound edges to migrate and proliferate, and synthesise, degrade and contract various elements of the extracellular matrix is critical to this process (Mehendale 2001). Failure or prolongation of any one of these phases results in either healing delay and/or subsequent non-closure of the wound.

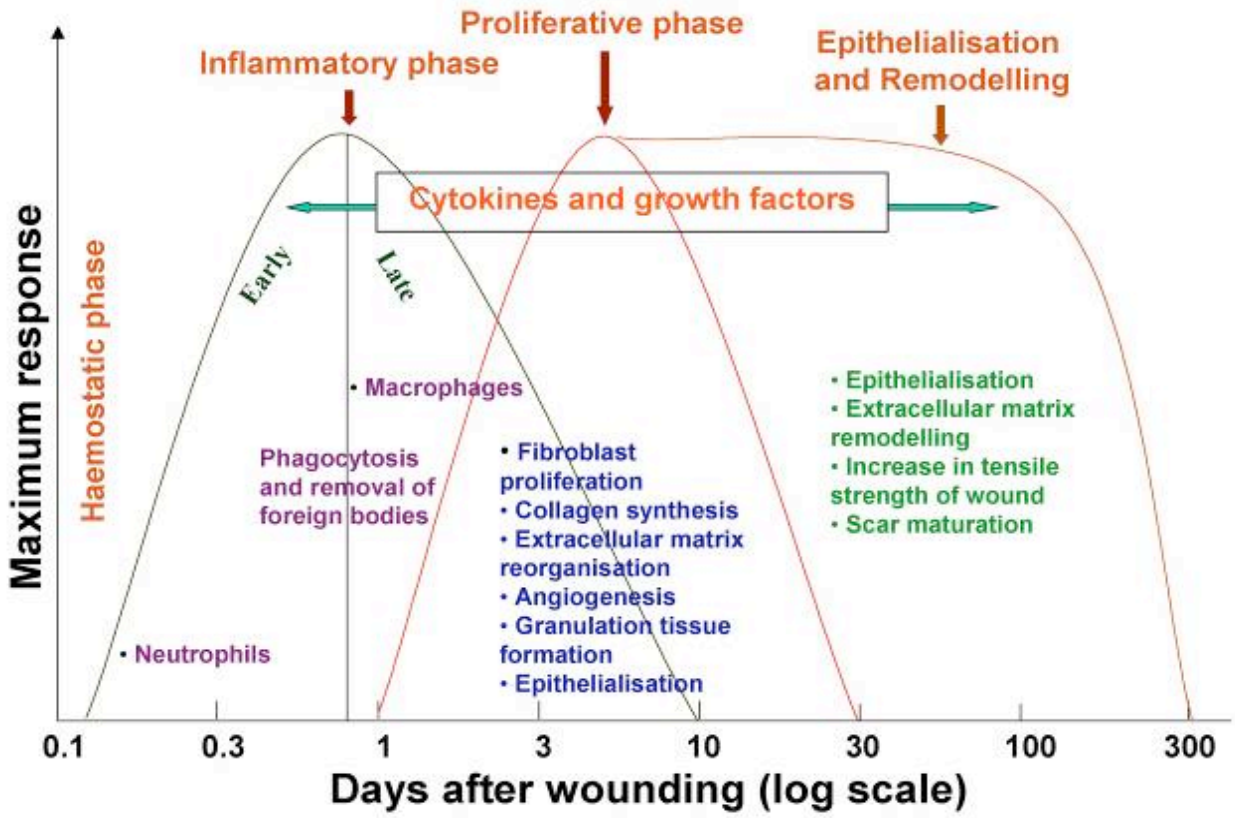


Figure 1.1: - The overlapping phases of wound healing, reproduced with pemission from (Enoch, Grey et al. 2008).

The relative importance of processes such as re-epithelisation and granulation tissue formation is dependant upon the degree of tissue loss and the wound depth. Partial thickness wounds are categorised by a loss of the entire epidermis but only a part of the dermis, so remnants of the epithelial derived structures such as hair follicles, sweat glands and sebaceous sweat glands are still present in the tissue left behind. These wounds generally tend to heal with minimal scarring as the re-epithelisation occurs from both the edges of the wounded dermis and also from the cut edges of the adnexal structures. However with increasing involvement of the dermis as is seen in full thickness wounds, results in progressively slower wound healing and increasing scar formation. As these wounds cannot heal by re-epithelisation alone, this requires

the formation of granulation tissue and subsequent wound contraction and decreasing the area of exposed wound and in turn leads to scar formation. This subsequent scarring contractures as for instance seen post major burns, especially in certain anatomical regions, such as joint surfaces and eyelids, can result in severe deformity leading to psychological, functional and cosmetic morbidity to the individual (Mehendale 2001).

1.2.1 Haemostasis

Shortly after wounding, normal haemostatic mechanisms arrest the haemorrhage from damaged blood vessels and a wound haematoma is formed. This in turn acts as a temporary shield, as the barrier effect of skin is lost secondary to the injury. The ruptured vessels result in blood filling the defect and exposing it to various components of the Extra Cellular Matrix (ECM) (Schultz and Wysocki 2009). Prothrombin can be activated via two pathways known as the extrinsic and intrinsic pathways. The extrinsic pathway is activated when blood is exposed to tissue factor after tissue injury, and plays a major role in the initiation of blood coagulation in wound healing. Once prothrombin is activated, fibrinogen is converted to fibrin, leading to the formation of a fibrin plug (Mackman, Tilley et al. 2007).

The haematoma consists of platelets and other blood cells enmeshed in a cross-linked fibrin fibres that are derived by thrombin cleavage of fibrinogen, along with smaller amounts of plasma fibronectin, vitronectin and thrombospadin (Singer and Clark 1999). Once exposed to collagen, platelets aggregate under the influence of a number of factors (such as adenosine diphosphatase (ADP)), forming a platelet plug

(Murugappan and Kunapuli 2006). Subsequent Serotonin (5-HT) release, leads to vasoconstriction and thus further reducing blood loss.

As well as aggregating within the clot platelets subsequently degranulate releasing a cocktail of growth factors and cytokines that act to stimulate other inflammatory cells to get recruited into the injured area thus initiating the cellular movements of re-epithelisation and connective tissue contraction thus stimulating the classic wound angiogenic response (Murugappan and Kunapuli 2006).

1.2.2 Inflammation

With the degranulation of platelets releasing their activated granular contents, a variety of chemotatic and growth stimulating cytokines including; factor X, platelet factor 4 (PF4), ADP, serotonin, thromboxane A2 (TX-A2), interleukin-1 (IL-1), Von Willebrand factor (vWF), platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Clark and Henson 1988). The above agents lead to an increase in the vascular permeability and thus affect chemotaxis and activation of pro inflammatory cells such as macrophages and polymorphonuclear leukocytes (PMNs or neutrophils), characterising the Inflammatory wound response (Witte and Barbul 1997).

Neutrophil infiltration of the wound site normally commences within a few minutes of wounding. They are the first immune cell type to arrive at a wound site, with their levels peaking around 24-48 hours subsequent to injury (Park and Barbul 2004). Their primary initial role is that of Phagocytosis in turn leading to wound debridement, though they are also a source of pro-inflammatory cytokines that

probably serve as early signals in a cascade to activate local fibroblasts and keratinocytes (Park and Barbul 2004). Neutrophils have a short half life, with their numbers reducing dramatically after a few days in the absence of Infection, and whilst neutrophils can reduce the likelihood of infection in a wound, they are not essential with their role of phagocytosis, being substituted by macrophages in their absence (Simpson and Ross 1972).

Macrophages, migrate into the wound peaking at 48 to 96 hours after injury. They are derived from monocytes and continue to conclude the inflammatory cascade (Witte and Barbul 1997). They also are pivotal in phagocytosis, as well as releasing oxygen radicals (ROS) and the production of collagenase and elastase enzymes thus incapacitating any bacteria that come into contact with the wound (Park and Barbul 2004). Microphages as well as neutrophils are also involved in the secretion of cytokines and growth factors thereby activating and recruiting various pro inflammatory cells, in turn influencing angiogenesis and extracellular matrix synthesis (Wahl 1985; Barbul 1990). A frequently cited study by Leibovich and Ross 1975 showed that by the depletion of macrophages in the serum and concurrent steroid treatment tissue repair could be seriously impaired, thus leading to the dogma that microphages were essential for wound healing. However as early embryos are able to heal in the absence of inflammatory cell infiltrate as demonstrated by (Hopkinson-Woolley, Hughes et al. 1994), this indicates that macrophages are perhaps not essential in the wound healing process.

This inflammatory response whilst normally regulated can be very beneficial to the wound healing process as outlined above, however the possibility that this response

can become detrimental is illustrated by various human inflammatory skin condition such as Pyoderma gangrenosum, in which there is as clear association between an excessive exaggerated inflammatory response leading to the impedence and failure of wounds to heal. In such conditions steroid treatment appears to suppress this inflammatory response thus allowing the wounds to heal (Schwaitzberg, Bradshaw et al. 1982).

1.2.3 Proliferative phase

As in the other phases of wound healing, steps in the proliferative phase do not occur in a series but rather partially overlap in time. The proliferative phase which occurs two to five days after injury, lasting approximately two weeks, involves predominantly fibroblasts (McAnulty 2007). The main role of fibroblasts is the synthesis and deposition of extracellular matrix components, along with the production of angiogenic and growth factors that regulate cell proliferation and angiogenesis. Fibroplasia ends two to four weeks after wounding (Midwood, Williams et al. 2004).

During the first two to three days after injury, fibroblasts mainly migrate and proliferate, while later, they become the main cell type to lay down the collagen matrix in the wound site, referred to as granulation tissue (Stadelmann, Digenis et al. 1998). These fibroblasts are thought to originate from the adjacent uninjured cutaneous tissue (although some new evidence links them to blood-borne, circulating adult stem cell precursors) (Song, Nguyen et al. 2010). Initially through the manipulation of fibrin cross-linking fibres the fibroblasts migrate across the wound, adhering subsequently to fibronectin, depositing ground substance and collagen into

the wound bed which they can adhere to for continued migration (Barrientos, Stojadinovic et al. 2008), (Schultz and Wysocki 2009). This tissue forms a scaffold through which angiogenesis can take place (Deodhar and Rana 1997).

Growth factors TGF-β, PDGF and fibronectin encourage proliferation and migration into the wound, and stimulate production of ECM molecules by fibroblasts. Epithelial cells are also attracted to the site by these growth factors secreted by the fibroblasts (Schultz and Wysocki 2009). Hypoxia also contributes to fibroblast proliferation and excretion of growth factors (Deodhar and Rana 1997).

The ECM forms the basis for the granulation tissue which continues growing until the wound bed is covered. Granulation tissue is composed of new blood vessels, inflammatory cells, endothelial cells, myofibroblasts, fibroblasts, and components of a new provisional extracellular matrix (ECM). The provisional ECM is different in composition from the ECM in normal tissue, Type III vs type I collagen (Schultz and Wysocki 2009). Such components include fibronectin, collagen, glycosaminoglycans, elastin, glycoproteins and proteoglycans (Song, Nguyen et al. 2010). The polysaccharides found in ECM are called proteoglycans (PGs) and glycosaminoglycans (GAGs), and include Hyaluronan, a key component in the wound healing process (Chen and Abatangelo 1999). Elastin fibres are also found in the ECM, and play an important structural role. Later this provisional matrix is replaced with an ECM that more closely resembles that found in non-injured tissue (Schultz and Wysocki 2009).

1.2.4 Angiogenesis

The process of angiogenesis (neovascularisation) occurs concurrently with fibroblast proliferation when endothelial cells migrate to the area of the wound (Kuwhara 2007). It is essential in supplying the oxygen and nutrients for the activity of fibroblasts and epithelial cells, required for successful synthesis or the various components of the ECM. The tissue in which angiogenesis has occurred typically looks red (is erythematous) due to the presence of capillaries (Kuwhara 2007), As a result angiogenesis and collagen deposition occur in a co-dependent manner in the proliferative phase (Risau 1997). Stem cells derived from endothelial origin, situated in parts of the uninjured blood vessels, develop pseudopodia and migrate through the ECM into the wound site to establish new blood vessels (Greenhalgh 1998). The endothelial stem cells produce a degradation enzyme, Tissue Plasminogen Activator and various collagenases in order to penetrate the ECM (Clark and Henson 1988; Folkman and Shing 1992).

Endothelial cells are attracted to the wound by fibronectin which is found on the fibrin scab and also chemotactically by angiogenic factors released by other cells, such as macrophages and platelets when in a low-oxygen environment. Hypoxia and presence of lactic acid in the wound also directly stimulates Endothelial growth and proliferation (Deodhar and Rana 1997). These two phenomena coupled together mean that when the tissue becomes adequately perfused, and the hypoxic and lactic acid filled environment is thus removed, the migration and proliferation of endothelial cells decreases due to a stoppage of angiogenic factor production. Eventually those blood vessels that are no longer needed die by apoptosis (Greenhalgh 1998).

When macrophages and other growth factor-producing cells are no longer in a hypoxic, lactic acid-filled environment, they stop producing angiogenic factors. These cytokines include interleukins (IL-1,-6), fibroblast growth factor a and b (aFGF, bFGF), tumour necrosis factor (TNF), transforming growth factor β (TGFβ, vascular endothelial growth factor (VEGF),) and interferons (Gill 1998; Eming, Brachvogel et al. 2007). Thus, when tissue is adequately perfused, migration and proliferation of endothelial cells is reduced. Eventually blood vessels that are no longer needed die by apoptosis (Deodhar and Rana 1997).

1.2.5 Epithelialisation

The formation of granulation tissue allows epithelisation to take place. Epithelialisation is the process by which keratinocytes migrate from the wound edge, subsequently proliferating and differentiating to eventually form a congruent layer of keratinocytes over the wound bed thus creating a barrier between the wound and the environment (Martin 1997). Basal keratinocytes from the wound edges and dermal appendages such as hair follicles, sweat glands and sebaceous (oil) glands are the main cells responsible for the epithelialization phase of wound healing (Deodhar and Rana 1997). The keratinocytes advance in a sheet across the wound site and proliferate at its edges, ceasing movement when they meet in the middle. The retraction of the intercellular monofilaments results in a loss of adhesion of the cells dissolving the intercellular desmosomes holding the cells together leading to an increase in mobility of the cells (Singer and Clark 1999). This dissolution of hemidesmosomal links between the epidermis and basement membrane ensures that epidermal and dermal cells no longer adhere to each other, in turn permitting lateral

movement of the epidermis (Singer and Clark 1999). This unfortunately results in scar formation as sweat glands and hair follicles do not form.

Integrins are thought to play a key role in keratinocyte migration, in particular β1 integrins. They are heterodimeric transmembrane proteins comprised of an α and β subunit that are vitally important in cell-cell and cell-matrix interactions (Grose, Hutter et al. 2002). These integrin receptors are expressed on epidermal cells allowing interaction with a variety of extra-cellular matrix proteins including laminectin, fibronectin and vitronectin that in turn are interspersed with collagen at the wound margin and interwoven with the fibrin on the wound bed (Larjava, Salo et al. 1993).

1.2.6 Wound contraction

Work by Gabbiani and colleagues has demonstrated that granulation tissue contraction is effected by transformed wound fibroblasts or myofibroblasts that express alpha smooth muscle actin and like smooth muscle cells are capable of generating strong contractile forces on the wound edges (Gabbiani, Ryan et al. 1971). The contraction process refers to the centripedal movement of wound edges that initiates and facilitates the closure of wound edges and peaks at 5-15 days following wounding. Two main theories that have been put forward to explain this process in which myofibroblasts exert their contractile activity, are the cell traction, or fibroblast theory (Ehrlich and Rajaratnam 1990) and the cell contraction, or myofibroblast theory (Gabbiani, Ryan et al. 1971).

In partial thickness wounds with predominantly epidermal loss this effect can be less dramatic, however in large full thickness wounds for example following extensive burn injury or large wounds left to heal by secondary intention this effect can be quite dramatic. Myofibroblasts have been implicated in a number of fibro proliferative disorders such as Dupytrens disease (Chiu and McFarlane 1978). Hypertrophic scars are also known to contain large quantities of myofibroblasts which may partially explain their tendency to contract, as well as their eventual resolution over time unlike keloid scarring, which do not contract or regress and have been shown to lack myofibroblast (Ehrlich, Desmouliere et al. 1994).

1.2.7 Maturation and wound remodelling

The maturation phase begins when the levels of collagen production and degradation equalize (Greenhalgh 1998). It's characteristic is the deposition of collagen in the wound space (Witte and Barbul 1997). With time, collagen becomes the predominant component of the wound matrix, and provides stiffness and tensile strength to the wound. During maturation, type III collagen, which is prevalent during proliferation, is replaced by type I collagen. It is a process that requires a fine balance between the synthesis and degradation of collagen, the later being regulated by collagenases (Theoret 2004). The initially disorganized collagen fibres are rearranged, cross-linked, and aligned along tension lines (Deodhar and Rana 1997). This remodelling process results in an increase in wound strength from 50% of normal at three months, to 80% of normal skin strength when fully mature (DiPietro and Burns 2003).

The onset of the maturation phase may vary extensively, depending on the size of the wound and whether it was initially closed or left open, (Deodhar and Rana 1997)

ranging from approximately 3 days to 3 weeks. The maturation phase can last for a year or sometimes longer, and similarly depend on the type of wound (Schein 2010). With the loss of activity at the wound site, the scar becomes more pale as blood vessels that are no longer needed are removed by apoptosis (Greenhalgh 1998).

1.3 Introduction to chronic wounds

1.3.1 Quantifying the chronic wound problem

Cost saving is gaining ever increasing importance within the NHS and wound management is one area in which it is possible to lower the financial burden of care and hence why research in this field is of ever increasing importance. At any given time 200,000 individuals in the UK have a chronic wound (mainly leg ulcers, pressure ulcers and diabetic foot ulcers) (Prosnett J 2007). These are mostly cared for by nurses in the patients' homes, in community-based clinics or in residential care homes (Drew, Posnett et al. 2007; Vowden and Vowden 2009). There are 24 000 admissions per year of patients with diabetic foot ulceration in the United Kingdom accounting for a cost to the NHS of £17 million (Currie, Morgan et al. 1998). With the cost of treating venous leg ulcers having been estimated to be at least £168 to £198 million per year (Posnett and Franks 2008). Similarly the cost of pressure sores is estimated to be between £1.8 and £2.6 billion (Posnett and Franks 2008). The direct cost to the NHS of caring for patients with chronic wounds has been estimated at £2.3-3.1 billion per year. This accounts for 3% of the overall NHS budget (Prosnett J 2007), with these figures still being an underestimate, as they do not include other associated costs to the economy and the wider reaching impact of chronic wounds which are harder to evaluate such as sick pay and as a result missed work opportunities. This is why

effective and timely diagnosis with treatment appropriate to the cause and condition of the wound, alongside active measures to avoid the incidence of wound complication and hospitalisation, can have major impact on both costs and patient quality of life (Vowden, Vowden et al. 2009).

In recent years Health-related quality of life (HRQoL) scores have been used in an attempt to quantify this known morbidity of chronic wounds. One such example of score is the Cardiff Wound Impact Schedule (CWIS) which has allowed physicians to gain insight into the challenges faced by individuals with chronic leg ulcers. These scores have been shown to be reliable in a clinical setting responding to changes in the clinical symptoms of the patient and assessing the patients' quality of life when faced with chronic wounds (Price and Harding 2004). This assessment is achieved by the incorporation of questions about the patients physical symptoms, general well-being and daily life as well as the impact on the individuals social life which are all known to be factors in the perception of morbidity by patients with chronic wounds. Price et al went on to illustrate this decrease in quality of life in patients with arterial and venous leg ulcers in a multinational study involving 2000 subjects. In this study symptoms such as impaired mobility, leakage, odour, dressing or bandage slippage and pain were rated as the symptoms which most affected patients with pain being ranked highest with 36.6% of patients listing this as their primary problem and source of their morbidity (Abdelgadir, Shebeika et al. 2009).

Chronic wounds can be a source of significant morbidity and even mortality, and can be very difficult to manage, thus they can represent a major challenge to the patient and physician alike (Harding, Morris et al. 2002)

1.3.2 Classification of chronic wounds

Wounds as a whole may broadly be classified into two groups, either acute or chronic, although there is a large area of overlap in this spectrum and most fall somewhere in between the two. The terms acute and chronic may be viewed as references to both the cause and the healing time frame of the wound. Acute wounds generally result from acute causes, such as trauma, surgical procedures, burns, insect bites, etc., and have an expected healing timeframe (Moreo 2005). Acute wounds are not usually complicated by localized or systemic impediments to healing and will generally achieve closure resulting in a healed wound. In contrast, chronic wounds do not follow the same pattern and typically some disruption to the body's normal cellular healing processes render them dysfunctional (Moreo 2005).

Even though the vast majority of wounds will heal within a normally recognised time frame those wounds that don't heal, heal slowly or heal and tend to recur and are known as chronic wounds. Some of the many causes of chronic wounds include trauma, burns, skin cancers, infection or underlying medical conditions such as diabetes. In fact there is no uniformity in the literature about what actually constitutes a 'chronic wound' with varying classifications in existence within the literature :

- Some authors classify chronic wounds as "wounds that have failed to return to functional and anatomical integrity in a timely fashion, or wounds that have proceeded through the repair process without a normal functional end result" (Telgenhoff and Shroot 2005).

- Whilst others propose definitive time-frames proclaiming a wound chronic after 6 weeks, or 3 month's as the suggested time period of failure to heal (Dale, Callam et al. 1983; Schultz, Barillo et al. 2004).

Another way to classify chronic wounds is by the underlying disease process leading to the development of that wound. The most prevalent disease processes responsible are ulcers secondary to diabetes, venous disease, arterial disease and pressure ulcers. Not infrequently ulcers may be of mixed aetiology. The primary site usually affected by diabetic, venous and arterial ulcers is the legs; whilst pressure ulcers more frequently affect the sacral region.

Cutaneous healing may be defined broadly as the interaction of a complex series of phenomena that eventuates in the resurfacing, reconstitution and proportionate restoration of tensile strength of wounded skin (Deodhar and Rana 1997). The process of epithelial resurfacing is critical in order for the wound to be considered `healed'. The initial event in epithelisation is the migration of undamaged epidermal (Keratinocyte) cells from the wound margins. This process occurs within hours of wounding and is a directed event that doesn't require an initial increase in cellular proliferation. After migration has begun, an increase in epithelial proliferation at the wound margins occurs to provide the additional cells needed for wound cover. Proliferation is maximal at 48 to 72 hours after wounding and is reflected by a 17 fold increase in mitosis and epithelial hyperplasia at the wound edges. Keratinocytes assist in the process of reepithelisation by producing fibronectin, collagenases, plasminogen activator, neutral proteases and type V collagen.

Many factors can be responsible for impairing wound healing. These are classified into Local factors affecting the wound itself and Systemic factors indirectly contributing to a failure of a wound to heal. It is therefore important to quantify the impact of disease states on healing and to target specific treatments correcting the underlying abnormality in affected patients. Although good clinical practice should aim to remove or reduce the impact of these factors, it is not always possible to do so (Harding, Morris et al. 2002).

1.3.3 Local versus systemic factors influencing healing

There can be various recognised reasons why wounds may fail to heal. Even though initially there is often an initiating factor causing or prolonging a wound (e.g. poor venous drainage), there can be many contributing factors involved in preventing wound healing and often multiple factors affecting the one wound. Traditionally these are sub- divided up into local (e.g. foreign body, infection, pressure, necrotic tissue) and systemic causes (e.g. renal disease, malnutrition, DM, advanced age) (Figure 1.2).

Examples of some more common causes include prolonged pressure or shear forces on the skin, poor tissue perfusion (such as that from oedema-related congestion), one or more underlying disease states that not only contribute to wound formation but also impede healing, and other multiple local or systemic factors. Chronic wounds may take an extended period to achieve an apparent healing, but the wound may recur, if it is unable to sustain closure.

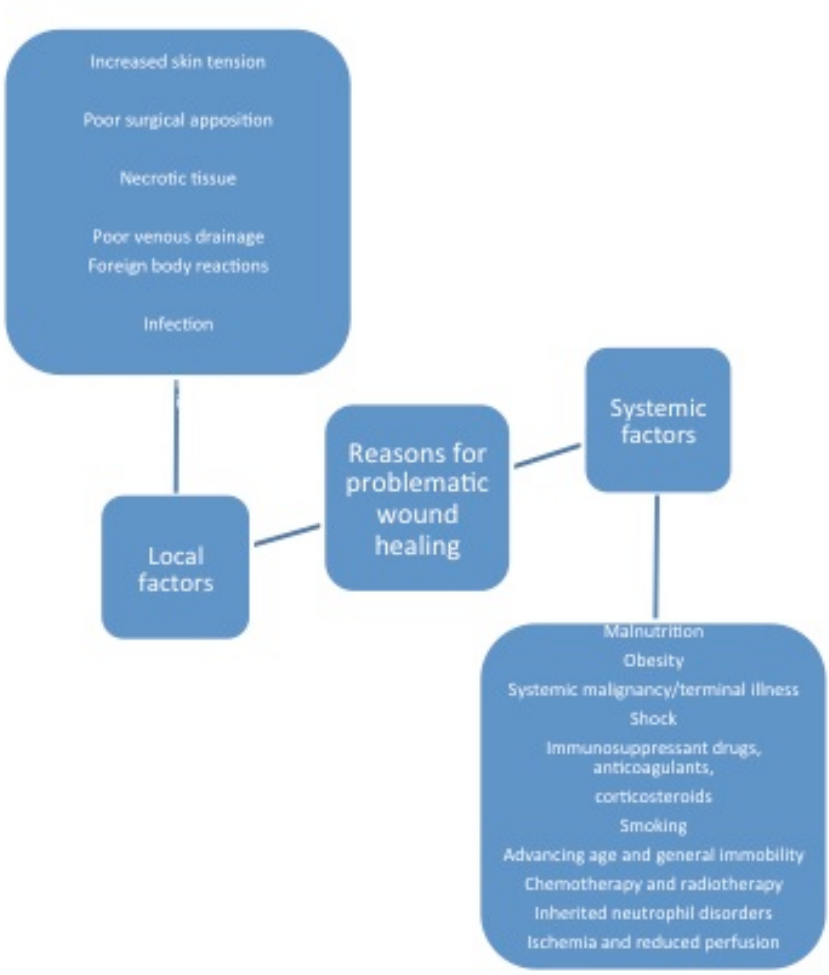


Figure 1.2: - Derived from (Grey, Enoch et al. 2006).

In addition to local and systemic factors that impair healing, reduction in tissue growth factors, an imbalance between proteolytic enzymes and their inhibitors, and the presence of senescent cells seem to be particularly important in chronic wounds (Harding, Morris et al. 2002).

With systemic factors it is important for the clinician to quantify the impact of these disease states on healing targeting specific treatments in order for the underlying abnormality to be corrected in individual patients. As hard as good clinical practice

aims to reduce or remove the impact of these factors, it is not always possible to do so (Harding, Morris et al. 2002).

Equally important clinically is the local management of the wound itself, with wound bed preparation proving key in the acceleration of endogenous healing and facilitate the effectiveness of other therapeutic measures. The key stages of local management of non-healing wounds include three key parameters; on-going wound debridement, management of exudate and resolution of bacterial imbalance (Schultz, Sibbald et al. 2003; Schultz, Barillo et al. 2004).

Despite differences in the causal aetiology, wounds inherently share many similarities at a molecular level (Medina, Scott et al. 2005). Reduced levels of active growth factors in the wound environment may partially explain why certain wounds fail to heal. Chronic ulcers are known to have reduced levels of platelet derived growth factor, basic fibroblast growth factor, epidermal growth factor, and transforming growth factor β in comparison with acute wounds (Harding, Morris et al. 2002). It has been suggested that the imbalance between proteinases and their inhibitors leads to trapping of growth factors by extracellular matrix molecules or an over-degredation by the overactive proteases, with ultimately both processes resulting in non-healing wounds (Chan, Zhang et al. 2008).

Excessive proteinase activity in chronic wounds, likely resultant from overexpression of matrix metallo-protein leads to abnormal degradation of the extracellular matrix (Ladwig, Robson et al. 2002; Lobmann, Ambrosch et al. 2002). As a result of these developments new treatment strategies directed at modifying this imbalance by; the topical application of proteinase inhibitors, inducing the expression of endogenous

inhibitors, or combining proteinase inhibitors with growth factors are currently being developed (Chan, Zhang et al. 2008).

Furthermore, chronic wound cells in particular dermal fibroblasts, keratinocytes and endothelial cells, have an age related decrease in proliferation potential, called senescence (Telgenhoff and Shroot 2005). It is thought that it is this impaired responsiveness to growth hormone, which may be due to an increased number of senescent cells within fibroblasts resident in chronic wounds (Stojadinovic, Pastar et al. 2008).

1.3.4 Relevance of gene signatures in chronic wounds

As well as the recognised influences of proteolytic enzymes and growth factors exerting effects on wound healing there has been a growing body of literature investigating the impact of genetic expression 'gene signatures' on wounds and their relevance in the hindrance of healing a wound (Cole, Tsou et al. 2001; Kirsner, Charles et al. 2008). The lack of understanding of these molecular mechanisms and pathogenesis as discussed above predisposes to serious health problems leading to limb amputations and associated morbidity secondary to chronic wounds.

The predominant cell type implicated in this process by Tomic-Canic et al is the Keratinocyte with its early response to injury and interactions with the extracellular matrix leading to migration and hyper-proliferation which is paralleled by changes in keratinocyte adhesion and cytoskeletal content (Stojadinovic, Brem et al. 2005). This transient healing response phenotypically resembles malignant transformation of keratinocytes during squamous cell carcinoma progression (Pedersen, Leethanakul et

al. 2003). With genetic analyses pertaining to cancer having already been significantly developed the past years it is only logical that science is now trying to establish the genetic and molecular mechanisms associated with chronic wounds and how these profiles differ and change following injury and chronicity of a wound. Tomic-Canic et al have already begun to establish and identified hundreds of potential target genes which could contribute and are involved in the wounding process (Tomic-Canic and Brem 2004). With the advancements of microarray gene technology this high volume analysis of genes has now become a reality. Cole et al looked at and compared normal skin tissue with acutely wounded tissue at set time intervals, with 3% of the 4000 identified genes undergoing a transformation from the baseline in their up or down regulation from non injured tissue (Cole, Tsou et al. 2001).

Stojadinovic et al analysed the effects of both c-myc and β-Catenin in relation to keratinocyte migration using a similar wound healing model, and investigated the levels of these genes at the edge of non-healing wounds (Stojadinovic, Brem et al. 2005).

Following on from this work Stojadinovic et al looked at microarray data from tissue samples sourced in a clinical setting from venous leg ulcers and compared them to normal skin. Their results were interesting in that genes pertaining to the cell cycle (required in a normal healing wound) such as p107, p130 and Rb were down regulated in venous ulcers, indicating a breakdown in this normal wound healing cycle (Stojadinovic, Pastar et al. 2008).

Also around the same time Charles at al analysed tissue samples obtained from the edges and the centre of chronic healing and non-healing venous leg ulcers. They too identified a number of genes that differed in between the two cohorts both with the respect to up and down-regulation (Kirsner, Charles et al. 2008).

These studies provide a template for analysis of identified specific genes known to affect wound healing allowing subsequent in depth analyses of their function and mechanism of action to be carried out.

1.4 Epithelial Protein Lost in Neoplasm (EPLIN)- molecular and clinical implications and relation to LIM domain family of proteins

There has been little knowledge on the role of EPLIN (Eplithelial Protein Lost in Neoplasm) in the healing process of humans and with EPLIN's relatively recent discovery it is only now slowly coming to light where this protein falls in terms of classification, function and relevance in vivo. The following chapter will examine the available literature to date on the topic looking at EPLIN classification as well as the molecular mechanisms through which it is thought to elucidate its function and its implications in human disease.

1.4.1 LIM domain

The LIM domain family of proteins was first described 15 years ago as a cysteine-rich sequence that was common to a small group transcription factors, it is now recognized as a tandemzinc-finger structure that functions as a modular protein-binding interface. LIM domains represent in many proteins that have diverse cellular roles as regulators of gene expression, cytoarchitecture, cell adhesion, cell motility and signal

transduction (Way and Chalfie 1988; Freyd, Kim et al. 1990). Protein-interaction domains, including the LIM domain, are now recognized as key components of the regulatory machinery of the cell (Pawson and Nash 2003).

In 1988, Way and Chalfie et al isolated and characterized cDNAs that encoded *Caenorhabditis elegans* MEC-3, required for the specification of mechanosensory neurons. They noted that MEC-3 contained a homeodomain as well as another sequence that had no similarity to any sequences known at this time. They subsequently encoded the *C. elegans* cell-lineage protein LIN- 11 by cloning of the gene responsible (Way and Chalfie 1988), and the insulin gene-enhancer-binding protein Isl1 (Stadelmann, Digenis et al. 1998). These discoveries led to the identification of a cysteine rich sequence that was common to all three of these proteins with the new protein motif being called the LIM domain. The name coming from the first letter of LIN-11, Isl1 and MEC-3, respectively (Kadrmas and Beckerle 2004). This LIM amino-acid sequence is believed to promote various diverse biological functions and is present is a wide variety of eukaryotic cells. It possesses features that promote the formation of a stable structural core, and variable features that impart high-affinity binding to many structurally and functionally diverse protein partners and it is through the specific binding of their targets that LIM proteins fulfil their array of biological functions (Kadrmas and Beckerle 2004).

Biological systems mix and match a restricted number of modular protein domains in a cassette-like fashion to generate a proteome with the requisite functional complexity. By virtue of their ability to recruit specific proteins, protein-interaction domains can localize these targets to discrete subcellular locations, modulate their

activities or assemble them into multi-component complexes. Therefore, these protein- interaction domains are essential for integrating diverse cellular circuits. Years after the discovery and initial characterization of the LIM domain, its importance as a facilitator of protein liaisons is beginning to be fully appreciated (Kadrmas and Beckerle 2004).

Through the binding of their partners, LIM proteins participate in an array of biological processes, encompassing aspects of cytoskeletal function and the control of gene expression (Zheng and Zhao 2007). LIM proteins are known to shuttle in between the nucleus and the cytoplasm predominantly with involvement in the integration and communication of the actin cytoskeleton and the nucleus (Kadrmas and Beckerle 2004).

Within the cytoplasm, several LIM proteins have been identified to directly regulate actin polymerization and depolymerisation reactions one of which is EPLIN. As mentioned above LIM domains represent in many proteins that have diverse cellular roles as regulators of gene expression, cytoarchitecture, cell adhesion, cell motility and signal transduction (Way and Chalfie 1988; Freyd, Kim et al. 1990) EPLIN (Epithelial Protein Lost In Neoplasm) which is located on the LIM domain containing gene and shows a down regulation in cancer is a cytoskeletal LIM protein believed to crosslink and depolarize actin filaments and so increasing the number of actin stress fibres at the detriment of more dynamic structures. Such an action means that a loss or downregulation of EPLIN is believed to result in the increased motility and invasiveness of tumour cells (Maul, Sachi Gerbin et al. 2001; Maul, Song et al. 2003).

As EPLIN is downregulated in tumour cells no knockout models are present investigating the effects of knockout of the gene.

1.4.2 EPLIN discovery and background

EPLIN (Epithelial Protein Lost In Neoplasm) is a cytoskeletal protein that is preferentially expressed in human epithelial cells (Maul and Chang 1999). EPLIN was initially discovered in 1998 by Chang *et al* in a study that searched for transformation related genes in oral cancer. The authors used cDNA representational difference analysis to identify genes that were differentially expressed between normal oral epithelial cells and HPV-immortalised oral epithelial cell lines and identified EPLIN as one such gene located in chromosome 12 LIMA1 12q13.12 (Figure 1.3) (Chang, Park et al. 1998). There are two known EPLIN isoforms, EPLIN α (a 600 amino acid) and EPLIN β (a 759 amino acid), both of which are detected in primary epithelial cells of oral mucosa, prostate and mammary glands (Chen, Maul et al. 2000). The human EPLIN gene contains 11 exons and spans more than 100kb, with transcription arising from different starting points accounting for the two EPLIN isoforms (Chen, Maul et al. 2000). Analysis of the predicted amino acid structure identified a central LIM domain (Maul and Chang 1999). Both EPLIN isoforms localise to filamentous actin and suppress cell proliferation, and most strikingly the migration when over expressed (Maul, Song et al. 2003; Han, Kosako et al. 2007; Leitner, Shaposhnikov et al. 2010). Early studies highlighted the localisation of both EPLINα and β have been observed in the cytoplasm with a fibrillar pattern similar to that of actin fibres and over-expression of EPLINα and β have been associated with reduced growth potentials (Maul and Chang 1999). For the purposes of the current

study EPLIN α was predominantly looked at as this is the isoform mostly associated and active in cancer.

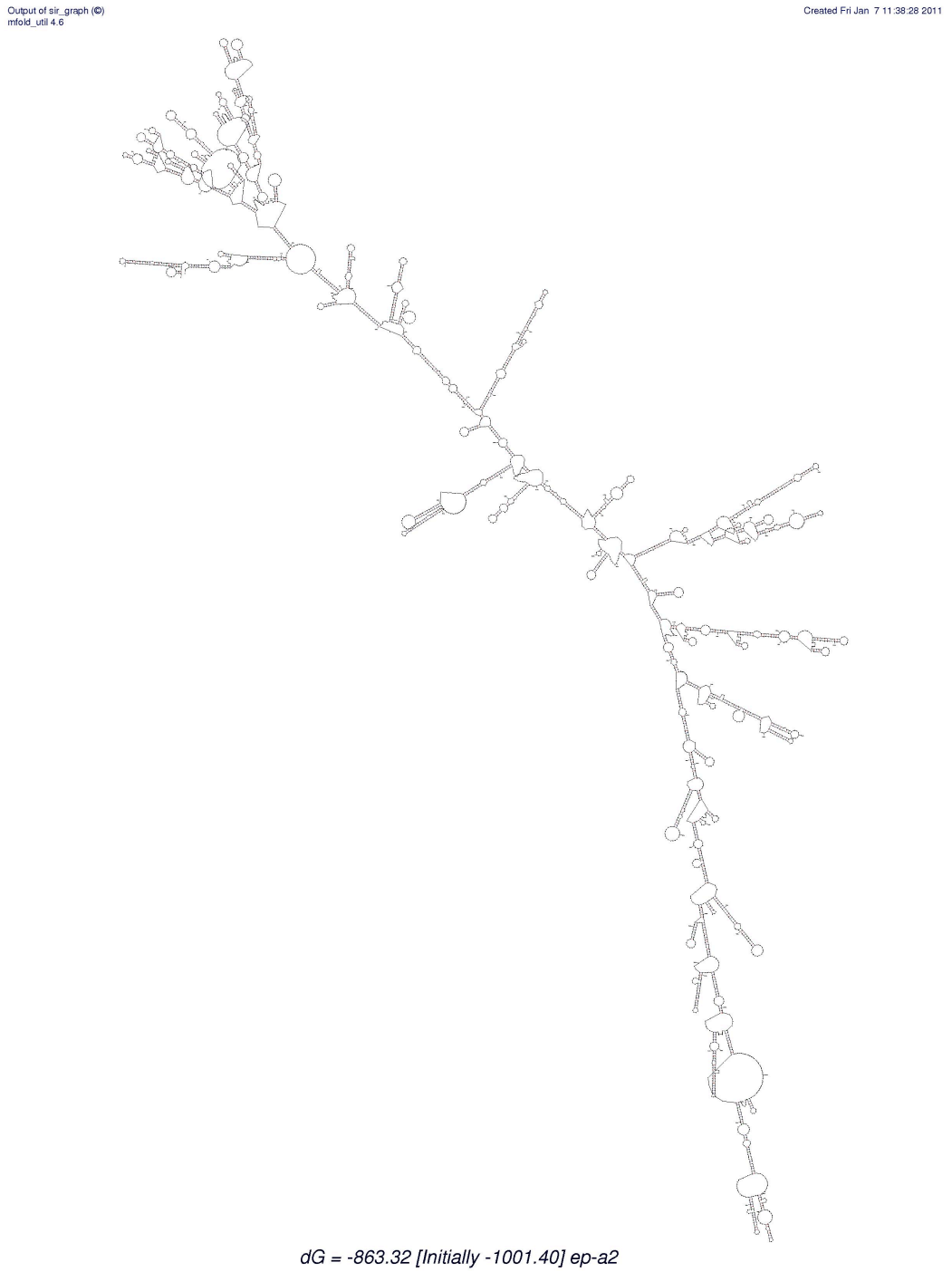


Figure 1.3: - The Secondary structure of human EPLIN mRNA.

EPLIN has been characterised as a cytoskeletal protein and studies have implicated its involvement in a variety of processes.

Maul *et al.,* demonstrated the ability of EPLIN to regulate actin structures, finding that expression of EPLINα can increase actin stress fiber numbers and can inhibit membrane ruffling mediated by Rac1. EPLINα is also able to bind actin monomers at both NH_2 and COOH with reduced binding efficiency being seen in truncated rather than full length EPLINα and suggesting at least two independent binding sites situated either side of the LIM domain. Similarly EPLINα is able to bind F-actin in a ratio of at least two actin molecules per EPLIN and can cause actin filament bundling. Further evidence for EPLINs role in actin stabilisation is demonstrated through the ability of EPLINα to delay actin filament depolymerisation but have little effect on actin polymerisation and that binding of EPLINα to actin filaments could prevent secondary activation of nucleation mediated by Arp2/3 complex (Maul, Song et al. 2003).

1.4.3 Molecular implications of EPLIN function

1.4.3.1 Cell to cell interactions

Throughout the lifetime of a cell they need to interact with one another. Their interactions can be short lived such as an immunological synapse and long lived such as a neuromuscular junction. The adhesion between the cells is mediated by various cell adhesion molecules or CAMss. The function of these is to mediate and allow binding to specific partner proteins thereby facilitatate interactions between two cells whether that be adhesion or migration amongst other cellular functions.

The CAMs are mainly composed of Immunoglobulin based molecules (IgCAMs), cadherins, integrins and selectins (Takai and Nakanishi 2003). The immunoglobulin function is predominantly to facilitate cadherin based cell junctions via proteins called Nectins (Takai and Nakanishi 2003). The Cadherins on the other hand are a large group of proteins consisting of more than 100 members and six sub families which perform a variety of important cellular functions. It is the type 1 and 2 cadherins that are well established in the processes of cell to cell adhesion and thus facilitate migration which is of relevance to our study (Niessen, Leckband et al. 2011). These two families exert their extra cellular interactions through multiple outermost cadherin repeats on the apposing cells (Harrison, Jin et al. 2011). The type 1 cadherins in particular also utilize molecules called catenins to facilitate this process of cell to cell binding, adhering to the long cytoplasmic tails of the catenin proteins. The catenins are important as they form a part of the bridge connecting adherens junctions to the cytoskeleton of the cell (Harrison, Jin et al. 2011). The third subfamily of CAMs the intergrins are primarily responsible for cell matrix interactions via their

interactions with extracellular proteins and also via their interactions with IgCAM's play an important role in mounting an immune response (de Fougerolles and Springer 1995). Finally the selectin members of this CAM family are responsible for P-selectin protein interactions in between endothelial cells (Lasky 1992).

In the process of cellular motility which underpins the essence of the current study, cells need to form junctions with one another. This is where these CAM molecules are of such importance as are required in the formations of junctions in between the cells, of which there are three main types in humans; Zona occludens or Tight junctions which feature in epithelial and endothelial cells and primarily function as diffusion barriers (Steed, Balda et al. 2010). Adherens junctions which regulate cell shape and through the translation of actinomyosin generated forces maintain tissue integrity and thereby are of paramount importance in the motility of a cell (Niessen, Leckband et al. 2011). The key component of adherens junctions are the transmembrane glycoproteins cadherins, which bind the intracellular proteins α and β-catenin. The understanding of the exact mechanism in which this occurs and the large framework of contributing proteins and molecules is still not fully understood (Yonemura 2011). Finally desmosomes are the last type of cellular junction providing mechanical stability to cells such as bladder and myocardium which are under a large amount of mechanical stress (Steed, Balda et al. 2010).

With the Adherens junctions (AJ) being of greatest interest in relation to our study in potentially understanding how EPLIN might elicit its function or where it might integrate into the process, it is important to briefly look at what is already understood with relation to the functioning of this process. The Adherens junction is not a static

entity, it is on the contrary a dynamic structure which is continuously assembled and disassembled in response to changing biochemical signals and mechanical tissue forces. The exact mechanism of their formation is still not fully understood however cytoskeletal adapter proteins, cadherins and catenins are believed to play a crucial role and provide the physical skeleton for the process (Green, Getsios et al. 2010). It is thought that AJ formation occurs in response to cells encountering each other, migrating or changing their shape with their turnover being of paramount importance to the homeostasis of epithelial tissues. With relation to migrating cells what is observed is that as they come into contact a spatiotemporal distribution of cadherins and catenins occurs, in which the cells 'reach out' to each other with these protein molecules in almost an exploratory filopodal manner and make contact with one another. The initial contact is short lived however they then go onto form a stable junction with one another, via a zipper like motion with cadherins and catenins clustering at the contact sites (Jacinto, Wood et al. 2000).

1.4.3.2 Mechanism of molecular function of EPLIN

Therefore as outlined above one of the primary roles of CAM complexes is to connect the actinomyosin network of one cell to that of neighboring cells and facilitate the generation and transduction of mechanical forces at their interface (Bershadsky, Balaban et al. 2003; Weber, Bjerke et al. 2011). All CAM complexes consist of an intercellular and cytoplasmic component. Intercellular interactions are predominantly driven by cell-adhesion molecules (CAMs) such as cadherin or nectin, often in a calcium-dependent process (Niessen and Gottardi 2008). However the function of the surrounding cytoplasmic region which is visualized as a dense plaque of proteins containing a mixture of a vast number of scaffolding and regulatory proteins is still to

be fully understood. This area is key to understanding how a cell goes about initiating and maintaining the integrity of the adherens junctions (Niessen and Gottardi 2008). It is believed that it is in this area that EPLIN exerts its function.

During development or any pathological processes such as cell repair epithelial sheets undergo dynamic cell rearrangement, such as epithelial-mesenchymal transition, convergent extension, migration and folding (Thiery 2002; Kang and Massagué 2004; Montell 2008). For this processes the regulation of the Adherens junction (AJ) is thought to be important (Perez-Moreno, Jamora et al. 2003). One of the key processes associated with the AJ related morphogenetics is that of wound healing, in which the epithelial sheets fuse to one another via AJ's (Jacinto, Wood et al. 2000; Vasioukhin and Fuchs 2001). This means that in wound closure there is a 'purse stringing' effect of the actinomyosin cables as they are organised along the margins of the leading edges of the cells. These gradually contract to close the open space (Franke, Montague et al. 2005; Tamada, Perez et al. 2007).

The zona adherens (ZA) which is a type of adherens junction (AJ) is believed to play a major role in cell to cell adhesions. It is thought that a variety of epithelial cells adhere to each other via this ZA consisting of E cadherin-catenin complexes and associated actin filaments, called circumferential actin cables and is located in the region near the apical end of lateral cell to cell contacts showing a closed ring configuration (Farquhar and Palade 1963; Boller, Vestweber et al. 1985; Cavey and Lecuit 2009; Meng and Takeichi 2009). It is thought that Alpha-Catenin which binds to E cadherin via Beta Catenin, mediates the actions between E cadherin–Beta Catenin complex and actin filaments (Kovacs, Goodwin et al. 2002; Nelson 2008;

Meng and Takeichi 2009; Sawyer, Harris et al. 2009; Kwiatkowski, Maiden et al. 2010). However, it is little known how this ZA is remodelled during epithelial reorganisation. Recent work by Katsutoshi et al 2011 showed that EPLIN acts to maintain the ZA via its association with Alpha- Catenin and that junctional tension was important to retain EPLIN at the AJ. This study pointed to the finding that epithelial cells remodel their junctional architecture by responding to mechanical forces, and that Alpha Catenin bound EPLIN was a mechanosensitive regulator in this process (Taguchi, Ishiuchi et al. 2011). Maul et al demonstrated that EPLIN has the ability to stabilise actin filaments (Maul and Chang 1999) and in turn this maintains the circumferential actin cables (Abe and Takeichi 2008).

Interaction of EPLIN found in the cadherin-catenin complex with interaction through α-catenin appears to be through both the N- and C- terminal domains involved in actin binding and the VH3-C region of α-catenin as deletion of any of these regions removed the interaction. EPLIN can then function to combine the cadherin-catenin complex to F-actin, through its binding with α-catenin and thus, link cadherin to F-actin. Whilst EPLIN is required to link cadherin to F-actin to form the adhesion belt it doesn't seem to be required for the interaction between cadherin and radial actin fibres, with EPLIN depletion resulting in adhesion belt conversion into zig-zag forms (Abe and Takeichi 2008).

The study demonstrates the importance of EPLIN in both linking the actin bundles to the cadherin-catenin complex and also in the stabilisation of these actin bundles and suggests that in cancerous cells, where EPLIN is frequently lost, cadherin-mediated cell adhesion may also be disrupted and could thus contribute to enhanced invasive behaviour.

Cells may use this potentially dynamic linkage of the two systems to transmit mechanical forces generated by the cytoskeleton to cell junctions and vice versa, so as to regulate cell shape and migration along with other cellular properties (Taguchi, Ishiuchi et al. 2011). It has also been proposed that EPLIN works as a linker between the Alpha Catenin and F Actin. Thus assisting in the ZA formation and hence emphasising its importance in the cellular processes such as migration and rearrangement of epithelial architecture at the periphery of the colonies (Taguchi, Ishiuchi et al. 2011).

In 2007 EPLIN was identified as an Extracellular Signal-Regulated Kinase (ERK) substrate by Han *et al.,* undergoing ERK phosphorylation at Ser360, Ser602 and Ser692 *in vitro* and in living cells thus ERK has been shown to phosphorylate EPLIN. This phosphorylation was found to decrease the C-terminal region affinity for actin filaments and it was discovered what a non ERK – phosphorylatable EPLIN mutant inhibited PDGF actin stress fiber disassembly, cell migration and membrane ruffling suggesting phosphorylation of EPLIN by ERK plays a role in EPLINs regulation of actin dynamics and motility (Han, Kosako et al. 2007).

1.4.4 EPLIN in human disease

EPLIN was initially discovered as a protein whose expression was reduced in transformed compared to normal oral cells (Chang, Park et al. 1998). In the last few years, there have been some reports to show the potential role of this protein in human cancer (Chang, Park et al. 1998; Maul and Chang 1999; Jiang, Martin et al. 2008). It is the acquisition of invasive and migratory capabilities by cancer cells that is the first step in cancer metastasis at the primary site (Fidler 2003). Efficient migration and

invasion require cancer cells to establish and maintain defined morphological features often lost with cell polarity, and although stabilisation of the actin cytoskeleton is important to the maintenance of the epithelial phenotype dynamic remodelling of the actin network is required for the invasive cancer cells to be able to spread further (Zhang, Wang et al. 2011).

1.4.4.1 EPLIN role in Prostate and Breast cancer

Since its discovery EPLIN, particularly EPLINα, levels have been shown to be frequently reduced or absent in a wide variety of cancer cells and tissues. Early studies identified EPLINα as being significantly reduced in aggressive PC-3 and DU-145 cell lines compared to prostate epithelial cells, similar to this in EPLINα levels are non-detectable in LNCaP and LAPC4 cells, whilst expression of EPLINβ was comparable to prostate epithelial cells. The same study identified similar trends in the MCF-7, T-47D and MDA-MB-231 breast cancer cell lines, where again EPLINα levels were either absent or reduced in the breast cancer cell lines and EPLINβ levels were unchanged or increased compared to the mammary epithelial cells (Maul and Chang 1999).

In keeping with these early results, work conducted within our laboratories has similarly reported differential expression of EPLIN in clinical prostate and breast cancer (Jiang, Martin et al. 2008; Sanders, Martin et al. 2011). Examination of EPLINα expression throughout a cohort consisting of 120 tumour and 32 normal breast samples revealed a reduction in EPLINα expression in breast tumour sections compared to normal breast sections following immunohistochemical analysis. Additionally, analysis of EPLINα transcript levels, using quantitative polymerase

chain reaction, in these samples also demonstrated a reduction in tumour versus normal breast samples. Interestingly, further correlation of EPLINα together with clinical patient data indicated that, within the breast cancer specimens, EPLINα expression reduced as cancer predictive factors, such as Nottingham Prognostic Index (NPI), grade and TNM stage increased and lower levels of EPLINα transcript could be associated with poorer patient prognosis and shorter overall and disease free survival rates (Jiang, Martin et al. 2008). A similar analysis of EPLIN expression was conducted in a prostate sample cohort consisting of 20 tumour and 11 normal prostate samples. Immunohistochemical analysis of these sections once again revealed a reduction in EPLIN expression in tumour sections compared to normal sections which, through quantification of staining intensity was found to be significant. However, due to the limited size of the prostate cohort, analysis of patient data did not demonstrate significant reductions between grade 6 and grade 7 samples and T1 compared to T2 stage samples, though it was noted that EPLIN levels were generally reduced in the higher stage and grade samples (Sanders, Martin et al. 2011).

A study by Chircop *et al.,* has linked EPLIN to cytokinesis, where EPLINs role in this process seems to be through its ability to associate with cytoskeletal systems needed for membrane ingression and formation of the cleavage furrow and depletion of EPLIN in HeLa cells resulted in a large number of multinucleated cells, characteristic of cytokinesis failure (Chircop, Oakes et al. 2009). This study provides additional implications for EPLIN in cancer as the failure of cells to undergo cytokinesis results in aneuploidy and genomic instability, a trend frequently seen in cancer cells.

1.4.4.2 EPLIN as a potential tumour/metastasis suppressor

To further examine the role of EPLINα in cancer progression Jiang et al generated a mammalian plasmid (pEF6/V5-His-TOPO, Invitrogen, Paisley, UK) containing the full coding sequence of EPLINα (Jiang, Martin et al. 2008). This EPLINα expression plasmid was subsequently used in a number of studies to assess the impact of enhancing EPLINα expression in a range of breast, prostate and endothelial cell lines (Jiang, Martin et al. 2008; Sanders, Ye et al. 2010; Sanders, Martin et al. 2011).

Overexpression of EPLINα in MDA-MB-231 breast cancer cells brought about a number of changes to this aggressive cell line. MDA-MB-231 cells overexpressing EPLINα became less invasive and were no longer responsive to the pro-invasive effect of Hepatocyte Growth Factor (HGF), which could substantially increase *in vitro* invasiveness in MDA-MB-231 wild type and plasmid control cells but had minimal effect of MDA-MB-231 cells transfected with the EPLINα expression plasmid. Additionally, overexpression of EPLINα reduced *in vitro* growth over three day incubation. This trend was also observed *in vivo* where MDA-MB-231 cells overexpressing EPLINα inoculated into CD-1 athymic nude mice produced significantly smaller tumours than those arising from inoculation of control MDA-MB-231 cells. Overexpression of EPLINα was also seen to have a profound effect on MDA-MB-231 motility, substantially decreasing migrational rates compared to control cells (Jiang, Martin et al. 2008). Similar to the trends seen in the breast cancer study, a decrease in aggressive traits was seen in PC-3 prostate cancer cells following enhancement of EPLINα expression. Overexpression of EPLINα in PC-3 cells similarly reduced cellular invasiveness and could negate the pro-invasive effect induced by HGF. Similar to the breast cancer cells, EPLINα over-expressing PC-3 cells displayed slower growth rates *in vitro* and produced significantly smaller

tumours *in vivo* following inoculation into nude mice. EPLINα also seemed to impact on cell-matrix adhesion where overexpression of this protein reduced the ability of PC-3 cells to adhere to an artificial basement membrane (Sanders, Martin et al. 2011).

In the breast and prostate cancer studies carried out by Jiang et al the overexpression of EPLINα reduced cell growth rates and *in vivo* development. This ability has similarly been observed in other studies (Maul and Chang 1999) and suggests that this protein may have some role in regulating cell growth in the tumour. This could have implication in tumorigenesis and the local growth and development of the tumour suggesting a potential tumour suppressive role for EPLINα. In addition to this, the ability of EPLINα to interfere with the processes of cellular invasion, migration and cell-matrix adhesion have implication on the metastatic escape of tumour cells from the primary tumour, indicating a potential metastasis suppressor role for EPLINα. In support of this the ability of EPLINα to reduce the sensitivity of breast and prostate cancer cells to the HGF molecule is interesting. HGF is widely recognised in the literature as enhancing the processes of tumorigenesis and enhancing aggressive traits required for cells to undergo metastasis (Jiang, Martin et al. 2005; Cecchi, Rabe et al. 2010; Martin and Jiang 2010; Nakamura, Sakai et al. 2010; Nakamura, Sakai et al. 2011). Thus, the observations reported in these two studies could indicate one potential mechanism through which EPLINα can act to slow cancer progression.

The ability of EPLIN to reduce aggressive traits in both breast cancer and prostate cancer cell lines implies that the loss of EPLIN may be a contributing factor in cancer development and progression and indeed other studies have proposed that the loss of

EPLIN and the associated cytoskeletal changes associated with this may aid to enhance the invasive migration seen in cancerous cells (Maul, Song et al. 2003).

Further evidence in support of a metastasis suppressive role for EPLIN has been recently provided by Zhang *et al*., in a study examining the potential of EPLIN to contribute to the Epithelial-Mesenchymal Transition (EMT) process utilising a previously established androgen refractory cancer of the prostate (ARCaP) cell lineage model that resembles characteristics of EMT and mimics the pathophysiology of prostate cancer metastasis (Zhang, Wang et al. 2011).

Zhang *et al.,* discovered that EPLIN expression was abundant in the epithelial like $ARCaP_E$ cells but was significantly reduced in the mesochymal like $ARCaP_M$ cells, similarly, IHC of tumours formed from subcutaneous inoculation of $ARCaP_E$ cells showed abundant EPLIN expression whereas those formed from inoculation of $ARCaP_M$ showed significantly reduced EPLIN levels (Zhang, Wang et al. 2011). Additionally, the depletion of EPLIN levels through siRNA or shRNAs in $ARCaP_E$ cells resulted in a loss of cell-cell contacts and the formation of spindle shape mesenchymal like morphology characteristic of EMT, induced Actin remodelling, enhanced migratory properties and the capability to infiltrate Matrigel. Depletion of EPLIN in $ARCaP_E$ cells resulted in a decreased expression of E-cadherin, increased expression of vimentin, brought about the translocation of β-catenin to the nucleus and activation of T-cell reporter presenting further evidence for a role for EPLIN in regulation of EMT. Reduced expression of EPLIN was also apparent in lymph node metastatic tumours of prostate, breast, colorectal and squamous cell carcinoma of the head and neck in comparison to the respective matched primary tumour, implicating

the downregulation of EPLIN as a potential indicator of clinical metastasis in a number of epithelial cancers (Zhang, Wang et al. 2011).

1.4.4.3. Similarities shared between wound healing and cancer

Tissue regeneration and tumorigenesis are both complex, adaptive processes influenced by cues from the host and tissue microenvironment with a variety of signals orchestrating the response to injury that results in regeneration and repair of a wound. Both tissue regeneration and carcinogenesis involve cell proliferation, survival, and migration that are controlled by growth factors and cytokines as well as inflammatory and angiogenic signals (Riss 2006).

As a result, cancers and wounds share a number of phenotypic similarities in cellular behavior, signaling molecules, and gene expression (Riss 2006). It was Haddow et al that first recognized the similarities in the processes between wound healing and carcinogenesis, with Dvorak et al being the first to describe cancer as wounds that would not heal (Haddow 1974; Dvorak 1986).

With the use of Microarray technology it has also been possible to conduct analysis and compare global gene expression between regenerating and malignant tissues predominantly in the keratinocyte cell type where similarities in gene expression have been found between squamous cell skin cancer and normally healing wound tissue (Pedersen, Leethanakul et al. 2003). Furthermore Chang et al. conducted a study looking at changes in the global gene expression profiles of fibroblasts exposed to serum *in vitro* and compared those profiles with the publicly available gene expression data for numerous tumours. The outcome was a striking similarity

between the gene expression profile of fibroblasts, compared with that of cancer, the fibroblast being one of the key cells in wound healing (Chang, Sneddon et al. 2004).

There have also been a number of studies that have put the in vitro and theoretical findings into practice, notably an in vivo study carried out by Dolberg et al showing that Chickens injected with the Rous sarcoma virus developed cancer exclusively at the site of injection even if wounded at different and distant sites (Dolberg, Hollingsworth et al. 1985). This pattern of wounding followed by a cancer stimulus developing into a tumour was also seen by Schuh et al who noted that transgenic mice which overexpressed the *Jun* oncogene developed dermal sarcomas following wounding (Schuh, Keating et al. 1990).

It is also well recognised that non healing ulcers are capable of progressing to malignant transformation, first recognised by pioneering clinician Virchow in 1863 where he noted a number of examples of chronic irritation predisposing to tumour formation (Schafer and Werner 2008). Following on from Virchow's initial observations modern medicine is aware of numerous examples of this phenomenon notably in venous ulcers progressing to SCC (so called 'Majorin ulcer'), inflammatory bowel disease predisposing to bowel cancer and gastric inflammation associated with H.Pylori infection progressing to gastric cancer.

Given this established relationship, it may be possible to infer how LIM proteins in general and EPLIN in particular, affect wound healing given their known role in cancer. And whether indeed the opposite phenomenon would be seen if an underexpression of a substance in cancer known to be an aggressive and proliferative

process would in fact be the opposite scenario in relation to say a non healing non proliferating wound.

1.5 The future of wound healing

With wound healing and cancer sharing so many cellular processes in common and the multitude of recent advances and developments in the field of genetics and molecular biology in cancer understanding it is perhaps not surprising that the genetic composition of wound tissue is gaining so much recent interest. This new direction has opened up the possibility of very exciting new discoveries and advances as we head into the future. The LIM family in general, and specifically the EPLIN protein coding sequence, could prove to be a highly relevant potential gene candidate for investigating in the context of wound healing with a known role in cancer, and effects on cellular migration analysing gene expression in tissues surrounding the edge of chronic wounds.

The ultimate aim of this research would be to develop a diagnostic test which following analysis of chronic wound samples would be able to predict with some accuracy those wounds capable of healing and the wounds which get stuck in the chronic phase and are unable to heal through the detection of the expression patterns, or signatures, of a group of identified genes known to influence and progress to chronicity in chronic wounds (such as the overexpression of EPLIN). This would be a very exciting development in the field as it would allow allocation of resources more accurately in the management of difficult wounds. Also through scientific investigation into potentially key molecules, such as EPLIN, and their importance in

the wound healing process we may gain vital information into the complex cellular and tissue events that may ultimately determine whether a wound will heal in a timely and organised fashion or not, giving rise to potential therapeutic intervention. Further advances in wound healing may include the development of diagnostic tests capable of accurately predicting wound healing rates based solely on genetic expression of wound-edge keratinocytes (and other cells). Already, similar technology is used as a prognostic tool in the context of breast cancer (Ross, Hatzis et al. 2008). A better understanding of aberrant genetic expression of various genes would allow a genetic test to be validated and used. Furthermore, understanding important protein pathways involved in wound healing may allow targeted treatments to be delivered.

1.6 Hypothesis and aims

Hypothesis: EPLIN is a key regulator in keratinocyte migration (and possibly proliferation) during wound re-epithelisation, which may be perturbed in non-healing chronic wound phenotype.

1- ***Determine if EPLIN expression has an influence on the migration of keratinocytes***

2- ***Examine the mechanism through which EPLIN impacts on keratinocyte cell function***

3- ***Define EPLIN expression in clinical wound tissue and potential links to healing profile***

CHAPTER 2 – MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

All standard chemicals and reagents, unless otherwise stated, were obtained from Sigma (Dorset, UK). The cell line used was the HaCaT cell line containing the EPLIN overexpression plasmid.

Table 2.1: General materials used

Material & Reagent	Supplier
10% foetal calf serum (FCS)	PAA Laboratories, Coelbe, Germany
Acetic acid	Fisher Scientific, Leicestershire, UK
Acrylamide mix (30%)	Sigma-Aldrich Co, Poole, Dorset, UK
Agarose	Melford Laboratories Ltd, Suffolk, UK
Ammonium persulfate (APS)	Sigma-Aldrich Co, Poole, Dorset, UK
Amphotericin B	Sigma-Aldrich Co, Poole, Dorset, UK
Bio-Rad DC Protein Colourimic Assay	Bio-Rad Laboratories, Hercules, CA, USA
Boric acid	Duchefa Biochemie, Haarlem, Netherlands
Bromophenol Blue	Sigma-Aldrich Co, Poole, Dorset, UK
$CaCl_2$	Sigma-Aldrich Co, Poole, Dorset, UK
Chloroform	Sigma-Aldrich Co, Poole, Dorset, UK
Commasine Blue	Sigma-Aldrich Co, Poole, Dorset, UK
DAB Chromogen	Vector Laboratories Inc, Burlingame, CA, USA
DEPC (Diethylpyrocarbonate)	Sigma-Aldrich Co, Poole, Dorset, UK
Dimethylsulphoxide (DMSO)	Fisons Scientific Equipment, Loughborough, UK
DMEM/Ham's F12 with L-Glutamine medium	PAA Laboratories, Coelbe, Germany
EDTA (Ethylenediaminetetraacetic acid)	Duchefa Biochemie, Haarlem, Netherlands
Ethanol	Fisher Scientific, Leicestershire, UK
Ethidium bromide	Sigma-Aldrich Co, Poole, Dorset, UK
FITC conjugated rabbit anti-goat IgG	Santa-Cruz Biotechnology, Santa-Cruz, CA, USA
FITC conjugated sheep anti-mouse IgG	Santa-Cruz Biotechnology, Santa-Cruz, CA, USA
Fluorescence mounting medium	CalBiochem, Nottingham, UK
Glycine	Melford Laboratories Ltd, Suffolk, UK
HaCaT cell line	Cancer Research Centre (DKFZ), Heidelberg, Germany
HCL	Sigma-Aldrich Co, Poole, Dorset, UK
Horse Serum	Sigma-Aldrich Co, Poole, Dorset, UK
Isopropanol	Sigma-Aldrich Co, Poole, Dorset, UK
KCl	Fisons Scientific Equipment, Loughborough, UK
KH_2PO_4	BDH Chemicals Ltd, Poole, England, UK
Mayers Htx	Sigma-Aldrich Co, Poole, Dorset, UK
Methanol	Fisher Scientific, Leicestershire, UK
NaCl	Sigma-Aldrich Co, Poole, Dorset, UK

Na_2HPO_4	BDH Chemicals Ltd., Poole, Dorset, UK
NaN_3	Sigma-Aldrich Co, Poole, Dorset, UK
NaOH	Sigma-Aldrich Co, Poole, Dorset, UK
Nitrocellulose membrane	Ammersham, Cardiff, UK
Penicillin	Sigma-Aldrich Co, Poole, Dorset, UK
Peroxidase conjugated goat anti-rabbit IgG	Sigma-Aldrich Co, Poole, Dorset, UK
Peroxidase conjugated rabbit anti-goat IgG	Sigma-Aldrich Co, Poole, Dorset, UK
Peroxidase conjugated rabbit anti-mouse IgG	Sigma-Aldrich Co, Poole, Dorset, UK
Ponceau S Stain	Sigma-Aldrich Co, Poole, Dorset, UK
Precision qScript™ RT PCR kit	Primerdesign LTD, Southampton, UK
REDTaq™ ReadyMix PCR reaction mix	Sigma-Aldrich Co, Poole, Dorset, UK
RNA extraction buffer	Advanced Biotechnologies Ltd, Epsom, Surrey, UK
SDS (Sodium dodecyl sulphate)	Melford Laboratories Ltd, Suffolk, UK
Serum bovine albumin	Sigma-Aldrich Co, Poole, Dorset, UK
Streptomycin	Sigma-Aldrich Co, Poole, Dorset, UK
SupersignalTM West Dura system	Pierce Biotechnology Inc., Rockford, IL, USA
TBS Automation Wash Buffer	Biocare Medical, Concord, CA, USA
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich Co, Poole, Dorset, UK
TRI Reagent	Sigma-Aldrich Co, Poole, Dorset, UK
Tris-Cl	Melford Laboratories Ltd, Suffolk, UK
TRITC conjugated goat anti-rabbit IgG	Sigma-Aldrich Co, Poole, Dorset, UK
Triton	Sigma-Aldrich Co, Poole, Dorset, UK
Trypsin	Sigma-Aldrich Co, Poole, Dorset, UK
Tween 20	Melford Laboratories Ltd, Suffolk, UK
Vectastain Universal ABC kit	Vector Laboratories Inc, Burlingame, CA, USA

Table 2.2: General hardware and software used

Hardware/Software	Supplier
0.4 μm filtration unit	Sigma-Aldrich Co, Poole, Dorset, UK
16-well chamber slide (for Immunohistochemistry)	Nalge NUNC International, Rochester, NY
$25cm^2$ and $75cm^2$ culture flasks	Cell Star, Germany
Amplifluort detection system	Intergen, England, UK
ECIS (Electrical Cell-Substrate Impedance Sensing)	Applied BioPhysics Inc., Troy, New York, USA
Electroporation cuvette	Euro Gentech, Southampton, UK
Fluorescent microscope	Olympus, Lake Success, NY, USA
iCycler iQt system	Bio Rad, Hercules, CA, USA
Image J	Public Domain
Lecia DM IRB microscope	Lecia GmbH, Bristol, UK
Microscope heated plate	Lecia GmbH, Bristol, UK
Microsoft Excel	Microsoft In., Redmond, WA, USA
Neubauer haemocytometer counting chamber	Reichert, Austria
Nitrocellulose membrane	Hybond C, Ammersham, Cardiff
Protein spectrophotometer	BIO-TEK, Wolf Laboratories, York, UK
RNA spectrophotometer	BIO-TEK, Wolf Laboratories, York, UK
Ultra-Turrax T8 Homogenizer	IKA Labortechnik, Staufen, Germany

UV light chamber	Germix
UVI-doc system	UVITech, Inc., Cambridge, England, UK
UVITech imager	UVITech, Inc., Cambridge, England, UK

2.1.1 Solutions for cell culture work

0.05M EDTA

One gram KCl (Fisons Scientific Equipment, Loughborough, UK), 5.72g Na_2HPO_4, 1g KH_2PO_4, 40g NaCl and 1.4g EDTA (Duchefa Biochemie, Haarlem, The Netherlands) was dissolved in distilled water to make a final volume of 5L. The solution was adjusted to pH 7.4 before autoclaving and storing for use.

Trypsin (25mg/ml)

Ten grams trypsin was dissolved in 400ml 0.05M EDTA. The solution was mixed and filtered through a 0.2µm minisart filter (Sartorius, Epsom, UK), aliquoted in 5ml samples and stored at -20^{o}C until required. For use in cell culture one 5ml aliquot was diluted in a further 100ml of 0.05M EDTA solution and used for cell detachment.

100X Antibiotics (Penicillin, Strpetomycin and Amphotericin B)

Five grams streptomycin, 3.3 grams Penicillin and 12.5mg Amphotericin B (previously dissolved in DMSO) were dissolved in 500ml of BSS. This solution was then filtered through a 0.2µm filter, aliquoted to 25ml portions and stored at -20^{o}C until required. Five millilitres of this solution was added to one 500ml bottle of DMEM giving 0.25µg/ml Amphotericin B, 0.1mg/ml streptomycin and 100U/ml penicillin.

Balanced Saline Solution (BSS)

Seventy nine and a half grams NaCl, 2.2g KCl, 2.1g KH_2PO_4, and 1.1g Na_2HPO_4 was dissolved in distilled water to make a final volume of 10L. pH was adjusted to 7.2 before use.

2.1.2 Solutions for use in RNA and DNA molecular biology

DEPC water

Two hundred and fifty microlitres diethyl pyroncarbonate (DEPC) was added to 4750µl distilled water. Solution was then autoclaved before use.

5X Tris, Boric acid, EDTA (TBE)

Five hundred and forty grams of tris-Cl (Melford Laboratories Ltd., Suffolk, UK), 275g Boric acid (Duchefa Biochemie, Haarlem, The Netherlands) and 46.5g of disodium EDTA was dissolved in distilled water and made up to a final volume of 10L. Solution was stored at room temperature and diluted to 1X concentrate prior to use in agarose gel electrophoresis.

Ethidum bromide

One hundred milligrams of ethidium bromide powder was dissolved in 10ml of distilled water. Container was wraped in aluminium foil to protect solution from sunlight and stored safely before use.

2.1.3 Solutions for protein work

Lysis Buffer

Two millimolar $CaCl_2$, 0.5% Triton X-100, 1 mg/ml leupeptin, 1 mg/ml aprotinin and 10 mmolar sodium orthovanadate was dissolved in distilled water and stored at 4^oC until required.

10% Ammonium Persulfate (APS)

One gram of ammonium persulfate was dissolved in 10ml of distilled water, separated into 2.5ml aliquots and stored at 4^oC until required.

10X Running buffer

Three hundred and three grams tris, 1.44Kg Glycine and 100g SDS was dissolved in distilled water to a final volume of 10L. Solution was further diluted to 1X concentrate before use.

Transfer buffer

Seventy two grams og glycine, 15.15g Tris and 1L Methanol (Fisher Scientific, Leicestershire, UK) were dissolved in distilled water to a 5L final volume.

10X TBS

One hundred and twenty one grams of tris and 400.3g NaCl were dissolved in distilled water, made up to a final volume of 5L and adjusted to pH 7.4.

Ponceau S stain

Supplied directly by Sigma.

Amido black stain

Two and a half grams of amido black (Edward Gurr Ltd., London, UK) was dissolved in 50ml acetic acid (Fisher Scientific, Leicestershire, UK) and 125ml ethanol (Fisher Scientific, Leicestershire, UK). Three hundred and twenty five millilitres of distilled water was added and solution mixed well.

Amido black destain

One hundred millilitres of acetic acid and 250ml ethanol were added to 650ml distilled water.

Coomasie blue stain

One gram coomassie blue, 400ml of methanol and 100ml of acetic acid were mixed and made up to a final volume of 1L in distilled water.

Coomasie blue destain

500 millilitres of methanol was mixed with 100ml of acetic acid and made up to a final volume of 1L in distilled water.

2.1.4 Solutions for cell and tissue staining

DAB chromagen

The DAB (Diaminobenzidine) chromagen was prepared by mixing the following reagents in order, 2 drops of wash buffer, 4 drops DAB (Vector Laboratories Inc., Burlingame, USA) and 2 drops of H_2O_2 to 5ml of distilled water. The mixture was shaken well after the addition of each reagent.

ABC Complex

The ABC complex was prepared using a kit obtained from Vector Laboratories Inc., Burlingame, USA. 4 drops of reagent A were added to 20ml of wash buffer, followed by the addition of 4 drops of reagent B and thorough mixing. The ABC complex was then left to stand for approximately 30 minutes before use.

2.2 General methods

2.3 Cell culture and storage

2.3.1 Preparation of cell medium

Cells were routinely cultured in DMEM / Ham's F12 with L-Glutamine medium (PAA Laboratories, Somerset, UK), supplemented with streptomycin, penicillin and 10% foetal calf serum (PAA Laboratories, Somerset, UK). Transfected cell lines, containing the pEF6 plasmid, were cultured initially in selection medium containing 5µg/ml blasticidin S and then later were routinely cultured in a maintenance medium containing 0.5µg/ml blasticidin S.

2.3.2 Revival of cells from liquid nitrogen

When required cells were removed from liquid nitrogen and resuscitated. Cells were thawed rapidly following their removal from liquid nitrogen by placing in a water bath at 37^{o}C. Once thawed, the outside of the cryotube was cleaned thoroughly with a steret sterile swab and placed in a universal container containing 10ml of pre-warmed medium to immediately dilute the DMSO present. The universal containers were centrifuged at 1600 RCF for 5 minutes to pellet the cells. The medium was aspirated to remove any traces of DMSO and the cell pellet was resuspended in 5ml of pre-warmed medium and placed into a fresh 25cm^2 tissue culture flask and incubated for 4 - 5 hours. Following incubation, the flask was examined under the microscope to visually confirm a sufficient number of healthy adherent cells had survived. The medium was changed to remove any dead cells which did not survive the freezing/resuscitation process. The flask was returned to the incubator and standard sub-culture techniques carried out when further necessary.

2.3.3 Maintenance of cells

Cells were maintained in supplemented DMEM medium prepared as described, and routinely sub cultured upon reaching 80 – 90% confluency. Confluence was assessed by visually assessing the approximate coverage of cells over the surface of the tissue culture flask using a light microscope. Cells were maintained and grown in either 25cm^2 or 75cm^2 tissue culture flasks (Greiner Bio-One Ltd, Gloucestershire, UK), in an incubator at 37.0^{o}C, 5% CO_2 and 95% humidity. All tissue culture techniques were carried out following aseptic techniques inside of a class II laminar flow cabinet.

2.3.4 Detachment of adherent cells and cell counting

Upon reaching approximately 80 – 90% confluency, medium was removed and the cells rinsed briefly with EDTA. Following this, adherent cells were detached from the tissue culture flask by incubating with trypsin/EDTA for several minutes. Once detached the cell suspension was placed in 20ml universal container (Greiner Bio-One Ltd, Gloucestershire, UK) and centrifuged at 1600 RCF for 5 minutes to pellet the cells. The cell pellet was resuspended, in an appropriate volume of fresh medium, before either determining cell numbers per millilitre (for use in cellular assays), or transferring a small volume of cell suspension into new tissue culture flasks.

A haemocytometer counting chamber allows the number of cells in a previously determined volume of suspension fluid to be calculated. Cells were counted on a haemocytometer counting chamber using an inverted microscope (Reichet, Austria) under 10 x 10 magnification. This allowed the number of cells per millilitre to be determined and accurate numbers of cells to be seeded in the *in vitro* and *in vivo* cellular functional assays. The dimensions of each 16 square area, containing cells to be counted, is 1mm x 1mm x 0.2mm which allowed the number of cells per millilitre to be determined using the following equation:

Cell number / ml = (number of cells counted in 16 squares ÷ 2) x (1×10^4)

Two 16 squared areas were counted to confirm consistent approximate cell density per millilitre between the two counts.

2.3.5 Storage of cell stocks in liquid nitrogen

Stocks of low passage cells were stored in liquid nitrogen. Cells were first detached from a large 75cm^3 flask using EDTA/Trypsin as described in section 2.3.4 and pelleted in a centrifuge. These cells were resuspended in the required volume (dependent on the number of samples to be frozen) of a protective medium consisting of 10% dimethyl sulphoxide (DMSO) in normal growth medium. Following resuspension, cells were alliquoted into pre-labelled 1.8ml cryotubes (Nunc, Fisher Scientific, Leicestershire, UK), in 1 ml volumes, attached to cryocanes, wrapped loosely in tissue paper and stored overnight at -81^{o}C in a deep freezer. Cells were later transferred to liquid nitrogen tanks for long term storage.

2.4 Synthesis of complementary DNA for use in PCR analysis

2.4.1 Total RNA isolation

RNA isolation was completed using the Sigma Total RNA Isolation Reagent (TRIR) Kit and protocol (Sigma, Dorset, UK) as described here. Cells were grown until 85-90% confluent, the medium was removed and 1ml of TRIR was added to the cell monolayer. The cell lysate was passed through a transfer pipette several times before being transferred into a 1.8ml eppendorf. The homogenate was kept at 4^{o}C for 5 minutes before adding 0.2ml chloroform and vigorously shaking the samples for 15 seconds, samples were centrifuged in a refrigerated centrifuge (Boeco, Wolf laboratories, York, UK) at 4^{o}C and 12,000g for 15 minutes. Following centrifugation, the upper aqueous phase containing RNA was carefully removed and added to a pre-labelled eppendorf containing an equal volume of isopropanol, the samples were then stored at 4^{o}C for 10 minutes before centrifuging at 12,000g and 4^{o}C for 10 minutes.

At this stage, RNA present in the sample will precipitate out of solution and will be visible as a pellet in the bottom of the eppendorf. The supernatum was discarded and the RNA pellet washed twice in 75% ethanol (prepared in a 3:1 ratio of absolute ethanol:DEPC water). Each wash consisted of the addition of 1ml of 75% ethanol, vortexing and subsequent centrifugation at 4^{o}C and 7,500g for 5 minutes. Following the final wash, as much ethanol as possible was removed from the eppendorf before briefly drying the pellet in a Techne, Hybridiser HB-1D drying oven (Wolf laboratories, York, UK) at 50^{o}C to remove any remaining ethanol. Finally the pellet was dissolved in 50 – 100µl (depending on pellet size) of DEPC water before proceeding to quantify the RNA present in the sample. DEPC water was used in RNA isolation to reduce the effects of any RNases that may be present. DEPC is a histidine specific alkylating agent and inhibits the action of RNases which rely on histidine active sites for their activity.

2.4.2 RNA quantification

Following isolation, RNA was quantified using a UV1101 Biotech Photometer (WPA, Cambridge, UK), that had been configured to detect ssRNA (µg/µl) in a 1 in 10 dilution based on the difference in absorbance at 260nm wavelength to a DEPC blank. All samples were measured in a Starna glass curvette (Optiglass limited, Essex, UK). So for our study I quantified the RNA level using a spectrophotometer and then standardised that to a set level throughout the samples (in this case it would either have been 250ng or 500ng of RNA). I then did the reverse transcription reaction and probed the resultant cDNA with GAPDH - this gives an indication of the quality and standardisation of the cDNA and thus the RNA.

If I was looking to check quality more thoroughly we can examine the ratio of the RNA between absorbance at 260nm and 280nm. Additionally, I ran the RNA on a gel to check the 28S and 18S bands.

2.4.2.1 Reverse transcription-polymerase chain reaction (RT-PCR) of RNA

Following RNA isolation and quantification, 250ng of RNA was converted into complementary DNA (cDNA) using an Enhanced Avian Reverse Transcriptase-PCR-100 kit (Sigma-Aldrich, Dorset, UK). RT-PCR was undertaken following the DuraScript Reverse Transcription for Two-Step RT-PCR protocol which is outlined below:

A sufficient volume of RNA suspended in DEPC water (isolated previously) to supply 250µg of RNA was added to a thin-walled 200µl PCR tube, additional PCR water was added to make the total volume of 8µl before adding 1µl of deoxynucleotide mix and 1µl of anchored oligo $(dT)_{23}$. The tube was then mixed gently and centrifuged before placing the mix in a T-Cy Thermocycler (Creacon Technologies Ltd, The Netherlands) and heating at 70^{o}C for 10 minutes. This initial step may denature the RNA secondary structure and allow more effective reverse transcription. Once this initial incubation step had finished, the tubes were removed from the thermal cycler and placed on ice before centrifuging and adding the following to each tube:

- 6µl – PCR water
- 2µl – 10X buffer for DuraScript RT

- 1µl – RNase inhibitor
- 1µl – Enhanced Avian Reverse Transcriptase (eAMVRT)

The tubes were mixed, centrifuged and placed back in the thermal cycler to be heated at 42^{o}C for 50 minutes. Once completed the cDNA generated was diluted with 1:3 with PCR water and tested using conventional PCR probing for β-actin expression to confirm successful reverse transcription. Samples were stored at -20^{o}C until further required.

2.4.3 Primers

The following primers were used in this thesis, purchased from Sigma-Aldrich, UK. Primer3 online programme was used to calculate PCR product size. Primers would have been designed using either Becon Designer or Primer3 site based on the sequence of the target gene. These were designed to span at least 2 exons (to enable identification of genomic DNA contamination) and have optimal Tm of 55^{o}C and 50% GC percentage. Once designed, primer specificity/ product size was checked using primer-BLAST.

Table 2.3: Primers used for PCR analysis

Primer	Forward	Reverse	PCR Products (bps)
EPLIN (PCR)	5'-GAAAATTGGCTGAGTGT	5'-ACTCAAGACCTTTTGCTCT	547

	ATG	T	
GAPDH (PCR)	5’-AGCTTGTCATCAATGGAAAT	5’-CTTCACCACCTTCTTGATGT	593
EPLIN (QRT-PCR)	5’-AAAGGGATTTGTTTCTGAC	5’-ACTCCAGGAACCGTACACAGAGTCGAGGAACTGGAG	93
GAPDH (QRT-PCR)	5’-CTGAGTACGTCGTGGAGTC	5’-ACTGAACCTGACCGTACAGAGATGATGACCCTTTTG	91
NWasp F8/R8	5'-AGTCCCTCTTCACTTTCCTC	5'-GCTTTTCCCTTCTTCTTTTC	457
Beta Catenin F11/R11	5’-GGTAGTCATGGAGAACTTAAA	5’-TTATAACGCGAACCACCAA	259
Alpha catenin F2/R2	5’-GCTATACTCATTCCTGCCGTGGG	5’-AGCGGCGTGTGCTTCCCATCAAATG	525
Gamma catenin F5/R4	5’-CTAGAACACAGTGGGGCTCAG	5’-CATCCCAGCGTTGTCCATCC	564
Erk F6/R7	5’-CCGGGACAACTACGAAA	ATTCACGTCCTCGTTATGC	547

	G		
FAK F1/R1	5'-AACGACAAGTTTCACCTCAG	5'-CATGTTCTCTGGACCGTGCTGTG	345
Paxillin F/R 22	5'-AAAGGTTCTACTGGGATTGA	5'-CCAGAGTGTCCAAAAAGAAC	223

2.4.4 Polymerase chain reaction (PCR)

PCR was carried out using a REDTaq ReadyMix PCR Reaction mix (Sigma, Dorset, UK). Sixteen microlitre reactions were set up for each sample to be tested as follows:

- 8µl - 2X REDTaq ReadyMix PCR Reaction mix
- 1µl – Specific forward primer
- 1µl – Specific reverse primer
- 5µl – PCR water
- 1µl - cDNA

Primers were designed using the Beacon Designer programme (Palo Alto, California, USA) and were synthesised by Invitrogen (Paisley, UK). Primers were diluted to a concentration of 10pM before being used in the PCR reaction. The PCR reaction was set up in a 200µl PCR tube (ABgene, Surrey, UK), mixed briefly and centrifuged

before being placed in a T-Cy Thermocycler and subjected to the following temperature shifts:

- Step 1: Initial denaturing period – 94°C for 5 minutes
- Step 2: Denaturing step – 94°C for 1 minute
- Step 3: Annealing step – reaction specific temperature for 2 minutes
- Step 4: Extension step – 72°C for 3 minutes
- Step 5: Final extension period – 72°C for 10 minutes

Steps 2 – 4 were repeated over 38 cycles. Specific reaction annealing temperatures together with primer sequence data is detailed in Table 2.3. Primer binding sites and predicted product sizes were verified using the Primer3 (v.0.4.0) software available online (http://frodo.wi.mit.edu/). RT-PCR products which corresponded with this predicted size following electrophoresis and staining were taken as being accurate. Positive and negative controls were also tested to verify RT-PCR primers. A collection of mixed RNA extracted from a large number of prostate cancer tissue sections and subsequently converted to cDNA was used as the positive control for most of the primer pairs

2.4.5 Agarose gel electrophoresis

Once the samples had completed the sufficient number of cycles in the thermocycler, the amplified DNA was separated according to size using agarose gel electrophoresis. Dependent on the predicted size of the DNA produced, the samples were loaded into either 0.8% (samples greater than approximately 500bp), or 2% (samples less than 500bp), agarose gels. Agarose gels were made by adding the required amount of

agarose (Melford Chemicals, Suffolk, UK) to TBE solution. This was then heated to fully dissolve the agarose, poured into the electrophoresis cassette and allowed to set around a plastic comb creating loading wells. Once set, the gel was submerged in TBE running buffer, 8µl of a 1Kb ladder (Invitrogen, Paisley, Scotland), or 10µl of sample was then added to the wells. The samples were then electrophoretically separated at 95V for a period of time to allow sufficient separation of the samples.

2.4.6 DNA staining and visualisation

Following successful electrophoresis, the gel was placed in ethidum bromide stain diluted in the TBE buffer used in the run. The gel was left to stain for 15 minutes before being visualised under ultra violet light using a UV illuminator (UVitech, Cambridge, UK) and capturing images using a UV camera imaging system (UVitech, Cambridge, UK). If necessary, the gel can be returned to the ethidum bromide stain for additional staining or to a container of distilled water for destaining to remove background. RT-PCR was repeated three independent times and representative data illustrating the expression pattern is presented.

2.5 Protein Detection Methodology SDS-PAGE and western blotting

cellular lysis and protein extraction

Upon reaching sufficient confluency, the cell monolayer was detached from the flask using a sterile cell scraper, both the detached cells and medium were then transferred to a universal container using a sterile transfer pipette. The cell suspension was centrifuged for 5 minutes at 1800 RCF to pellet cells and protein at the bottom of the universal container. Following centrifugation, the medium was aspirated and the cells

were lysed in 200 – 250µl (depending on pellet size) of lysis buffer (see section 2.1.4), before being transferred to a 1.8ml eppendorf (A Laboratories, Hampshire, UK) and placed on a Labinco rotating wheel (Wolf laboratories, York, UK) for 1 hour. The lysis solution was then spun at 13,000 rpm in a microcentrifuge for 15 minutes to remove any insolubles and the supernatant was transfer to a fresh eppendorf to await quantification.

2.5.1 Antibodies used

The following antibodies were used during this study

Table 2.4: Antibodies used for Western Blot Analysis (all antibodies used at concentrations recommended by the manufacturer as optimum levels)

Name	Animal Source	Molecular weight (kDa)	Commercial Origin	Product Code
Anti-EPLIN	Mouse	57.6	Santa Cruz	SC-28635
Anti PPaxillin	Rabbit	34	Santa Cruz	SC-45673
Anti-FAK	Goat	44.8	Santa Cruz Biotechnology, Inc.	SC-10127
Anti-Vimentin	Goat	56	Santa Cruz	SC-45621
Anti-βCatenin	Rabbit	85.5	Santa Cruz Biotechnology, Inc.	SC-2206
Anti-PAXILLIN	Goat	99.7 & 58.6	Santa Cruz Biotechnology, Inc.	SC-14236 and SC-14234
Anti-GAPDH	Mouse	38	Santa Cruz	SC-47724

			Biotechnology, Inc.	
Anti-FAK	Rabbit	46.7	Santa Cruz Biotechnology, Inc.	SC-9166
Anti-PFAK	Goat	29.7	Santa Cruz Biotechnology, Inc.	SC-8832
Anti-ERK	Mouse	78.5	Santa Cruz Biotechnology, Inc.	SC-21737
Anti-N-WASP	Goat	54.8 & 65	Santa Cruz Biotechnology, Inc.	SC-10122
Anti-PLCγ	Goat	148.5	Santa Cruz Biotechnology, Inc.	SC-31748

2.5.2 Protein quantification

Protein quantification was undertaken as one method to standardise the concentrations of the protein samples prior to their use in Western blotting. Protein concentration was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad laboratories, Hammelhempstead, UK), following the microplate method as outlined here. Firstly a range of standard samples of known concentrations were set up using a serial dilution of a 10mg/ml bovine serum albumin (BSA) standard (Sigma, Dorset, UK), standards were serially diluted from 10mg/ml to 0.005mg/ml in lysis buffer. Five microlitres of either the sample or standard was pipetted into a fresh well before adding 25µl of 'working reagent A' followed by 200µl of reagent B. 'Working reagent A' was prepared by combining each millilitre of reagent A with 20µl of reagent S and was used as samples contained detergent. Following addition of reagent B, samples were mixed briefly and then left for approximately 45 minutes to allow the colorimetric

reaction to fully occur. Absorbance of samples and standards at 620nm was then read using an ELx800 plate reading spectrophotometer (Bio-Tek, Wolf laboratories, York, UK). A standard curve was constructed based on the absorbances of the BSA standards and used to determine sample concentration. All samples were then normalised to the desired final concentration of between 1.0 – 1.5mg/ml through dilution in an appropriate amount of lysis buffer and further diluted in a 1:1 ratio with 2x Lamelli sample buffer concentrate. Samples were then boiled and stored at -20^{o}C prior to use.

2.5.3 Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to detect the presence, or absence, of specific proteins. Cells were grown in 75cm^2 tissue culture flasks to confluence. SDS-PAGE was undertaken using an OmniPAGE VS10 vertical electrophoresis system (OminPAGE, Wolf Laboratories, York, UK). Resolving gels of a certain percentage (depending on the predicted size of the protein of interest) were made up in a universal container and added in-between glass plates held in place in a loading cassette. The amount of each ingredient required to make up 15ml (enough for two gels) for both 8% and 10% resolving gels is indicated below:

Component	**8% gel**	**10% gel**
Distilled water	6.9ml	5.9ml
30% acrylamide mix	4.0ml	5.0ml
1.5M Tris (pH 8.8)	3.8ml	3.8ml
10% SDS	0.15ml	0.15ml
10% ammonia persulphate	0.15ml	0.15ml

TEMED	0.009ml	0.006ml

Once the resolving gel had set, the stacking gel was prepared and added to the top of the resolving gel. A plastic comb was placed in the unset stacking gel and the mixture was left to harden. The components and quantities required to prepare 5ml of stacking gel solution (enough for two gels) are shown below:

Component	**Stacking gel**
Distilled water	3.4ml
30% acrylamide mix	0.83ml
1.0M Tris (pH 6.8)	0.63ml
10% SDS	0.05ml
10% ammonia persulphate	0.05ml
TEMED	0.005ml

Once both resolving and stacking gels had set, the loading cassette was placed into an electrophoresis tank and covered in 1X running buffer. The combs were carefully removed and 8-10µl of molecular weight marker or 18µl of protein samples was added to the wells. The proteins were then separated according to molecular weight using electrophoresis at 125V, 40mA and 50W for a varying length of time (dependent on protein size and gel percentage).

8% gels were used to detect proteins with a molecular weight ranging between 50 – 100kDa and 10% gels were used when protein sizes were predicted to be between 20 – 90kDa.

2.5.4 Western blotting

Following PAGE of the protein samples they were transferred to a nitrocellulose membrane using Western blotting. Gels were removed from the electrophoretic tank and unclipped from the loading cassette; the stacking gel was cut away and the resolving gel placed on top of a Hybond nitrocellulose membrane (Amersham Biosciences UK Ltd, Bucks, UK) in a SD10 SemiDry Maxi System blotting unit (SemiDRY, Wolf Laboratories, York, UK). The following arrangement was set up for protein transfer from the gel to the membrane:

negative electrode : 3X blotting paper : membrane : gel : 3X blotting paper : positive electrode

3mm chromatography paper (Whatman International Ltd., Maidstone, UK) was used as blotting paper. Electoblotting was undertaken at 15V, 500mA, 8W over a 1 hour period. Once complete, membranes were removed and stored at 4^{o}C in 10% skimmed milk, 0.1% polyoxyethylene (20) sorbitan monolaurate (Tween 20) in TBS until required for specific antibody probing.

2.5.4.1 Staining of proteins

2.5.4.1.1 Nitrocellulouse membrane staining

Membranes were stained prior to probing with specific antibodies to confirm successful transfer or to aid in the sectioning on the membrane. Membranes were placed in Ponceau S solution for several minutes to allow visualisation of protein bands on the membrane, the membrane was then cut into the required number of sections before washing off the stain several times in 10% milk solution.

Following completion of antibody probing membranes were stained in Amido black solution for several minutes before being removed and placed in Amindo Black destain solution to remove background staining. The membranes were left overnight to dry completely and retained as a record of the analysis.

2.5.4.1.2 Polyacrilamide gel staining

Following electroblotting, the gels were stained in Coomasie blue protein stain to confirm that successful protein transfer from the gel had occurred. Gels were placed in Coomasie blue stain for several minutes before being placed into Coomasie blue destain and viewed.

2.5.4.2 Detection of proteins using specific antibody probing

Membranes were transferred to 50ml falcon tubes (Nunc, Fisher Scientific, Leicestershire, UK), placed in fresh 10% milk blocking solution and left to rotate on a

roller mixer (Stuart, Wolf laboratories, York, UK) for 1 hour. After this initial blocking period the 10% milk solution was removed and a specific primary antibody made up in 3% milk solution (3% milk powder, 0.1% Tween 20 in TBS) was added to the falcon tube to wash over the membrane for 1 hour, (specific details on antibodies are outlined in Table 2.4). After the 1 hour incubation period, the primary antibody solution was removed and the membrane subjected to three 15 minute washes with 3% milk to ensure complete removal of the primary antibody. Following these washes the secondary antibody of the same species as the primary was added in 3% milk solution to the membrane at a concentration of 1:1000 and left for 1 hour. The membrane was subjected to two 10 minute washes with tween TBS (TBS containing 0.1% tween 20), followed by two 10 minute washes with TBS. Following these washes chemiluminescent detection of the antibody-antigen complex was undertaken**.**

2.5.4.3 Chemiluminescent detection of antibody-antigen complex

Once specific antibody binding had taken place, the membranes were placed in a weighing boat and covered in Supersignal West Dura system reagents (Pierce Biotechnology, Rockford, Illinois, USA). The two reagents were added in a 1:1 mixture and left covering the membrane for five minutes. Excess reagent was then removed and the membrane was placed inside an UVITech imager (UVITech, Inc., Cambridge, UK). The imager contains both a camera and an illuminator and is linked to a computer that captures the image. The membranes were subjected to a number of exposure times and images captured. These images were then further analysed using the UVIband software package (UVITEC, Cambridge, UK), which allows the protein bands detected on the screen to be quantified. The expression of the housekeeping GAPDH was detected in conjunction to the other proteins of interest.

2.6 Keratinocyte cell functional assays

2.6.1 Transfection of cells with EPLIN expression plasmid

The full length human EPLINα cDNA sequence was isolated and amplified from normal tissues and subsequently inserted into a pEF6 plasmid as described previously (Jiang, Martin et al. 2008). This expression plasmid was used to transfect the HaCaT keratinocyte cell line, which express low levels of this molecule. Following transfection, the cells underwent blasticidin selection and successful over-expression of EPLINα was verified. HaCaT cells containing the expression plasmid and displaying amplified levels of EPLINα expression were termed $HaCaT^{EPLIN\ EXP}$ and were compared throughout to wild type cells ($HaCaT^{WT}$) and cells containing an empty pEF6 plasmid ($HaCaT^{pEF6}$). Cells were trypsinised, resuspended in complete medium and prepared at a density of 1million cells/ml. Approximately 800µl of this cell suspension was added to an electroporation curvette (GeneFlow, Staffordshire, UK) and mixed with 5µg of plasmid DNA. The cells were electroporated at either 290V or 310V and 1500 capacitance in an electroporator (Easyject, Flowgene, Surrey, UK). Following electroporation, the cell suspension was rapidly transfered to a $25cm^2$ tissue culture flask containing 10ml of pre-warmed complete medium and allowed to recover overnight. Following recovery, cell viability and density was assessed under the microscope and if sufficient cells were immediately placed into blasticidin selection medium (10µg/ml) for 5 - 10 days. Following this time, resistant cells that contained the plasmid (encoding blasticidin resistance) were placed in a less harsh maintenance medium (0.5µg/ml blasticidin) and grown up to sufficient denisity

to allow verification of over-expression sequence and cryopreservation of successfully transfected, over-expression lines.

2.6.2 *In vitro* keratinocyte cell growth assay

Cells were detached from the culture flask and cell density (per millilitre) was established as described previously. Cells were then seeded into a 96 well plate (Nunc, Fisher Scientific, Leicestershire, UK) at a seeding density of 3,000 cells in 200µl of normal medium. Triplicate plates were set up to obtain a cell density reading following 1, 3 and 5 day incubation periods. Following the appropriate incubation period, the medium was removed and cells were fixed in 4% formaldehyde in BSS for at least 5 minutes before rinsing and staining in 0.5% (w/v) crystal violet in distilled water, for 5 minutes. The stain was then extracted from the cells using 10% acetic acid and cell density determined by measuring the absorbance at 540nm on a plate reading spectrophotometer (ELx800, Bio-Tek, Wolf laboratories, York, UK). This experimental protocol has been modified from that previously described (Bonnekoh, Wevers et al. 1989). Cell growth was presented as percentage increase and calculated by comparing the absorbances obtained for each incubation period using the following equation:

Percentage increase = ((day 3 or 5 absorbance) – day 1 absorbance) / day 1 absorbance) x 100 [in %]

Within each experiment at least four duplicate wells were set up and the entire experiment protocol was repeated four times. This protocol is frequently used within the department to assess cell growth and has been well documented within the

literature. This protocol was thus chosen to the method to assess cell growth over other well documented methods.(Sanders, Ye et al. 2010)

2.6.3 *In vitro* keratinocyte cell migration (wound healing) assay

A wounding/migration assay was also used to assess the migratory properties of the HaCaT Keratinoytes cells This technique has been modified from a previously described method (Jiang, HIscox et al. 1999).

Cells were grown in a 24 well plate and, upon reaching confluence, the monolayer of cells was wounded with a 21G needle. After wounding the cells were given 15 minutes to recover before the closure of the wound via the migration of cells into the wound was tracked and recorded using a CCD camera attached to a Lecia DM IRB microscope (Lecia GmbH, Bristol, UK) to capture images at specific intervals over a 120 min period. The 24 well plate was placed on a heated plate (Lecia GmbH, Bristol, UK) to maintain a constant temperature of 37^{o}C. Cell migration was measured using Optimus 6 motion analysis software. The tape was played back and images saved at 0, 30, 60, 90 and 120 minute time points. The distance between the two wound fronts at several random points per incubation time was calculated using the ImageJ software; the arbitrary values obtained were converted into µm by multiplying the value by 0.8 as previously calibrated using a calibration grid. The distance that the wound fronts had migrated into the wound at each time point could then be determined by subtracting the distance between the two fronts at any given time point from that at the initial 0 minute experimental start point. The experimental procedure was repeated three independent times.

2.6.4 ECIS detection of cell attachment and migration

Cell adhesion and migrational rates following over-expression of EPLIN expression was assessed using the Electric cell-substrate impedance sensing (ECIS) model system. Cells were seeded into the ECIS array at a density of 200,000 cells per well and the array connected to the ECIS system. Changes in impedance were then recorded on the system as the cells attached to the electrodes present within the arrays. Cells were incubated for sufficient periods to allow complete adherence and formation of a confluent monolayer before undertaking migrational analysis (Figure 2.1). For migrational analysis, the cell monolayer was electrically wounded by applying a 3000 uA of for 30 secs per well (Figure 2.2). This was sufficient to kill the cells attached to the electrode, creating a break or "wound" in the monolayer. Resistance and impedance changes were then recorded as the cells migrated back into this wound. This protocol has been previously described in the analysis of EPLIN in human breast cancer cell line (Jiang, Martin et al. 2008).

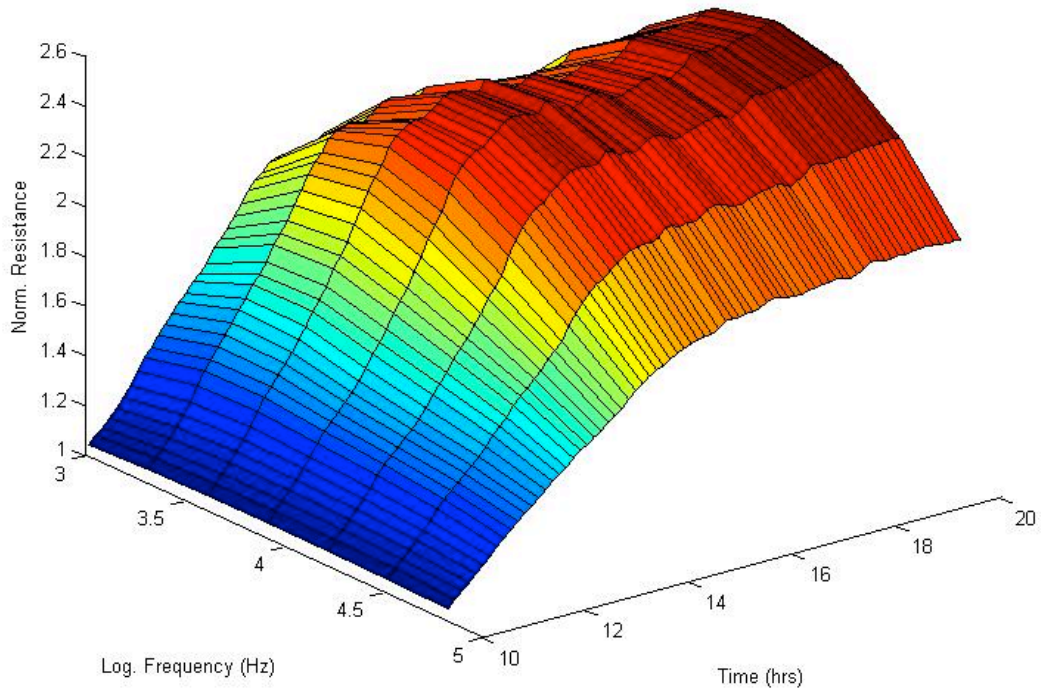


Figure 2.1: - Representative data readout from ECIS assay of initial attachment of cells in one of the wells with the associated change in impedance.

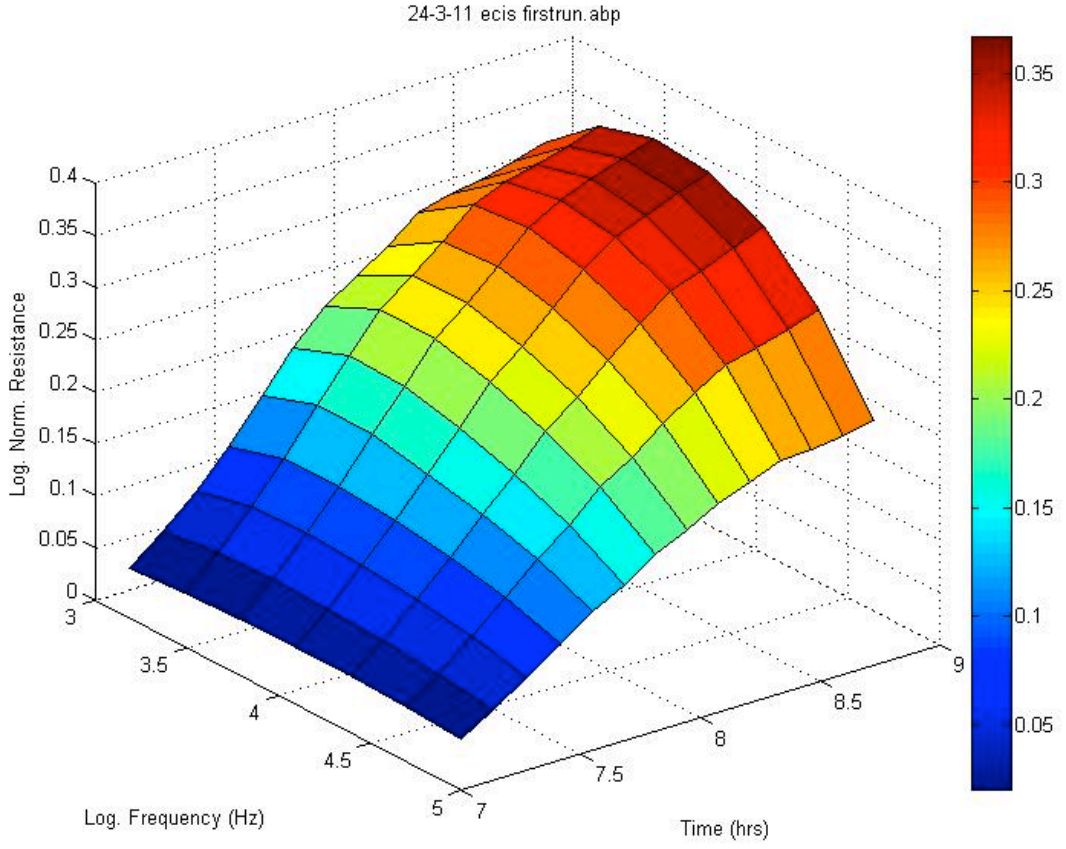


Figure 2.2: - Representative data readout from ECIS assay showing change in impedance over time accords the electrodes of one well following 'wounding' of monolayer.

2.6.4.1 Statistical analysis

Comparisons were drawn between HaCaT$^{EPLIN\ exp}$ cells and the empty plasmid HaCaT^{pEF6} control cells to determine significance in the *in vitro* functional assays. Either the parametric two sample, two tailed t-test or the non-paramentric Mann_Whitney test was used to compare two groups. In the case of ECIS migration analysis, a two way ANOVA was carried out on the SigmaPlot 11 statistical package.

Values were taken as significant at $p < 0.05$ and a minimum of three independent repeats of experimental procedures were undertaken.

2.7 Analysis of EPLIN in wound tissue

We utilised a pre-existing tissue bank for analysis of EPLIN transcript in wound tissues. The human skin and wound tissues were collected from patients who participated in wound clinics at the University Hospital of Wales or from healthy volunteers, with consent under the approval of the South East Wales Local Research Ethics Committee. The core biopsies were immediately stored in -80^{o}C and subsequently in liquid nitrogen, until processing. Three types of wound samples were collected as previously reported (Conway, Ruge et al. 2007).

2.7.1 Chronic wound tissue

Fourteen biopsies were collected from patients with chronic venous wounds. The following inclusion criteria were used in assessing patient suitability:

- No evidence of healing occurring 6 weeks prior to biopsy
- Wound present minimum of 6 months
- Evidence of venous disease as diagnosed by duplex ultrasonography
- A minimum surface area of 4cm^2 prior to biopsy
- Clinically free from infection.

2.7.2 Acute wound tissue

Ten biopsies collected were from patients with acute surgical wounds after undergoing excision of pilonidal disease (without primary closure, i.e. left to close via secondary intention). These wounds were judged to be clinically free from infection. The biopsies site was the edge of the healing wound within 6 weeks from the surgical excision.

2.7.3 Biopsy process

The following steps were used in collecting tissue:

- An aseptic technique was used throughout the procedure
- Local anaesthetic (1% Lidocaine), injected into the biopsy site
- 6mm punch biopsies (3mm punch biopsies for 'Normal Wound Tissue') were taken from the wound margin, incorporating epidermis and dermis at the wound edge with adjacent granulation tissue.

The lack of a power calculation was a limitation to the study and was the case as larger number of patients were not available for the study analysis.

2.7.4 Analysis of wound tissues

Individual biopsies were placed in separate Appendorf tubes and rapidly thawed. The biopsy was then homogenized using the Ultra-Turrax T8 homogenizer (IKA Labortechnik, Germany) in an RNA extraction buffer (Advanced Biotechnologies Ltd, UK). Total cellular RNA was quantified using a spectrophotometer.

2.7.4.1 Quantitative RT-PCR (Q-RT-PCR)

Q-RT-PCR is a sensitive technique that is capable of detecting very small quantities of cDNA within a sample. The cDNA for use in Q-RT-PCR was generated as described in the sections above; this cDNA was then used to make up a master reaction mixture containing the following amounts of each component per reaction:

- Forward Z primer – 0.3µl (1pmol/µl)
- Reverse primer – 0.3µl (10pmol/µl)
- Q-PCR Master Mix – 5µl
- Probe Ampiflour – 0.3µl (10pmol/µl)
- PCR H_2O – 2.1µl
- cDNA – 2µl

In each reaction, one of the primer pairs (Table 2.3), will contain a Z-sequence (ACTGAACCTGACCGTACA) at a 1/10 concentration of the other primer and the probe. The Amplifluor system was used to detect and quantify transcript copy number. The ampliflour probe consists of a region specific to the Z-sequence together with a hairpin structure labelled with a fluorescent tag (FAM). Whilst in the hairpin structure this fluorescent tag is effectively quenched and produces no signal. However, the specificity of the 3' region of the ampilflour probe to the Z-sequence causes the incorporation of this uniprimer. Subsequent DNA polymerisation, following incorporation, results in the disruption of the hairpin structure and effective signalling of the fluorescent tag within this structure. The degree of fluorescence within each sample compared to a range of standards of known transcript copy number allows the calculation of transcript copy number within each sample.

Detection of β-actin copy number within these samples was subsequently used to allow further standardisation and normalisation of the samples. Figure 2.3 (A) and Figure 2.3 (B) show the detection of a range of standard samples (10^8 to 10^2 copy number) and the subsequent generation of a standard curve from these samples.

Sample cDNA was amplified and quantified over a large number of shorter cycles using an iCycler^{IQ} thermal cycler and detection software (BioRad laboratories, Hammelhempstead, UK) and experimental conditions are outlined below:

- Step 1: Initial denaturing period – 94^{o}C for 5 minutes
- Step 2: Denaturing step – 94^{o}C for 10 seconds
- Step 3: Annealing step – 55^{o}C for 15 seconds
- Step 4: Extension step – 72^{o}C for 20 seconds

Step 2 – 4 was repeated over 60 cycles. The camera used in this system is set to detect signal during the annealing stage. The experimental procedure was repeated twice and data representative of the expressional trends is presented. It should be noted that the high number of cycles was not necessarily needed to detect the product, as calculation of the transcript number arises based on when fluorescent detection reaches a certain threshold point. Furthermore, in this established method, approximately 20 cycles are required for the generation of Z-tagged products (Nazarenko *et al*., 1997). This is illustrated in Figure 2.3 where the 10^8 sample was similarly run at a high number of cycles but reaches threshold relatively early on the (approx 18 cycles). Subsequently, calculation of sample copy number will depend on

point at which the sample reaches threshold cycle (TC) in comparison to the standards, automatically generated by the instrument software. Specific Q-PCR primers were verified using a positive control known to express the molecule of interest and a negative control, where PCR water replaced cDNA, was also included to rule out contamination of the reaction.

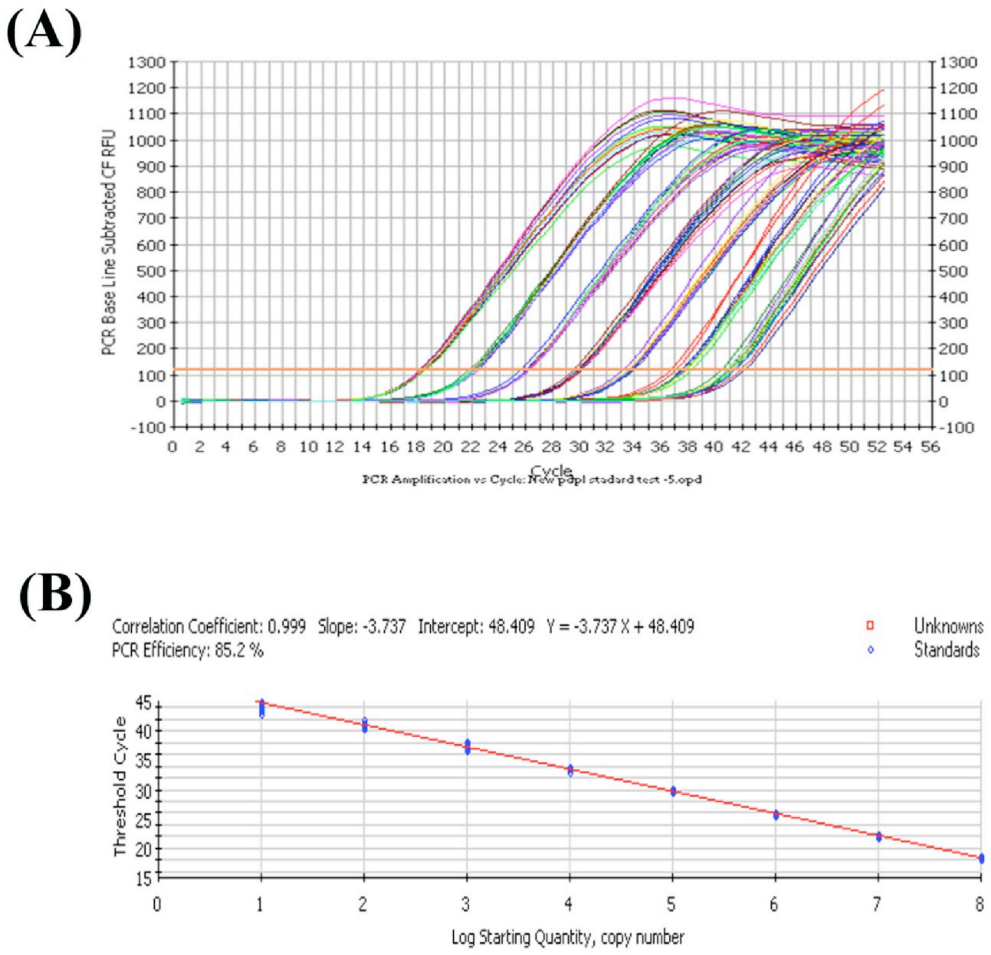


Figure 2.3: - Analysis of standards used in Q-RT-PCR.

(A) Detection of the range of Q-RT-PCR standards used over the experimental procedure. (B) Each standard was tested multiple times (n=12) and used to generate a standard curve.

CHAPTER 3 -

Expression of EPLIN with basic cellular functions

3. Introduction

All cells depend on varying vital biological processes to establish themselves in their environment, and to subsequently mobilise. The most important of these include aberrations in cell proliferation, adhesion and migration. Cells can influence these processes in several ways, most significantly by altering the expression of molecules that play key roles in controlling these cellular functions.

In the current chapter, EPLIN was overexpressd in the keratinocute cell line (HaCaT) utilising a plasmid coding for the EPLIN sequence, with the effect this had on cellular functions subsequently investigated.

3.1 Results

3.1.1 Expression profile of EPLIN in human wound tissue

Even though it is possible to hypothese that EPLIN plays an important role in wound healing in vitro, it was really important to establish this link in actual human tissue. Wound tissue was obtained over several months in outpatient clinics and EPLIN mRNA transcript data were analysed in wound tissues taken from the edge of healing (surgical) and non healing (chronic ulcers) wounds (Figure 3.1). Samples were analysed using qRT-PCR, as described in section 2.7 Data obtained was normalised to CK19. Subsequently, normalised EPLIN transcript levels were assessed in both non-healing and healing chronic wound tissues. As the data was not found to be normally distributed, a Mann-Whitney non-parametric test was used to test for significance. EPLIN levels were tended to be elevated in the non-healed tissues

(median value = 2607) compared to the healed tissues (median value = 1420), however, this difference was not found to be statistically significant (Figure 3.1). Even though the sample size was relatively small (healed n=10, non healed n=14) and as a result a statistically significant difference could not be established it is clearly visible looking at the median values of the two samples that there is a substancially increased EPLIN expression in the non-healed as compared with the healed samples. This correlates with our in vitro findings of EPLIN overexpression negatively affecting migration and motility of keratinocyte cells, as one would expect keratinocyte cells at the periphery of a non healing wound to be relatively dormant and not migrating as would have been the cells on the peripheries of a healed wound. This further provides evidence that EPLIN is a pivotal regulatory factor in the migration of keratinocyte cells in a real world clinical setting.

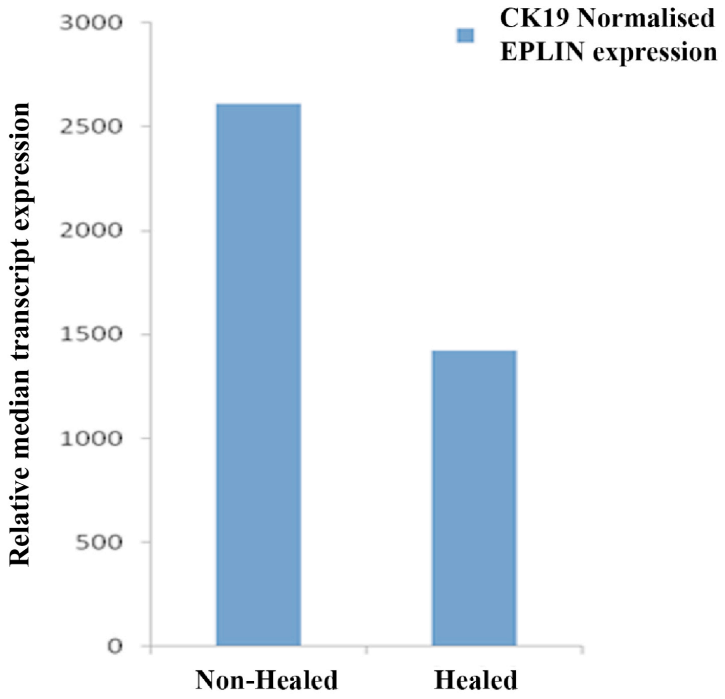


Figure 3.1: - qRT PCR analysis of healed vs non-healed wounds normalised to CK19. Median CK19 normalised EPLIN expression values in non-healed (n = 14) and healed (n = 10) clinical samples.

3.1.2 Imuno-histochemical staining of EPLIN in wound cohort

Imunohistochemical staining was also performed on the non-healed and healed tissue samples to see wherther there would be any apparent visible differences between the two sample groups when the samples were probed for EPLIN. Again a small difference in expression was visible between the two sample groups. The resultant differences in expression were not large enough to be statistically significant on densitometry analysis using ImageJ, but on reviewing multiple samples a clear trend of increased expression and positive staining was observed in the non healing compared with the healing wound groups with the non healing groups staining darker for EPLIN expressio (Figures 3.2 A and B).

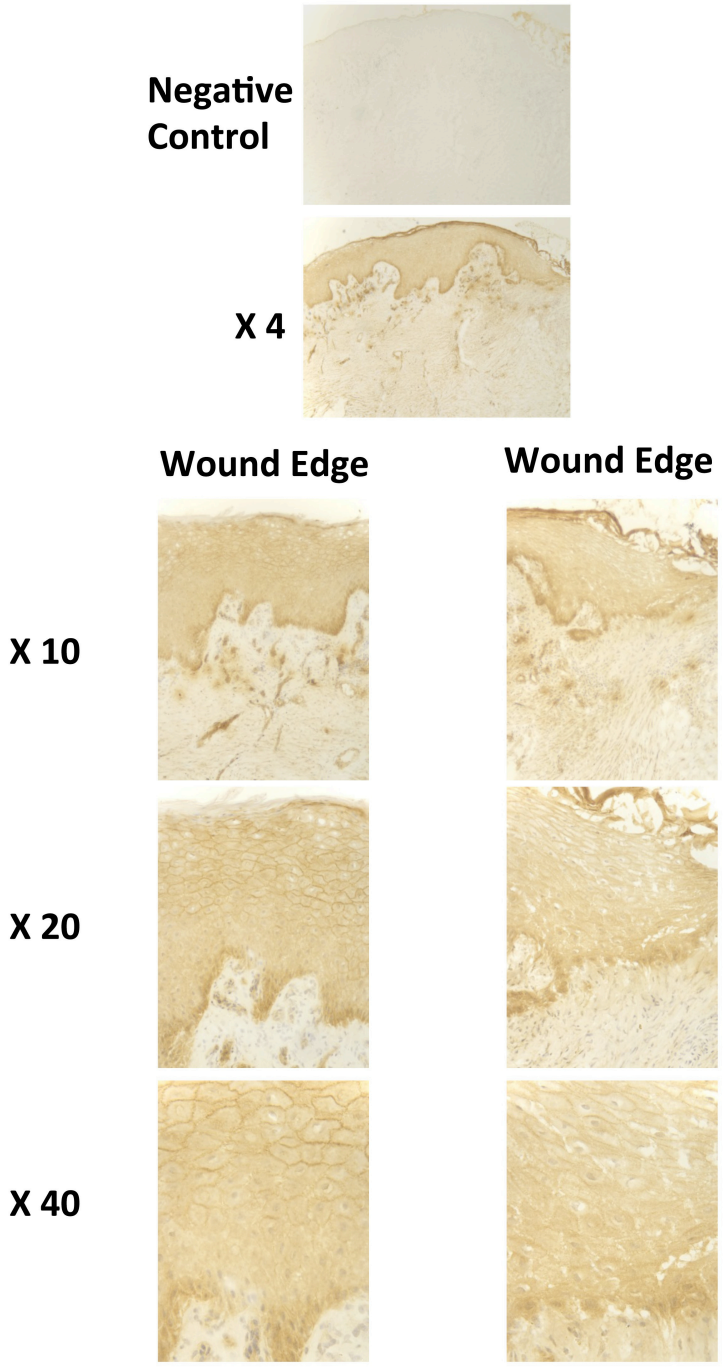


Figure 3.2 A: - Immunohistochemical staining of tissue collected for the Wound cohort in non-healing wounds. (Brown colour indicating presence of EPLIN protein). Representative data is shown with samples taken at two separate points along the wound edge of the biopsy sample.

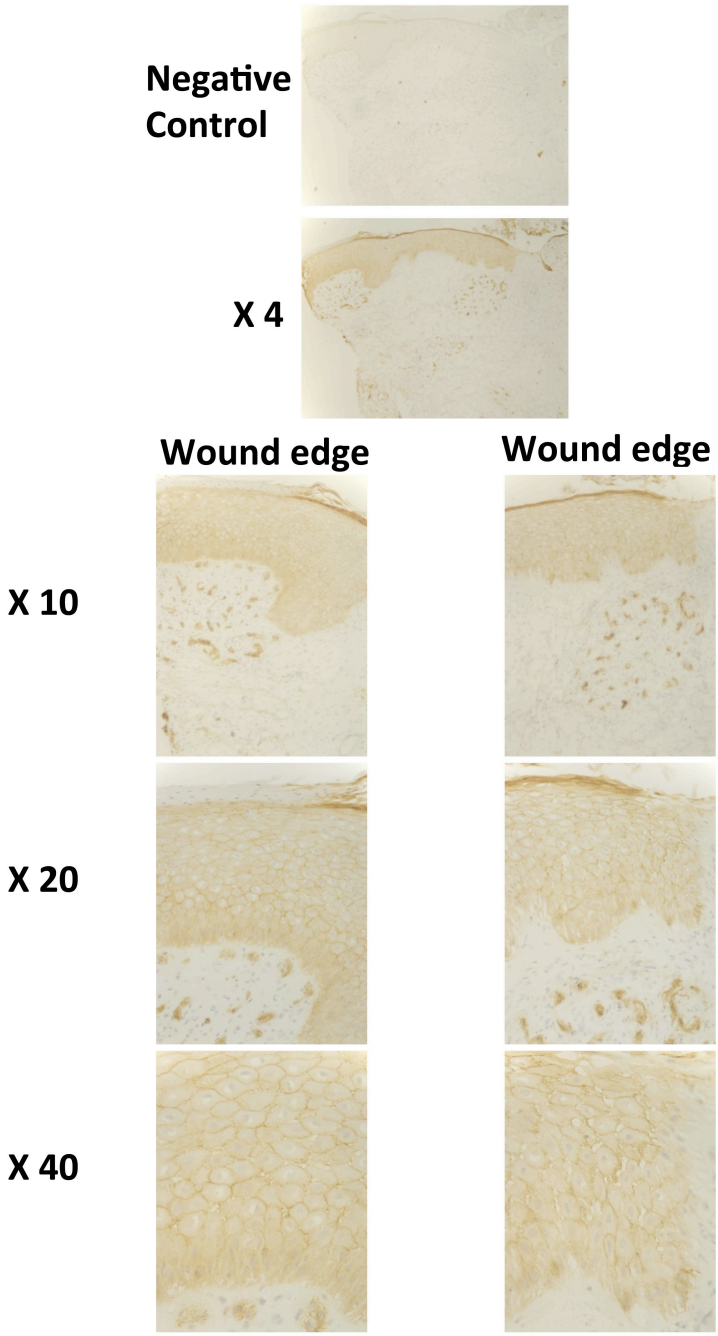


Figure 3.2 B: - Immunohistochemical staining of tissue collected for the Wound cohort in healing wounds. (Brown colour indicating presence of EPLIN protein) Representative data is shown with samples taken at two separate points along the wound edge of the biopsy sample. Negative control were sections stained with no primary antibody (substituted with PBS plus serum), these only received the

secondary antibody to show no cross reactivity and background staining with this secondary antibody.

3.2 Verification of EPLIN overexpression in HaCaT keratinocytes

3.2.1 Screening for EPLIN in HaCaT cells

To investigate the effects of EPLIN further, human keratinocytes and endothelial cells (HaCaT and HECV cells) were screened for the presence of EPLIN. We found both cell lines were very weak in their expression of EPLIN (Figure 3.3) Tissue biopsies (as detailed in section 2.7) are composed predominantly of keratinocytes, endothelial cells and fibroblasts (Normand and Karasek 1995), and thus in theory either cell type would be appropriate for further study. Both HaCaT and HECV cells are readily available, however as the host laboratory had previous experience using keratinocyte HaCaT cells in the genetic models and previous wound healing studies (Jiang, Ye et al. 2010) and a previous study on the impact of EPLIN in HECV endothelial cells had recently been conducted (Sanders 2010). I selected to investigate the HaCaT cell line in the context of wound healing in human tissue and, as EPLIN was only weakly expressed in this cell line, used an overexpression model to examine EPLINs importance in HaCaT cells.

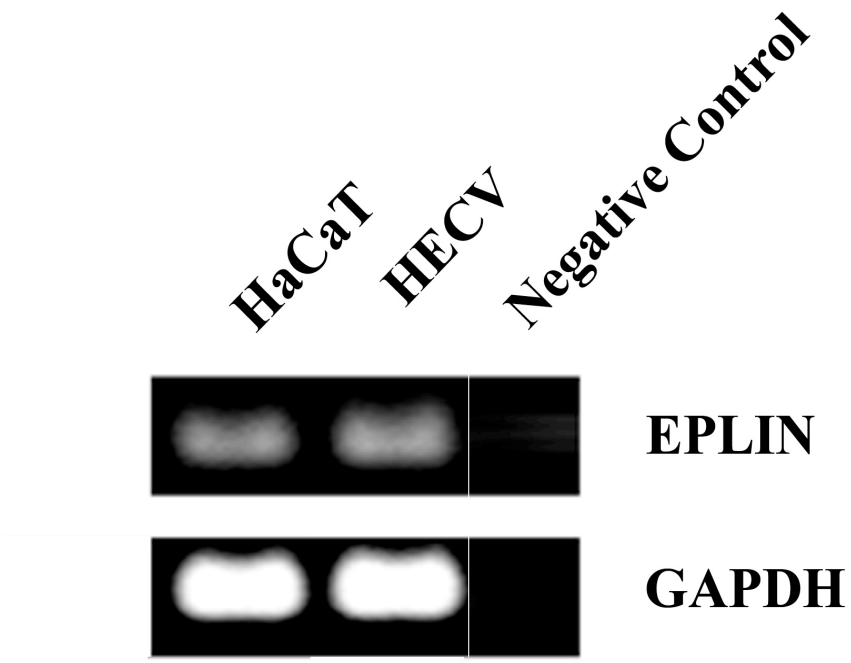


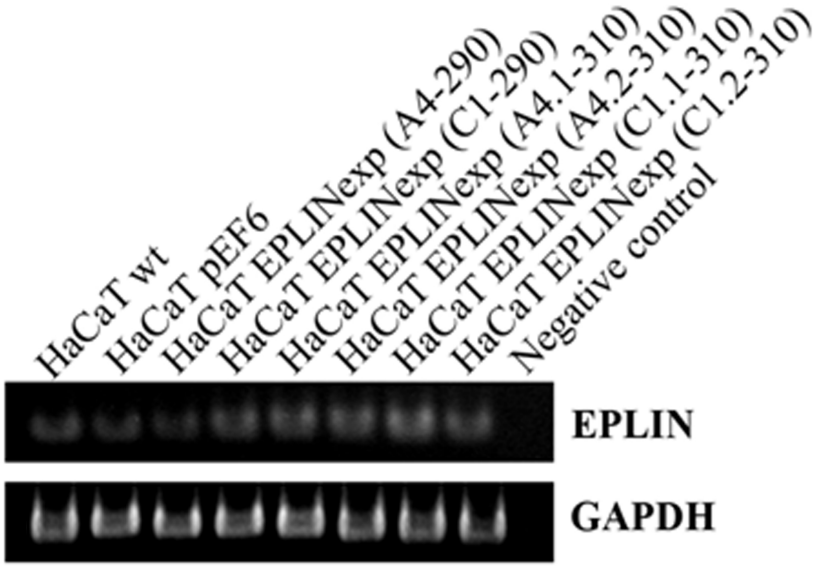
Figure 3.3: - Western blot screening for EPLIN expression in human keratinocyte (HaCaT) and human endothelial (HECV) cell lines. EPLIN expression is very weak in both HaCaT and HECV cell lines. The HaCaT cell line was selected for further evaluation as the host laboratory had more experience in this model and work had previously been completed on HECV cells.

3.2.2 Transfection with EPLIN coding sequence

As EPLIN was established to be relatively weakly expressed in the HaCaT cell line I chose to overexpress the EPLIN coding sequence and thereby increase the amount of the protein within the cells, which could subsequently be functionally investigated.

HaCaT cells were transfected, using electroporation, with varying plasmid clones (A4 or C1.1), plasmid volumes (5µl or 10µl) and electroporation conditions (240V or 310V). A4 and C1.1 where the two variant clones available in plasmid form ready for transfection from previous work done in the host laboratory on cancer cell types. (Jiang, Martin et al. 2008) Following transfection, cells containing the plasmid were selected in 10µg/ml Blasticidin (as per manufacturers recommendation for cell selection and our own laboratory experience) for 7 - 10 days before culturing in a maintenance medium containing 0.5µg/ml Blasticidin. (Sanders, Ye et al. 2010) The medium changes during the selection process were carried out every 48hrs and the cells maintained at approx 20% confluence to maximise the chances of successful selection and by maintaining the cells at low confluence level minimised resistance to the antibiotic being passed onto the non transfected cells.

Once transfected RNA and cDNA were generated and used to do an initial RT-PCR screen to find optimal overexpression line that displayed the most convincing over expression and could subsequently be used in all future experimentation to maintain experimental consistency (Figure 3.4). RT-PCR and semi-quantitative analysis illustrated that the C1.1 line displayed the most substantial overexpression of EPLIN, with large increases seen in comparison to the wild type and plasmid control (pEF6) cell lines.



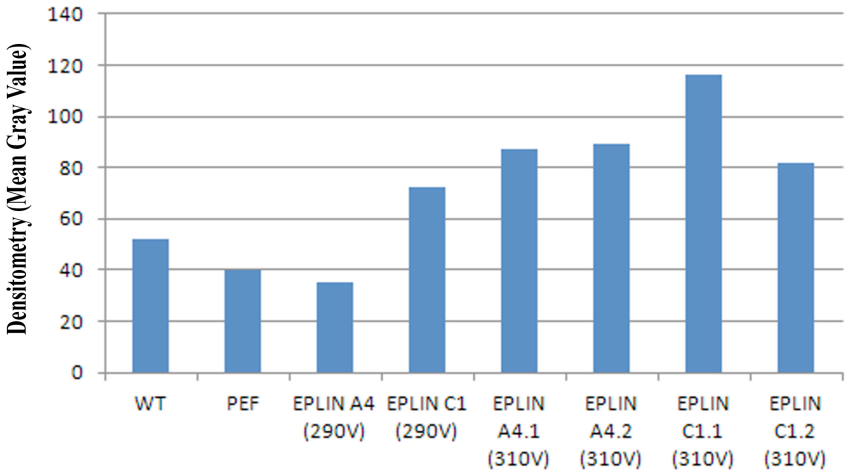


Figure 3.4: - RT-PCR screen and semi-quantitative analysis of EPLIN overexpression following various transfection conditions. The C1.1 cell line was found to have the largest degree of EPLIN overexpression in comparison to control

cell lines following RT-PCR. Semi-quantatitive analysis and normalisation against GAPDH expression demonstrates a similar trend.

3.2.3 Confirmation of EPLIN overexpression at mRNA level

Following this initial screen and a few RT-PCR repeat runs to minimise error in selection, one would be expect the vast majority of cells to contain the active plasmid, resulting in the presence of a plasmid containing the EPLIN sequence in these cells. EPLIN expression would therefore be increased at both the mRNA and protein level. The previous initial screen has demonstrated that this is largely the case, with the vast majority of transfections resulting in a reasonable level of EPLIN overexpression.

Continuing on from the initial screen, additional RT-PCR was conducted to confirm this overexpression (Figure 3.5). As can be seen in the expression picture the HaCaT cells transfected with the EPLIN expression plasmid showed a substantial degree of EPLIN overexpression at the mRNA level when compared with pEF6 and WT control cells.

To quantify the difference in these bands obtained using RT-PCR we used the software Image J. The rectangular selection tool was used to select a single band, large enough to encompass each band being directly compared, but no bigger than required to minimise interference from background 'noise'. Integrated Density of the selected rectangle is calculated by Image J as the product of the area and mean grey value of the selection**.** This process was repeated for the GAPDH control bands. Data was normalised to GAPDH to account for any. slight differences in starting cDNA concentration.

The C1.1 cell line was chosen for all future experiments as the most convincing and reliable HaCaT overexpressing cell line, thus optimal electroporation conditions having been established at 10µl of plasmid at 310V. This cell line was subsequently cultured in maintenance medium to provide a large quantity of cells which were all frozen down in liquid nitrogen and would provide the cell line used in all future experiments ensuring consistency of results in relation to the cells used for experimentation. Likewise the cells used for control in the experiments also came from the original frozen batch of cells to maintain result reproducibility. Subsequently this cell line will be referred to as HaCaT EPLIN exp or $HaCaT^{EPLIN}$ with the controls refered to as $HaCaT^{wt}$ (cells containing no plasmid) and $HaCaT^{PEF}$ (cells containing the empty plasmid with no coding sequence).

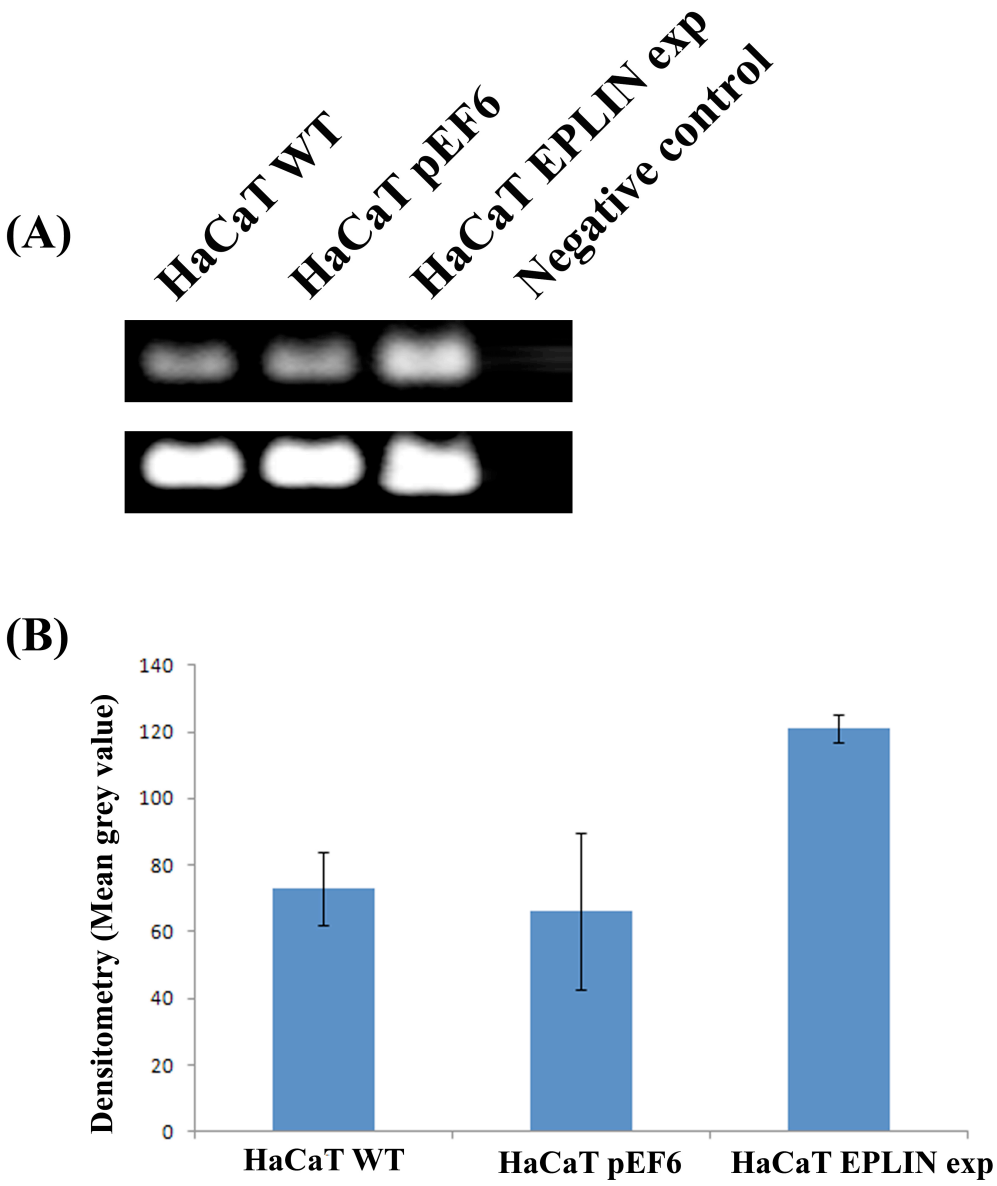


Figure 3.5: - Confirmation of EPLIN overexpression at the mRNA level. (A) RT-PCR showing that transfection of HaCaT cells with the EPLIN overexpression plasmid resulted in a large increase in EPLIN transcript levels. (B) Semi-quantitative analysis of these bands and normalisation against GAPDH levels similarly illustrates a substantial increase in EPLIN expression in HaCaT EPLIN exp cells. Y-axis is mean grey value of EPLN PCR bands when standardised for any GAPDH variation in between the samples.

3.2.4 Confirmation of EPLIN overexpression at the protein level

As well as confirming expression at an mRNA it was necessary to establish whether the cells were indeed expressing EPLIN at a protein level. For this to be done a Western blot analysis was also performed (Figure 3.6). Samples were collected and run as detailed in methodology with resultant data analysed using Image J software as detailed above.

Similar to the RT-PCR data, the figure demonstrates increased expression of EPLIN protein in the overexpression cell line compared to the empty plasmid control and wild type cells.

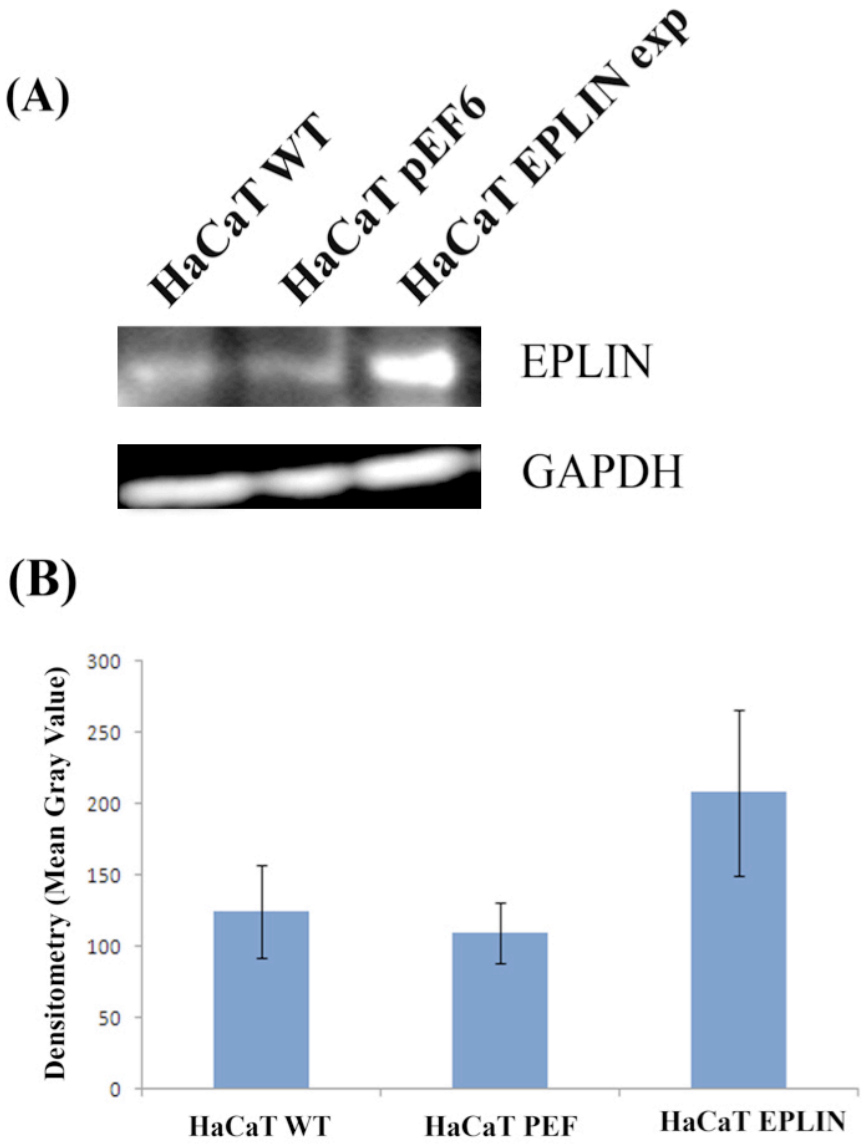


Figure 3.6: - A) Western blot analysis demonstrating the enhanced expression of EPLIN in cells transfected with the EPLIN expression plasmid in comparison with empty plasmid (pEF6) and no plasmid (WT). (B**) Semi-quantitative analysis using band densitry confirming overexpression** in the HaCaT EPLIN exp cells following normalisation against GAPDH levels.

3.2.5 Confirmation of EPLIN overexpression using ICC

Immunocytochemical staining (ICC) was also used as a further technique to visually quantify the overexpression of EPLIN in the transfected cells (Figure 3.7). The procedure used was as per the one outlined in the methods section for ICC. As expected, and in line with the RT-PCR and Western blot data, a higher proportion of HaCaT EPLIN exp cells showed brown staining confirming presence of EPLIN. This proportion of cells reacting positively for EPLIN was higher than in the control pEF6 and wild type cells.

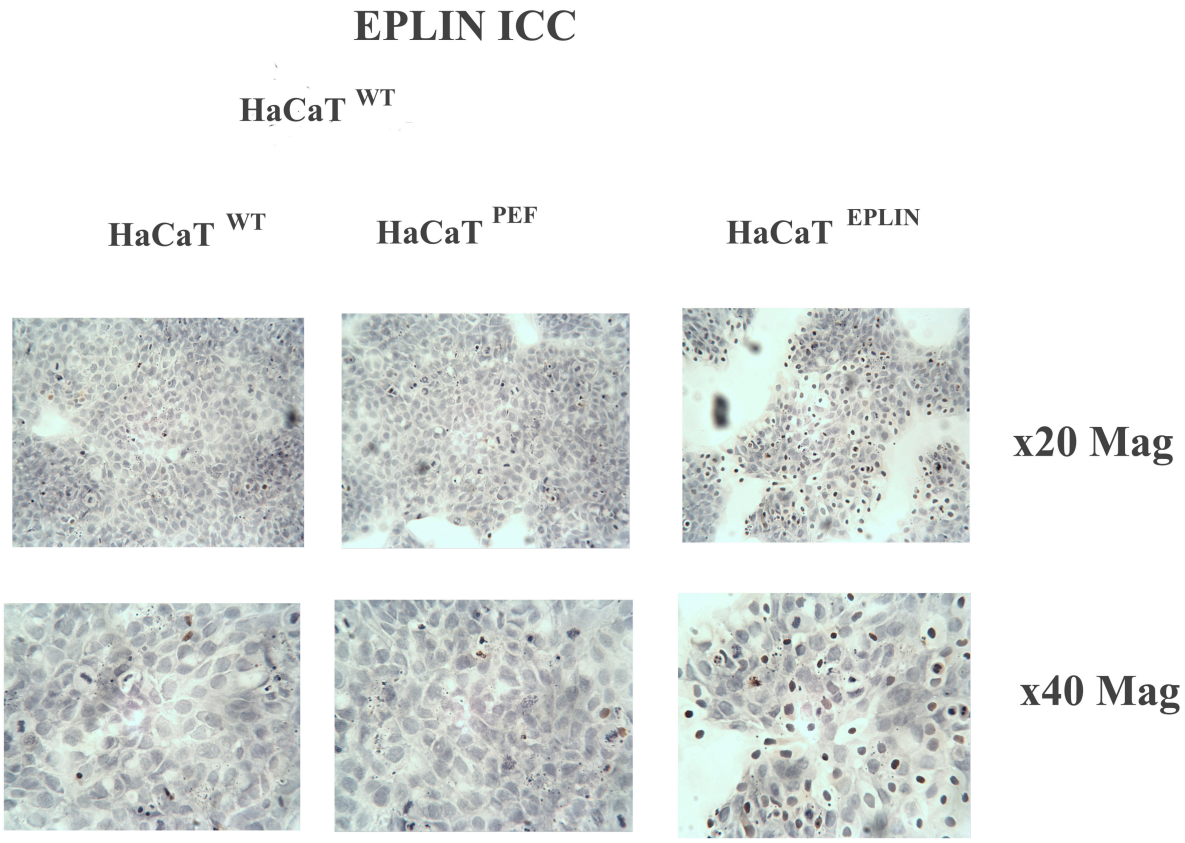


Figure 3.7: - ICC of WT, pEF6 and EPLIN overexpressing HaCaT cells showing the brown nuclear staining for a positive stain. Note the increased number of positively stained cells in the EPLIN plasmid cell line verifying overexpression.

3.3 Basic cellular functions

In order for us to study the effects of proteins on cell behaviours an overexpression model cell line such as the one derived above can be used to establish an effect of a specific protein (In this case EPLIN). In such a model any changes noted in the overexpressing cell line can provide insight into the role and function of the protein in the model system.

3.3.1 Impact of EPLIN overexpression on HaCaT migration (scratch wounding assay)

Initially a standard scratch wounding assay was used to assess cellular migration (Figure 3.8). As outlined in the methodology, cells were seeded at varying concentrations on a 24 well plate. Following establishment of a confluent monolayer, a 21G needle was passed over the surface to create 3 parallel wounding lines. The medium used for the experiment was changed to pre-warmed HEPES medium, and following 15 minutes cell recovery time, the cells were transferred onto a heated plate (Lecia GmbH, Bristol, UK) with progress of wounded lines tracked using a camera attached to a Lecia DM IRB microscope (Lecia GmbH, Bristol, UK). Results were repeated independently and results shown are the mean of 3 repeats. As is evident from the data overexpression of EPLIN brought about a significant decrease in cell migration rates closure of the artificial wound, through migration of the two fronts, was significantly retarded, particularly in the later stages (90 mins and 120 mins, $p < 0.05$ vs HaCaT pEF6).

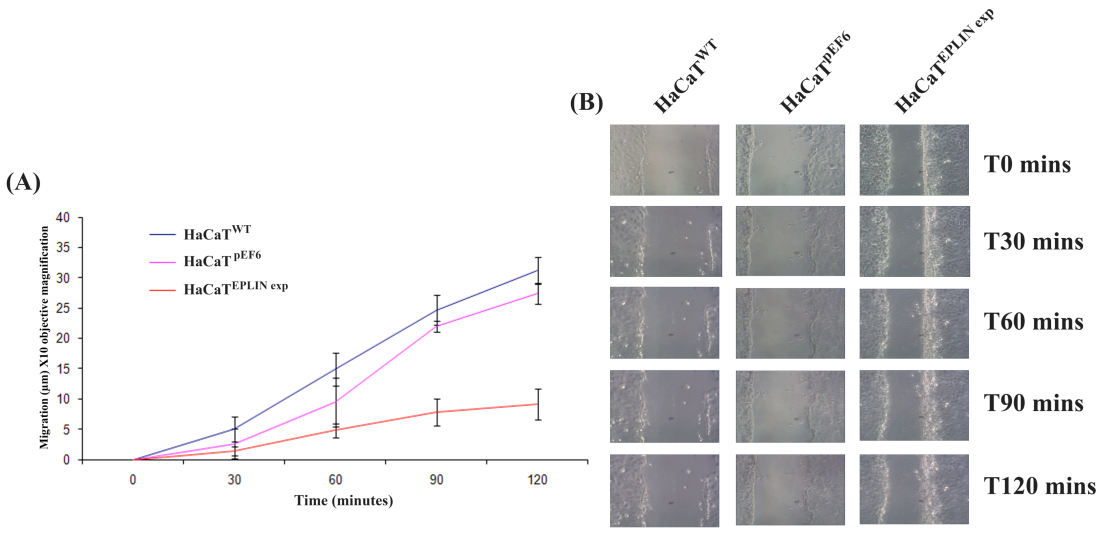


Figure 3.8: - Scratch wounding assessment of cell migration. (A) Following transfection with the EPLIN expression plasmid, HaCaT$^{EPLIN\ exp}$ cells migrated at a substantially reduced rate compared to control cells. Significant differences were seen between closure rates of the wounds following 90 and 120 minutes (* $p < 0.05$, Mean values of 3 independent repeats shown +/- SEM) using two sample, two tailed t-test at each time point between pEF6 control and EPLIN overexpression cells. (B) Representative images of wound closures.

3.3.2 Impact of EPLIN overexpression on HaCaT motility using ECIS model

Electrical Cell-Substrate Impedance Sensing (ECIS) is an sophisticated system using changes in electrical impedance to monitor adherence and migration of cells in culture. Advantage of such a system is that cells can be cultured in optimum conditions with measurements collected in 'real time'. This system also makes it much easier to perform numerous different experiments with the same cell line simultaneously ensuring uniformity in the cell to cell comparisons and uniformity in the results.

The raw data output from the ECIS apparatus consists of a graph showing all the different wells (each assigned a different colour) and their resistance changes over time from adhesion (attachment of the cells onto the plate forming a confluent monolayer), wounding (passing of a high voltage current through the electrodes) and 'wounding' the cells overlying the electrodes (forming a gap in the monolayer) and migration (ie the resistance changes over the electrode as the cells migrate to fill this gap) (Figure 3.9). Each well contains either the study or the control group which all run in parallel, but can be separated at the end of the experiment and separately analysed. As each well has its own intrinsic resistance the values will start at slightly different points on the scale (usually between 1500-3500Ω). With cells attaching onto the electrode this results in a changing resistance measured over the electrode, thus the gradient of the curve increases.

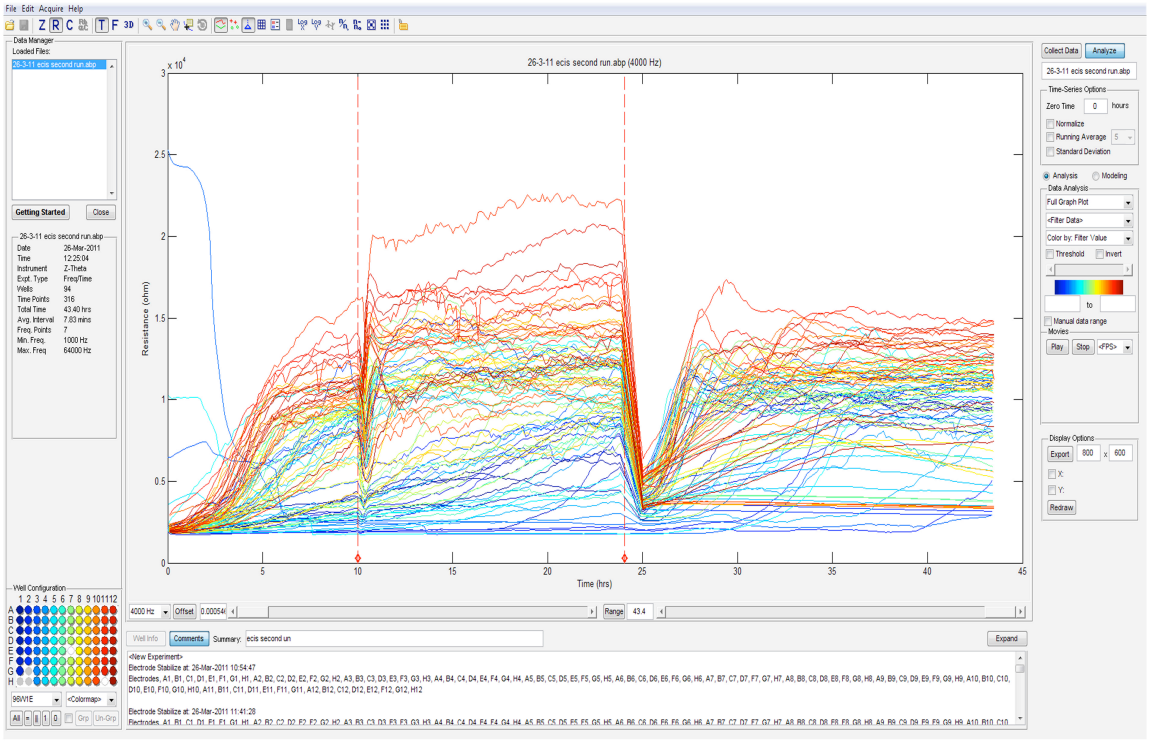


Figure 3.9: - Figure showing a typical readout from the ECIS machine following a run with two separate woundings visible as two distinct dips in the line.

Usually two 'woundings' are carried out during the period of each experiment, however these have to be visually inspected as sometimes secondary to insufficient cell number, leakage and electrode dysfunction the graphs do not behave in the expected way thus need to be discarded.

As with all other experiments the data has to be normalised prior to analysis to allow comparison of the different runs of the same experiment, as well as comparison of the different cell lines within the same experiment. Normalisation takes place by selecting the initial resistance reading (the lowest recorded resistance value within the first 15 minutes of the experiment), subsequent values are then subtracted from this reading at this initial time point. What we end up with is a normalised T0, which is assigned the

value 0 Ω. The time points chosen for analysis were T1 (1Hour), T2 (2Hours), T3 (3Hours) and T4 (4 Hours).

ECIS data was analysed through the calculation of change in resistance. Following wounding, the resistance values for each well were noted and further changes from this values at the desired time points calculated and taken as an indication of cellular migration onto the electrode. This method allowed for normalisation of the data should there be variations in the initial resistance of wells within the array.

3.3.2.1 Impact of EPLIN overexpression on HaCaT cell-matrix adhesion using ECIS model

For epithelial cells to migrate across the extracellular matrix, adhesion to this surface has to take place, the process seen during the re-epithelisation phase of wound healing. (Suzuki, Saito et al. 2003; Kanchanawong, Shtengel et al. 2010). Thus it was important to assess adhesion in our cell line and see what effect overexpressing EPLIN in our cells had on the adhesion process. The adhesion was determined by the change in resistance across the ECIS electrode as the free floating cells in medium slowly adhered to the surface over the electrode causing a change in absolute resistance (total resistance minus the intrinsic resistance). Measurements were taken at time points T1 (1 Hour), T2 (2 Hours), T3 (3 Hours) and T4 (4Hours), with resistance calculated from readings taken at 4,000 Htz, as this has been the determined frequency that best distinguishes between cell free and a cell covered electrode (Wegener, Keese et al. 2000). EPLIN overexpression results in a significant reduction in cellular adhesion across all four time points measured (Figure 3.10).

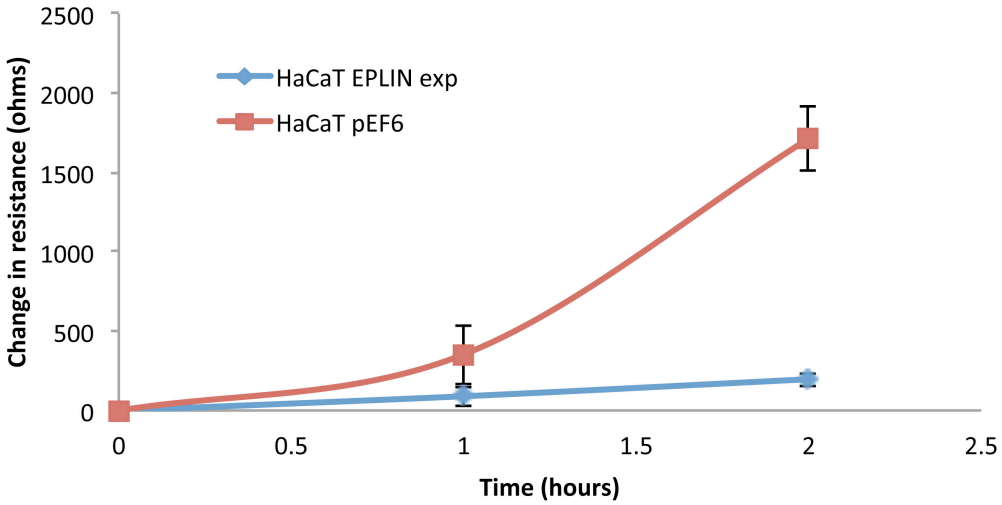


Figure 3.10 - ECIS detection of cell attachment. HaCaT cells overexpressing EPLIN had a reduced capacity to adhere to the array electrodes compared to plasmid control cells.

3.3.2.2 Impact of EPLIN overexpression on HaCaT cell-matrix migration using ECIS model

As outlined in the section above the data at the four time points (1 Hour), T2 (2 Hours), T3 (3 Hours) and T4 (4Hours) was normalised against the intrinsic resistance and values measured against T0 base resistance. The average changes in resistance of three independent runs were then subsequently plotted. Migration was also assessed at 4,000 Htz. EPLIN overexpression resulted in reduced migration across all four time points (Figure 3.11). Overexpression of EPLIN in HaCaT cells significantly reduced migration in comparison to the control cells over the 4 hour period ($p < 0.05$).

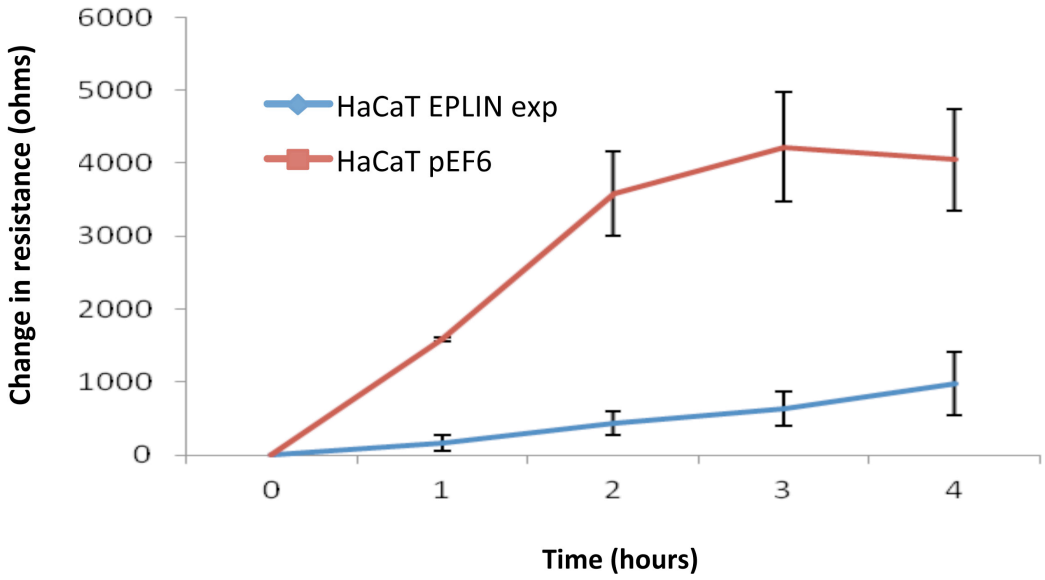


Figure 3.11: - ECIS detection of cell migration. HaCaT control cells migrate at a steady pace to recover the monolayer following electrical wounding, in contrast to this HaCaT$^{EPLIN\ exp}$ cells display a reduced capacity to migrate onto and re-colonise the electrode.

3.3.3 Impact of EPLIN overexpression on HaCaT growth

Cell growth was assessed using a colorimetric assay. In this technique, absorbance is used to represent a measurement for cell number with the advantage of minimisation of human error (as the absorbance is more consistent than repeated cell counts) and that more repeats of an experiment can be carried out due to the increased speed of the data analysis. Absorbance is calculated with total well absorbance subtracting the empty well absorbance. The data has to then be normalised to account for any minor discrepancies in seeding, which achieved by normalising the wells from day 3 and 5 to the wells in day1 to represent a relative increase in cell number. Results shown are the percentage of the 'true increase'. Therefore, by day 5 if there was no growth

compared to day 1, this would correspond to '0'. Conversely if the absorbance had doubled, this would be recorded as 100% increase on the baseline value.

Overexpression of the EPLIN protein in the HaCaT cell line was seen to have no impact on the cell growth rate of the HaCaT cell line using this assay method and no significant differences were observed between the plasmid control and the EPLIN overexpressing lines at either day 3 or day 5 (Figure 3.12).

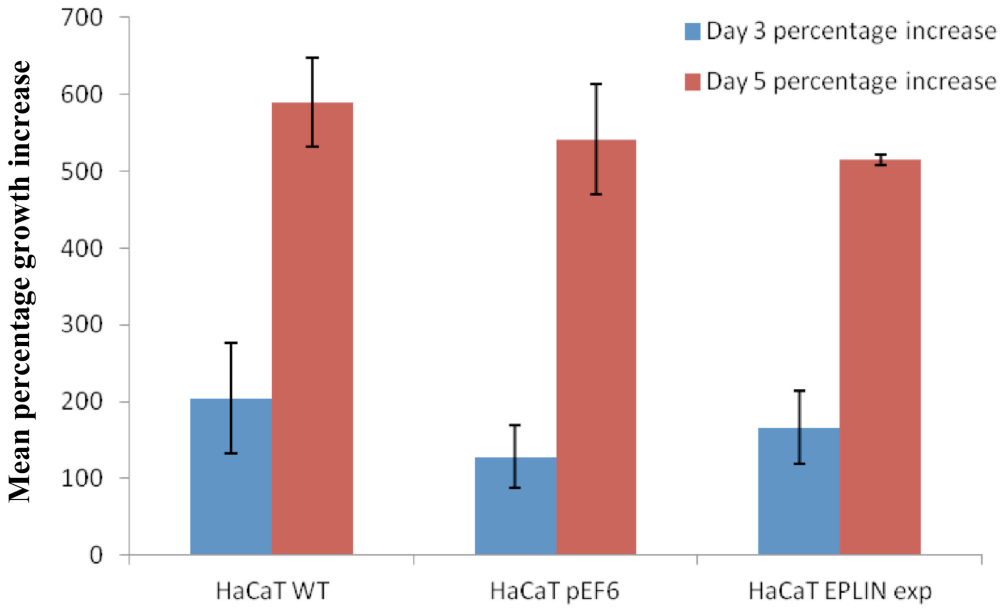


Figure 3.12: - Colorimetric analysis of cellular growth. This figure shows that that HaCaT cells overexpressing EPLIN have no significant effect on cellular growth. Measurement of cellular growth rate is an important aspect of examining the effect of a particular protein on cellular activity.

3.3.4 Discussion

In light of the similarities shared between wound healing and cancer touched upon in the introduction, it is perhaps unsurprising that a protein known to be lost from various aggressively migrating neoplastic cell lines (EPLIN) appears to impact on wound healing in the opposite way. From this chapter we can see that EPLIN levels are found in greater quantity in Non-Healing as opposed to Healing wounds, with this being visually apparent from the IHC staining (representative data only shown). Additionally, analysis of EPLIN transcript levels within the tissues revealed that EPLIN levels were elevated in non-healing tissues as opposed to healing tissues, however this was not statistically significant. This result gives indications that higher levels of EPLIN in wound tissues seems to be associated with a non-healing state in chronic wound tissues. This trend would be expected, given EPLIN's role as a potential anti-migratory protein, where high levels of EPLIN in non-healing tissue could interfere with the re-epithelialisation process and block the migration of epithelial cells. However, despite this trend, the Q-PCR results did not result in a significant p value. This could potentially be due to the relatively small sample size of this initial study, expanding this healing, non-healing cohort, together with testing additional types of wound tissues should be investigated in the future and will aid in the determination of EPLINs role in clinical wound healing.

HaCaT cells when overexpressing EPLIN significantly reduce cell motility both with respect to adhesion, and migration compared to WT and PEF control cells. Overexpression of EPLIN however has no effect on cellular growth. Thus we can conclude that EPLIN may act as a anti-migratory protein, without affecting growth.

From this data the effects of EPLIN indicate that it potentially plays an important effect in wound healing. With the previously identified similarities and overlapping phases in wound healing and cancer development it is perhaps not surprising that a protein which is lost in cancer (turning it into a pro-migratory process) would then be overexpressed in keratinocyte cells, shown to have an opposite effect with the slowing of migration. In fact as hypothesised the overexpression of EPLIN proved to have an anti migratory influence on HaCaT cellular motility. This trend was observed in both the conventional scratch assay as well as the more sophisticated ECIS assay with the results being comparable. As the trends were so similar and the ECIS method allows for processing of larger quantities of data as well as upon experimental set up the process is automated with 'real time' monitoring eliminating human error. The results contained within this thesis are consistent and reproducible across a number of separate motility based assays.

Previous work carried out in the host laboratory demonstrated a similar effect once EPLIN was overexpressed in a breast cancer cell line and an endothelial cell line (Jiang, Martin et al. 2008; Sanders 2010).

As EPLIN expression has an effect on motility but not on growth further supports existing literature and validates the hypothesis of the protein being an anti-migratory protein, as growth is a process primarily present in the later stages of wound healing (increasing cell number in later stages) where as motility plays a role during the re-epithelisation stage. It is here that EPLIN is thought to exert its effect in the context of wound healing.

The Process of wounding keratinocytes promotes them to transform from a sedentary cell to a migratory one. In the context of wound healing the keratinocytes need to detach from the basement membrane and migrate before re-attaching to the extracellular matrix. This transformation is a key component of successful wound healing. In actual fact the forward motion of these keratinocytes in the process of wound healing results in multiple attachments and detachments driving the cells forward. As a result of this it is perhaps unsurprising that any factors affecting this process result in a reduction of motility (Suzuki, Saito et al. 2003; Chen, Hughes et al. 2011).

CHAPTER 4 -

Interaction between ERK, FAK, PLC γ and NWASP and EPLIN

4. Introduction

In the above results chapter I have shown that EPLIN is upregulated in terms of expression in non-healing in comparison with healing wounds. EPLIN overexpression results in reduced adhesion and migration without affecting growth. It is most likely that it is through its effect on adhesion and migration that EPLIN has its effect on wound healing.

So that further clarification of the mechanisms through which EPLIN has its effect can take place, it is necessary to explore EPLIN protein interactions in greater detail. In this chapter I have used a variety of inhibitors in combination with function assays to identify potential pathway interactions in regards to the anti-migratory function of EPLIN discovered previously.

4.1 Inhibitor studies to explore potential roles of EPLIN in cell pathways

In recent literature there has been an increase with the use of inhibitors with ECIS to evaluate the effect the various inhibitors have on a variety of cell lines (Garcia, Liu et al. 2001; Sanders, Ye et al. 2010; Sanders, Martin et al. 2011; Xue, Chow et al. 2011).

The use of Inhibitors on the EPLIN overexpressing cell line can give an indication which other proteins are likely to be involved with or affect the functioning of this protein. This method is a recognised practical way of screening a number of pathways for evidence of interaction with the study protein of interest. It is not possible to draw concrete conclusions from inhibitor work regarding the upstream and

downstream signalling mechanisms, however it does identify potential areas of further study to allow focused protein work to subsequently be undertaken.

Through analysis of the available body of literature relating to what proteins and their inhibitors have been found to have interactions with EPLIN in a variety of contexts (predominantly in the setting of cancer) coupled with the host laboratories own preliminary works, a number of proteins of interest were identified to provide the backbone for analysis which would fit into the scope of the study. The proteins chosen and their potential relevance to the study at hand are outlined below.

4.1.1 ERK (Extracellular signal-regulated kinases) background

Extracellular-signal-regulated kinases (ERKs) or classical MAP kinases are widely expressed protein kinase intracellular signalling molecules that are involved in functions including the regulation of meiosis, mitosis, and post mitotic functions in differentiated cells. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens, activate the ERK pathway (Boulton and Cobb 1991).

Activation of ERK occurs in response to growth factor stimulation through the Ras-Raf-MEK pathway, and activated ERK translocates from the cytoplasm to the nucleus, where it phosphorylates several protein kinases, nuclear transcription factors, and other proteins (Pearson, Robinson et al. 2001; Kondoh, Torii et al. 2005). Certain substrates like focal adhesion kinase (Hunger-Glaser, Fan et al. 2004) and Paxillin (Liu, Yu et al. 2002) are known to function in ERK mediated cell migration.

ERKs are known to activate many transcription factors, such as ELK1, and some downstream protein kinases. Disruption of the ERK pathway is common in cancers, especially Ras, c-Raf and receptors such as HER2 (Rao and Reddy 1994). In addition to its role in the nucleus, recent data show that ERK is involved as an essential component in the migration of cells from many different organisms (Fincham, James et al. 2000).

Phosphorylation of the C-terminal region of EPLIN inhibits its actin-binding activity. Stimulation with platelet derived growth factor (PDGF) induces stress fiber disassembly and localization of phosphorylated EPLIN to peripheral and dorsal ruffles. Furthermore, expression of a non-ERK-phosphorylatable mutant of EPLIN prevents PDGF-induced membrane ruffling as well as cell migration. These results suggest that phosphorylation of EPLIN by ERK leads to reorganization of actin filaments and stimulation of cell motility (Han, Kosako et al. 2007).

EPLIN protein was actually phosphorylated by ERK thus indicating that its effect is downstream to that of EPLIN. I thought it would be interesting to include this as one of the inhibitors to see what effect it had on EPLIN if any in the context of assessing migration with the ECIS array. Han et al hypothesised that phosphorylation of EPLIN by ERK was required for cell migration. Dynamic phosphorylation and dephosphorylation of cytoskeletal proteins is required for essential cell motility (Han, Kosako et al. 2007). However the precise mechanism by which ERK promotes cell migration via phosphorylating EPLIN remains unclear.

In addition our host laboratory have previously looked at the relationship between ERK and EPLIN in breast cancer and endothelial cells, demonstrating potential relationships in migration and angiogenic functions (Jiang, Martin et al. 2008; Sanders, Ye et al. 2010).

4.1.2 FAK (Focal Adhesion Kinase) background

Focal Adhesion Kinase (FAK) is a protein that, in humans, is encoded by the *PTK2* gene (Andre and Becker-Andre 1993) PTK2 is a focal adhesion-associated protein kinase involved in cellular adhesion and motility (Leevers and Marshall 1992). It has been shown that when FAK was blocked, breast cancer cells became less metastatic due to decreased mobility (Kyriakis, App et al. 1992).

FAK tyrosine phosphorylation is induced by adhesion of cell surface integrins to extracellular matrix and by a variety of other extracellular factors including the ligands for receptor tyrosine kinases and for seven transmembrane domain G-protein-coupled receptors (RS, Y et al. 2003) FAK can associate with multiple cellular components including other focal adhesion associated proteins and signalling molecules. FAK is localized to focal adhesion and is centrally implicated in the regulation of cell motility and adhesion. Knocking out the FAK gene in mice prevents normal embryonic development and is associated with loss of mesenchymal cell motility (Fan, Zhao et al. 2013).

The finding that FAK mRNA and protein is up regulated in metastatic and invasive tumours compared to non-invasive adenocarcinomas (Abedi and Zachary, 1995) suggests that increased FAK expression may play a role in tumour invasiveness. It

can be hypothesized that up regulation of FAK expression may accompany other disease states in which aberrant cell migration occurs. I thought that it would be interesting to see whether FAK inhibitor had any effect on EPLIN overexpressing cells.

4.1.3 PLC Gamma background

Phosphoinositide specific-phospholipase C (pi-PLC) is a membrane bound protein which hydrolyses PIP2 to diacylglycerol (which activates phospholipid-dependent protein serine/threonine kinase and protein kinase C) and inositol 1,4,5-triphosphate (which promotes release of Ca2+ from intracellular stores). Together, these changes promote cellular motility growth and differentiation, which has been noted to be of particular importance in the context of cancer metastasis (Kassis, Moellinger et al. 1999; Martin, Davies et al. 2008; Reynisson, Court et al. 2009).

PLCγ is one of six subfamilies of pi-PLC. Recently our host laboratory demonstrated that knock down of PLCγ in human prostate cancer cells results in cells with a reduced invasive and motile phenotype, without affecting cellular growth (Sewell, Smyth et al. 2005; Martin, Davies et al. 2008). In wider literature its importance in enhancing cellular migration and invasiveness in breast cancer has also been shown (Falasca, Sala et al. 2008). As yet the role it plays in wound healing is not fully understood, however given the similarities between wound healing and cancer, one would expect PLCγ to act as an enhancer to wound healing.

4.1.4 NWASP (Neuronal Wiskott - Aldrich Syndrome Protein) background

NWASP (neuronal Wiskott - Aldrich syndrome protein) is a member of the Wiskott-Aldrich syndrome (WAS) family of proteins shown to play a role in the transduction of signals from receptors on the cell surface to the actin cytoskeleton (Ochs and Thrasher 2006). WASP is a key regulator of actin polymerization in hematopoietic cells with 5 domains involved in signalling, motility/migration, immune synapse formation and in facilitating the nuclear translocation of nuclear factor kappaB. Mutations of WASP result in patients with X-linked neutropenia and Wiskott-Aldrich syndrome (thrombocytopenia, infection susceptibility, eczema and bloody diarrhoea) symptoms varying with the level of the mutation (Ochs and Thrasher 2006).

WASP is a 65kDa protein, amino acid sequence was approximately 50% homologous to Wiskott-Aldrich syndrome protein (WASP) and this novel protein was thus termed NWasp predominantly present in brain tissue (Miki, Miura et al. 1996). This increased activity results in membrane ruffling (Zalevsky, Lempert et al. 2001). Interestingly the reduction in expression of NWASP is associated with a greater malignant potential (Baluk, Hashizume et al. 2005) in the context of breast cancer, via its role in cellular migration (Martin, Pereira et al. 2008). This is interesting as our study protein has very similar characteristics. For this reason I thought it would be worthwhile analysing this protein in association with EPLIN to see whether there are any links between the two.

The role of NWASP in wound healing has only very recently been described, and is consistent with its effect in breast cancer. The study of the effect of NWASP in previous studies was done using the inhibitor Wiskostatin which has shown to

increase keratinocyte migration *in vitro* (Jiang *et al,* unpublished data). Furthermore our host laboratory uncovered that it is overexpressed in patients with chronic ulcers, compared to patient with acute wounds. The inhibition of NWasp resulted in enhanced wound closure rates when applied topically and via the intraperitoneal route to mice (Jiang et al, unpublished data).

With establishing that EPLIN has an effect on both cellular adhesion and migration in the previous chapter, I looked at how some specifically chosen inhibitors affected these functions noting any differences between the study and control cell groups.

4.2 Materials and methods.

4.2.1 Cell lines

Cells derived from the human keratinocyte cell line (HaCaT) were used in the study. EPLIN HaCaT, WT HaCaT and PEF HaCaT cells were routinely cultured in DMEM-F12 medium as described in section 2.3.

4.2.2 Inhibitors used

Inhibitors for PLC, ERK, NWASP and FAK were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). All the primers used were synthesised and provided by Invitrogen (Paisley, UK). Primer sequences are shown in Tables 2.3.

4.2.3 ECIS (Electric Cell-Substrate Impedance Sensing)

Cells were counted and suspended in HEPES medium. The appropriate numbers of cells were seeded into each well (60,000 cells in 400µl HEPES medium for the 8 and

16 well plates, 50,000 cells in 200µl HEPES medium for the 96 well plates). Inhibitors were added at an appropriate concentration as specified by manufacturer. The loaded wells of the ECIS plate were loaded with HEPES medium (400µl for the 8 and 16 well plates, 200µl for the 96 well plates) and the plate transferred to the incubator. Once attached to the recording software, a calibration run was performed to ensure the wells were all working and resistance change recorded.

The ECIS readings were started immediately afterwards. Wounding was performed using the manufacturers 'Electroporate' function once the cells had reached a monolayer, approximately 8-12 hours after seeding to ensure adequate monolayer formation. A second wounding took place after the cells had recovered from the first. All data was normalised to baseline impedance and analysed in Excel®.

4.3 Results

4.3.1 Inhibitor studies using ECIS (Electrical Cell-Substrate Impedance Sensing)

Analysis of migration was carried out with the seeding of the ECIS wells with either normal control medium or medium containing a predetermined concentration of inhibitor. Data obtained from the ECIS readout was then analysed at time points (T1 Hour), T2 (2 Hours), T3 (3 Hours) and T4 (4Hours).

With PLCγ has previously been shown to promote cellular motility particularly in the context of cancer metastasis, we examined what effect inhibiting this protein in the keratinocyte cell line would have both in the control cell group and the EPLIN over expressing cells if any (Kassis, Moellinger et al. 1999; Martin, Davies et al. 2008;

Reynisson, Court et al. 2009). PLCγ inhibition caused an increase in migration of the EPLIN cell line, with interestingly the slowing of migration in the WT control group. It also appears that when the inhibitor is applied to both cell lines, with increase in migration of EPLIN transfected cells and decrease of migration of WT cells they come closer together in terms of their rate of migration almost following the same pattern. The inhibitory migratory effect seen on WT cells following treatment with PLCγ inhibitor did not quite reach significance, though was close ($p = 0.084$) (Figure 4.1). However, treatment of EPLIN exp HaCaT cells with the PLCγ inhibitor caused some increase but was again not statistically significant ($p = 0.239$)

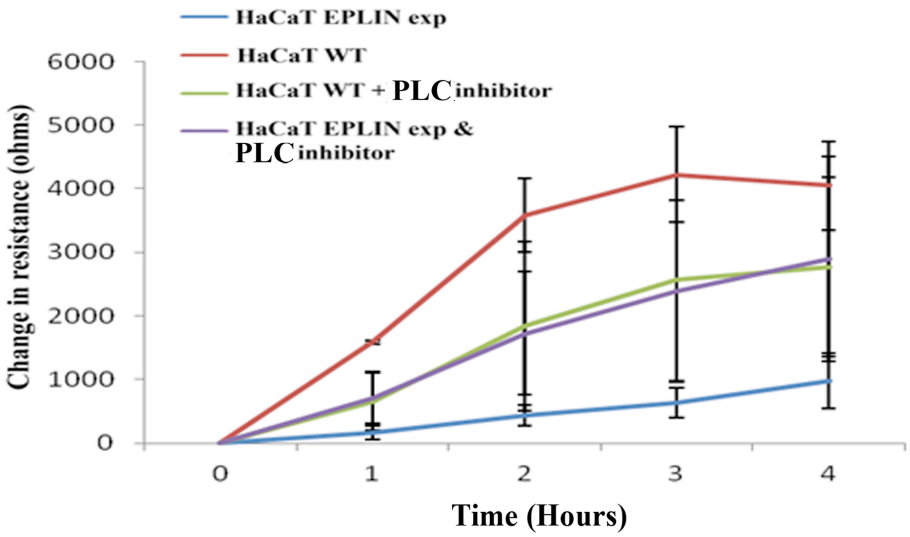


Figure 4.1: - Data from the ECIS assay evaluating migration of EPLIN over expressing cell line and WT HaCaT call line following exposure to PLCγ inhibitor. Results are given as absolute resistance (mean of 3 repeats +/- SEM) Inhibition of EPLIN overexpressing cells with the PLCγ inhibitor resulted in an

increase in detectible migration of the cells with an unexpected slowing of the migration of the WT control cell line.

Treatment of HaCaT WT cells with the ERK inhibitor brought about significant reductions in rates of migration over the 4 hour time period ($p = <0.01$) (Figure 4.2). However, when EPLIN overexpressing cells were treated with the ERK inhibitor no significant differences were seen in the migratory rates ($p = 0.611$). This is perhaps not surprising considering that EPLIN is a downstream regulator of ERK.

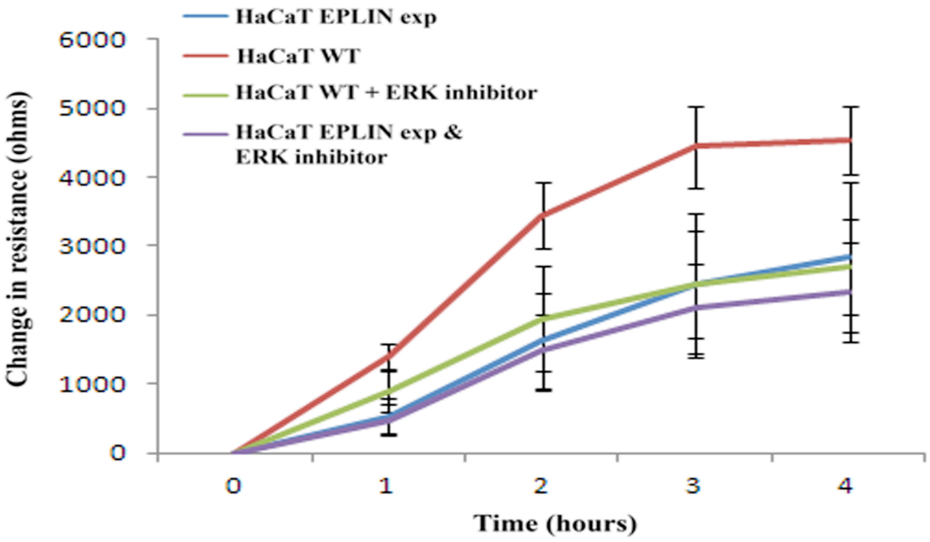


Figure 4.2: - Data from the ECIS assay evaluating migration of EPLIN over expressing cell line and WT HaCaT call line following exposure to ERK inhibitor. Results are given as absolute resistance (mean of 3 repeats +/- SEM) at time points T0 to T4 respectively encompassing a period of 4 hours from the start of the inhibitor exposure. This figure shows the effect of ERK inhibitor treatment on both EPLIN overexpressing and control cells, showing a significant reduction in the

migration of control cells ($p<0.01$) however minimal effect on the EPLIN over expressing keratinocytes.

Similarly, HaCaT WT cells responded differently than EPLIN overexpressing cells to treatment with Wiskostatin, a NWASP inhibitor. Treatment of WT cells with this inhibitor again resulted in a decreased rate of migration, though this trend did not quite reach significance ($p = 0.079$) (Figure 4.3). However, when EPLIN overepxressing cells were treated with Wiskostatin, migrational rates significantly increased over the 4 hour period to a level similar to that of the WT cells treated with Wiskostatin ($p = 0.035$).

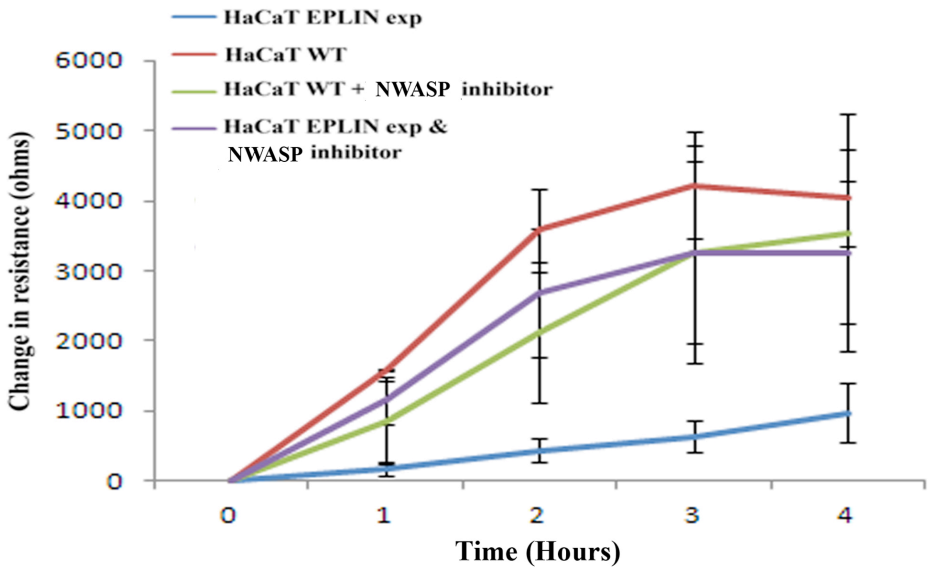


Figure 4.3: - Data from the ECIS assay evaluating migration of EPLIN over expressing cell line and WT HaCaT call line following exposure to NWASP inhibitor. Results are given as absolute resistance (mean of 3 repeats +/- SEM) at time points T0 to T4 respectively encompassing a period of 4 hours from the start of

the inhibitor exposure. This figure shows the effect of NWASP inhibitor treatment on both EPLIN overexpressing and control cells, showing a significant increase in the migration of EPLIN overexpressing cells ($p<0.035$) with minimal effect on control cells.

Treatment of WT or EPLIN overexpressing cells with the FAK inhibitor again resulted in differential effects on cellular migration. Inhibition of FAK in WT cells significantly decreased the rate of migration over a 4 hour period ($p < 0.05$), whereas no significant differences in the migration rates of treated and untreated EPLIN overexpressing cells was seen ($p = 0.54$) and migrational rates tended to be somewhat elevated. (Figure 4.4).

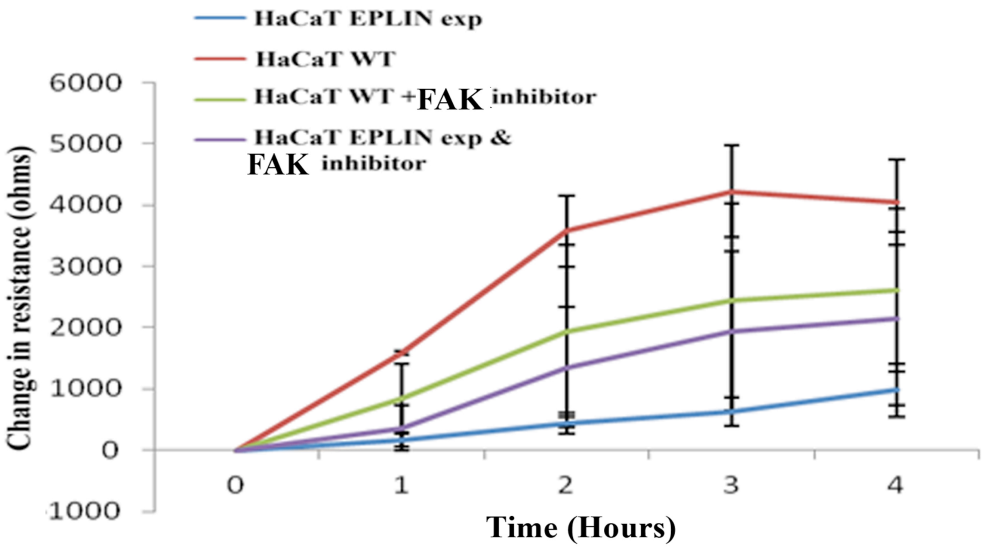


Figure 4.4: - Data from the ECIS assay evaluating migration of EPLIN over expressing cell line and WT HaCaT call line following exposure to FAK

inhibitor. Results are given as absolute resistance (mean of 3 repeats +/- SEM) at time points T0 to T4 respectively encompassing a period of 4 hours from the start of the inhibitor exposure. This figure shows the effect of FAK inhibitor treatment on both EPLIN overexpressing and control cells, showing a significant decrease in the control cells ($P<0.05$) but little effect on in the migration of EPLIN overexpressing cells.

4.4 Discussion of inhibitor work

Currently, the ECIS system is being used within our laboratories to detect and examine a number of cellular functions such as cellular attachment and migration. The ECIS system presents a number of advantages over the older conventional methodologies previously used to detect cell migration, such as the scratch wounding assay. Older methodologies such as the scratch wound assay previously used by our department (Jiang, Hiscox et al. 1999) were time consuming; requiring the tracking of wound fronts over several hours and subsequently the quantification and calculation of wound closure over time. The ECIS system of detection is fully automated and with the development of 8 and, more recently, 96 well arrays allows rapid generation of data simultaneously across large numbers of test samples. This methodology was explored in two recent studies looking at the differences and benefits of using the ECIS automated system of data collection on comparison with a standard more conventional scratch wounding assay, in both instances looking at wound migration (Jiang, Martin et al. 2008; Sanders, Ye et al. 2010). Both studies highlight the importance of EPLINα in the process of cell migration in cancer cells, however, the high throughput ECIS method of data acquisition and analysis facilitates easy

applications of multiple treatments or inhibitors to cell cultures, allowing for enhanced scrutiny of cell attachment or migratory responses.

Recently a number of candidate genes have been explored in this way using cancer cells to observe protein interactions (Jiang, Martin et al. 2008; Davies and Jiang 2010; Ablin, Kynaston et al. 2011), and with the similarities shared between wound healing and cancer makes this the logical choice for exploring similar functions within keratinocyte cells in the context of wound healing.

In order to map out potential protein interactions, inhibitors can be used to explore potential pathways. Once a large database of proteins is screened for small molecules for effects on a cellular function (e.g. migration (Yarrow, Totsukawa et al. 2005) potential proteins can start to be identified likely to exert some effect on each other and then subsequently studied to establish whether this is indeed the case .

The way inhibitor studies work is that they give us an idea when used in conjunction with knockdown or overexpressing cell line whether there are any potential interactions between the study molecules and thus subsequently indicate potential pathways worthy of further study and investigation (Pollard, Nolen et al. 2009).
Within this thesis it has been hypothesised that a relationship can be inferred from the effects of an inhibitor on both the study (overexpressing) and control cell lines.

In this chapter I demonstrate the differential impact of a number of pathway inhibitory molecules on wild type cells and cells overexpressing EPLIN-α, with respect to the keratinocyte cell lineage potentially translatable to wound healing. Treatment of

HaCaT cells with inhibitors to the PLCγ, ERK, NWASP and FAK pathways all resulted in significant or close to significant inhibitory effect on cell motility in control cells ($p = 0.084$, $p < 0.01$, $p = 0.079$ and $p = 0.05$ respectively). In contrast to this, responses to these inhibitors following treatment of the overexpression EPLIN-α cell line were quite different ($p = 0.239$, $p = 0.611$, $p < 0.05$, $p = 0.54$). In HaCaT cells overexpressing EPLIN-α treatment with the inhibitors brought about slight increases in migratory rates or had not significant effects. This differential response of WT or EPLIN exp cells to these inhibitors implicates potential associations between these pathways and EPLIN.

Table 4.1: - Summary the trends of the inhibitors on the EPLIN overexpressing and WT control HaCaT cells.

	Effect on EPLIN HaCaT	Effect on WT HaCaT
PLCγ	↑	↓
ERK	↔	↓
NWASP	↑	↔
FAK	↑	↓

A study by Jiang et al used a similar methodology to analyse the impact of EPLIN overexpression on the breast cancer cell lines in vitro (Jiang, Martin et al. 2008). Similarly to the current study the authors used a variety of inhibitors associated with pathways that are linked to cell motility in order to establish any links with the EPLIN protein in the breast cancer cell line. They included ROCK inhibitor, JAK3 inhibitor, JNK inhibitor, PI3K inhibitor, PKC inhibitor and ERK inhibitor. In this study the authors found that EPLIN overexpressing cells slowed the migration of the MDA–MB-231 (Breast cancer) cell line. Once treated with the ERK inhibitor the study cells appeared to slightly increase their rate of migration bringing them back to control cell levels. This is really interesting as in the current study I also found that treatment with ERK inhibitor of the overexpressing keratinocyte (HaCaT) cell line resulted in the rate of migration increased marginally, closer to the control cell group. This perhaps indicates that these two proteins share a common pathway in the context of keratinocytes and wound healing. The study by Han et al 2007 also demonstrated a relationship between ERK and EPLIN, highlighting the ability for ERK to phosphorylate EPLIN and linking it to EPLINs potential to influence migration (Han, Kosako et al. 2007). Sanders et al demonstrated in their study an effect of EPLIN on endothelial cell migration and potential links with ERK and EPLIN in regulation of angiogenic traits. The data presented in this chapter also indicates that this pathway may be key in the EPLINs role to regulate keratinocyte migration.

Other authors have also used overexpression of genes in combination with inhibitors, and measured primary outcomes such as speed of migration. Charvat et al (Charvat, Le Griel et al. 1999) used the HaCaT-*Ras* cell line (overexpressing Ras gene) in combination with the Matrix metalloproteinase (MMP) inhibitor Marimastat. Ras

overexpression resulted in faster migration, but this effect was blocked by Marimastat, resulting in slowing migration rates similar to the wild type (WT) HaCaT cell line (Control cells). The authors hypothesised that Ras mode of action was via its effect on MMPs. Using this methodology only shows a potential link between Ras and MMPs, as the outcome is a measure of migration, not of downstream proteins.

Recently in our host laboratory Jiang et al noted that NWASP inhibitor results in a substantial increase in HaCaT keratinocyte migration (Jiang et al unpublished data), with a plan to further utilise this knowledge into the in vivo treatment of chronic wound patients. Thus this previous observation of NWASP resulting in the increased cellular migration and adhesion when applied to current study results one notes a marked increase in migration of the EPLIN overexpressing cells with little change to the control cell group. As the inhibitor effect on the EPLIN overexpressing cells was greater than that of the controls, it can be hypothesised that the two proteins share an interaction with one another and possibly a common pathway. For this link to be accurately established further analysis and study is required including immunopercipitation studies thus this was outwith the scope of the current work.

From recent literature it has been noted that PLCy inhibition has a negative impact on cellular migration in cancer cells (Sewell, Smyth et al. 2005). Looking at the effect observed from this study this would be a consistent trend seen in keratinocytes as the control cells did indeed display a reduction in migration when treated with the PLCy inhibitor, supporting the suggested trend observed in cancer cells. However the treatment of the EPLIN overexpressing cell line with the PLCy inhibitor resulted in an

increase on migration of the cells. This would indicate a synergy between these two proteins and perhaps a common pathway shared by both.

Thus EPLIN-α appears to be powerful regulator of the cellular motility of keratinocyte cells. Keratinocyte cells overexpressing EPLIN-α are less motile. We have shown that treatment with a number of inhibitors resulted in the alteration of this inhibitory effect on migration indicating that there are a number of pathways which could play a role in this process, namely the ERK, FAK and NWASP pathways and potentially PLCγ. Together with the clinical relevance as demonstrated in the present study, EPLIN-α could have value as an important prognostic indicator as to whether chronic wounds are likely to heal and may be an important target when considering gene therapies. For us to be able to draw a more concrete conclusion as to the effects of these inhibitors the study could have gone on the analyse protein Western Blot) and RNA (PCR) expression in the overexpressing EPLIN cell lines in comparison to controls to see what if any effect on the expression the inhibitors had on the cells in conjunction with analysing the migration effects. Unfortunately to to time constraints this was not possible in the current study and hence is a study limitation however would certainly be an interesting avenue to explore in any future work on this subject.

Further study is also required to examine the exact molecular pathways involved in EPLIN-α mediated cell migration, particularly focusing on these proposed molecules however that level of detail was outwith the scope of the current study. In order to clarify the interaction of these proteins with EPLIN it was necessary to undertake protein work to establish expression levels in each cell line, the results of which are presented in the following section.

CHAPTER 5 -

Protein expression study of FAK, Paxillin, ERK, PLCγ and NWASP in EPLIN overexpressing keratinocyte cell line

5. Introduction

In addition to the inhibitor work further study was necessary to establish protein expression levels in each cell line. Western blots were carried out on the relevant proteins identified as potentially having an effect on and interacting with EPLIN quantifying their presence within the cell lines. These were further confirmed using Immunocytochemistry and Immunoflourescence studies to get a full picture and the distribution of the protein expression within the target protein group.

Focal Adhesion Kinase (FAK) is a focal adhesion-associated protein kinase involved in cellular adhesion and motility (Leevers and Marshall 1992). What is interesting is that both EPLIN and FAK have been shown to have a role in Breast cancer. Whilst a loss of EPLIN protein leads to an increased motility and metastasis potential within breast cancer, though opposite is true for FAK with a blockage of the protein resulting in a decreased mobility and metastatic potential (Kyriakis, App et al. 1992). We know that FAK can associate with multiple cellular components including other focal adhesion associated proteins and signalling molecules, thus the question arises whether this signalling molecule associated with migration in breast cancer cells would have any interaction and effect within the human keratinocyte cell line.

With PLC together, these changes promote cellular motility growth and differentiation, which has been noted to be of particular importance in the context of cancer metastasis (Kassis, Moellinger et al. 1999; Martin, Davies et al. 2008; Reynisson, Court et al. 2009). As noted in chapter above a knock down of PLC led to a reduced cell motility and invasiveness in prostate cancer cells (Sewell, Smyth et al. 2005; Martin, Davies et al. 2008), which would be the exact opposite effect as seen by

that in EPLIN knockdown. It will be interesting to see whether this is mirrored in the protein expression analysis in the current study.

As touched upon in the previous chapter the connection between ERK and EPLIN has already been established in the literature and here a connection in terms of protein expression will be investigated (Han, Kosako et al. 2007).

Furthermore to the proteins examined with inhibitor studies in the previous chapter the Protein Paxillin was also analysed for its influence with relation to EPLIN over expression. In the recent study by Sanders *et al* immunofluorescence staining was performed to examine the paxillin adhesion molecule. Paxillin staining in EPLIN over-expressing PC-3 cells seemed enhanced compared to that in control cells and paxillin staining was observed around the cellular periphery. EPLIN overexpression in human HECV endothelial cells also suggests a similar relationship between EPLIN, cell-matrix adhesion and paxillin expression (Sanders, Ye et al. 2010). In addition, this study implicated a link and a frequent overlap between EPLIN and paxillin staining, and suggested that EPLIN may also be present in focal adhesion plaques (Maul and Chang 1999). EPLIN has been linked to cell-cell adhesion through its interaction with cadherin-catenin complex binding to F-actin (Abe and Takeichi 2008). It can therefore be assumed that there is some sort of link present between these molecules and paxillin adhesion molecule and may also have a role in regulating cellular adhesion to the extracellular matrix, although further research is required to fully examine this potential (Sanders, Ye et al. 2010). With such a link already established in the prostate cancer cell line we analysed this in the Keratinocyte cell line to try and see whether a similar pattern would emerge.

5.1 Materials and methods

5.1.1 Cell lines

Cells derived from the human keratinocyte cell line (HaCaT) were used in the study. EPLIN HaCaT, WT HaCaT and PEF HaCaT cells were routinely cultured in DMEM-F12 medium as described in section 2.3.

5.1.2 Antibody and Primers

Polyclonal rabbit anti-FAK antibody (SC-28450), goat anti-PLCy (SC 27898), rabbit anti-ERK (SC 25678), rabbit anti-Paxillin (SC 26787) and mouse anti-NWASP (Wiskostatin) (SC 27898) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). All the primers used were synthesised and provided by Invitrogen (Paisley, UK). Primer sequences are shown in Section 2.4.3, Tables 2.3. Due to various lab constraints not all of the phosphorylated protein variant antibodies could be obtained thus only those available were examined in this study.

5.1.3 Protein extraction, SDS-PAGE, and western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked, and probed with the specific primary and the corresponding peroxidise-conjugated secondary antibodies (1:1000). All of the antibodies used in this study are listed in Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

5.1.4 Immunochemical staining (ICC) of EPLIN HaCaT cell line

Immunochemical staining for PLCγ, FAK, pFAK, ERK and NWASP in EPLIN overexpressing and control cells lines (HaCaT) were carried out using specific primary antibody for the protein, followed by secondary antibody for visualisation under staining. For the detailed procedure refer to Section 2.4.6.

5.1.5 Immunoflourescent staining (IFC) of EPLIN HaCaT cell line

Immunoflurescent staining for PLCγ, FAK, ERK, pFAK and NWASP in EPLIN overexpressing and control cells lines (HaCaT) were carried out using specific primary antibody for the protein, followed by secondary antibody for visualisation under light microscopy. For the detailed procedure refer to Section 2.4.6.

5.2 Results

Following are the results of the protein analyses. As a result of various lab constraints the phosphorylated variants of only the FAK and Paxillin proteins were available and hence analysed. Similarly not all of the molecular antibodies were available to carry out IFC on all the proteins, and as such only the available ones were analysed.

5.2.1 Impact of EPLIN overexpression on expression profile of FAK

Looking at the western blot data for FAK (Figure 5.1) we can see that there appears to be little difference in the level of expression of FAK in the EPLIN overexpressing cell line in comparison to the control cells. This trend is further evident in the IFC results (Figure 5.2) where no visible difference in expression of the protein appears detectible between the cell lines. However a slightly unusual trend was seen in the ICC staining of the cells (Figure 5.3), with an apparent increase in positive staining of the nuclei of

the EPLIN overexpressing cell line in comparison with the controls. This is a trend that would not be expected based on the available literature and raises the question of whether the interaction between these two proteins is different in the context of cell wound healing in comparison to that observed in breast cancer cell lines.

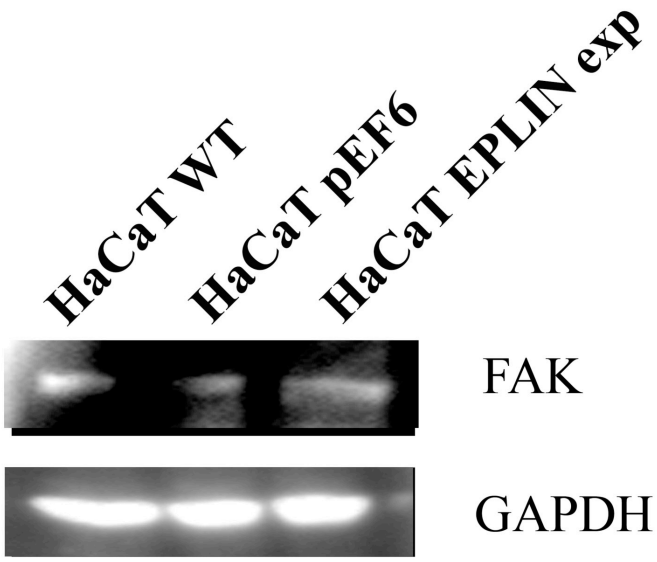


Figure 5.1: - Western blot for FAK using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. EPLIN overexpression results in a marginal increase of to FAK when standardised with GAPDH levels, especially with the pEF6 control cell line.

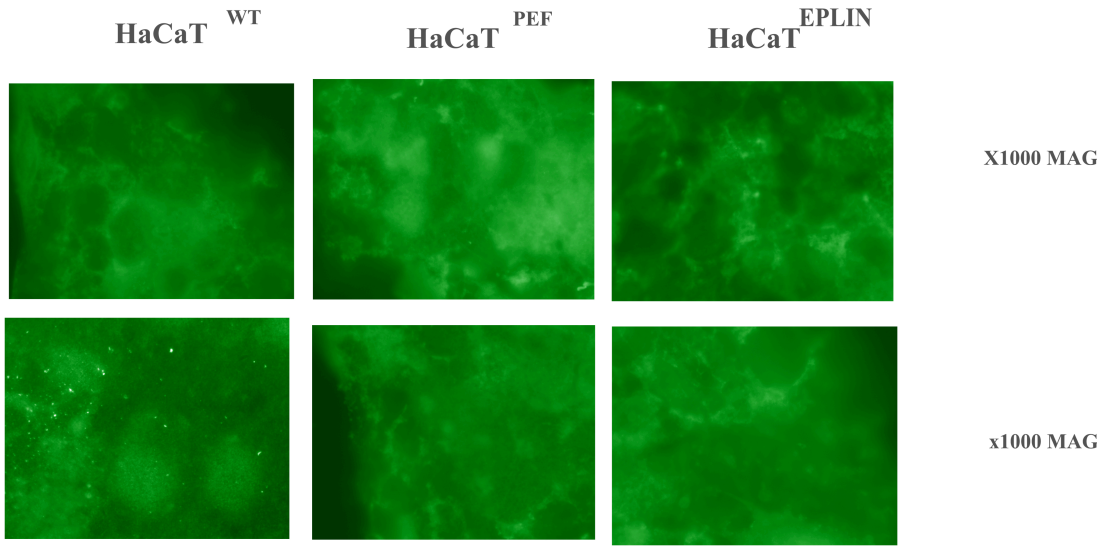


Figure 5.2: - IFC staining probing for FAK protein in EPLIN overexpressing HaCaT cells. Figure shows FAK expression around cell peripheries with little notable difference in the fluorescence levels between EPLIN overexpressing cells and control cells.

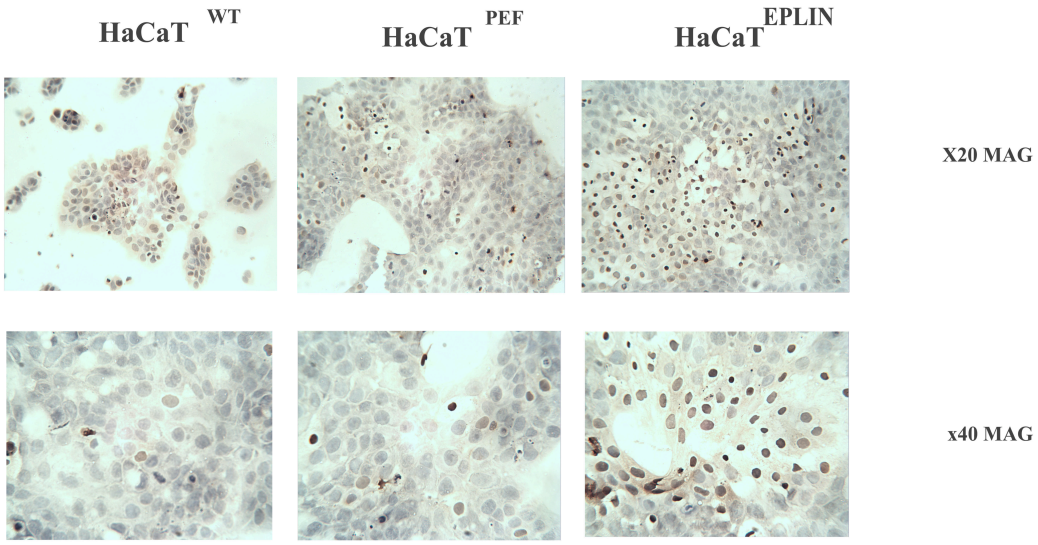


Figure 5.3: - ICC (Immuno cyto-chemical) staining of the cells probing for FAK in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows increased expression of FAK in EPLIN overexpressing cell line in compared with WT and PEF HaCaT cell controls, an increased percentage of HaCaT EPLIN exp cells stained positive for FAK.

5.2.1.1 Impact of EPLIN overexpression on expression profile of phosphorylated FAK

As the protein quantification results of the FAK molecule expression within EPLIN overexpressing cells were not consistent and the trends in particular that of the ICC not in keeping with the current body of literature; I additionally went on to examine the expression of the protein in its phosphorylated state to try and see whether any further revelations would come to light as a result of this.

Analysis of the western blot data for pFAK (Figure 5.4) showed that there was little difference in the protein detection in between the study and control cell lines. This trend is further evident in the IFC results (Figure 5.5) where no visible difference in expression of the protein appears detectible between the cell lines. However similarily to the non phosphorylated varient of the protein there was a similar increase in ICC staining observed in the study (EPLIN HaCaT) relative to the control cell lines (WT, PEF HaCaT). This is a trend is again inconsistent with the available literature.

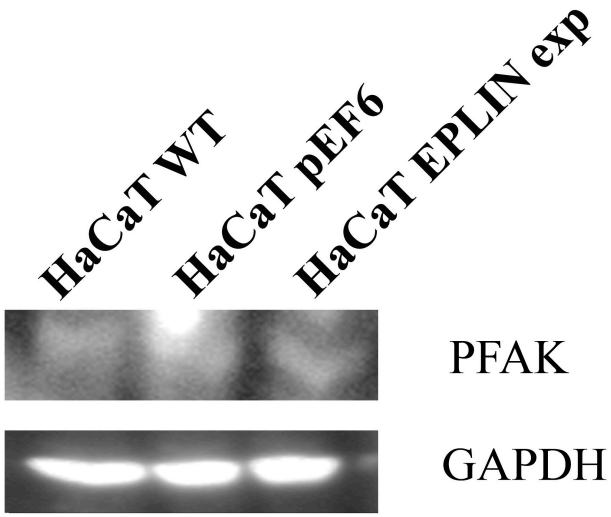


Figure 5.4: - Western blot for the phosphorylated FAK protein using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. The figure shows that in its phosphorylated state the FAK protein expression is not increased in between the study and control cell lines with no apparent difference as demonstrated in the Western blot.

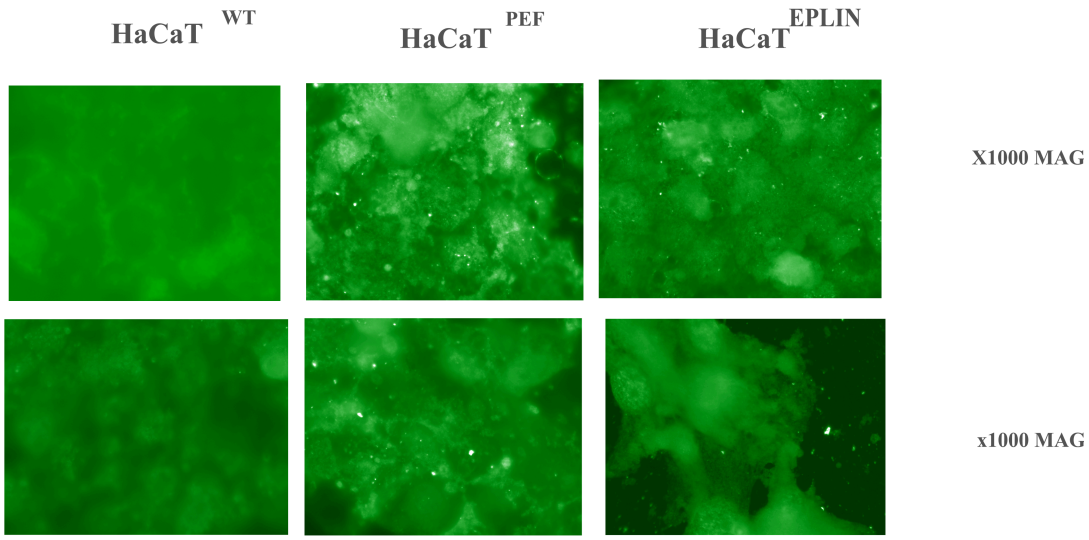


Figure 5.5: - IFC staining probing for the phosphorylated variant of the FAK protein in EPLIN overexpressing HaCaT cells. Figure shows that in its phosphorylated state there are no apparent detectable differences of pFAK expression around cell peripheries with little notable difference in the fluorescence levels between EPLIN overexpressing cells and control cells.

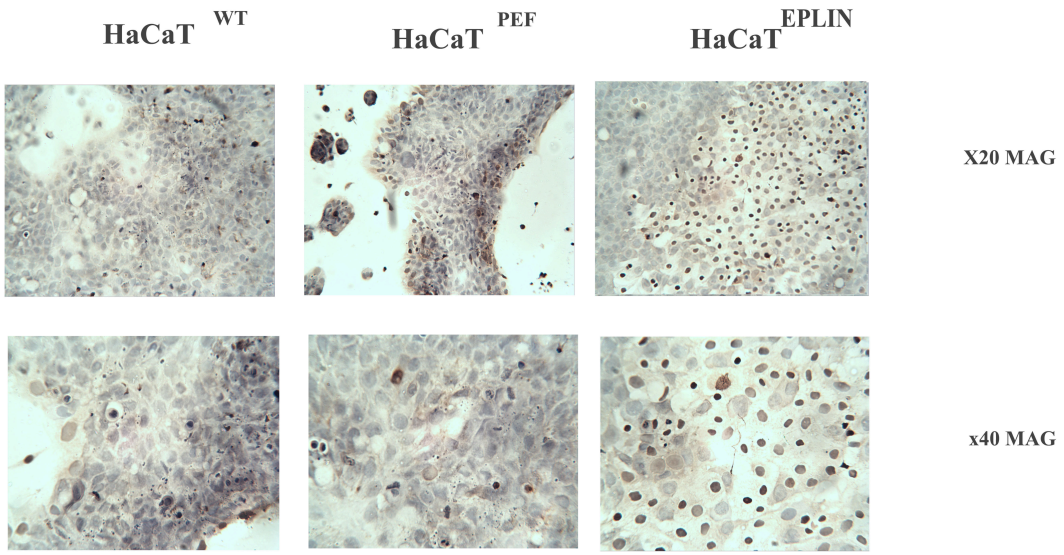


Figure 5.6: - ICC (Immuno cyto-chemical) staining of the cells probing for phosphorylated variant of the FAK protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows increased expression of phosphorylated FAK in EPLIN overexpressing cell line in compared with WT and PEF HaCaT cell controls. With an increased number of HaCaT EPLIN exp cells staining positive (brown) for presence of pFAK. This is similar to the trend observed in the non phosphorylated FAK protein.

5.2.2 Impact of EPLIN overexpression on expression profile of Paxillin

From the western blot data for paxillin (Figure 5.7) we can see that there appears to be little difference in the level of expression of Paxillin in the EPLIN overexpressing cell line in comparison to the control cells. This trend is further evident in the IFC results (Figure 5.8) where no visible difference in expression of the protein appears detectible between the cell lines. The ICC staining however indicates that there in an upregulation of the paxillin protein at the very edges of the stained sample in the EPLIN overexpressing cell lines (Figure 5.9), which is interesting as this would be the leading migratory edge of the cellular monolayer and thus have the most actively proliferating cells.

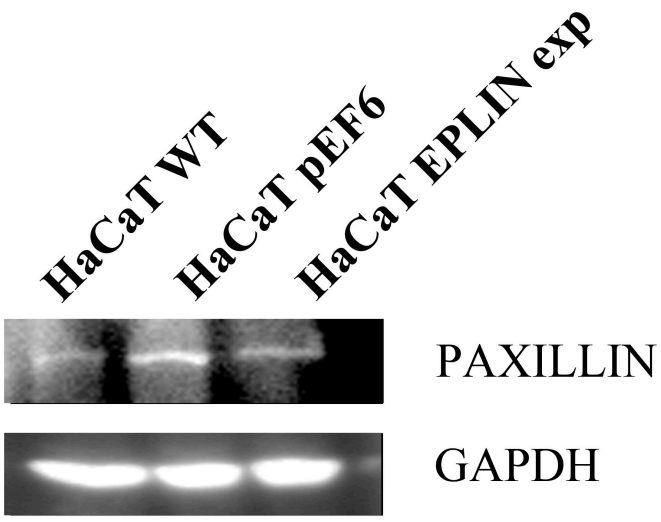


Figure 5.7: - Western blot for Paxillin using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. EPLIN overexpression does not appear to impact paxillin levels within the HaCaT cells when comparing study and control groups.

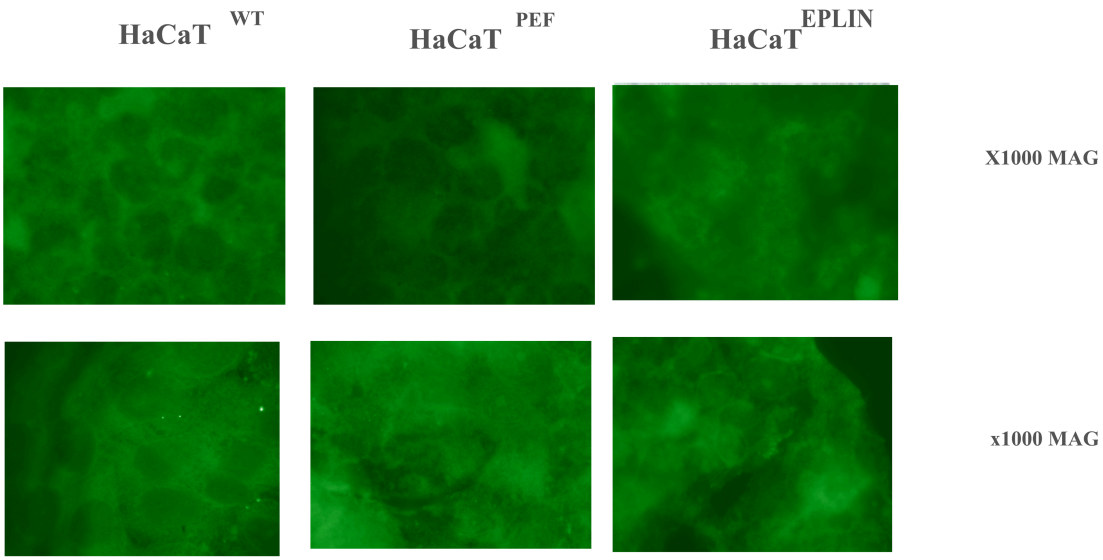


Figure 5.8: - IFC staining probing for the paxillin protein in EPLIN over expressing HaCaT cells. Figure shows that no apparent detectable differences of paxillin expression around cell peripheries exist with little notable difference in the fluorescence levels between EPLIN overexpressing cells and control cells.

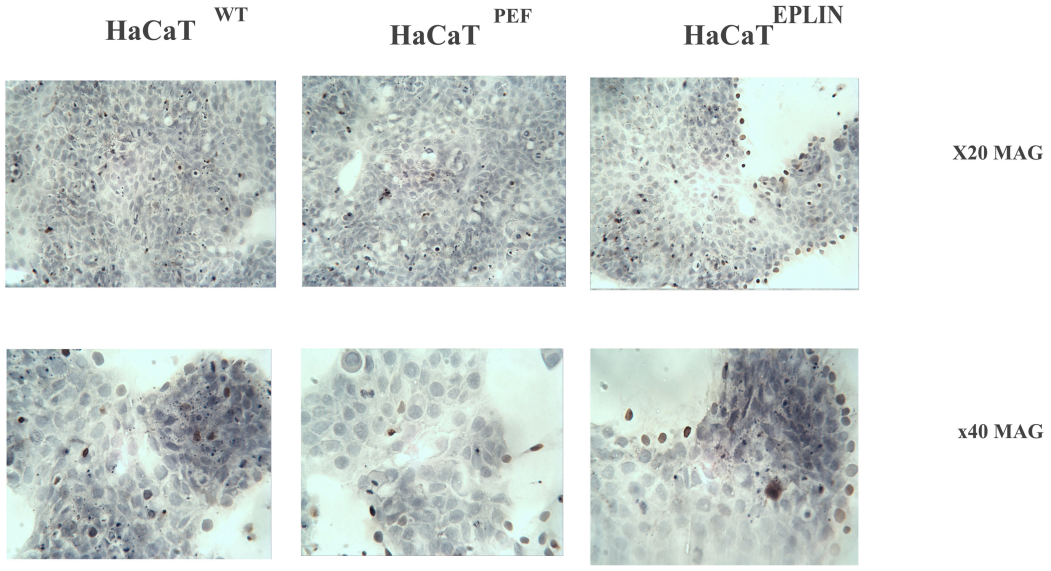


Figure 5.9: - ICC (Immuno cyto-chemical) staining of the cells probing for paxillin protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows increased expression of paxillin staining in the study cells in particular around the peripheries of the cells in the EPLIN over expressing HaCaT cells with a notable decrease in both of the control cell groups. This would be the region most notably involved in migration as this was the leading cell edge in the stained sample when the cells were fixed prior to staining.

5.2.2.1 Impact of EPLIN overexpression on expression profile of phosphorylated Paxillin

In the western blot data probing for paxillin (Figure 5.10) we can see that there is a definitive downregulation and reduced expression of this protein in the study (EPLIN HaCaT) compared with the control (WT, PEF HaCaT) cells. This trend can also be seen on the IFC staining for the protein as seen in (Figure 5.11). The ICC staining however shown no differences in the protein expression in the study and control groups (Figure 5.12).

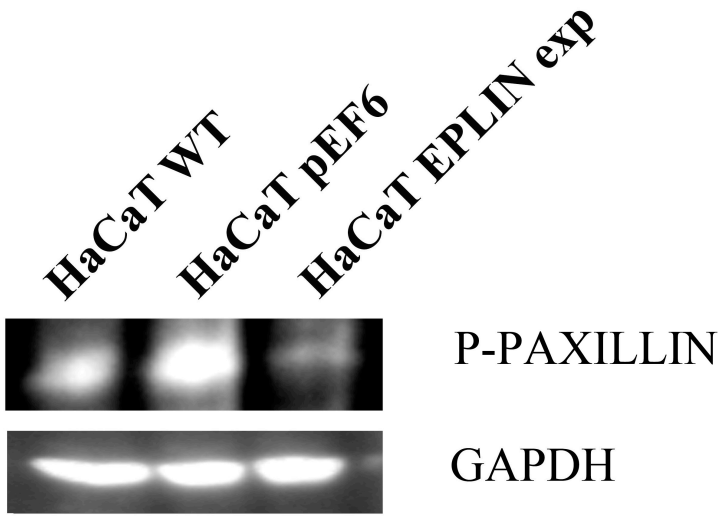


Figure 5.10: - Western blot for phosphorylated Paxillin protein varient using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. EPLIN overexpression appeared to cause a down regulation of the phosphorylated paxillin protein on western blot.

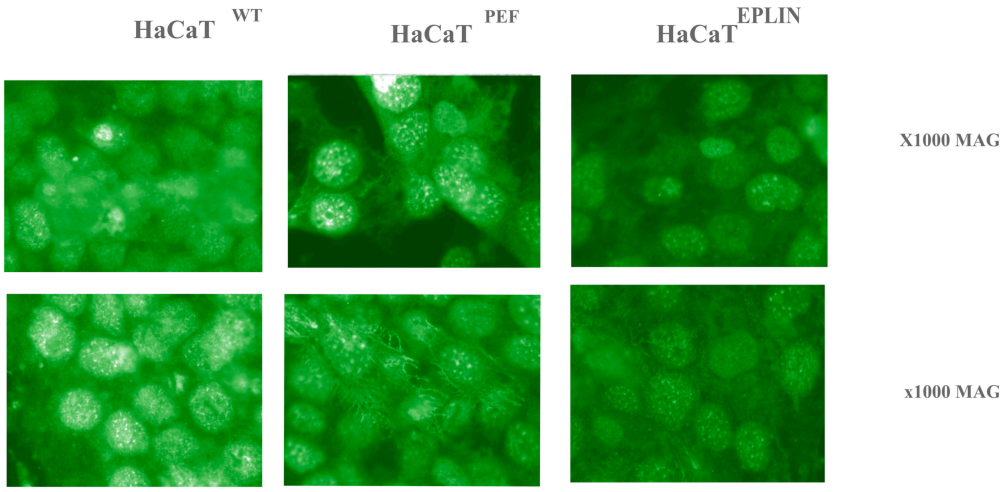


Figure 5.11: - IFC staining probing for the phosphorylated variant of the paxillin protein in EPLIN overexpressing HaCaT cells. Figure shows a decreased p-paxillin protein expression in between EPLIN overexpressing and control cells.

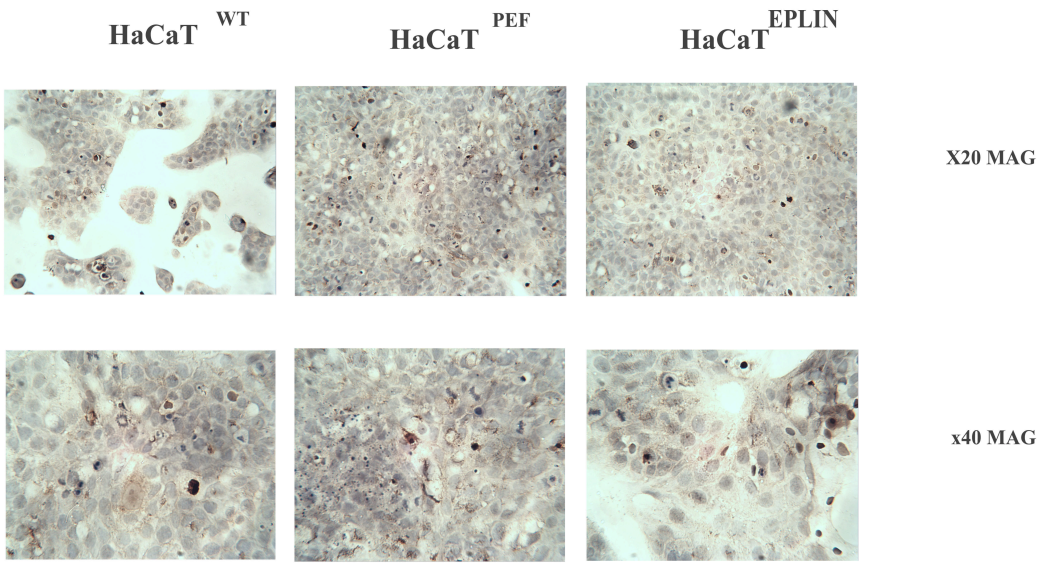


Figure 5.12: - ICC (Immuno cyto-chemical) staining of the cells probing for phosphorylated variant of the paxillin protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows little noticeable difference of the phosphorylated paxillin expression in EPLIN overexpressing cell line in compared with WT and PEF HaCaT cell controls.

5.2.3 Impact of EPLIN overexpression on expression profile of ERK

From the western blot data for paxillin (Figure 5.13) it is evident that there appears to be little bit of a reduced expression of the ERK protein in the EPLIN overexpressing cell line in comparison to the control cells. This trend is marginally evident also in the ICC staining of the protein (Figure 5.14).

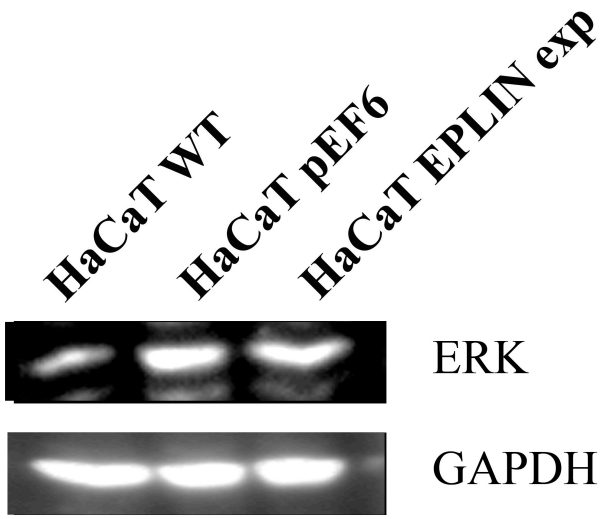


Figure 5.13: - Western blot probing for ERK protein using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. EPLIN overexpression appeared to have no effect on regulation of the ERK protein on western blot analysis. Marginal difference seen in between WT and study group however presence of the plasmid alone in PEF cells does not account for this difference therefore no firm conclusions can be drawn.

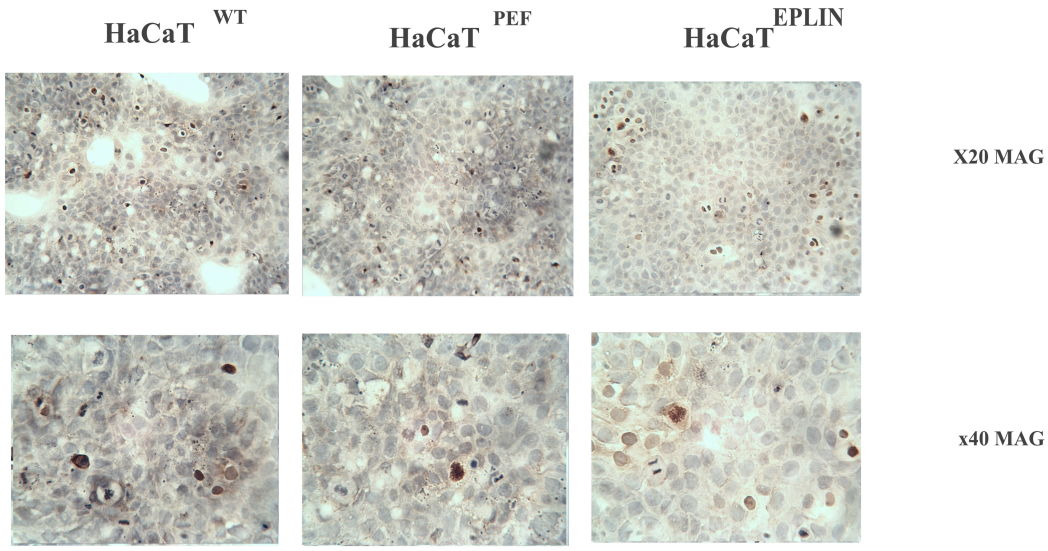


Figure 5.14; - ICC (Immuno cyto-chemical) staining of the cells probing for ERK protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows marginally reduced expression of ERK staining in the EPLIN over expressing HaCaT cells in particular compared with the WT control cell groups.

5.2.4 Impact of EPLIN overexpression on expression profile of PLCγ

From the western blot data for PLCγ (Figure 5.15) it is evident that there appears to be a reduced expression of the PLCγ protein in the EPLIN overexpressing cell line in comparison to the control cells. This is particularily evident when comparig the EPLIN HaCaT and the WT HaCaT cell lines. However the results are unlikely to be of significance with the lack of plasmid being the only difference between the two control groups, this alone is unlikely to account for the observed difference in expression. Although when coupled with the ICC data also showing a reduced expression of the protein this is likely to be a true trend and an accurate reflection of the reduced PLCγ protein expression in HaCaT cells overexpressing EPLIN (Figure 5.16).

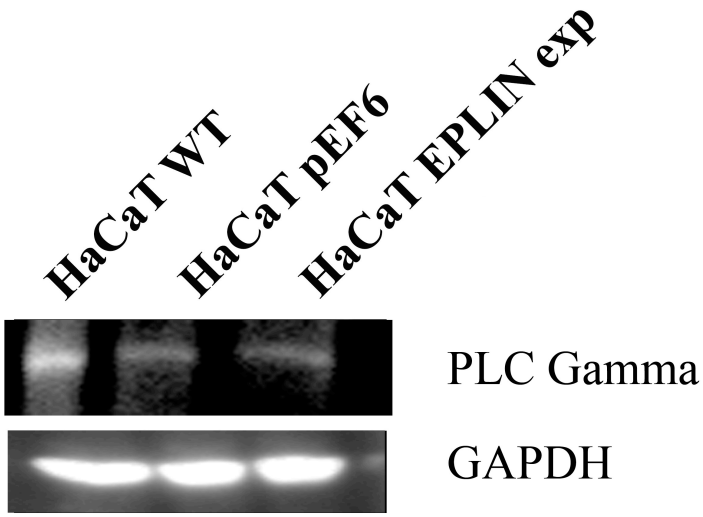


Figure 5.15: - Western blot probing for PLCγ **protein using EPLIN overexpressing HaCaT cells compared with WT and PEF controls.** EPLIN overexpression appeared to cause a down regulation of the PLCγ protein on western blot analysis, particularily when compared with WT HaCaT cell line. However with

the PEF HaCaT cells failing to display the same trend, the absence of the plasmid alone is unlikely to account for the observed difference.

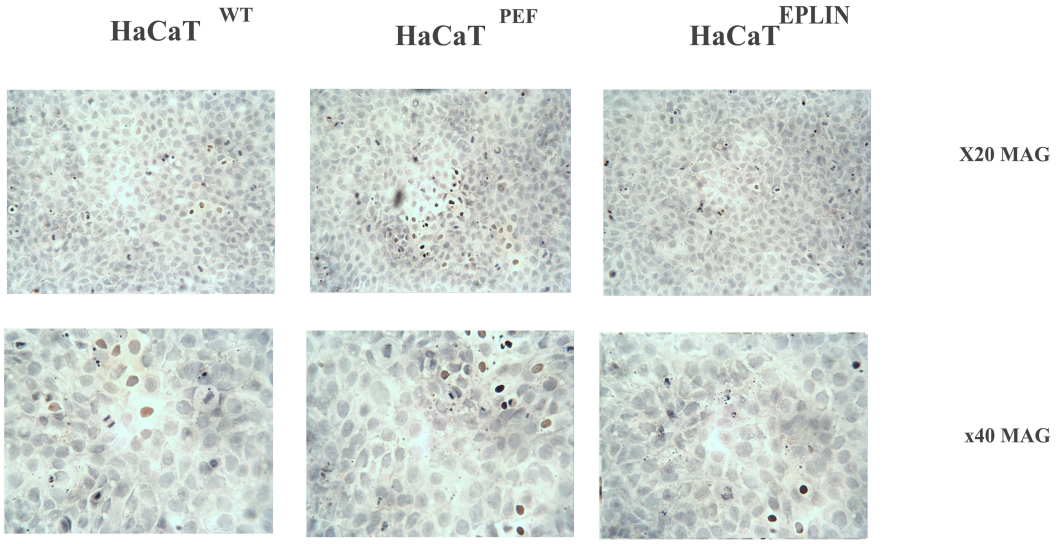


Figure 5.16: - ICC (Immuno cyto-chemical) staining of the cells probing for PLCγ protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows a mild reduction in expression of PLCγ in the study cell group in compared with controls. This observation coupled with the strong reduction in expression observed in the western blot between the EPLIN HaCaT and the WT HaCaT cell groups makes this more likely to be a 'true' result and a significant observation.

5.2.5 Impact of EPLIN overexpression on expression profile of NWASP

From the western blot data for NWASP (Figure 5.17) it is evident that there appears to be a marginal reduced expression of the NWASP protein in the EPLIN overexpressing cell line in comparison to the control cells. This is particularily evident when comparig the EPLIN HaCaT and the PEF HaCaT cell lines. It is unlikely that the observed difference would have been as a result of the plasmid absence alone, so when data is coupled with the ICC data showing no demonstratable differences in expression the conclusion has to be drawn that this result is simply an anomaly and unlikely to be significant (Figure 5.18).

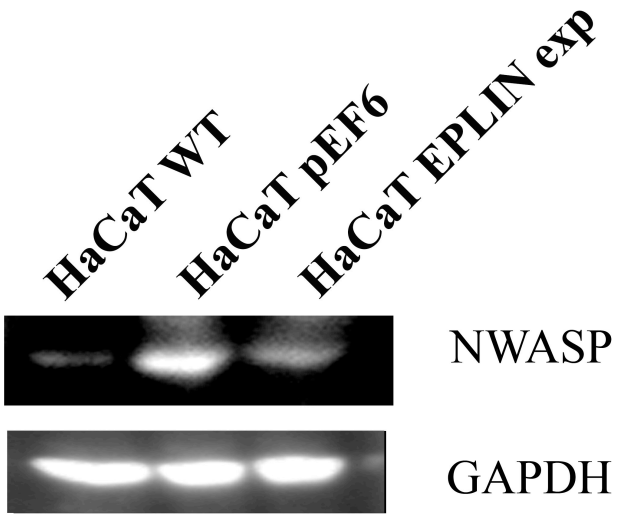


Figure 5.17: - Western blot probing for NWASP protein using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. There appeared to be a marginal reduction in protein expression of the EPLIN HaCaT cell line in comparison to the PEF (plasmid containing) HaCaT cell control, however no such difference was observed in the WT HaCaT cell line.

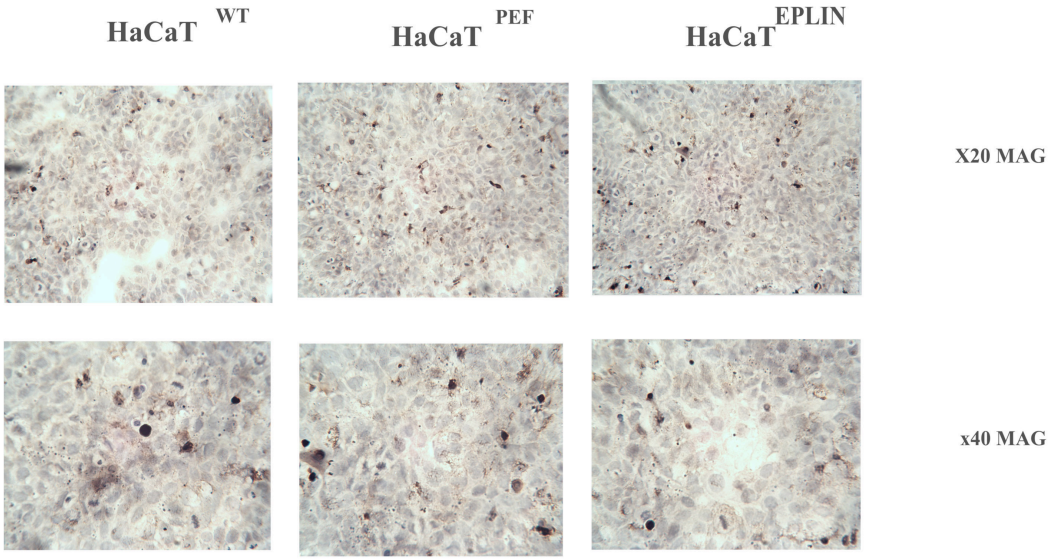


Figure 5.18: - ICC (Immuno cyto-chemical) staining of the cells probing for NWASP protein in EPLIN overexpressing keratinocyte (HaCaT) cells. Figure shows no difference in expression of NWASP in the study cell group in compared with controls.

5.3 Discussion

The finding that FAK mRNA and protein is up regulated in metastatic and invasive tumours compared to non-invasive adenocarcinomas (Abedi and Zachary, 1995) suggests that increased FAK expression may play a role in tumour invasiveness. Also apparent is that knocking out the FAK gene in mice prevents normal embryonic development and is associated with loss of mesenchymal cell motility (Fan, Zhao et al. 2013). It can thus be hypothesized that up regulation of FAK expression may accompany other disease states in which aberrant cell migration occurs. By looking at the interaction between FAK inhibitor and EPLIN overexpressing keratinocyte cells it would be interesting to see whether any link existed between the two and whether they two could be linked in some way.

EPLIN overexpression in HaCaT cells did not seem to have any substantial effects on FAK staining and localisation using IFC. However, potential differences in FAK levels between control and EPLIN overexpression lines became apparent following Western blot and, particularly, ICC staining. Using these assay, it appeared that the levels of FAK were slightly enhanced in EPLIN overexpressing HaCaT cells. In addition, similar results were indicated when examining the phosphorylated version of FAK, where IFC and Western blot analysis did not seem to indicate vast differences in expression levels between control and overexpression lines but ICC indicated an increase in pFAK in HaCaT overexpression cells. Taken together, this data could suggest that there is a relationship between EPLIN and the FAK protein and that EPLIN may regulate somehow the expression and phosphorylation state of this molecule. However, as this trend does not appear to be clear cut across all the methodologies used the significance of this is limited and it may be that if there is an

association between FAK and EPLIN that this is not necessarily the key role. Further work into this area of study are needed to form solid conclusions regarding FAK and EPLIN interactions and indeed if such interactions could account for the effects mediated by EPLIN on HaCaT migration seen in earlier chapters.

In the recent study by Sanders et al immunofluorescence staining was performed to examine the paxillin adhesion molecule. Paxillin staining in EPLIN overexpressing PC-3 cells seemed enhanced compared to that in control cells and paxillin staining was observed around the cellular periphery. EPLIN overexpression in human HECV endothelial cells also suggests a similar relationship between EPLIN, cell-matrix adhesion and paxillin expression (Sanders, Ye et al. 2010). In addition, this study implicated a link and a frequent overlap between EPLIN and paxillin staining, and suggested that EPLIN may also be present in focal adhesion plaques (Maul and Chang 1999). As discussed in the next chapter EPLIN has been linked to cell-cell adhesion through its interaction with cadherin-catenin complex binding to F-actin (Abe and Takeichi 2008). It can therefore be assumed that there is some sort of link present between these molecules and paxillin adhesion molecule and may also have a role in regulating cellular adhesion to the extracellular matrix, although further research is required to fully examine this potential (Sanders, Ye et al. 2010) .

The levels of paxillin examined in control and EPLIN overexpression HaCaT cells. Both IFC and ICC demonstrated a similar level of paxillin in the wild type, plasmid control and EPLIN overexpression cells with little difference seen on western blot. Analysis of the phosphorylated version of paxillin in the cells similarly demonstrated no substantial differences in p-paxillin levels between control and EPLIN transfected

cells using ICC. However, western blot analysis and IFC indicated that levels of p-paxillin may be reduced somewhat in HaCaT EPLIN exp cells compared to controls. The current data suggests that in HaCaT keratinocytes, this association seen in prostate and endothelial cells may not be as important as overexpression of EPLIN did not largely alter the expression or localisation of paxillin in HaCaT cells. However, the reduction of phosphorylated paxillin in EPLIN overexpressing cells does indicate that some relationship may exist, though further study will be required to clarify this and its importance to EPLINs role in keratinocyte functions such as migration.

Although no notable changes in expression were seen in the HaCaT study vs control cells in terms if the quantity of expression when probed for Paxillin, an interesting trend was observed when ICC staining was done, in that the pattern of expression appeared to be different with an increase in the cells overexpressing Paxillin at the very edge of the stained samples. This would be the region most notably involved in migration as this was the leading cell edge in the stained sample when the cells were fixed prior to staining. This observation suggests that even though no clear link could be demonstrated at a protein expression level, it does not mean that these two molecules have no effect on each others functioning but further in depth study would be needed to determine this possible relationship.

Table 5.1: - Summary of the trends in protein expression between the study (EPLIN HaCaT) and control cell lines (WT/PEF HaCaT) when looking at western blot results. The results presented are representative data based on 3 independent repeats and show the observed differences in band width in between the different cell lines. On the whole with the exception of p-paxillin the results are difficult to interpret as differences were not consistent in between both of the controls when compared with the study group so conclusions have to be drawn when piecing the results together in concordace with the IFC/ICC results and looking at the overall picture of protein expression.

	WT	PEF	EPLIN
FAK			
PFAK			
PAXILLIN			
P-PAXILLIN			
ERK			
PLC			
NWASP			

In the literature, links between ERK and EPLIN have been established (Han, Kosako et al. 2007) and appear to be important in process of migration and tubule formation (jiang 2010; Sanders 2012). ERK has been shown to phosphorylate EPLIN acting upstream of EPLIN. The observations shown here indicate that EPLIN itself does not have a direct effect on ERK expression in keratinocytes

NWASP (neuronal Wiskott - Aldrich syndrome protein) is a member of the Wiskott-Aldrich syndrome (WAS) family of proteins shown to play a role in the transduction of signals from receptors on the cell surface to the actin cytoskeleton (Ochs and Thrasher 2006). The reduction in expression of NWASP is associated with a greater malignant potential (Baluk, Hashizume et al. 2005) in the context of breast cancer, via its role in cellular migration (Martin, Pereira et al. 2008). This is of particular relevance as our study protein has very similar properties. With the recent observation that NWASP inhibitor results in a substantial increase in HaCaT keratinocyte migration in the host laboratory (Jiang et al unpublished data), it was expected for there to be a strong collation between the two proteins.

The role of NWASP in wound healing has only very recently been described, and is consistent with its effect in breast cancer. The study of the effect of NWASP in previous studies was done using the inhibitor Wiskostatin which has shown to increase keratinocyte *migration in vitro* (Jiang *et al,* unpublished data). Furthermore our host laboratory uncovered that it is overexpressed in patients with chronic ulcers, compared to patient with acute wounds. The inhibition of NWASP resulted in

enhanced wound closure rates when applied topically and via the intraperitoneal route to mice (Jiang et al, unpublished data). Perhaps thus it was a little disappointing that in these study only a very weak association could be demonstrated between these two protein with a slight reduction of protein expression in the EPLIN overexpressing cells in comparison to the PEF controls with no apparent demonstrable link on ICC staining.

Future studies are warranted that focus on the generation and efficacy of a recombinant form of EPLIN to treat non healing wounds in vitro and in vivo as well as large-scale studies of its reliability as a biomarker that is lost in cancer progression and healing wounds.

CHAPTER 6 -

Protein expression of cadherins and catenins in the EPLIN overexpressing cell line

6. Introduction

6.1 Impact of EPLIN overexpression on expression profile of catenins and cadherins

The cadherin–catenin complex is the major machinery for cell– cell adhesion and motility in many animal species. This complex in general associates with actin fibres at its cytoplasmic side, organizing the adherens junction (AJ) (Abe and Takeichi 2008). Adherens junctions are required for vascular endothelium integrity. These structures are formed by the clustering of the homophilic adhesive proteins including cadherin, which recruits intracellular partners, such as alpha and beta catenins, vinculin, and actin filaments (Song, Maul et al. 2002). In epithelial cells, the AJ encircles the cells near their apical surface and forms the ‘‘zonula adherens’’ or‘‘adhesion belt.’’ The mechanism as to how the cadherin–catenin complex and F-actin cooperate to generate these junctional structures, however, remains unclear. EPLIN (epithelial protein lost in neoplasm), an actin-binding protein, couples with Alpha-catenin and, in turn, links the cadherin–catenin complex to F-actin (Abe and Takeichi 2008). Recently, epithelial protein lost in neoplasm (EPLIN) has been proposed as a possible bond between the E-cadherin-catenin complex and actin in epithelial cells, believed to be expressed at similar levels in endothelial and epithelial cells (Song, Maul et al. 2002). Without EPLIN, this linkage is unable to form and when depleted in epithelial cells, the adhesion belt is disorganized and converted into zipper-like junctions in which the actin fibres are radially arranged (Song, Maul et al. 2002). However, non-junctional actin fibres are not particularly affected by EPLIN depletion. As EPLIN is known to have the ability to suppress actin depolymerization, suggests that EPLIN functions to link the cadherin–catenin complex to F-actin and

simultaneously stabilizes this population of actin fibres, resulting in the establishment of the adhesion belt. Near the apical surface of the cells, a class of cell–cell junction structures, the adherens junction (AJ), develops (Mège, Gavard et al. 2006). The AJ encircles the cells, together with a bundle of cortical actin filaments, organizing the ‘‘zonula adherens’’ or ‘‘adhesion belt.’’ This actin bundle, called the circumferential actin belt, is known to play a number of roles in epithelial morphogenesis (Lecuit and Lenne 2007), (Pokutta and Weis 2007), For example, the contractility of this belt contributes to the constriction of the apical end of epithelial cells (Hildebrand 2005; Fernandez-Gonzalez and Zallen 2009) and the rearrangement of cells undergoing convergent extension (Bertet, Sulak et al. 2004). The AJ comprises cadherin, catenins, and other associated proteins. Cadherins interact homophilically via their extracellular domain, functioning as a physical linker between the confronting cell membranes. The cytoplasmic region of cadherin binds beta catenin; and this catenin, in turn, associates with alpha catenin. Alpha catenin is indispensable for cadherin-mediated cell adhesions (Hirano, Kimoto et al. 1992).

In a Matrigel assay, EPLIN depleted endothelial cells exhibited a reduced capacity to form pseudocapillary networks because of numerous breakage events. Thus it can be modelled that EPLIN establishes a link between the cadherin-catenin complex and is necessary for stabilization of capillary structures in an angiogenesis model (Song, Maul et al. 2002).

With respect to the above previously described association between EPLIN and the Cadherin- Catenin complex I thought to investigate this association in Keratinocyte cells in order to establish whether cells overexpressing EPLIN have altered quantities

of Alpha, Beta, Gamma Catenin and E Cadherin. I did this by Staining the cells for IFC and quantifying at a mRNA level using RT-PCR and protein level using a Western Blot.

6.2 Materials and methods

6.2.1 Cell lines

Cells derived from the human keratinocyte cell line (HaCaT) were used in the study. EPLIN HaCaT, WT HaCaT and PEF HaCaT cells were routinely cultured in DMEM-F12 medium as described in Section 2.3.1.

6.2.2 Antibody and Primers

Polyclonal mouse anti-alpha catenin antibody (SC-24550), rabbit anti-beta catenin (SC 2206), rabbit anti-gamma catenin (SC 25538), rabbit anti-e-cadherin (SC 24567) (Santa Cruz, California, USA). All the primers used were synthesised and provided by Invitrogen (Paisley, UK). Primers sequences are shown in Tables 2.3.

6.2.3 Protein extraction, SDS-PAGE, and western blot analysis

Protein was extracted and was then quantified using the DC Protein Assay kit (BIO-RAD, USA). After SDS-PAGE, the proteins were transferred onto nitrocellulose membranes which were then blocked, and probed with the specific primary and the corresponding peroxidise-conjugated secondary antibodies (1:1000). All of the antibodies used in this study are listed in Section 2.51, Table 2.4. The protein bands were eventually visualised using the chemiluminescence detection kit (Luminata, Millipore).

6.2.4 Immunoflourescent staining (IFC) of EPLIN HaCaT cell line

Immunoflurescent staining for alpha, beta and gamma catenin as well as E-cadherin in EPLIN overexpressing and control cells lines (HaCaT) were carried out using specific primary antibody for the protein, followed by secondary antibody for visualisation under light microscopy.

6.3 Results

The following section looks to examine the protein link between the EPLIN over expressing cell line and the cadherin and catenin family of proteins, in particular alpha, beta, gamma catenin and e- cadherin.

6.3.1 Impact of EPLIN overexpression on expression profile of alpha- catenin

From the western blot data for alpha-catenin (Figure 6.1) it is evident that there appears to be a slight increase in protein expresion in the EPLIN overexpressing cell line in comparison to the control cells. This is particularily evident when comparig the EPLIN HaCaT and the WT HaCaT cell lines. These results are confirmed on the IFC stain likewise showing a deeper and brighter staining at the cell edges for the alpha catenin protein abundant in the EPLIN over expressing cell line (Figure 6.2). This interestingly is what we would expect to find following the recent revelations in literature of EPLIN association with alpha catenin and their proposed interaction in cell adhesion and migration.

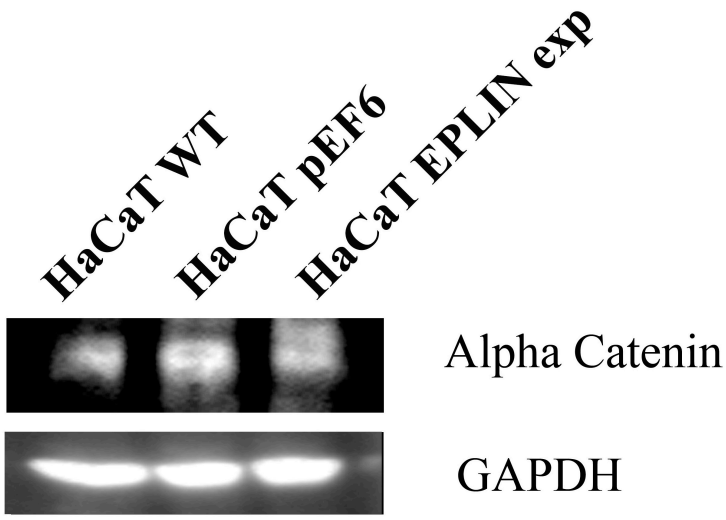


Figure 6.1: - Western blot for alpha catenin using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. EPLIN overexpression results in a marginal increase of to aplha-catenin expression when standardised with GAPDH levels, when looking at control and study cell lines (especially with the WT control cell line).

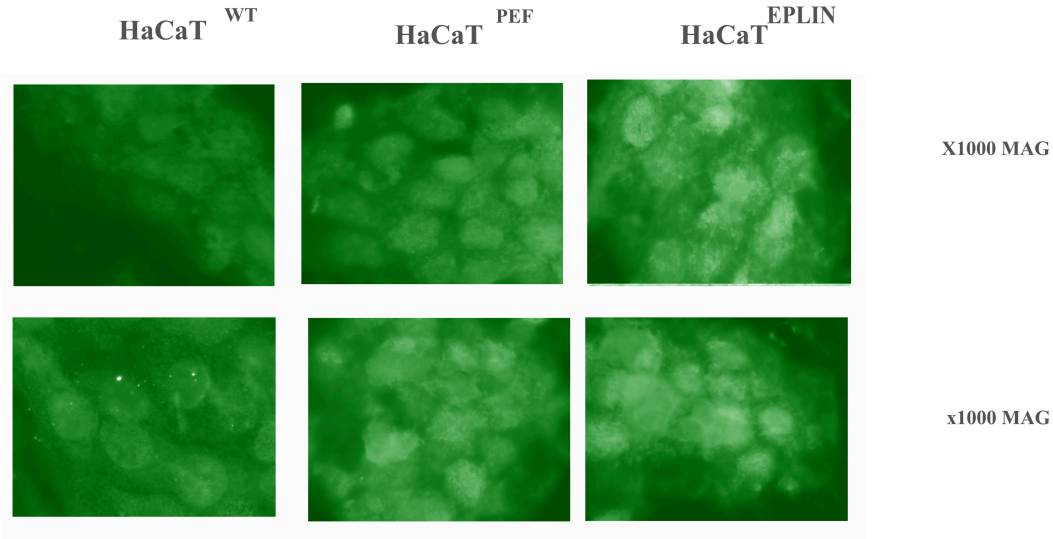


Figure 6.2: - IFC staining probing for the alpha catenin protein in EPLIN over expressing HaCaT cells. Figure shows an increased alpha catenin protein expression in between EPLIN overexpressing and control cells, with brighter more vivid fluorescent staining clearly visible.

6.3.2 Impact of EPLIN overexpression on expression profile of beta - catenin

Looking at the data in relation to the beta catenin protein it appears that there perhaps is a slight increase in protein expression in the study cells (EPLIN HaCaT) in particularily compared with the WT HaCaT cell type. It is unlikely that the observed difference would have been as a result of the plasmid absence alone, so when data is coupled with the IFC data also showing no demonstratable differences in expression the conclusion has to be drawn that this result is simply an anomaly and unlikely to be significant

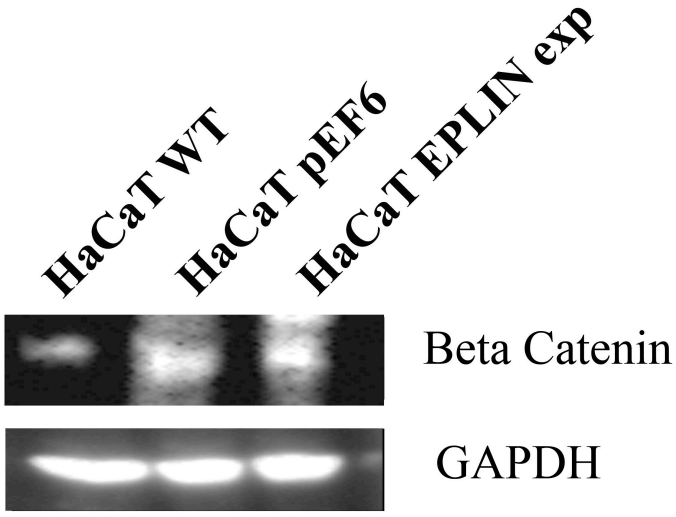


Figure 6.3: - Western blot for beta catenin using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. There appears to be a slight upregulation of beta-catenin protein expressin in EPLIN HaCaT in comparison with WT HaCaT cells, however result is unlikely significant as the same trend is not also seen in the pEF cell line**.**

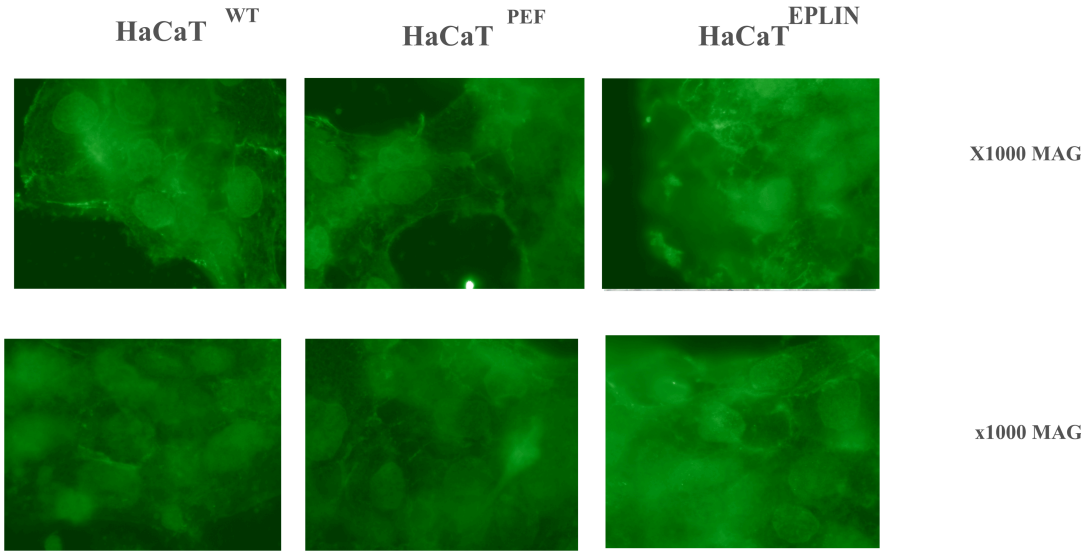


Figure 6.4: - IFC staining probing for the beta-catenin protein in EPLIN over expressing HaCaT cells. Figure shows no demonstrable difference in beta-catenin protein expression in between EPLIN overexpressing and control cells.

6.3.3 Impact of EPLIN overexpression on expression profile of gamma - catenin

The western blot data for gamma-catenin (Figure 6.5) shows an apparent marginal upragulation of the protein in the EPLIN study HaCaT cells, predominantly in relation to the WT control cells. Alone this result would be of borderline significance however when coupled with the clear increase in IFC staining of the study cells in comparison to both the control subjects one must deduct that the result is indeed significant with the overexpression of EPLIN clearly leading to an upregulation of the gamma catenin protein (Figure 6.6). This interestingly is what we would expect to find following the recent revelations in literature of EPLIN association with the cahderin-catenin family.

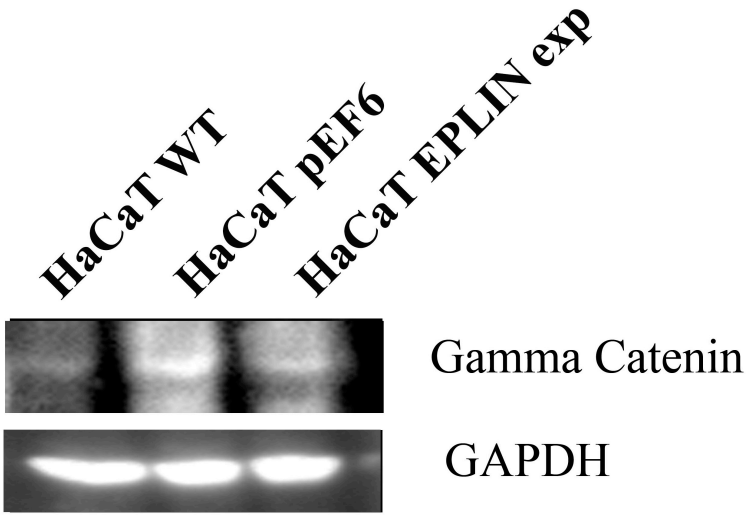


Figure 6.5: - Western blot for gamma - catenin using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. There appears to be a slight upregulation of gamma-catenin protein expressin in EPLIN HaCaT in comparison with WT HaCaT cells, likely significant coupled with IFC stain**.**

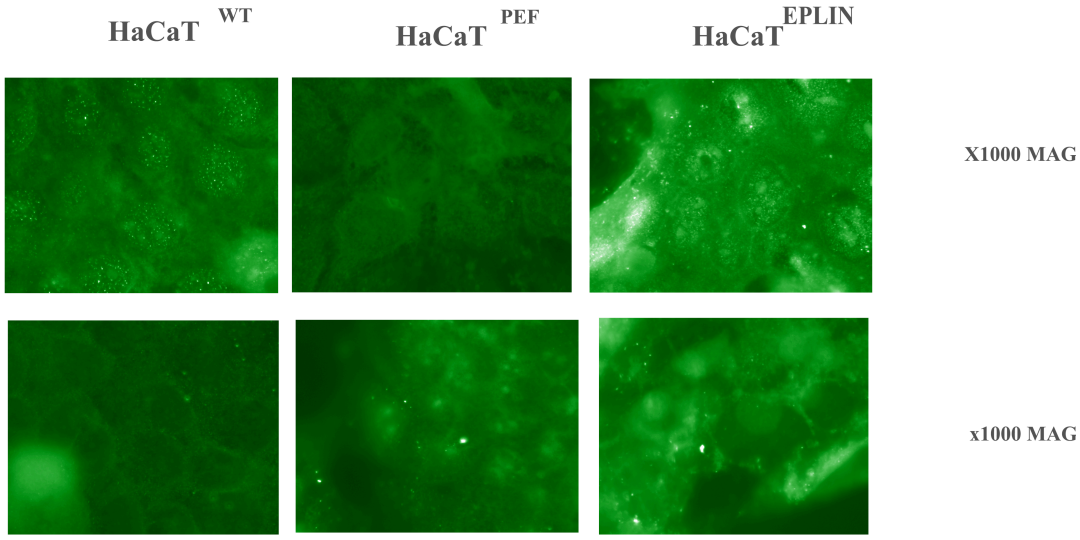


Figure 6.6: - IFC staining probing for the gamma-catenin protein in EPLIN over expressing HaCaT cells. Figure shows an increased gamma-catenin protein expression in between EPLIN overexpressing and control cells, with brighter more vivid fluorescent staining clearly visible.

6.3.4 Impact of EPLIN overexpression on expression profile of E - Cadherin

Finally E-cadherin yielded some excellent convincing results. In the western blot a clear upregulation of the protein is seen in the EPLIN overexpressing cell line in comparison to both the control cell lines (Figure 6.7). This is even more exciting when coupled with the beautifully enhanced IFC stain probing for the e-cadherin molecule in the studycell line (Figure 6.8). This confirms the association of these two proteins in the keratinocyte cell lines which is applicable to wound healing. It is highly likely with these results and coupled with current recent literature that these wo proteins share a common patway and aid one another in the motlity of cells. This again cements the previous results from IFC (Anderson, Wong et al. 2009).

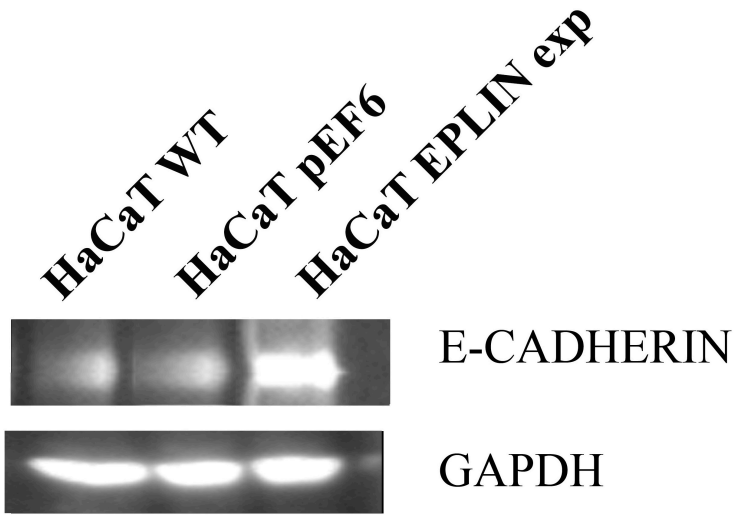


Figure 6.7: - Western blot for e-cadherin using EPLIN overexpressing HaCaT cells compared with WT and PEF controls. There is a convincing upregulation of the e cadherin in the EPLIN HaCaT study cell line in comparison to the control.

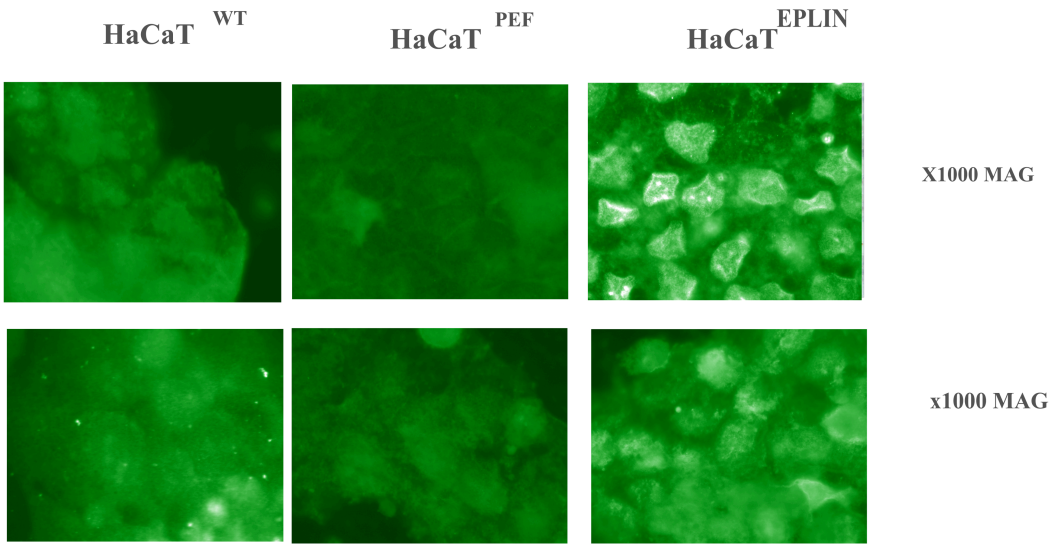


Figure 6.8: - IFC staining probing for the e-cadherinn protein in EPLIN over expressing HaCaT cells. Figure shows a clear up regulation of the e cadherin in the EPLIN HaCaT study cell line in comparison to the controls.

6.4 Discussion

These results are very interesting as they tie in with recent literature linking EPLIN with the Cadherin-Catenin complex, where EPLIN was proposed to as a possible bond between the cadherin-catenin complex and the actin in epithelial cells. As described above this is the key cell machinery affecting cell-cell motility and adhesion (Abe and Takeichi 2008).

Our results show that in keratinocyte cells overexpressing the EPLIN protein resulted in no substantial effects on the expression of beta and gamma catenin, both as illustrated in the IFC assay, RT-PCR and Western blot analysis. However I did note a small difference in fluorescence on analysis of Alpha catenin in the EPLIN overexpressing cell line in comparison with the control cells. Unfortunately this same trend was not seen in the Western blot and RT PCR analysis. Where the results became extremely interesting was the markedly increased florescence of cells overexpressing the EPLIN plasmid in comparison to control cells when stained for E-Cadherin. This same trend was also seen in the Western blot analysis with a much stronger band of expression when probed for E cadherin seen in the EPLIN overexpressing cells when compared with that of control cells.

These findings suggest that EPLIN is strongly associated with the cadherin-catenin complex and in particular E-Cadherin and is consistent with what would be expected from literature in other epithelial cell lines, further cementing the findings made in other literature as to the association between EPLIN and E cadherin and its importance in establishing links between the cadherin-catenin complex. This could be a way in which EPLIN exerts its effect on cell migration and adhesion and

subsequently, when overexpressed, reduced both of those cellular functions. As Abe et al showed in their work, EPLIN to be associated between E- cadherin and actin with the forming of the adhesion belt.(Abe and Takeichi 2008)

Table 6.1: - Summary the trends in CATENIN-CAHDERIN protein expression between the study (EPLIN HaCaT) and control cell lines (WT/PEF HaCaT).

	WT	PEF	EPLIN
ALPHA-CATENIN			
BETA-CATENIN			
GAMMA-CATENIN			
E-CADHERIN			

It is possible that in the keratinocytes over-expressing EPLIN more of the Cadherin-catenin complexes might become saturated and hence account for why these cells subsequently migrate slower than the control cells. In these cells, enhanced VE-

cadherin could increase cell-cell adhesion resulting in tightly bonded cells that are unable to migrate sufficiently. It would certainly be worth further study to investigate this association and pathway further to work out how EPLIN exerts its effect on this cadherin-catenin complex and in turn how this affects actin binding and motility.

The endothelium forms a semi-permeable barrier that regulates the passage of macromolecules and circulating cells between blood and tissues. Even though traditionally viewed as passive vascular lining, vascular endothelium is in-fact a dynamic tissue undergoing continuous remodelling required for physiological and pathological processes, such as wound healing and angiogenesis (Liebner, Cavallaro et al. 2006; Aird 2007). This protective barrier function of the endothelium is in large part regulated by adherens junctions, which comprise the transmembrane adhesive receptor Ecadherin. This is the main protein involved in homophilic/ homotypic cell-to-cell adhesion (Vestweber 2008; Gavard 2009). E Cadherin is thought to recruit other intracellular proteins which then help to links with the actin cytoskeleton inherently stabilizing the interendothelial junctions (Shapiro and Weis 2009). Alpha catenin plays a key role in strengthening E cadherin-mediated adhesion by promoting anchorage to the actin cytoskeleton (Song, Maul et al. 2002).

Alpha catenin is known to interact with multiple actin binding proteins (Kobielak and Fuchs 2006). The above results tie in with this and link EPLIN which is a known actin binding protein with an overexpressioin of Alpha catenin. Abe et al suggest that EPLIN can bind and stabilize apical actin bundles even in the absence of Alpha-catenin. However, it is likely that EPLIN normally controls the dynamics of actin fibres at the AJ by associating with the cadherin–catenin complex. However, despite

the reported actin-stabilizing ability of EPLIN, depletion of this protein does not cause the entire disruption of actin networks in cells (Abe and Takeichi 2008).

The data in this chapter highlights EPLINs role in regards to the cadherin-catenin complex in keratinocytes. The results demonstrate a potential mechanism of action for EPLINs effects on cell migration, through its interaction with E-cadherin. This data presents an interesting avenue for future research down this potential mechanism warranting further study.

Some work on E- cadherin has in fact been done in the HaCaT cell line. In a study by Scholtz et al (2005) cells were observed to display cleavage and a loss of cell-cell contacts, with e-cadherin and dislocation of B-Catenin when exposed to ADAM 10 protein. The authors concluded that ADAM was responsible for mediating the epithelial cell-cell adhesion and E-cadherin localisation. This study is an example of how hypotheses can be drawn based upon protein to protein interaction and their consequent effect on migration and expression. We have to remember however that using this methodology only shows a possible link between two study proteins, with the outcome measure being migration and not of downstream proteins (Maretzky, Reiss et al. 2005).

E cadherin is well known to stabilise cellular movements by binding to actin via alpha, beta and gamma catenin complex. This can be seen by the increase in cellular movement when this process is artificially disrupted as with the use of HGF chemokine (Muller, Bain et al. 2002). Therefore by not permitting excess cellular movements this catenin-cadherin complex can be thought of as a tumour suppressor.

It is extremely exciting then that in the current study the results indicate a strong expression of e-cadherin in EPLIN overexpressing cells in relation to controls. This implicates EPLIN as another protein integral to this process as we already know it to be a tumour suppressor. It would be interesting in the future to investigate the interaction of EPLIN and e- cadherin in greater detail within the HaCaT keratinocyte cell line and see whether this could be a potential pathway by which wounds develop chronicity and fail to heal by overexpressing these anti-migratory molecules.

Beta-Catenin has been linked in the literature with playing a role in a number of different cancer types predominantly its role in promoting gene transcription and cell cycle control leading to an up regulation of colorectal cancers via the wnt pathway (Giles, van Es et al. 2003), a reduction in hepatocellular tumour growth and improved survival rates (Hsu, Jeng et al. 2000), and no apparent effect on gastric cancer (Nabais, Machado et al. 2002). With these inconsistent results with regard to beta-catenin effects on cancer cells it was difficult to predict what the expected finding was in regards to protein expression in the study cells. In a study by Stojadinovic et al (2005) the effect of beta-catenin on keratinocyte cells was investigated in vivo using mice models. Their study showed that an increased expression of beta catenin was associated with higher levels of chronicity, delayed healing and reduced keratinocyte motility (Stojadinovic, Brem et al. 2005). From our results we see that there was no obvious effect in terms of protein expression exerted by beta-catenin indicating perhaps that this is not one of the proteins interacting with EPLIN to regulate cell adhesion and motility. This perhaps indicates that EPLIN is not a part of the wnt pathway which is well recognised as a molecular contributor to the development of

many disease state including its role in cancer cells, and exerts its effect on cancer via an alternate pathway (Giles, van Es et al. 2003).

CHAPTER 7- GENERAL DISCUSSION

7.1 Discussion

It has been accepted that cell migration plays a key role in re-epithelialisation of clinical wounds during the proliferative phase of the wound healing process and it is suggested that EPLIN could be of paramount importance in contributing to this process.

EPLIN is a cytoskeletal molecule which is believed to be involved in regulating actin dynamics and hence influencing processes such as cell migration. The role of EPLIN in cancer cells and endothelial cells is beginning to be identified, with this protein being down-regulated in cancer cells and tissue compared to normal cells and tissues, reducing the aggressive nature of cancer cells and having an anti-angiogenic effect.

To date there is no information regarding EPLIN role in wound healing. Currently, the data obtained has implicated this protein to play a role in keratinocyte cell attachment and migration following wounding, showing decreased attachment and slowed migration when overexpressed. This is consistent with the clinical data that we have obtained showing an increased expression of this protein in chronic non healing wounds.

As noted previously EPLIN is a novel protein that is able to bind both Alpha- catenin and actin filaments (Song, Maul et al. 2002). EPLIN as discussed above belongs to the family of LIM domain proteins as it contains a centrally located LIM domain known to form two closely packed zinc-binding subdomains (Zheng and Zhao 2007). Additionally, EPLIN exhibits two functional acting binding sites, one on each side of the centrally located LIM domain, that give it the ability to cross-link and bundle actin

filaments (Maul, Song et al. 2003). *In vitro*, EPLIN stabilizes actin filaments by preventing their depolymerisation and blocks the formation of branched filaments by inhibiting actin nucleation by Arp2/3 (Maul, Song et al. 2003). Based on these properties, EPLIN is implicated in different actin-related processes, such as cell motility and migration, cytokinesis, and intercellular junction (Han, Kosako et al. 2007; Jiang, Martin et al. 2008). Subsequent *in vivo* studies confirmed the down regulation of EPLIN in a number of human epithelial cancer cells and tissues, suggesting that the loss of EPLIN could contribute to the transformed phenotype. This indicates that EPLIN may act as a tumour suppressor (Jiang, Martin et al. 2008).

The ability of EPLIN to suppress growth effectively may be linked to its location within the cells and a previous study has shown the inhibition of anchorage independent growth of EPLIN expressing NIH3T3 transformed with Cdc42 or the chimeric nuclear oncogene EWS/Fli-1, where EPLIN remains localised to actin filaments, but not in Ras transformed NIH3T3 cells, where EPLIN is distributed heterogeneously throughout the cytoplasm.

Overexpression of EPLINα in MDA-MB-231 breast cancer cells has previously been shown to reduce *in vitro* and *in vivo* growth, invasiveness and motility. In addition, overexpression of EPLINα also seems to inhibit the pro-invasive effect of the hepatocyte growth factor (HGF) cytokine on PC-3 cells (Sanders, Martin et al. 2011). The importance of HGF in cancer and its capacity to enhance various aggressive pro-tumorigenic traits is well established. The precise mechanism through which EPLINα interferes with this or whether EPLINα contributes directly to HGF signalling is currently unknown. Previous studies have suggested that the interaction of EPLIN

with cytoskeletal components such as actin filaments may act to inhibit cell movement and invasion. Thus, it may be that EPLIN's inhibitory role in these processes may indirectly oppose the pro-invasive effect of HGF though further work down this line is necessary to highlight potential mechanisms.

The previous reports in existing literature have shown that EPLIN is only weakly expressed at a protein and mRNA level in Arterial (Sanders, Ye et al. 2010) and venous endothelial cells (Maul and Chang 1999; Jiang, Martin et al. 2008).

Abe et al showed that EPLIN is mostly associated with the plasma membrane and more particularly at cell-cell junctions where it co-localizes with Alpha-catenin in agreement with a recent study on epithelial cells. Additionally, this work proved that EPLIN associates with alpha-catenin tethered to the E-cadherin adhesive complex. Thus, providing evidence that EPLIN participates in the linkage of the E-cadherin-alpha-catenin complex to the cortical actin fibres via alpha- catenin and also predicting that EPLIN functions similarly in endothelial cells and in epithelial cells (Abe and Takeichi 2008).

Overexpression of EPLIN in some transformed cells reverses their capacity to grow in an anchorage-independent way, indicating that EPLIN may function in growth control by associating with and regulating the actin cytoskeleton (Song, Maul et al. 2002). Frequent overlap between EPLIN and paxillin staining has been reported and suggests that EPLIN may also be present in the focal adhesion plaques. EPLIN has been linked to cell-cell adhesion through its interaction with cadherin-catenin complex binding to F-actin. It would be reasonable to suggest that this molecule may have

some relationship with the paxillin adhesion molecule and may also play a role in regulating cellular adhesion to extra cellular matrix, though further research is required to fully examine this potential.

The ECIS system is proving to be a suitable methodology for the high throughput screening of cellular migration and attachment with both 8 well and 96 well arrays currently commercially available. The data obtained can be used to aid in the analysis of the function of various proteins and their roles in these processes in cancer or other cell types. This data, when combined with additional information can be useful in providing insight into if certain proteins of interest could have metastasis suppressive properties or may promote the likelihood of cellular metastasis.

Within the short life of the ECIS system we have already seen advancements in the technology and software with production of 96 well arrays enhancing the potential of this system for screening purposes. Further advances or fine tuning of methodologies and software in the future may enhance the efficacy of this system further and may extend the potential of this system to detect other metastatic traits on a large scale basis.

The inhibitor data shows the effects of PLCy, ERK, NWASP and FAK on EPLIN overexpressing keratinocytes. From the data we can see NWASP had the most profound effect substantially increasing the migration rates of EPLIN expressing cells whilst at the same time reducing the migration rates in the control cells. This implies a solid link between the two proteins and EPLIN and further study is warranted to establish whether indeed they are linked, perhaps by some common as yet unexplored

pathway. The ERK inhibitor slowed the migration of the control cells whilst not really affecting the EPLIN study group. As ERK is known to phosphorylate EPLIN this could have accounted for this result as EPLIN is downstream of ERK.

With the well documented effects of EPLIN on human cancer and the similarities between the two disease processes share, this work represents yet another piece of evidence linking the two. As such it was initially hypothesized that EPLIN would have an inhibitory effect in wound healing when overexpressed because of the opposite effect observed in cancer cells. This proved to be the case with EPLIN both slowing migration and adhesion in keratinocyte cells. It was also noted to display a lower expression in healed vs non-healed wounds supporting that hypothesis in vivo as well.

The work, throughout this study is focused on highlighting potential mechanisms through which EPLIN may bring the above noted effect and improved understanding of cellular and subcellular physiology and may in turn lead to new or better forms of therapy for patients with acute, chronic, and surgical skin wounds.

In conclusion, EPLIN-α is a powerful regulator of the cellular motility of keratinocyte cells. Keratinocyte cells expressing EPLIN-α are less motile and grow slowly In *vivo*. Together with the clinical relevance as demonstrated in the present study, EPLIN-α is and important prognostic indicator as whether chronic wounds are like to heal and may be an important target when considering therapies. Further study is required to examine the molecular pathways involved in EPLIN-α mediated cell migration,

although touched upon in this thesis further study is needed so that these pathways may be understood in more depth.

7.2 Summary of findings

There has been little knowledge on the role of EPLIN (Eplithelial Protein Lost in Neoplasm) in the healing process of humans and with EPLIN's relatively recent discovery it is only now slowly coming to light where this protein falls in terms of classification, function and relevance in vivo.

It is also well recognised that non healing ulcers are capable of progressing to malignant transformation, first recognised by pioneering clinician Virchow in 1863 where he noted a number of examples of chronic irritation predisposing to tumour formation (Schafer and Werner 2008). Given this established relationship, it may be possible to infer how LIM proteins in general and EPLIN in particular, affect wound healing given their known role in cancer. And whether indeed the opposite phenomenon would be seen if an underexpression of a substance in cancer known to be an aggressive and proliferative process would in fact be the opposite scenario in relation to say a non healing non proliferating wound.

As well as the recognised influences of proteolytic enzymes and growth factors exerting effects on wound healing there has been a growing body of literature investigating the impact of genetic expression 'gene signatures' on wounds and their relevance in the hindrance of healing a wound (Cole, Tsou et al. 2001; Kirsner, Charles et al. 2008). The lack of understanding of these molecular mechanisms and

pathogenesis as discussed above predisposes to serious health problems leading to limb amputations and associated morbidity secondary to chronic wounds.

The predominant cell type implicated in this process by Tomic-Canic et al is the keratinocyte with its early response to injury and interactions with the extracellular matrix leading to migration and hyper-proliferation which is paralleled by changes in keratinocyte adhesion and cytoskeletal content (Stojadinovic, Brem et al. 2005). This transient healing response phenotypically resembles malignant transformation of keratinocytes during squamous cell carcinoma progression (Pedersen, Leethanakul et al. 2003). With genetic analyses pertaining to cancer having already been significantly developed the past years it is only logical that science is now trying to establish the genetic and molecular mechanisms associated with chronic wounds and how these profiles differ and change following injury and chronicity of a wound. Tomic-Canic et al have already begun to establish and identified hundreds of potential target genes which could contribute and are involved in the wounding process (Tomic-Canic and Brem 2004). With the advancements of microarray gene technology this high volume analysis of genes has now become a reality.

As such, it was hypothesised in this study that EPLIN would be a negative regulator of wound healing, and it would most likely have its effect via cellular migration. Inferring from the tissue cohort of patients with healing and non healing wound transcript was noted to be higher in non-healed wounds. Assuming its effect was at least partially due to keratinocyte migration, a stably transfected overexpression cell line was created for further investigation. EPLIN overexpression resulted in a marked reduction in keratinocyte migration and adhesion with no change in growth rates.

Thus EPLIN-α appears to be powerful regulator of the cellular motility of keratinocyte cells. Keratinocyte cells overexpressing EPLIN-α are less motile. I have shown that treatment with a number of inhibitors resulted in the alteration of this inhibitory effect on migration indicating that there are a number of pathways which could play a role in this process. Namely the ERK, FAK and NWASP pathways and potentially PLCγ. Together with the clinical relevance as demonstrated in the present study, EPLIN-α could have value as an important prognostic indicator as to whether chronic wounds are like to heal and may be an important target when considering gene therapies.

Focal Adhesion Kinase (FAK) is a focal adhesion-associated protein kinase involved in cellular adhesion and motility (Leevers and Marshall 1992). The study only demonstrated a weak association between EPLIN and FAK which is a shame considering the link established in Breast cancer. With regards to Paxillin, as demonstrated in a recent study by Sanders et al a relationship was discovered between Prostate cancer and endothelial EPLIN overexpressing cells in the context of increased staining for paxillin, particularly around the wound edges. Unfortunately the current study was unable to translate this finding to keratinocyte cells with if anything a slightly decreased staining observed, however the increased staining around the wound edge was observed particularly on ICC indicating that some weak relationship may indeed exist. Similarly very little relationship in the keratinocyte could be demonstrated between EPLIN and ERK despite the strong links in literature (Han, Kosako et al. 2007) (jiang 2010; Sanders 2012).

With the recent observation that NWASP inhibitor results in a substantial increase in HaCaT keratinocyte migration in the host laboratory (Jiang et al unpublished data), it was expected for there to be a strong colleration between the two proteins. The reduction in expression of NWASP is associated with a greater malignant potential (Baluk, Hashizume et al. 2005) in the context of breast cancer, via its role in cellular migration (Martin, Pereira et al. 2008). This was of particular relevance as our study protein has very similar properties. The host laboratory uncovered that it is overexpressed in patients with chronic ulcers, compared to patient with acute wounds with the inhibition of NWASP resulting in enhanced wound closure rates when applied topically and via the intraperitoneal route to mice (Jiang et al, unpublished data). I was unable to establish this strong link in terms of the increased protein expression of NWASP in overexpressing EPLIN keratinocytes however I did find a statistically significant increase in the migration of the EPLIN overexpressing cells when treated with the NWASP (Wiskostatin) inhibitor. This is consistent with findings in our host laboratory with other cell types (Jiang *et al,* unpublished data). To establish the exact nature of the link between these two proteins in the keratinocyte cell line further research is required namely immunopercipitation studies thus this was outwith the scope of the current work.

The results in this study show that in keratinocyte cells overexpressing the EPLIN protein resulted in no substantial effects on the expression of alpha, beta and gamma catenin, both as illustrated in the IFC assay and Western blot analysis. However a markedly increased florescence level of cells overexpressing the EPLIN plasmid was seen in comparison to control cells when stained for E-Cadherin. This same trend was also demonstrated in the Western blot analysis with a much stronger band of

expression when probed for E cadherin seen in the EPLIN overexpressing cells when compared with that of control cells.

These findings as supported by surrounding literature suggest that EPLIN is strongly associated with the cadherin-catenin complex; in particular E-Cadherin, further cementing the findings of the importance in establishing links between the cadherin-catenin complex with this most likely providing the mechanism through which EPLIN exerts its effect on cell migration and adhesion and subsequently, reducing both of those cellular functions.

7.3 Future work and study Implications

As mentioned throughout this thesis the similarities between wound healing and cancer are undeniable. Thus identifying EPLIN and the mechanism via which it exerts is effect is important to allow the scientific community to build on previous work and knowledge as to the key molecular pathways that predispose to poor wound healing. Of the vast body of work carried out pertaining to cancer and metastasis a large number of target and potentially significant molecules are identified but not taken further in the context of wound healing where they could be of great significance and potentially very clinically relevant. Of course the inverse is also true in that the information presented in this thesis could hold significance when related back to cancer in reverse and that the relationships between molecules identified in this thesis could be worth exploring in relation to the cancers where EPLIN has been found to be an important factor namely breast, prostate and oral, as well as others as yet unexplored.

With regards to tissue biopsies (as detailed in section 2.7) which are composed predominantly of keratinocytes, endothelial cells and fibroblasts (Normand and Karasek 1995), it would be interesting to see how the other cell types namely endothelial and fibroblast relate in their expression of EPLIN and its interactions with other proteins investigated in this thesis and this could certainly be scope for further study.

EPLIN has been linked to cell-cell adhesion through its interaction with cadherin-catenin complex binding to F-actin (Abe and Takeichi 2008). The data in this chapter 6 highlights EPLINs role in regards to the cadherin-catenin complex in keratinocytes. The results demonstrate a potential mechanism of action for EPLINs effects on cell migration, through its interaction with E-cadherin. This data presents an interesting avenue for future research down this potential mechanism warranting further study.

There is very exciting work directly following on from this study coupled with a number of other molecules identified in our host laboratory. The idea is through using the knowledge developed of which genes are responsible for wounds developing chronicity and entering a non healing state that a 'gene signature' is developed which will 'predict' which patients wounds will and which will not heal on presentation to the wound clinic based on which of these genes their wounds are over or under expressing. This would be a huge breakthrough as it would allow clinicians to focus their time and NHS resources on wounds that have the best chance of healing and thus avoiding unnecessary and costly therapies for wounds that are unlikely ever to heal. There is a patent for just such a novel gene signature molecular personalised medicine for patients with wounds, developed by the host laboratory awaiting clinical trials.

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