Ex Vivo and *In Vitro* Culture Models of Human Osteoblast-Like Cells and the Effects of TGFβ$_3$ and Serum-Free Culture

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Ich widme diese Arbeit meinen geliebten Eltern

Ursula und Bernhard Jähn.

Commander Pavel Chekov: 'Course heading, Captain?'

Captain James T. Kirk: 'Second star to the right and straight on 'til morning.'

from Star Trek VI: The Undiscovered Country (1991)

Thesis Summary

The loss of viable osteocytes in the elderly is a critical issue upon the development of osteoporosis. Previous work in our group suggested a positive osteocyte survival effect by $TGF\beta_3$ supplemented to a serum containing medium during long-term *ex vivo* culture of human cancellous bone explants. In this study, no beneficial effect by $TGF\beta_3$ addition to serum-free cultured *ex vivo* osteocytes was observed. Therefore, the effect of $TGF\beta_3$ on osteocyte survival was further investigated *in vitro*.

The most commonly used culture model for osteoblast-lineage cells is 2D/monolayer culture. However, monolayer culture only resembles the *in vivo* situation of osteoblast-lineage cells in bone to a limited extent, omitting the extensive 3D cell-to-cell and cell-to-matrix interconnectivity. In order find a more relevant culture model to be used for *in vitro* investigations of osteoblast biology; this study evaluated a variety of cell culture systems.

In all systems evaluated, the effect of $TGF\beta_3$ was strongly dependent upon the presence of serum. Only in the presence of serum did $TGF\beta_3$ increase osteocyte survival both *ex vivo* and *in vitro*. These investigations, also, led to the generation of different 3D *in vitro* culture models. Within a multilayer and pellet culture model for human primary osteoblasts, a reduction of initial proliferation in combination with a more rapid cell differentiation in comparison to monolayer culture was identified. Such 3D culture models are of great benefit compared to the existing *in vitro* osteoblast monolayer culture as they create more active/mature osteoblasts or potentially early osteocytes in a shorter time course.

This study showed, firstly $TGF\beta_3$ is an *in vitro* survival factor for osteocytes, if cultured in the presence of serum, and secondly the choice of 3D culture models for osteoblast-lineage cells should be intended more frequently to properly evaluate cellular reactions prior to conducting animal studies.

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Abbreviations

aBMD	Areal bone mineral density
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
αMEM	Alpha-MEM developed 1971 from MEM, differs as vitamin B12, vitamin C, non-essential amino acids, sodium pyruvate, lipoic acid, biotin and ribo- and deoxyribonucleotides are added. αMEM has been used to culture bone marrow cells.
ARS	Alizarin Red S staining
BCP	1-Bromo-3-chloropropane
BGJb	Modified BGJ medium (developed by Biggers, Gwatkin, and Judah) for supporting cultures of cartilaginous embryonic bone.
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSP	Bone sialoprotein
cAMP	Cyclic adenisine monophosphate
2D	Two-dimensional
3D	Three-dimensional
cDNA	Complementary DNA
CO_2	Carbon dioxide
CPC	Cetylpyridinium chloride monohydrate
CT	Computed tomography
DAB	3-3'-Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DEPC	Diethylpyrocarbonate
DPBS	Phosphate Buffered Saline supplemented with 2 mM NaCl
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked-immunosorbent-assay
ESA	European space agency
EtOH	Ethanol
FCS	Foetal calf serum
Gla	Y-carboxyglutamic acids
GLAST	Glutamate/aspartate transporter

HBSS	Hank's buffered salt solution
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidise
i.e.	For instance
INT	2-p-Iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride
ISS	International space station
ITS	Insulin-transferrin-selenium
LAP	latency-associated peptide
LTBP	latent-binding protein
MAPK	Mitogen-activated protein kinase
MH1/2	Mad-homolgy domain
MMP	Matrix metallo protease
NADH	Nicotine amid adenine dinucleotide
NBT	Nitroblue tetrazolium
NTx	N-terminal telopeptide of collagen I
OCN	Osteocalcin
OPN	Osteopontin
p	Probability value
PBE	Phosphate buffer containing 10.68 g/l NaH_2PO_4 $2H_2O$, 8.45 g/l Na_2HPO_4 $7H_2O$ and 3.36 g/l Disodium-EDTA in Millipore water (pH=6.5)
PBS	Phosphate-buffered saline
PBT	1x PBS containing 'Tween 20'
PI3-K	Phosphatidylinositol-3 kinase
PMMA	Poly methyl methacrylate
POD	Peroxidase
ProCI	C-terminal propeptide of collagen I
qPCR	Quantitative polymerase chain reaction
RANK/L	Receptor activator of nuclear factor κ B / ligand
rER	Rough endoplasmatic reticulum
RGD	Sequence of the 3 amino acids arginine, glycine, aspartic acid, common binding site for integrins
RNA	Ribonucleic acid
Runx2	Runt-related transcription factor-2 also named Cbfa1 (core binding factor alpha-1, or Osf2 (osteoblast-specific transcription factor-2)
SMAD proteins	Homologs of both the *drosophila* protein mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA
SF	Serum free (FCS replaced by BSA, lipids, and ITS)
TCNT	Tissue-culture-non-treated
TNSALP	Tissue-non-specific alkaline phosphatase (the ALP present in bone)
TGFβ	Transforming growth factor β

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Background and Aim

During the course of any research investigation, *in vitro* cell cultures are performed prior to *in vivo* investigations using animal models. This practice aims to reduce the number of questions addressed within *in vivo* studies and, therefore, to reduce the number of animals needed. Yet, in order to gain conclusive results from *in vitro* investigations, the culture model needs to be relevant to the aim of the biological question.

The most common method for *in vitro* studies using bone-derived cells is two-dimensional (2D) monolayer culture. This 'straightforward' method enables investigations on individual bone cell types in a controlled environment. While being most favourable for investigations on cellular responsiveness of osteoblast-lineage cells to material surfaces, 2D monolayer culture of osteoblastic cells resembles the *in vivo* situation of osteoblast-lineage cells only to a very limited extent. Osteoblasts, but also osteocytes, in 2D culture lack the three-dimensional (3D) engagement with neighbouring cells, as well as the 3D attachment of the cells to the extracellular matrix (ECM) that occur *in vivo*.

Several approaches can be carried out to potentially improve cell culture of osteoblastic cells. Mechanical load or fluid flow can be applied to *in vitro* cultured osteocytic cells and provide the required phenotypic stimulus. Biological stimulation of osteoblast-lineage cells by the addition of growth factors or hormones to culture medium can also aid the phenotypic behaviour *in vitro*. Especially the biological approach is frequently used to mediate *in vitro* differentiation or activation of primary osteoblast-lineage cells, which tend to loose their phenotypic characteristics during culture. A further development is the use of bone explant culture. *Ex vivo* bone explant culture systems can supplement the *in vitro* methods, as bone cells are cultured in their 3D biological extracellular environment and the application of mechanical load is possible. Yet, phenotypic maintenance and long-term cell viability are still considered

problematic during mature bone explant culture, and most likely prevented the more frequent use of this culture method.

This study aims to improve and supplement the commonly accepted methods of osteoblastic cell culture. Therefore, multiple culture models for osteoblast-lineage cells were evaluated. A 3D pellet culture model and a multilayer culture model for the human primary osteoblast *in vitro* culture were characterised for their potential to rapidly create a more mature osteoblast or osteocytic phenotype *in vitro* compared to monolayer culture. The *ex vivo* culture of mature human cancellous bone explants was optimised in order to increase long-term central cell survival.

During the optimisation of *ex vivo* culture of human cancellous bone explants, two further objectives of this study arose. One was to investigate a possible osteocyte survival effect caused by $TGF\beta_3$. This is of great clinical interest, as the loss of viable osteocytes in the elderly is a critical issue in the development of osteoporosis. Therefore, the cellular effects of $TGF\beta_3$ on cultured osteoblast-lineage cells, with major focus on osteocyte survival, were investigated in more detail *in vitro* within monolayer culture, multilayer culture, as well as within an osteoblast-osteocyte co-culture model. The second further objective was to set the effects by $TGF\beta_3$ in relation to the presence of serum proteins. Foetal calf serum (FCS) is frequently used to supplement the culture medium. However, it is a non-defined source of a variety of growth factors, hormones, lipids and vitamins that potentially affect the cellular response to a single factor application. Contrary effects caused by $TGF\beta_3$ were seen in this study depending upon culture in serum-containing or serum-free (SF) medium, highlighting the importance to conduct biological studies on single factor effects in a defined culture milieu.

Chapter 1: Introduction

1.1. What is bone?

Bone can be described very differently, depending on personal specialisation. In a material or physical view, it is likely described as a composite material that is made up of from an organic component, consisting of collagenous and non-collagenous proteins, and a mineral phase (Cowin 2001). From a biological view, bone is often described as a complex living tissue whose ECM is mineralised and maintained by four different cell types, namely osteoblasts, bone-lining cells, osteocytes and osteoclasts (Bilezikian *et al.* 2002).

Bone is a highly specialised connective tissue found in all higher vertebrates that serves as an endoskeleton to the body. Within the skeleton, bones fulfil a variety of functions. Some bones have developed to protect internal organs, as the skull protects the brain. While other bones sustain the weight of the body and aid locomotion by networking with ligaments, tendons, muscles and joints. Bone further serves as a storage place for growth factors such as members of the TGFβ superfamily which regulate the events of bone turnover (Bonewald 2002). Bone also stores a variety of inorganic ions as well as fatty acids, the later of which are stored within the fatty (yellow) bone marrow. The haematopoietic (red) bone marrow, which is found in the medullary cavity of long bones or in the interstices of cancellous bone, is responsible for another important function of bone – the process of haematopoiesis and the production of red blood cells (Bilezikian *et al.* 2002).

Even though the function of bones can be generalised, the specific purpose of an individual bone very much depends on its location within the body. The need that is to be fulfilled within the body is reflected by the macroscopic shape of the individual bone. Accordingly, there are four groups of bones existing in the body (Gray 1973; Marks & Odgren 2002) [Fig. 1.1A]. The group of long bones are found almost exclusively in the limbs. The tibia and the femur are representative members of this family of bones, which have the main function of serving the

body as a system of levers. The second group of bones is composed of short bones (i.e. carpus). This group of bones is predominantly found in regions where strength and compactness are needed. Therefore, the short bones are characterised by a thin cortex of cortical bone and an interior which is completely occupied by cancellous bone. Flat bones such as sternum and patella are the third group of bones. Bones of flat shape are found where muscle attachment and protection of internal organs are needed. The last group of bones contains the residual shapes. This mixed group is known as irregular bones (i.e. sacrum) (Gray 1973).

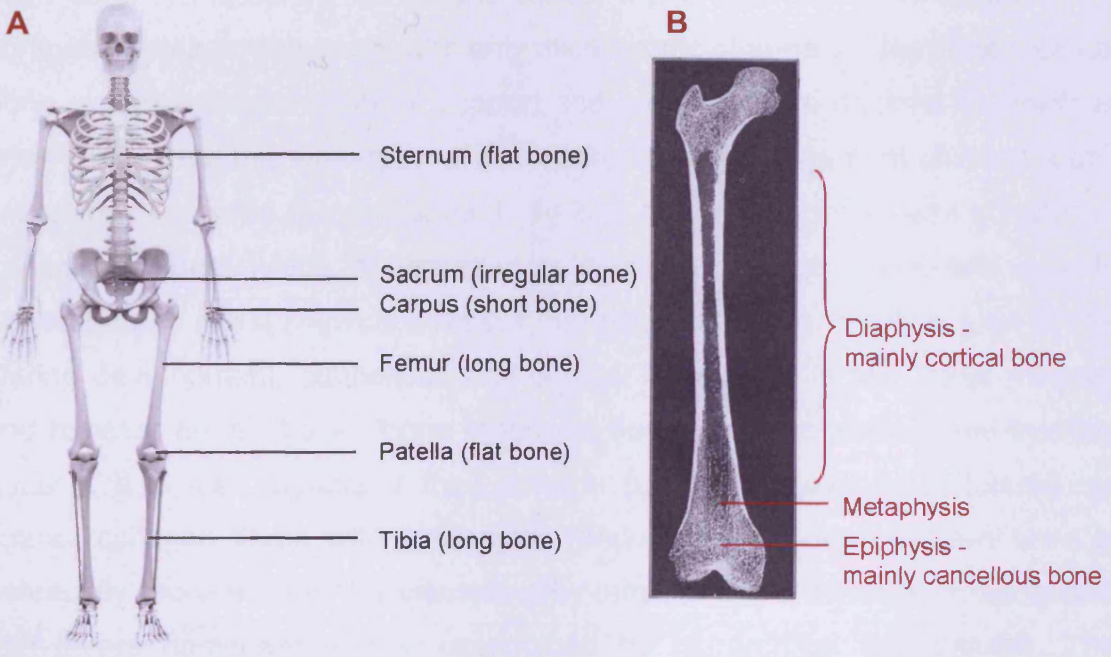

Fig. 1.1: A: Schematic image of a human skeleton adapted from: www.aofoundation.org. Radiographic micrograph of a human femoral head (B) adapted from 'Gray's Anatomy' (B) (Gray 1918).

Long bones serve as a classical model for the macroscopic structure of bone [Fig. 1.1B]. They consist of a central diaphysis (cylindrical shaft) with two rounded ends - the epiphyses. The Epiphyses are connected with the diaphysis via metaphyses. At a younger age, a cartilage composed growth plate separates epiphysis from metaphysis and allows the bone to grow. Some epiphyses are broader than the diaphysis and are used for joints, for example in the articular joints of tibia or femur. Therefore, they are covered with articular cartilage to withstand the load during movement. The outer surfaces of bones

that are not covered in cartilage, or where tendons and ligaments are present, are covered with a fibrous membrane called periosteum. The periosteum is a sheet of fibrous connective tissue with an inner layer of undifferentiated cells that has the potential to build bone during growth or fracture healing. The internal bone surface is called endosteum. It is a membrane of bone surface cells (Gray 1973; Jee 2001).

Two different structures of the macroscopic arrangement of bone matrix are present in mature bones [Fig. 1.2]. The classification of cortical/compact bone or cancellous/spongy bone is mainly based on porosity. Cortical bone is the main bone of the diaphyses of long bones. It is characterised by a dense and compact matrix arrangement with only microscopic channels. Therefore, cortical bone is found in areas, where support and protection are desired. Cancellous bone represents the main type of the macroscopic arrangement of bond found within the epiphyses of long bones. Only 20% of the total bone mass consists of cancellous bone, which is arranged as a lattice of large plates and rods to withstand and resist physical stresses that occur during locomotion (Jee 2001). During development, cancellous and cortical bone exist in two types - woven and lamellar bone. Woven bone is formed during embryogenesis and fracture healing. It is less organised than lamellar bone and consists of interwoven coarse collagen fibres with osteocytes randomly distributed. Woven bone is eventually replaced during remodelling by lamellar bone which is composed of unit layers (lamellae) and is determined by hierarchical organisation. The fundamental structure of this hearchical organisation in cortical bone is the osteon (Haversian system). Osteons consist of circular rings of lamellae, which surround a central canal of blood vessels and nerve fibers that supplies the bone. Outward from the canal embedded inside the lamellae are osteocytes within their lacunae. The canalicular system of the osteocytes connects the different cells with each other and the canal. The trabeculae of cancellous bone are structurally similar to osteons. In cancellous bone these fundamental structures are called hemiosteons (Jee 2001).

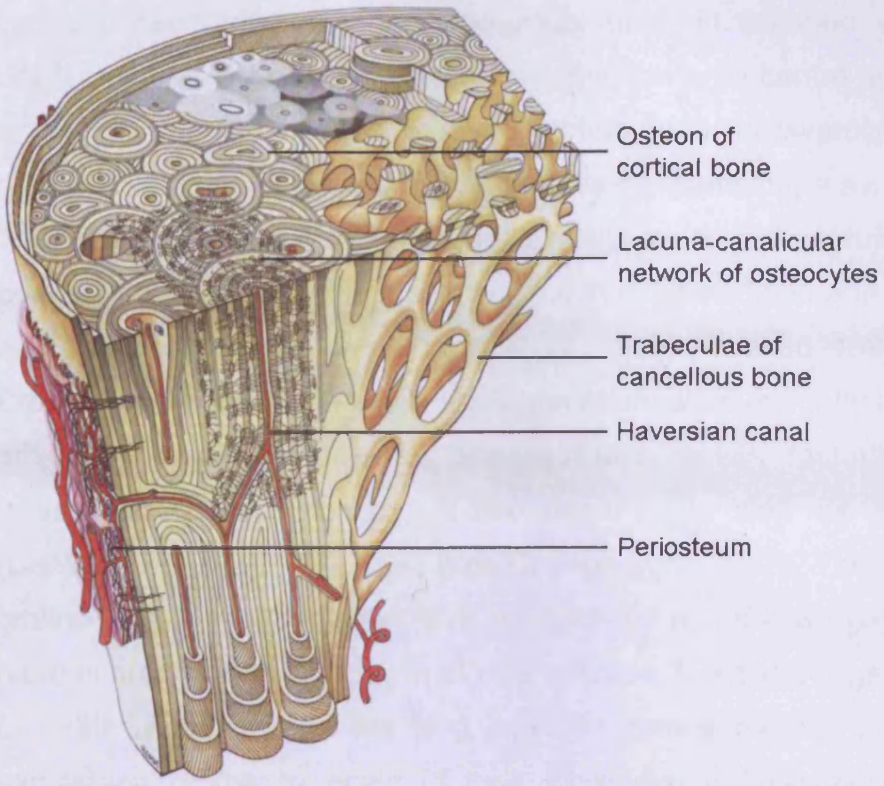

Fig. 1.2: Schematic image of a cross-section through a layer of cortical and cancellous bone. The presence of osteons in demonstrated with the haversian canals in their centre. Adapted from (Gray 1918).

1.1.1. The Extracellular Matrix of Bone

The extracellular matrix (ECM) of bone consists of 65-70 % mineral, 22-35 % organic material and less than 5 % water. The main component of the organic ECM is type I collagen. Within this study, mRNA expression as well as protein expression and degradation of type I collagen will be frequently investigated. Therefore, a main focus of this introduction section is set to describe type I collagen.

Type I collagen is fibril-forming protein, which is essential for bone quality and tensile strength (Rossert & de Crombrugghe 2002; Viguet-Carrin *et al.* 2006). However, it is not exclusively found within bone. Type I collagen is one of the most abundant proteins in vertebrates and is present in skin, tendons and ligaments. Each type I collagen molecule is composed of three polypeptide chains, one α_2 and two α_1 chains. Each α chain forms a left-handed helix. The three single α chains are organised as a right-handed triple helix, which is due

to the conserved amino acid sequence found in collagen: glycine-X-Y. The small amino acid glycine is positioned within the helix centre and the residues X and Y, which in most cases represent proline and hydroxyproline, are present at the helix surface. The helix forms in a self-assembling way, probably due to 'nucleated growth' as seen during crystallisation. It is stabilised by disulfide bonds as well as inter-chain connections from unmodified and deaminated lysyl and hydroxylysyl residues (Calvo *et al.* 1996; Prockop 1990; Rossert & de Crombrugghe 2002). The type I collagen expression is tightly regulated through different cytokines, homones, vitamins and growth factors influencing the transcriptional gene levels, mRNA stability, as well as protein synthesis (Centrella *et al.* 1994; Rossert & de Crombrugghe 2002). The two α polypeptide chains of the type I collagen fibril are encoded by different genes, the α_2 chain gene is found on the long arm of chromosome 7, while the gene coding for the α_1 chain is situated on the long arm of chromosome 17. In this study gene expression of the α_1 chain of type I collagen will be evaluated in several osteoblast culture models.

During protein synthesis, type I collagen is translated into an immature collagen molecule containing an N-terminal signal sequence as well as N- and C-terminal pro-peptides [Fig. 1.3]. The signal sequence will be cleaved when the polypeptide enters the rough endoplasmatic reticulum (rER). The pro-collagen I then undergoes a variety of post-transcriptional modifications, the most important one being hydroxylation by specific ascorbic acid dependent hydroxylases. Hydroxyproline within the collagen molecule is required for the stable triple helical conformation. After hydroxylation and glycosylation at the rER, the triple helix is formed, beginning with the formation of inter-chain disulfide bonds at the C-terminal pro-peptide. Hsp47 a molecular chaperone stabilises the newly formed triple helix, which is then transported to the Golgi apparatus, packed into vesicles and secreted into the extracellular space. During fibril maturation of type I collagen, specific endo-peptidases cleave the N-, and the C-terminal ends of the pro-collagen I, releasing pro-peptides into the circulation (Calvo *et al.* 1996). The C-terminal pro-peptide of type I collagen (ProCI) is measured within serum as one of the markers for *in vivo* bone

formation (Calvo *et al.* 1996; Eriksen *et al.* 1993; Robins & Brady 2002). The event of bone resorption by osteoclasts leads to further release of type I collagen fragments. These pyrodinoline-containing segments can differ in their range of size or their collagen domain origin (Eyre *et al.* 1984). The N-terminal telopeptide of collagen I (NTx) is a direct product of osteoclastic bone resorption and does not require further metabolism *in vivo* to be cleared by the kidney (Calvo *et al.* 1996). Both, the ProCI and NTx will be used within this study to determine bone turnover in an *ex vivo* bone explant culture situation.

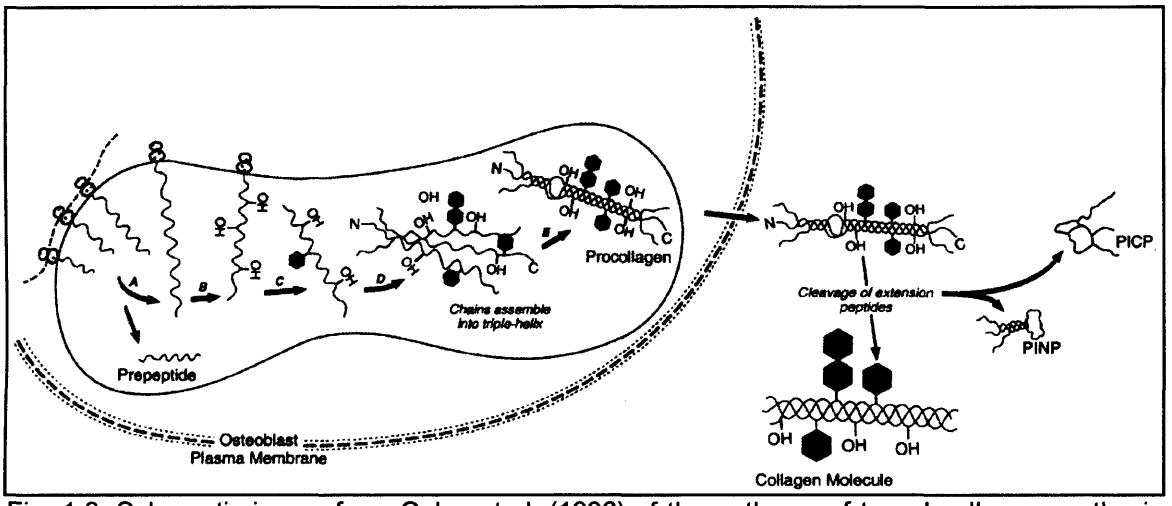

Fig. 1.3: Schematic image from Calvo et al. (1996) of the pathway of type I collagen synthesis (Calvo *et al.* 1996). A: Type I collagen polypeptides are synthesized as pre-pro-α-chains that contain. B: Many of the prolyl and lysyl residues are hydroxylated before translation is complete. C: The resulting hydroxylysyl residues are glycosylated. D: Three pro-α-chains are associated into a triple helical molecule that is stabilised by disulfide bonds. E: The helical folded molecule is transported to the Golgi apparatus for subsequent secretion. After secretion, the N- and C-terminal pro-peptides are cleaved by specific peptidases and enter the circulation.

Other proteins present within the bone matrix are so called 'non-collagenous' proteins. Their specific function is still not completely understood. One of the most abundant non-collagenous proteins is osteocalcin (OCN). This small 49 amino acid protein contains, in most species, an uncommon posttranscriptional modification of three γ-carboxyglutamic acids (Gla). These calcium-dependent residues lead to the formation of 'Gla'-helices which bind to the hydroxyapatite of the bone matrix (Calvo *et al.* 1996). The function of OCN is not fully defined. OCN is expressed during the late stage of osteoblast differentiation *in vitro* (Lian & Stein 1992). Moreover, in embryonic bone it is up-regulated with the early onset of hydroxyapatite crystal formation (Rossert & de Crombrugghe 2002).

However, it seems also to be involved in the recruitment of bone resorbing cells (Calvo *et al.* 1996). Therefore, OCN has been suggested to be one of the main regulators of bone turnover, and mineralisation (Ivaska *et al.* 2004). Recent findings also suggest a role for this calcium-binding protein within the hormonal regulation of glucose and fat metabolism (Ferron *et al.* 2008).

Osteopontin (OPN) is another abundant non-collagenous protein found in bone (Denhardt & Guo 1993). Due to its distribution in other tissues, like brain, kidney and uterus, OPN likely fulfils a variety of functions. Different splicing variants, as well as a change in the posttranscriptional modification of OPN, lead to modification of the protein properties and results in an alteration of the biological effects of OPN (Sodek *et al.* 2000). In bone, as the name osteopontin implies, this extracellular protein builds bridges between the ECM and bone cells through binding to integrin receptors and CD44 (Noda & Denhardt 2002; Oldberg *et al.* 1986). Potential functions of the protein range from the prevention of mineral crystal formation and growth, a chemo-attractant for macrophages, and activation of osteolastic bone resoption due to unloading (Ishijima *et al.* 2001).

Another major non-collagenous protein found in the ECM of bone is bone sialoprotein (BSP). After OPN, BSP was the second sialoprotein identified in bone. In contrast to OPN, BSP occurrence is more tightly bound to bone tissue, where its expression is associated osteoblast attachment through its RGD sequence (Wuttke *et al.* 2001), as well as with the onset of matrix mineralisation (Bianco *et al.* 1991; Bianco *et al.* 1993; Robey 2002).

The ECM of bone is characterised by the incorporation of a specific calcium phosphate mineral. The mineral part of the bone is an analogue of the naturally occurring hydroxyapatite which forms small crystals of the size of approximately 20 x 40 x 200 Å. The difference of bone hydroxyapatite to geologic apatite is the impurity of the bone mineral. Within the lattice and on the surface of the crystals in bone other substitutes such as CO_3^{2-}, $H_2PO_4^-$, Mg^{2+}, Na^+ are found (Boskey 2001). Mineralisation in bone is thought to be associated with type I collagen.

The long axis of the mineral crystals appears to be parallel to the collagen fibril axis. Therefore, type I collagen is thought to be the biological nucleator of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Cowin 2001). Crystal formations starts with collision of component lattice ions or clusters of these ions and remain together in the final orientation of the lattice. After the formation of a stable nucleus the crystal grows. The tissue-nonspecific alkaline phosphatise (TNSALP) is involved in the mineralisation process *in vivo* (Anderson *et al.* 2004). Osteoblasts express TNSALP, which is believed to play a key role in human skeletal mineralisation. ECM vesicles, where the early phase of mineralisation takes place and the first hydroxyapatite crystals arise, are rich in TNSALP (Ali *et al.* 1970; Anderson 1969).

Different biological functions for ALP in bone are proposed. The two main hypotheses are the hydrolysis of phosphoesters such as phosphoethanolamine or pyridoxal-5'-phosphate to provide a source of P_i for mineral deposition (Whyte *et al.* 1995). Moreover, ALP may hydrolyse pyrophosphate, an inhibitor of mineralisation *in vivo* (Whyte 1994). Unequivocal evidence in the case of hypophosphatasia condition demonstrates, however, the essential role of TNSALP for proper mineralisation of the skeleton (Anderson *et al.* 2004; Whyte 2002). Investigations on ALP activity in 2D and 3D culture models for osteoblast-like cells will be presented in this study.

1.1.2. Bone Cells

1.1.2.1. The Osteoblast-Lineage

The osteoblast is commonly known as the 'bone forming' cell and is characterised by its unique ability to form a type I collagen-rich ECM that finally mineralises. Osteoblasts were first named by Gegenbaur in 1864 (Gegenbaur 1864), and since then their function as major protein expressing cells in bone has been widely studied. In total four maturation stages of the osteoblast-lineage are recognised – preosteoblasts (immature), osteoblasts, osteocytes and bone lining cells (Aubin & Liu 1996). The processes of osteoblast differentiation starting with a progenitor cell can be subdivided in three stages –

proliferation, matrix maturation and matrix mineralisation (Aubin & Triffitt 2002; Lian & Stein 1992). Osteoblasts arise from mesenchymal progenitor cells which are located at the periosteum, the endosteum, and the marrow stroma (Aubin & Triffitt 2002; Burger *et al.* 1986). The differentiation process, but also osteoblast recruitment, as well as rate and duration of osteoblast activity are highly regulated by transcription factors, hormones and growth factors. The so called 'Master control gene' of osteoblast differentiation is Runx2 (Runt-related transcription factor-2; also named Cbfa1 for core binding factor alpha-1; or Osf2 for osteoblast-specific transcription factor-2) (Ducy *et al.* 1997; Schinke & Karsenty 2002). Runx2 is known to control osteoblast differentiation by subsequently activating the expression of osteoblast phenotype-specific genes such as osteocalcin and osterix (Ducy 2000; Nakashima *et al.* 2002). The importance of this 'Master control gene' is highlighted by a mice model from Komori *et al.* (1997), where targeted disruption of Runx2 resulted in early death just after birth due to the complete absence of endogenous ossification (Komori *et al.* 1997).

Osteoblasts possess a round – cuboidal cell shape, they contain large nuclei, as well as abundant endoplasmatic reticula and enlarged Golgi apparatus (Majeska 2001). Mature osteoblasts secrete mainly type I collagen, but also a number of non-collagenous proteins to form unmineralised ECM in the osteoid (Hauschka *et al.* 1989; Oldberg *et al.* 1986). This structure forms a seam between the osteoblasts and the mineralised matrix. Furthermore, osteoblasts regulate the assembly of the ECM and finally facilitate mineral deposition.

The mature differentiated stage of the osteoblast-lineage is apparent, when osteoblasts become surrounded by mineralised matrix. Osteoblasts then undergo morphological changes and are called osteocytes [Fig. 1.4]. These star-shaped cells are located in lacunae and are the most abundant cell type found in bone (Parfitt 1977). They are known to show less remodelling activity than osteoblasts and morphologically have lost many of their cytoplasmic organelles (Aubin & Liu 1996). Yet recently the interest in the functionality of this cell type has been increased. The proposed main function of osteocytes is

to sense mechanical strain distribution and amount that is applied to the bone (Klein-Nulend *et al.* 1995; Knothe Tate 2003). Therefore, they are best placed in the load-bearing ECM of bone (Lanyon 1993). The transfer of the strain information between osteocytes is realised through their long slender-like processes - the most dramatic morphological feature of osteocytes - which are placed within small fluid-filled canaliculi. The death of osteocytes caused due to aging as been widely recognised (Dunstan *et al.* 1993; Frost 1960). The maintenance of osteocyte viability will, therefore, be a major interest in this study.

Other cells derived from the osteoblastic-lineage are bone lining cells. They are thought to be either resting osteoblasts or pre-osteoblasts, and cover almost all surfaces in adult bone to build a connective tissue barrier. Bone lining cells are active cells and are known to participate in bone resorption, as they digest surface osteoid, and in bone formation, as they are involved homeostatic processes and are thought to be part of the strain sensing network (Majeska 2001). Additionally, lining cells may be one of the major sources of osteoblasts or osteoblast-like cells under the influence of the right stimulus. This theory was proposed by Dobnig *et al.* (1995), where intermittent treatment of adult rats with parathormone (PTH) led to a reactivation of bone lining cells to bone forming osteoblasts and to increased bone formation (Dobnig & Turner 1995).

1.1.2.2. The Osteoclast

The cells that resorb the ECM of bone are called osteoclasts. They arise from the haematopoietic cell line due to fusion of individual progenitor cells (Takahashi *et al.* 2002). The co-stimulating molecule RANKL (RANK ligand), which is expressed by osteoblasts, is the most commonly known cellular activation pathway of osteoclast-precursors and the osteoclastogenic cascade of transcription factors (Matsuo & Irie 2008; Takahashi *et al.* 2002). Osteoclasts are very rare in bone, only 2-3 cells per μm^3 bone can be found (Meunier *et al.* 1980). The active form of these multinucleated giant cells is present in specialised cavities on the bone surface, known as Howship's lacunae.

Osteoclasts show high polarisation when attached to the bone surface. The most characteristic feature in this state is the ruffled border, which consist of finger-like cytoplasmic projections (Roodman 1996) and is turned towards the bone surface. The osteoclast seals the cavity around its ruffled border and then starts secrete protons and a variety of proteolytic enzymes i.e. collagenases, gelatinases into the cavity, to carry out the organic breakdown of the bone ECM (Hill 1998).

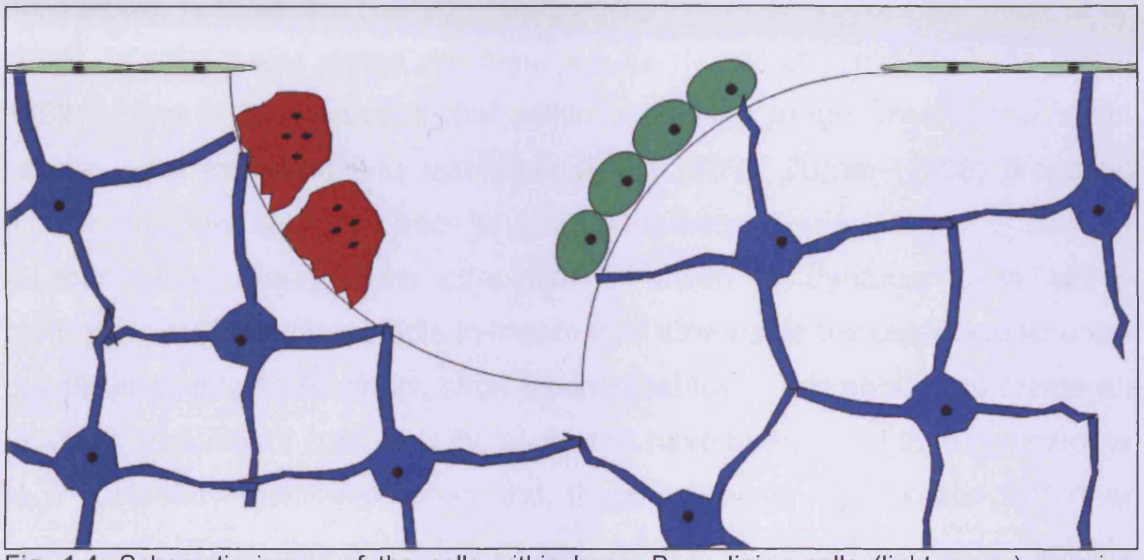

Fig. 1.4: Schematic image of the cells within bone. Bone lining cells (light green) covering quiescent surfaces, while osteoblasts (green) lay down new ECM, following bone resorption by osteoclasts (red). Osteocytes (blue) lay within the ECM of bone and demonstrate high interconnectivity.

1.2. Bone Adaptation to Mechanical Stimulation

In load-bearing bones, mechanical loading plays an important role in the control of bone architecture and mass. This is not the case for nonload-bearing bones (i.e. in the skull) as their architecture is believed to be directly influenced by the genetic code (Cowin 2001; Lanyon 1987). Whereas with load-bearing bones only their general morphology and development are directly specified by the genome, the particular features to withstand functional loading are the result of an adaptive mechanism (Lanyon 1987; Lozupone *et al.* 1996; Rawlinson *et al.* 1995). In 1892 it was stated that there is a functional adaptation in bone (Wolff 1892). Frost (1988) believed that within a specific range, mechanical strain affects bone formation and resorption (Frost 1988). Turner (1998) proposed three commonly accepted rules of bone adaptation to mechanical stimulation (Turner 1998). Firstly, bone adaptation is driven by dynamic loads, which contrary to static loads are able to induce fluid flow inside the osteocyte lacunar-canalicular system. Secondly, short mechanical loads are enough to create an anabolic stimulus for cells. Thirdly, bone cells have been found to accommodate to a customary mechanical load and, therefore, need re-activation to further build bone. Within this study the *ex vivo* culture of cancellous bone explants with a daily application of a dynamic compressive loading regime will be investigated, and the impact of an unconventional loading of high frequency low amplitude will be tested within a near orbit space mission experiment.

1.2.1. Hypothesis of Mechano-Sensing in Bone

To react to mechanical loading, one population of bone cells needs to be sensitive to at least one of the physical properties of strain: strain distribution, rate of change or magnitudes. All cells within bone are known to react to mechanical stimulation but osteocytes are thought to have the best characteristics to fulfil the strain sensing function (Lanyon 1987; Noble *et al.* 2003). Due to their development, osteocytes are placed within the bone matrix and are connected with each other and the bone remodelling cells via long slender-like cell processes.

Gap junctions at the end of these processes enable the osteocytes to communicate. Gap junctions are specialised cell-cell-contact points which are formed by members of the connexin protein family (Cherian *et al.* 2003; Jiang *et al.* 2007; Lanyon 1987). Six of these trans-membrane proteins are joined across the extracellular gap between two adjacent cells to form a channel structure allowing the passage of small metabolites, ions and intracellular signalling molecules below a size of 1 kDa. But what causes these conducted intracellular signals due to mechanical stimulation? In theory there are at least three different options to act on bone cells – hydrostatic pressure, direct cell strain and fluid flow induced shear stress (Cherian *et al.* 2003). The most commonly accepted theory is load-induced fluid flow (Cherian *et al.* 2003; Klein-Nulend *et al.* 1995; Knothe Tate 2003). Interstitial fluid is squeezed through the porous ECM and the lacunar-canaliculi system in response to bone deformations by physiological loading (Burger & Klein-Nulend 1999). As the osteocytes lie in the fluid filled lacunar-canalicular system, shear stresses can be caused by fluid flow and then act directly on the outer surfaces of the cells (Weinbaum *et al.* 1994).

1.2.2. Cellular Mechano-Transduction

The cellular structure that reacts to the load-induced fluid flow is of major interest to the research community and several candidates have been shown to be load or fluid flow responsive in osteoblasts and osteocytes. Vatsa *et al.* (2006) demonstrated that both cell body and cell processes of individual MC3T3-E1 and MLO-Y4 cells respond to mechanical stimulation by optical tweezers in an up-regulation of nitric oxide (NO) production (Vatsa *et al.* 2006). Yet, other candidates at the cellular surface have been proposed. Recent ultra-structural studies by You *et al.* (2004) revealed the existence of transverse tethering filaments attaching the osteocyte processes to the canalicular wall (You *et al.* 2004). Due to their connection to the cytoskeleton of the cells these filaments could be responsible for the sensing of fluid flow. However the tissue-level strains in whole bone caused by locomotion are typically an order of

magnitude smaller than needed for intracellular biochemical responses. Han and others (2004) proposed a finite element model by which the required strain amplification needed for bone adaptation can be easily explained (Han *et al.* 2004).

Another candidate to sense fluid flow within bone, are cilia, a cell organelle composed of microtubules, which are attached to the cell membrane of eukaryotic cells. Cilia are known to possess a high concentration of receptors for signalling molecules in the ciliary membrane and are known to be inducible by fluid flow in kidney cells (Malone *et al.* 2007). Both filaments and cilia are tightly linked to the cytoskeleton of the cell which seems transmit the strain-induced information possibly actin-dependent. A further tight link to the cytoskeleton is permitted via integrins. Integrins are major cell attachment receptors found in a variety of mammalian cells. Structurally, these heterodimeric transmembrane proteins are built out of non-covalently linked α and β polypeptide chains (Horton *et al.* 2002). Integrins are linked to the cytoskeleton via interaction of the β subunit with actin-binding proteins. Their role in bone mechano-transduction has been proposed by several authors (Aarden *et al.* 1996; Horton *et al.* 2002; Plotkin *et al.* 2005). Further candidates to directly react to an applied mechanical stimulus are mechano-sensitive ion channels. One of the early responses of osteoblasts to *in vitro* applied fluid shear stresses is an intracellular increase in Ca^{2+} (Hung *et al.* 1995). Cheng *et al.* (2000) demonstrated that *in vitro* cultured MC3T3-E1 cells react to fluid shear stresses with increased intracellular calcium levels, which was at least partly due to mechano-sensitive ion channels (Chen *et al.* 2000).

Several signalling pathways have been proposed to be involved in the mechano-transduction of osteoblasts and, or more relevantly, of osteocytes. Their potential importance *in vivo*, as well as interplay is still to be defined. The work of Klein-Nulend *et al.* (1995) demonstrated that pulsating fluid flow directly induces prostaglandin E_2 (PGE_2) release from cultured primary foetal chicken osteocytes *in vitro* (Ajubi *et al.* 1999; Klein-Nulend *et al.* 1995). Prostaglandins belong to the prostanoid subclass of fatty acid derivatives (Simmons *et al.*

2004). They are known to act as hormones in an auto- or paracrine mechanism, modulating the action of secondary messenger systems. Ajubi and coworkers (1999) proposed a possible signal transduction pathway for the mechano-transduction in osteocytes [Fig. 1.5]. Starting with the fluid flow induced cellular deformation of osteocytes, possibly sensed by integrins, actin-dependent mechano-sensitive calcium channels are opened and the intracellular calcium level increases. The increase in calcium level activates phospholipase C (PLC) causing the hydrolysis of the membrane lipid phosphatidylinositol to produce the secondary messenger molecules diacylglycerol (DAG) and D-myo-inositol 1,4,5-triphosphate (IP_3). IP_3 also activates intracellular calcium release from internal stores such as the endothelial reticulum (ER). Calcium and DAG can activate protein kinase C (PKC) which can in turn enhance phospholipase A_2 (PLA_2) activity. PLA_2 and DAG-lipase induce an increased production of arachidonate (AA) - a prostaglandin substrate.

Nitric oxide (NO) is another secondary messenger seems to be involved in the production of prostaglandins. Intracellular NO arises from the catalytic action of NO synthases, some of which are calcium-dependent (Salvemini *et al.* 1993). Therefore, the enhanced cellular calcium level after physiological loading is predicted to increase NO production. NO mediates the majority of its effects by interacting with iron-haeme containing enzymes. One of these is COX. The catalytic action of COX uses AA to produce prostaglandins. The increased production of AA by PLA_2 and DAG-lipase activity and the possible NO-induced activation of COX result in an intracellular increase in PGE_2 production (Ajubi *et al.* 1999; Salvemini *et al.* 1993). PGE_2 is known to have anabolic effects on cortical and cancellous bone *in vivo*; inducing a variety of effects such as stimulation and recruitment of cells of the osteoblast cell lineage as well as modulation of gap junction function (Bakker *et al.* 2003; Cherian *et al.* 2003; Machwate *et al.* 2001).

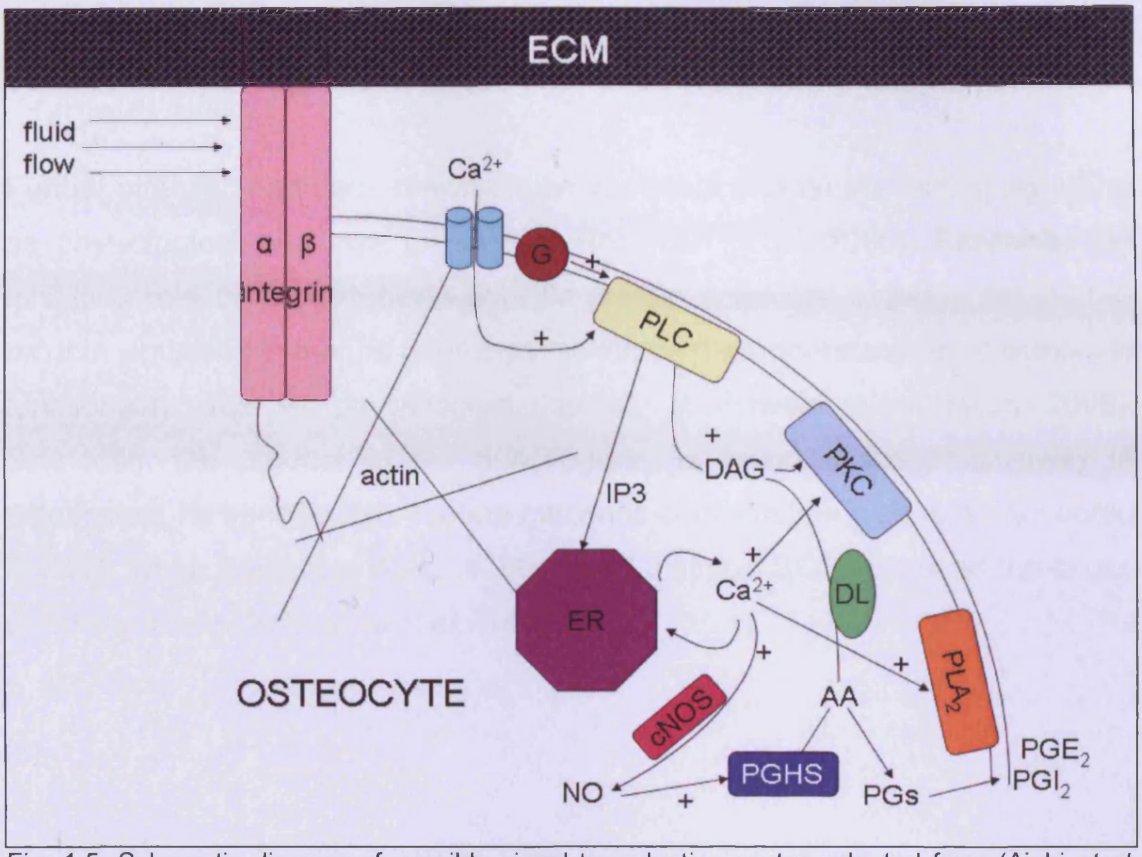

Fig. 1.5: Schematic diagram of possible signal transduction routes adapted from (Ajubi *et al.* 1999) involved in the mechanical activation of osteocytes. The osteocyte is attached via integrins to the surrounding ECM. Fluid shear stresses, resulting from load-induced fluid flow (parallel arrows), activate cellular components either directly (membrane perturbation) or through force transmission via integrin-cytoskeletal structures, leading eventually to enhanced prostaglandin synthesis. PLA2: cytosolic phospholipase A2; PLC: phospholipase C; ER: endoplasmic reticulum; DAG: diacylglycerol; DL: diacylglycerollipase; PKC: protein kinase C; G: G-protein; IP3: inositol trisphosphate; cNOS: constitutive nitric oxide synthase; NO: nitric oxide; PGHS: prostaglandin H synthase; AA: arachidonic acid; PGs: prostaglandins; PGE2 and PGI2: prostaglandin E2 and I2.

Other signalling molecules and pathways have been considered to be responsible for the transmission of the mechanical signal by osteocytes. Mason *et al.* (1997) was the first to describe the importance of glutamate/aspartate transporters (GLAST) in bone (Mason *et al.* 1997). The group demonstrated that GLAST-1, which is expressed in the plasma membrane of osteocytes, is trans-located to the cell membrane in response to extracellular glutamate concentrations (Huggett *et al.* 2002). The proposed mechanism of action is due to the function of GLAST-1 as co-transporter for 3 Na^+ into the cell and 1 K^+ out of the cell for each transported glutamate molecule, leading to the formation of electrochemical gradient. The down-stream signalling is suggested as mitogen-

activated protein kinase (MAPK) pathway, which involves the subsequent activation of specific protein kinases (Mason 2004).

Further attention had been drawn on the importance of Wnt/β-catenin signalling as physiological response to loading (Robinson *et al.* 2006). Especially the inhibiting role of the osteocyte specific protein sclerostin towards the Wnt/β-catenin signalling in mature osteoctes increased the understanding of osteocyte functionality due to physiological loading (Bonewald & Johnson 2008). Sclerostin, the product of the SOST gene, is found almost exclusively in osteocytes. Its specific role in bone mechano-transduction was first discovered in 2001, when Balemans W. *et al.* described mutated SOST gene as the cause of sclerosteosis (Balemans *et al.* 2001).

1.3. TGFβ – a Family of Growth Factors Influencing Bone Locally

Bone remodelling processes are not only influenced by mechanical stimuli. A wide variety of hormones i.e. parathormone, oestrogen influence the balance of bone turnover processes. Local regulation of bone turnover is achieved by growth factors i.e. the transforming growth factor-βs (TGFβs). The superfamily of TGFβ cytokines includes members of the activins and inhibins, the Müllerian inhibitory substance, growth differentiation factors, bone morphogenetic proteins (BMPs) and the TGFβs themselves (Bonewald 2002). The effects of $TGF\beta_3$ on cultured osteoblast-lineage cells will be investigated in this study. A major interest is to evaluate the potential of the growth factor, to inhibit osteocyte cell death (see Chapters 4 and 5). Therefore, this introduction section will describe in detail the members of the TGFβ family and well published cellular effects.

The TGFβ isoforms are known to be potent regulators of cell growth, differentiation, matrix production and apoptosis in a range of tissues and cell types (Bonewald 2002; Centrella *et al.* 1994). The most abundant TGFβ isoforms in bone are the homodimers, namely $TGF\beta_1$, $TGF\beta_2$ and $TGF\beta_3$ (Centrella *et al.* 1994; Khalil 1999). The biological effects of TGFβs are thought to be comparable, but the expression patterns differ throughout developmental stages. All three isoforms can be detected at sites of endochondral and intramembranous bone formation in human. The isoforms are encoded by different genes, $TGF\beta_1$ (NC_000019.9) is lying on chromosome 19, $TGF\beta_2$ (NC_000001.10) is found on chromosome 1 and $TGF\beta_3$ (NC_000014.8) on chromosome 14 (Pubmed 2010). Moreover, the promoters of the three genes consist of different elements. For instance the promoter of $TGF\beta_1$ only contains an activator protein type 1 (AP-1) enhancer element, while the promoter regions of $TGF\beta_2$ and $TGF\beta_3$ are more complex and contain a TATA box, a cAMP responsive element and AP-2, implicating that the regulation differs between isofoms (Centrella *et al.* 1994; Roberts *et al.* 1991).

TGFβ proteins are unique among the superfamily of TGFβ cytokines, as they are synthesised as latent precursor proteins that consist of an N-terminal latency-associated peptide (LAP) region and a C-terminal mature TGFβ (Khalil 1999). Therefore, these cytokines offer an additional regulation level, which is permitted through the latency. Bone cells, however, can produce two forms of latent TGFβ (Bonewald & Dallas 1994). While the first form ('small latent complex') only contains the LAP region within the precursor protein, the second form ('large latent complex') contains an additionally covalently bound protein, called the latent-binding protein (LTBP) (Bonewald 2002; Bonewald & Dallas 1994). The LTBP protein is not required for latency and its specific function in the regulation of TGFβ functionality is, therefore, controversially discussed. It appears that the 'small latent complex' is the circulating form, which makes TGFβ more readily available for cells. Moreover, TGFβ in its latent form showed an extended half-life in an *in vivo* rat model compared to its active form (Wakefield *et al.* 1990). Within bone, TGFβ is stored within the ECM. The LTBP targets the 'small latent complex' to the matrix for storage (Taipale *et al.* 1994). This mechanism of storage of an inactive protein is suggested to have developed to allow a quicker supply of TGFβ compared to activation of transcription and translation of the gene. Active TGFβ proteins need to be separated from LTBP, if present, and LAP to permit biological activity of the cytokine. Several ways to activate latent TGFβ have been suggested ranging from extreme pH values, to heat, chaotropic agents and γ-irraditation (Brown *et al.* 1990; Centrella *et al.* 1994; Ehrhart *et al.* 1997). Most likely latent TGFβ is released from the ECM due to an acidic microenvironment as produced within the sealing zone of bone resorbing osteoclasts (Baron *et al.* 1985). Yet, other candidates for 'natural' *in vivo* bone activation of latent TGFβ, are the proteolytic cleavage by plasmin (Dallas *et al.* 1995; Nunes *et al.* 1997), the conformational change of LAP due to thrombospondin-1 interaction (Crawford *et al.* 1998), as well as the proteolytic cleavage by matrix metallo proteinases (MMPs) (Karsdal *et al.* 2002).

1.3.1. Signal Transduction

The active TGFβ$_{1/2/3}$ monomeric peptide is characterised by a length of 112-114 amino acids. Nine cysteine residues are conserved among the growth factor family, 8 residues enable the formation of a cysteine-knot structure via disulphide bonds, with the 9th cysteine available to form a bond between two monomers (Lin *et al.* 2006). The monomer secondary structure is characteristically compared to a hand. Two pairs of anti-parallel β-strands build fingers that extend from an α-helix that builds the wrist region. In general a dimer is formed by hydrophobic interactions in combination with the inter-subunit disulphide bonding. The 25kD active homodimer of TGFβ$_{1/2/3}$ binds with high affinity to type II receptors (TGFβR-II) and type I receptors (TGFβR-I/ALK5) (Heldin *et al.* 1997), glycoproteins of 70kD and 55kD respectively, which interact upon ligand binding. It has been suggested that TGFβR-III (betaglycans) make TGFβ more accessible for its binding to TGFβR-II. Yet, only receptor I and II contain intrinsic serine/threonine kinase activity, which is important for the downstream signal transduction (Centrella *et al.* 1994; Heldin *et al.* 1997).

The first step is the interaction of active TGFβ dimer with two TGFβR-II monomers, prior to auto-phosphorylation of type II receptor and the formation of a TGFβR-I dimer complex. Therefore, the TGFβ$_{1/2/3}$ ligands posses two hydrophobic areas, the concave type I receptor binding interface (the knucle of the hand structure) and the convex type II receptor binding interface (fingertip of the hand structure) (Lin *et al.* 2006). Type I receptor is phosphorylated by the intrinsic kinase of type II receptor on its conserved GS-domain (named after the 'TTSGSGSG' amino acid sequence found at the receptor core), which in turn activates its own kinase activity (Cox 1995; Heldin *et al.* 1997). The downstream signalling mechanism is complex and more than one pathway is used. The most commonly studied, and most likely important mechanism, starts with the activation of the receptor-associated SMADs Smad2 and 3 by the kinase activity of TGFβR-I [Fig. 1.6] (Inman 2005). SMADs contain of the Mad-homology domains MH1 and MH2 connected via a linker domain. The phosphorylated Smad molecules get translocated into the nucleus. In

mammalian cells, Smad4 seems to be involved in this pathway as it is believed to form complexes with Smad2 and 3 after their activation (Heldin *et al.* 1997). This heteromeric complex (dimer or trimer) of Smad2, 3 and 4 can induce the transcription of different target genes most likely due to conformational opening of the MH1 domain in relation to the MH2 domain, however, transcriptional activation function of the linker region of Smad 4 and 3 has been recently discovered (de Caestecker *et al.* 2000; Wang *et al.* 2005).

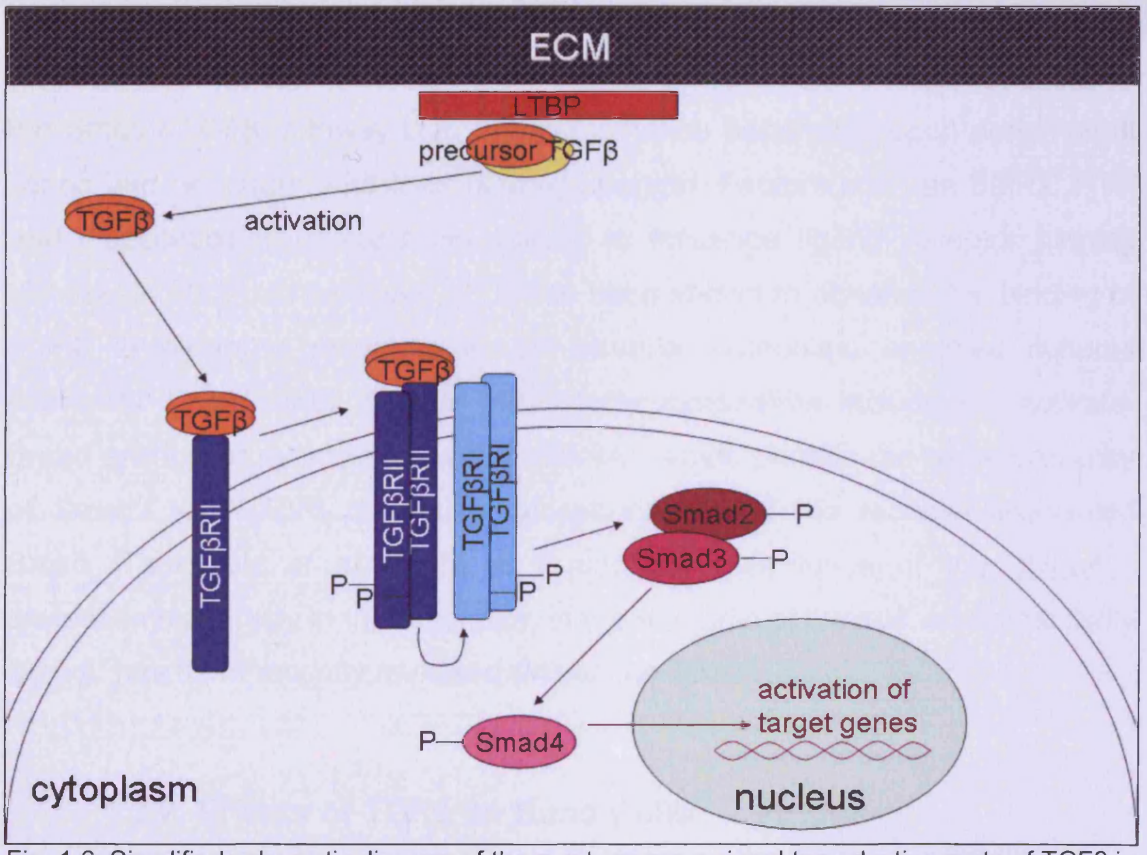

Fig. 1.6: Simplified schematic diagram of the most common signal transduction route of TGFβ in bone. Latent TGFβ is produced by osteoblasts and can be stored in the ECM. Due to osteoclast activity, plasmin activity or others, TGFβ is activated and can bind to TGFβRII. This binding leads to complexing of TGFβRII and TGFβRI, whereby TGFβRI is phosphorylated by RII due to serine/threonin kinase activity. Downstream signalling is achieved through activation of receptor-associated SMAD molecules (Smad2, 3), which then activate the coSMAD Smad4. Smad 4 is translocated into the nucleus and activates the transcription of target genes.

Non-SMAD TGFβ signals have been elucidated in several studies and their importance seems to lie partly in the differential cellular effects caused by TGFβ (Bonewald 2002; Centrella *et al.* 1994; Moustakas & Heldin 2005). Some of these signalling cascades following binding of TGFβ to its receptors, include the MAP (Mitogen-activated protein) kinase pathway (Hofmann *et al.* 2003), or

phophatidylinositol 3-kinase (PI3-K) (Dufour *et al.* 2008). However, G-protein mediated and ras signalling pathways have, also been connected to TGFβ signalling (Bonewald 2002). The potential involvement of the later pathways in the Smad / TGFβ pathway and their specific interplay in permitting the cellular effects by TGFβ is complex and is not completely unravelled for each cell type and differentiation stage (Guo & Wang 2009; Moustakas & Heldin 2005). Other regulators of the Smad / TGFβ pathway are the TGFβ isoforms (at least 1 and 2) themselves, which have been shown to activate and cross-activate their own and other TGFβ isoform expression in different *in vitro* cell culture models (Bascom *et al.* 1989; Kim *et al.* 1990). The effectiveness and responsiveness of the Smad / TGFβ pathway is in the first instance dependent upon presence of ligand and receptors, and their binding strength. Factors such as BMP2, PTH and glucocoticoids demonstrate activity to influence ligand receptor binding (Bonewald 2002). In particular, PTH has been shown to enhance the binding of TGFβ to all three receptors in rat calvarial osteoblast enriched cultures (Centrella *et al.* 1988). Further intracellular modulators include the activator Smad anchor for receptor activation (SARA), which permits the close proximity of Smad2 to TGFβRI to enable phosphorylation of the receptor-associated Smad (Tsukazaki *et al.* 1998). But also the importance of the ubiquitin-proteasome pathway in the regulation of the amounts of Smad4, and most likely Smad2 has been recently reviewed (Miyazono 2000).

1.3.2. Effects of TGFβ on Bone Cells

The best studied among the TGFβs is $TGF\beta_1$ which is used as a prototype for the investigations of the biological effects of the growth factor (Centrella *et al.* 1994; Wakefield *et al.* 1990). $TGF\beta_1$ was originally isolated 1983 from human platelets (Assoian *et al.* 1983). *In vivo* studies using local administrations of TGFβ ($TGF\beta_1$) in close proximity to bone demonstrated new bone formation (Mackie & Trechsel 1990; Marcelli *et al.* 1990; Noda & Camilliere 1989). Noda and colleges (1998) were the first to show periosteal woven bone formation on the parietal bone of neonatal rats induced by 12 daily local injections of $TGF\beta_1$. Moreover, in 1991 Beck *et al.* demonstrated that repeated doses of $TGF\beta_1$ were

capable to induce the closure of a non-healing skull created in an adult rat model (Beck *et al.* 1991). Transgenic mice models were created to investigate the involvement of the three main TGFβ isoforms in bone development. The immuno deficient $TGF\beta_1$ null mice demonstrated defects in mineralisation of both bone and teeth, as well as a size-reduction of 50-80% compared to their non-knockout littermates (D'Souza *et al.* 1998), while depletion of $TGF\beta_2$ and $TGF\beta_3$ in developing mice cause a range of skull defects with loss of $TGF\beta_2$ leading to cranial defects (Sanford *et al.* 1997) and loss of $TGF\beta_3$ resulting in failure of the palatal shelves to fuse (Proetzel *et al.* 1995).

To further evaluate the precise cellular effects of the TGFβs on bone cells *in vitro* studies were performed. It has been elucidated by Pfeilschifter *et al.* (1990) that osteoblast-like ROS 17/2.8 cells, as well as rat calvarial cells, are chemotactically attracted by $TGF\beta_1$ (Pfeilschifter *et al.* 1990). The chemotactic epitope in $TGF\beta_1$ was discovered 5 years later (Postlethwaite & Seyer 1995). Therefore, it was suggested that TGFβ plays an important role very early during recruitment of pre-osteoblasts or progenitor cells. Most likely, latent-TGFβ stored in the ECM is released by osteoclast activity and then permits its chemotactic activity to couple bone formation to bone resorption. Further investigations on osteoblasts or osteoblast-like cells led to rather controversial results in their aim to assess the underlying biochemistry of the *in vivo* effects. The effects on osteoblast replication for instance seem to be dependent on the differential state of the cultured cells. In osteoblast-enriched cultures from foetal tissue TGFβ may act as a mitogen, but using osteosarcoma cells, which represent a more differentiated osteoblast phenotype, TGFβ inhibits DNA synthesis (Centrella *et al.* 1994). The main effect of TGFβ is to induce osteoblasts to differentiate and produce extracellular matrix. However, even though TGFβ increases osteoid production, it inhibits mineralisation of the produced matrix (Kato *et al.* 1988). Consequently, type I collagen expression is stimulated by TGFβ while osteocalcin expression is inhibited by TGFβ (Noda 1989). TGFβ is known to influence Runt domain transcription factors, such as Runx1, 2 and 3, through activated Smad proteins (Ito & Miyazono 2003). Recently, the influence of $TGF\beta_1$/Smad3 on Runx2 was shown to repress the

osteocalcin promoter and therefore inhibit osteoblast differentiation of rat calvaria cells *in vitro* (Kang *et al.* 2005). Again these results suggest the influence of differentiation state of cultured cells on the final result using TGFβ *in vitro*. Another important effect of TGFβs has been pioneered by the group of Karsdal *et al.* (2002 and 2004), who demonstrated that the growth factor is able to maintain osteoblastic cell survival in the event of apoptotis (Karsdal *et al.* 2002; Karsdal *et al.* 2004)

1.3.3. Biological Effects of the TGFβ$_3$ Isoform

TGFβ$_3$ was first isolated from porcine and human cDNA libraries (Derynck *et al.* 1988). TGFβ$_3$ has been shown to play important roles according its expression pattern *in vivo*. In embryonic heart, TGFβ$_3$ mediates epithelial-mesenchymal transformation. TGFβ$_3$ also takes part during skin repair and keratinocyte differentiation and is expressed in the intact nervous system and some cancer cells. Additionally, it was shown to be a potent regulation of bone formation (Cox 1995).

Ten Dijke *et al.* (1990) compared the biological effects of recombinant TGFβ$_1$ and TGFβ$_3$ in osteoblast-enriched bone cell cultures (primary cells) *in vitro* (ten Dijke *et al.* 1990). Both transforming growth factors had a stimulatory effect on mitogenesis, as well as type I collagen protein expression but an inhibitory effect on alkaline phosphatase activity. TGFβ$_3$ was proven to be more potent for the different tested biological effects. Additionally, it was shown that TGFβ$_3$ binds to the three known transforming growth factor receptor types with a three- to fourfold-greater affinity than TGFβ$_1$.

Hering *et al.* (2001) were able to extract large quantities of TGFβ$_3$ protein from human bone samples (30-66 ng/g bone) (Hering *et al.* 2001). During this study, the expression of TGFβ$_3$ mRNA and protein were investigated in human bones and sera from different donors. No site specific distribution of TGFβ$_3$ protein in bone was observed; suggesting that the mechanical environment does not alter the protein level. Yet, the gene expression of TGFβ$_3$ and, therefore, the mRNA

amounts differed in various sites of the skeleton. It was proposed that the modulation of TGFβ$_3$ gene expression is induced by short-term alterations of the surrounding bone environment, such as mechanical loading. Since the protein concentration of TGFβ$_3$ in bone was remarkably higher than the concentration in sera, the authors concluded that circulating TGFβ$_3$ derives from bone. A clear distinction of the origin of circulating TGFβ$_3$ from synthesis or bone resorption processes could not be drawn.

Hering *et al.* (2001) were unable to detect a sex specific distribution of TGFβ$_3$ mRNA or protein. Oestrogen is thought to enhance TGFβ expression, resulting in a stimulatory effect of bone formation (Hering *et al.* 2001; Sowa *et al.* 2003). Another interesting result was the observation of an age dependent decline of TGFβ$_3$ mRNA from bone and TGFβ$_3$ serum protein. Therefore, TGFβ$_3$ was proposed as marker for osteoporosis as a decline in TGFβ$_3$ expression could result in decrease of osteoblastic activity seen during osteoporosis. However, the investigation of osteoarthritic bone showed declined TGFβ$_3$ protein levels but increased TGFβ$_3$ mRNA in bone, in combination with increased TGFβ$_3$ serum levels. The authors suggested, that the capacity of TGFβ$_3$ storage in bone was reduced, yet, TGFβ$_3$ synthesis was up-regulated and led to increased serum levels (Hering *et al.* 2001). Therefore, TGFβ$_3$ seems to be a potential serum marker for age-related bone diseases.

1.4. Osteoblast-Lineage Cell Culture Systems

For decades, study of bone cell biology has routinely been performed outside of the body. Cellular mechanisms, reactions to growth factor addition or to application of a mechanical stimulus can be investigated without the disturbance of unwanted cell types or systemic factors. These studies benefit additionally from the controlled environment in which they are performed in, the culture temperature, pH, as well as supplied nutrients can be regulated and maintained (Majeska & Gronowicz 2002). The aim of these studies is to reduce the number of questions addressed in the following animal studies and, therefore, to reduce the number of animals that have to be euthanised. There are several methods to culture bone cells outside of their natural *in vivo* environment. The most obvious difference between the culture methods is the culture of isolated cells, generally termed *in vitro* cell culture, and the culture of whole bone explant pieces termed *ex vivo*.

1.4.1. *In Vitro* Cell Culture Methods

Different cell types are utilised frequently for the *in vitro* investigation of bone cell biology. The use of homogenous immortalised cell lines or cells derived from cancers further simplifies *in vitro* investigation, as these cells represent a relatively uniform cell population (Di Silvio & Gurav 2001; Lian & Stein 1992). Several cell lines have been established. Commonly used osteosarcoma cell lines include the ROS 17/2, the SaOS-2 and the MG-63 (Kartsogiannis & Ng 2004). The ROS 17/2 cell line is derived from a spontaneaous tumor in a rat model (Majeska *et al.* 1980). These cells react with adenlylate cyclase activity due to exposure to PTH, as well as demonstrate a high ALP activity due to PTH exposure (Majeska & Rodan 1982). The subclone ROS 17/2.8 was further elucidated and demonstrated high osteocalcin expression, as well as responsiveness to TGFβ with an increase in ALP, type I collagen, osteonectin and osteopontin mRNA and a decrease in osteocalcin gene expression (Noda *et al.* 1988; Noda 1989; Noda & Rodan 1987). SaOs-2 and MG-63 cells are examples for human-derived osteosarcoma cell lines. Both cell lines were

characterised and compared to human primary derived osteoblasts (Clover & Gowen 1994; Murray *et al.* 1987; Pautke *et al.* 2004). In general SaOS-2 cells seem to represent a mature osteoblastic phenotype, while MG-63 cells are considered younger (immature or less differentiated) osteoblasts. The MC3T3-E1 cell line belongs to the group of clonal non-transformed cell lines. These cells were established from newborn mouse calvaria and demonstrate, if cultured in monolayer, typical spindle-shaped osteoblast morphology, increased ALP activity due to confluency, as well as the potential to form mineralising nodules (Sudo *et al.* 1983). Another important cell line for osteoblast-lineage investigations was developed from the group of Bonewald in 1997 (Kato *et al.* 1997). These experimentally immortalised cells were derived from transgenic mice over expressing T-antigen driven by the osteocalcin promoter. MLO-Y4 in culture show extensive and complex dendritic processes. The cells are positive for osteopontin, CD44, and connexin 43 (protein found in gap junctions). Furthermore, as expected from osteocytes (Kartsogiannis & Ng 2004), MLO-Y4 produce large amounts of osteocalcin, but low amounts of alkaline phosphatase and collagen 1 compared to *in vitro* cultured osteoblasts (Kato *et al.* 1997).

Primary osteoblasts can be harvested from a variety of origins and donors. The isolation methods for osteoblasts are well established and range from enzymatic digestion methods using trypsin or collagenase, to the mechanical isolation method with the intention to collect 'out migrating' osteoblasts (Di Silvio & Gurav 2001). Primary human osteoblasts can be isolated from i.e. femoral heads of hip replacement patients. Another common model are foetal calvaria-derived primary cell cultures. These cells are often used to mimic osteoblast differentiation *in vitro* (Boskey & Roy 2008; Kartsogiannis & Ng 2004). In general these cells demonstrate a rather heterogenous cell population of osteoblasts, precursor cells, fibroblasts and even osteocytes. Nijweide and others (1981) demonstrated that different cell isolation populations derived from foetal chick calvaria via enzymatic digestion resemble different osteoblastic cells ranging from osteoblasts to osteocytes (Nijweide *et al.* 1981).

The most frequently used culture method for any of these cells during *in vitro* investigations, disregarding tissue engineering applications to produce bone, is 2D monolayer culture (Kartsogiannis & Ng 2004). Therefore, the individual cell type is cultured on polystyrene (cell culture plastic), which has been either treated commercially to increase the hydrophilicity of the surface, or which has been additionally coated with type I collagen to permit growth of osteocytic cells (Boskey & Roy 2008; Gallagher 2003; Nijweide *et al.* 2010). Preliminary attempts to culture osteoblastic cells in 3D have been undertaken and range from micromass cultures (Bellows *et al.* 1986) to culture with type I collagen gels (Karsdal *et al.* 2002). Yet, their application for *in vitro* bone biology investigations is still limited. Within this study, several 3D culture methods for osteoblastic cells will be introduced and the cell phenotype within these systems will be compared to the generally accepted monolayer culture for their *in vitro* differentiation state.

1.4.2. *Ex Vivo* Bone Organ Culture

It is obviously important to investigate some cellular reactions *in vitro* using a single cell type. However, in order to resemble the *in vivo* situation of bone, monolayer *in vitro* cell cultures lack important features of the complex bone tissue. Bone is characterised by a large amount of mineralised extracellular matrix. Different cell types are present in bone and are in contact with each other and to the ECM that engulfs them (Bilezikian *et al.* 2002). Bone organ cultures present the naturally *in vivo* occurring 3D cell-to-cell and cell-to-matrix interconnectivity. The culture of a 3D tissue with its architectural intact hierarchy, therefore, provides more physiological information without the interference of systemic factors *in vivo* (Meghji *et al.* 1997; Silbermann & Maor 1984).

The first described culture of embryonic femora and limb-buds goes back to 1929 where Fell and Robinson studied growth, development and phosphatase activity of these avian embryonic bones (Fell & Robinson 1929). Therefore, tissue fragments were placed on top of a 'clot' (achieved by mixture of blood

plasma and embryo extract) and cultured inside a moist chamber. Most of the later bone organ culture systems aimed to investigate bone developmental issues of bone formation and resorption processes using embryonic organ rudiments, foetal long bones, or neonatal calvaria (Bingham & Raisz 1974; Meghji *et al.* 1997). In general, these non-mineralised embryonic limbs or calvaria were placed on grids and maintained in tissue culture medium for relatively short periods of times (Boskey & Roy 2008). Some of these organ culture systems were also used for mechanical testing purposes (Brown 1995; Rodan *et al.* 1975). Similar foetal or neonatal organ cultures were used for *ex vivo* investigations of cellular responses to vitamin, hormone, lipid or growth factor treatment (Klein-Nulend *et al.* 1991; Manzi *et al.* 1994; Raisz *et al.* 1993; Simmons & Raisz 1991).

For further studies that involve the response of mature bone cell types to a treatment, the use of a mature bone explant becomes more appealing. However, the culture of mature organs *ex vivo* can be problematic, due to the high mineral content of the ECM, but also due to the size of the mature bone. Oxygen availability, the supply of nutrients and the removal of waste products remain challenging tasks (Trowell 1961). Either a small mature bone is chosen for *ex vivo* culture, or perfusion systems and dynamical compressive loading have to be applied during culture of prepared bone explant pieces. The group of Lanyon (1995) demonstrated very elegantly the first possibility of mature organ culture (Rawlinson *et al.* 1995). The group maintained calvaria and ulna derived from male rats *ex vivo* for less than 24 h and exposed the individual bone explants to compressive strain using an apparatus that placed two pneumatic-driven pistons around the explant. They pointed out, that early cellular responses between mechanically loaded calvariae and limb bones are different.

Yet, the culture of human bone explant pieces *ex vivo* remains a very uncommon tool for bone biology investigations. Previously, a perfusion culture and bioreactor system was designed to enable the performance of long-term studies on *ex vivo* maintained mature cancellous bone biopsies from different donor species (Jones *et al.* 2003). The system offers the opportunity to perfuse

culture medium along the explant surfaces and therefore increase nutrient availability for the explant. Moreover, the ZetOs bioreactor is designed to allow the application of a defined mechanical load of naturally occurring load patterns, which on one hand increases nutrient supply but furthermore creates an anabolic stimulus for the cultured bone explants. A naturally occurring loading pattern of a complete jump [Fig. 1.7] was chosen to simulate the *in vivo* load-bearing situation. This loading pattern also governs the feature of a high impact exercise, which was shown to be more potent for building bones (Rubin & Lanyon 1985). Therefore, daily dynamic mechanical loading was performed to apply 4000 µstrain to each bone explant. With the physiological parameters of 1 Hz frequency (Hsieh & Turner 2001) and 300 cycles (Hsieh & Turner 2001) the daily loading regime was completed. In earlier studies using this bioreactor system, ECM and cell integrity after culture of bovine, ovine and human cancellous bone cores was demonstrated as being comparable to the *in vivo* situation (Davies 2005). The presence of non-collagenous proteins, such as osteopontin, osteonectin and osteocalcin could be localised by immunohistochemistry on embedded non-decalcified bone. Cell activity by the determination of [^{3}H]-glycine incorporation during protein synthesis was shown during 14 days *ex vivo* culture (Davies 2005).

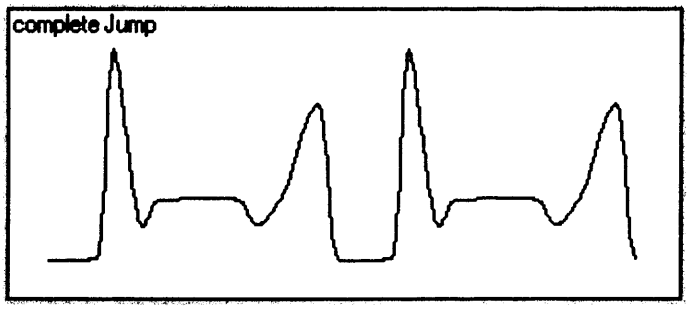

Fig. 1.7: Loading pattern of the complete jump waveform applied during daily loading onto cancellous bone explants cultured in the ZetOs system.

- Within this study, the *ex vivo* culture of cancellous bone explants within ZetOs system will be further improved. The focus will be placed on phenotypic maintenance, as well as cell survival in culture. Moreover, the effects of defined SF and TGFβ_3 will be investigated within this system.

Chapter 2: Materials and Methods

2.1. Materials

- Alexa Fluor® 488 phalloidin (Invitrogen, A12379)
- Alexa Fluor® 594 goat-anti-mouse IgG (Invitrogen, A11005)
- Alkaline buffer solution 1.5M (Sigma, A9226)
- L-Ascorbic acid-2-phosphate magnesium salt n-hydrate (Wako, 013-12061)
- BGJb medium (Gibco, 074-90938A)
- 1-Bromo-3-chloropropane for molecular biology (BCP; Sigma B9673)
- BSA fraction V (Sigma, A3059)
- Calf thymus DNA (Invitrogen)
- Chemically defined lipids (Gibco, 11905-031)
- 'Cell Titer-Blue® Cell Viability Assay' (Promega)
- Collagen type I from rat tail (Sigma, C7661)
- Collagenase type II 327 U/ml (Worthington Biochemical Corporation, 4177)
- 'DEAD End Fluorometric TUNEL System' (Promega, G3250)
- DeoxyNTPs mixture - 0.5 mM each dNTP (Applied Biosystems, N8080260)
- Diethanolamine 98.5% (Sigma, D0681)
- Disulphine blue VN150 (Merck, 42045)
- DMEM high glucose powder (Gibco, 12800-058)
- Dimethyl sulfoxide (DMSO; Fluka, 41640)
- DNase I (Promega, M6101)
- DPX mountant (Fluka, 44581)
- EDTA (Sigma, E5134)
- Ethanol absolute (Alcosuise)
- Ethanol for molecular biology (Merck, UN1170)
- Eosin Yellowish (Fluka, 45380)
- Foetal calf serum 'new' (biowest, S1810 Lot. S05084S1810)
- Foetal calf serum 'old' (Biochrom AG, S 0113/5 Lot.615B)
- Formic acid 85% (Fluka, 06460)

- Gentamycin (50 mg/ml; Gibco, 15750)
- Giemsa solution (Fluka, 48900)
- 'Glucose Assay Kit' (BioVision, K606-100,)
- Glycerol-2-phosphate disodiumhydrate (Sigma, G9891)
- Glycyl-glycine (Gly- Gly Powder, Sigma, G3915)
- Goat-anti-rabbit antibody (VectorLabs, BA-1000)
- Goat serum (Sigma, G9026)
- Haematoxylin solution according to Mayer (Fluka, 51275)
- HBSS (Gibco, 2008)
- HEPES (Sigma, H-3375)
- Hoechst 33258 (Polysciences Inc., 09460)
- Horse-anti-goat antibody (DSHB, BA-9500)
- Horse-anti-mouse/rabbit antibody (DSHB, BA-1400)
- Horse serum (Vector Laboratories, S2000)
- Human TGF-β_3 ELISA Development kit (R&D Systems, DY 243)
- Hydromount (National Diagnostics, HS-106)
- Image-iT™ FX Signal Enhancer (Invitrogen Molecular Probes, I36933)
- 100x Insulin-Transferrin-Selenium (Gibco, 51500-056)
- Isopropanol BioUltra for molecular biology (Fluka, 59304)
- 'Lactate Assay Kit' (Biomedical Research Service Center University at Buffalo, A-108L)
- Lactic acid (Sigma, 69771)
- Leukocyte ALP kit (Sigma)
- Magnesium chloride (Sigma, M8266)
- Magnesium chloride solution 25 mM (Applied Biosystems, N8080010)
- Monoclonal Mouse anti-connexin 43 (Chemicon Internation, MAB3068)
- Monoclonal mouse-anti-human C-terminal propeptide of type I collagen (DSHB, M-38)
- Monoclonal mouse-anti-human MMP13 (R&D Systems, DHD024111)
- Monoclonal mouse-anti-human osteocalcin (abcam, OC4-30)
- MultiScribe Reverse Transcriptase 50 U/µl (Applied Biosystems, 4311235)
- Nicotinamide adenine dinucleotide (Sigma, 43410)
- Nitroblue tetrazolium (NBT; Sigma N5514)

- p-Nitrophenol solution 10 mM (Sigma, V7660)
- 'Osteomark® NTx Serum Kit' (Wampole Laboratories)
- Phosphate-buffered saline (PBS; Sigma, P4417 tablets)
- PCR Buffer II 10 x (500 mM KCl, 100 mM Tris/HCL, pH 8.3 ;Applied Biosystems)
- Penicillin/Streptomycin (10 kIU/ml; Gibco, 2802)
- Formalin solution 36.5% (Sigma, 33220)
- Polyacryl carrier (Molecular Research Center, Inc., PC152)
- Polyclonal goat-anti-human MMP9 (R&D Systems, TQ053101)
- Polyclonal rabbit-anti-human OSF-2 (BioVendor, RD-719)
- Polyclonal rabbit-anti-human TGFβRI (Abgent)
- Polyclonal rabbit-anti-human TGFβRII (abcam, ab61213)
- Polypep (Sigma, P5115)
- Procollagen Type I C-Peptide EIA Kit (Takara, MK101)
- Prolong Gold antifade reagent with DAPI (Invitrogen Molecular Probes, P36935)
- Protease inhibitor cocktail (Sigma, P8340)
- Random Hexamer 50 µM (Applied Biosystems, 8080127)
- RNase Inhibitor 20 U/µl (Applied Biosystems, N8080119)
- RNaseZap® (Sigma, R2020)
- Saline (Fresenius Kabi, NI2516)
- Sodium hydrogen carbonate (Merck, 6329)
- D(+)Sucrose (Fluka, 84100)
- Sodium hydroxide (Fluka, 71692)
- Sodium hypochlorite solution 12% (Roth, 9062.1)
- Superscript III Rtase 10k Units (Invitrogen, 18080-044)
- Sybr Green master mix (Sigma Aldrich, S4438-500RXN)
- 'Technovit 9100 New' kit (Kulzer GmbH)
- 8.8 mg/ml TGFβ_3 solution (kindly provided by Novartis Pharma AG)
- Tissue freezing medium (Jung, 020108926)
- TRI Reagent® (Molecular Research Center, Inc., TR118)
- Tris-EDTA 100x (SIGMA cat. # T-9285)
- Triton-X-100 SigmaUltra (Sigma, T9284)

- TRIZMA® base (Sigma, T6066)
- Trypsin*EDTA 0.5% = 10x (Invitrogen, 15400-054)
- Tween 20 (Fluka, 93773)
- Type I collagen from rat tail Bornstein and Traub 1 (Sigma, C8897)
- 'VECTASHIELD® + DAPI' (Vector Laboratories, H-1500)
- 'VECTASTAIN ABC Mouse *Elite* IgG Kit' (Vector Laboratories, PK 6102)
- Xylene (Brenntag AG)

2.2. Methods

2.2.1. Preparation of Cancellous Bone

Cancellous bone specimens were used for the 3D *ex vivo* culture experiments. Due to the porosity of the cancellous bone, diffusion of fluids and, therefore, nutrient supply can be achieved easier than using 3D cortical bone specimens. Two different model systems were utilised: bovine distal metacarpals from 3-5-month-old calves which were supplied by the local slaughterhouse and osteoarthritic human femoral heads which were received from hip replacement patients within the Chur and Davos hospitals (approved by Ethic Commission Graubünden 18/02). Human donors filled out a patient consent form; their sex, age, weight and height were recorded. They were asked about smoking and drinking habits as well as use of cortisone, bisphosphonates, or parathormone medications prior to operation. The orthopaedic surgeon operating on the patient was asked about the medical reason for the hip replacement. Only femoral heads from donors that had osteoarthritis (no rheumatoid arthritis or necrosis) were used for experiments. Biosafety level 2 standards were followed at all times during preparation and culture. The operators wore mouth protection masks as well as hair protection during bone preparation for reasons of their own safety. The work was performed using aseptic technique in order to avoid fungal or bacterial contamination. Consequently, metal equipment used was sterilised with vapour at temperatures of 134°C for 5 min and polymer equipment was exposed to either 121°C for 20 min, or ethylene oxide for 4 h followed by degassing under vacuum. Surfaces and gloves were sprayed with 70 % ethanol. Equipment was cleaned with Hibitane solution (Hibitane 5% concentrate, medical solution gmbh), water, 70% ethanol, and deionised water. Human waste tissue was discarded according to biosafety level 2 and was, therefore, either bleached using sodium hypochlorite solution (12%) or autoclaved with steam at high temperatures (121°C for 20 min).

The bovine material was supplied as a complete cow foot including the fetlock joint. All soft tissues - skin, muscle and tendons - were removed from the bones by the use of forceps and scalpels. The metacarpal bone was separated from

the other elements and the cancellous bone from the distal end was used for the experiments. Human femoral heads and bovine metacarpals were then processed as previously described (Davies 2005; Davies *et al.* 2006). With the use of an 'Exakt 300' band saw (Exakt Apparatebau GmbH & Co. KG) the bones were cut into 7 mm-thick sections [Fig. 2.1A, B]. Therefore, the bone was attached to the clamp and secured in place. The arm of the band saw was adjusted so that the first small segment of bone could be cut. This piece was discarded. Only sections containing cancellous bone throughout the centre were collected and these were placed in sterile Petri dishes (TPP) containing sterile HBSS. These sections were then used to drill out cancellous cores of 10 mm diameter. Subsequently, the section was placed onto a Teflon table of an EcoMac 212 bench drill containing a Synthes drill bit (Ref: 387.661, Synthes, Bettlach, Switzerland). The cores were obtained after manual slow drilling into the bone by pushing out from the drill bit. Cores were then cut parallel to 5 mm height with a Leica annular saw (Leica AG, Glattbrugg, CH) [Fig. 2.1C; red arrow marks bone cores]. During all cutting and drilling procedures, the bone explants were irrigated with sterile pre-cooled (4°C) saline (0.9% sodium chloride solution) to reduce the formation of bone debris and heat-induced cell death.

Adherent bone debris was removed by washing each explant twice in 10 ml HBSS (Hank's buffered salt solution) at 4°C using an overhead shaker from Heidolph REAX 2. The third wash was performed for 30 minutes and included 1000 IU Penicillin/Streptomycin and 1.5 mg Gentamycin within 10 ml HBSS per bone explant. Each cancellous bone explant was then inserted inside a culture chamber (Mathys, CH) under sterile conditions [Fig. 2.1D]. Each culture chamber was composed of a titanium or stainless steel piston [Fig. 2.1D 1] which was placed inside the whole of the polycarbonate-polyurethane midpiece [Fig. 2.1D 2]. The bone explant was then positioned inside this midpiece and the culture chamber was closed by screwing a titanium or stainless steel baseplate [Fig. 2.1D 3] onto the midpiece. Rubber rings between the piston, midpiece and baseplate sealed the culture chambers.

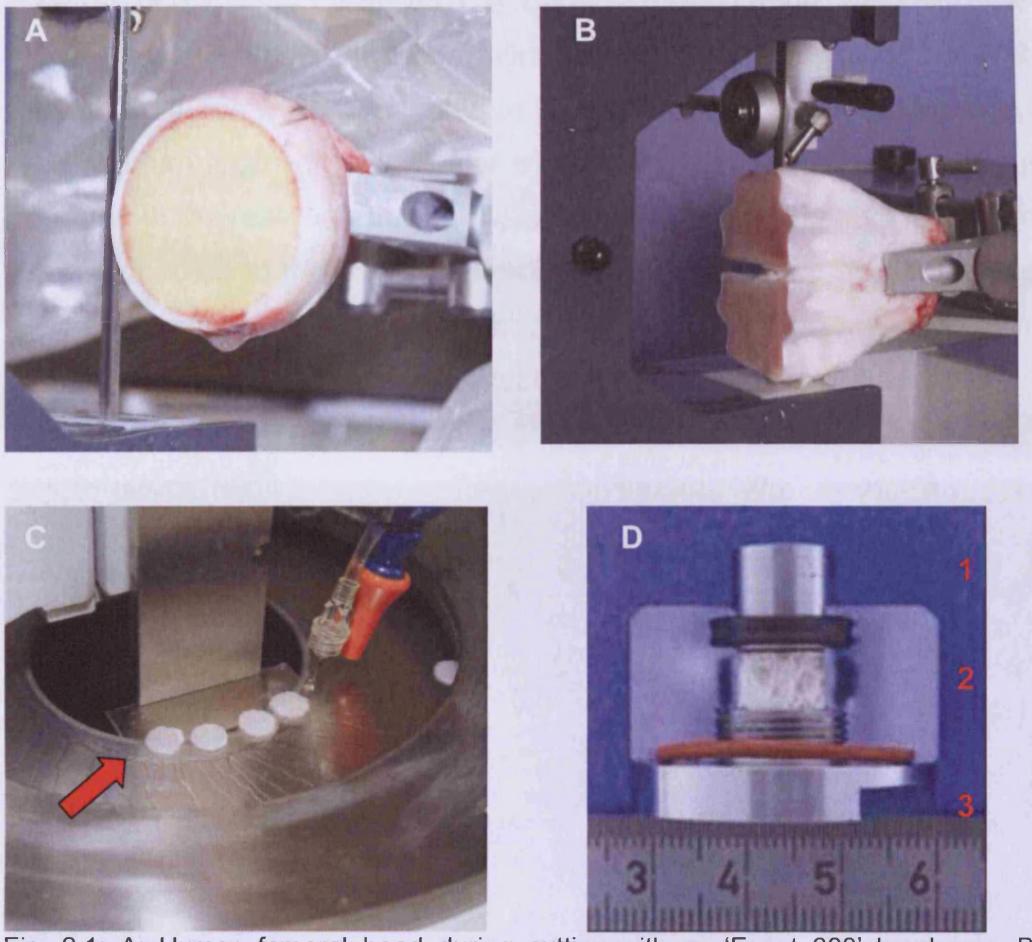

Fig. 2.1: A: Human femoral head during cutting with an 'Exact 300' band saw. B: Bovine metacarpal bone during cutting with an 'Exact 300' band saw. C: Human cancellous bone cores of 10 mm diameter during cutting with a Leica annular saw. D: Schematic inside view of a cancellous bone explant within a culture chamber. Chambers consist of a titanium or steel piston (1), middle piece (polycarbonate-polyurethane) (2) and titanium or steel baseplate (3).

2.2.2. Bone Explant Culture and Maintenance

2.2.2.1. Culture and Maintenance

The culture of cancellous bone explants within the 'ZetOs' bioreactor (Jones *et al.* 2003) system was performed within a 37°C air-conditioned room. Consequently, all of the equipment needed, apart from the operation computer was placed within the culture room. Static culture is known to increase central necrosis during *ex vivo* culture of organs or tissues. In order to maintain a culture environment with a continuous supply of nutrients as well as removal of metabolic waste products, each bone explant [Fig. 2.2A 2] was perfused with its individual culture medium using an 'Ismatech' pump (IPC-ISM934) at a constant

flow rate of 0.1 ml/min [Fig. 2.2A 3]. Each culture chamber was connected by an inflow and an outflow pipe consisting of Masterflex® Tygon® and Pharmed® tubes. The tubes were connected to the chambers and then to the 50 ml-falcon tube medium reservoir with a luer system [Fig. 2.2A 4]. Medium was pumped from and to the reservoir through silicon tubes (Fisher Bioblock Scientific) which were connected to the reservoir lids and the chamber in- and out-flow pipes. A low perfusion rate of the 'Ismatech' pump was chosen to minimise disturbance of the bone marrow within the interstitial spaces of the trabeculae.

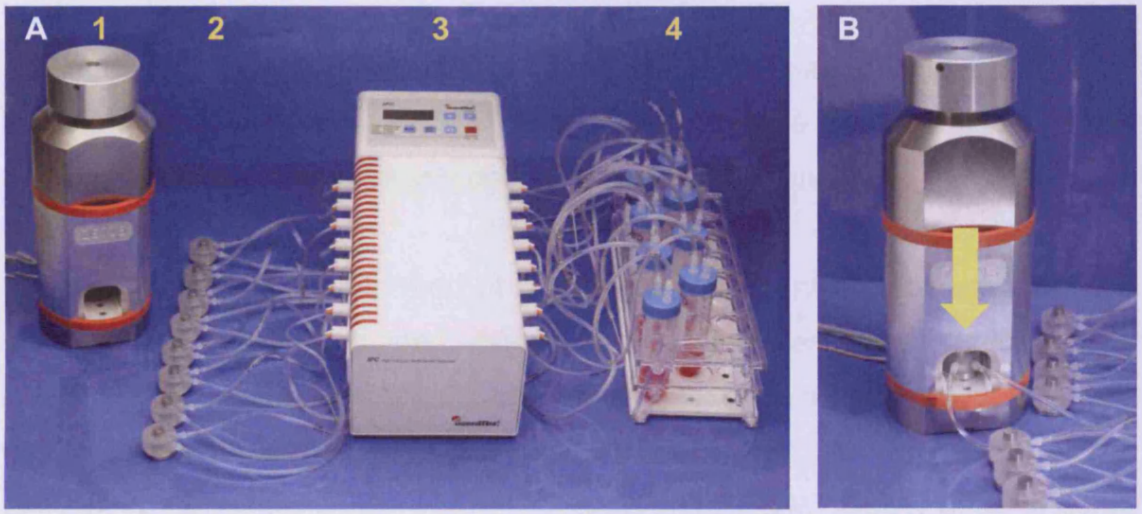

Fig. 2.2 A: 'ZetOs' culture system consisting of the bioreactor (1), the culture chambers (2), an 'Ismatech' pump (3) and the medium reservoirs (4). B: 'ZetOs' bioreactor with an inserted culture chamber. The compressive load will be applied in the direction of the yellow arrow.

Bone cores were loaded daily within the 'ZetOs' loading device [Fig. 2.2B] (Jones *et al.* 2003). Due to the application of a constant compression onto the explant which is followed by a relaxation phase after loading, the renewal of culture medium is supported. Moreover, mechanical strain is one of the main factors that influences bone architecture and mass (Lanyon 1987). The correct dosage of an applied mechanical load can act as an anabolic stimulus on bone. A naturally occurring loading pattern of a complete jump was chosen to simulate the *in vivo* load-bearing situation. Prior to each daily performed loading the 'ZetOs' device was calibrated using a defined steel chamber. Only if the Young's modulus of this chamber was around 1.3 GPa +/- 0.2 and 3 measurements resulted in a 0.91-1.09 correction factor, the loading of the cancellous bone explants was performed. To apply mechanical loading to the

explants, each culture chamber was placed within the 'ZetOs' loading device [Fig. 2.2B, yellow arrow] and a manual preload from 30 to 15 N was performed to ensure bone-piston contact prior to loading (Jones *et al.* 2003). Loading was then performed automatically. Contrary to static load, cyclic loading induces fluid flow inside the lacunar-canalicular system, which can create shear stress on surfaces of loaded osteocytes - the required bone adaptation stimulus (Rubin & Lanyon 1984). Physiological parameters of 1 Hz frequency (Hsieh & Turner 2001), 300 cycles/d (Hsieh & Turner 2001), 4000 µstrain (Rubin & Lanyon 1985) and a complete jump wave-form are applied during daily cyclic loading (Jones *et al.* 2003). The Young's modulus was measured during selected experiments. Therefore, bone explants were placed inside the 'ZetOs' loading device, a manual pre-load was applied and the Young's modulus was determined by automatic steady compression until 20 µm.

All experiments were performed at least twice, or otherwise as stated. For the first 3 experiments performed, bone explants were assigned randomly into different groups. Each group consisted of 2 to 3 bone cores. The supplied medium was either DMEM / BGJb containing 10% FCS, or DMEM / BGJb SF containing 15 ng/ml TGFβ_3. For further experiments, the number of bone cores per group was increased to 6 cores. As human femoral heads are internally variable (Davies 2005), group assignment of each bone core was performed according to the measured Young's modulus (previously described method (Davies *et al.* 2006)). All cores were cultured for 14 days within the system.

2.2.2.2. Preparation of the Culture Medium for *Ex Vivo* Experiments

DMEM (Dulbecco's Modified Eagle Medium) and BGJb (Modified BGJ medium developed by Biggers, Gwatkin, and Judah) media were prepared according to the same basic protocol:

	13.5 g/l	**DMEM high glucose powder**
or	**19.8 g/l**	**BGJb powder**
	2.38 g/l	**HEPES**
	0.12 g/l	**Sodium bicarbonate**
	1.08 g/l	**Glycerol-2-phosphate disodiumhydrate**
	10 mg/l	**L-Ascorbic acid-2-phosphate magnesium salt n-hydrate**

HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) is a buffering substance as experiments were performed under atmospheric oxygen concentrations (Davos, CH, 1500 m above sea level). Sodium hydrogen carbonate was added to supply cells with the essential carbonate ions. To enhance calcification and collagen I synthesis, β-glycerol-2-phosphate and L-ascorbic acid were added. The media ingredients were dissolved in appropriate volume Millipore deionised water, leaving at least 50 ml to adjust the pH to 7.25 using a 5 M sodium hydroxide solution.

The serum-containing media was prepared by the addition of 5 kIU/l Penicillin/Streptomycin to the medium. Medium volume was adjusted to it's final volume and mixed. Ten percent of the medium was discarded prior to the addition of foetal calf serum (FCS). Finally, the medium was sterile filtered under a sterile hood using a 0.22 µm filter and a sterile glass bottle.

Serum-free (SF) medium was prepared by addition of 5 kIU/l Penicillin/Streptomycin and:

1.35 ml/l	**7.5% BSA (bovine serum albumin) solution**
10 ml/l	**100x Chemically defined lipids**
10 ml/l	**100x ITS (insulin-transferrin-selenium)**

The BSA (bovine serum albumin) solution was prepared by dissolving the appropriate amount of BSA in 1x phosphate-buffered saline (PBS). ITS is a mixture of insulin, which enables the uptake of glucose by the cells, the iron carrier transferrin, and the essential micronutrient selenium. Some of the SF medium was completed with the addition of 15 ng/ml TGFβ_3 (Transforming growth factor beta 3). Therefore, the stock TGFβ_3 (8.8 mg/ml) was diluted 1:1000 in a 5% BSA solution and sterile filtered. From this solution, 170 µl per

100 ml prepared medium were added after sterile filtration of the medium to achieve a final concentration of 15 ng TGFβ_3 per ml medium.

2.2.3. Lactate Dehydrogenase Viability Assay

Stoddart *et al.* established a modified version of the LDH viability method to be used on *ex vivo* cultured bone explants (Stoddart *et al.* 2006). Lactate dehydrogenase (LDH, EC 1.1.1.27) is an ubiquitinous cytoplasmic enzyme present in almost all living cells in a variety of organisms. The major advantage of this assay is the long stability of the LDH enzyme after cell death – up to 36 hours - eliminating any false negative results due to cutting of the sample prior to analysis. The LDH assay was used to determine the viability of long term cultured bone explants. Therefore, explants were harvested and cut into 250 µm sections using a Leica annular saw until the explant centre (Leica AG, Glattbrugg, CH). To maintain a wet and chilled condition during the cutting procedure, explants were irrigated with sterile saline at 4°C. Sections were kept in a sterile 24-well plate (TPP) and covered with HBSS prior to LDH staining.

A base solution was prepared containing 5% Polypep, 0.75% sodium chloride and 2 mM Glycyl-glycine. Polypep is a mixture of peptides, which when dissolved in water results in a viscous solution able to stabilise tissue sections. For 100 ml LDH solution, 0.66 g of the buffering substance glycyl-glycine (active range between pH 7.5-8.9 and a pKa of 8.25) and 50 ml deionised water was added. The solution becomes isotonic by the addition of 0.75% sodium chloride. The pH of the solution was adjusted to pH 8.0 using sodium hydroxide solution. Five grams of Polypep were added to the solution and left to stir for 1-2 h at ambient temperature, before adjusting to the final volume to 100 ml using deionised water. The prepared base solution can be stored up to 6 months if kept at 4°C.

The LDH solution was prepared on the day of the assay by adding 60 mM lactic acid and 1.75 mg/ml nicotine amide adenine dinucleotide (NAD^+) to the base solution. The pH was re-adjusted to 8.0 prior to the addition of 3 mg/ml nitroblue

tetrazolium (NBT). NBT is a light-sensitive substance and it is, therefore, necessary to protect the solution from light during the residual procedure. Mix the solution well to ensure complete dissolving of the NBT. Both lactate and NAD^+ are substrates in the lactate dehydrogenase catalysed reaction resulting in the reduced form of NAD^+ (NADH) and pyruvate. NADH is oxidised by the reaction with the third substrate NBT to finally produce a purple, water-insoluble formazan salt. These crystals can be detected in the cytoplasm of viable cells containing the essential metabolic enzyme LDH.

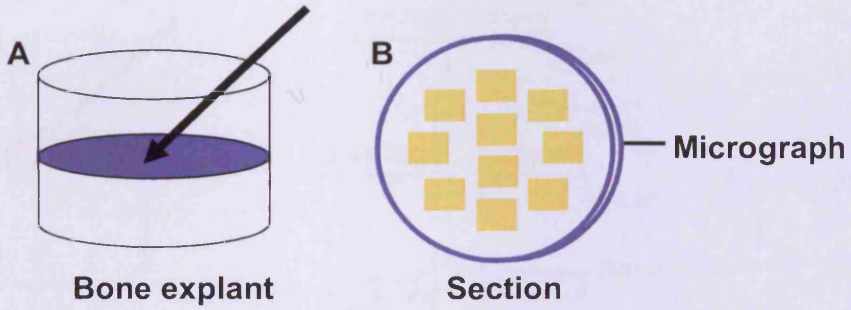

Fig. 2.3: Schematic diagram of a bone explant (A), the centre section is marked blue. This section was used for LDH assay (B). Middle region was visualised and analysed.

The LDH assay was performed on the surface and centre sections from each bone core. Therefore, the medium was aspirated and sections were incubated with 500 µl LDH solution for 4 h at 37°C in the dark. Following this incubation, sections were washed twice with PBS and fixed in 4% phosphate-buffered formalin at 4 °C. Prior to visualisation, the fixed sections were washed shortly in deionised water and placed onto a microscopic glass slide. The section was covered with Hydromount and a coverslip was applied onto the section and left to dry for 2-4 h at ambient temperature. Osteocyte viability within the bone matrix of LDH stained sections was analysed as previously described (Stoddart *et al.* 2006). The LDH stained sections were visualised using an Axioplan microscope (Zeiss). Slides were viewed at 20x lens magnification with a Zeiss filter set #10 (excitation BP450-490 nm, beam splitter FT510 nm, emission BP515-565 nm) to visualise the dark-stained osteocytes blocking the green background autofluorescence of bone. LDH stained centre-located sections were evaluated for osteocyte viability by randomly taking a minimum of 5

micrographs using fluorescence microscopy from the middle area of each section [Fig. 2.3].

The LDH stained surface sections were cut in halves. One half was turned up-side down to analyse the upper-most surface of the explant, and the 250 µm deeper surface from the other half. The lower surface [Fig. 2.4, blue] was used to determine osteocyte viability; micrographs were randomly taken from the middle area of that surface.

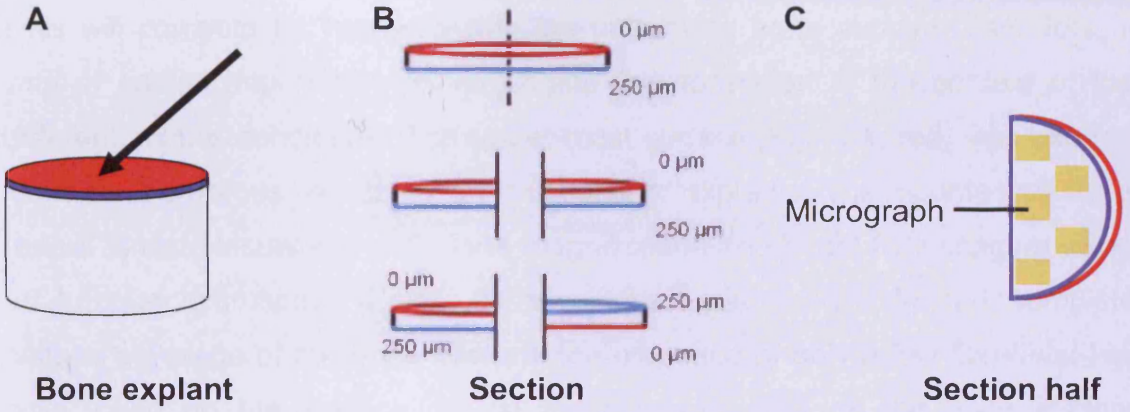

Fig. 2.4: Schematic diagram of a bone explant (A), the upper section was stained for LDH. Prior to visualisation, the section was cut in two and one side was turned up-side down (B). The marked red (upper-most surface) was visualised for fibrous tissue formation, while the blue surface (250 µm lower surface) was used for LDH viability analysis. Therefore, the middle region of the lower surface was visualised and analysed (C).

Quantification of viable osteocytes per bone matrix area on the processed 250 µm thick bone sections was possible due to the small achieved depth field of 4.12–4.52 µm. Using Axiovision software, the extracellular bone matrix area per micrograph was marked using the outline tool. The dark-stained osteocytes within this area were determined and the ratio of viable osteocytes per mm^2 bone matrix area was calculated.

The LDH assay was further used qualitatively on cryo-sections of cell pellets, as well as other culture systems. Therefore, cryo-sections from unfixed samples were prepared. Sections had to be prepared within 24 h of sample harvest. LDH staining could be performed at a later time point, as LDH activity is not lost during storage of cryo-sections at -20°C prior to the assay. LDH assay was

performed as described above. However, in order to stabilise the thin (12 µm) sections a 40% Polypep base solution was prepared with 40 g Polypep per 100 ml solution. Consequently, no addition of NaCl was needed.

2.2.4. Fibrous Membrane Quantification

Methods were performed to quantify the formation of the fibrous membrane, which was found on some of the cultured bone explants. On the affected explants, the density of the formed membrane was uneven. The fibroblast-like cells will compete for nutrients with the underlying bone explant. Therefore, it was of crucial importance to investigate this formation in the context of the different culture conditions. The upper-most surface [Fig. 2.4, red] was used to visualise the fibrous membrane on the cultured explants. The mounted sections (see 2.3) were visualised at 5x lens magnification and bright field imaging using an Axioplan microscope (Zeiss). Three different grades were defined; complete cellular coverage of the bone explant, the presence of only a few fibroblast-like cells found on the surface, or no fibroblast-like cells on the bone explant surface. Fibrous membrane quantification was performed during 5 human loading experiments (♀ 55 years, ♀ 75 years, ♂ 71 years, ♂ 72 years, ♂ 78 years).

As this methodology could only determine different grades of the fibrous membrane formation and was not 100% conclusive about the amount of the fibrous membrane layer, another method was investigated for possible quantification in two experiments using bovine cancellous bone from 3-5-month-old calves. Therefore, cancellous bone explants were statically cultured in 50 ml-falcon tubes (TPP) using DMEM + 10% FCS as culture medium. After 7 days culture, explants were harvested, washed once for 10 min in 1x HBSS (Ca^{2+}-, and Mg^{2+}-free) at 4°C prior to digestion in 7 ml Trypsin (0.05%) / Ethylenediaminetetraacetic acid (EDTA) solution (in HBSS) per bone explant. Digestion times varied from 0 min, 15 min, 30 min, to 60 min (each group contained 4 bone explants). Digestion was stopped by the addition of FCS. One bone explant was heat-inactivated at 56°C (overnight) prior to 7 days culture

and used as a 'dead control' explant. The supernatant after digestion and HBSS incubation was collected and cell counts were performed using a 'Neubauer haemocytometer' (method described in 2.2.14.1). Surface sections were cut from each side of the bone explant and used for LDH assay to visualise for presence of fibrous tissue. A later experiment used increased trypsin digestion times (60 min, 75 min or 90 min), as well as (for some explants) a pre-digestion using 25 U/ml collagenase II in SF medium for 2 h at 37°C prior to trypsin digestion.

2.2.5. Technovit Embedding of Bone Explants

2.2.5.1. Dehydration

All processes were performed at 4°C unless otherwise stated. Xylene and Technovit solutions can dissolve plastic, therefore glass vials were used to embed the bone explants. Cancellous bone explants were 'fixed' in 70 % ethanol for at least 7 days. The fixation solution was replaced several times to ensure that a final concentration of 70 % ethanol was present throughout the whole explant. The explants were further 'fixed' by the addition of first 80 % ethanol for 24 h, followed by 96 % ethanol for 24 h, and 100 % ethanol for 48 h.

2.2.5.2. Embedding Procedure

During infiltration and embedding, bone explants were covered with approximately 5 ml of the different solutions. All work was performed under a fume hood as xylene and Technovit solutions are harmful and hazardous. The Technovit embedding (Yang *et al.* 2003) was initialised with infiltration of the intermediate substance xylene for 8 h at 4°C. Afterwards, explants were left overnight in TMA I, a mixture of 1 part xylene and 1 part stabilised basic solution. TMA II was applied for the next 24 h (100 ml stabilised basic solution + 0.5 g hardener 1). Before the next infiltration step using TMA III, 500 ml of the stabilised basic solution needed to be destabilised. Therefore, stabilised basic solution was incubated with aluminium oxide within a 50 ml syringe, which was filled with cotton wool to allow mixture of the solution and the aluminium oxide prior to collection of the resulting destabilised solution. The destabilised, clear

solution was collected in a dark glass bottle until 500 ml were produced. The next infiltration step used 100 ml destabilised basic solution and 0.5 g hardener I (TMA III) for an infiltration time of 24 h. TMA IV was prepared using 100 ml destabilised basic solution, 8 g of Poly-methyl methacrylate (PMMA) powder and 0.4 g hardener I (stirring overnight was needed). Cores were left in this solution for 5 days to ensure a complete infiltration. The presence of hardener, PMMA and destabilised basic solution within the core centres, should enable a more even polymerisation process. The polymerisation was performed in special plastic molds (Semadeni AG, Switzerland, product no. 1661) which resist Technovit. For the final embedding, 2 solutions were prepared. Poly-A was composed of 120 ml destabilised basic solution, 19.2 g PMMA powder and 0.72 g hardener I and was stirred overnight. Poly-B was prepared by adding 533 µl of regulator to 13.3 ml destabilised basic solution, 1.07 ml hardener II were added drop-wise. Explants were covered with a 9:1 mix of Poly-A and B and left at -20°C to allow polymerisation of the Technovit. All TMA contaminated glassware was rinsed first with xylene which was removed with other xylene waste in an organic solvent waste container, the second rinse was performed with ethanol which was discarded in the sink.

2.2.5.3. Preparation of Thick Sections

Thick sections were prepared from embedded tissue blocks using a Leitz 1600 saw microtome. Therefore, the block was firmly clamped inside the block holder. The final section thickness was set to 200 µm (500 µm blade thickness were added) and the motor switched on. The block was brought to the blade prior to adjusting the speed of trimming to a slow rate (12). The diamond-coated blade trimmed through the block, while the blade was constantly cooled with running tap water. Sections were collected and glued individually onto white opaque Plexiglas slides. The thickness of the sections was the reduced with the use of an Exakt micro grinding system. Therefore different sandpapers from high roughness (P800) to low roughness (P4000, all from Exakt) were applied to the grinding machine. Grinding and Polishing of the sections was performed until 120 µm section thickness.

2.2.6. Cryo-Sectioning of Bone Explants

2.2.6.1. Decalcification

Human cancellous bone cores were decalcified using 12.5 % EDTA solution. The chelating agent EDTA binds calcium ions from the bone matrix and removes them. A 1.25 % NaOH-solution was added to the decalcification solution to prevent an acidic environment which would destroy the tissue integrity during decalcification. The explants were wrapped in macro-porous cotton material, connected to a cotton string, and placed in a 2-litre glass beaker containing the decalcification solution. Every week this solution was refreshed. To visualise the decalcification process, radiographical micrographs were taken before, and during the decalcification. Therefore, bone explants were washed in deionised water to remove adhering EDTA-calcium complexes, positioned on a radiograph film (Structurix D4DW from AGFA) and placed within a 'Faxitron X-ray' machine. Images were taken using an exposure time of 3 min with 30 kV of energy. The radiograph films were developed in the dark inside a 'STRUCTURIX NDT Meco' machine. Decalcification was confirmed as successful when X-rays were not sufficiently blocked by any mineralised ECM within the bone explants. After successful decalcification, bone explants were washed with deionised water for 1.5 hours to remove any remaining EDTA-calcium complexes. Explants were then re-'fixed' in 70% ethanol.

2.2.6.2. Cryo-Sectioning

To remove the fixation solution within the decalcified cancellous bone explants, the specimens were rinsed with deionised water for 30 min. Explants were then placed in 5 % sucrose + PBS solution overnight to allow infiltration of the viscous solution. On the next day, this solution was replaced by tissue freezing medium, a similar solution to the sucrose + PBS, and left to incubate for at least 1 h. Prior to cryo-sectioning the soaked bone explants were placed centred on the specimen holder and frozen in the 'Cryostat-Microtome HM 500 OM' at app. -20°C. After 30 min of freezing, the specimens were cut. Consequently, the specimen holder was placed behind the microtome blade (r35 from Feather) and timed by using the hand wheel until a plane bone surface was visible. Using the automatic cutting drive, 12 µm thin cryo-sections were cut. The sections

were carefully removed from the blade onto adhesive 'SuperFrostPlus' slides (85-0911-00 from Menzel-Gläser). The slides were stored at -20°C prior to immunochemical analysis.

2.2.7. Immunohistochemistry and Immunocytochemistry

2.2.7.1. Immunohistochemical / Immunocytochemichemical Labelling

Cryo-sections were used to perform immunohistochemical labelling detecting the C-terminal propeptide of collagen I (ProCI) and osteocalcin (OCN) [see Table 2.1].

Table 2.1: Primary and secondary antibodies, as well as appropriate blocking reagent used for immunochemistry.

Antigen	Blocking Reagent	Primary Antibody	Dilution & Incubation	Secondary Antibody
C-terminal propeptide of collagen I (ProCI)	1:20 Diluted horse serum	Monoclonal; mouse-anti-human; M38; DSHB	10 µg/ml; 30 min; R.T.	horse-anti-mouse/rabbit; BA-1400; VectorLabs
Osteocalcin	1:20 Diluted horse serum	Monoclonal; mouse-anti-human; OC4-30; abcam	10 µg/ml; 30 min; R.T.	horse-anti-mouse/rabbit; BA-1400; VectorLabs

Slides were defrosted for 5 min and a fat border was drawn around the sections using a 'Dako pen' to prevent section drying during the labelling procedure. Sections were rehydrated for 5 min in 1x PBS containing 0.1 % 'Tween 20' (PBT). Bone is a source of endogenous peroxidases. The activity of these enzymes can lead to false positive labelling results using a horseradish peroxidase (HRP) based detection system. Endogenous peroxidase activity present within the bone sections was, therefore, inactivated using 0.3 % hydrogen peroxide in methanol; incubation was performed for 30 min. After a 5 min wash with PBT, slides were incubated with the appropriate blocking reagent, which was chosen according to the origin of the secondary antibody [see Table 2.1]. Serum used for blocking was diluted 1:20 in PBT and applied for 1 h to the sections to mask nonspecific binding sides. Immediately after

blocking, the primary antibody was applied. Specific dilution and incubation times are given in Table 2.1. Some slides were used as negative controls and were not exposed to the primary antibody. All sections were washed 3 times for 5 min each with PBT to remove non-specifically bound antibodies. Sections were then incubated with the appropriate secondary antibody [see Table 2.1] for 30 min, followed by 3 wash steps with PBT.

'VECTASTAIN ABC Elite IgG Kit' was used to detect the antibody-antigen reaction. Therefore, the 'AB complex' was prepared by mixing 1 ml PBT with 20 µl 'ABC-A' and 20 µl 'ABC-B'. Solution A contains avidin which binds irreversiblly to the biotinylated secondary antibody. Solution B consists of the biotinylated HRP. As avidin contains four binding sites for biotin, the signal will be effectively enhanced. The 'AB complex' solution was prepared prior to used and left at 4°C for 20-30 min to allow complete association of the complex. The secondary antibodies were then marked with the 'AB complex' for 30 min, which results in HRP-labelled antigens. Slides were washed 3 times with PBT to remove any access of the 'AB complex'. The detection solution was prepared immediately prior to use. As containing HRP- substrate 3-3'-diaminobenzidine (DAB) was used, producing a brown-coloured product [Fig. 2.5].

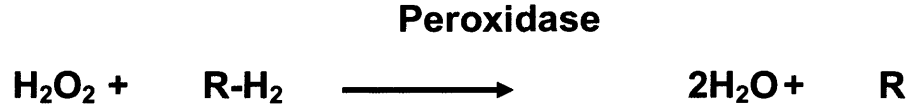

Fig. 2.5: Peroxidase catalysed reaction using hydrogen peroxide and a general substrate.

The DAB solution was prepared as followed by avoiding direct light exposure. Fifteen ml deionised water were mixed with 8 drops of the 'VECTASTAIN ABC Elite IgG Kit' buffer, 12 drops DAB substrate solution, 6 drops hydrogen peroxide and 6 drops Ni^{2+} to enhance the colour reaction resulting in a black-coloured product. This solution was applied for 4 min to the sections.

For one set of experiments (2.2.16.5), labelling was performed using fluorescence labelling of β-actin (phalloidin; Alexa 488), connexin 43 and 4',6-diamidino-2-phenylindole (DAPI) nuclear staining was performed on fixed cryo-

sections. Therefore sections were treated as described above. However, primary antibody was either phalloidin or anti-connexin 43. Phalloidin is already fluorescent and does not require a secondary antibody; mouse anti-connexin 43 was bound to an anti-mouse Alexa 594 secondary antibody to be detected. Double labelling of both antibodies was possible due to the different emission colours of Alexa 488 (green) and Alex 594 (red). Therefore 'Image-iT™ FX Signal Enhancer' was used as blocking agent. Blocking of unspecific binding sites was performed for 20 min at ambient temperature. Anti-connexin 43 was then applied at 1.25 µg/ml and was incubated for 1 h at ambient temperature on the sections. Excess antibody was removed by washing in PBT as described above. Two µg/ml of the secondary anti-mouse Alex 594 antibody was incubated for 1 h at ambient temperature with the sections. Excess antibody was removed by washing and the sections were incubated with phalloidin for 30 min. Excess was removed and sections could be mounted using 'Prolong Gold antifade reagent with DAPI'. Visualisation was performed using an Axioplan microscope (Zeiss).

2.2.7.2. Counterstaining and Section Embedding

Haematoxylin counterstaining was performed on some sections to achieve an easier visualisation of the bone cell nuclei. In order to avoid too dark a background staining which could complicate the detection of positive stained sections, slides were exposed to the solution for only 10 s. Mayer's Haematoxylin solution was filtered prior to use. The slides were washed twice in deionised water to remove any access DAB staining. Sections were dehydrated in increasing ethanol concentrations of 70%, 80%, 96%, and twice 100% (5 min each). The sections were then transferred to a fume hood and incubated twice for 5 min with the intermedium xylene. Sections were permanently mounted by adding 1 drop of the embedding medium DPX on a glass coverslip and carefully placing the slide containing a section on top. The construct was turned upside down after 2 s and slides were left under the fume hood for up to 4 days until polymerisation was completed. Visualisation was performed using an Axioplan microscope (Zeiss).

2.2.8. Histological Staining

2.2.8.1. Giemsa Eosin

Thick Technovit-embedded sections from cancellous bone explants were stained with Giemsa eosin. While the eosin staining labels all positively charged surfaces in red, the Giemsa does label negatively charged surfaces in blue. Therefore, the mineralised ECM of bone stains red, while the cytoplasm of bone and soft tissue cells and the osteoid stains blue. Staining was started with 30s incubation with 1% formic acid to softly etch the section surface. Acid was removed by incubation for 5 min under running tap water. Sections were then rinsed in deionised water and blotted dry. Sections were stained for 10 min in a 15 % Giemsa solution at 57°C on top of a heat block. Therefore, the Giemsa solution supplied by Fluka was diluted from 100% to 15% with deionised water. This solution was heated in a microwave at 700 W for 2 min prior to use. Excess staining was removed by a rinse in deionised water, and section were blotted dry. A 1% eosin solution was prepared by adding 1 g eosin yellowish to 100 ml deionised water. From this stock solution, a working solution was prepared where to 100 ml eosin solution approximately 2 ml of glacial acetic acid were added drop-wise. This 1% eosin working solution is stable for 4 weeks at ambient temperature. The sections were stained with the Eosin solution for 2.5 min at ambient temperature. Immediately afterwards, sections were incubated in 70% and 96% ethanol for 10s each to differentiate the colour of the staining. Dehydration was completed by 2 min incubation in absolute ethanol. Stained sections were blotted dry and were imaged using an Axioplan microscope (Zeiss).

2.2.8.2. Haematoxylin and Eosin

Cryo-sections were stained with haematoxylin and eosin. Therefore, sections were thawed and rehydrated with PBS. Staining was performed using Mayer's haematoxylin for 5 min incubation. Excess staining was removed by a 5 min wash in hand-warm tap water. Sections were rinsed in deionised water and incubated for 1.5 min with 1% eosin working solution (preparation see 2.2.8.1). Staining was differentiated by incubation in tap water, and dehydrated in a

ascending series of alcohols. Sections were then incubated in the intermedium xylene and embedded in DPX mountant as described in 2.2.7.2.

2.2.9. Medium Distribution and Penetration Investigation

The distribution of culture medium in cancellous bone explants during culture under different conditions was investigated and carried out in triplicate. Cancellous bone explants from bovine metacarpal bones (3-5-month-old calves) were prepared and placed inside a culture chamber. Explants were cultured overnight and perfused with DMEM + 10% FCS using an 'Ismatech' pump (IPC-ISM934). On the second day, cancellous bone cores were perfused with the same medium additionally containing 10 % disulphine blue for 14 min. After harvest, images were taken immediately with a 'Konica Minolta Dynax 7D' from the bone explant surfaces exposed to baseplate or piston. Moreover, bone explants were cut in halves and images were taken from this middle cut to investigate medium penetration into the cultured explants.

2.2.10. Media Analyses

The culture medium of *ex vivo* cultured cancellous bone explants was investigated during some loading experiments. Media was collected during media exchange after 48 h of culture. The samples were frozen at -80°C prior to analyses, which were performed according to manufacturers instructions.

2.2.10.1. N-Terminal Telopeptide of Type I Collagen - Competitive-Inhibition Enzyme-Linked Immunosorbent Assay

Using the 'OSTERMARK® NTx-Serum' osteoclast-dependent release of the N-terminal telopeptide of collagen I (NTx) was detected in the culture medium of cultured bone explants of one human loading experiment (♂ 78 years) and the FreqBone experiment using bovine bone (chapter 3). Samples, as well as kit reagents were brought to ambient temperature prior to use. 'Assay calibrators' containing 0-40 nM BCE (bone collagen equivalent), as well as two supplied control samples were mixed and diluted 1:5 in 'Specimen Diluent' according to

manufacturers instructions. The culture medium samples were diluted 1:1 in 'Specimen Diluent'. Samples, 'Assay calibrators', and control samples were to the appropriate wells of antigen-coated 96-well plates with 100 µl per well. To each well, 100 µl monoclonal murine anti-human NTx antibody was added. Plates were gently swirled and incubated for 90 min at ambient temperature. During this reaction time, the bound NTx antigen on the bottom of the micro-well and the media sample NTx will compete for the amount of antibodies added. Wells were washed five times with 350 µl 'wash solution' prior to incubation with 200 µl diluted 'chromogen reagent/buffered substrate' (containing 3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxide) for 30 min. The reaction was stopped by addition of 100 µl 1 N sulfuric acid. The optical density of the colour reaction was determined at 450 nm using a 'HTS 7000 Bio Assay Reader' (Perkin Elmer).

2.2.10.2. Procollagen Type I C-Peptide Enzyme Immunoassay Kit

The procollagen type I C-peptide EIA was used to determine the type I collagen synthesis in the culture medium of *ex vivo* cultured cancellous bone explants of the FreqBone experiment using bovine bone (chapter 3). The kit detects the instable C-terminal propeptide (ProCI) which is released during type I collagen synthesis. The samples and all constituents of the kit were brought to ambient temperature prior to use. At first, 100 µl 'antibody-POD conjugate solution', which comprises horseradish peroxidase (POD) conjugated with murine monoclonal antibody to ProCI, were added to each well of an antibody pre-coated 96-well plate. Subsequently, 20 µl sample or standard (0-640 ng ProCI/ml) were added to the appropriate wells. The plate was hand-shaken to ensure mixing of the solutions, then sealed and left to incubate for 3 h at 37°C. During this time, added ProCI in the samples will bind to the antibody attached to the well-plate surface, and to the POD-conjugated antibody supplied with the 'antibody-POD conjugate solution'. After incubation, the wells were emptied and washed four times with 400 µl PBS ensuring that each well was completely emptied during the washing steps to remove all non-bound antibodies. The substrate solution containing hydrogen peroxide and tetramethylbenzidine was

then added with 100 µl per well and left to incubate at ambient temperature for 15 min. The reaction was stopped by the addition of 100 µl 1 N sulphuric acid per well. The absorbance of the product was measured at 450nm using a 'HTS 7000 Bio Assay Reader' (Perkin Elmer).

2.2.10.3. Glucose and Lactate Measurements

The metabolic state of cultured human bone explants was investigated in two human loading experiments (♂ 71 years, ♂ 78 years) by the detection of glucose and lactate media levels. Assays were performed during two experiments. Glucose concentration was determined using the 'Glucose Assay Kit'. Media samples were diluted 1:3 in 'glucose assay buffer'. From this dilution, 1 µl was added the appropriate well containing 49 µl 'glucose assay buffer'. Standards in the range from 0-10 nmol glucose per well were also prepared diluting the pre-prepared 1 nmol/µl glucose standard in the appropriate volume of 'glucose assay buffer'. The reaction mix was prepared and 46 µl 'glucose assay buffer', 2 µl 'glucose probe' containing hydrogen peroxide, and 2 µl 'glucose enzyme mix' containing a glucose-oxidase per well. The plate was mixed, sealed and then incubated at 37°C for 30 min and the colorimetric product was measured at 550 nm using a 'HTS 7000 Bio Assay Reader' (Perkin Elmer).

Media lactate levels were determined with a 'Lactate Assay Kit', which is based on the reduction of the tetrazolium salt INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) in a NADH-coupled enzymatic reaction to a water-insoluble formazan precipitate. For this measurement, medium samples were diluted 1:30 in deionised water. Lactate standards were pre-prepared and comprised 50-250 µM lactate. Blank, standards and samples were added to the appropriate wells (20 µl per well). In total, 50 µl 'lactate assay solution' per well was added. The plate was mixed, sealed and the reaction was incubated for 45 min at 37°C. The reaction was stopped by the addition of 50 µl 0.5 M acetic acid. Optical density was determined at 492 nm using a 'HTS 7000 Bio Assay Reader' (Perkin Elmer) to detect the formation of the formazan product.

2.2.11. Performance and Analysis of µ-Computed Tomography (µCT)

Detection of bone mass changes during *ex vivo* culture of bone explants is of crucial importance. The µCT 40 from Scanco Medical [Fig. 2.6A] was used for measurements of the 'FreqBone' bovine bone explant samples (chapter 3). Bone explants were placed inside a µCT sample holder [see Fig. 2.6B] and covered with 2-4 ml 70 % ethanol. The tube holder was placed inside the µCT for measurement. The bone explants had been fixed in place with pieces of plastic in order to prevent them from moving inside the sample holder during measurement.

The measurement parameters were as followed - 70 kV beam energy, 10 µm resolution, 300 ms ms integration time, 114 µA; and 500 to 250 projections. After the measurement, the data were analysed for bone volume/total volume, trabeculae number and trabeculae thickness. In order to prevent the inclusion of bone debris in the analysis, which arose through explant preparation, a smaller volume than the explant itself was analysed. For all analyses, the software supplied from Scanco Medical was used. An important analysis factor is the threshold of bone versus not-bone tissue. A lower threshold of 250 was used, as the application of this threshold resulted in a good correlation of bone tissue versus non-bone tissue.

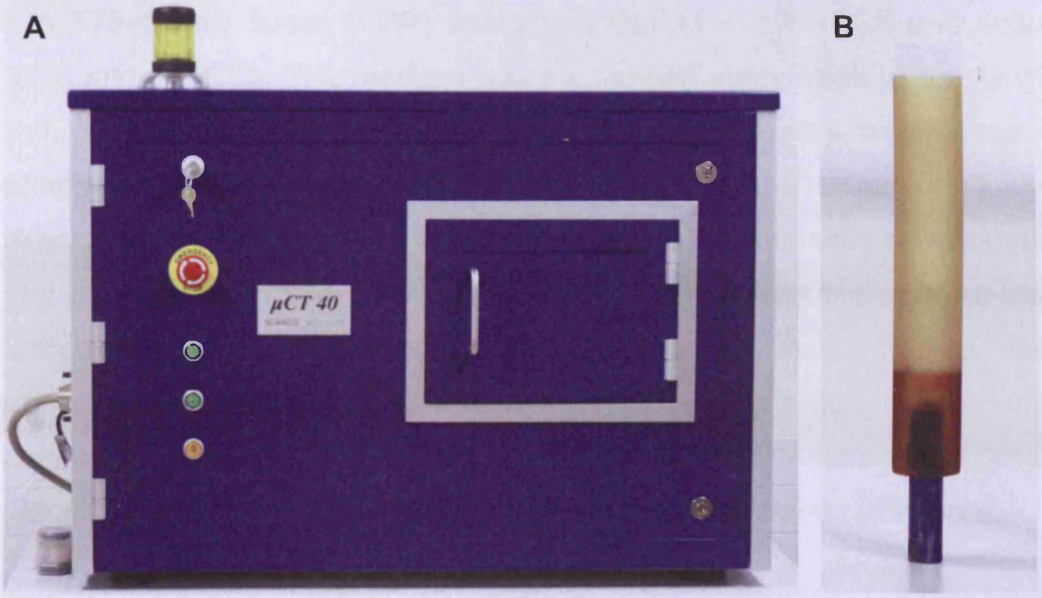

Fig. 2.6: Images of the μCT 40 device (A) and the μCT sample holder into which the bone explants were inserted (B).

2.2.12. Isolation of Primary Human Bone-Derived Cells

2.2.12.1. Osteoblast Isolation

Osteoarthritic human femoral heads obtained from hip replacement patients within the Spital Chur and Spital Davos (approved by Ethic Commission Graubünden 18/02), were used for the isolation of primary osteoblasts (Di Silvio & Gurav 2001; Poulsson 2007). The femoral head was placed inside a sterile hood and wrapped in several layers of sterile gauze, sterile latex gloves, and sterile bags. A package was formed and closed using elastic tape. The bone was taken out of the sterile hood and compressed under a hydraulic press (Collin) at 50 bar. The crushed bone was then placed back into the sterile hood and carefully unwrapped. With the use of bone cutters, the crushed femoral head was cut into small pieces (approximately 1-3 cm^3). Only pieces of cancellous bone were used for cell isolation. Areas of cysts were excluded from the collection. Bone pieces were washed in sterile PBS to remove fat and blood cells. Subsequently, pieces were distributed between several 50 ml falcon tubes and PBS was added to pieces. Falcon tubes were closed and hand-shaken vigorously to mechanically remove the unwanted cells. The PBS was renewed several times until no fat cells were detected in the wash-out and the cancellous bone pieces were free of red marrow. The washed bone chips were distributed

into T75-culture flasks (TPP) containing DMEM + 10% FCS and cultured at 37°C and 5% CO_2. The medium was exchanged every week to supply the cells with enough nutrients, but also to avoid unnecessary disturbance of the attaching cells that might be caused during medium exchange. Cells were allowed to migrate out of the bone chips until 80% confluence was reached. The first migration normally took 2-3 weeks. Isolated osteoblasts were detached using trypsin-EDTA solution and seeded into new culture flasks at approximately 8000 to 10000 cells per cm^2. Osteoblasts were cultured until 80% confluence, detached and frozen down to -196°C as described in 2.2.15. Bone pieces were cultured further and osteoblasts were isolated twice more.

2.2.12.2. Osteocyte Isolation

After three cell isolations, bone pieces were used for one further cell isolation. Nijweide's group (Nijweide *et al.* 1981, van der Plas & Nijweide 1992) isolated osteocytes from the calvaria of 18-day-old chicken embryos. Based upon these methods, bone pieces were incubated for 1 h at 37°C with 3 mg/ml collagenase II in SF medium. The digestion solution was removed from the chips and isolated cells were collected from the solution by centrifugation at 500xG (10 min). The cells were then counted using a 'Neubauer haemocytometer' as described in 2.2.14.1. Isolated cells were frozen to -196°C as described in 2.2.15. Bone pieces were discarded after this final cell isolation according to biosafety 2 standards (incubation with bleach for 48 h).

2.2.13. Culture of Human Osteoblasts

Cell culture experiments were performed under sterile conditions. All experiments were performed at least three times. All liquids exposed to the cultured cells were sterile filtered prior to use. Culture vessels were only opened under a sterile hood. Biosafety level 2 procedures were followed at all times. Therefore, all materials in contact with human-derived cells were either bleached or autoclaved prior to disposal.

Human primary osteoblasts were cultured in TPP culture vessels. $8x10^3$ to $10x10^3$ cells / cm^2 were seeded. Cells were cultured until 80% confluence prior

to passaging. Cells were used up to passage 7 for experiments to ensure a high number of cells, but preventing de-differentiation of the cultured cells which is a common effect seen with primary cells at higher passages (Kartsogiannis & Ng 2004). The general culture medium during expansion is DMEM + 10% FCS, 2.2 g/l sodium bicarbonate, 10 mg/l L-Ascorbic acid-2-phosphate magnesium salt n-hydrate and 50 kIU/l Penicillin/Streptomycin. Medium pH was adjusted to 7.25 and the medium was sterile filtered using a 0.22 µm filter prior to use. Cell cultures were performed in an incubator set to 37°C and 5% CO_2. Medium was renewed every 2 days.

2.2.13.1. Passaging

The culture medium was removed from the cells and cells were washed twice with pre-cooled (4°C) sterile PBS to wash off cellular debris and attached serum proteins. Cells were detached using 0.05% trypsin-EDTA in PBS. The pre-warmed (37°C) trypsin solution was added to the cells with only enough volume to cover the cells and then incubated for 5 min at 37°C. Detachment was registered by light microscopy using an Axiovert 40 CFL microscope (Zeiss). Trypsin was inhibited by the addition of culture medium containing 10% FCS, as FCS contains trypsin inhibitors (Freshney 2000b). The detached cells were washed off the cell culture plastic using FCS-containing medium. The cell suspension was collected and centrifuged at 250xG for 8 min. The supernatant was removed and cells were resuspended in a small volume of culture medium (1-10 ml). A manual cell count was performed and cells were seeded.

2.2.14. Determination of Cell Growth

2.2.14.1. Manual Cell Count

In order to determine the number of cells, and to distinguish between dead and alive cells, the trypan blue method for manual cell counting was used. This manual cell count was performed during passaging of cells, freezing of cells or cell seeding during an experiment. Therefore, the collected cells resuspended in a small volume of culture medium. Trypan blue was mixed 1:1 with the 50 µl cell suspension and filled into the reservoirs of a 'Neubauer haemocytometer'.

Trypan blue is a diazo-dye which cannot pass through the cell membrane of viable cells. Therefore, viable cells appear unstained while dead cells with a ruptured cell membrane are stained blue. Using an Axiovert 40 CFL microscope (Zeiss) a cell count was performed. The average from the 2 reservoirs was used for cell number calculation using the following formulation:

Total cell number = Average cell number
÷ Number of counted large squares
x Dilution of the cell suspension with trypan blue
x Media volume in ml x 10^4

2.2.14.2. 'Cell Titer-Blue' Assay

The 'Cell Titer-Blue® Cell Viability' assay is a fluorometric/colorimentric assay based on the metabolic-dependent reduction of the redox indicator dye reazurin which turns from non-fluorescent and blue into the fluorescent, red resorufin. The assay has been widely used to measure cell growth and has been shown to be non-cytotoxic for cells (O'Brien *et al.* 2000). As the 'Cell Titer-Blue®' assay detects the activity of cultured cells rather than the actual cell number, conditions have to be found where actual cell number correlates with cell activity determined in the assay. For the use of 'Cell Titer-Blue®' during the culture of human primary osteoblasts, Poulsson (2007) optimised the assay protocol (Poulsson 2007). According to her work, the 'Cell Titer-Blue®' was used in a 10% concentration in DMEM + 10% FCS medium. This mixture was sterile filtered prior to use. The culture medium of the cells was removed and the cells i.e. seeded in a well of a 24-well plate were incubated with 400 µl of the 'Cell Titer-Blue®' mixture for 4 h at 37°C. The mix was removed from the cells after incubation, transferred to a 96-well plate and used for absorbance measurements at 595 nm which is the absorbance peak of reazurin, and 545 nm which resembles the maximum for resorufin. The amount of reduced indicator dye, resorufin was calculated using the following equation:

$$Percent\_resorufin = \frac{(117.216)\cdot(A_{545nm}) - (80.586)\cdot(A_{595nm})}{((155.677)\cdot(Acontrol_{545nm}) - (14.652)\cdot(Acontrol_{595nm}))} \cdot 100\,\%$$

The absorbance (A) of the individual samples at 545 nm and 595 nm are given as A_{545nm} and A_{595nm}. The 'Cell Titer-Blue®' without cells incubated for 4h at 37°C was used as control sample, the absorbance values at 545 nm as well as 595 nm are given as $Acontrol_{545nm}$ and $Acontrol_{595nm}$. The different values within the equation are the molar extinction coefficients (ε) of the oxidised and reduced 'Cell Titer-Blue®' at 545 nm and 595 nm:

$\varepsilon_{reazurin\ at\ 545\ nm}$ **= 80.586** $\varepsilon_{reazurin}$ **at 595 nm = 117.216**

$\varepsilon_{resorufinn\ at\ 545\ nm}$ **= 155.677** $\varepsilon_{resofurin}$ **at 595 nm = 14.652**

After the 'Cell Titer-Blue®' was removed the cells were washed with sterile PBS to remove any residual dye. Cells were then either cultured further or used for Alizarin Red S staining or Hoechst DNA analysis.

2.2.14.3. Hoechst DNA Assay

Another method to determine cell number during culture is the Hoechst DNA assay. The Hoechst 33258 dye (bisbenzimide) binds to the minor groove of deoxyribonucleic acid (DNA). Due to the binding, the fluorescence of the dye is enhanced and can be detected using a plate reader (Labarca & Paigen 1980). The intercalating characteristic of the Hoechst dye also determines its dangerous potential for the user and care must be taken working with this carcinogenic and toxic dye. Prior to the performance of the assay, a PBS containing 3.36 g/l disodium-EDTA (PBE) with pH=6.5 was prepared. Whole samples are washed with PBS and a proteinase K solution containing 0.5 mg proteinase K per ml PBE was added to the cells. The volume was chosen according to the size of the culture wells i.e. 300 µl were added to a well of a 96-well plate. The cells were incubated with the proteinase K solution for 12 h at 56°C. During this time, the serine protease proteinase K digests proteins and removes contaminations from the nucleic acid preparation. After incubation, the digest can be stored at -20°C prior to measurement. The samples of one experiment were thawn and measured at the same day. DNA standards were prepared from calf thymus DNA. The assay can be performed using a standard DNA range from 12.5 ng/µl, 6.25 ng/µl, 3.125 ng/µl, 1.56 ng/µl, 0.78 ng/µl, to 0.39 ng/µl, or an extended DNA range from 50 ng/µl, 25 ng/µl, 12.5 ng/µl, 6.25 ng/µl, 3.125 ng/µl, to 1.56 ng/µl. Therefore, the appropriate volumes of a 100

µg/ml DNA stock solution were added to the PBE buffer. Fourty µl of samples, standards and a blank control, all measured in duplicates, were transferred in to well of a white opaque 96-well plate (Falcon® Becton Dickinson Labware). To each well, 160 µl assay solution were added. For the standard DNA range, the assay solution contained 10 µl Hoechst stock and 100 ml DPBS. The extended DNA range assay solution contained 10x more Hoechst per DPBS than the standard range. DPBS is PBS supplemented with 2.0 M NaCl and is used to enhance the fluorescence of the Hoechst dye. After 20 min incubation in the dark, the plate was used for fluorometric measurement at 360 nm excitation and 465 nm emission using a VICTOR$^3{}_{TM}$ plate reader (Perkin Elmer).

2.2.14.4. Terminal Deoxyribonucleotidyl Transferase Mediated 2´-Deoxyuridine-5´-Triphosphate (dUTP) Nick-End Labelling (TUNEL)

Formalin fixed slides were used for the determination of dead cells using the 'DEAD End Fluorometric TUNEL System'. This assay specifically labels nicks in the DNA sequence. The terminal deoxyribonucleotidyl transferase adds fluorescein-12-dUTP to the ends of fragmented DNA. The assay is designed to detect apoptotic cells only; however, it is known to label necrotic cells as well (de Torres *et al.* 1997). We, therefore, used the assay to detect cell death in general.

Slides were immersed in PBS for 5 min at ambient temperature. Cells were then permeabilized for 5 min in 0.2% Trixon-X solution, and rinsed for 5 min in PBS. An 'equilibration buffer' was applied for 10 min to acclimatise the cells for the reaction. The supplied 'nucleotide mix' and the transferase were thawed on ice and an incubation buffer containing 45 µl 'equilibration buffer', 5 µl 'nucleotide mix' and 1 µl transferase was prepared. Slides were incubated with the buffer for 1 h at 37°C in a humidified atmosphere. A plastic coverslip was applied to the sections to prevent them from drying-out and to allow the use of a relatively small volume of the incubation buffer per slide. After incubation the enzyme was denaturated by 15 min incubation in 2x saline-sodium citrate (SSC) buffer.

Slides were washed three times with PBS to remove remaining buffer and were mounted in 'VECTASHIELD® + DAPI' mountant.

A positive and a negative control were always performed during the performance of the TUNEL assay. For the negative control slide, the transferase enzyme was not added to the incubation buffer. The positive control was incubated with 10 U/ml DNase I for 10 min at ambient temperature prior to incubation in equilibration buffer.

2.2.15. Freezing and Thawing of Cells

If the detached cells after trypsinisation were required for further culture, cells were collected after centrifugation as described in 2.2.13.1 and resuspended in 10% DMSO (Dimethylsulfoxide) in FCS. A cell number volume ratio of 1-2 million cells per ml DMSO-FCS was used. DMSO as a cryo-protectant reduces the ice crystal formation during freezing. These ice crystals that are normally formed could be lethal for the cells (Freshney 2000a). Cells in DMSO-FCS were filled into 2 ml-cryo-tubes™ vials (Nunc™ Apogent) and then placed into a 'Mr. Frosty' (Nalgene Labware), which is filled with isopropanol which freezes at a constant rate of 1°C per min during initial freezing to -80°C. For long-term storage, the cell suspension was placed into liquid nitrogen (-196°C).

In order to re-vitalise cells, the frozen cell suspension was removed from the liquid nitrogen and quickly thawed using a water bath at 37°C. The cryo-tube with the cell suspension was then transferred to a sterile hood and opened. Pre-warmed culture medium was added drop-wise to the thawed cell suspension until the initial volume of the cell suspension was doubled. The cell suspension was then transferred into a 50 mn tube and about 8 ml pre-warmed medium was added drop-wise. Cells were seeded into culture flasks containing pre-warmed culture medium to allow adsorption of serum proteins prior to cell seeding. The cell density seeded after re-vitalisation was increased up to 15000 cells/cm^2.

2.2.16. *In Vitro* Culture Methods for Cells of Osteoblast-Lineage

2.2.16.1. Multilayer Cultures of Primary Human Osteoblasts

Human primary osteoblasts were used for multilayer culture experiments. Each treatment group consisted of 3-4 seeded wells. Within a pre-experiment 3 different cell densities were investigated for their increase in cell density over a culture period of 16 days. Ten thousand cells/cm^2 (seeded into 6-well plates with 9 cm^2/well) as typical monolayer seeding density, were compared to 120000 cells/cm^2 (seeded into 48-well plates with 0.75 cm^2/well) as medium densitiy, and a high cell density of 290000 cells/cm^2 (seeded into 96-well plates with 0.31 cm^2/well). Due to the use of different well plates, 90000 cells per well could be seeded for each cell density.

For all later experiments 120000 cells/cm^2 at passage 6-7 were seeded into 96-well plates. Experiments were performed for up to 6 days and in four different media groups of DMEM+FCS+/-TGFβ_3, DMEM SF+/-TGFβ_3 were compared. Cell growth by Hoechst DNA assay, ribonucleic acid (RNA) isolation and quantitative polymerase chain reaction (qPCR), as well as ARS staining of deposited ECM were determined on day 2, 4 and 6. Relative fold-changes in gene expression were normalised to the level of expression by osteoblasts on day 2 cultured in DMEM + FCS. Statistical analysis was performed on the dCT values.

Some multilayer cultures were performed for only 120h (5 days) and were investigating medium starvation, as well as serum starvation and the potential of TGFβ_3 to prevent osteoblast cell death in this specific culture model. The different media groups are shown in Table 2.2 and include DMEM+FCS, DMEM SF, plus a diluted version of these media with PBS with or without the addition of TGFβ_3. One group was incubated with 1 µM staurosporine, a natural alkaloid from *Streptomyces staurosporeus*, which used for research purposes to induce apoptosis in osteoblastic cells (Chae *et al.* 2000). DNA amounts were quantified every 24 h using the Hoechst method.

Table 2.2: Multilayer culture groups used for medium and serum starvation

Group	Media	Dilution	Addition
1	DMEM+FCS	-	
2	DMEM+FCS	1:2 with PBS	
3	DMEM+FCS	1:2 with PBS	15 ng/ml TGFb3
4	DMEM SF	-	
5	DMEM SF	1:2 with PBS	
6	DMEM SF	1:2 with PBS	15 ng/ml TGFb3
7	DMEM+FCS	-	1 uM Staurosporine

2.2.16.2. Pellet Cultures of Primary Human Osteoblasts

Human primary osteoblasts were used for 3D pellet cultures. For the first 2 sets of pre-experiments different culture conditions were evaluated. The initial experiment investigated in which way osteoblast pellets could be generated. Therefore, 90000 or 36000 cells per well were seeded and cultured in V-shaped tissue-culture-non-treated sterile 96-well plates. Four different pellet preparations were tested, each containing up to 23 pellets per group [Table 2.3]. Group A pellets were cultured in SF medium, pellet formation was achieved by overnight culture on an orbital shaker (Labnet Orbit300) at 40 rpm. Pellet formation in group B was achieved by initial centrifugation at 500 xG for 10 min, culture medium was SF. The pellets of group C and D were allowed to form on their own. Group C contrary to D was cultured SF in order to reduce the coating of the culture well walls with FCS proteins. Pellets were cultured for 2 days and visualised using an Axiovert 40 CFL microscope (Zeiss).

Table 2.3: Pellet culture cell seeding plan for experiment 1.

Group	Cells	Centrifugation	Shaking	Medium day 1
A	36000 or 90000	No	Yes	SF
B	36000 or 90000	Yes	No	SF
C	36000 or 90000	No	No	SF
D	36000 or 90000	No	No	FCS

A further pre-experiment was performed using 36000 or 19000 cells for initial seeding. Each group consisted of 3 pellets. Eppendorf tubes were used as

culture vessels to increase media volume to 750 µl. Cells were seeded into SF medium for day 1, some cell pellets were cultured in DMEM + FCS from day two [Table 2.4, groups A-D]. Pellet groups E-H were supplemented with 2.25 cm^2 or 0.75 cm^2 '2D MicroHex' micro-carriers. Cell growth and viability were determined by the trypan blue method. Cultures were performed until day 5.

Table 2.4: Pellet culture cell seeding plan for experiment 2.

Group	Cells	Centrifugation	Micro carrier
A	19000 or 36000	Yes	-
B	19000 or 36000	Yes	-
C	19000 or 36000	Yes	-
D	19000 or 36000	Yes	-
E	36000	No	2.25 cm^2
F	36000	No	0.75 cm^2
G	19000	No	2.25 cm^2
H	19000	No	0.75 cm^2

For all further experiments, cells at passage 6 were seeded for pellet cultures into 96-V-shaped-non-tissue-culture coated well plates at 36000 cells per well. Pellet formation was achieved by centrifugation at 500x G for 10 min. Loosely formed cell pellets were transferred individually into 1.5 ml Eppendorf tubes and cultured overnight under continuous orbital shaking at 40 rpm in 750 µl SF medium per pellet. All cultures were performed in the absence of β-glycerol phosphate and dexamethasone. Medium was replaced on day 1 and then every 48 h with DMEM + 10% FCS. Comparative monolayer cultures were performed in 12-well plates with 36000 cells per well. Pellets were analysed on day 3, 5 and 7 for cell growth using Hoechst DNA assay, RNA isolation followed by qPCR, ARS staining and immunocytochemistry. For qPCR analysis, relative fold-changes in gene expression were made relative to gene expression levels in monolayer at culture day 1. Statistical analysis was performed on the fold-change data.

2.2.16.3. Co-Culture of MG-63 Human Osteoblasts and MLO-Y4 Mouse Osteocytes

Co-culture experiments investigating the effect of serum-containing versus defined SF culture medium and TGFβ$_3$ were performed. The co-culture system was established by Mason (2008) using MG-63 osteoblasts and MLO-Y4 osteocytes (Mason *et al.* 2008). The human osteoblast cell line MG-63 was cultured in monolayer until confluence. Cells from passage 16-20 were used for co-culture experiments. The mouse osteocyte cell line MLO-Y4 was kindly provided by Prof. L.F. Bonewald (Kato *et al.* 1997). Cells were cultured in monolayer as reported (Kato *et al.* 1997), and used for experiments between passage 39 and 44. MG-63 - MLO-Y4 co-cultures were prepared in type I collagen gels as previously described (Mason *et al.* 2008). Therefore, 50 mg type I collagen was dissolved over night at 4°C in 7 mM acetic acid to produce 2.5 mg type I collagen per ml solution. DMEM (10x) was mixed 1:1 with deionised water containing 22 g/l $NaHCO_3$. The so produced 5x DMEM was added 1:4 to the 2.5 mg/ml type I collagen on ice. The mixture was neutralised with 1 M Trisbase (pH=12.6) prior to the addition of cells. MLO-Y4 cells were seeded at a density of $1.5x10^6$ cells/ml in 250 µl gels. Each gel was prepared within a well of a 24 well plate. Gels were left to polymerise at 37°C for 1 h. A layer of $1.6x10^5$ MG63 was seeded on top of each gel. Co-cultures were performed for 6 days in either αMEM + 5% FCS (other supplements as in 2.2.12), or αMEM SF (supplements as in 2.2.2) with or without the addition of 15ng/ml TGFβ$_3$. One ml media was supplied per gel. Media was changed every 48h. The co-cultures were harvested on day 2, 4 and 6. RNA isolations and qPCRs were performed. Gene expression levels were made relative to 18SrRNA levels. Relative fold-changes in gene expression were normalised to the level of expression by osteoblasts on day 2 cultured in αMem + FCS. Statistical analysis was performed on the dCT values. Cultures were also investigated with LDH and TUNEL assay for viable and dead cells. Immunochemical analyses were performed.

2.2.16.4. Culture of Primary Human Osteocytes

Primary human osteocytes were cultured to investigate the influence of TGFβ$_3$ on viability. Cells at passage 2 were used and seeded in monolayer on top of a type I collagen-coated Lab-Tek™ - chamber slides™. The coating was prepared using a type I collagen solution (1 mg/10 ml), which was prepared in 0.1 M acetic acid and sterile filtered prior to use. The chamber slides were incubated with the solution for 4 h at ambient temperature. Prior to cell seeding at 8000 cells/cm^2, chamber slides were cleared from the solution and washed twice with sterile PBS. Cells were seeded in DMEM + 10% FCS and left overnight in this medium, prior to culture in α-MEM + 10% FCS. α-MEM contains more amino acids, vitamins (most importantly it already contains vitamin C), lipoic acid, as well as ribo- and deoxyribonucleotides in its medium formulation than DMEM. Additionally, to the general media constituents described in 2.2.12, this medium preparation contained 5 mM β-glycerolphosphate, which is thought to be a necessary substrate for calcification probably due to the hydrolysis via TNSALP (Whyte 1994). Cell death was quantified during 7 days culture using the TUNEL assay.

2.2.17. Quantification of Relative Gene Expression

2.2.17.1. RNA Isolation using TRI Reagent®

RNA was isolated from cell cultures using the Chomczynski method (Chomczynski 1993). TRI reagent® is a very efficient cell lysis agent and contains phenol and guanidine thiocyanate which efficiently block RNase activity. As the name implies, TRI reagent® can be used to isolate not just RNA, but also DNA and proteins from a sample. Only RNA isolation was performed in this study with the use of the reagent. All solutions, Eppendorf tubes and pipette tips were used RNase-free. Gloves and surfaces were treated with either RNaseZap or 70% ethanol to inactivate RNase activity.

The RNA isolation started with the removal of the culture medium from the cells. One ml TRI reagent® per well (containing up to 5 million cells maximal) was added. Cells were incubated with the reagent for 2 min; cell lysis was completed

by up-and-down pipetting. The cell lysate was then transferred to an RNase-free Eppendorf tube containing 5 µl polyacryl carrier Polyarcryl carrier consists of acryl polymer which was designed for use in the isolation of small amounts of RNA. In this form the cell lysate was stored at -80°C prior to RNA isolation of all samples from 1 experiment. On the day of RNA isolation, the cell lysates were thawed at ambient temperature and vortexed for 10 s using a MS2 Minishaker (IKA®) to ensure complete cell disruption of cell aggregates. The lysate was left for 10 min at ambient temperature, to allow the disaggregation of the nucleoprotein complexes. Phase separation was achieved by the addition of 0.1 ml 1-bromo-3-chloropropane instead of chloroform which may decreases the possibility of contaminating RNA with DNA (Chomczynski & Mackey 1995). The samples were immediately shaken vigorously for 15 s and phase separation was left to form for 10 min at ambient temperature. To ensure complete separation, the samples were centrifuged at 16000 xG for 15 min at 4°C using a Eppendorf centrifuge 5415R. It is important to perform this centrifugation at 4°C, to prevent that DNA dissolves in the aqueous phase. After centrifugation, the Eppendorf tubes were collected very carefully from the centrifuge to avoid disturbance of the phase separation. Approximately 80% of the upper aqueous phase containing the RNA was transferred to a new RNase-free Eppendorf tube without touching the DNA-containing interphase or the lower protein-containing organic phase. The RNA was precipitated from the aqueous phase using 0.5 ml ice-cold isopropanol per sample. The tube containing the sample was closed and turned up-side-down several times to ensure complete distribution of the isopropanol. Precipitation was completed at -20°C. After 20 min the tubes were transferred to the centrifuge and the RNA precipitate was collected at 16000 xG for 20 min. The supernatant was carefully removed from the precipitate and the RNA was washed twice with a total volume of 1.2 ml ice-cold 70% ethanol. During this step salts will be removed from the precipitate. Each washing step involved vortexing of the precipitate with the ethanol, collection of the precipitate by centrifugation at 7500 xG for 5 min and removal of supernatant. Finally the RNA was air-dried at ambient temperature for 10 min. The isolated RNA was then solubilised in 15-50 µl of Diethylpyrocarbonate- (DEPC) treated RNase-free water. Solubilisation was performed for 10 min at 56°C. From this point

onward the RNA samples should be treated with great care. They were kept at -80°C for storage and were thawed slowly on ice prior to use.

The amount and purity of the isolated RNA was then determined using a Nano Drop spectrophotometer (ND-1000, witec ag). The concentration was determined at 260 nm wavelength; purity was evaluated from the 260 nm / 280 nm ratio and the 260 nm / 230 nm ratio. While the first ratio describes the amount of nucleic acids versus proteins in the sample, the second ratio is characterised by the amount of nucleic acids versus salts in the sample. Both ratios should be higher than 1.7.

2.2.17.2. Reverse Transcription (RT)

The isolated RNA was transcribed into complementary DNA (cDNA) using a 'MultiScribe Reverse Transcriptase'. In total 0.5 µg RNA per sample were used in the 20 µl reaction volume. All reagents for the RT reaction were kept at -20°C and should be thawed slowly on ice. The reverse transcriptase should be removed from the -20°C freezer only prior to use. A master mix was prepared. All reagent volumes needed were multiplied with the number of samples plus 4 to ensure that enough volume for all reactions is available. For the master mix 2 µl 10x 'PCR II Buffer', 4.4 µl 25 mM magnesium chloride, 4 µl deoxy nucleotide triphosphates (dNTP) mix (each 2.5 mM), 1 µl random hexamers (50 µM), 0.4 µl RNase inhibitor (20 U/µl) and 0.5 µl 'MultiScribe Reverse Transcriptase' (50 U/µl). From this master mix 12.3 µl were added per reaction tube (0.2 µl RNase-free Eppendorf tube). From each sample the appropriate amount of RNA was added into its individual tube. DEPC-treated water was used to fill up the volume to a total 20 µl reaction mix. The tubes were shortly centrifuged to ensure that all reagents are in the bottom of the tube. The reverse transcription was performed using an Eppendorf Mastercycler gradient (Faust Laborbedarf AG). The reaction was performed with 10 min at 25°C for primer incubation, 30 min at 48°C for reverse transcription, 5 min at 95°C for reverse transcriptase inactivation. The samples were then left at 4°C prior to the addition of 20-40 µl of 1x Tris-EDTA buffer (prepared from 100x concentrate with DEPC-treated

water). The buffer will stabilise the pH of the cDNA if thawed and therefore stabilises the samples. Storage temperature of the cDNA is -20°C.

For one set of experiments (2.2.16.5), the RT reactions were performed using Superscript III reverse transcriptase according to manufacturers protocol.

2.2.17.3. Quantitative Polymerase Chain Reaction (qPCR)

The prepared cDNA was used for qPCR with the TaqMan© method. The method utilises the 5'-nuclease activity of the DNA polymerase to hydrolyse a hybridisation probe bound to its target amplicon.

For each gene to be investigated, one forward and one reverse primer was designed with the 'Perl Primer' program. To avoid unspecific amplification of remaining DNA in the cDNA sample, one primer was designed to overlap with an exon-exon-boundary. The primers had a length of approximately 20 base pairs and a melting temperature of 59 +/-1°C. The TaqMan© probe was designed with a length of 13-30 base pairs and a melting temperature of 69 +/- 1°C. The probe contained a 5'-fluorochrome FAM (absorbtion = 520 nm, emission = 494 nm) and a 3'-quencher TAMARA (absorbtion = 588 nm, emission = 556 nm). After annealing of the probe to the specific amplicon, the DNA polymerase will amplify the cDNA supplied form the 3'-primer end onwards, which will lead to displacement and cleavage of the probe. This separates the 3'-quencher from the 5'-fluorochrome of the probe and fluorescence can be detected during the 60°C polymerisation step.

The 20 µl qPCR reaction mix contained 900 nM forward and 900 nM reverse primer, together with 250 nM probe, 10 µl '2x TaqMan© reaction mix' containing the DNA polymerase, 2 µl cDNA and DEPC-treated water to fill up the volume to 20 µl. However, primer and probe concentration were tested for optimal amplification conditions. Therefore, a cDNA dilution series was prepared prior to first use. Diluted, 1:3, 1:9, 1:27 and to 1:81 diluted cDNa in TE buffer was prepared. Primers and probes designed for the amplicon were tested in several concentrations against the 'housekeeping' gene primers and probe set. The

dCT was calculated from each cDNA dilution by subtracting the CT value of the gene to be tested from the 'housekeeping' gene CT. The CT value is the threshold cycle number at which the amplified target reaches a fixed threshold. The CTs of the dilution series of one set of primers and probe concentrations were blotted against the logarithm of dilution. The amplification efficiency of a primers and probe set should be around 100% and can be calculated from the linear regression with the following equation:

$$Efficiency = \left(\left(10^{\frac{-1}{growth}} \right) - 1 \right) \cdot 100\%$$

Moreover, the dCT of the gene of interest and the house-keeping gene can be blotted against the logarithm of dilution. If the growth of the linear regression is below 0.1 the primers and probe concentrations used for both genes show similar amplification efficiencies. Relative quantification of gene expression by TaqMan© was performed for the following genes of interest, primer and probe sequences are given in Table 2.5. The 18S-rRNA primers-probe TaqMan© mix was bought from Applied Biosystems and was used as 'housekeeping' gene overall human primary osteoblast cultures.

All qPCR reactions were pipetted into optical 96-well plates (ABgene® PCR plates, Thermo Scientific). Plates were sealed (Absolute QPCR Seal, Thermo Scientific) and the qPCR was run with an 'AB 7500 Real Time PCR System' (Applied Biosystems) with the following settings – the hot-start DNA polymerase was activated at 95°C for 10 min and then 40 cycles of 15 s at 95°C followed by 1 min at 60°C were run.

All plates of one experiment were then imported into 1 relative qPCR study using the Applied Biosystems software. Therefore, the system automatically calculated an averaged threshold per gene for all plates within the study / experiment. This threshold CT was used for dCT calculation by subtracting the CT from the gene of interest from the CT of the 'housekeeping' gene of that sample. Moreover the ddCT was determined by defining one sample (day 1 or untreated) as the internal calibrator. The dCT of that sample was subtracted from each individual dCT of all samples to create the ddCT. The relative fold

change in gene expression was determined by calculating the 2^{-ddCT} per sample (Livak & Schmittgen 2001).

Table 2.5: RT-qPCR was performed to investigate relative expression levels of Runx2, osterix, type I collagen, osteocalcin and E11. The table shows the primer and probes sequences used.

Gene	Forward primer	Reverse primer	Probe
human Runx2	AAG CAG TAT TTA CAA CAG AGG GTA CAA G	GGT GCT CGG ATC CCA AAA	CAT CAA ACA GCC TCT TCA GCA CAG TGA CAC
human osterix	CCT GCT TGA GGA GGA AGT TCA	GGC TAG AGC CAC CAA ATT TGC	TCC CCT GGC CAT GCT GAC GG
human type I collagen	CCC TGG AAA GAA TGG AGA TGA T	ACT GAA ACC TCT GTG TCC CTT CA	CGG GCA ATC CTC GAG CAC CCT
human osteocalcin	AAG AGA CCC AGG CGC TAC CT	AAC TCG TCA CAG TCC GGA TTG	ATG GCT GGG AGC CCC AGT CCC
human E11/podoplanin	GGT ACT CGC CCT AAA GAG CTG AA	GCA CAG AGT CAG AAA CGG TCT TTT	TTA CGC CCT GCT GCC AAC GTG C

For one set of experiments (2.2.16.5), qPCRs were performed using the Sybr Green method. Therefore, instead of using TaqMan© probes, the nucleic acid stain (cyan dye) Sybr Green was added to the reactions in the form of 'Sybr Green master mix'. The primer sequences for this set of experiments are shown in Table 2.6. The PCR setting were the same as with the TaqMan© method. Primer optimisation was performed by preparation of individual serial primer dilutions from 800 nM to 200 nM. Each forward primer dilution was then combined with each reverse primer dilution. PCR was performed and a melting curve was included at the end. An optimal melting curve should only show 1 product peak, which characterises the product amplified. The optimal primer concentrations were determined from the combinations resulting in good melting curves. From these, the one primer dilution combination with the lowest

CT value in combination with a high peak value was determined as optimal. Moreover, this primer concentration combination was tested for efficiency as described above using serial cDNA solutions.

Table 2.6: RT-qPCR was performed to investigate relative expression levels of human GAPDH, human Runx2 and human osteocalcin in MG-63 cells, as well as mouse 18SrRNA, mouse E11 and mouse osteocalcin in MLO-Y4. The table shows the primer sequences used.

Gene	Forward primer	Reverse primer
human GAPDH	GGT ATC GTG GAA GGA CTC ATG A	GGC CAT CCA CAG TCT TCT G
human Runx2	GTG GAC GAG GCA AGA GTT TC	TTC CCG AGG TCC ATC TAC TG
human osteocalcin	GGC AGC GAG GTA GTG AAG AG	GAT CCG GGT AGG GGA CTG
human type I collagen	CCC TGG AAA GAA TGG AGA TGA T	ACT GAA ACC TCT GTG TCC CTT CA
mouse 18SrRNA	GCA ATT ATT CCC CAT GAA CG	GGC CTC ACT AAA CCA TCC AA
mouse E11	AAG ATG GCT TGC CAG TAG TCA	GGC GAG AAC CTT CCA GAA AT

2.2.18. Analysis of Alkaline Phosphatase (ALP) Activity

2.2.18.1. Quantitative Alkaline Phosphatase Activity Determination

Prior to the performance of the ALP assay, the culture medium was removed from the cells and they were washed once with ice-cold PBS. The cells were incubated with 300 µl lysis buffer consisting of 0.1% Triton-X in 10mM Tris-HCl at pH 7.4 and protease inhibitor cocktail (1:1000) for 3 h on an orbital shaker. Samples were then stored at -20°C until analysis. All samples of 1 experiment were thawed and analysed at the same time. Several buffer had to be prepared for the ALP assay.

The alkaline buffer was prepared by 1:7 dilution of the stock to a final concentration of 1.5 M using deionised water. The pH was adjusted to 10.3. The alkaline buffer was ready for use. The substrate solution was prepared using a diethanolamine buffer. Therefore, 1 M diethanolamine were prepared in a 0.5mM magnesium chloride solution. For the preparation of around 4.5 ml diethanolamine buffer 4 ml of a 0.553 mM magnesium chloride solution were mixed with 424 µl of diethanolamine. The pH of the final solution was adjusted to 9.8. The substrate solution was completed by the addition of 25 mg NBT per 1 ml of the diethanolamine buffer. Due to the light sensitivity of the NBT substrate all further steps were performed avoiding direct light exposure. A standard curve was prepared diluting the p-Nitro phenol standard (10 mM) 1:10 with 0.1 % Triton-X in 10mM Trizma-Base to a final concentration of 1 mM. From the diluted standard solution 0, 10, 20, 30, 40, 50, 60 and 70 µl were added to Eppendorf tubes and made up to 100 µl with 0.1 % Triton-X in 10mM Trizma-Base. The standards were then treated as the samples.

Cell lysate samples were thawed on ice and 200 µl per sample and only 100 µl standards (+ 100 µl deionised water) were added to Eppendorf tubes. Then 250 µl of alkaline buffer and 50 µl substrate solution were added. During this addition the reaction mix was kept on ice. The reaction was started simultaneously for all samples with incubation at 37°C. During the incubation the reactions will develop a yellow colour which correlates with the activity of the sample ALP. Standards were incubated for exactly 15 min, while samples were incubated up to 45 min if ALP activity was very low. The enzyme was inactivated by the addition of 500 µl 0.1 M NaOH. Aliquots of each sample and standard were measured in duplicates (250 µl each) at 405 nm absorbance using a PE HTS 7000 Bio Assay Reader.

2.2.18.2. Qualitative Alkaline Phosphatase Activity Staining

The qualitative analysis of ALP activity was performed using the 'Leukocyte ALP kit'. Therefore cells washed with ice-cold PBS and then 'fixed' in 60% citrate-buffered acetone for 5 min at ambient temperature. Excess fixative was washed from the cells with deionised water. The alkaline-dye mixture was

prepared according to manufacturers instructions. One capsule containing 'Fast Blue RR' was added to 48 ml deionised water. The solution was mixed well and 2 ml 'Naphthol AS-MX Phosphate Alkaline Solution' were added. The alkaline-dye mixture was ready for use and cells were incubated for 45 min with the solution avoiding direct light exposure. The dye solution was removed from the cells and excess was washed off with deionised water. The cells could then be treated for mounting or in the case of cell pellets for cryo-sectioning.

2.2.19. Determination of Calcium Deposition

2.2.19.1. Alizarin Red S Staining

Cells were washed with PBS prior to fixation with pre-cooled (4°C) 4% neutrally phosphate-buffered formalin for 15 min. The fixative was removed from the cells and cells were washed with Millipore water for 5x. The Alizarin Red S staining was performed in the dark to protect the light-sensitive substance. Therefore, 200 µl Alizarin Red S stock solution per 96-well, or 500 µl per 24-well or per 48-well or per pellet were used. The staining solution contained 40 mM Alizarin Red S and was buffered with ammonium hydroxide to pH = 4.2 (Puchtler *et al.* 1969). Alizarin Red S is a Ca^{2+}-chelating agent and stains calcified extracellular matrix in a bright red colour. Staining was performed at ambient temperature for 1 h on an orbital shaker. Afterwards, the onbound excess dye was removed by repeated washing with deionised water and 15 min incubation with PBS. The time for water incubation was 1 h for monolayer and overnight for all 3D cultured constructs – multilayer and pellet. The stained cell cultures could then be stored dry at ambient temperature until quantification. If required, they were used for visualisation with an Axioplan microscope (Zeiss).

2.2.19.2. Quantification of Bound Alizarin Red S

The calcium staining with Alizarin Red S was dissolved in 300 µl 10% w/v cetylpyridinium chloride monohydrate (CPC) solution (pH = 7) (Gregory *et al.* 2004). Monolayer cultures were dissolved for 1h. However, all 3D cultured constructs – multilayer and pellet were dissolved overnight on an orbital shaker to ensure complete dissolving of the bound Alizarin. Alizarin Red S standards

were prepared from the stock solution. The concentration ranged from 1 mM to 5 µM in CPC. The CPC blank, the samples and the standards were measured in duplicates. Therefore, twice 140 µl were transferred into wells of a 96-well plate (TPP) per sample. Absorbance was measured at was performed at 545 nm using a VICTOR$^3{}_{TM}$ plate reader (Perkin Elmer). The amount of bound Alizarin Red S was made relative to the amount of DNA in the sample.

2.2.20. Statistical Analyses

Data collected underwent statistical analysis using SPSS 16.0 software package. Data sets were investigated for normal distribution by the use of a histogram, a normality-probability plot, and the Kolmogorov–Smirnov test.

For normally distributed data, Student's t-test was used to compare 2 groups. If the experiment consisted of more than 2 groups, one-way (univariant) analysis of variance (ANOVA) and Tukey post-hoc test were used. Some data sets were not normally distributed. Therefore, data transformation using logarithm or fraction calculation was performed to gain normal data distribution.

In some cases normal distribution was not achieved. For this data non-parametric tests were used. Statistical significance was determined using the Kruskal-Wallis test for more than 2 groups and the Mann-Whitney test with Bonferroni correction to determine the correct probability value (p). For non-normally distributed data involving only 2 groups, the Mann-Whitney test was used.

For all investigations statistical significance was set as $p \leq 0.05$

Chapter 3: The Effect of Dynamic Loading on Bone Mass – a Micro-Gravity Experiment

Missions to space have been a dream of mankind for decades. With the start of the first manned missions in the 1960s through the Russian and American space agencies, negative effects on the human body were recognised and their investigation as well as intervention are of great interest (Baisden *et al.* 2008). Several physiological problems might occur in response to weightlessness. The heart muscle suffers during prolonged exposure to weightlessness due to the redistribution of body fluids away from the extremities during a space mission, causing changes in cardiovascular physiology. Further impacts are made on the neurovestibular system. Astronauts may have problems standing up, stabilising their gaze, walking and turning immediately after landing and returning to gravity. The loss of the 24-hour day/night cycle can cause disruption of the circadian rhythm leading to sleep loss and increased levels of stress. Moreover, the space radiation caused by heavy ions present is one of the major risks on astronauts health and remains a main challenge during prolonged missions outside of the earth protective magnetic field (Baisden *et al.* 2008). Concerning the musculoskeletal system, weightlessness has a huge impact on muscle mass and strength. Trappe *et al.* showed that astronauts during a 6-month space mission on the International Space Station (ISS) experience a 13 +/- 2% decrease in calf muscle volume and an overall 32% lower peak power in comparison to pre-flight situation (Trappe *et al.* 2009). In relation to the loss of muscles which can be regained within 30-60 days post-flight, one major problem during manned space missions is the loss of bone mass by the astronauts (LeBlanc *et al.* 2000). The phenomenon is similar to the loss of bone that can be seen in patients experiencing prolonged bed rest (LeBlanc *et al.* 2007). Data from spaceflights to the Russian space station Mir and the ISS demonstrate the impact of weightlessness on bone volume, especially considering that astronauts perform a controlled exercise during missions using a treadmill, a stationary bicycle, and a bowflex. LeBlanc *et al.* demonstrated an overall monthly loss in areal bone mineral density (aBMD) of 0.3%, with 97% of

that occurring in the pelvis and legs after a Mir space mission (Lang *et al.* 2004; LeBlanc *et al.* 2000). The variation of bone loss in this study was high. Some astronauts lost half of their BMD that would be lost during normal life time, while others experienced only a small BMD reduction. The investigation of Lang *et al.* (2004) on ISS crew members concentrated on relevant areas of frequently seen osteoporotic bone fractures in the elderly – spine and hip – where he showed a decreased aBMD per month of 0.9% and 1.5% respectively (Lang *et al.* 2004). This study also used CT measurements in combination with DXA (dual energy X-ray absorptiometry), and was, therefore, able to recognise that bone is lost in cancellous and cortical areas. These effects on bones seen during spaceflights might not have direct implications for astronauts, but could lead to increased fracture risk and an early onset of age-related osteoporosis (Lang *et al.* 2004).

In bones which have to sustain weight and, therefore, bear load during locomotion or during normal tension, mechanical load plays an important role in the control of bone architecture and mass (Cowin 2001; Lanyon 1987). In load-bearing bones, only the general morphology and development are directly specified by the genome, but the particular features to withstand functional loading are the result of an adaptive mechanism (Lanyon 1987; Lozupone *et al.* 1996; Rawlinson *et al.* 1995). Frost (1987) believed that in a specific range, mechanical strain affects bone formation and resorption (Frost 1988). It seems that the experience of weightlessness at micro-gravity is one of the factors influencing the maintenance of bone, as it is been proposed by Klein-Nulend *et al.* (Klein-Nulend *et al.* 2003). To overcome bone loss during space missions a specific exercise or medication is needed to support the astronauts during a long-term mission. The pharmacokinetics in weightlessness of applied medicines, i.e. bisphosphonates to reduce bone resorption in space, are not yet completely understood (Shapiro 2006). As strain is a major stimulator of bone remodelling (Lanyon 1987), physical exercise during space missions is, therefore, of great interest to counteract the loss of bone. Contrary to static mechanical loading, cyclic loading induces fluid flow inside the lacunar-canalicular system (Rubin & Lanyon 1984). The fluid flow is thought to be responsible for the translation of exercise/mechanical loading to a remodelling

stimulus by the creation of shear stresses on the surfaces of loaded osteocytes (Rubin & Lanyon 1984). The correct load conditions will influence bone remodelling processes and possibly create an anabolic stimulus to prevent the bone loss experienced at micro-gravity.

An unmanned Foton-M3 mission was launched from Kazakhstan by the European Space Agency (ESA) in corporation with the Russian Federal Space Agency (Roscosmos) at 13:00 central European summer time on the 14th September 2007. The Foton capsule [Fig. 3.1 right side] carried 40 European experiments – a total of 400 kg experiment payload. Experiments from different research areas were part of the mission, ranging from fluid physics, biology, crystal growth, radiation exposure and exobiology. The mission duration was 12 days in weightlessness – where the Foton was in a near circular orbit around the earth inclined at 63° with maximum and minimum attitude of 305 – 260 km (which resembles to around 9% loss of earth's gravity and is generally termed 'micro-gravity'). A total of 190 orbits were completed by the Foton each lasting 90 min. The Foton-M is an improved version of a spacecraft that offers the advantages of thermal control and increased data flow. Therefore, it was possible to implement biological experiments into these mission (www.esa.int 2009).

Fig. 3.1: Image of the Foton spacegraft (right side) inside and next to the Soyuz-U launcher unit prior to launch. The Foton consisted of 3 modules – the battery module which provided the payload with 500 W daily (A), the re-entry module that housed the scientific payload (B) and the service module that contained the attitude control system (C). Image source www.esa.int.

The FreqBone experiment was part of the Foton mission. The aim of the experiment was to investigate the effect of mechanical high-frequency (30 Hz), low amplitude (30 N) loading on bone remodelling at normal and micro-gravity (Jacobs *et al.* 1998). The planning and performance of the FreqBone experiment was done exclusively by the Biomechanics and Engineering Design Section at the Catholic University Leuven (Belgium) under the supervision of Prof. Jos Vander Sloten with his co-workers Dr. Maarten Van Guyse and Dr. Pieter Spaepen. Prof. Harry van Lenthe together with Dr. Antonia Torcasio are responsible for the finite element analysis of the applied strains within the bone explants. The biological analysis within this study was performed at the AO Research Institute, and the results will be presented in this chapter. Moreover, in preparation for the FreqBone space experiment, a team of collaborators were brought together by the ESA partnership agreement 'AO-99-122' to fulfil specified work packages. Active partners of the agreement were the Biomechanics and Engineering Design Section at the Catholic University Leuven (Belgium) under Prof. Jos Vander Sloten, the group of Prof.

David Jones at Phillipps University Marburg (Germany), the group of Prof. Laurence Vico at Jean Monnet University St. Etienne (France), the Novartis Institute for Biomedical Research represented by Dr. Jürg Gasser, and the group of Prof. Geoff Richards from the AO Research Institute in Davos (Switzerland). Every group contributed experimental data of their individual expertise using a similar bioreactor and culture system to the one that was used for the FreqBone experiment. The results of our group on certain work packages concerning the effect of $TGF\beta_3$ on cultured bone explants, the change in loading regime, the investigation on osteocyte viability, the use of biochemical markers during culture and the improvement of culture conditions will be presented and discussed in Chapter 4.

The FreqBone experiment consisted of four different groups each containing 6 bovine cancellous bone explants (sternum from 1-year old cow). Two groups were cultured at micro-gravity and were either loaded dynamically using a sinusoid curve (30 Hz frequency and 30 N amplitude), or experienced only a static preload of 30 N. Two control groups were cultured at normal gravity and experienced either static or dynamic load as the groups at micro-gravity. The preparation of the bovine cancellous bone explants, as well as the culture medium (DMEM + 10% FCS) was performed at the ESA Research and Technology Centre in Noordwijk (Netherlands) on the 8th September 2007. The FreqBone culture and bioreactor system containing the bone samples was transported on the 9th of September 2007 to Samara (Russia) first, and the onwards to Baikonur (Kazakhstan) by plane. The ground model with the control samples was sent to Leuven (Belgium) by car. All bone explants and culture medium were kept at 10°C for 6 days prior to launch / start of experiment. During the 12 days experimental time at either micro-gravity or at ground level, all bone explants were maintained at 37°C and experienced a daily mechanical load. The culture medium provided was kept at 10°C and was only pre-heated prior to bone explant contact. Medium was exchanged once during the 12 days at micro-gravity, each explant was cultured with 20 ml medium for 6 days prior to exchange. After the experiment, bone explants were stored for 2 more days at 10°C prior to 'fixation' in 70% ethanol. A schematic illustration of the

FreqBone bioreactor and culture system designed by the group of Jos Vander Sloten is shown in figure 2. The culture chambers each containing a 10 mm x 5 mm bone explant are visible in the front illustration (Fig. 3.2A). Culture chambers (black arrow) are in circular arrangement with a circulating loading stack in the centre. Controlling electronics are on the right side of the front illustration. The back illustration (Fig. 3.2B) shows the medium reservoirs (yellow arrow) and the pump system.

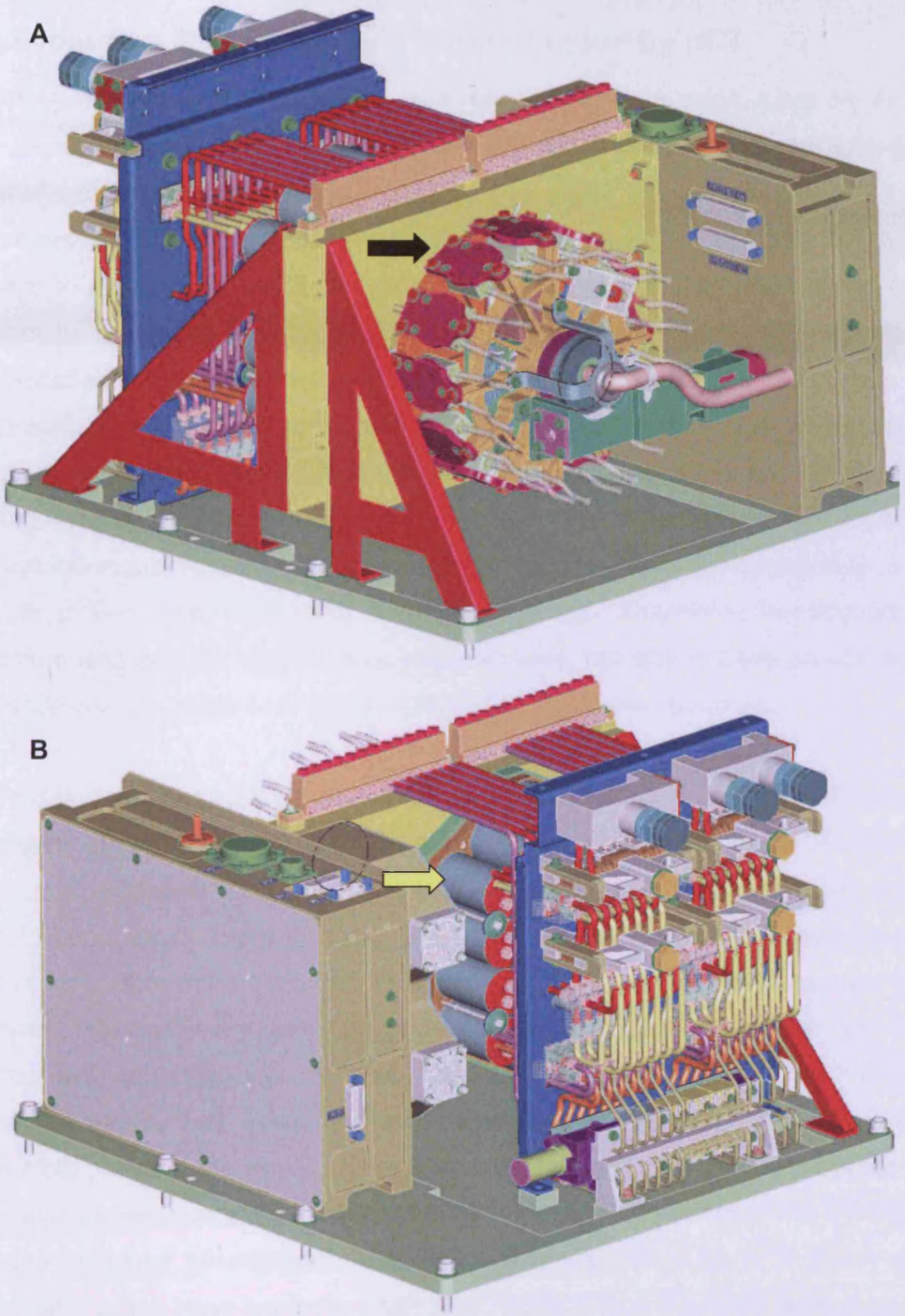

Fig. 3.2: Schematic illustration of the FreqBone bioreactor and culture system from front (A) and back (B) property of the Catholic University Leuven (Belgium). The front side shows the culture chambers (black arrow) that are arranged in a circle with a rotating loading stack in the centre. The yellow arrow on the back side highlights the medium reservoirs. Electronics are visible on the right side of the front illustration. The pump system is shown on the right side of the back illustration.

3.1. The Influence of Micro-Gravity and Dynamic Compressive Loading on Bone Volume – Determination by µCT

There are different possibilities to detect changes in bone mass in *ex vivo* cultured bone explants. The performance of micro-computed tomography (µCT) analysis is an important tool in any *in vivo* study that aims to investigate the processes of bone formation or resorption (Ruegsegger *et al.* 1996). CT uses X-rays to generate cross-sectional images of an object. By rapid rotation of the sample holder or the X-ray tube itself, images are obtained and can be translated into a 3D reconstruction of the measured sample. As X-rays pass through a sample, they are absorbed according to the density of the tissue. The 3D reconstruction of a tissue created from a µCT measurement, relates to the density differences within the sample. Due to this characteristic, it is possible to distinguish soft tissue from mineralised bone. µCT offers the opportunity to work with a very high resolution in the µm range. Therefore, investigations of differences in bone matrix thickness or volume, but also in bone volume versus tissue volume, number of trabeculae and thickness are possible.

To determine possible changes in bone volume between the different treatment groups of the FreqBone experiment, the ethanol-fixed bone explants were used for µCT measurements. A µCT 40 (Scanco Medical) device was used with the following measurement characteristics - 10 µm resolution, 70 kV beam energy, 114 µA, 300 ms integration time and a total of three measurements for averaging. Analysis parameters were bone volume per total volume, trabeculae thickness and trabeculae number. The analyses included a maximum volume of each explant. The edge regions, containing bone debris derived from the sample preparation, were excluded from the analyses resulting in a cylinder of 8 mm diameter and a total of 200 voxels. The defined volume was exactly the same between all explants. The data are presented in figure 3. Every group consists of six bone explants. Data sets for the different groups were compared using one-way ANOVA and Tukey post-hoc test as samples were normally distributed. No statistical significance could be determined. However, there was a trend that dynamic loading under weightlessness resulted in increased bone volume versus total volume [Fig. 3.3A]. The same was true for trabeculae

number and thickness [Fig. 3.3B, C]. This trend correlates with the expected results for the application of an anabolic dynamic loading applied to cancellous bone. Moreover, the unloaded samples (with only a static preload applied) at weightlessness showed a trend towards a decrease in trabeculae thickness compared to the unloaded samples at normal gravity [Fig. 3.3C]. Again, this trend correlated with the expected result, that non-loading in weightlessness leads to loss of bone volume.

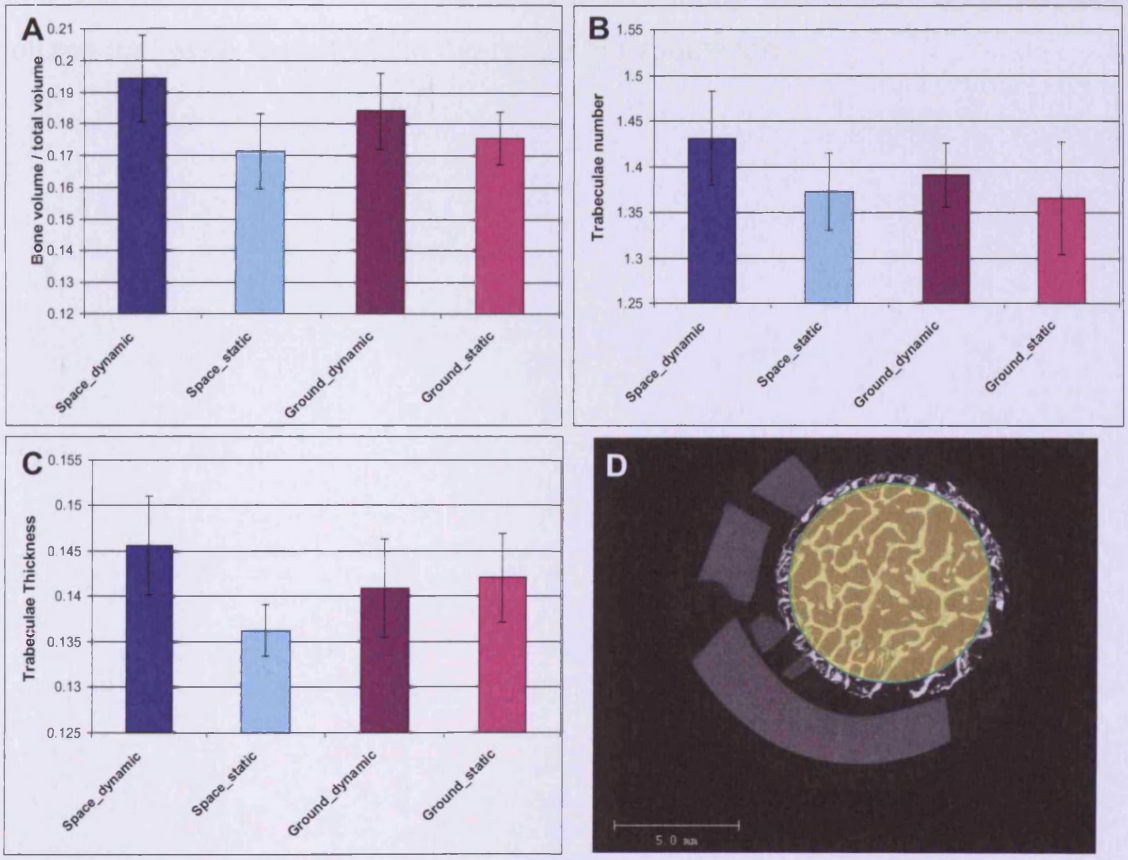

Fig. 3.3: µCT analyses of whole bovine cancellous bone explants (200 voxels, excluding edge regions - D) after harvest from the FreqBone experiment. Bar charts show the mean values for bone volume / total volume both in mm^3 (A), trabeculae number (B) and trabeculae thickness in mm (C) with standard error of the mean. The bone explants of 2 groups were cultured at micro-gravity and either dynamically loaded ('space_dynamic') or experienced static loading ('space_static'). The 2 remaining groups were cultured at normal gravity and either dynamically loaded ('ground_dynamic') or statically loaded ('ground_static'). No significant differences were detected.

To possibly reach statistical significance within the data sets, the original cylinder was divided into a centre cylinder of 5 mm diameter and the remaining outer ring. In theory, the central area should have experienced a slight reduction in nutrients in comparison to the outer ring. The data sets for outer ring [Fig. 3.4A, C, E] and central area [Fig. 3.4B, D, F], however, did not show statistical significance either. The trends for trabeculae number varied between centre and outer ring, which might be due to the small amount of volume investigated in each group. Yet, the trends in trabeculae thickness and bone volume versus total volume were the same as seen investigating the 'whole' bone explant volume and were, therefore, in the range of expectation.

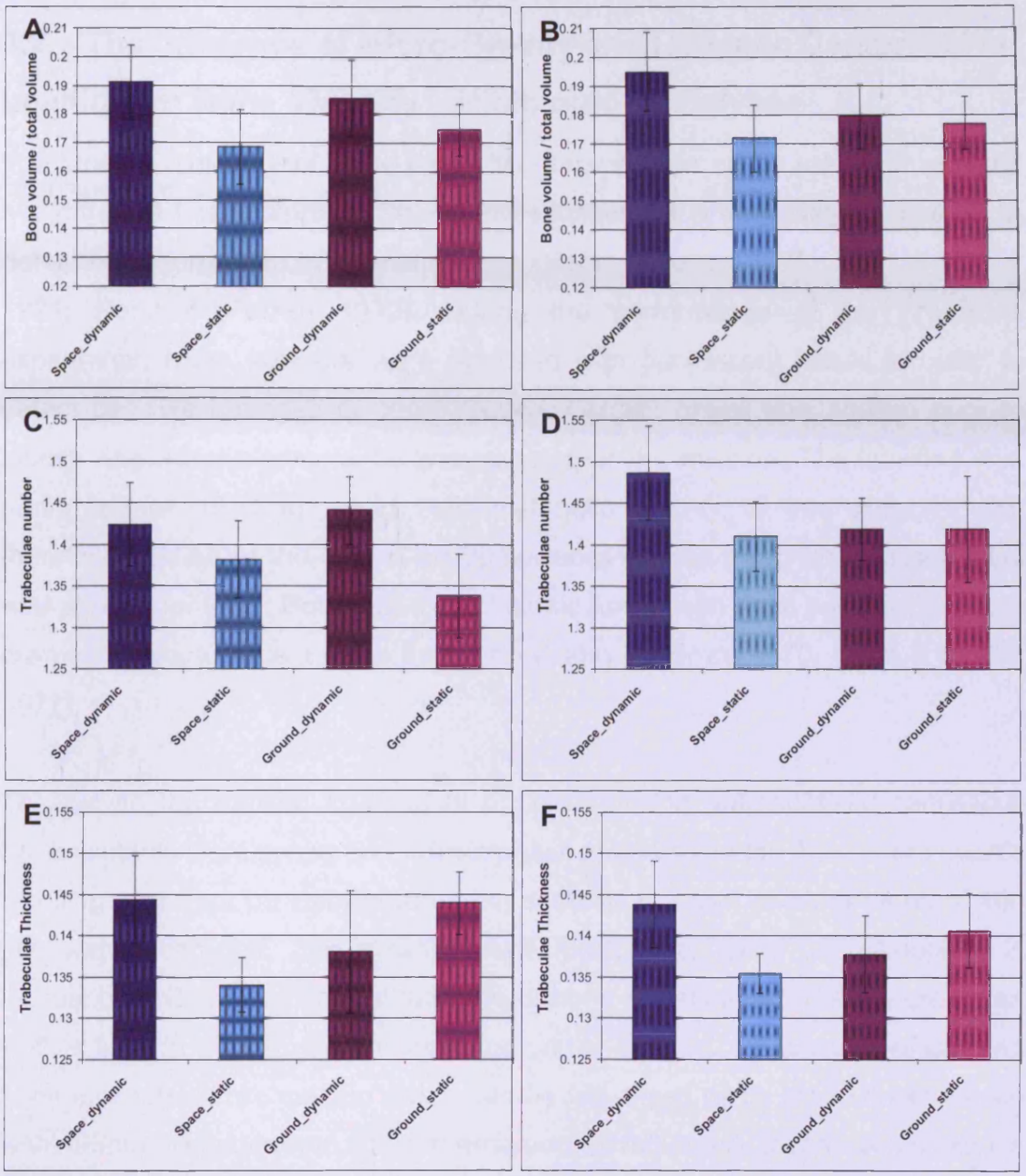

Fig. 3.4: µCT analyses of centre region (B, D, F) and outer ring (A, C, E) of bovine cancellous bone explants after harvest from the FreqBone experiment. Bar charts show the mean values for bone volume / total volume both in mm^3 (A, B), trabeculae number (C, D) and trabeculae thickness in mm (E, F) with standard error of the mean. The bone explants of 2 groups were cultured at micro-gravity and either dynamically loaded ('space_dynamic') or experienced static loading ('space_static'). The 2 remaining groups were cultured at normal gravity and either dynamically loaded ('ground_dynamic') or statically loaded ('ground_static'). No significant differences were detected.

3.2. The Influence of Micro-Gravity and Dynamic Compressive Loading on Bone Viability – Histological Analyses

The 'double labelling' of bone using fluorescent dyes which are given at least two different time points during an investigation, is a very elegant method to detect bone formation in animal studies (Rahn & Perren 1970; Rahn & Perren 1971; Rahn & Perren 1972). During the performance of the FreqBone experiment, bone explants were perfused with fluorescent labels in order to detect possible changes in bone volume. Calcein green was applied prior to launch and alizarin complexon was given after the mission. The labelling was performed at 10°C to avoid high metabolic activity of the explants and, therefore, uptake of the dye at active surfaces (Harris *et al.* 1962). Each label was applied for 24 h. Both fluorescent labels have been used successfully over the past years to detect bone formation (Rahn & Perren 1970; Rahn & Perren 1972).

To analyse the applied fluorescent double labelling, ethanol-fixed cancellous bone explants were prepared for histological analysis. As the fluorescent double labelling could not be visualised in thin sections (12 µm), thick sections of 100 µm were prepared. Visualisation was performed using an 'Axioplan 2' microscope (Zeiss). Individual labelling of bone surfaces with calcein green, as well as alizarin complexon was detected on the surface of trabeculae of cultured bone explants. While calcein green can be visualised by its green fluorescence with 495nm excitation and 515 nm emission wavelengths, alizarin complexon is of red fluorescence and can be visualised at 520 nm excitation and 625 nm emission wavelengths [Fig. 3.5A, B]. However, it needs to be considered that both applied labels bind to all 'free calcium binding sites'. This means on one hand, that labels can bind during bone formation but also during bone resorption processes when free calcium binding sites are made available by the activity of osteoclasts. Direct labelling of resorption sites can thus come about because the osteoclasts expose binding sites for the fluorochromes. Labelled resorption lacunae remain visible in the section, but only when the resorption process does not proceed further after application of the fluorochrome (Hulth & Olerud 1962; Olerud & Lorenzi 1970; Rahn 2003). Due to the absence of the

vascular system, removal of unspecific precipitated dyes or non-bound dyes is not optimal in the *ex vivo* culture situation of bone organ culture (Davies 2005). This effect was demonstrated by the immense bone edge labelling of drilled or cut bone explant surfaces, as well as the extensive labelling of bone debris that derived from preparation [Fig. 3.5C, D].

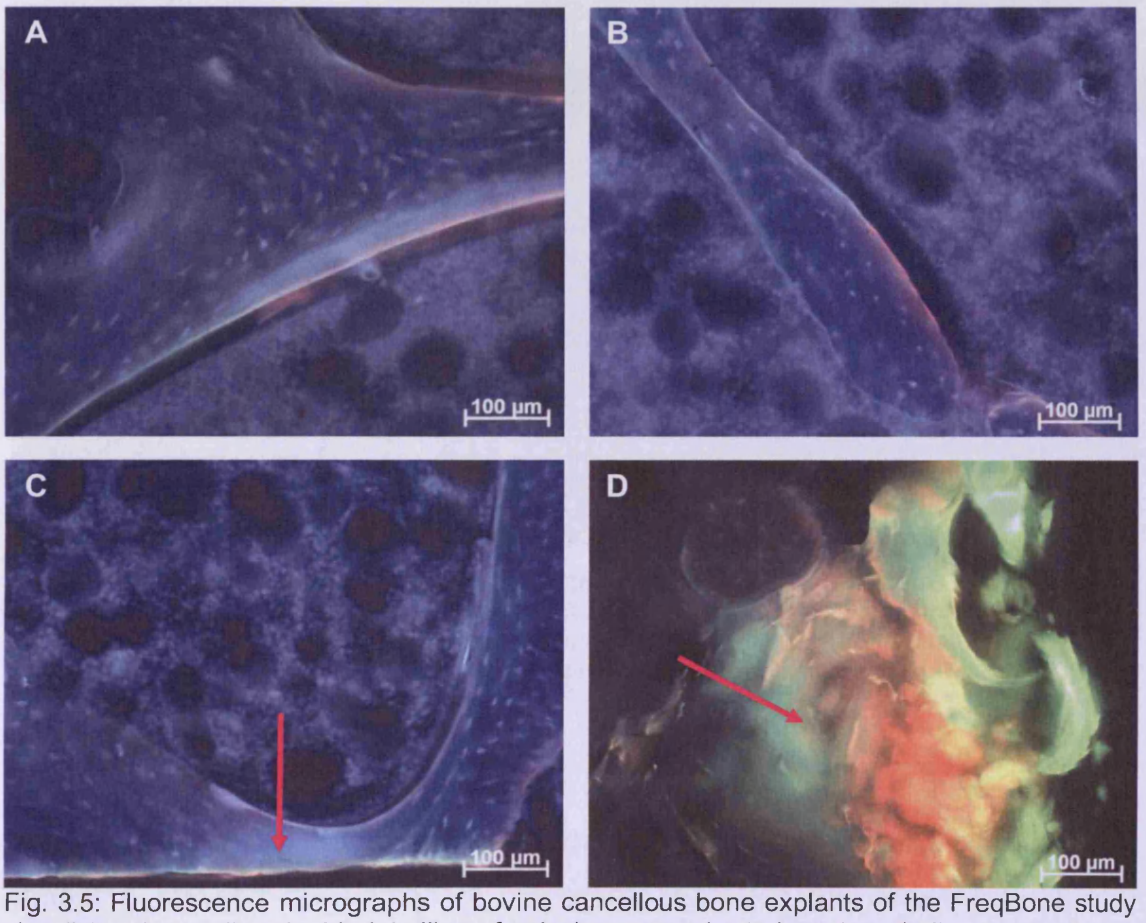

Fig. 3.5: Fluorescence micrographs of bovine cancellous bone explants of the FreqBone study visualising the applied double labelling of calcein green prior to launch and alizarin complexon after recovery of the explants. Individual green and red labelling of bone surfaces is shown in **A** (explant nr. 5; flight_dynamic) and **B** (explant nr. 12; ground_static). Additionally, unspecific labelling (marked by pink arrows) of drilled, edge surfaces (**C**; explant nr. 3; flight_static) and of bone debris can be found (**D**; explant nr. 21; flight_dynamic).

The analysis of bone formation rate and mineral apposition was not possible as a distinct double label of one alizarin complexon band on top of one calcein green band was not found. Only single bands of either fluorochrome or a yellow band created by the overlapping of both fluorochrome bands could be detected. To evaluate cell activity of cells inside the bone explants, label penetration into the bone explants was measured [Fig. 3.6]. Therefore, fluorescent micrographs

from the whole area of the centre section from each explant were taken. The single micrographs were stitched together to represent this section of each explant. The maximal label penetration was then measured at the diameter penetration site, as well as height penetration side.

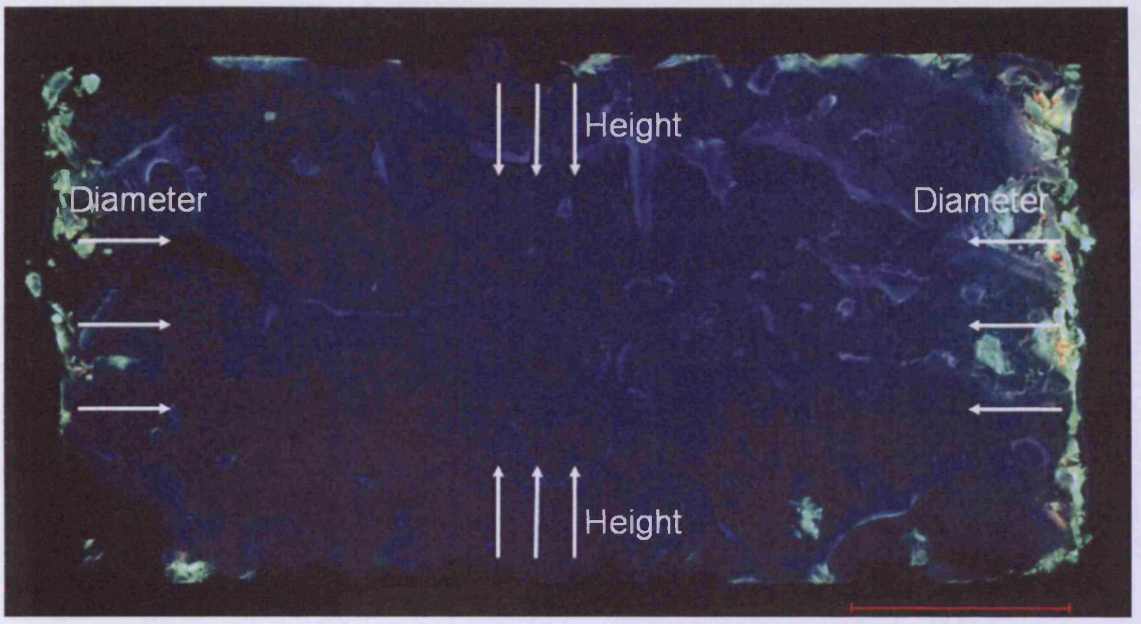

Fig. 3.6: Fluorescence micrographs stitched together to show one complete section of a bovine cancellous bone explant (nr. 21, flight_dynamic). Maximal label penetration of calcein green and alizarin complexon inside the section was measured and classified to 2 different regions – bone explant height and diameter. Scale bar represents 2 mm. Strong labelling of free binding sites on the core surface and debris is evident

The comparison of label penetrations of the single labels (calcein green or alizarin complexon) at either penetration site (explant height or explant diameter) showed no significant differences between the different FreqBone experimental groups. However, the overall calcein penetration depth at either diameter or height penetration side was significantly greater (one-way ANOVA and Tukey post-hoc test) compared to alizarin penetration ($p=0.001$) [Fig. 3.7]. The average calcein diameter penetration was 1.65 mm (+/- 0.45 mm) while alizarin diameter penetration was only 0.93 mm (+/- 0.18 mm) on average ($p \leq 0.0001$). Height penetration was lower with 1.33 mm (+/- 0.39 mm) on average for calcein green and 0.69 mm (+/- 0.33 mm) for alizarin complexon ($p \leq 0.0001$). Both labels represent experimental time points, calcein green specifies the time point zero prior to launch, while alizarin complexon specifies the end point of the experiment after explant recovery. Therefore, the decrease

in label penetration of alizarin complexon in comparison to calcein green describes a reduction in label penetration over time.

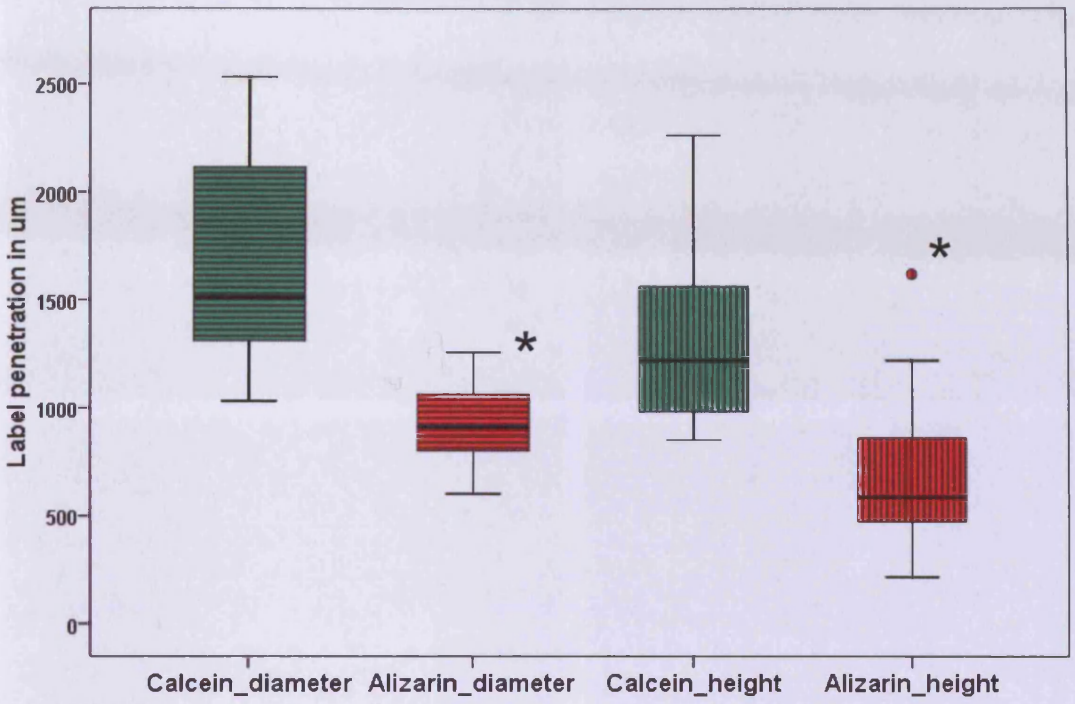

Fig. 3.7: Calcein green and alizarin complexon penetration depth into bovine cancellous bone explants from the FreqBone experiment at two penetration sites (explant height and explant diameter). Calcein green penetration, which can be defined as time point zero, was significantly increased (one-way ANOVA and Tukey post-hoc test; * $p \leq 0.01$) compared to the alizarin complexon penetration (end time point). Box plots show the median line, the 25% and the 75% quartiles which define the box, the 1.5x interquartile-range whiskers, as well as outliers (○).

To investigate the morphological state of the cultured bone explants, sections used for the visualisation of the fluorescent double labelling were stained with Giemsa and eosin. The presence of mainly red bone marrow was detected in the samples [Fig. 3.8E]. Moreover, the presence of an *ex vivo* organ 'culture phenomenon' - the surface fibrous membrane could be visualised additionally [Fig. 3.8F]. This layer of fibroblast-like cells can cover the surface of a cultured organ to a varying extent and its importance and quantification will be discussed in Chapter 4. Furthermore, several intact osteoclasts were detected after 20 days FreqBone experimental culture time [Fig. 3.8A, C]. Osteoclasts as multinucleated mature bone-resorbing cells have a life span of only 12.5 days *in*

vivo (Hill 1998). The presence of osteoid seams - newly deposited, non-mineralised matrix – could also be visualised [Fig. 3.8B, D].

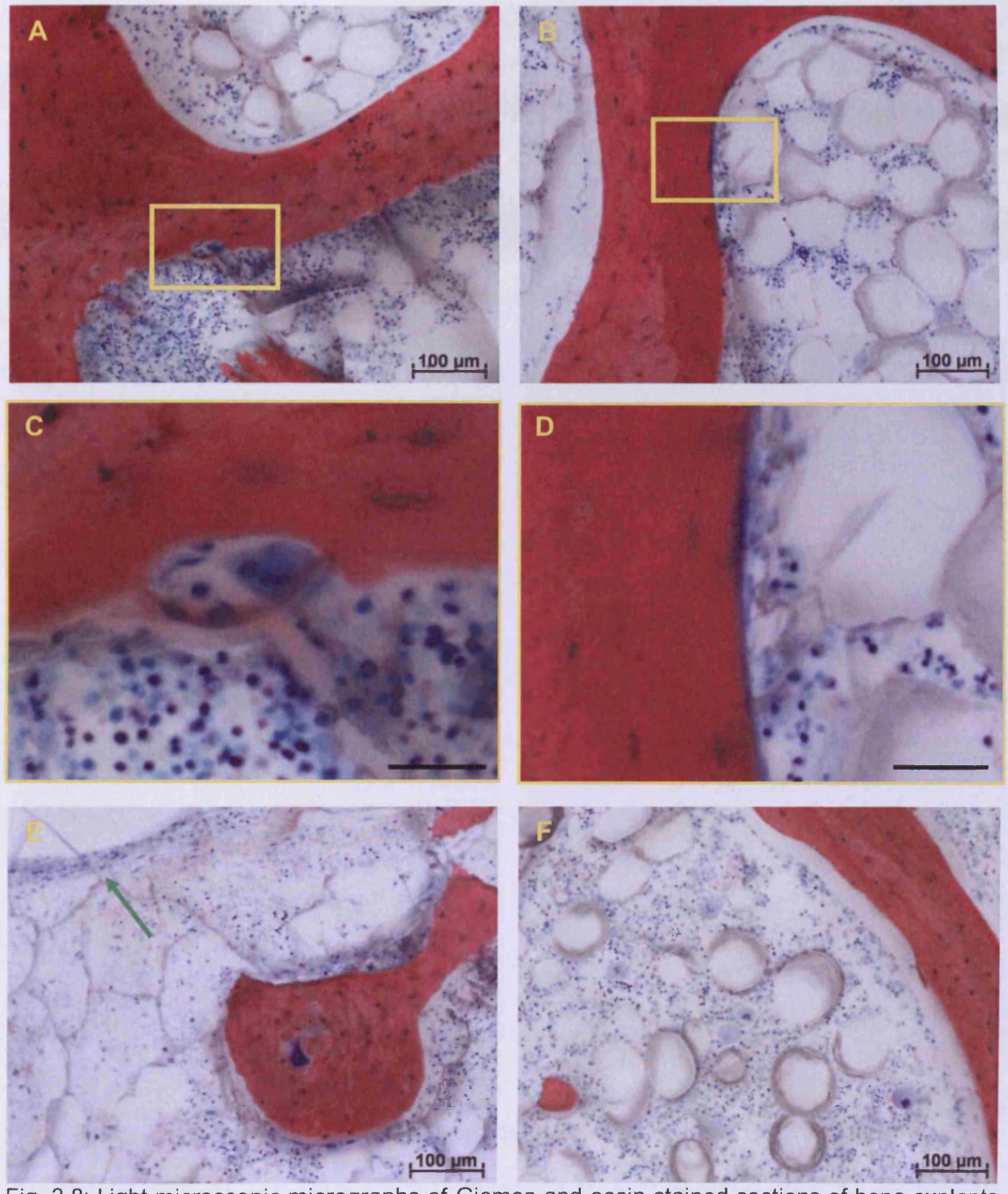

Fig. 3.8: Light microscopic micrographs of Giemsa and eosin stained sections of bone explants from the FreqBone experiment. A: Micrograph shows bone matrix labelled red, haematopoietic bone marrow (blue), and the presence of a multinucleated osteoclast (blue) lying in its resorption lacunae (C close-up of osteoclast). B: Micorgraph shows bone matrix (red), haematopoietic bone marrow (blue), and the presence of an osteoid seam (blue; D close-up of osteoid). Close-up scale bar represents 25 µm. E: The fibrous membrane is demonstrated on the surface of the cancellous bone section (green arrow). F: Micrograph shows the predominance of the red, haematopoietic bone marrow (blue) in between the cancellous bone trabeculae (red).

It was possible, to correlate areas of bone formation and resorption activities seen by the detection of osteoid seams and intact osteoclasts respectively with fluorescent double labelling in order to demonstrate that labelling was specific to the cellular activity during FreqBone mission. The four micrographs in figure 3.9 show the overlapping osteoclast presence inside its resorption lacuna and alizarin complexon labelling [Fig. 3.9] demonstrating that bone resorption can be correlated with the labelling. The presence of osteoid and overlapping alizarin complexon labelling [Fig. 3.10] highlights the significance of the calcium labelling also to bone formation.

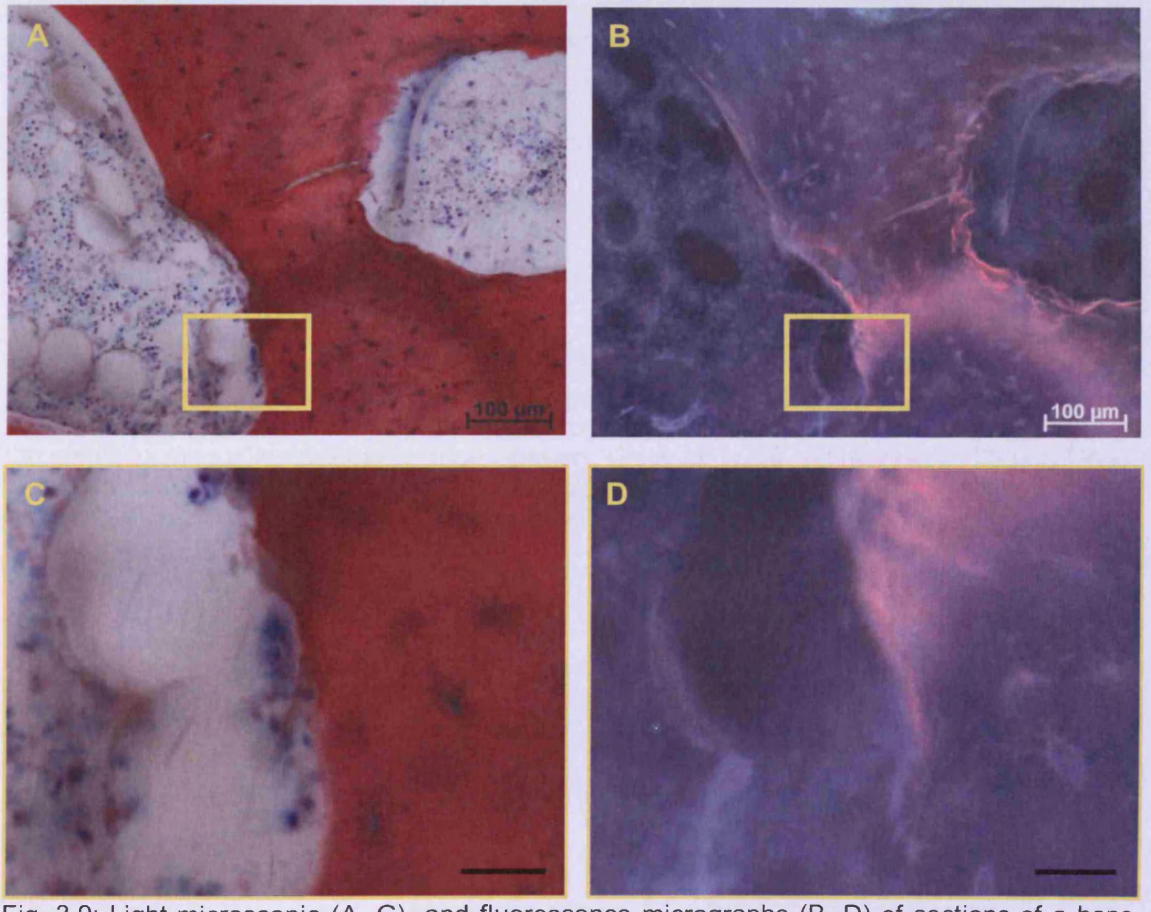

Fig. 3.9: Light microscopic (A, C), and fluorescence micrographs (B, D) of sections of a bone explant (number 21 flight_dynamic) of the FreqBone experiment. A and C close-up: Micrographs show an osteoclast within its resorption lacunae. B and D close-up: Micrographs show the same area of cell activity with the corresponding alizarin bone surface labelling. Close-up scale bar represents 25 µm.

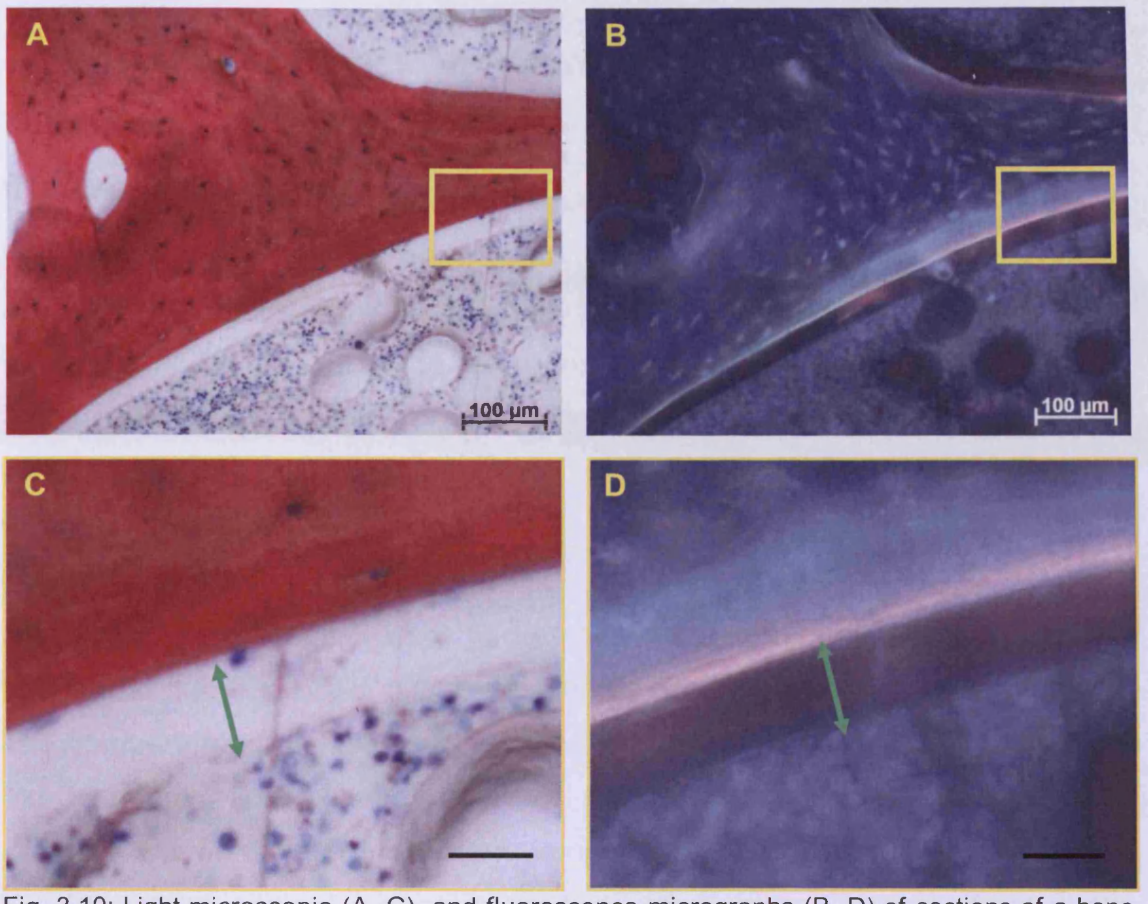

Fig. 3.10: Light microscopic (A, C), and fluorescence micrographs (B, D) of sections of a bone explant (number 5 flight_dynamic) of the FreqBone experiment. A and C close-up: Micrographs show an osteoid seam. B and D close-up: Micrographs show the same area of cell activity with the corresponding alizarin bone surface labelling. Close-up scale bar represents 25 µm. Green arrows in close-up micrographs (C, D) highlight a shrinkage artefact which occurred due to polymerisation problems with the Technovit used for bone explant embedding.

3.3. The Influence of Micro-Gravity and Dynamic Compressive Loading on Collagen Turnover – Investigations Using the Culture Medium

The culture medium of all bone explants was collected during the FreqBone mission. The analysis of soluble products that arise from matrix breakdown or formation will be present in the culture medium and can be used to quantify the processes of bone formation and resorption. As one medium change was performed during the experiment, two medium samples per explant could be used for analyses within this study.

All medium investigations were performed on the same day to avoid repeated freeze and thawing of the precious samples and to keep analysis consistency. The first parameter investigated was the pH (the negative decadic logarithm of the hydronium ion concentration) of the medium samples. Therefore, all samples were thawed to ambient temperature. The medium pH will affect cellular activity within the bone explants and vice versa. The pH of the media from explants cultured at normal gravity was significantly higher with an average pH of 6.96 than the pH of explants cultured at micro-gravity, which had an average pH of 6.88 (Student's t-test; $p \leq 0.0001$) [Fig. 3.11A]. However, the change in pH calculated from the first to the second medium sample taken per explant, showed no significant differences between samples of different gravities and, or load [Fig. 3.11B].

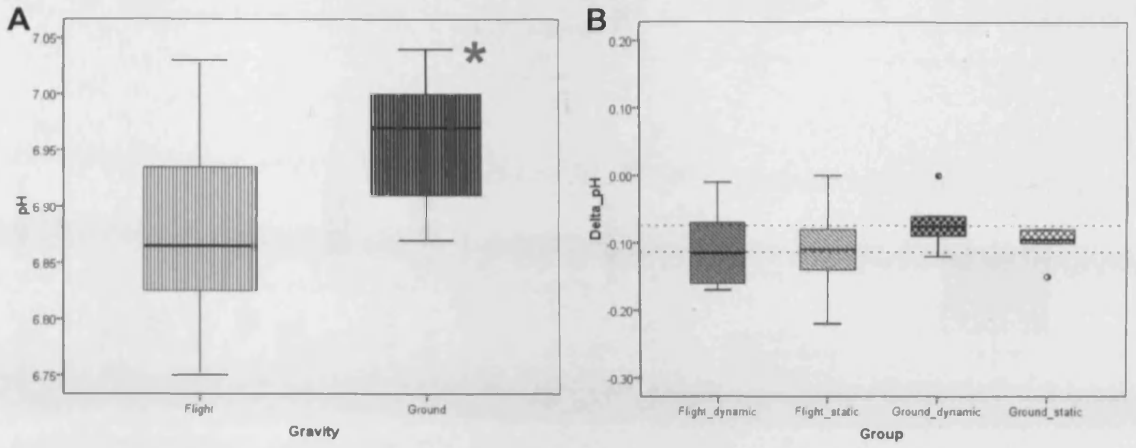

Fig. 3.11: Measurements of the medium pH of samples from bone explants of the FreqBone experiment cultured either at normal (Ground) or micro-gravity (Flight). Explants were either daily dynamical loaded or experienced only static load. A: Significant difference in medium pH was detected between explants cultured at normal or micro-gravity (one-way ANOVA and Tukey *post-hoc* test; * $p \leq 0.01$). B: The pH change over time did not differ significantly between the different treatment groups. Box plots show the median line, the 25% and 75% quartiles, the 1.5x interquartile-range whiskers, as well as outliers (○).

Further analysis of the medium samples involved the detection of the C-terminal propeptide of type I collagen (ProCI) as a bone formation marker. The raw data was normally distributed and, therefore, analysed with either Student's t-test or one-way ANOVA and Tukey post-hoc test. The blot of the data over all experimental groups, as well as media exchanges, indicated increased type I collagen production with culture time from the first to the second medium sample per explant [Fig. 3.12A]. This is summarised in figure 3.12B, where all first medium samples (889 µM +/- 246 µM) and all second medium samples (1245 µM +/- 380 µM) from all explants are summarised. The media gained from the second media exchange showed significantly higher ProCI level than the first media samples ($p \leq 0.0001$). However, the increase of type I collagen production over culture time plotted over gravity, revealed only a significant increase for the samples cultured at normal gravity with an average of 906 µM ProCI (+/- 322 µM) on the first and 1540 µM ProCI (+/- 327 µM) on the second medium exchange ($p \leq 0.0001$) [Fig. 3.12C, Ground]. This result would be expected for space missions, where loss in bone mass seems to be related to lower osteoblast activity.

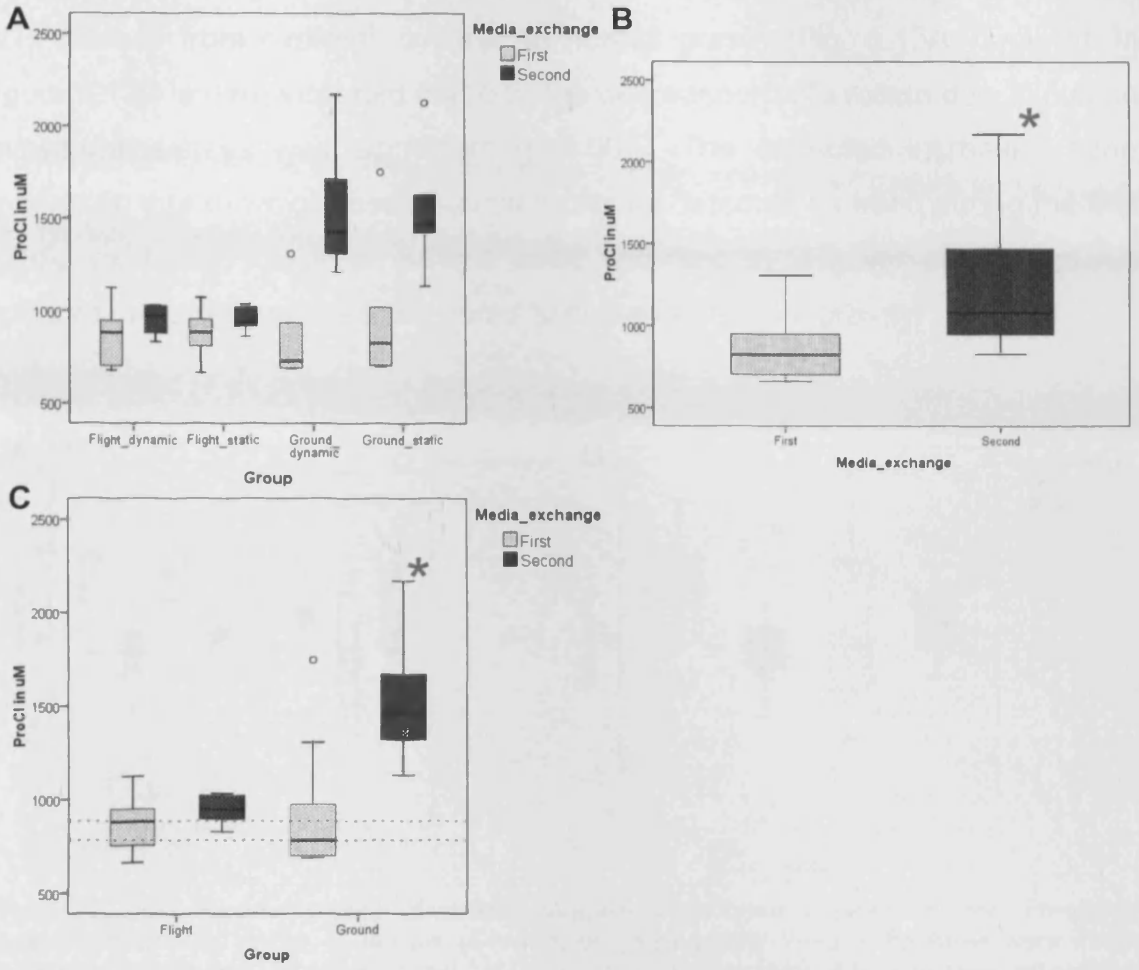

Fig. 3.12: ProCI measurements of media samples from bone explants of the FreqBone experiment cultured either at normal (Ground) or micro-gravity (Flight). Explants were either daily dynamical loaded or experienced only static load. A: Raw data of ProCI release indicated an increase over culture time. B: The increase in collagen I production was significant over all treatment groups (Mann-Whitney test * $p \leq 0.01$). C: If ProCI release was plotted over gravity, only the explants cultured at normal gravity showed a significant increase in collagen I production. Box plots show the median line, the 25% and 75% quartiles, the 1.5x interquartile-range whiskers, as well as outliers (○).

The amount of released N-terminal telopeptide of type I collagen (NTx) was measured in all media samples as indicator of bone resorption. The event of bone resorption by osteoclasts leads to release of type I collagen fragments (Eyre *et al.* 1984). NTx is a direct product of osteoclastic bone resorption and does not require further metabolism *in vivo* to be cleared by the kidney (Calvo *et al.* 1996). Contrary to the ProCI measurement which was presented in µM, the amount of NTx released is given in nM. The raw data of NTx was normally distributed and, therefore, analysed with one-way ANOVA and Tukey post-hoc test. The NTx data indicated a decrease in NTx release from the explants

cultured at micro-gravity over time, while the opposite was suggested for the NTx release from explants cultured at normal gravity [Fig. 3.13A, Ground]. In figure 3.13B is demonstrated that only the decrease in NTx relase due to culture in weightlessness was significant (p=0.002). The expected increased bone resorption due to weightlessness could only be detected as trend during the first media exposure, however, seems to be reflected by a lower pH in explants cultured in weightlessness compared to culture at normal gravity.

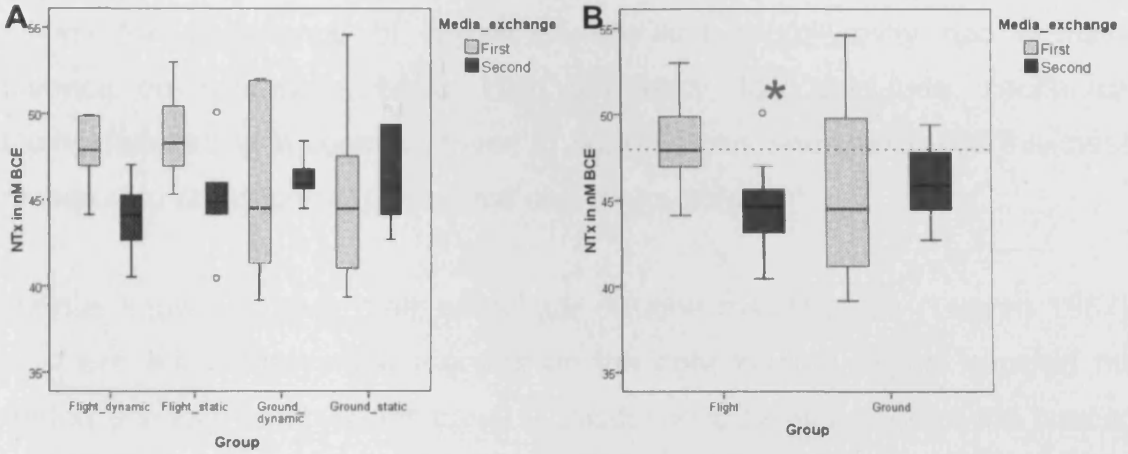

Fig. 3.13: NTx measurements of media samples from bone explants of the FreqBone experiment cultured either at normal (Ground) or micro-gravity (Flight). Explants were either daily dynamical loaded or experienced only static load. A: Raw data of NTx release indicated a decrease for explants cultured at micro-gravity, and an increase for explants cultured at normal gravity over culture time. B: These opposite trends of NTx release at the different gravities did not reach significance. Box plots show the median line, the 25% and 75% quartiles, the 1.5x interquartile-range whiskers, as well as outliers (○).

3.4. Discussion

A micro-gravity experiment was performed by the Catholic University Leuven in collaboration with the ESA to investigate the effectiveness of high frequency, low amplitude mechanical loading on the maintenance of cancellous bone volume in weightlessness. The AO Research Institute had no influence on the study group size. The cancellous bone explants of the space mission were analysed using µCT, histology and culture medium analyses in this study and the results were presented in this chapter. Under the performed experimental settings the experience of weightlessness and micro-gravity had a major influence on cancellous bone. High frequency, low amplitude mechanical loading resulted in a slight increase in bone volume and trabeculae thickness compared to static loading at normal and micro-gravity.

Strain is known to be a main stimulator of bone maintenance (Lanyon 1987), yet, there are controversial theories on the right loading regime causing the needed anabolic stimulus for bone. Considering only frequency of the loading applied, the stimulus could be either at low frequencies of around 1-2 Hz relating to physiological locomotion, or within the high frequency range of around 15-30 Hz relating to posture (Cowin 2002). It has been recognised that the later high frequency loading in combination with low amplitudes is present in the daily muscle contraction strain energy spectrum regardless of activity (Fritton *et al.* 2000). Opposing the school of high peak strain loading with low frequency (Rubin & Lanyon 1984) – which is already not successfully applied during space missions by the use of tread milling – the FreqBone experiment investigated the possible effect of high frequency, low amplitude loading on bone mass maintenance in weightlessness. However, the µCT results showed no significant differences between the treatment groups, yet, the trends observed correlate with the expected results at micro-gravity, where bone loss is still a main issue in space missions (LeBlanc *et al.* 2007). In this study, weightlessness in combination with only a static load resulted in slightly decreased bone volume versus total volume, as well as slightly decreased trabeculae thickness in comparison to ground levels. Moreover, the application

of a dynamic high frequency, low amplitude load resulted in slightly increased level of newly deposited mineralised bone. This would implicate that this loading regime had an anabolic stimulus on the cultured cancellous bone explants. However, it appeared that the used experimental set-up was not optimal to detect significant changes of bone volume due to weightlessness. The low sample number is one problem and in combination with the natural diversity of cancellous bone does not ease evaluation of group differences by the end-point determination using µCT. We performed a post-hoc power analysis of the data gained by µCT, in order to determine the least amount of samples needed per group to allow a statistical power of at least 0.81. It could be determined, that the statistical power of the performed experiment was only 0.263. A total of 100 samples evenly distributed over the 4 groups would have been needed to allow for the determination of a statistical significance of $p \leq 0.05$. Due to the spatial limitations of the Foton, this amount of samples would have not been realistic to include in the mission. The unavailability of comparable studies also complicated prospective power analysis. Further to be taken into consideration is the fact that in this study cancellous bone from the bovine sternum was used. It has been shown by David *et al.* that sternum cancellous bone explants do respond with increased bone formation in response to the low frequency, high amplitude loading detected i.e. using µCT to evaluate trabeculae thickening (David *et al.* 2008). However, the negative changes in BMD during weightlessness and micro-gravity were primarily detected in weight-bearing bones (LeBlanc *et al.* 2000; Trappe *et al.* 2009). The sternum does experience loading by breathing activity and posture, however it does not belong to the group of load-bearing bone which are found in the lower extremities or the pelvis. This fact might also be accounted for the non-significance of the µCT results.

Problematic issues with the use of calcium-binding fluorescent dyes should be mentioned. No real 'double labelling' with distinguishable calcein green and alizarin complexon lines was detectable, both dyes showed low penetration into the bone explant centres, and massive explant surface and debris labelling was detected. Yet, active bone surface labelling by calcein green and alizarin

complexon was detected. Bone surfaces were labelled prior to and after the space mission. Labelling significance was supported by the presence of intact osteoclasts situated in their resorption lacunae, as well as non-mineralised osteoid fronts on overlapping areas of fluorescent labelling. The qualitative analyses of histomorphological parameters clearly demonstrate the viability of cultured bone explants. Since osteoclasts have a limited life-span of around 12.5 days (Hill 1998), the detection of intact mature cells after 20 days bone explant culture indicates viability of possibly osteoclast-precursor cells as well as the mature osteoclasts themselves during FreqBone culture. The quantitative analysis of fluorochrome labelling fronts revealed no significant differences between the loading groups, and the gravity groups. However, label penetration significantly increased during culture time. Calcein green label penetration inside the cancellous bone explants was significantly higher than the later applied alizarin complexon penetration. Two interpretations of the label penetration differences are possible. The decreased penetration depth seen with the smaller and less charged molecule alizarin complexon (385 g/mol, charge 2^-) compared to the penetration of the larger and higher charged calcein green (622 g/mol, charge 6^-) might be explained with a loss of cellular activity and, therefore, a loss in calcium deposition or resorption at the central areas of the bone explants with time. However, the contrary interpretation is also possible - due to 10°C maintenance of the explants prior to launch, cell activity was reduced and calcein green could penetrate further into the explants, while explants were cultured at 37°C during the space mission, the resulting higher cell activity could cause higher 'uptake' of alizarin complexon by the explant edge areas leading in a decreased alizarin complexon penetration depth.

This hypothesis is supported by the significant increase in ProCI release and, therefore, type I collagen production during culture, which is most likely accounted to greater osteoblast cellular activity. The separation of the data sets for first and second culture medium exchange into normal and micro-gravity culture lead to a significance of the increased type I collagen production only at normal gravity. However, the decreased penetration of alizarin complexon may have been derived from partial blockage/uptake by surface cells of the fibrous

membrane. These fibroblast-like cells could also increase the production of type I collagen during culture, as fibroblasts, like osteoblasts produce this protein.

It seems clear that micro-gravity / weightlessness leads to an uncoupling of bone remodelling processes (LeBlanc *et al.* 2007). Serum data of astronauts reveal increased levels of NTx and decreased levels of ProCI due to micro-gravity missions (LeBlanc *et al.* 2007). Our results from *ex vivo* bone explants partly confirm these *in vivo* findings. Yet, the NTx data showed only a trend in increased NTx release by culture in weightlessness compared to culture at normal gravity. Moreover, the significant increase in bone formation over culture time, which was seen at normal gravity, could not be detected at micro-gravity. Carmeliet *et al.* demonstrated a change in osteoblast gene expression and morphology at micro-gravity leading to a less differentiated osteoblast phenotype (Carmeliet & Bouillon 1999). The lower culture medium pH at micro-gravity compared to normal gravity correlates with the NTx results of slightly increased bone resorption during the first 6 days of the FreqBone mission. The interaction of low pH and osteoclastic activity seems to be a feedback-loop response. Osteoclasts resorb bone due to the release of hydronium ions into the resorption cavity and, thereby, lowering the pH in the milieu (Roodman 1996). On the other hand, low medium pH has been shown to increase activity of mature osteoclasts (Meghji *et al.* 2001). The release of NTx into the culture medium is of nM magnitude, therefore, the trends in NTx release are negligible compared to the µM levels of ProCI media release and might be accounted for the missing detection of bone loss at weightlessness by µCT. However, it needs to be considered for all created results within the study, that the experimental time may have been too short to reach significance. It was shown by LeBlanc *et al.* that during a 17-day micro-gravity mission loss in aBMD could not be detected in astronauts (LeBlanc *et al.* 2000). Astronauts experienced only a loss in fat and muscle mass during these short-term missions. Moreover, Smith *et al.* showed that a significant increase in NTx urine level after 14 days at micro-gravity can only be detected if the NTx level was normalised to creatinine (Smith *et al.* 2005). It seems likely that an increase in bone resorption at micro-gravity can only be detected at a much later time point than investigated in this

ex vivo culture study. Therefore, the intervention by the loading regime applied could possibly demonstrate significant results at a later stage.

In summary, the analysis of the FreqBone experiment demonstrated the impact of weightlessness and reduction in gravity on the activity of bone cells. The application of a dynamic high frequency, low amplitude load on bone turnover processes at micro-gravity looks promising but further studies need to extend the experimental time and increase sample number. Additional research needs to be conducted to prevent bone loss during space mission. Applications of artificial gravity might be a possible future research candidate.

Chapter 4: The Influence of Culture Conditions on the *Ex Vivo* Culture of Cancellous Bone Explants

The long-term *ex vivo* culture of mature bone explants remains a challenging task. Contrary to the culture of embryonic explants, which are grown and differentiated *ex vivo*, the aim of the culture of fully differentiated explants is to maintain their histological characteristics and cell functionality *ex vivo*. These features of mature explant maintenance are, however, problematic. Trowell discussed the issue of mature organ culture in 1961 (Trowell 1961). He pointed out that non-specific tissue growth, as well as cell migration and de-differentiation during culture have to be actively prevented to maintain the natural histology of cultured organs. Moreover, the importance of oxygen supply during culture was discussed and defined as (one) size-limiting factor of organ culture.

The first and major issue of mature organ cultures is to prevent central necrosis within *ex vivo* cultured explants. Due to the loss of blood supply, such constructs mainly depend on diffusion for their nutrient supply as well as their waste removal. The negative influence on osteocyte survival caused by preparation-derived bone matrix micro-cracks (Noble & Reeve 2000) eliminates the possibility of size-reduction of the bone explants. Gentle convection of the culture medium has been shown to increase the availability of nutrients as well as the removal of waste products. A recent review discussed the significance of mass transport in cultured tissue engineering constructs (Muschler *et al.* 2004). Convection seems to be of crucial importance for the renewal of nutrients, as well as removal of metabolic waste products during the culture of cells embedded in dense extracellular matrix. Convection can be permitted by several methods. The perfusion culture is one of them and is mostly investigated for tissue engineering applications such as for bone replacement constructs (Janssen *et al.* 2006; Volkmer *et al.* 2008). During medium perfusion through porous material i.e. cancellous bone or polymeric scaffolds, the rate of fluid flow should be high enough to enable mass transfer, but slow enough to prevent damage or cellular washout (Trowell 1961). Another possibility to

enable convection *in vitro* is by compression, through which fluid is forced out of the explant (Muschler *et al.* 2004). Explant relaxation, which follows mechanical compression, leads to fluid flow back into the explant. A fluid exchange is created which renews the culture medium in the cultured explant and therefore increases nutrient supply.

The ZetOs bioreactor and culture system that was used within this study to culture human and bovine cancellous bone possesses several features to ease the long-term *ex vivo* bone explant culture (Jones *et al.* 2003). The system offers the opportunity to culture cancellous bone explants under constant medium perfusion while also enabling the application of a defined mechanical compressive load during explant culture. However, the evaluation of cell viability, after 14 days *ex vivo* culture of mature human cancellous bone explants in the ZetOs system, revealed the great need to improve culture conditions. Simpson demonstrated the occurrence of central cell death by comparing viability of surface to central region of cultured explants (Simpson 2006). Simpson was able to show that central bone marrow and osteocyte viability is diminished due to 14 days long-term culture [Fig. 4.1].

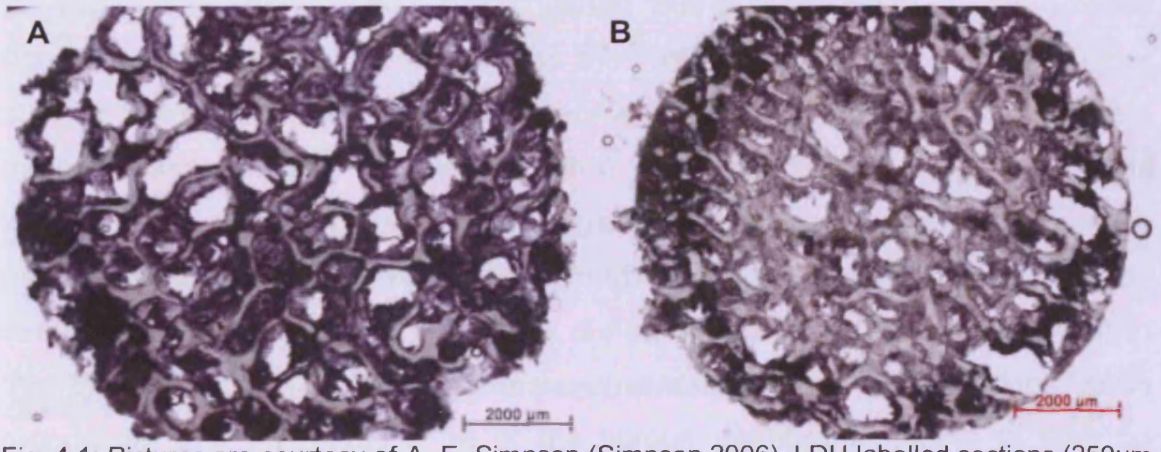

Fig. 4.1: Pictures are courtesy of A. E. Simpson (Simpson 2006). LDH labelled sections (250µm thick) taken from human cancellous bone explants. A: Even LDH staining of bone marrow can be detected on this human cancellous bone section (54 years, ♂) directly after in vivo harvest. B: The LDH staining on human cancellous bone section (66 years, ♀) after 14 days of cullture and daily mechanical loading within the ZetOs system does show that the outer region of the of the section was labelled more intensely than the central area.

Another previous study investigated the competence of medium perfusion to increase central explant nutrient availability. However, it was demonstrated that medium perfusion is not optimal in the ZetOs system, and that it occurred almost entirely around the circumference of the cultured explants (Davies *et al.* 2006; Koller 2004). Moreover, it was shown that the reduction of medium distribution along the baseplate- and piston-exposed bone explant surfaces, which was suggested to have occurred due to the use of flat-surfaced culture chambers (Davies *et al.* 2006). All of these points emphasise the need to improve *ex vivo* culture conditions.

The second major issue of mature organ culture is the preservation of the natural histology of the tissue. Davies *et al.* demonstrated the maintenance of bone histology even after 21 days *ex vivo* culture in the ZetOs system (Davies 2005). However, it was recognised during the study that this was only true for the central region of the explant. The surface of many cultured explants demonstrated the formation of a thick non-physiological cell layer engulfing the inner explant [Fig. 4.2].

The phenomenon of this so-called surface fibrous membrane is not uncommon for organ cultures. These cells covering the outer surface of the explant decrease the nutrient availability for the residual cells, which might further support the development of central necrosis during *ex vivo* culture. There are many possible causes for the formation of the fibrous membrane, such as increased cell migration towards the region of highest nutrient availability – the explant surface. Alternatively, the formation of fibroblast-like cells may be caused by dedifferentiation processes during culture, which could occur due to the lack of an *in vivo* differentiation/maintenance stimulus. It has been recognised that the occurrence of the fibrous membrane seems to also be related to the addition of serum to the culture medium. This might further lead to the conclusion that increased proliferation caused by the several proliferation stimulating factors i.e. endothelial growth factor, or fibroblast growth factor within serum are the third possible cause for the fibrous membrane formation (Barnes & Sato 1980; Freshney 2000b).

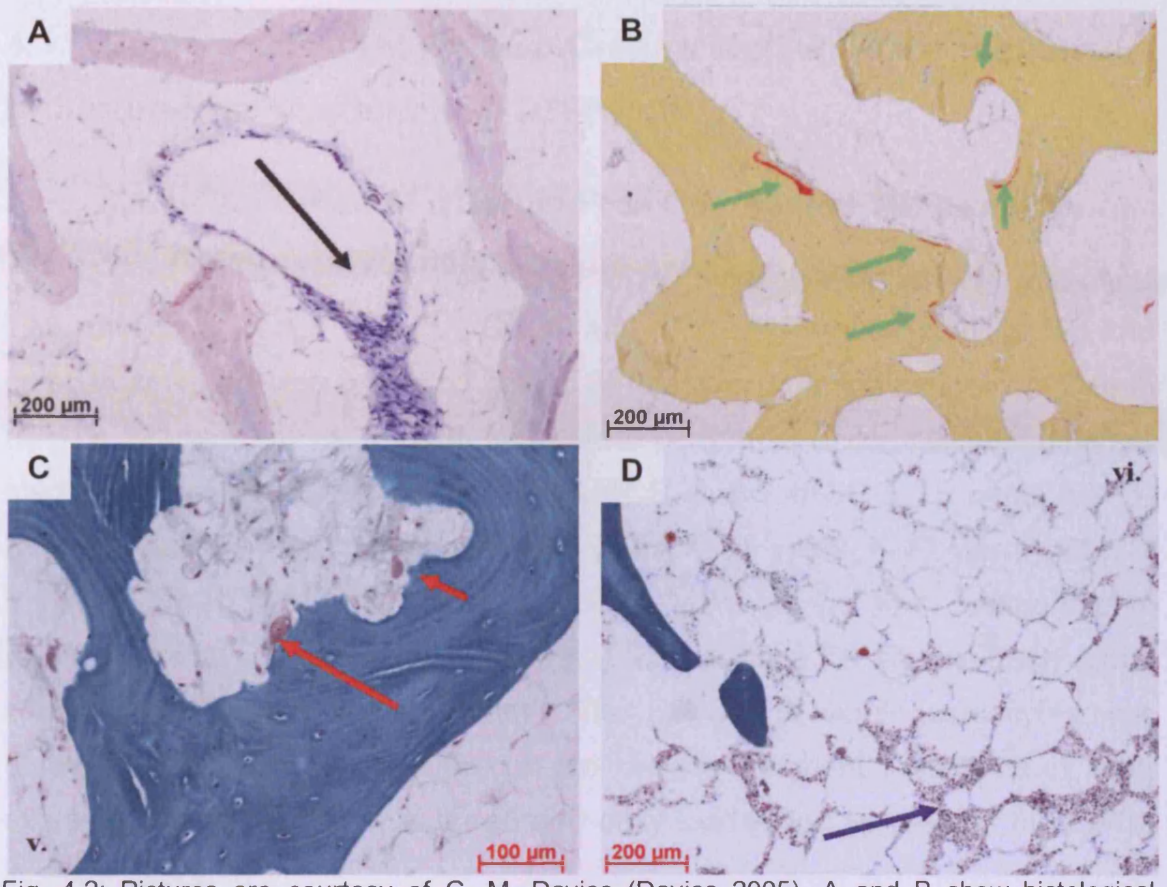

Fig. 4.2: Pictures are courtesy of C. M. Davies (Davies 2005). A and B show histological sections of cancellous bone explants human (71 years, ♂) cultured and daily mechanical loaded within the ZetOs system for 15 days. A: Giemsa staining demonstrating the presence of fibrous cells on the surface of the bone marrow (black arrow). B: Morvat staining demonstrating red labelled osteoid seams (green arrows) on the bone surface. C and D show histological sections of human cancellous bone explants (81 years, ♂) cultured and daily mechanical loaded within the ZetOs system for 21 days. C: Masson staining demonstrating osteoclasts present in Howship's lacunae (red arrows). D: Masson staining demonstrating red marrow presence together with adipocytes (blue arrow).

This chapter will present and discuss new culture conditions to improve the existing *ex vivo* culture of cancellous bone explants within the ZetOs system. The increase of *ex vivo* cell survival will be attempted using a new culture chamber design to improve nutrient availability for the cultured explants. This chapter will also investigate the effect of SF medium on the occurrence of the surface fibrous membrane. The influence of this membrane on central cell viability as well as medium penetration will be presented and discussed. To overcome a possible loss in cell viability due to the continuous use of a SF culture medium, we supplied the SF medium with a potential 'osteocyte survival factor' $TGF\beta_3$ (Simpson *et al.* 2009).

4.1. Using a Different *Ex Vivo* Culture Medium – the Influence of Serum-Free Medium and TGFβ$_3$

4.1.1. The Effect of a Serum-Free Culture and TGFβ$_3$ on Osteocyte Viability

Two different media types - BGJb and DMEM - were used during the comparison of serum-containing versus SF medium. Loading experiments, each using a different human femoral head, were performed to compare the different media supplementations. BGJb + 10% FCS or BGJb SF + 15 ng/ml TGFβ$_3$ were compared in 3 experiments (♀ 55 years, ♀ 75 years, ♂ 72 years). DMEM + 10% FCS or DMEM SF + 15 ng/ml TGFβ$_3$ were compared in 5 experiments (♀ 55 years, ♀ 75 years, ♂ 71 years, ♂ 72 years, ♂ 78 years). Each group consisted of 3 to 6 bone explants. The number of viable osteocytes was quantified (Stoddart *et al.* 2006) on the surface and centre sections of bone explants, which had been cultured and daily loaded for 14 days in the ZetOs system [Fig. 4.3].

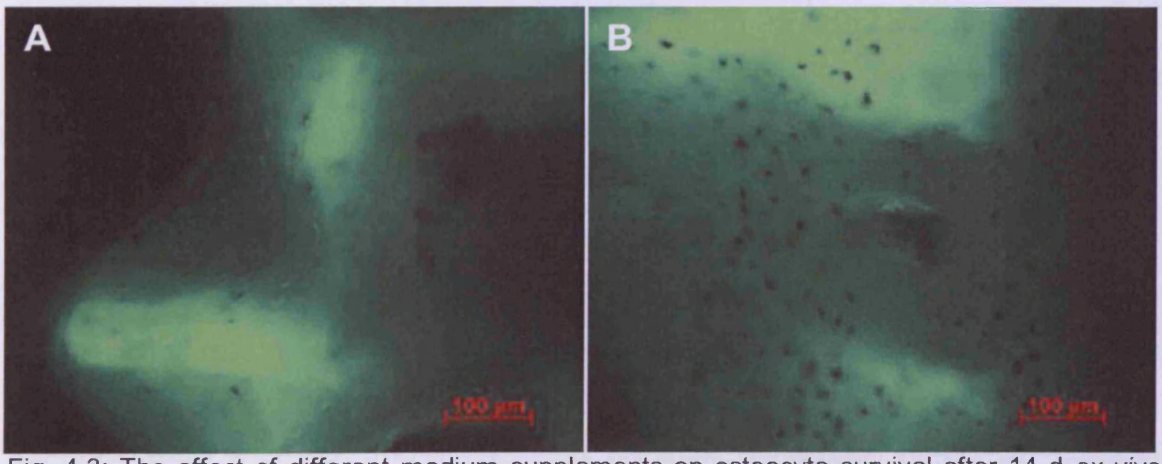

Fig. 4.3: The effect of different medium supplements on osteocyte survival after 14 d *ex vivo* culture. Two representative micrographs from LDH labelled centre sections (centre region) of one human (♀ 75 years) loading experiment visualised with Axioplan (Fluorescence 515-565 emission filter). **A**: Explant cultured in DMEM + 10% FCS; **B**: Explant cultured in DMEM SF + 15 ng/ml TGFβ$_3$. The micrographs suggest that culture of human cancellous bone cores in DMEM SF + 15 ng/ml TGFβ$_3$ instead of DMEM + 10% FCS increases osteocyte survival.

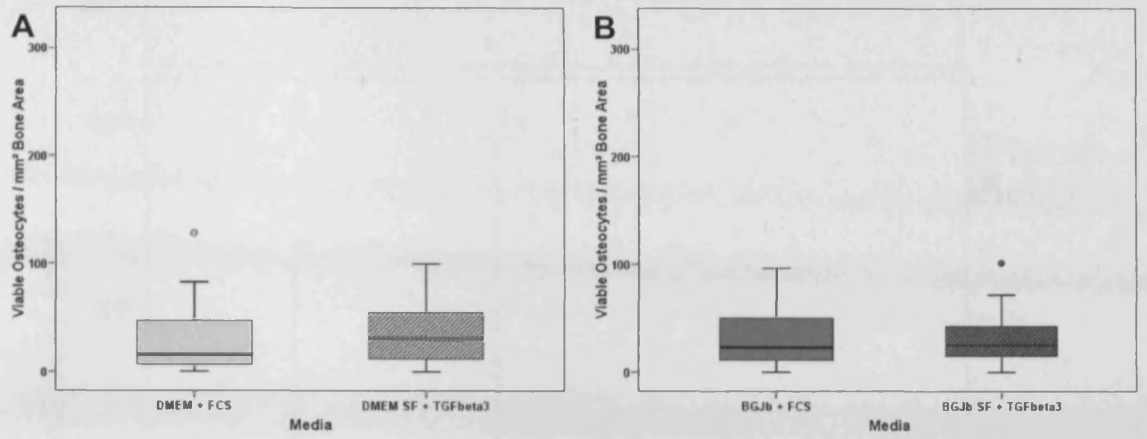

Fig. 4.4: Loading experiments investigating the effects of media supplements on osteocyte survival. The number of viable osteocytes per bone matrix area was determined in the centre of LDH stained surface sections (250 µm) taken from daily loaded human cancellous bone explants cultured for 14 d in either DMEM + 10% FCS vs. DMEM SF + 15ng/ml TGFβ$_3$ (**A**; ♀ 55 years, ♀ 75 years, ♂ 71 years, ♂ 72 years, ♂ 78 years), or in BGJb + 10% FCS vs. BGJb SF + 15ng/ml TGFβ$_3$ (**B**; ♀ 55 years, ♀ 75 years, ♂ 72 years). Box plots show the median line, the 25% and the 75% quartiles which define the box, the 1.5x interquartile-range whiskers, as well as outliers (○). Mann-Whitney test and Bonferroni correction were used to compare groups ($p \leq 0.05$). Viability was not significantly different between FCS-containing and SF DMEM or BGJb medium.

Osteocyte survival on the surface of cultured bone explants did not differ significantly if a serum-containing or SF medium was used [Fig. 4.4]. However, significant differences in cell survival were found in the critical central regions of cultured bone explants, demonstrated using Mann-Whitney test and Bonferroni correction [Fig. 4.5]. The use of BGJb SF + TGFβ$_3$ showed improved central osteocyte survival (38.7 +/- 28.7) compared to the use of FCS with BGJb (24 +/- 18.5; p=0.038). Also, the use of DMEM SF + TGFβ$_3$ showed improved central osteocyte survival (67.2 +/- 58.3 viable osteocyte/mm^2 bone area) compared to the use of all other media / supplements ($p \leq 0.002$). Due to this positive viability result using DMEM SF + TGFβ$_3$, DMEM was chosen as primary culture medium in later experiments. Moreover, osteocyte survival in edge regions of central sections from cultured bone explants showed increased osteocyte viability maintenance compared to the centre regions of that section in all medium groups ($p \leq 0.003$), thus highlighting that there is a discrepancy between core edge and core centre viability even with the use of SF medium. However, the results clearly demonstrate that the use of SF culture improves the survival of central osteocytes during long-term culture of human cancellous bone explants in the ZetOs system.

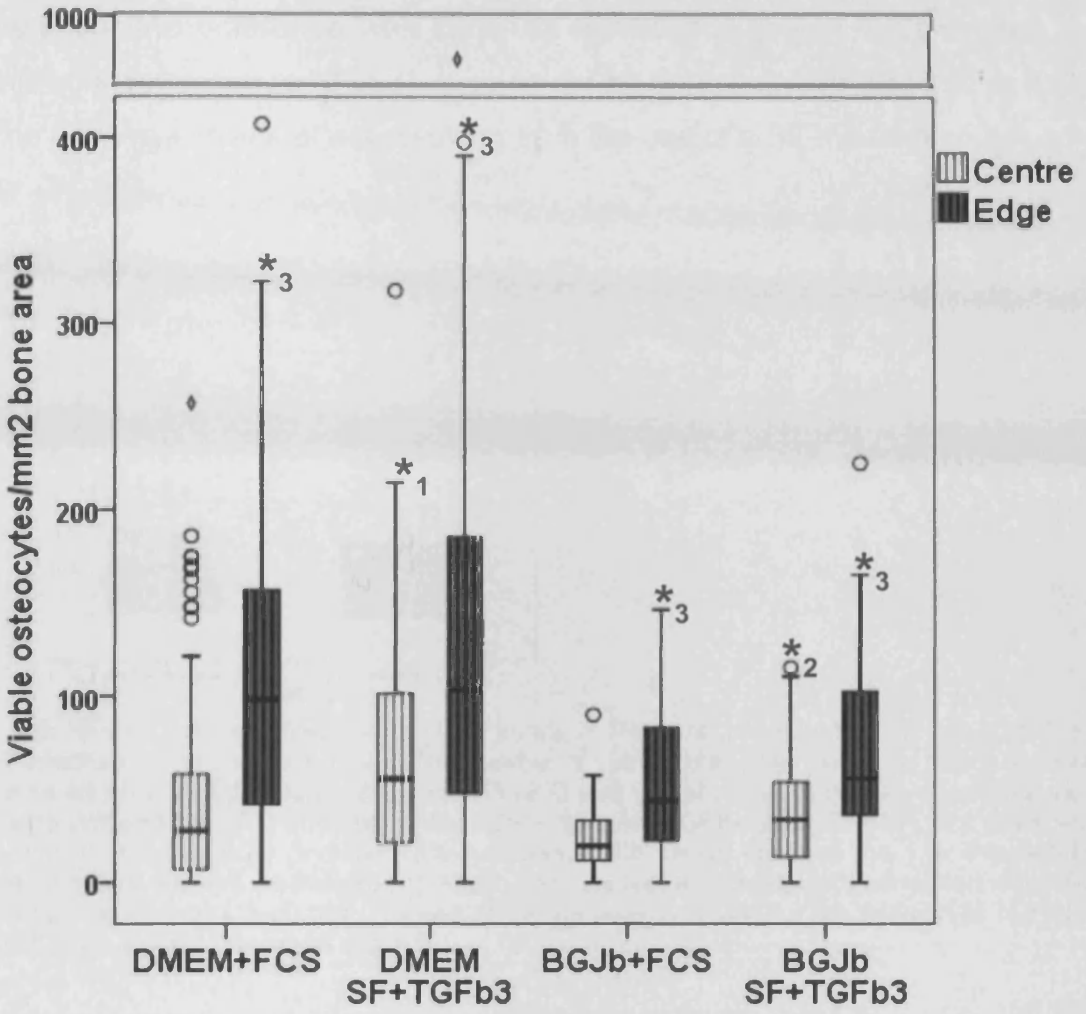

Fig. 4.5: Loading experiments investigating the effect of media supplementation on osteocyte survival on centre sections of daily loaded human cancellous bone explants, which were cultured for 14 d in either BGJb + FCS / SF + TGFβ_3 (♀ age = 55 years, ♀ age = 75 years, ♂ age = 72 years), or DMEM + FCS / SF + TGFβ_3 (♀ 55 years, ♀ 75 years, ♂ 71 years, ♂ 72 years, ♂ 78 years). Box plots show the median line, the 25% and the 75% quartiles which define the box, the 1.5x interquartile-range whiskers, as well as outliers (○) and extremes (◊). Kruskal-Wallis Test was used as comparison between groups; Mann-Whitney test and Bonferroni correction were used to compare centre and edge regions within groups (* $p \leq 0.05$). *1: The use of DMEM SF + TGFβ_3 showed increased central osteocyte survival over all groups. *2: The use of BGJb SF + TGFβ_3 showed increased central osteocyte survival over BGJb + FCS. *3: Osteocyte survival was higher in edge than centre regions of the centre section of cultured bone explants from all groups.

To investigate if the positive effect upon cell survival arose from the use of a SF medium in general, or from the addition of TGFβ_3 to the medium, a further set of human loading experiments was performed. DMEM was utilised as culture medium, SF supplementation with or without 15 ng/ml TGFβ_3 was compared. Each group consisted of 6 bone explants. Survival of osteocytes in the critical centre of *ex vivo* cultured human cancellous bone explants was not significantly different if DMEM SF was supplemented with TGFβ_3 or not [Fig. 4.6]. In one

experiment the difference was close to significance (Mann Whitney test and Bonferroni correction, p=0.058). It could not be demonstrated that TGFβ$_3$ added to the positive survival effect resulting from the use of a SF medium.

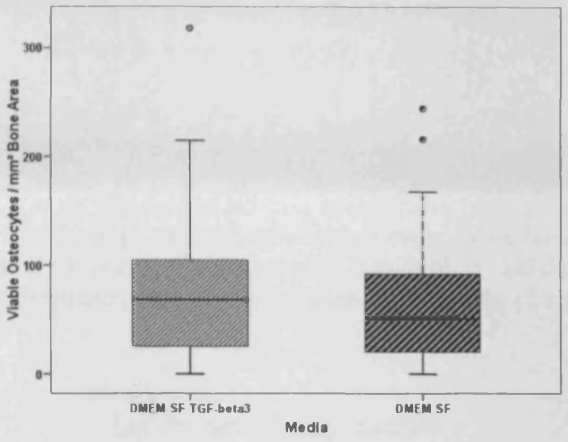

Fig. 4.6: Human loading experiments (♂ 71 years, ♂ 78 years) investigating the effect of TGFβ$_3$ in SF medium on osteocyte survival. The number of viable osteocytes per bone matrix area was determined on LDH labelled centre sections (250 µm) of daily loaded human cancellous bone explants cultured for 14 d in either DMEM SF + 15ng/ml TGFβ$_3$ or DMEM SF. Box plots show the median line, the 25% and the 75% quartiles which define the box, the 1.5x interquartile-range whiskers, as well as outliers (○). Mann-Whitney test and Bonferroni correction were used as statistical analysis (* $p \leq 0.05$). The use of SF media with or without the addition of TGFβ$_3$ did not influence central osteocyte survival.

4.1.2. The Effect of Serum-Free Medium on the Formation of the Surface Fibrous Membrane

The formation of a fibrous membrane was detected on the surface of some of the *ex vivo* cultured cancellous bone explants [Fig. 4.7]. The amount of fibroblast-like cells, as well as the density of the formed membrane layer, was inconsistent from bone explant to bone explant. The occurrence of the surface fibrous membrane during *ex vivo* culture (♀ 55 years, ♀ 75 years, ♂ 71 years ♂ 72 years, ♂ 78 years) was investigated in relation to the use of FCS or SF medium supplementation. Explants were either cultured in FCS-containing or SF medium plus TGFβ$_3$. Bone explants were divided into one of three categories - fibrous membrane formation, only a few fibroblast-like cells on the surface, or no surface fibrous membrane [Fig. 4.8].

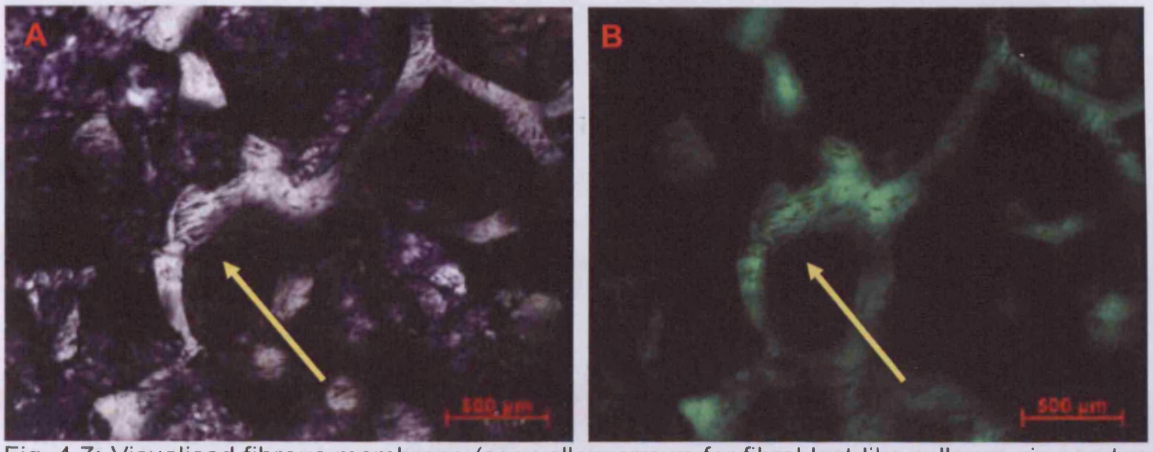

Fig. 4.7: Visualised fibrous membrane (see yellow arrows for fibroblast-like cells growing on top of cancellous bone trabeculae) on a LDH labelled surface section of one human cancellous bone explant (♂ 64 years) cultured in BGJb + 10% FCS. **A:** Bright field micrograph. **B:** Micrograph using fluorescence microscopy (515–565 nm emission filter).

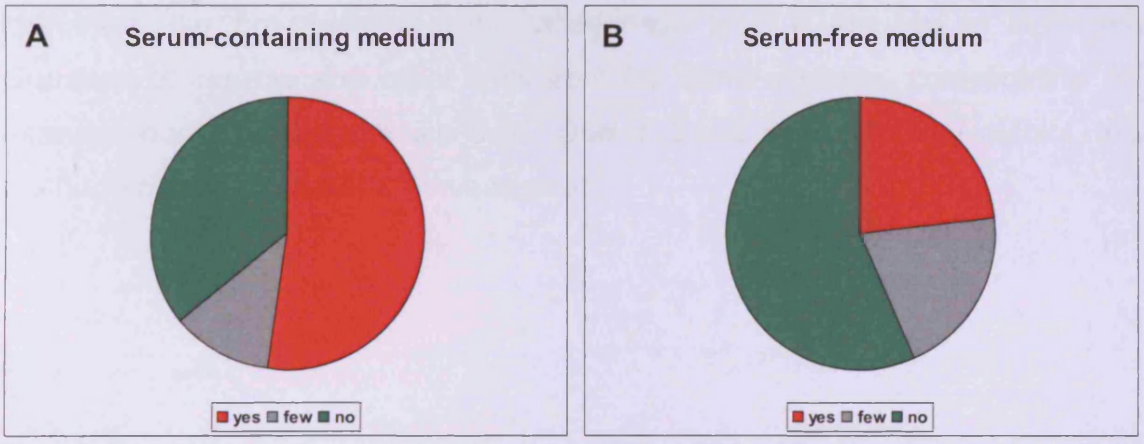

Fig. 4.8: Fibrous membrane formation determined in 5 human loading experiments (♀ 55 years, ♀ 75 years, ♂ 71 years ♂ 72 years, ♂ 78 years). The occurrence of the fibrous membrane was reduced from 52% using an FCS-containing medium to 23% by the use of a SF culture medium.

A total of 25 explants were cultured in serum-containing medium, 13 (52% of all explants cultured with serum) showed the formation of a fibrous membrane on the surface. On the surface of 3 explants, few fibroblast-like cells were detected, while 9 explants cultured with serum showed no fibrous membrane formation. Thirty cores were cultured SF (+ $TGF\beta_3$), 7 explants (23% of all explants cultured SF) had the formation of a surface fibrous membrane, 6 explants showed only few fibroblast-like cells on the surface and 17 explants had no fibrous membrane. Therefore, the formation of a surface fibrous tissue was clearly reduced using a SF culture medium.

However, only different grades of the fibrous membrane formation were detected. To quantify the absolute amount of fibrous membrane per bone explant another set of experiments using bovine bone (3-5-month-old calves) was performed. In the first trial the membrane was attempted to be digested from the bone explants (method described in 2.2.4). The visualisation of LDH labelled surface sections revealed the presence of fibroblast-like cells on top of bone explants even after 60 min enzymatic digestion using trypsin, demonstrating an incomplete removal of the surface cells by this method [Fig. 4.9]. Therefore, a second experiment used a pre-digest with collagenase type II (Worthington Biochemical Corporation) prior to trypsin incubation [see Table 4.1 for results of both experiments]. Collagenase type II pre-treatment did not lead to a complete removal of the surface fibroblast-like cells from the explants. Moreover, the pre-digestion with collagenase type II resulted in increased digestion of marrow and other cells from the bone explants, complicating the manual count of fibroblast-like cells. Due to these unsatisfactory results, this methodology was not further investigated.

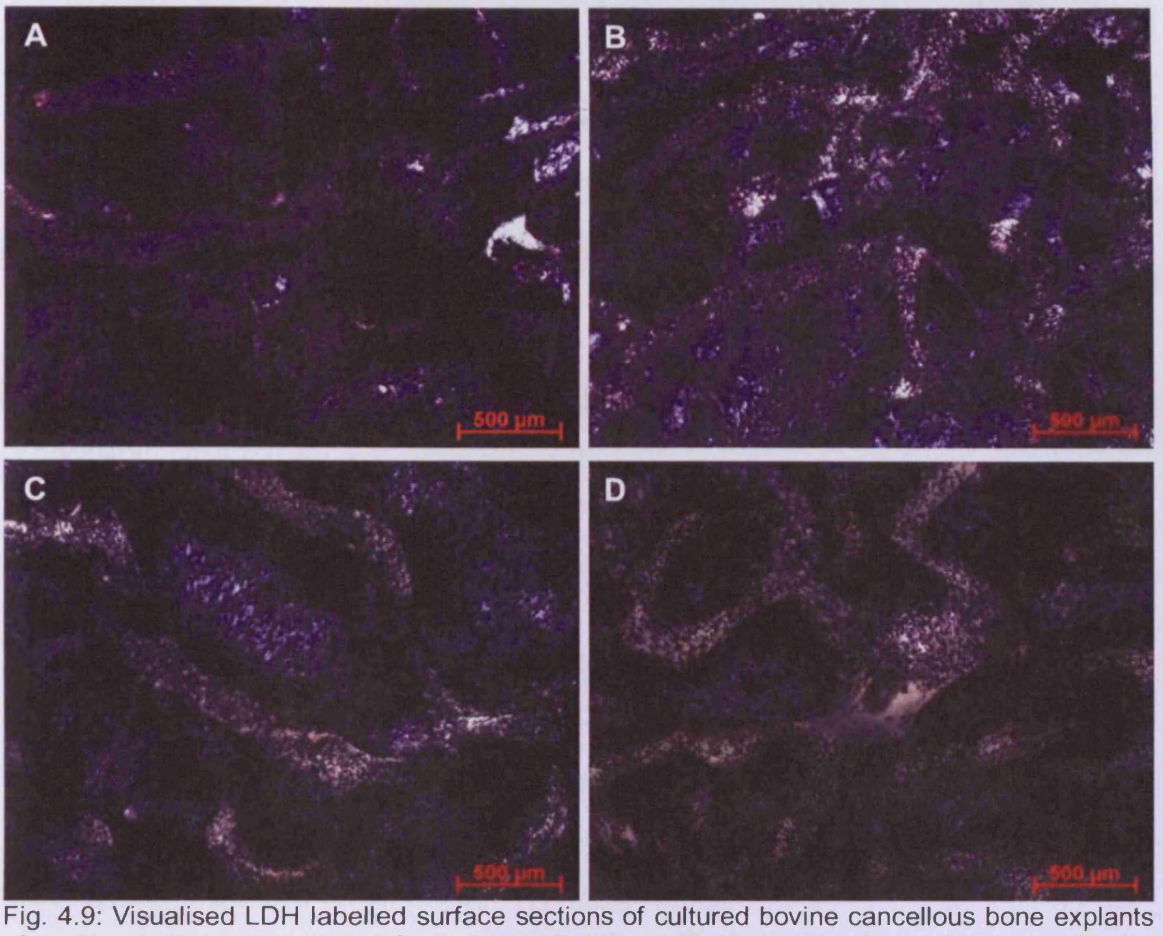

Fig. 4.9: Visualised LDH labelled surface sections of cultured bovine cancellous bone explants after digest with trypsin solution for A: 0 min, B: 15 min, C: 30 min, D: 60 min. Even after 60 min trypsin incubation fibroblast-like cells were still covering the bone explant surfaces.

Table 4.1: Results of the enzymatic digests of the surface fibrous membranes formed on top of cancellous bone explants during 2 bovine (3-5-month-old calves) culture experiments. Trypan blue cell counts are presented.

Bovine bone	Collagenase	Trypsin	Alive cells	Dead cells	All Cells	Percentage alive
1	No	15 min	56250	16875	73125	76,92
	No	30 min	73125	5625	78750	92,86
	No	60 min	101250	30000	131250	77,14
2	No	60 min	38333	23333	61667	62,16
	Yes	60 min	46667	16667	63333	73,68
	No	75 min	21667	18333	40000	54,17
	Yes	75 min	20000	22500	42500	47,06
	No	90 min	34167	23333	57500	59,42
	Yes	90 min	18333	35000	53333	34,38

4.1.3. Metabolic Investigations of Cultured Bone Explants using Different Medium Supplements

To investigate if *ex vivo* cultured cells demonstrate a change in cellular metabolism due to use of different medium supplements, glucose consumption, lactate production as well as culture medium pH were quantified in a set of human loading experiments. Glucose levels were measured to detect the consumption of this main sugar source. Moreover, the level of the potential end product – lactate – was investigated. The ratio of glucose consumption/lactate production could then be used to determine if cultured explants were using aerobic or anaerobic respiration. Both glucose consumption and lactate production were made relative to the level of the 'dead control' explant medium. Lactate medium levels were ascending over the culture period [Fig. 4.10A]. As figure 4.10B demonstrates, the overall lactate medium levels were significantly higher in the presence of FCS in culture medium than using a SF culture medium (ANOVA + Tukey post-hoc test, $p \leq 0.017$), while 116 +/- 72.5 mM lactate was detected in DMEM + FCS, only 73.3 +/- 49 mM lactate on average was detected in both SF media. The consumption of glucose by the cultured bone explants stayed at extremely low levels [Fig. 4.10C, D]. With an average of 0-1 mM glucose consumption during a 48 h period, over 96 % of the supplied glucose was not used by cells. Yet, significant differences in glucose consumption could be detected. Generally, glucose consumption was lowest in DMEM SF over the other 2 medium supplements (ANOVA + Tukey post-hoc test, $p \leq 0.008$).

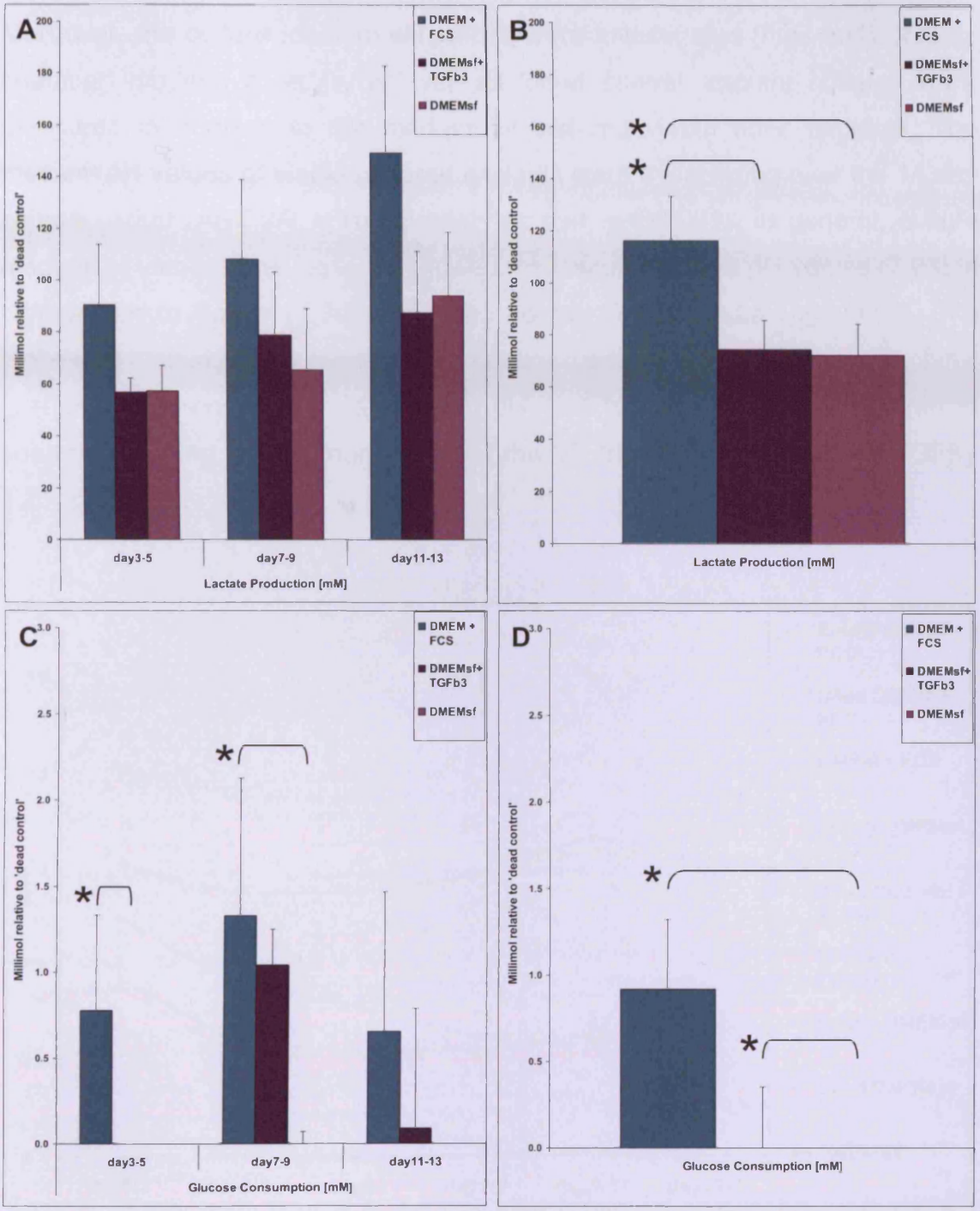

Fig. 4.10: Lactate production and glucose consumption of *ex vivo* cultured human cancellous bone explants (♂ 71 years; ♂ 78 years) cultured in DMEM + FCS, DMEM SF + TGFβ_3 or DMEM SF. Six bone explants per media group were investigated at three different time points (day 3-5; day 7-9; and day 11-13). Graphs indicate the mean glucose or lactate levels [mM] and the standard error of the mean A, C: Data arranged according to individual time points. B, D: Summarised medium supplement data for lactate production and glucose consumption. ANOVA and Tukey post-hoc test were used for statistical analysis (* $p \leq 0.05$). Lactate production was significantly higher in DMEM + FCS than other medium supplements, while glucose consumption was lowest using DMEM SF.

Moreover, the culture medium pH values were investigated [Fig. 4.11]. 'Empty chamber' medium controls, as well as 'dead control' explant cultures were measured in addition to the medium of cultured viable bone explants. The medium pH values of viable cultured explants were descending over the 14 day culture period (ANOVA + Tukey post-hoc test, $p \leq 0.0001$). In general, culture medium of viable bone explants showed the lowest average pH values (7.23) in comparison to empty (7.36) and dead control (7.43) media ($p \leq 0.0001$). The highest pH values were measured in culture medium from dead bone explants ($p=0.003$). Moreover, culture medium pH was higher in explants cultured in serum-containing (7.25), than in any of the SF media (SF: 7.22, SF + TGFβ_3: 7.21; $p \leq 0.017$).

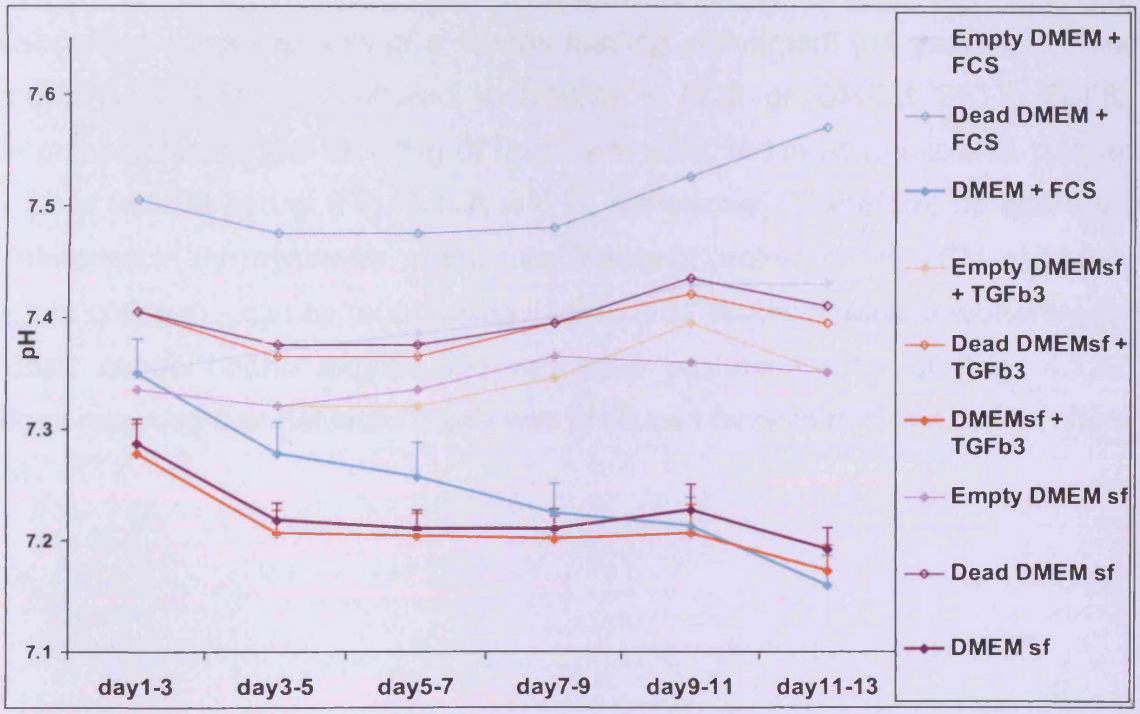

Fig. 4.11: Medium pH of culture medium received after 48 hour culture periods of human cancellous bone explants (♂ 71 years; ♂ 78 years). Graph shows the average medium pH values measured on 6 different time points during culture in DMEM + FCS, DMEM SF, or DMEM SF + TGFβ_3 and the standard error of the mean (n=12). ANOVA and Tukey post-hoc test were used for statistical analysis (* $p \leq 0.05$). The medium pH of viable cultured bone explants was descending during culture and showed in comparison to 'empty chamber' controls (n=2) and 'dead explant' controls (n=2) the lowest pH values.

4.1.4. The Effect of Serum-Free Medium on Type I Collagen Synthesis *Ex Vivo*

Ex vivo type I collagen synthesis was evaluated qualitatively by immunohistochemistry, using ProCI, which is often used for clinical investigations (Calvo *et al.* 1996). ProCI is a relatively instable pro-peptide released during type I collagen synthesis (Calvo *et al.* 1996; Miyahara *et al.* 1982). During the *in vivo* situation of new bone formation, produced ProCI is degraded within minutes. The stability of ProCI in cell culture is not known. Therefore, its detection on cultured bone explants implies type I collagen synthesis during *ex vivo* culture.

Type I collagen synthesis was investigated after 14 days culture of human cancellous bone explants of a human loading experiment (64 years, ♂). Bone explants were either cultured in DMEM + FCS or DMEM SF + TGFβ_3. Immunohistochemical labelling of ProCI was detected in bone explants cultured with or without serum [Fig. 4.12A and C; red arrows]. Therefore, no qualitative difference in the synthesis of the most frequent protein in the ECM of bone – type I collagen - can be found using FCS or a SF medium. Most importantly, the 'dead control' bone explant did not label positive for ProCI [Fig. 4.12E], demonstrating that detected ProCI was produced by cells during *ex vivo* culture.

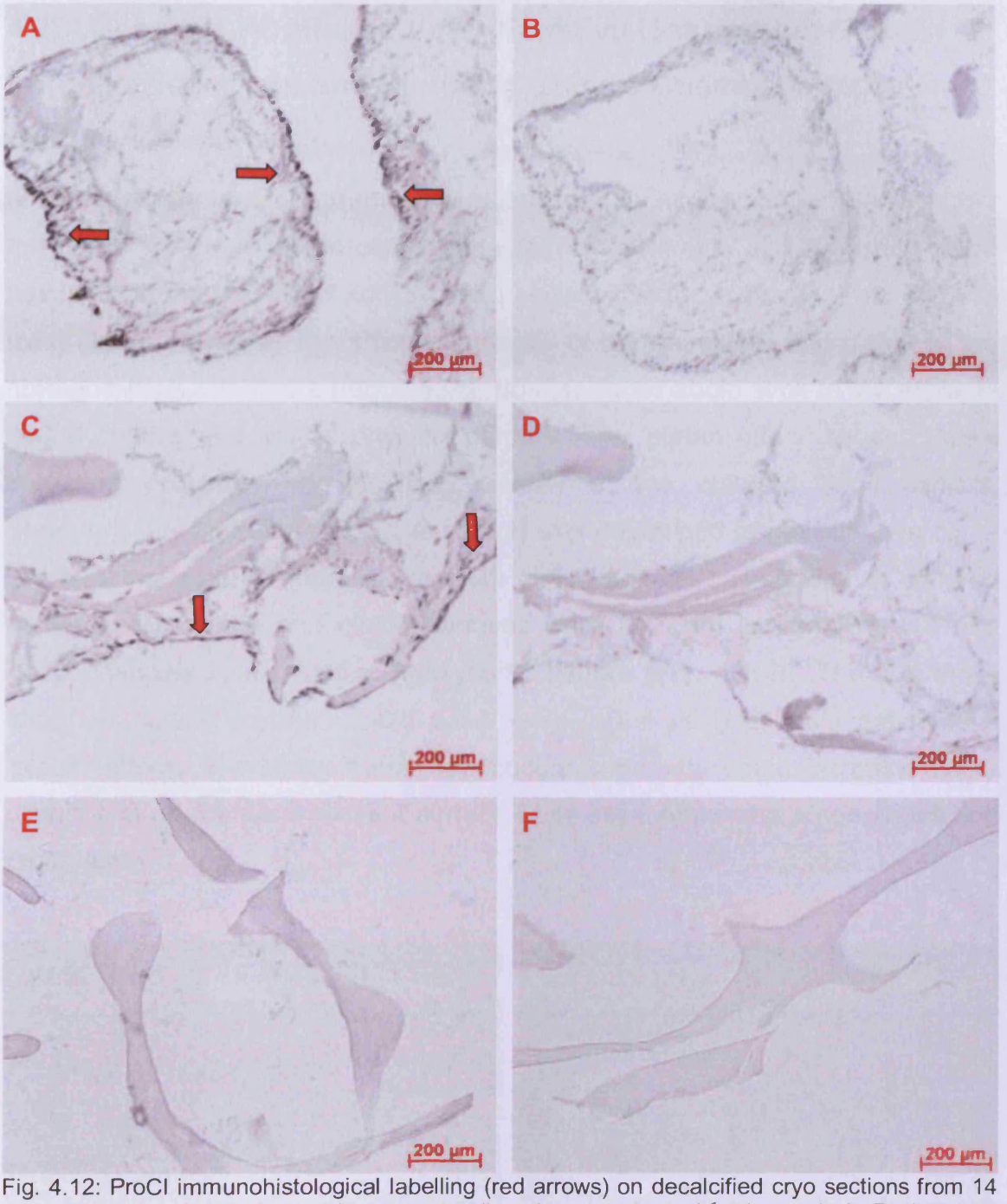

Fig. 4.12: ProCI immunohistological labelling (red arrows) on decalcified cryo sections from 14 days cultured and daily loaded human cancellous bone explants (♂ 64 years). A: Explant was cultured in DMEM + 10% FCS (B: Control without primary antibody). C: Explant was cultured in DMEM sf + TGFβ3 (D: Control without primary antibody). E: Dead bone explant - heat-inactivated at 56°C for 18 h prior to culture. (F: Control without primary antibody).

4.2. Does the Application of a Negative Honeycomb-Pattern onto the Baseplate and Piston of Culture Chambers Improve Culture Conditions?

A common phenomenon during the culture of cancellous bone explants within the ZetOs system is the so called 'platen effect' (Spilker *et al.* 1990) seen at the baseplate-exposed and piston-exposed surfaces of the explants. This effect is most likely caused by the smooth surfaces of the baseplate and piston of the normal culture chambers [Fig. 4.13A]. Both surfaces could stick to the explants during culture and act as physical barriers. The 'platen effect' causes media perfusion problems and declines viability of the cultured bone explant. Therefore, a new culture chamber design was developed to possibly overcome the effect or at least increase medium distribution on the surface of cultured explants. Baseplate and piston surfaces were modified by applying 0.2 mm deep channels in a negative honeycomb pattern [Fig. 4.13B]. Through these channels culture medium could pass, even in the case of the creation of a 'platen effect'. Therefore, these new culture chambers could increase media distribution on the bone explant surface while still maintaining a normal loading procedure.

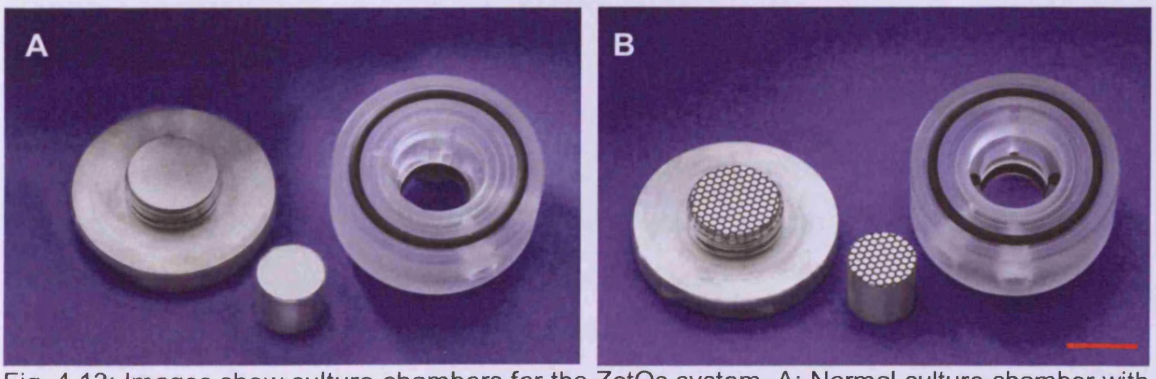

Fig. 4.13: Images show culture chambers for the ZetOs system. A: Normal culture chamber with smooth baseplate and piston surface. B: Honeycomb-surfaced chamber design with applied 0.2 mm deep channels on baseplate and piston surface. Scale bar represents 1 cm.

4.2.1. The Effect of Honeycomb-Surfaced Culture Chambers on Culture Medium Distribution

To investigate if the honeycomb chambers increase the distribution of the culture medium on the surface of bone explants, 3 experiments were performed using disulphine blue containing medium. Medium distribution was investigated using bovine cancellous bone explants (3-5-month-old calves). All cores were unloaded and no static preload was applied. Three bovine experiments were performed consisting of two groups. A total of 12 explants were cultured within normal chambers and 12 explants within honeycomb chambers. Table 4.2 shows the results gained from these experiments. Medium distribution was evaluated on baseplate and piston exposed bone explant surface, while medium penetration was evaluated after the explants were cut in halves. 'Plus' indicates an explant with even staining. 'Minus' defines an uneven staining seen on one bone explant. In total 11 from 12 explants in honeycomb chambers demonstrated even medium distribution, while all 12 showed an even medium penetration. Contrary, only 2-4 out of 12 explants in normal chambers showed even medium distribution and 5 out of 12 even medium penetration. Figure 14 shows representative images of both culture chamber groups. Uneven medium distribution is demonstrated on one surface of an explant cultured in a normal culture chamber [Fig. 4.14C: baseplate surface]. The explant cultured in a honeycomb chamber demonstrates an even medium distribution on both surfaces in combination with an even medium penetration [Fig. 4.14B, D, F]. Additional analysis via blind review by 3 independent evaluators revealed, that images from the group cultured in honeycomb chambers showed more even media penetration into the explants. Moreover, all 3 evaluators agreed that the occurrence of the 'platen effect' was seen more frequently on bone explants cultured in normal chambers. Therefore, the use of honeycomb chambers sufficiently reduced the 'platen effect'.

Table 4.2: Medium distribution and penetration investigation of normal and honeycomb culture chamber design using disulphine blue containing medium.

chamber	penetration	surface distribution	surface distribution
	middle cut	baseplate surface	piston surface
honeycomb	++++++++++++	++++++++++++ -	++++++++++++ -
normal	+++++ - - - - - - -	++ - - - - - - - - - -	++++ - - - - - - - -

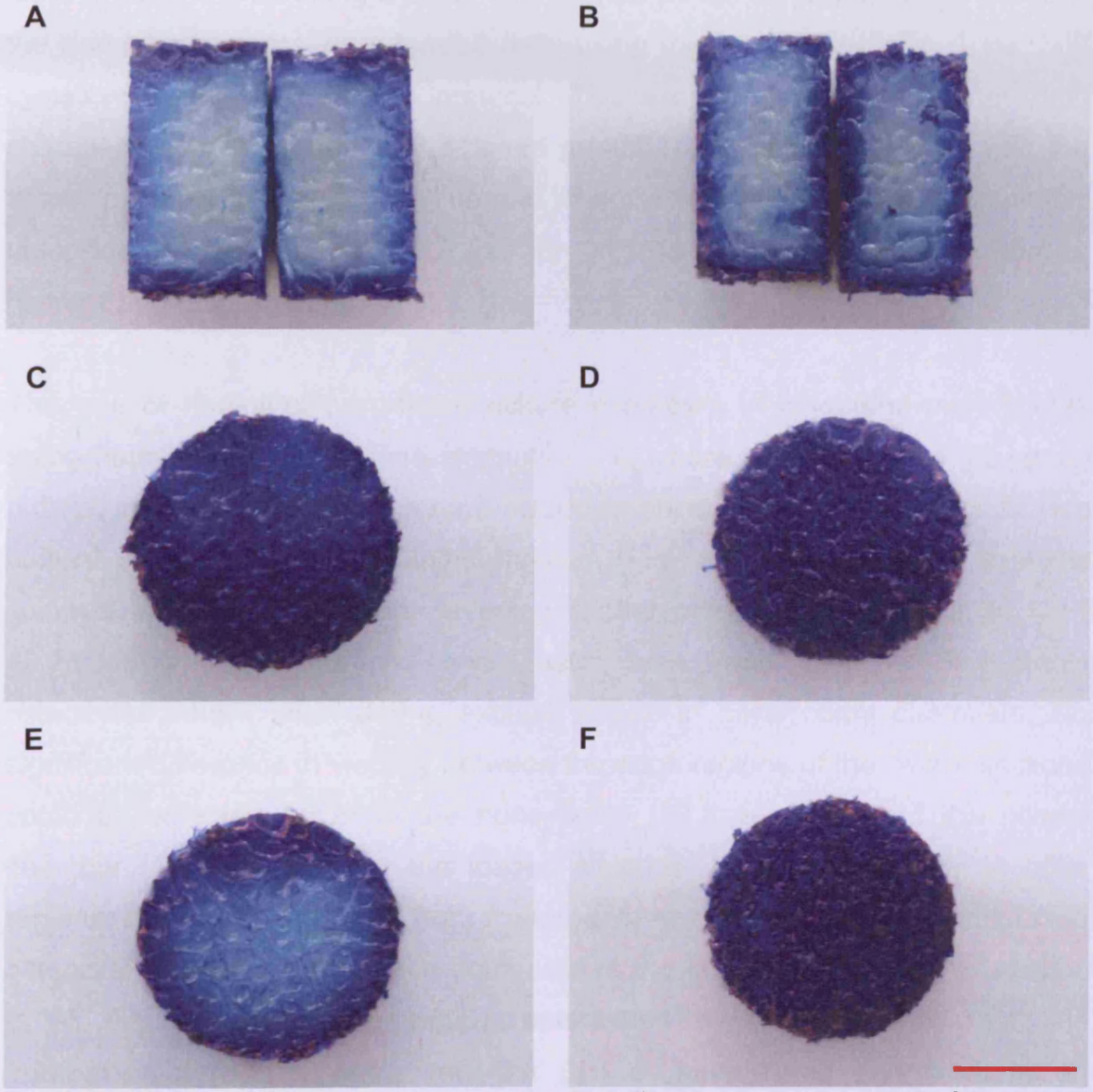

Fig. 4.14: Medium distribution and penetration investigation on bone explants from bovine metacarpal bone (3-5-month-old calf). Explants were cultured overnight in the ZetOs system and perfused with medium containing disulphine blue for 14 min. Images show the middle cut regions (A, B), the baseplate surface (C, D) and the piston-exposed surface (E, F) of 2 representative explants. A, C, E: Core within a normal chamber. B, D, E: Core within a honeycomb chamber. The 'platen effect' is demonstrating using the normal culture chamber. Scalebar represents 5 mm.

4.2.2. The Effect of Honeycomb-Surfaced Culture Chambers on Osteocyte Viability

The culture of human cancellous bone explants within honeycomb-surfaced culture chambers was compared to culture in normal culture chambers. The effect of both culture chambers on osteocyte viability was investigated in human loading experiments using DMEM + 10 % FCS as the culture medium. Cores in the normal chambers were loaded daily using the 'complete jump', 1 Hz, 300 cycles, 5 min, 4000 µstrain protocol. The explants inside the honeycomb chambers were assigned to 2 different groups, one was loaded daily with the same protocol used for the normal chambers, and one was completely unloaded. All explants were cultured for 14 days in the ZetOs system prior to harvest and LDH staining.

The use of honeycomb-surfaced culture chambers in 3 loading experiments using human cancellous bone explants (♀ 75 years, ♂ 36 years, ♂ 72 years) did not increase the critical central osteocyte survival during long-term *ex vivo* culture using a serum-containing medium [Fig. 4.15]. Daily loaded explants cultured in normal chambers revealed central osteocyte survival of 30.1 +/- 46.28 viable osteocytes/mm^2 bone matrix area, while 29.9 +/- 30.9 viable osteocytes where seen during explant culture in honeycomb chambers. No significant difference in viability between the edge regions of the centre sections could be detected between the honeycomb (57.3 +/- 37.5) and the normal chamber (46.4 +/- 37.4) in the loaded situation. Osteocyte survival in edge regions of central sections from cultured bone explants showed increased osteocyte viability maintenance compared to the centre regions of that section in all medium groups ($p \leq 0.005$), determined by Mann-Whitney test and Bonferroni correction. Moreover, the use of honeycomb chambers in an unloaded situation led to increased osteocyte survival at the edge region of the centre sections from 46.4 +/- 37.4 viable osteocytes/mm^2 bone matrix area achieved with normal chambers under loaded situation to 76.9 +/- 45 ($p \leq 0.0001$). Therefore, the absence of the 'platen effect' using honeycomb culture chambers might lead to increased cell survival at the edge region of the

cultured explants, however, the critical central osteocyte survival can not be increased using this chamber design with a serum-containing medium.

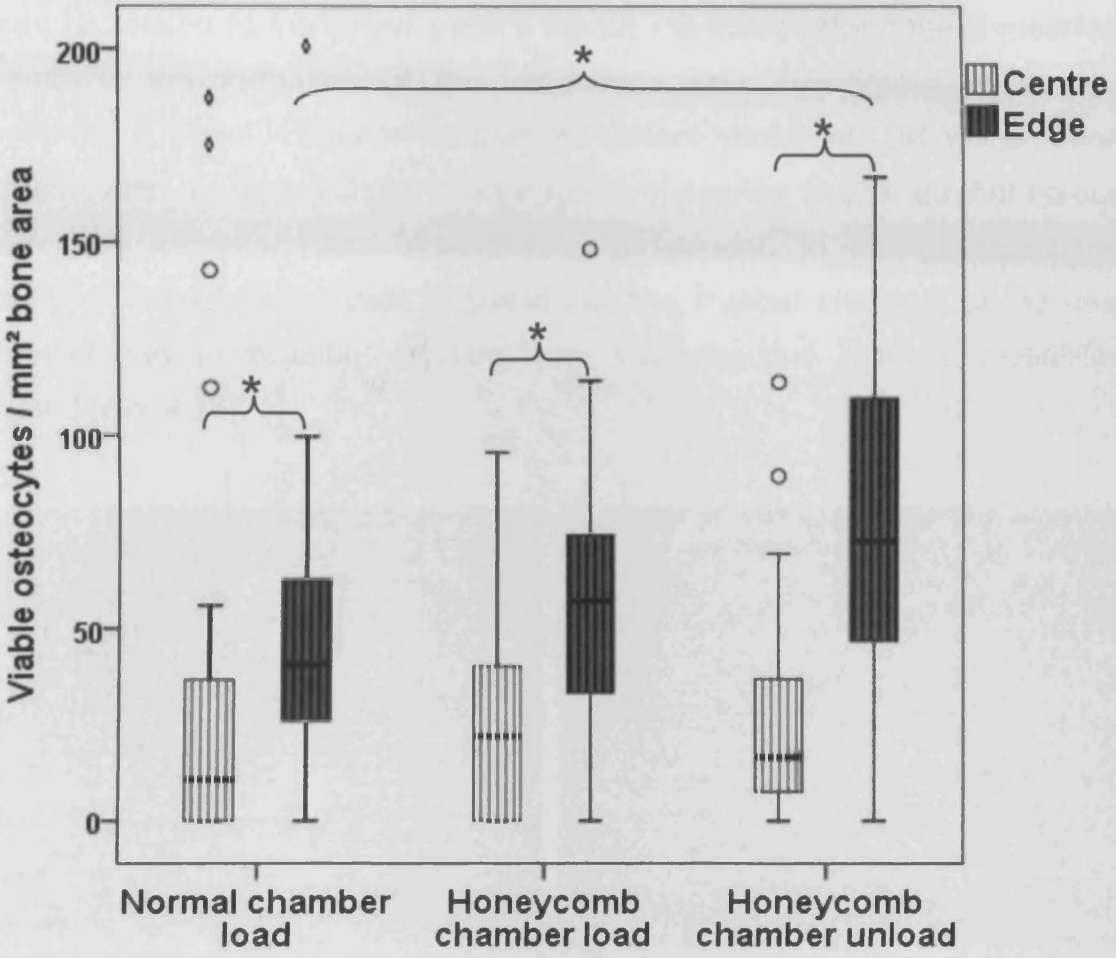

Fig. 4.15: The graph shows the effect of different culture chambers on osteocyte viability evaluated on the centre section of human bone explants (♀ 75 years, ♂ 36 years, ♂ 72 years) after 14 d culture within the ZetOs bioreactor. Three different groups were investigated – normal chamber with daily load, honeycomb chamber with daily load, and honeycomb chamber unloaded. The centre and edge region of the sections were evaluated. Box plots show the median line, the 25% and 75% quartiles, the 1.5x interquartile-range whiskers, as well as outliers (○) and extremes (◊). Kruskal-Wallis Test was used as comparison between groups, Mann-Whitney test and Bonferroni correction were used to compare centre and edge regions within groups (* $p \leq 0.05$). Osteocyte viability was significantly higher on edge regions in all groups. Moreover, osteocyte viability was significantly higher in the edge region of the honeycomb chamber unloaded group than in the normal chamber loaded group.

4.2.3. The Effect of Honeycomb-Surfaced Culture Chambers on the Formation of the Surface Fibrous Membrane

To investigate if the unimproved osteocyte survival in honeycomb chambers might be related to increased surface fibrous membrane using these modified chambers, the formation of this membrane was investigated in loading experiments. Some images taken from the surface section of LDH stained bone explants after 14 days culture in honeycomb chambers suggested that fibrous membrane formation was increased in comparison to normal chambers. Moreover, fibroblast-like cells migrated into the created channels of the new chamber design possibly blocking any advantageous nutrient availability created [Fig. 4.16].

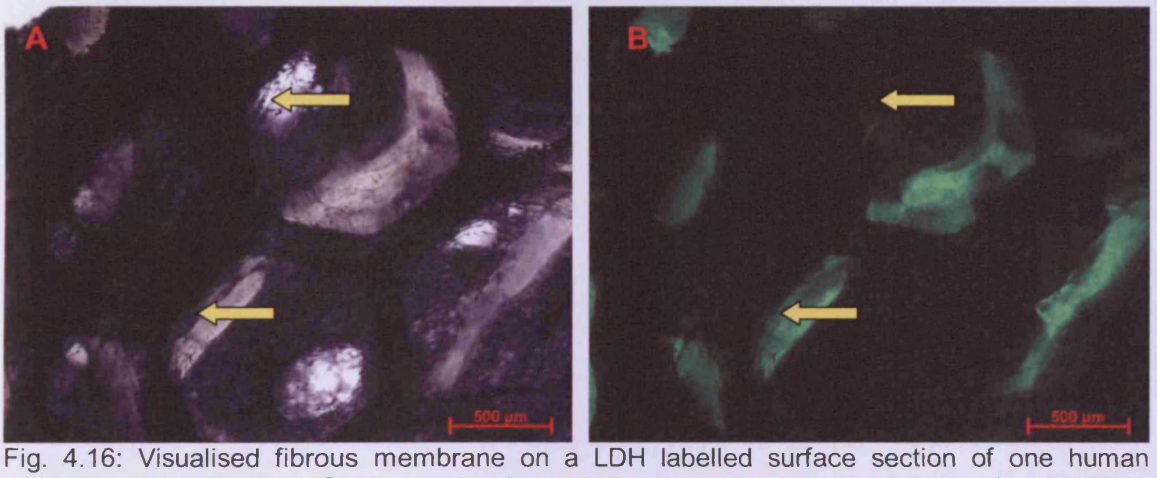

Fig. 4.16: Visualised fibrous membrane on a LDH labelled surface section of one human cancellous bone explant (♀ 55 years) cultured in honeycomb culture chamber using DMEM + 10% FCS. **A:** Bright field micrograph. **B:** Micrograph using fluorescence microscopy (515–565 nm emission filter). The fibrous membrane grown inside the honeycomb channels of the culture chamber is marked by yellow arrows.

A total of 25 explants were cultured in serum-containing medium, 13 (52% of all explants cultured with serum) showed the formation of a fibrous membrane on the surface. On the surface of 3 explants, few fibroblast-like cells were detected, while 9 explants cultured with serum showed no fibrous membrane formation. Six explants were cultured in honeycomb chambers under loaded condition, 4 (66.7%) showed the formation of a fibrous membrane on the surface. On the surface of 1 explant few fibroblast-like cells were detected, while only 1 explant cultured in a honeycomb chamber under loaded condition showed no fibrous membrane formation. Using honeycomb chambers in an unloaded situation

increased fibrous membrane occurrence to 83% (5 out 6 explants). Therefore, honeycomb chambers increased the formation of surface fibrous tissue in comparison to culture in normal chambers. This may have an effect central osteocyte survival in honeycomb chambers.

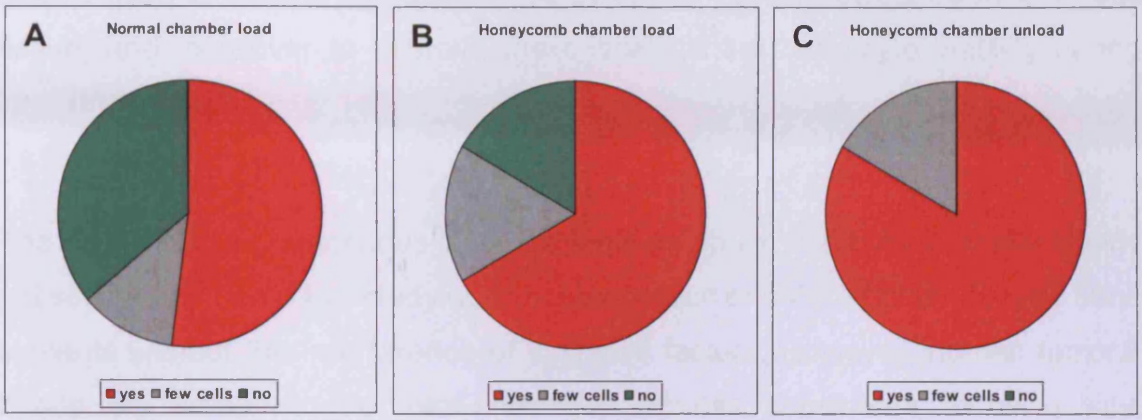

Fig. 4.17: Fibrous membrane formation determined in 3 human loading experiments (♀ 75 years, ♂ 36 years, ♂ 72 years). Explants were either cultured in normal culture chambers and loaded daily (A), in honeycomb-surfaced culture chamber and loaded daily (B), or were cultured in honeycomb-surfaced culture chamber non-loaded (C). The occurrence of the fibrous membrane was increased from 52% using a normal culture chamber under loaded condition to 67% using honeycomb culture chambers loaded, and up to 83% using honeycomb chambers in unloaded condition.

4.3. Discussion

This chapter is dedicated to the need to improve *ex vivo* culture conditions for cancellous bone explants within the ZetOs system. Attempts to increase central cell survival have been demonstrated. The use of a SF culture medium has been shown to reduce the occurrence of the non-physiological surface fibrous tissue, and moreover to improve the critical central osteocyte viability during long-term culture.

The use of human cancellous bone explants as an *ex vivo* culture model clearly possesses the benefit of studying 3D osteoporotic or osteoarthritic-derived bone explants without the interference of systemic factors. However, human femoral heads are known to be intra-/internally variable considering bone quality, structure, stiffness, and material density (Li & Aspden 1997). Therefore, culture conditions should be consistent and defined in order to achieve reasonable results. SF medium enables the culture of cells within a defined culture milieu (Barnes & Sato 1980). Yet, serum starvation is suspected to induce osteocyte apoptosis *in vitro* (Bakker *et al.* 2004). The replacement of viability maintaining factors in FCS was attempted in this study by the addition of insulin-transferrin-selenium, lipid supplements, and TGFβ_3. It is known from the studies of Karsdal *et al.* that TGFβ_1 is able to rescue matrix metalloprotease-induced apoptosis of MC3T3-E1 cells, a clonal non-transformed cell line established from newborn mouse calvaria (Karsdal *et al.* 2002). In 2001, the same group showed that TGFβ could activate the p38 MAPK in osteoblasts. This MAP kinase pathway seems to be involved in anti-apoptotic effects of TGFβ_1 on osteoblasts (Karsdal *et al.* 2001). Moreover, MT1-MMP activation of latent TGFβ_1 seems to be responsible for the maintenance of osteoblast viability during trans-differentiation into osteocytes (Karsdal *et al.* 2004). The second member of the TGFβ superfamily - TGFβ_2 - has been shown to have a protective role in osteoblast apoptosis (Dufour *et al.* 2008). The influence of TGFβ_3 on *ex vivo* osteocyte survival was recently demonstrated in the ZetOs culture system for human cancellous bone (Simpson *et al.* 2009). However, to our knowledge, all cell culture experiments were performed in the presence of serum. In the

current study, we successfully used a SF culture medium with TGFβ$_3$ for long-term culture of human cancellous bone explants. Central osteocyte survival of 14 days SF cultured bone explants was significantly increased compared to the use of FCS-containing media. Yet, no superior viability effect of TGFβ$_3$ could be detected within a SF medium. Further investigations on the effects of SF culture and TGFβ$_3$ will be presented in Chapter 5.

The depletion of serum from culture medium has also been thought to decrease the unwanted formation of a non-physiological surface fibrous membrane (Barnes & Sato 1980; Bingham & Raisz 1974; Trowell 1959). These fibroblast-like cells covering the explants during culture will compete for nutrients with the underlying cells. Moreover, these cells might influence the culture conditions by the secretion of unwanted factors stimulating unintended cellular responses by the explants cells. Within this study, we were able to demonstrate a positive effect of SF medium on the culture of human cancellous bone explants. The results further suggest that the most likely justification for the positive viability results using a SF medium is due to the reduction of the non-physiological surface fibrous tissue, which then allows for improved nutrition in the central areas of the bone core. Moreover, *de novo* type I collagen synthesis *ex vivo* did not differ between FCS or SF culture, suggesting, that at least qualitatively SF medium supplementation did not change this important cellular expression characteristic. Osteoclast-dependent *ex vivo* type I collagen resorption was determined by release of NTx into the culture medium during one human loading experiment (78 years, ♂) (data not shown). From this preliminary data, the use of SF medium independent of the addition of TGFβ$_3$ significantly increased NTx release ($p \leq 0.05$) compared to the use of serum-containing medium. However, this method can not prove if this preliminary result is equivalent to increased osteoclast activity, or improved osteoclast viability in SF medium. Yet, taken into consideration, that both bone formation and resorption could be detected after 14 days of *ex vivo* culture of human cancellous bone explants, cellular activity resembles at least to a certain extent the *in vivo* situation of bone turnover.

In a set of human loading experiments (♂ 71 years, ♂ 78 years) osteocyte survival prior to long-term culture was compared to viability after 14 days culture and daily loading within the ZetOs system. In these experiments the loss of viability due to culture in DMEM + FCS was 75% on average (day 0 average = 180 +/- 92.7; d14 DMEM + FCS average = 46.8 +/- 47.9 viable osteocytes / mm^2 bone matrix area). This dramatic decrease in the critical central survival region of *ex vivo* cultured human cancellous bone explants was maintained at a significantly higher level using DMEM SF+ TGFβ_3 as culture medium (Mann Whitney test and Bonferroni correction, $p \leq 0.0001$). With an average of 76.2 +/- 62 viable osteocytes per mm^2 bone matrix area the loss in osteocytes was about 58%. Therefore, the use of SF medium increases *ex vivo* viability maintenance, achieved by serum-containing medium, about 62%. However, the amount of dying osteocytes during culture was still large and further improvements were considered.

The use of a new culture chamber design with applied channels on baseplate and piston surface was investigated to increase nutrient availability for cultured explants. It was demonstrated that this design prevented the 'platen effect' on bone explants surfaces and therefore increased medium distribution on the surface of cultured explants, as well as medium perfusion into the explants. Yet, this improvement had no influence on central osteocyte survival and might be at least partly due to increased occurrence of surface fibrous membrane formation using honeycomb chambers. However, the increased surface fibrous tissue formation in honeycomb chambers in an unloaded situation did not decrease osteocyte survival at the edge of cultured explants, suggesting that these fibroblast-like cells are not the only reason for a reduced central cell survival. A set of experiments was intended to investigate the new chamber design using SF medium; yet, this could not be completed due to the absence of the ZetOs system in the second half of this study. Further changes were intended, to gain appropriate cell viability maintenance during long-term culture of human cancellous bone explants. The insertion of short rest periods (seconds) within the daily loading regime to increase fluid flow and therefore nutrient availability

during culture was intended, and a pre-experiment was performed (data not shown). As the 'standard' loading procedure used includes one complete jump waveform after the other, the aim of inserted rest periods as to improve fluid flow through the lacuno-canalicular system by allowing the bone explant to relax between individual loading cycles. Even though the insertion of a 7 s rest period into a 5 min daily loading diminished the significant difference between central and edge osteocyte survival of central sections of bone explants, no significant improvement of central osteocyte survival compared to 'standard' loading could be reached.

Another idea was to supply the culture medium with an oxygen carrier to increase the solubility of this critical metabolic substrate for the cells. Radisic *et al.* have recently shown that the use of a perfluorocarbon (PFC) as synthetic oxygen carrier increases DNA synthesis as well as contractile properties of a heart muscle tissue engineering construct (Radisic *et al.* 2008) The use PFC in an *ex vivo* setting for cancellous bone explants might have been advantageous for the cultured cells. Oxygen measurements using oxygen sensors in the culture medium would have been needed prior to PFC application, in order to ensure that oxygen is the limiting factor in this culture system, as the results presented in this chapter concerning the metabolic state of cultured explants, did not reveal the expected ratio of lactate/glucose levels for anaerobic (glycolysis) respiration. Mature organs are known to predominantly rely on aerobic respiration (Trowell 1961). To our surprise, the explants did not use the supplied glucose in the culture medium, but produced high levels of lactate at the same time, indicating that some cultured cells, might have been hypoxic and underwent anaerobic respiration possibly metabolising another energy source i.e. pyruvate from the medium to produce lactate. Yet, it seems likely, that some cells metabolised aerobically (oxidative phosphorylation) possibly other energy sources than sugar, i.e. fatty acids. However, no generalised conclusions can be drawn from the data.

The medium pH of cultured bone explants decreased over the culture period, which is due most likely due to the ascending production of metabolic products, i.e. lactic acid (Borle *et al.* 1960). The medium pH in SF culture medium was significantly lower than in FCS-containing medium. Taking into consideration that osteocyte survival is increased under the depletion of FCS. We could speculate that more osteoclasts were also kept alive under SF conditions, leading to decrease in pH due to osteoclastic bone resorption activities. In the appendix a pre-experiment on the release of NTx under FCS-containing or SF medium condition is presented, which suggests that bone resorption is increased due to serum depletion.

In summary, this chapter showed improvements made that increased *ex vivo* culture conditions of cancellous bone explants. The use of a SF culture medium did result in superior viability and reduced surface fibrous membrane formation during culture. However, more investigations on the role of SF medium on *ex vivo* osteoclast survival as well as metabolism in comparison to culture under the presence of FCS would have been essential.

Chapter 5: The Cellular Effects of TGFβ$_3$ in the Presence or Absence of FCS - *In Vitro* Investigations Using Different Culture Methods of Osteoblast-Lineage Cells

Members of the TGFβ super-family are generally known to mediate key events in the growth and development of a variety of species. The main effect of TGFβ is to cause osteoblasts to differentiate and produce extracellular matrix (Bonewald 2002). However, the cellular effects of TGFβs on osteoblast-like cells seem to be dependent on the state of cell differentiation (Bonewald & Dallas 1994). In osteoblast-enriched cultures from foetal tissue, TGFβ may act as a mitogen, but using osteosarcoma cells that represent a more differentiated osteoblast phenotype, TGFβ inhibits DNA synthesis (Centrella *et al.* 1994). Therefore, it is of crucial importance to use an appropriate *in vitro* culture model with the required state of osteoblast differentiation for the intended investigation. The results presented in Chapter 4 demonstrated that the cellular effect by TGFβ$_3$ on osteocyte viability in cancellous bone explant culture seems to depend on the presence or absence of serum in the culture medium. Due to the absence of the ZetOs bone organ culture system in the second half of this study, we decided to further concentrate on *in vitro* culture methods to study the cellular effects caused by TGFβ$_3$.

In vitro studies on the cellular effects by TGFβs on osteoblast-lineage cells have frequently been carried out using 2D monolayer culture of osteoblasts. From these studies, a huge amount of knowledge has been acquired, increasing the understanding of the role of the TGFβ growth factors in bone biology.

The generally accepted culture method for osteoblasts is 2D monolayer culture (Di Silvio & Gurav 2001; Majeska & Gronowicz 2002). During this culture method osteoblasts start to differentiate and lay down ECM. Lian and Stein described the processes of *in vitro* differentiation of monolayer-cultured rat

calvaria derived osteoblasts in detail (Lian & Stein 1992). Osteoblast behaviour in 2D culture generally goes through three phases – proliferation, matrix maturation and matrix mineralisation. At the onset of *in vitro* differentiation spindle-shaped osteoblasts proliferate to form a dense multilayer. During this stage the cells undergo morphological changes and express high levels of type I collagen, the most abundant protein in the extracellular matrix of bone. The start of the second phase – matrix maturation – is characterised by an up-regulation of alkaline phosphatase activity (Lian & Stein 1992). Reaching a constant cell number, characterised by a balance between cell proliferation and cell death, osteoblasts start to produce non-collagenous extracellular matrix proteins, such as osteopontin (Oldberg *et al.* 1986) and osteocalcin (Hauschka *et al.* 1989). The maturation of the synthesised ECM is finalised with the incorporation of hydroxyapatite crystals within the matrix. This step can be visualised by the formation of mineralised nodules *in vitro*.

One of the main problems using adult human osteoblasts even at low passage number is their inadequate ability to maintain their differentiation stage *in vitro*, as well as to differentiate further into more mature osteoblasts and later to osteocyte-like cells. To induce *in vitro* differentiation of primary osteoblasts in monolayer culture and, therefore, to create *in vitro* osteocyte-like cells, medium additives such as β-glycerolphosphate, dexamethasone or specific nutrient enrichment are commonly used (Di Silvio & Gurav 2001; Gallagher 2003). Yet, ECM deposition and calcification by primary osteoblasts in 2D culture is limited and can take up to 30 days *in vitro* (Di Silvio & Gurav 2001), resulting in a prolonged experimental time to allow osteoblast differentiation to the desired level. Also, *in vivo* active osteoblasts are defined by a cuboidal cell shape, while the resting bone lining cells or inactive osteoblasts demonstrate a spindle-shaped morphology *in vivo* as seen during the initial proliferation period of osteoblast monolayer culture *in vitro*.

For the *in vitro* monolayer culture of primary osteoblasts, the results of Owen *et al.* (1990) suggest a functional relationship between the inhibition of proliferation

and the induction of genes associated with cell differentiation and matrix maturation (Owen *et al.* 1990). Using foetal rat calvaria cells, the group showed that the inhibition of proliferation by hydroxyurea resulted in a subsequent up-regulation of alkaline phosphatase followed by up-regulation of osteopontin expression and, therefore, differentiation of osteoblasts *in vitro*. Moreover, it is well known that the formation of nodules, which simulates a micromass, during osteoblast monolayer culture leads to a rapid osteoblast differentiation *in vitro* (Bellows *et al.* 1986). Taken from these results, we hypothesised that mature/late osteoblasts, maybe even early osteocytes can be generated *in vitro* using a culture system that reduces the initial cell proliferation.

This chapter will, therefore, investigate the cellular effects of TGFβ3 in the presence or absence of serum using different *in vitro* osteoblast culture models. One part of this chapter will investigate the effects of TGFβ3 on human osteoblasts cultured in monolayer. While, in addition, a multilayer and a pellet culture model for human primary osteoblasts will be introduced. Within these culture models we were aiming to generate *in vitro* a population of more mature osteoblasts (up to early osteocytes), which can then be used for the *in vitro* investigation of cellular effects caused by growth factor addition. Moreover, the *in vitro* simulation of the *in vivo* bone organ situation will be taken a step further by the use of a 3D type I collagen gel-based osteoblast-osteocyte co-culture model (Mason *et al.* 2008). Within this model system, the two cell types are cultured interconnected with osteocytes being embedded within the gel and osteoblasts cultured on top. The multilayer culture model and the co-culture model will then be further used for the investigation of the cellular effects of TGFβ3.

5.1. The Effect of TGFβ3 and SF Medium on Monolayer Cultured Osteoblasts

The effect of TGFβ3 addition (5, 15, or 50 ng/ml) in the presence or absence of FCS was investigated in a set of experiments using human primary osteoblasts (♀ 80 years, ♂ 59 years; ♂ 62 years). Osteoblasts were isolated as described in 2.2.12.1. However two different sets of isolated osteoblasts were compared in this experiment. The first out-migrating population of cells from the bone pieces were termed early isolated osteoblasts, and the third out-migrating population of cells from the bone pieces were called late isolated osteoblasts. All cells were cultured in monolayer as described in 2.2.13 and exposed to the different culture supplements for 14 days culture.

We hypothesised, that early isolated osteoblasts will react differently to the treatments than late isolated osteoblasts. More specific, we speculated that early isolated osteoblasts would mimic 'younger' osteoblasts, as the bone pieces were not enzymatically treated prior to cell isolation. Moreover, we speculated that late isolated osteoblasts will behave more similar to more mature osteoblasts / early osteocytes, as these cells are potentially derived from further inside the bone matrix, and were only released due to the experience of two short enzymatic digestions using trypsin. Monolayer culture experiments of these cell populations were used to increase our knowledge on potential differences in cellular responses to TGFβ3 treatment depending on the presence or absence of FCS within culture medium. Investigations in this set of experiments will evaluate proliferation, cell activity, mineralisation, as well as expression of osteoblast marker genes.

Representative micrographs of early and late isolated osteoblasts (♀ 80 years) cultured in monolayer for 2 days are shown in figure 5.1 and 5.2 respectively. Morphologically, both cell populations appear very similar if cultured in DMEM + FCS. The cell population demonstrates the presence of cells with a spindle-shaped morphology and some with a rather cuboidal morphology [Fig. 5.1A, 2A;

see blue arrows]. Possibly the occurrence of long cell processes was higher in later isolated osteoblasts than in early isolated ones [Fig. 5.2A; see yellow arrow]. The addition of TGFβ$_3$ to FCS-containing medium resulted the generation of areas of increased cell density in both cell populations [Fig. 5.1C, 1E, 1G, 2C, 2E, 2F; green arrows]. Moreover, it appeared that cells which were not entrapped in these areas of high cell density demonstrated very long slender-like cell processes to connect with other cells (yellow arrow). The depletion of FCS resulted in both cell populations in a domination of spindle-shaped and very elongated cells [Fig. 5.1B, 2B]. The addition of TGFβ$_3$ to SF medium seemed to morphologically change the cells into more flat and spread-out cells [Fig. 5.1D, 1F, 1H, 2D, 2F, 2H].

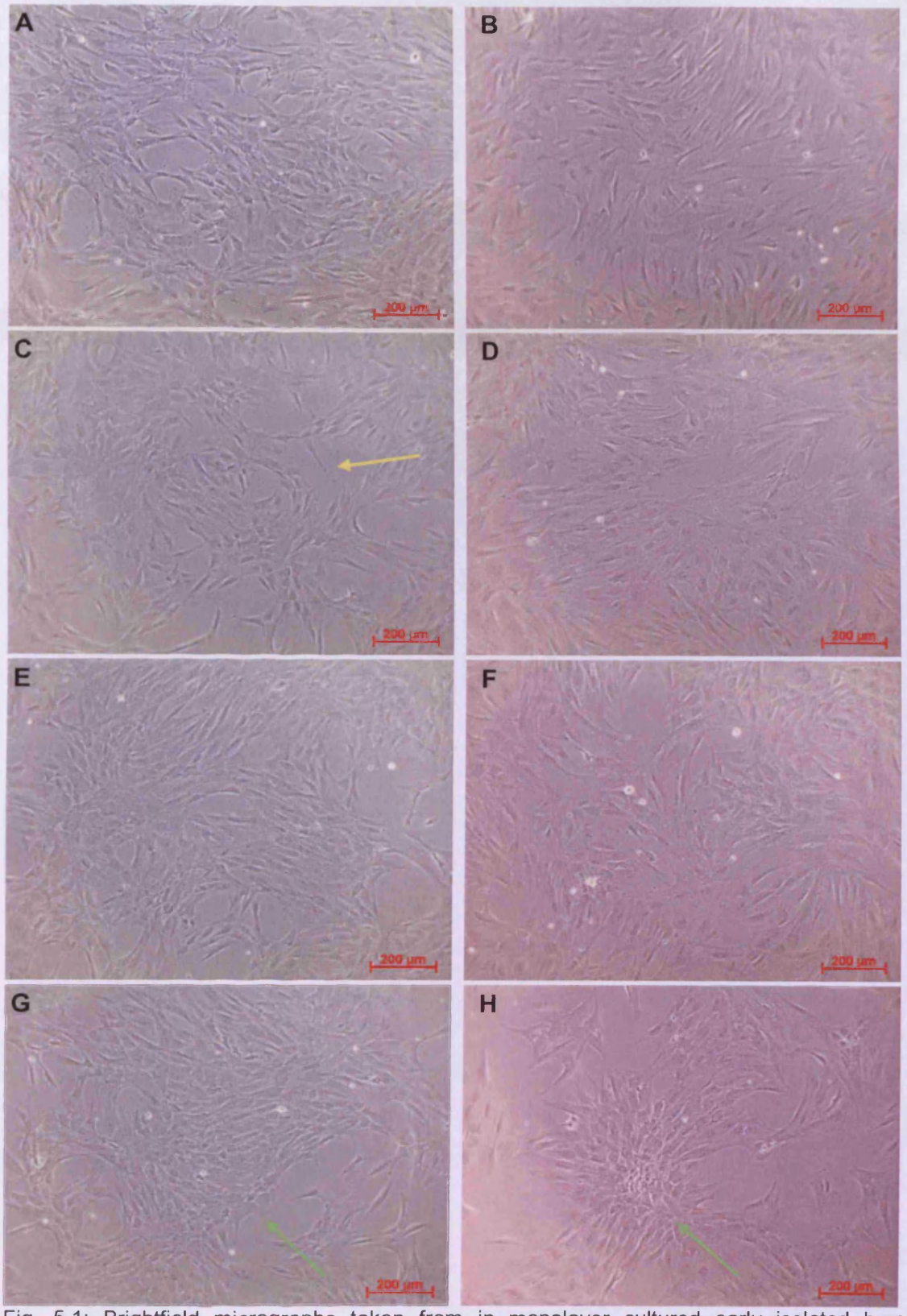

Fig. 5.1: Brightfield micrographs taken from in monolayer cultured early isolated human osteoblasts (♀ 80 years). A: DMEM+FCS; C: DMEM+FCS+5 ng/ml TGFβ$_3$; E: DMEM+FCS+15 ng/ml TGFβ$_3$; G: DMEM+FCS+50 ng/ml TGFβ$_3$; B: DMEM SF; D: DMEM SF+5 ng/ml TGFβ$_3$; F: DMEM SF+15 ng/ml TGFβ$_3$; H: DMEM SF+50 ng/ml TGFβ$_3$. Yellow arrow indicates long cell processes, green arrows show areas of high cell density.

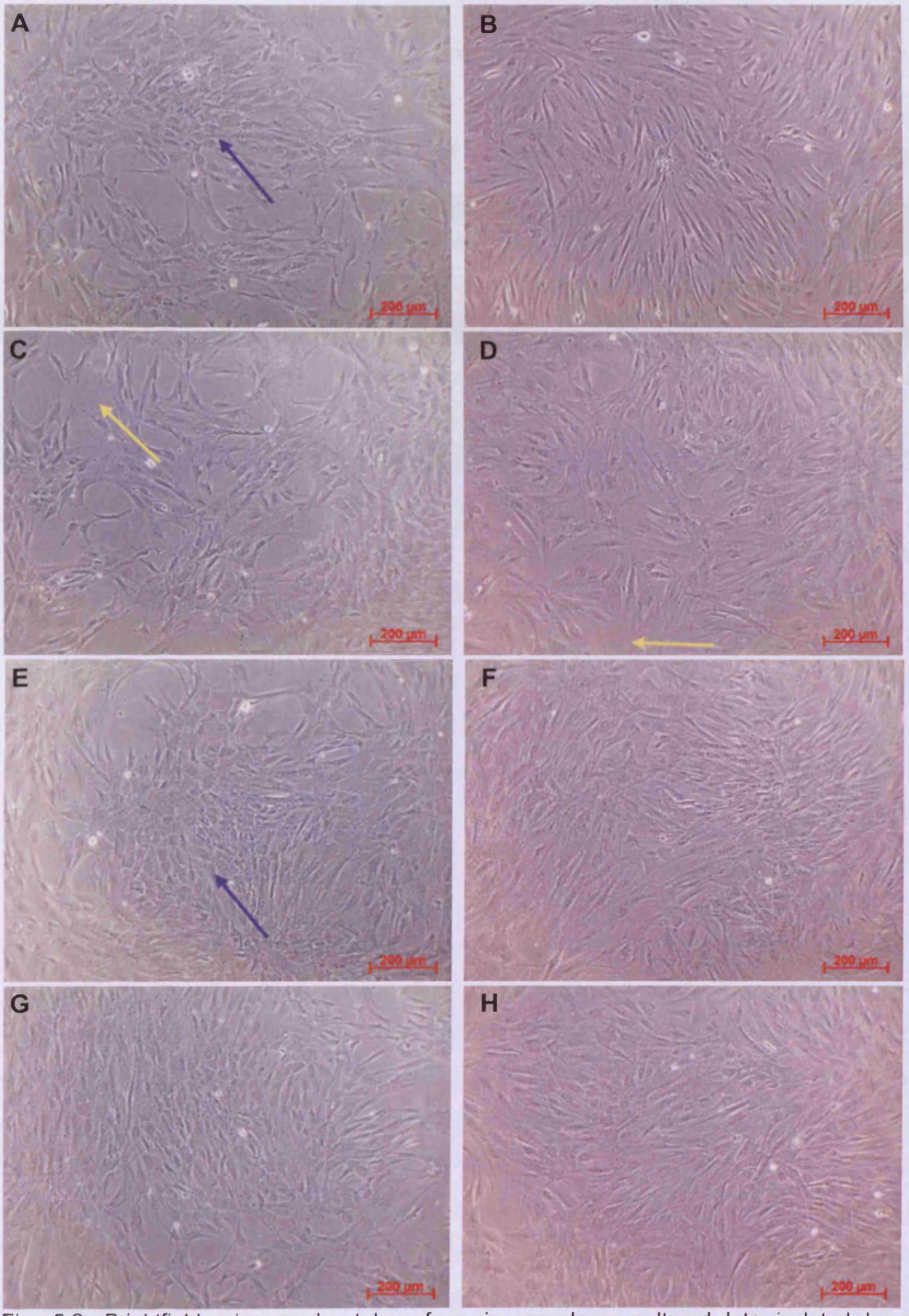

Fig. 5.2: Brightfield micrographs taken from in monolayer cultured late isolated human osteoblasts (♀ 80 years). A: DMEM+FCS; C: DMEM+FCS+5 ng/ml TGFβ$_3$; E: DMEM+FCS+15 ng/ml TGFβ$_3$; G: DMEM+FCS+50 ng/ml TGFβ$_3$; B: DMEM SF; D: DMEM SF+5 ng/ml TGFβ$_3$; F: DMEM SF+15 ng/ml TGFβ$_3$; H: DMEM SF+50 ng/ml TGFβ$_3$. Yellow arrows indicate long cell processes, blue arrows show areas of cuboidal cells.

5.1.1. Proliferation of Human Primary Osteoblasts Cultured in Monolayer – the Effect of TGFβ3 and FCS

Cell proliferation during the culture of early and late isolated osteoblasts was investigated using the Hoechst DNA assay (2.2.14.3). As it would be expected for monolayer cultured osteoblasts, the amount of DNA increased significantly from day 2 to day 14 ($p \leq 0.0001$). This effect was true for all media supplements during culture of both osteoblast isolation stages [Fig. 5.3], confirming that the monolayer culture model itself behaves as commonly accepted (Lian & Stein 1992).

The addition of TGFβ3 to FCS-containing medium had no significant effect on the amount of DNA in early isolated osteoblasts [Fig. 5.3A]. In late isolated osteoblasts, the addition of 5 and 15 ng/ml TGFβ3 to FCS-containing medium significantly increased the amount of DNA after 14 days of culture ($p \leq 0.033$) [Fig. 5.3C]. All osteoblasts cultured with TGFβ3 in SF medium revealed increased DNA amount compared to SF alone [Fig. 5.3B, D]. This effect was seen in early isolated osteoblasts over all culture days when 50 ng/ml TGFβ3 were added ($p=0.007$), while in late isolated osteoblasts this effect was already seen with doses as low as 15 ng/ml TGFβ3 ($p \leq 0.001$).

The comparison of SF versus FCS-containing medium exposure [Fig. 5.4A, C] revealed significant differences in DNA amount. In general, SF medium showed lower average DNA content than FCS-containing medium ($p \leq 0.0001$). This effect was the same for both populations of human primary osteoblasts. On day 14 the difference between early isolated osteoblasts cultured in FCS-containing media +/- TGFβ3 compared to culture in its SF opponent was 3778 ng +/- 375 ng on average, and for late osteoblasts 4155 ng +/- 658 ng DNA. Interestingly, early isolated osteoblasts demonstrated higher mean DNA amount than later isolated osteoblasts, but only in the presence of FCS [Fig. 5.4B], suggesting that either earlier isolated osteoblasts need less stimulus - only FCS, not FCS and TGFβ3 - to proliferate at a higher level, or that the two cell populations have different proliferation potential ($day \geq 8$, $p=0.018$).

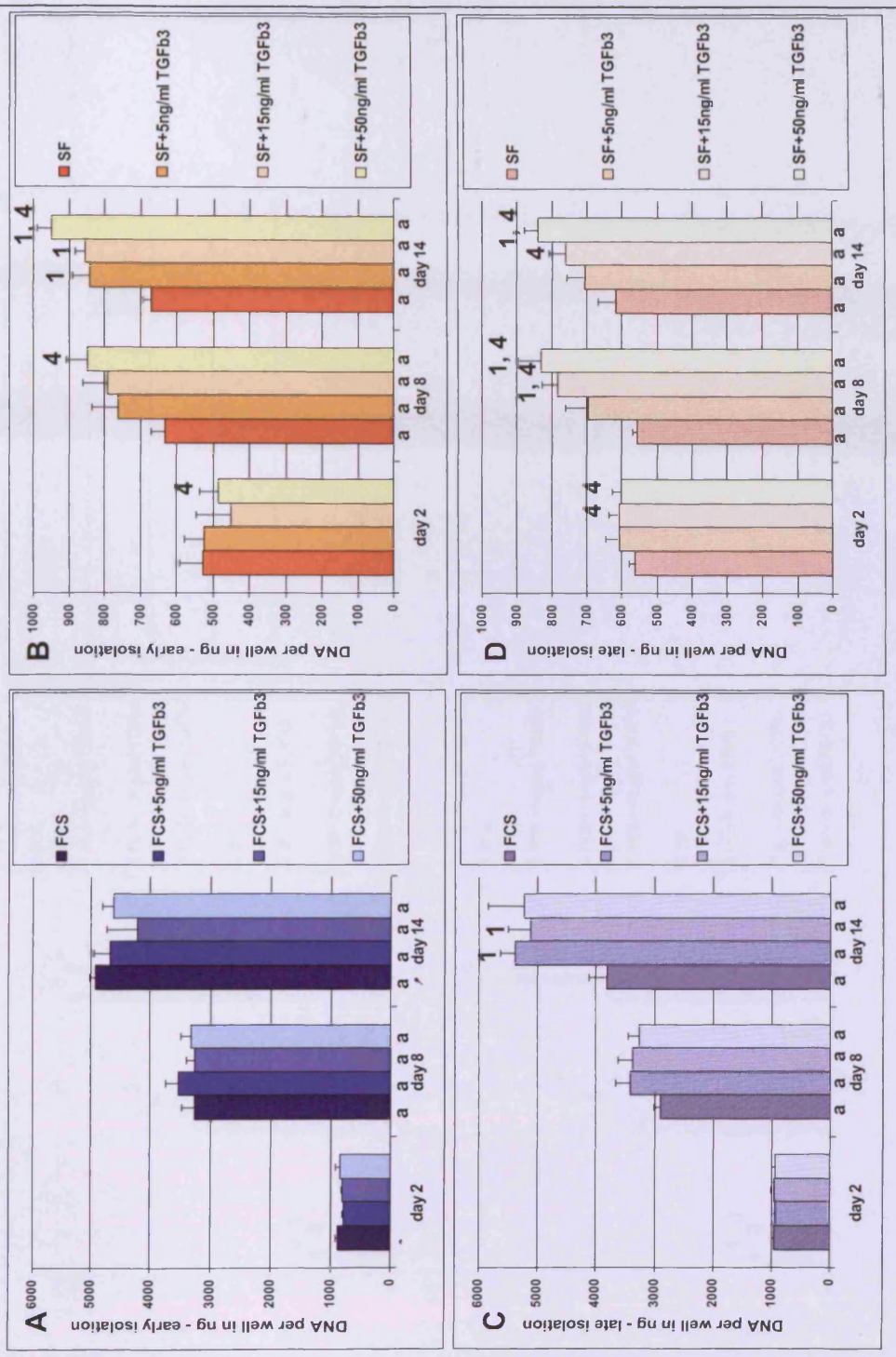

Fig. 5.3: Diagrams show the amount of mean DNA (Hoechst) with the standard error of the mean, quantified during the culture of early (A, B; non-stiped bars) and late (C, D; striped bars) isolated human primary osteoblasts in monolayer culture (3 experiments, n=9). A, C: Cells were cultured with FCS-containing medium (blue bars; not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni). B, D: Cells were cultured SF (orange bars; normally distributed, one-way ANOVA with Tukey). Statistical significance was determined as $p \leq 0.05$; 1: compared to TGFβ$_3$-free medium on that day; 4: compared to TGFβ$_3$-free medium over the whole culture period. The amount of DNA increased during culture (a: $p \leq 0.0001$). Significant increased DNA amount was detected due to the addition of TGFβ$_3$ to FCS medium only in late isolated osteoblasts (C: day 6; $p \leq 0.033$), as well for all osteoblasts cultured with the addition of TGFβ$_3$ to SF medium (B, D: $p < 0.048$).

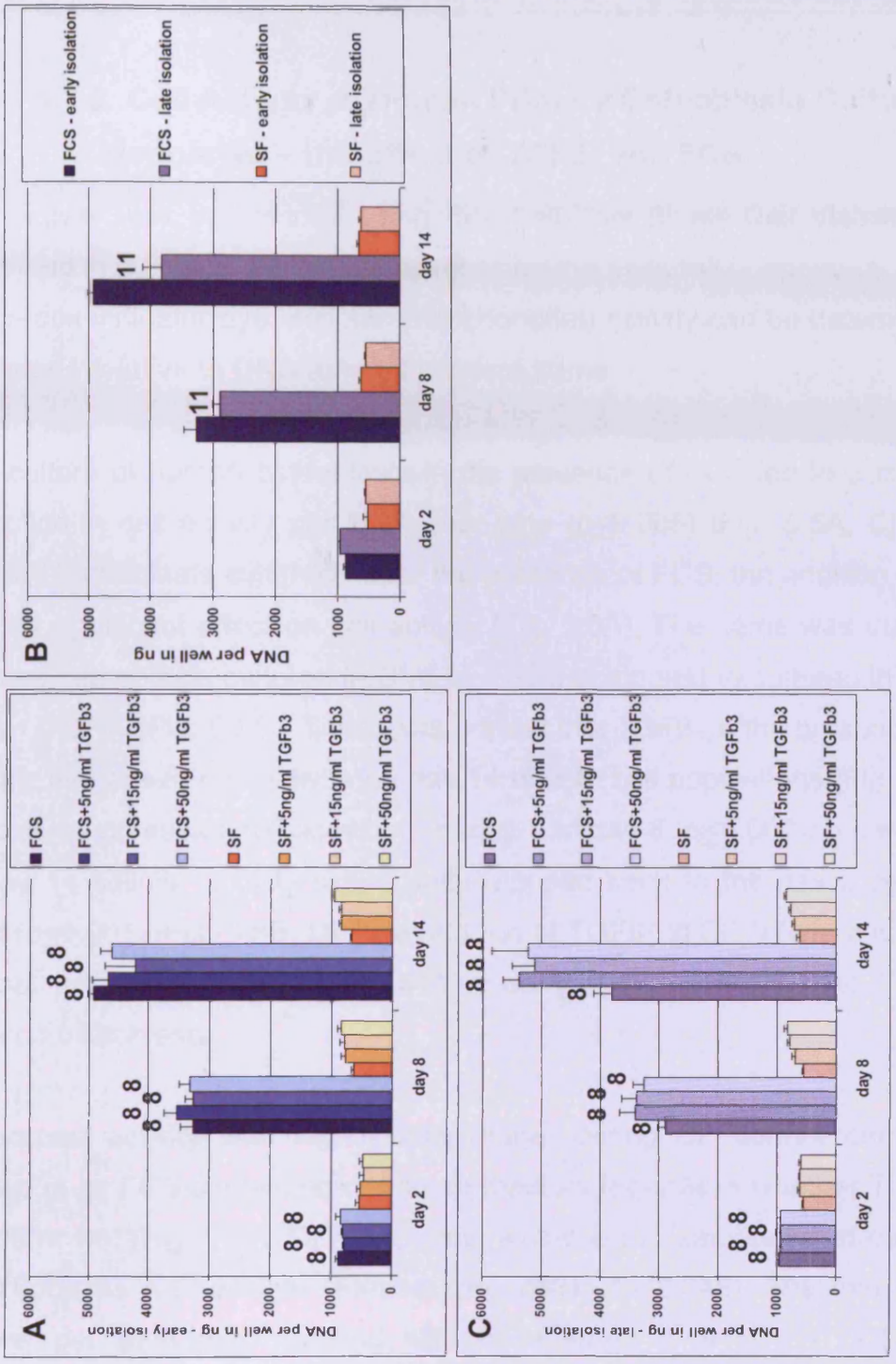

Fig. 5.4: Diagrams show the amount of mean DNA (Hoechst) with the standard error of the mean, quantified during the culture of human primary osteoblasts in monolayer culture (3 experiments, n=9). A: Early isolated osteoblasts. B: Early and late isolated osteoblasts cultured in TGFβ$_3$-free medium. C: Late isolated osteoblasts. A, C, early osteoblasts of B: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. B – late osteoblasts: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; 8: compared to SF medium counterpart with same amount of TGFβ$_3$ over all days; 11: compared to early isolated osteoblasts day≥8. Significant increased DNA amount was detected due to the culture in FCS medium (A, C: day 6; $p<0.0001$). Early isolated osteoblasts showed increased DNA if cultured in DMEM + FCS compared to late isolated osteoblasts (B, $p=0.018$).

5.1.2. Cell Activity of Human Primary Osteoblasts Cultured in Monolayer – the Effect of TGFβ3 and FCS

Cell activity was determined using the 'Cell Titer-Blue® Cell Viability' assay, described in 2.2.14.2. As the assay relies on the metabolic-dependent reduction of a redox indicator dye, cellular (mitochondrial) activity can be determined and was made relative to DNA amount in micrograms.

The culture of human osteoblasts in the presence of FCS led to a continuous reduction in cell activity per DNA over time ($p \leq 0.005$) [Fig. 5.5A, C]. In early isolated osteoblasts cultured under the presence of FCS, the addition of TGFβ3 had no significant effect on cell activity [Fig. 5.5A]. The same was true for late isolated osteoblasts cultured in DMEM + FCS compared to cultured in DMEM + FCS + TGFβ3 [Fig. 5.5C]. There was a trend that TGFβ3 in the presence of FCS slightly decreased cell activity on day 14 in both cell populations [Fig. 5.5A, C]. SF culture increased cell activity from day 2 to day 8 ($p \leq 0.007$), but from day 8 to day 14 cellular activity significantly dropped back to the day 2 cell activity level ($p \leq 0.001$) [Fig. 5.5B, D]. The addition of TGFβ3 to SF culture had no effect on cell activity per amount of DNA in early [Fig. 5.5B] and late [Fig. 5.5D] isolated osteoblasts.

Osteoblast activity was significantly higher during SF culture compared to culture in its FCS-containing opponent medium regardless whether TGFβ3 was added or not [Fig. 5.6A, C]. This effect was seen in early isolated osteoblasts ($p \leq 0.036$), as well as late isolated osteoblasts ($p \leq 0.048$). The maximum cell activity per DNA was reached with an average of 91% +/- 12% reduced Celltiter/µg DNA using DMEM SF + 15 ng/ml TGFβ3 for the culture of early isolated osteoblasts [Fig. 5.6A]. No significant difference in cell activity between early and late isolated osteoblasts was observed [Fig. 5.6B].

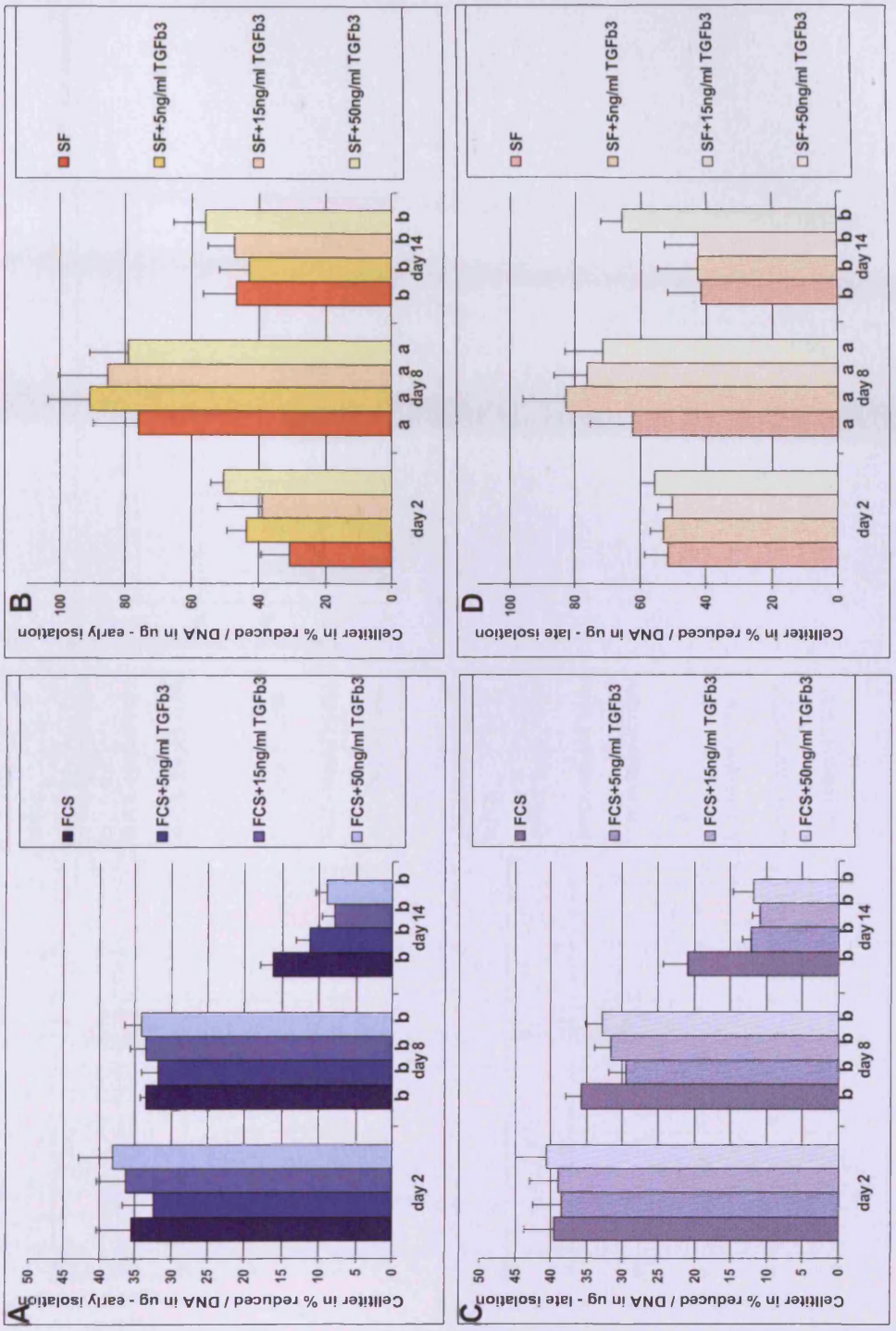

Fig. 5.5: Diagrams show the amount of mean reduced Cell Titer-Blue® per DNA with the standard error of the mean, quantified during the culture of early (A, B) and late (C, D) isolated human primary osteoblasts in monolayer culture (3 experiments, n=9). A, C: Cells were cultured with FCS-containing medium (not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni). B, D: Cells were cultured SF (normally distributed, one-way ANOVA with Tukey). Statistical significance was determined as $p \leq 0.05$. The amount of reduced Celliter per DNA decreased during culture in FCS-containing medium (b: $p \leq 0.005$). The amount of reduced Celliter per DNA increased from day 2 to day 8 (a: $p \leq 0.007$), and then declined from day 8 to day 14 in SF culture (b: $p \leq 0.001$).

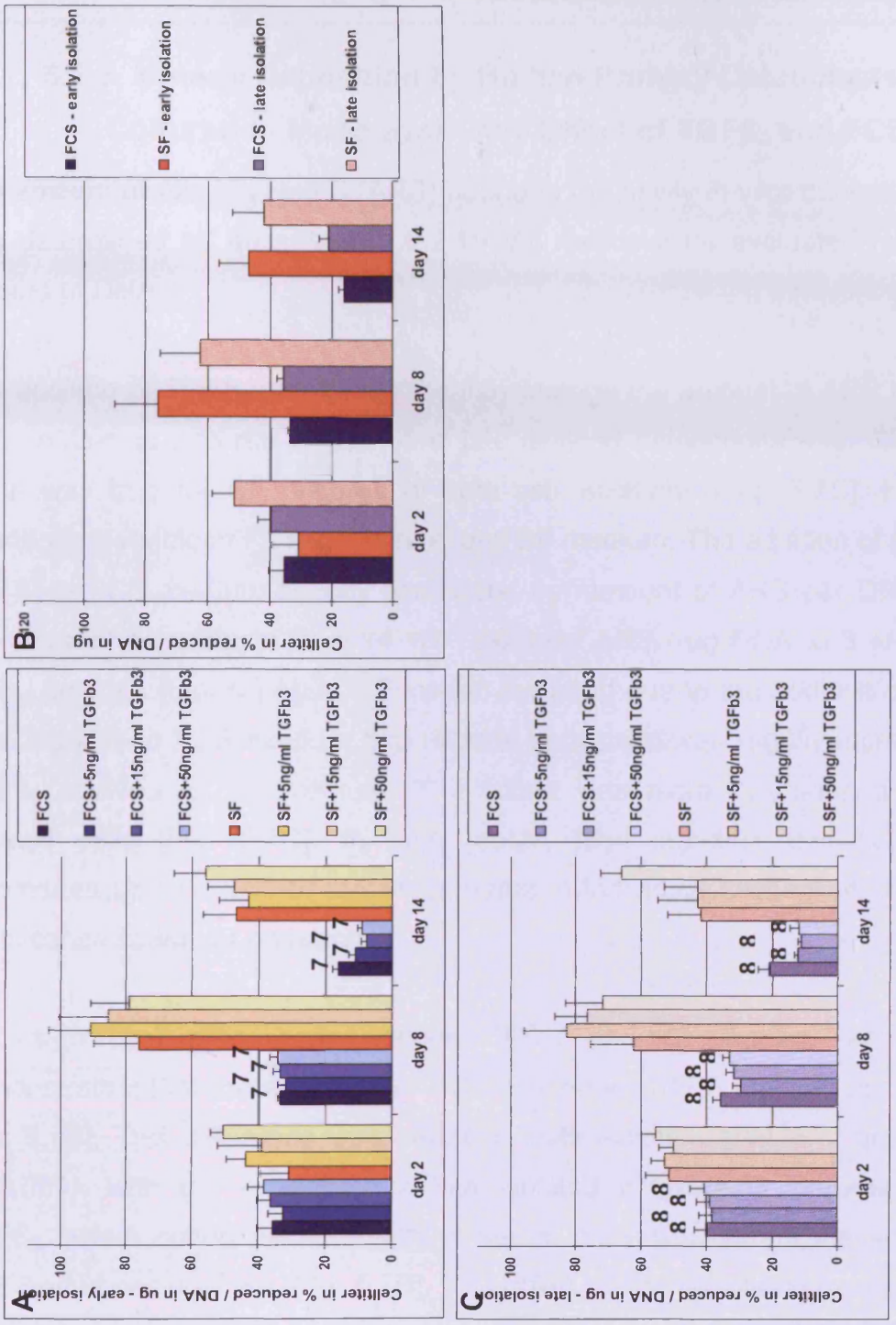

Fig. 5.6: Diagrams show the amount of mean reduced Cell Titer-Blue® per DNA with the standard error of the mean, quantified during the culture of human primary osteoblasts in monolayer culture (3 experiments, n=9). A: Early isolated osteoblasts. B: Early and late isolated osteoblasts cultured in TGFβ3-free medium. C: Late isolated osteoblasts. A, C: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. B: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; 7: compared to SF medium counterpart with same amount of TGFβ3 day≥8; 8: compared to SF medium counterpart with same amount of TGFβ3 over whole culture period. Significant increased reduced Celltiter per DNA was detected due to SF culture (A, C: $p \leq 0.048$).

5.1.3. Mineral Deposition by Human Primary Osteoblasts Cultured in Monolayer – the Effect of TGFβ3 and FCS

The amount of Alizarin Red S (ARS) bound to the newly *in vitro* deposited ECM was determined as described in 2.2.19. All results were evaluated relative to amount of DNA.

The addition of TGFβ3 did not significantly change the amount of ARS bound in FCS-containing cultures of early and late isolated osteoblasts [Fig. 5.7A]. The same was true for SF cultures of both cell isolations [Fig. 5.7C]. However, trends were visible in FCS-containing and SF medium. The addition of 50 ng/ml TGFβ3 to FCS medium slightly decreased the amount of ARS per DNA i.e. in late isolated osteoblasts from 14 +/- 12.6 pmol ARS / µg DNA to 3 +/- 1 pmol ARS / µg DNA [Fig. 5.7A]. In SF media, the trend due to the addition of TGFβ3 was opposite to FCS medium and mineral deposition was slightly increased by TGFβ3 addition to SF medium. This effect was more pronounced for late isolated cells [Fig. 5.7C]. In both media, high standard deviations were determined up to 100% of the mean value in individual cases and, therefore, significance could not be reached.

Yet, significant differences between FCS and SF culture were seen, demonstrating that the depletion of FCS caused less ARS staining per µg DNA [Fig. 5.7B]. This difference was visible in both isolation stages of osteoblasts ($p \leq 0.001$), with the exception of late isolated osteoblasts cultured without TGFβ3, where culture with or without serum did not influence the amount of ARS bound per µg DNA [Fig. 5.7B]. This effect might have been caused due to a slight increase in ARS bound in late osteoblasts cultured with FCS (13.9 +/- 12.6 pmol ARS/ µg DNA) compared to early ones (9.7 +/- 8.6 pmol ARS/ng DNA), while SF culture caused a slight decrease in bound ARS by late osteoblasts compared to early isolated osteoblasts from 24.5 +/- 9.4 pmol ARS/ µg DNA to 19.2 +/- 7.1 pmol ARS/ µg DNA [Fig. 5.7D]. However, these trends were not significant.

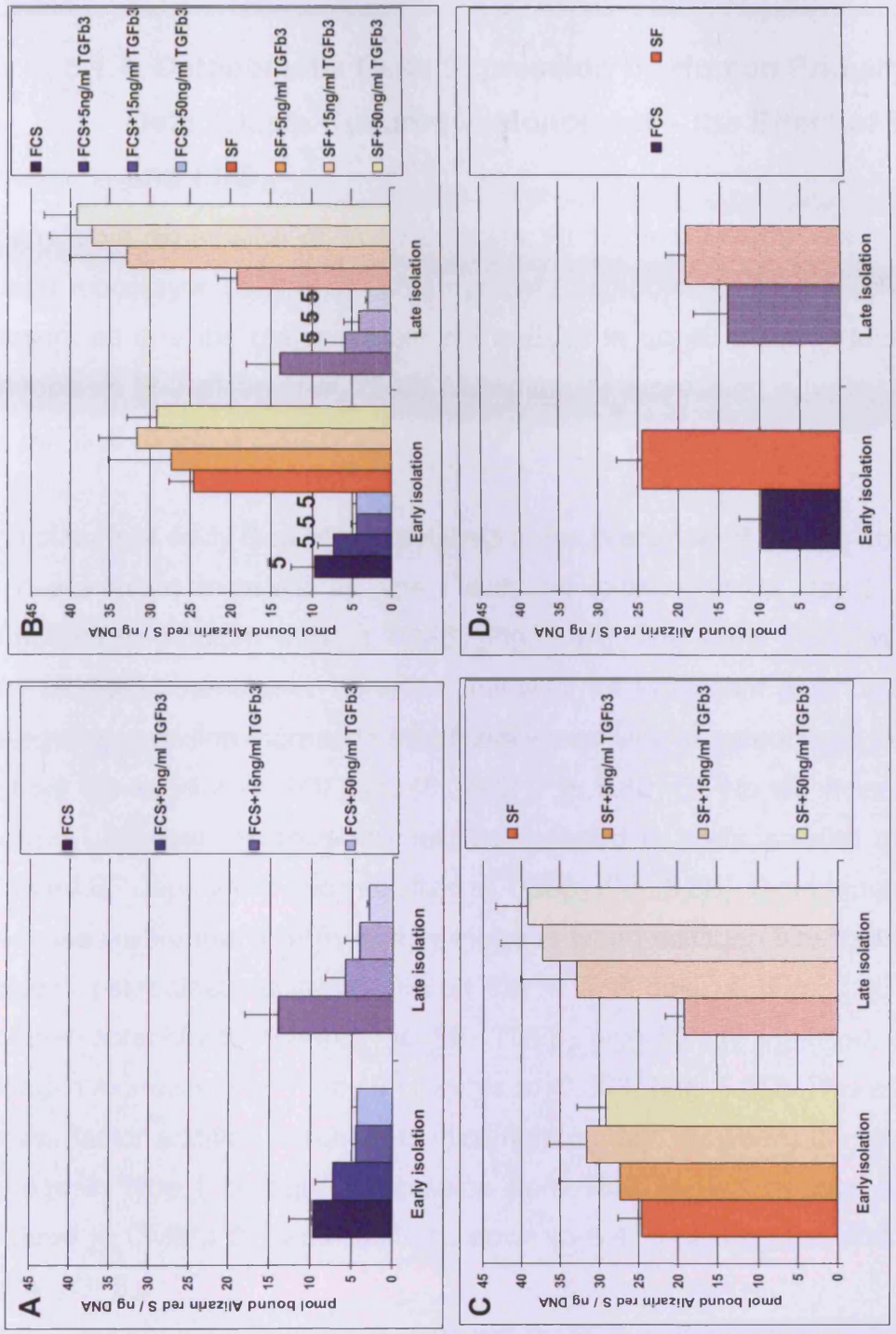

Fig. 5.7: Diagrams show the amount of bound Alizarin Red S staining per DNA with the standard error of the mean, quantified at day 14 of human primary osteoblast culture in monolayer (3 experiments, n=9). A: Cells were cultured with FCS-containing medium. C: Cells were cultured SF. A, C: normally distributed, one-way ANOVA with Tukey). B: All groups. D: Early and late isolated osteoblasts cultured in TGFβ3-free medium. B, D: Transformed to ln, normally distributed, one-way ANOVA with Tukey). Statistical significance was determined as $p \leq 0.05$. 5: compared to SF medium counterpart with same amount of TGFβ3. Significant increased bound Alizarin per DNA was detected due to SF culture (C: $p \leq 0.001$) with the exception of late isolated osteoblasts cultured without TGFβ3.

5.1.4. Osteoblastic Gene Expression by Human Primary Osteoblasts Cultured in Monolayer – the Effect of TGFβ3 and FCS

The relative expression of type I collagen, Runx2 and osterix was investigated during monolayer culture of human primary osteoblasts. Type I collagen was chosen, as it is the major protein of the ECM in bone, which is laid down by osteoblasts (Bilezikian *et al.* 2002). Moreover, its expression is highly influenced by TGFβ (Bonewald 2002).

The culture of early isolated osteoblasts in the presence of TGFβ3 and FCS led to a significant increase in type I collagen expression on day 2 ($p \leq 0.005$) compared to medium without TGFβ3 [Fig. 5.8A]. The same trend was seen in later isolated osteoblasts; however, this was not significant [Fig. 5.8C]. Type I collagen expression increased significantly over time in osteoblasts cultured SF without the addition of TGFβ3 ($p \leq 0.0001$) [Fig. 5.8B, D]. No significant changes in type I collagen expression could be detected in early isolated osteoblasts cultured SF dependent upon addition of TGFβ3 [Fig. 5.8B]. Even though, a trend was also visible that TGFβ3 slightly inhibited type I collagen expression in early isolated osteoblasts cultured SF on day 8 and day 14 [Fig. 5.8B]. In later isolated osteoblasts cultured as SF, TGFβ3 significantly inhibited the type I collagen expression from day 8 onwards ($p \leq 0.003$) [Fig. 5.8D]. This effect of the growth factor addition reached its maximum on day 14, where the relative fold-change in type I collagen expression from 15.6 +/- 8.3 by late osteoblasts cultured in DMEM SF was declined down to 6.4 +/- 2.8 by the addition of 50 ng/ml TGFβ3.

Type I collagen expression was lower in FCS cultures than using the SF equivalent medium ($p \leq 0.026$) [Fig. 5.9A, C]. This effect was independent of the use of early or late isolated osteoblasts. Comparing the different cell isolation stages, type I collagen expression was significantly higher in late isolated osteoblasts, than in early isolated ones independent of the use of FCS-containing or SF medium ($p \leq 0.024$) [Fig. 5.9B].

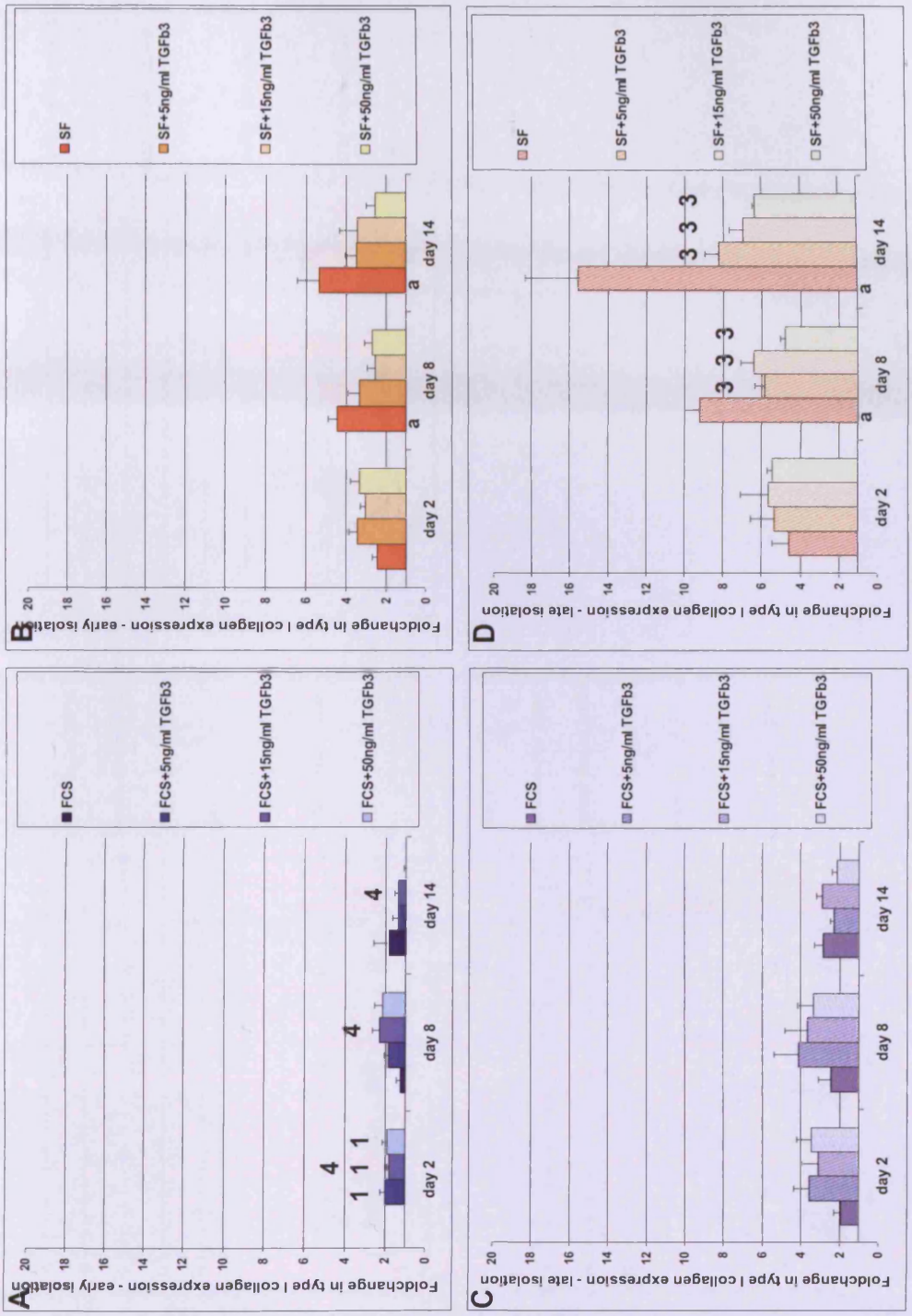

Fig. 5.8: Diagrams show the mean fold-change in relative type I collagen expression with the standard error of the mean, quantified by qPCR during the culture of early (A, B) and late (C, D) isolated human primary osteoblasts in monolayer culture (3 experiments, n=9). Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM+FCS at culture day 2. A, C: Cells were cultured with FCS-containing medium. B, D: Cells were cultured SF. A, B: normally distributed, one-way ANOVA with Tukey. C, D: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$; 1: compared to TGFβ3-free medium on that day; 3: compared to TGFβ3-free medium day≥8; 4: compared to TGFβ3-free medium over the whole culture period. Type I collagen expression increased over time in SF culture (a: $p \leq 0.0001$). Its expression was higher with the addition of TGFβ3 to FCS medium on day 2 in early isolated osteoblasts (A: $p \leq 0.005$). In late isolated osteoblasts TGFβ3 inhibited type I collagen expression in SF medium (D: $p \leq 0.003$).

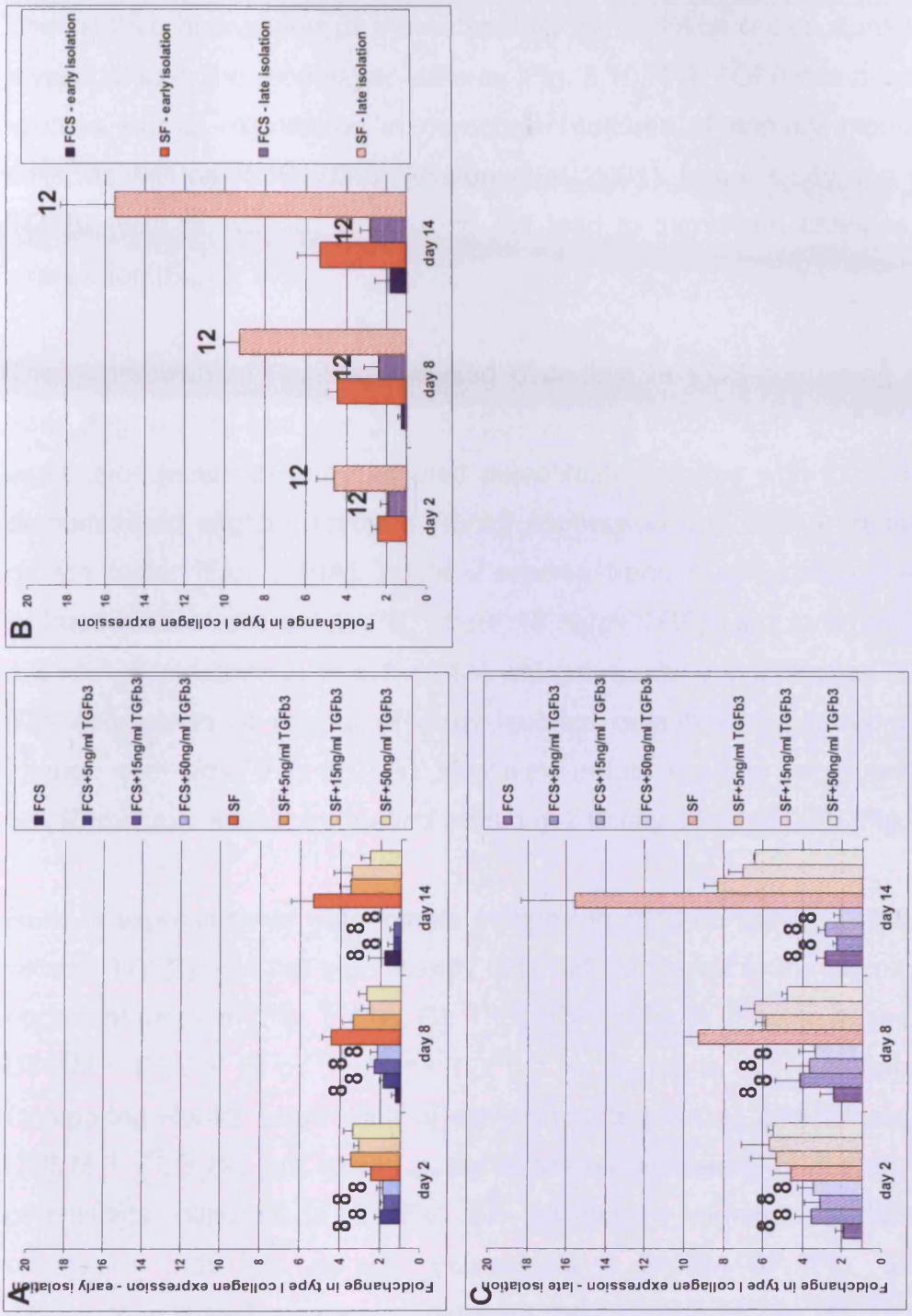

Fig. 5.9: Diagrams show the mean fold-change in relative type I collagen expression with the standard error of the mean, quantified by qPCR during the culture of human primary osteoblasts in monolayer culture (3 experiments, n=9). A: Early isolated osteoblasts. B: Early and late isolated osteoblasts cultured in TGFβ$_3$-free medium. C: Late isolated osteoblasts. Early isolated osteoblasts: normally distributed, one-way ANOVA with Tukey. Late isolated osteoblasts: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$; 8: compared to SF medium counterpart with same amount of TGFβ$_3$ over whole culture period. 12: compared to early isolated osteoblasts over the whole culture period. SF medium increased type I collagen expression (A, C: $p \leq 0.026$). Late isolated osteoblasts showed increased type I collagen expression compared to early isolated osteoblasts (B: $p \leq 0.024$).

The relative expression of the osteoblast transcription factor Runx2 was also investigated in the monolayer-cultures [Fig. 5.10, 11]. TGFβ has been shown to repress Runx2 expression in monolayer cultures of primary mouse calvaria cells, as well as ROS17/2.8 (Alliston *et al.* 2001). In our study, the addition of TGFβ3 (with or without serum) did not lead to significant changes in Runx2 expression [Fig. 5.10].

The expression of Runx2 increased over time in FCS-containing cultures of early [Fig. 5.10A] and late [Fig. 5.10C] isolated osteoblasts ($p \leq 0.011$). Runx 2 expression levels of early isolated osteoblasts cultures with FCS and TGFβ3, demonstrated slightly inhibited Runx2 expression due to the presence of the growth factor [Fig. 5.10A]. While a reverse trend may be suggested for late isolated osteoblasts on day 8, where 15 ng/ml TGFβ3 led to an increase from 1.8 +/- 1.4 fold-change to 5.1 +/- 8.4 without reaching significance [Fig. 5.10C]. The expression of Runx2 of early isolated osteoblasts cultured SF did not change over time [Fig. 5.10B]. However, in late isolated osteoblasts cultured SF, Runx2 expression increased from day 2 to day 14 ($p \leq 0.022$) [Fig. 5.10D].

Runx 2 expression of osteoblasts cultured in FCS-containing medium with or without TGFβ3 was not significantly different compared to the culture in the SF opponent medium [Fig. 5.11A, C]. This might at least in some cases (day 8 for DMEM + FCS + 15 or 50 ng/ml TGFβ3) be related to the high variation seen. Comparing Runx2 expression of early and late isolated osteoblasts, culture in DMEM + FCS did not result in significant differences [Fig. 5.11B]. Yet, early osteoblasts cultured in DMEM SF showed a significantly lower Runx2 expression than late isolated osteoblasts in DMEM SF [Fig. 5.11B]. This difference in Runx2 expression between cell populations was visible from day 8 onwards and was highest on day 14 with an average of 2.1 +/- 1.3 relative fold-change in Runx2 expression in early osteoblasts SF compared to 4.4 +/- 2.2 in late osteoblasts SF ($p \leq 0.002$).

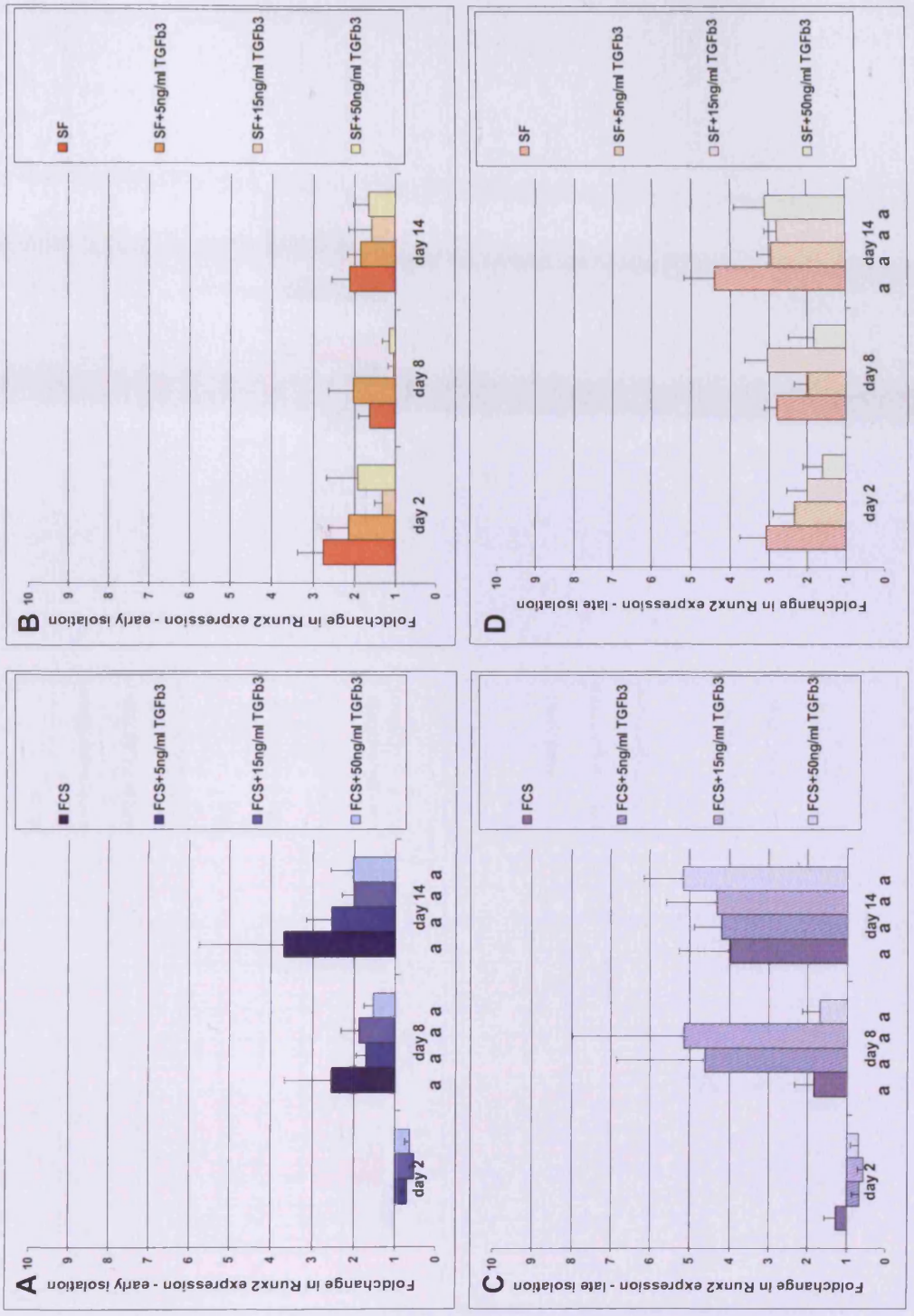

Fig. 5.10: Diagrams show the mean fold-change in relative Runx2 expression with the standard error of the mean, quantified by qPCR during the culture of early (A, B) and late (C, D) isolated human primary osteoblasts in monolayer culture (3 experiments, n=9). Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM+FCS at culture day 2. A, C: Cells were cultured with FCS-containing medium. B, D: Cells were cultured SF. A, C, late isolated osteoblasts of B: normally distributed, one-way ANOVA with Tukey. B – early isolated osteoblasts: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$. The expression of Runx2 increased during culture in FCS medium for both cell isolations, as well as SF medium if cells were late isolated osteoblasts (a: $p \leq 0.022$).

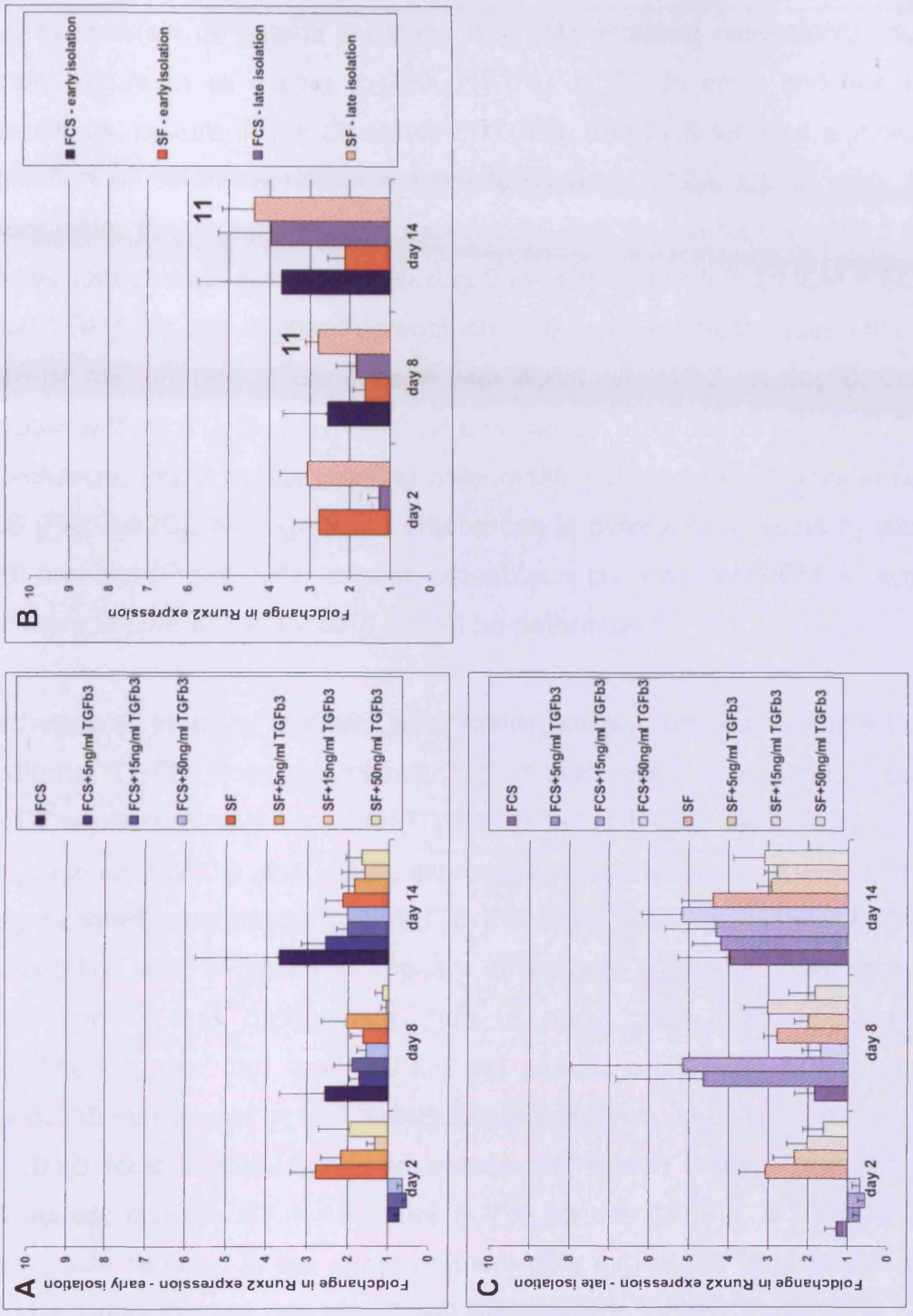

Fig. 5.11: Diagrams show the mean fold-change in relative Runx2 expression with the standard error of the mean, quantified by qPCR during the culture of human primary osteoblasts in monolayer culture (3 experiments, n=9). Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM+FCS at culture day 2. A: Early isolated osteoblasts. B: Early and late isolated osteoblasts cultured in TGFβ3-free medium. C: Late isolated osteoblasts. A, B: normally distributed, one-way ANOVA with Tukey. C: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$; 11: compared to early isolated osteoblasts day$\geq$8. Runx2 expression was increased in late osteoblasts if cultured SF (B: day$\geq$8, $p \leq 0.002$).

The expression of osterix by early and late isolated osteoblasts increased during culture in all media ($p \leq 0.0001$) [Fig. 5.12]. In early and late isolated osteoblasts, culture in the presence of TGFβ3 and FCS led to a significant up-regulation of osterix expression ($p \leq 0.0001$) [Fig. 5.12A, C]. In early isolated osteoblasts, the highest osterix expression with an average of 353.7 +/- 217 relative fold-change was found on day 8 in cells cultured in DMEM + FCS + 15 ng/ml TGFβ3. In late isolated osteoblasts, the same medium caused the highest average fold-change in expression with 366.4 +/- 346.3 on day 8. Culture in medium without TGFβ3 only reached a maximum increase of 4.3 +/- 3.5 relative fold-change, found in late isolated osteoblasts cultured for 14 days in DMEM + FCS [Fig. 5.12C]. No significant differences in osterix expression by early [Fig. 12B] and late [Fig. 5.12D] isolated osteoblasts cultured in DMEM SF compared to culture in DMEM SF + TGFβ3 could be determined.

Furthermore, in early isolated osteoblasts, osterix expression levels in FCS medium + TGFβ3 were significantly higher than levels expressed by culture in the SF equivalent media ($p \leq 0.0001$) [Fig. 5.13A, C]. Late osteoblasts cultured in the presence of FCS and TGFβ3 expressed similar levels of osterix mRNA than early isolated osteoblasts cultured in the same medium. However, TGFβ3 in combination with SF medium appears to promote a greater increase in osterix expression in late osteoblasts than in early ones. This implication was underlined by the fact that the highest osterix expression in SF medium + TGFβ3 (15 ng/ml) was in late isolated osteoblasts on day 14. On average 224.8 +/- 104.8 relative fold-change in osterix expression were expressed by late osteoblasts cultured SF + 15 ng/ml TGFβ3 on day 14 [Fig. 5.13C], while early osteoblasts cultured in the same medium only expressed an average of 146.2 +/- 192.4 [Fig. 5.13A]. No significant difference in osterix expression between early and late isolated osteoblast populations without the addition of TGFβ3 could be detected [Fig. 5.13B].

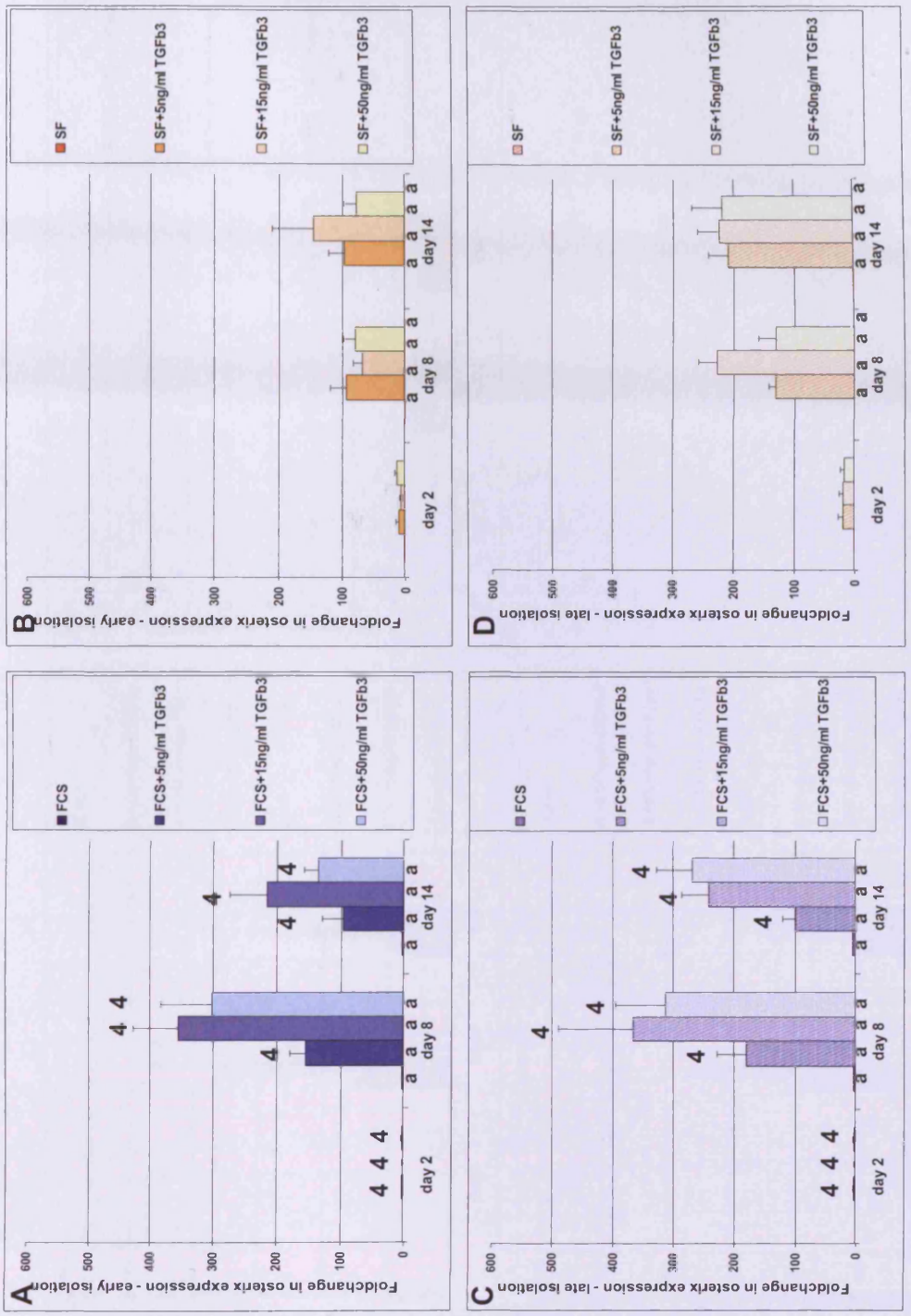

Fig. 5.12: Diagrams show the mean fold-change in relative osterix expression with the standard error of the mean, quantified by qPCR during the culture of early (A, B) and late (C, D) isolated human primary osteoblasts in monolayer culture (3 experiments, n=9). Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM+FCS at culture day 2. A, C: Cells were cultured with FCS-containing medium. B, D: Cells were cultured SF. A, C: normally distributed, one-way ANOVA with Tukey. B, D: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$. 4: compared to TGFβ3-free medium over the whole culture period. The expression of osterix increased over time (a: $p \leq 0.0001$). The culture of early and late isolated osteoblasts in DMEM+FCS+TGFβ3 led to an up-regulation of osterix expression ($p \leq 0.0001$).

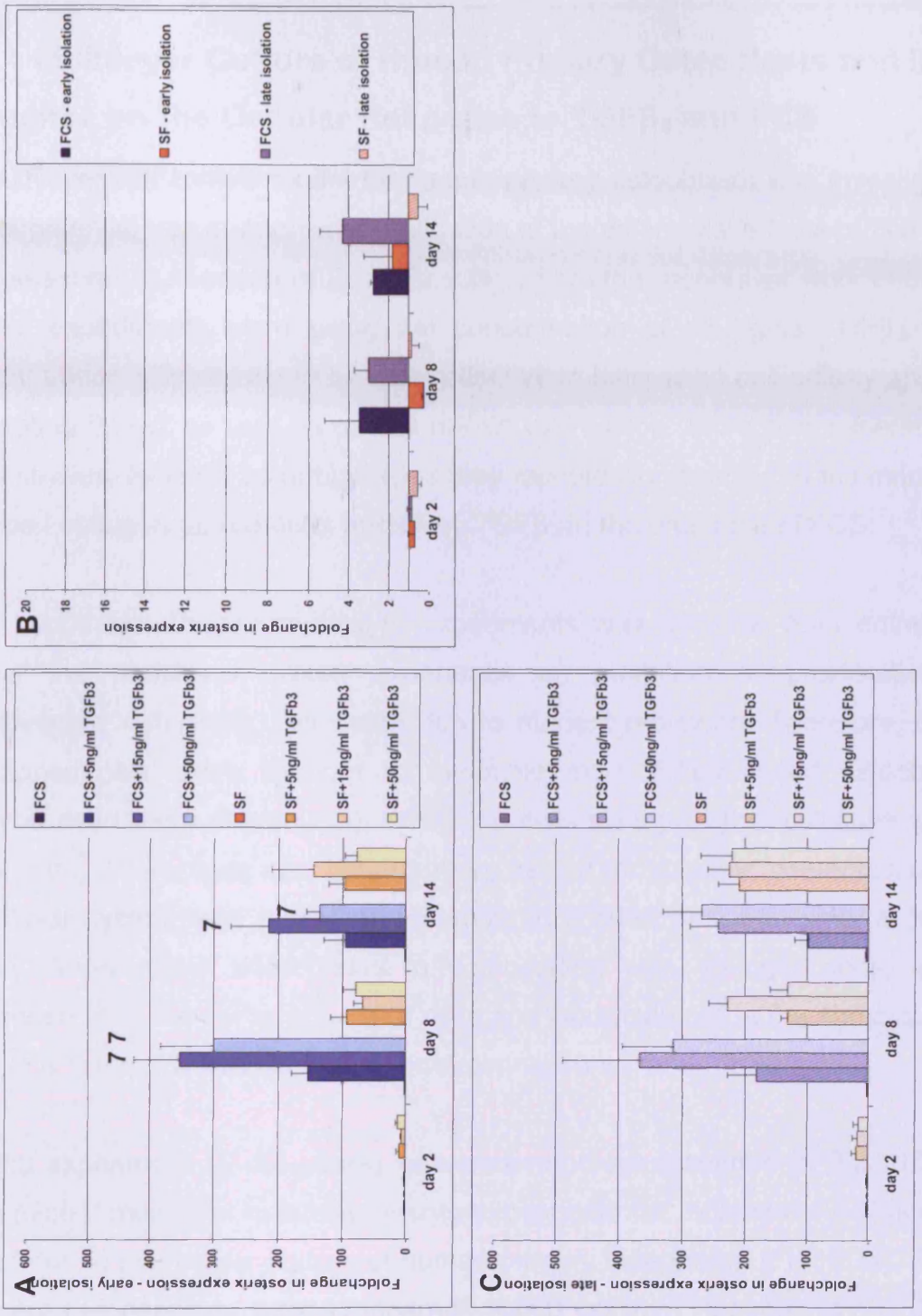

Fig. 5.13: Diagrams show the mean fold-change in relative osterix expression with the standard error of the mean, quantified by qPCR during the culture of human primary osteoblasts in monolayer culture (3 experiments, n=9). Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM+FCS at culture day 2. A: Early isolated osteoblasts. B: Early and late isolated osteoblasts cultured in TGFβ3-free medium. C: Late isolated osteoblasts. A, B, C: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni. Statistical significance was determined as $p \leq 0.05$; 7: compared to SF medium counterpart with same amount of TGFβ3 day≥8. The expression of osterix increased during culture ($p < 0.000$). Osterix expression increased by addition of at least 15 ng/ml TGFβ3 to DMEM+FCS to higher levels than its SF opponent only in early isolated osteoblasts (A: day≥8, $p < 0.000$).

5.2. Multilayer Culture of Human Primary Osteoblasts and its Influence on the Cellular Response to TGFβ3 and FCS

A multilayer cell culture model for human primary osteoblasts was investigated for its potential use during the investigation of the differential effects of TGFβ3 in the presence or absence of FCS. Resulting from the monolayer work in 5.1 all further experiments were using the concentration of 15 ng/ml TGFβ3. This concentration was shown to be most effective in increasing cell activity and up-regulating Runx2 as well as osterix mRNA expression. Moreover, experiments will use early isolated osteoblasts, as they reacted significantly on the induction of type I collagen, and osterix mRNA by TGFβ3 in the presence of FCS.

The hypothesis for the multilayer experiments was that the cells entrapped within the multilayer would experience an inhibition of proliferation in combination with some cell death due to nutrient reduction. Therefore, these entrapped cells were thought to resemble more differentiated osteoblasts (maybe even early osteocytes). While the cells on top of the multilayer would proliferate, differentiate and behave more similar to 'younger' osteoblasts. The multilayer system was aiming to resemble in a short period of time, a mix of highly differentiated osteoblasts in combination with younger ones, which originated from the same source of cells and were cultured in the same culture dish in a manner which allowed for interconnections.

A pilot experiment (♀ 53 years) was performed as described in 2.2.16.2., to determine if multilayer culture prevents the exponential increase of cell number found during monolayer culture of human primary osteoblasts [Fig. 5.14]. Three different cell densities were compared, 10000 cells/cm^2 (seeded into a 6-well plate) as typical monolayer seeding density, 120000 cells/cm^2 (seeded into a 48-well plate) as medium density, and a high cell density of 290000 cells/cm^2 (seeded into a 96-well plate). Due to the use of different well plates, 90000 cells per well could be seeded for each cell density. The quantification of the number of viable cells over a culture period of 16 days revealed the expected result of exponential proliferation for the monolayer cell density. The highest cell density

led to a huge decrease in cell number from day 0 to day 16 from 90000 cells to an average of 24810 cells +/- 2360 cells. This resembled a mean loss in cell number of 74%. Considering that surface cells were most likely proliferating during culture, the level of cell death in this model was very high. The medium cell density of 120000 cells/cm^2 did show an initial loss of cell number of about 37% on day 2. This result would suggest that cells experienced an inhibition of proliferation in combination with cell death. However, on culture day 10 and 16 the total cell number levelled out to 75360 cells +/- 3290. Therefore, all further described multilayer culture model used the multilayer culture with the medium cell density of 120000 cells/cm^2.

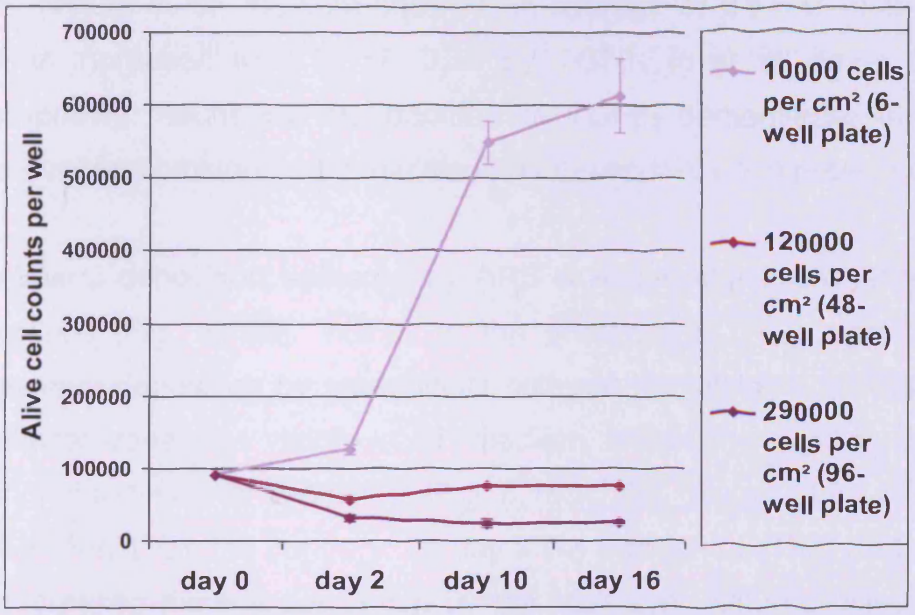

Fig. 5.14: Multilayer culture pre-experiment for human primary osteoblasts (♀ 53 years). The diagram shows the average number of viable osteoblasts and the standard error of the mean for each cell-density group during the 16 culture periods determined by manual counting using trypan blue. No increase in cell density could be observed in the 2 multilayer cultures (120000 cells/cm^2 seeded and 290000 cells/cm^2 seeded).

5.2.1. The Differentiation of Human Primary Osteoblasts Cultured in Multilayer and the Effect of TGFβ3 and FCS

The differential effects of 15 ng/ml TGFβ3 and FCS were investigated in a set of multilayer experiments using human primary osteoblasts (♀ 73 years, ♂ 59 years; ♂ 62 years). The total DNA amount per well, bound ARS staining per

DNA and gene expression levels of type I collagen, Runx2, osterix, osteocalcin and E11 were investigated in the presence or absence of TGFβ3 and FCS.

The amount of DNA increased from day 2 till day 6 ($p<0.0001$) under all media conditions [Fig. 5.15A]. DMEM + FCS was found to be the most potent culture medium to increase the total amount of DNA during culture ($p\leq0.032$). Thus by day 6, osteoblasts cultured in DMEM + FCS possessed an average DNA content of 1.64 +/- 0.95 µg DNA, while osteoblasts cultured in the same media with the addition of 15 ng/ml TGFβ3 demonstrated only 1.3 +/- 0.58 µg DNA (over all days: $p=0.032$). This inhibition of proliferation caused by TGFβ3 in the presence of FCS was the opposite if TGFβ3 was added to SF medium. By day 6, culture in SF medium showed an average of 0.83 +/- 0.49 µg DNA, which was increased to 1.14 +/- 0.46 by TGFβ3 (over all days: $p=0.001$). These opposing results by the addition of TGFβ3 demonstrate that the effect on osteoblast proliferation in multilayer is dependent upon presence of FCS.

Mineral deposition detected by ARS revealed significant differences between groups [Fig. 5.15B]. TGFβ3 in the presence of FCS significantly increased mineral deposition by osteoblasts cultured in multilayer on day 4 ($p\leq0.005$). A similar trend was visible in SF medium, where the addition of TGFβ3 slightly increased mineral deposition [Fig. 5.15B]. Yet, this result for SF culture was not significant. On the contrary, on day 6 the addition of TGFβ3 to SF medium led to decreased mineral deposition by the multilayer cultured osteoblasts ($p=0.026$) [Fig. 5.15B]. A similar trend was visible in culture in the presence of FCS [Fig. 5.15B]. However, the reduction of mineral deposition by the addition of TGFβ3 to FCS-containing medium on day 6 was not significant.

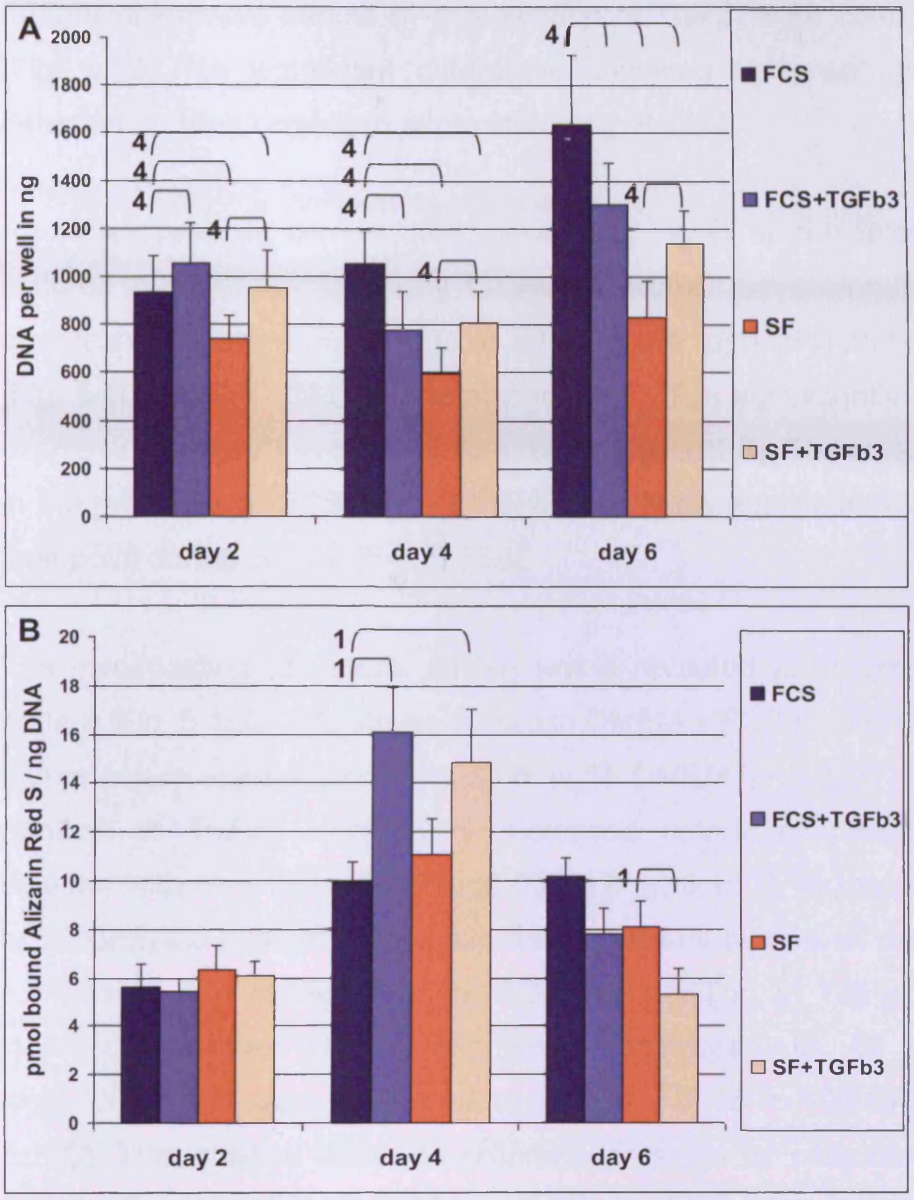

Fig. 5.15: Human primary osteoblasts were cultured in multilayer under the influence of different culture media. A: The diagram shows the amount of mean DNA together with the standard error of the mean, which was quantified by Hoechst assay. B: The diagram shows the amount of bound Alizarin Red S per DNA (♀ 73 years, ♂ 59 years; ♂ 62 years). Data was normally distributed: one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; 1: significant on that day; 4: significant over the whole culture period. The amount of DNA increased over time ($p \leq 0.0001$), while the amount of bound Alizarin per DNA increased from day 2 to day 4 and then declined from day 4 to day 6 ($p \leq 0.0001$). Culture in DMEM + FCS demonstrated higher DNA amount compared to all other media ($p \leq 0.032$), the addition of TGFβ3 increased DNA amount only if added to SF medium ($p \leq 0.001$). Alizarin staining was higher if TGFβ3 was added compared to FCS medium on day 4 ($p \leq 0.005$). The addition of TGFβ3 to SF medium decreased bound Alizarin on day 6 ($p \leq 0.026$).

Further differential effects by the addition of TGFβ3 were determined by qPCR [Fig. 5.16]. No significant differences between treatment groups could be detected for type I collagen expression [Fig. 5.16A].

Runx2 expression, on the other hand, was highly up-regulated in osteoblasts cultured in SF DMEM from day 4 onwards. Runx2 expression in SF DMEM was significantly higher compared to all other media ($p \leq 0.004$) and peaked on day 4 [Fig. 5.16B]. In SF DMEM, the addition of TGFβ3 significantly reduced Runx 2 expression on day 4 and day 6 ($p=0.004$) [Fig. 5.16B]. The culture of multilayers in the presence of FCS and TGFβ3 did not show significant differences at any time point during culture [Fig. 5.16B].

The investigation of osterix mRNA levels revealed an interesting expression pattern [Fig. 5.16C]. Multilayer culture in DMEM + FCS resulted in a significantly higher osterix expression than culture in SF DMEM ($p \leq 0.0001$) [Fig. 5.16C]. The addition of TGFβ3 significantly increased osterix expression in multilayer cultures with or without FCS ($p \leq 0.0001$) [Fig. 5.16C]. In the presence of FCS and TGFβ3 the highest osterix expression was measured on day 6 with 11.3 +/- 8.8 relative fold-change [Fig. 5.16C]. The addition of TGFβ3 to SF medium increased relative osterix expression compared to SF medium alone ($p \leq 0.0001$), and also compared to DMEM + FCS + TGFβ3 ($p \leq 0.0001$) [Fig. 5.16C]. This positive effect on osterix expression by osteoblasts in multilayer due to the addition of TGFβ3 to SF medium was even more dramatic, considering that osterix expression by osteoblasts in DMEM + FCS was generally greater than in DMEM SF ($p \leq 0.0001$) [Fig. 5.16C].

Osteocalcin expression of human primary osteoblasts cultured in multilayer was investigated. Significant differences between treatment groups were determined [Fig. 5.16D]. The highest osteocalcin expression was found during multilayer culture in SF DMEM ($p \leq 0.002$) [Fig. 5.16D]. However, osterix expression in DMEM SF significantly decreased during the 6 day culture period ($p \leq 0.0001$) [Fig. 5.16D]. The addition of TGFβ3 significantly decreased osteocalcin expression in multilayer cultures with or without FCS ($p \leq 0.028$) [Fig. 5.16D].

The expression of the early osteocytic marker gene, E11, also demonstrated significant differences during multilayer culture [Fig. 5.16E]. As for the osteocalcin expression, the highest E11 expression was found during culture in SF DMEM ($p \leq 0.0001$) [Fig. 5.16E]. Multilayer culture of osteoblasts in all other media led to a significant down-regulation of E11 ($p \leq 0.001$) [Fig. 5.16E]. The addition of TGFβ$_3$ significantly decreased E11 expression in DMEM + FCS, as well as in DMEM SF ($p \leq 0.0001$) [Fig. 5.16E].

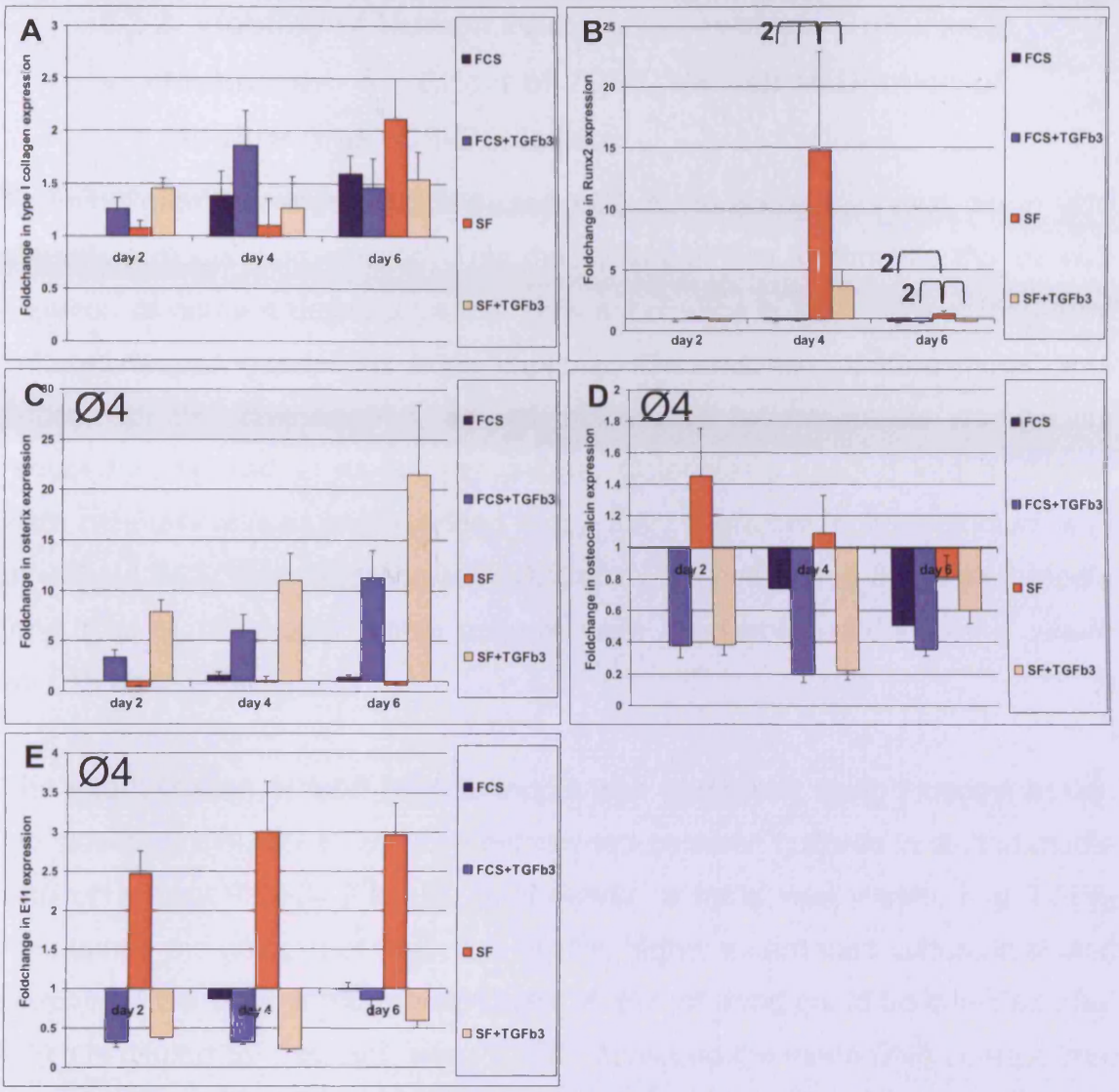

Fig. 5.16: Comparative qPCR of type I collagen (A), Runx2 (B), osterix (C), osteocalcin (D) and E11 (E) was performed during the 6 day culture period of osteoblasts cultured in multilayer (♀ 73 years, ♂ 59 years; ♂ 62 years; n=11). Diagrams show the mean fold change in gene expression and the standard error of the mean. Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in DMEM + FCS at culture day 2. A, B, C, E: not normally distributed: Kruskal-Wallis, Mann-Whitney test with Bonferroni correction. D: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; 2: significant on day≤4; 4: significant over the whole culture period; Ø4: all media significant to each other. Runx2 expression were increased by the use of DMEM SF over all other media by day 4 ($p \leq 0.016$). The addition of TGFβ3 to FCS-containing and SF medium significantly increase osterix expression ($p \leq 0.0001$), while the use of SF media + TGFβ3 increased osterix expression to an even higher level than FCS + TGFβ3 ($p \leq 0.001$). DMEM SF declined the reduction in osteocalcin expression over culture time seen in all other media ($p \leq 0.002$). Moreover this medium also led to the highest E11 expression by the cells ($p \leq 0.001$).

5.2.2. Viability of Human Primary Osteoblasts Cultured in Multilayer - the Effect of TGFβ3 as well as Dilution of Medium and FCS-Depletion

To investigate the effect of TGFβ3 and FCS on osteoblast survival, an *in vitro* starvation model was created. This model was aiming to simulate the *ex vivo* situation of nutrient deprivation that cells experience in the centre of long-term cultured human cancellous bone explants. The multilayer culture model was chosen for this investigation, as cell proliferation by osteoblasts was greatly reduced compared to monolayer culture. Osteoblasts seeded into multilayer were medium-starved as described in 2.2.16.2. Therefore, culture medium with or without FCS was diluted with PBS. Cells were cultured in the diluted media for a total of 120 hours. Some cultures were supplemented during the culture with 15 ng/ml TGFβ3.

The quantification of total DNA amounts was performed using Hoechst assay. No statistical difference could be determined between cultures in diluted media with or without TGFβ3 [Fig. 5.17]. However, a trend was visible, that TGFβ3 maintained the amount of DNA to a slightly higher extent than culture in diluted DMEM + FCS without the growth factor. A similar trend could be detected after 120 h in diluted SF medium, were TGFβ3 increased the mean DNA content from 396 +/- 229 ng to 466 +/- 246 ng DNA. Yet, only the positive treatment control, staurosporine, showed a significant result compared to all other groups. Total DNA amount in the staurosporine group was decreased compared to all other cultures ($p \leq 0.0001$). However, due to the non-conclusive results gained, the starvation model was not investigated further.

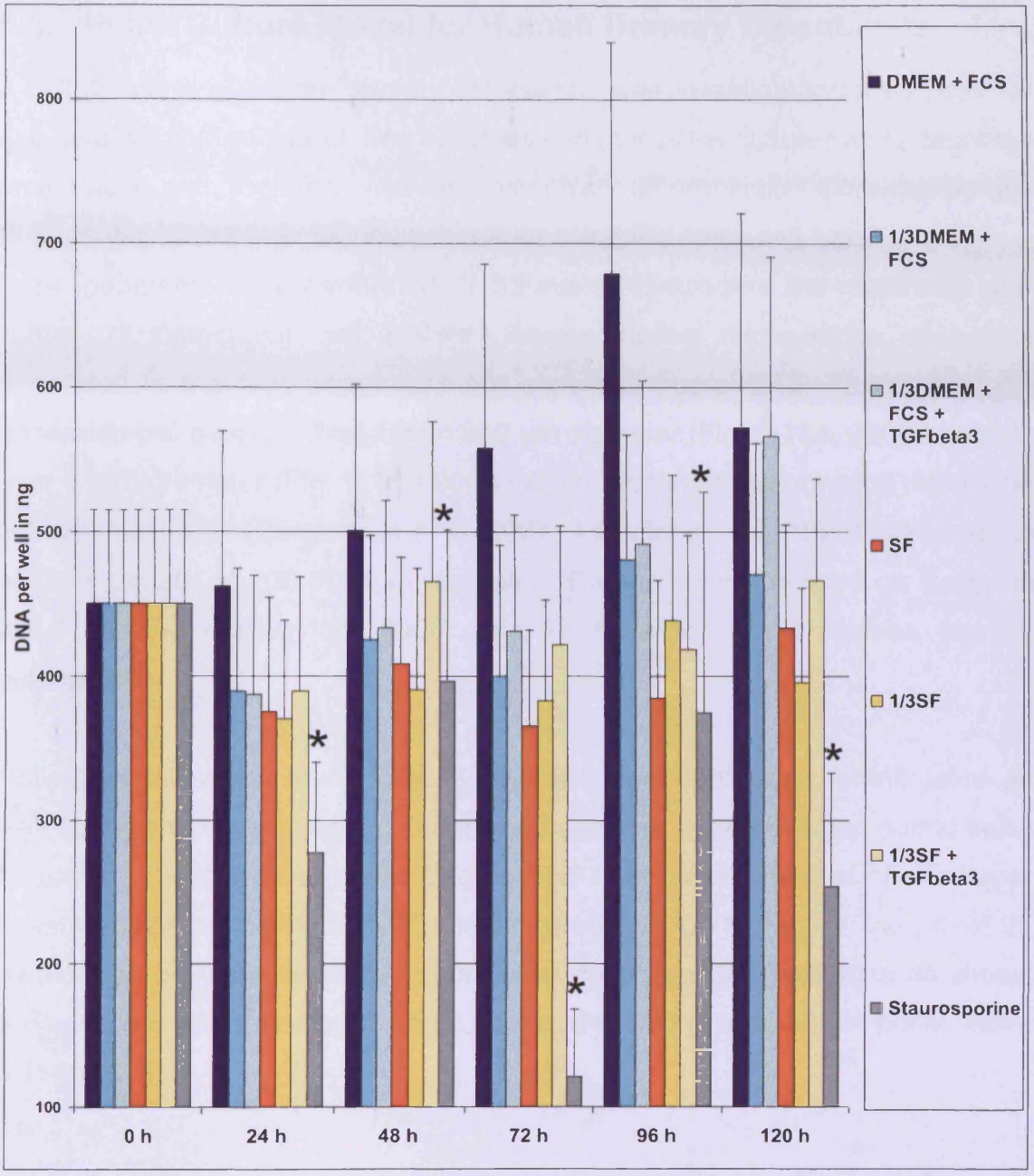

Fig. 5.17: Graph shows the average amount of DNA, as well as the standard error of the mean quantified during starvation experiments (♀ 73 years, ♂ 48 years; ♂ 59 years). Data was normally distributed: ANOVA and Tukey post-hoc test were used for statistical analysis. Probability of ≤ 0.05 was determined as statistical significant. No difference between treatment groups could be determined. Only the positive control with staurosporine resulted in significantly decreased DNA amount over the whole culture period (*$p \geq 0.0001$).

5.3. Pellet Culture Model for Human Primary Osteoblasts

A pellet culture of human primary osteoblasts was investigated for its potential use as a 3D culture model. We hypothesised that pellet culture would decrease proliferation and, therefore, increase osteoblast differentiation more rapidly and to a greater extent than monolayer culture using the same cell type.
Pre-experiments were performed (♀ 53 years) to optimise the generation and culture of osteoblast cell pellets. Representative micrographs of pellets generated in the first experiment are shown in figure 5.18. The size of the generated cell pellets differed from 800 µm diameter [Fig. 5.18A, 36000 cells] to over 1 mm diameter [Fig. 5.18B 90000 cells]. According to previous reports on cell pellet cultures (Carpenedo *et al.* 2007; Johnstone *et al.* 1998), we aimed to achieve pellets of 200-800 µm diameter. Therefore, we decided on using an initial seeding density of 36000 cells for pellet culture of human primary osteoblasts.

Pellet generation was found to be highly dependent upon initial centrifugation as well as the presence of serum. If seeded cells were not centrifuged during pellet formation, the chance of generating several small pellets instead of one larger pellet was approximately 50% [Table 5.1: groups A, C]. Moreover, the use of SF medium, in combination with a short initial centrifugation resulted in an almost 90% success of generating a single, round, and evenly shaped cell pellet [Table 5.1: group B].

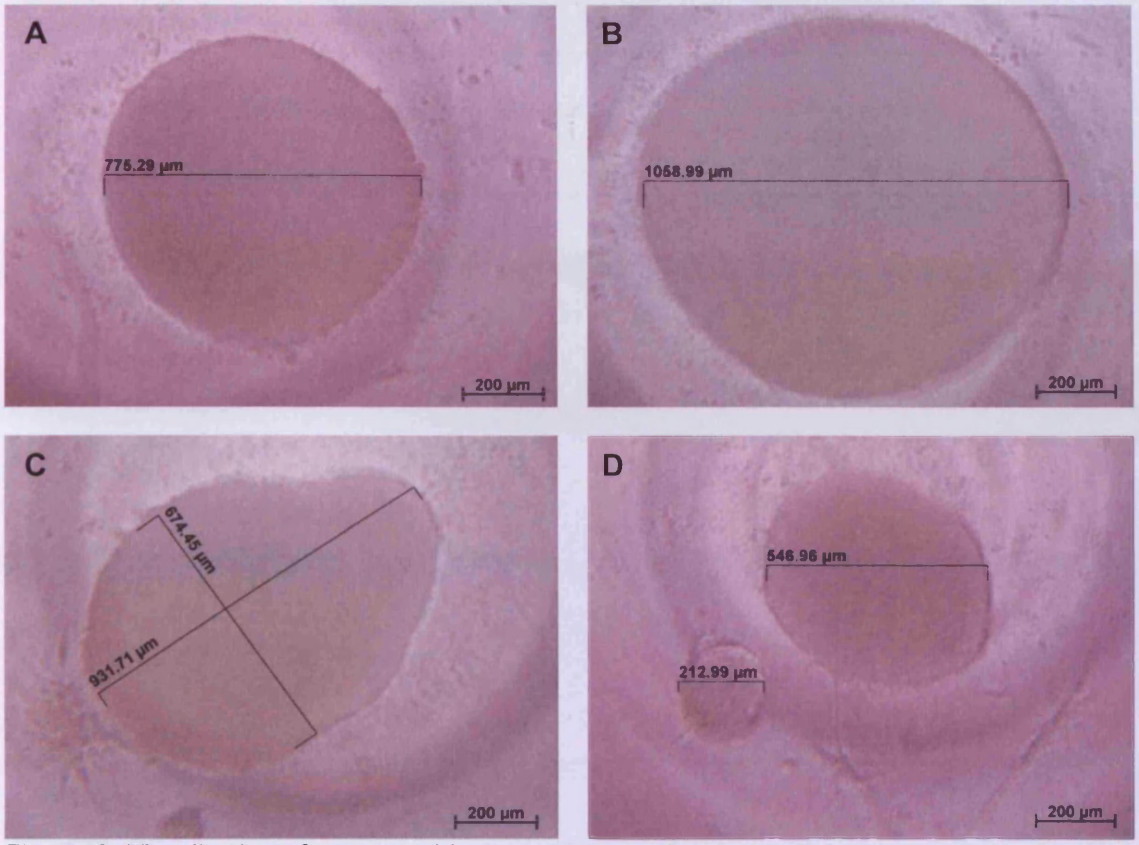

Fig. 5.18: Visualisation of generated human primary osteoblast pellet cultures on day 2, which were generated in the first experiment (♀ 53 years). A: An 800 µm (diameter), evenly round pellet was created seeding 36000 cells in addition to short exposure to 500xG and culture in DMEM SF. B: The pellet created by increasing only the amount of cells to 90000 was evenly round and approximately 1050 µm in diameter. C: The culture of a 36000 cell-pellet in DMEM + FCS, even with initial short centrifugation, resulted in an unevenly shaped pellet. D: If 36000 cells were only seeded without centrifugation at 500xG, various cell pellets were gained in one well.

To increase nutrition for the pellets during culture, further experiments used Eppendorf tubes as culture vessels after initial pellet formation. This led to an increase of medium volume per pellet from 250 µl to 750 µl. Moreover, overnight pellet formation was performed using SF medium in order to reduce adsorption of serum proteins to the tube walls making them less 'attractive' to adhering osteoblast cells.

Table 5.1: Morphologic analysis of cell pellets generated from human primary osteoblasts (♀ 53 years) within the first pre-experiment.

Group	Cells seeded	Centrifugation	Shaking	Medium day1	One round and even pellet	Pellet shape irregular	Additional small pellets / blebbing
A	90000	No	Yes	SF	9	-	9
	36000				9	-	10
B	90000	Yes	No	SF	11	7	-
	36000				**16**	**2**	**-**
C	90000	No	No	SF	12	-	10
	36000				18	-	5
D	90000	No	No	FCS	13	4	-
	36000				8	8	-

5.3.1. Viability Investigations on Osteoblast Pellet Cultures

A long-term pilot pellet experiment (♂ 62 years) was performed to investigate how long human primary osteoblasts could be kept alive in pellets. Therefore, pellet cultures were taken up to 27 days. Qualitative viability analysis was performed by LDH assay (Stoddart *et al.* 2006). Until culture day 7, an equal distribution of viable cells throughout the whole cultured pellet was detected [Fig. 5.19A-C]. However, extended culture of osteoblast cell pellets resulted in central areas of cell death [Fig. 5.19D]. Furthermore, the formation of a surface fibrous-tissue layer surrounding the inner cells of the pellets was detected by day 19. As the pellet culture model was changing its morphology so dramatically from day 7 to day 27, further experiments were performed only till day 7.

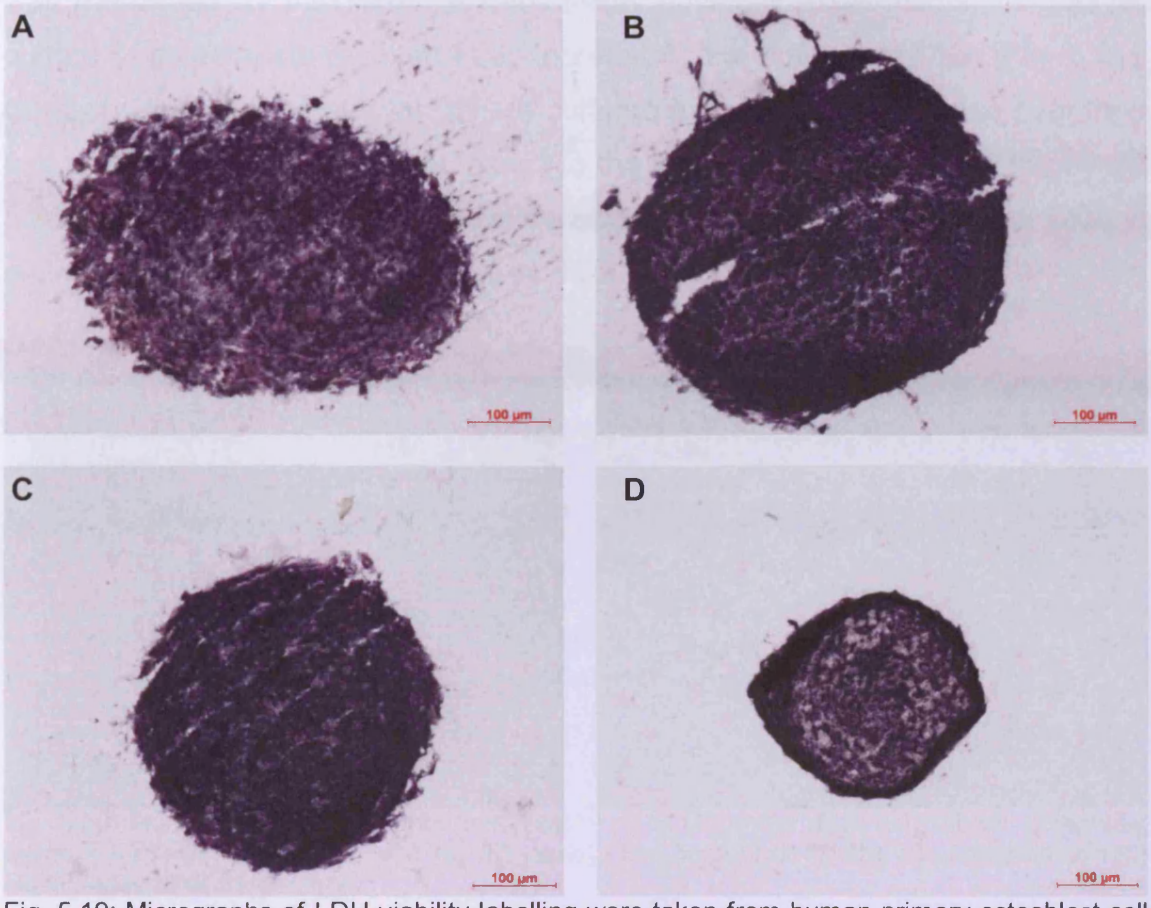

Fig. 5.19: Micrographs of LDH viability labelling were taken from human primary osteoblast cell pellets (♂ 62 years) cultured for 1 day (A), 3 days (B), 7 days (C) or 19 days (D). Evenly distributed LDH activity throughout the pellets was presented till day 7. The pellet on day 19 was dominated by a huge fibrous capsule and central areas of cell death.

From the results gained in the pre-experiments, four pellet culture experiments using human primary osteoblasts were performed (2 ♂ and 2 ♀; average age = 56 years; age range = 49-64 years) as described in 2.2.16.3. These experiments aimed to compare this new culture model in its potential to differentiate human primary osteoblasts in comparison to monolayer culture without using medium additives.

Qualitative evaluation of cell death was determined during the 7-day culture period using the TUNEL assay. By day 1 a small number of cells inside the pellet labelled positive for TUNEL [Fig. 5.20A, green cells]. However, the number of dead cells increased during the 7 day culture, with more cells labelled positive for TUNEL on day 7 than on day 1 [Fig. 5.20B].

The quantification of relative cell number during pellet and monolayer culture was performed by the Hoechst DNA assay. Over all experiments, monolayer culture of osteoblasts resulted in an increase in the amount of DNA [Fig. 5.21]. Contrary, the amount of total DNA in cultured pellets did not increase over time and was significantly lower compared to the monolayer cultures over the whole culture period ($p \leq 0.0001$). Moreover, a slight decrease in the amount of DNA in pellets was determined over 7 culture days.

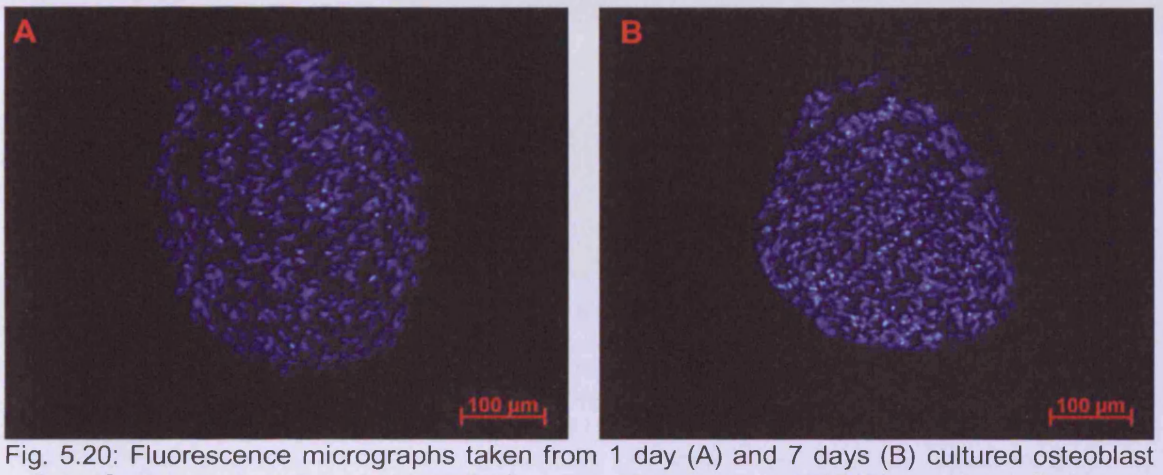

Fig. 5.20: Fluorescence micrographs taken from 1 day (A) and 7 days (B) cultured osteoblast pellets (♀ 73 years) analysed with TUNEL assay. The amount of TUNEL positive cells (green) did increase over culture time.

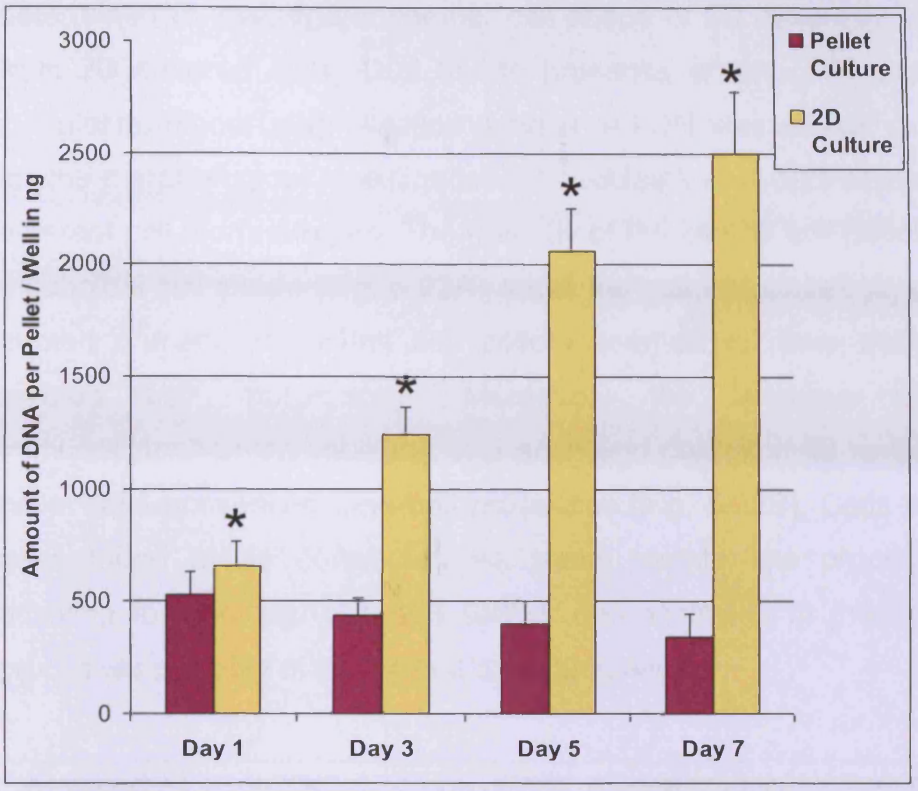

Fig. 5.21: The diagram shows the amount of mean DNA and the standard error of the mean quantified by Hoechst assay during culture of human primary osteoblasts in 2D or pellet (♀ 53 years, ♀ 73 years, ♂ 49 years, ♂ 59 years; n=14). Data was not normally distributed: Kruskal-Walis and Mann-Whitney-U test with Bonferroni correction were used as statistical analyses with statistical significance at $p \leq 0.05$. An exponential increase in DNA amount was detected during 2D culture, osteoblasts cultured in pellets showed a slight decrease in DNA over culture time. The amount of DNA in monolayer culture was significantly different to the amount in pellets (*$p \leq 0.0001$).

5.3.2. Three-Dimensional versus Two-Dimensional Culture of Human Primary Osteoblasts – Evaluation of Cell Functionality

In monolayer cultures, human primary osteoblasts display a fibroblast-like morphology (Kartsogiannis & Ng 2004). This spindle-shaped cell morphology is demonstrated *in vivo* only for inactive or resting osteoblasts/bone lining cells. It is known that during terminal differentiation of cuboidal-shaped, active osteoblasts to osteocytes, cell morphological changes are apparent; the cell shape of osteoblasts is transformed into star-shaped osteocytes. Due to the presence of the ECM, osteocytes are forced into developing long cellular processes which connect embedded osteocytes to their neighbouring cells (Nijweide *et al.* 2002). The cell morphology of osteoblasts in cell pellets was

determined to investigate whether cell shape of 3D cultured osteoblasts differs from 2D cultured cells. Due to the presence of the LDH enzyme within the cytoplasm, higher magnification images of LDH stained cell pellets were used for the morphological investigation. Osteoblasts in pellets revealed at least two different cell morphologies. The majority of the central cell population presented a cuboidal cell shape [Fig. 5.22A], while the outer most cell layer of the cultured human primary osteoblast cell pellets seemed to have maintained spindle-shaped cell morphology. Moreover, the analysis of the double immunocytochemical labelling of β-actin and connexin 43 revealed that central pellet cells possessed long cell processes [Fig. 5.22B]. Cells within the pellets were found to be connected via these slender-like processes. A positive labelling for connexin 43 was further demonstrated in the centre of the cell processes possibly enabling cell communication.

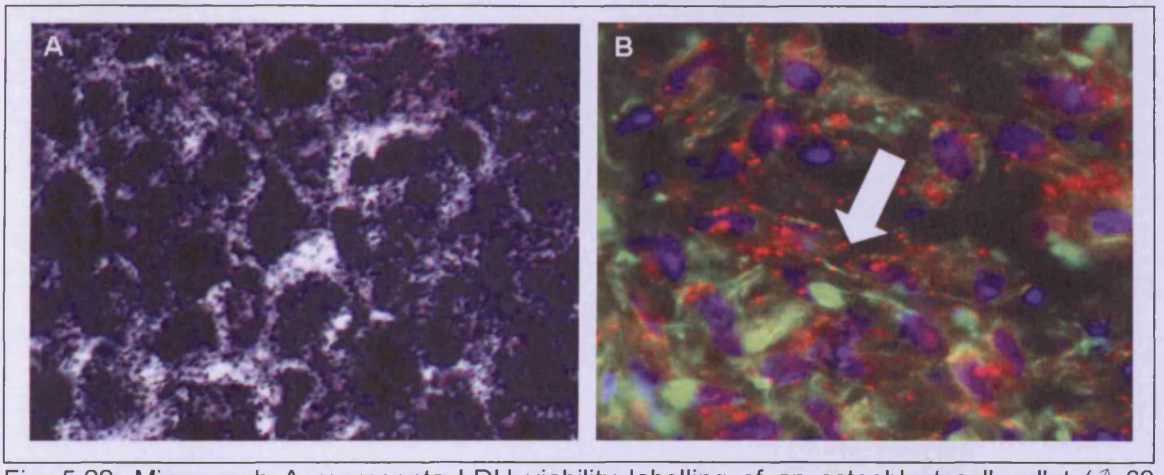

Fig. 5.22: Micrograph A represents LDH viability labelling of an osteoblast cell pellet (♂ 62 years) cultured for 3 days. Central cells demonstrate cuboidal cell morphology. Micrograph B shows an immuno-double labelling for β-actin (Alexa 488, green) and connexin 43 (Alexa 598, red) - in combination with a DAPI staining - of a 5 days cultured osteoblast pellet. Cell processes and gap junctions (white arrow) are present between central osteoblasts in pellets.

The activity of the tissue-non-specific form of ALP is seen during the early onset of *in vitro* differentiation of osteoblasts in monolayer culture. ALP activity is known to be of crucial importance for the maturation and calcification of the ECM. The enzyme is thought to be responsible for the generation of P_i from di-phosphate sources (Whyte *et al.* 1995). Lian and Stein have shown that the activity of the enzyme in rat calvaria osteoblast monolayer cultures peaks at around culture day 14 prior to the onset of osteoblast differentiation *in vitro* (Lian

& Stein 1992). The activity of ALP assessed in monolayer culture revealed an even staining of positive cells, and the amount increased as the number of total cells increased [Fig. 5.23A, B]. To determine the location of ALP activity during pellet culture of human primary osteoblasts, qualitative analysis of the enzyme activity was performed. Cell pellets demonstrated during the early stage of pellet culture, day 1 to 3, ALP activity almost exclusively at the outer surface cell layer surrounding a central population of non-active ALP osteoblasts [Fig. 5.23C]. This distinct pattern of the location of ALP activity shifted during pellet culture. By day 7, ALP activity was found futher into the pellet, with only the most central area being negative [Fig. 5.23D].

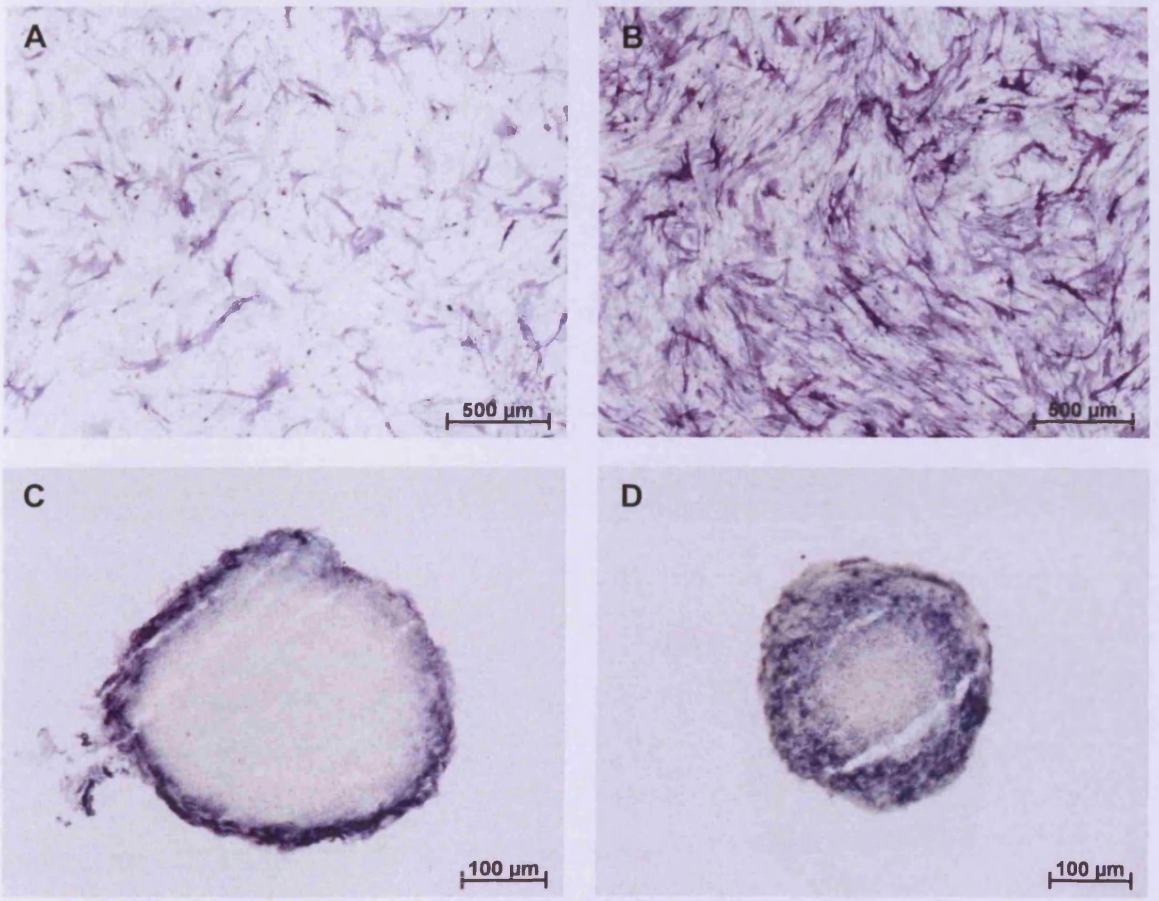

Fig. 5.23: Micrographs represent ALP activity labelling of human primary osteoblasts (♀ 73 years) cultured in monolayer (A, B), or pellets (C, D), cultured for 3 days (A, C) or 7 days (B, D). Monolayer culture showed even activity labelling, while pellets demonstrated concentrated areas of ALP activity predominantly on adjacent surface cells by day 3. On day 7 ALP activity in pellets was more evenly distributed in the centre of the pellet.

Type I collagen is the main protein produced by osteoblasts at an early stage of differentiation. It accumulates in the ECM of bone and serves as a construct of the developing matrix during bone formation. To determine possible protein expression differences of 2D and 3D cultured human primary osteoblasts, immunocytochemical analyses during culture were performed detecting the presence of the unstable C-terminal propeptide of type I collagen – ProCI - (Calvo *et al.* 1996). The immunocytochemical analysis of ProCI in monolayer cultures revealed even cell staining [Fig. 5.24A, B], similar to osteoblasts cultured in pellets [Fig. 5.24C, D]. However, by culture day 7, the endpoint of the pellet culture, the labelling for ProCI was found more intense at the surface layer of cultured pellets than in the centre, and for both monolayer and pellet the staining generally appeared more intense [Fig. 5.24D].

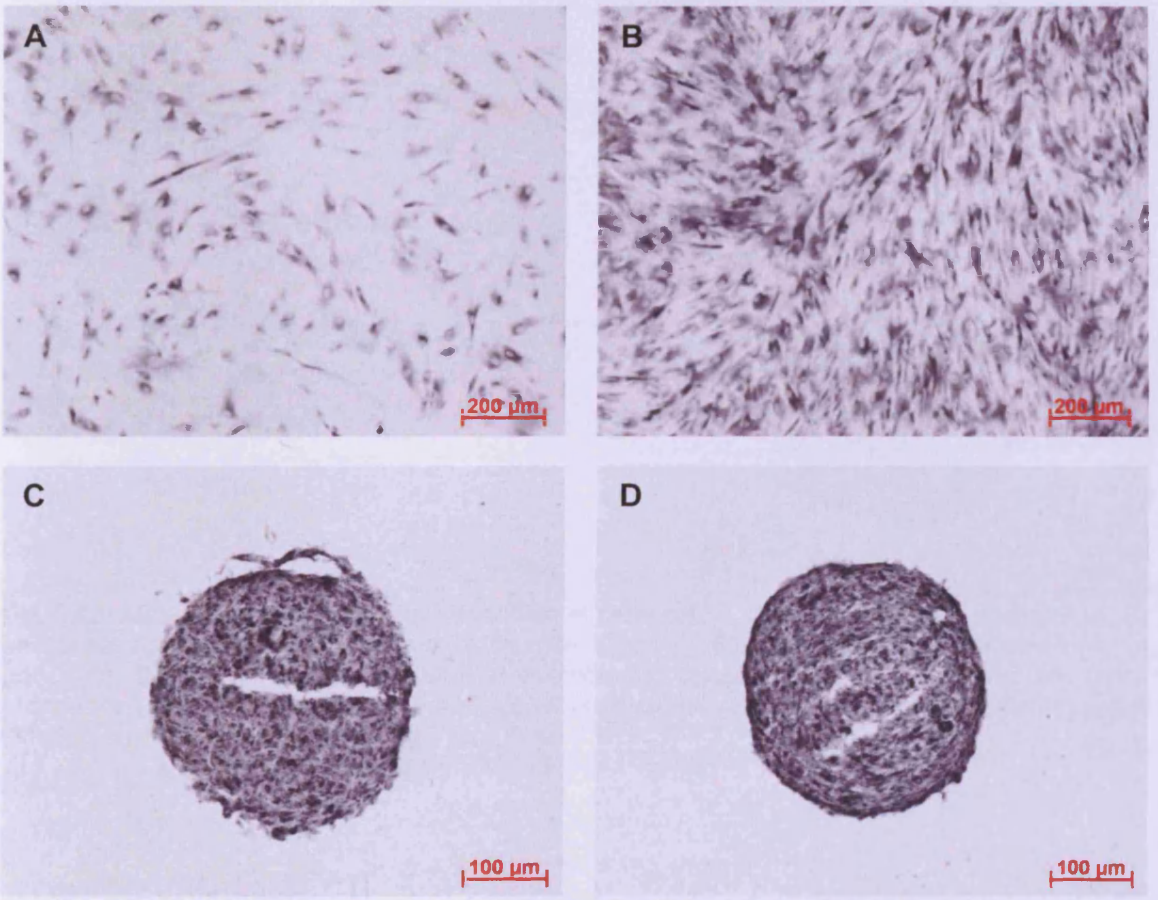

Fig. 5.24: Micrographs show immuno-labelling of the C-terminal propeptide of type I collagen, detected during monolayer (A, B) and pellet culture (C, D) of human primary osteoblasts (♂ 59 years) at culture day day 1 (A, C) and 7 (B, D). Even cellular staining could be detected throughout. However, by day 7 type I collagen synthesis in pellets was more dominant at the surface cell layer of pellets.

Immuno-labelling for the ECM protein osteocalcin during monolayer and pellet culture of human primary osteoblasts was performed. While in monolayer, cells showed a very slight cellular labelling on day 1, this was diminished on day 7 [Fig. 5.25A, B]. Cells cultured in 3D pellets demonstrated cellular labelling from day 1 till 7 [Fig. 5.25C, D]. On day 7, even a slight ECM deposition of osteocalcin was detected in osteoblast pellets.

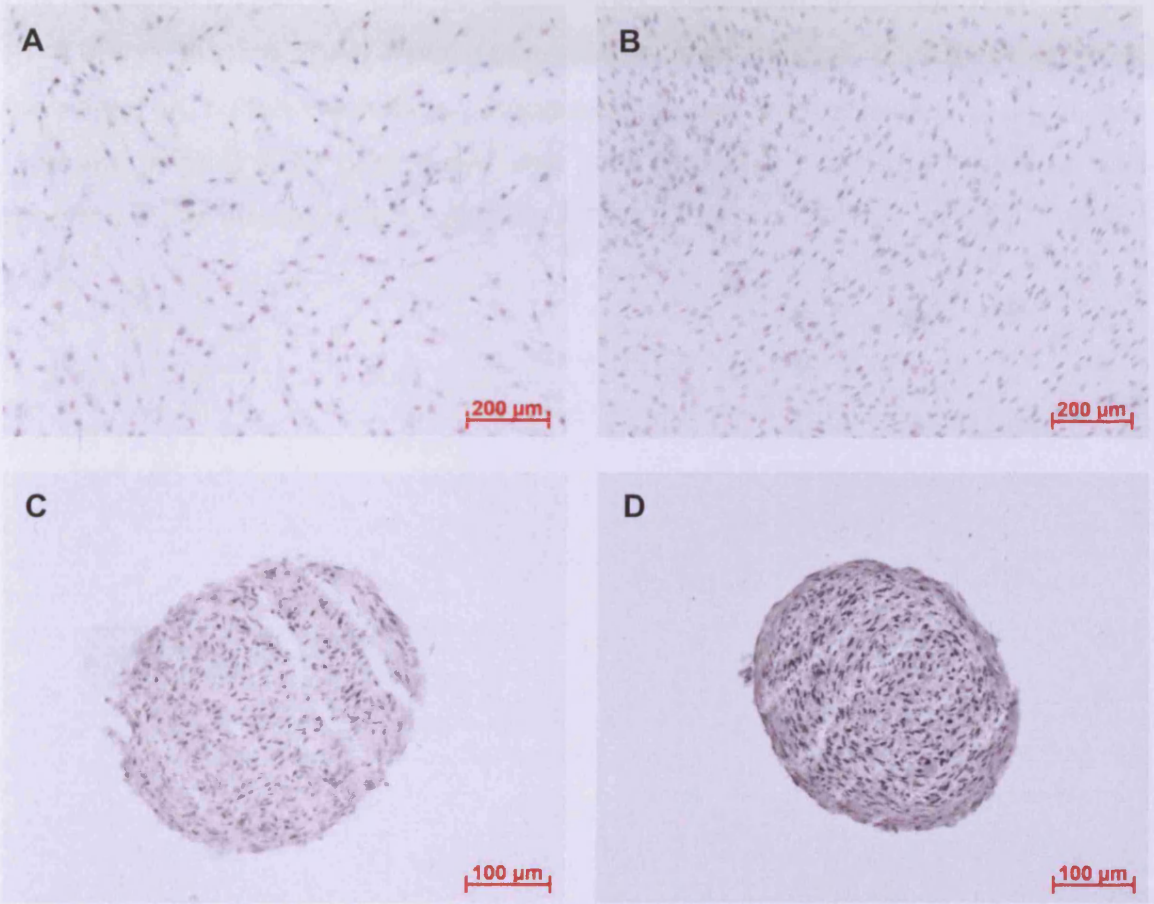

Fig. 5.25: Micrographs show immuno-labelling of osteocalcin, detected during monolayer (A, B) and pellet culture (C, D) of human primary osteoblasts (♂ 59 years) at culture day day 1 (A, C) and 7 (B, D). For in monolayer cultured osteoblasts, cellular osteocalcin labelling was more pronounced at day 1 than 7, while cellular osteocalcin labelling as well as deposition in pellet cultures seemed to increase during 7 day culture time.

The quantification of mineral deposition during the 3D and 2D culture of human primary osteoblasts was performed using the ARS assay. The results are shown in figure 5.26. Alizarin staining per DNA was significantly different between monolayer and pellet culture of human primary osteoblasts (2 ♂ and 2 ♀; average age = 56 years; age range = 49-64 years) on day 1 and day 5 of culture ($p \leq 0.042$) [Fig. 5.26A]. While ARS / DNA in monolayer cultures decreased from day 1 to day 5, and on day 7 reached again a similar level to day 1, ARS / DNA in pellets significantly increased from day 1 to day 7 ($p \leq 0.0001$) [Fig. 5.26A]. This demonstrates that mineral deposition per cell increased in pellets with time. Monolayer culture demonstrated a significant increase in total ARS bound per well over time [Fig. 5.26B], a response well reported in the literature (Lian & Stein 1992).

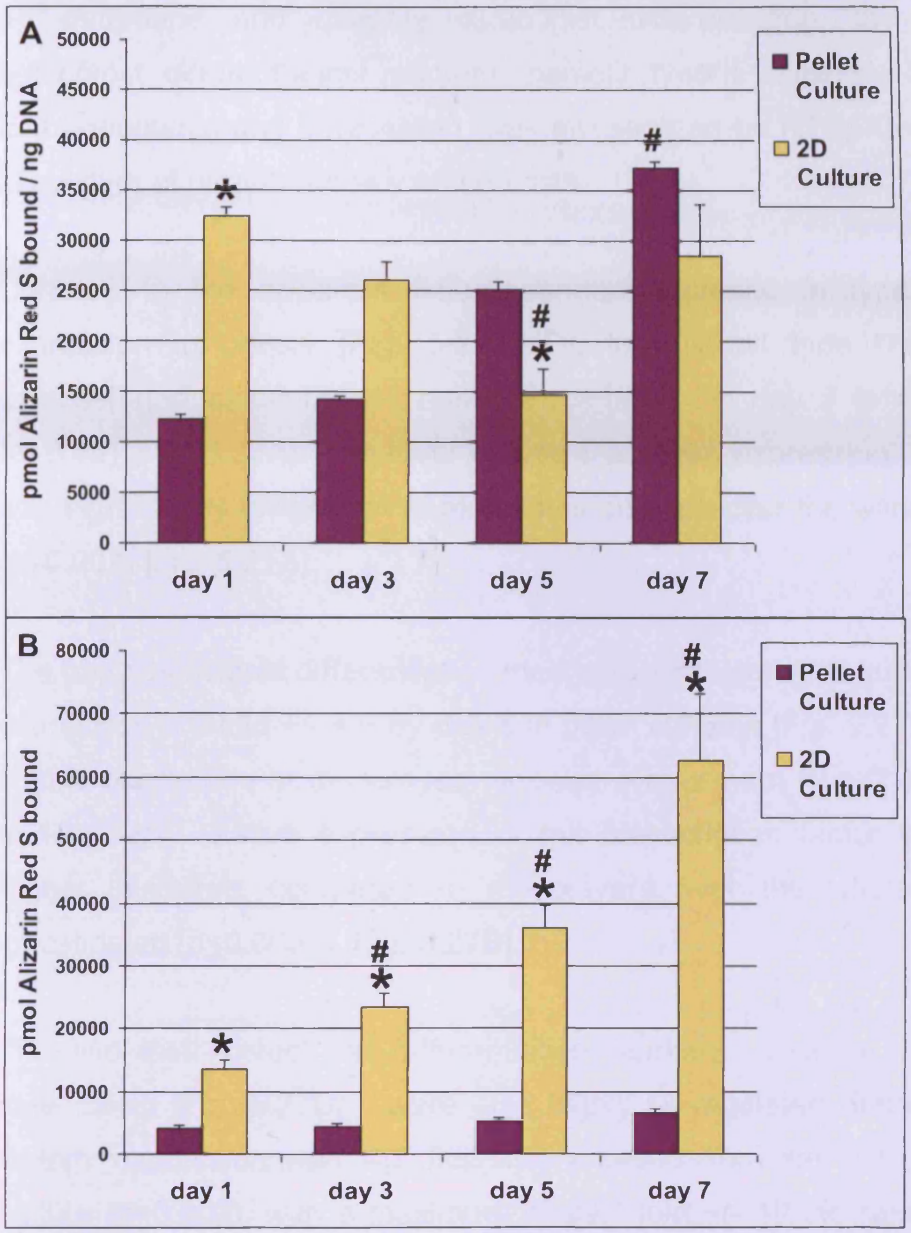

Fig. 5.26: Human primary osteoblasts were cultured in monolayer or pellets (♀ 53 years, ♀ 73 years, ♂ 49 years, ♂ 59 years; n=14). Diagram A shows the amount of mean ARS staining per total amount of DNA together with the standard error of the mean. Diagram B represents the total amount of Alizarin Red S bound per well or per pellet. Data was normally distributed: one-way ANOVA and Tukey were used. Statistical significance was determined as $p \leq 0.05$. *: significantly different compared to pellet culture; #: significantly different compared to the same culture on day 1. While ARS / DNA in pellet cultures significantly increased with culture time, the amount of ARS / DNA in monolayers decreased from day 1 to 5 and by day 7 was comparable to day 1 again. Total ARS in monolayers significantly increased during culture.

To determine and quantify osteoblast differentiation, the expression of osteoblast differentiation markers, namely type I collagen, Runx2, osterix, E11/podoplanin and osteocalcin, was investigated by RT-qPCR during 3D and 2D culture of human primary osteoblasts.

Contrary to the apparent time-dependent increase in type I pro-collagen expression in pellets [Fig. 5.24C, D], the overall type I collagen mRNA expression significantly decreased in pellets from day 3 to day 7 of culture ($p=0.05$) [Fig. 5.27A]. The level of type I collagen expression in pellet cultures was significantly lower than in monolayer cultures over the whole culture period ($p=0.007$) [Fig. 5.27A].

The early osteoblast differentiation marker Runx2 was up-regulated in its mRNA expression 6.6-fold +/- 4.6 by day 5 in pellet cultures [Fig. 5.27B]. Even though Runx2 expression in monolayers showed a maximum Runx2 expression of 2-fold +/- 2.2, relative expression of this transcription factor was significantly higher in pellets compared to monolayers over the whole culture period investigated ($p \leq 0.0001$) [Fig. 5.27B].

The two later osteoblast differentiation markers - osterix [Fig. 5.27C] and osteocalcin [Fig. 5.27D] - were also highly up-regulated during pellet culture. Osterix expression was significantly increased from day 1 to day 7 of pellet culture ($p=0.002$), with a maximum of 29.1-fold +/- 19 increase by day 7 [Fig. 5.27C]. The significant up-regulation in osterix mRNA in pellets was higher than osterix expression in monolayer culture during the whole culture period ($p \leq 0.0001$) [Fig. 5.27C].

Osteocalcin mRNA level was also significantly higher during pellet culture compared to monolayer culture ($p \leq 0.0001$) [Fig. 5.27D]. Yet, osteocalcin mRNA expression decreased from day 3 to day 7 in pellets ($p=0.002$). By day 7, pellets showed an average osteocalcin expression of 3.2-fold +/- 1.6 [Fig. 5.27D].
The most dramatic increase in relative mRNA expression during pellet culture of osteoblasts compared to monolayer culture was seen in E11/podoplanin. The

expression of this early osteocyte marker was dramatically increased in pellets compared to monolayers ($p \leq 0.0001$) [Fig. 5.27E]. Moreover, E11 expression in pellets increased during culture from day 1 to day 7 ($p=0.001$), with a maximal expression of 82.7-fold change +/- 114 by day 7. The maximum E11 expression in monolayers was found on day 5 with only 2-fold change +/- 2.1 [Fig. 5.27E].

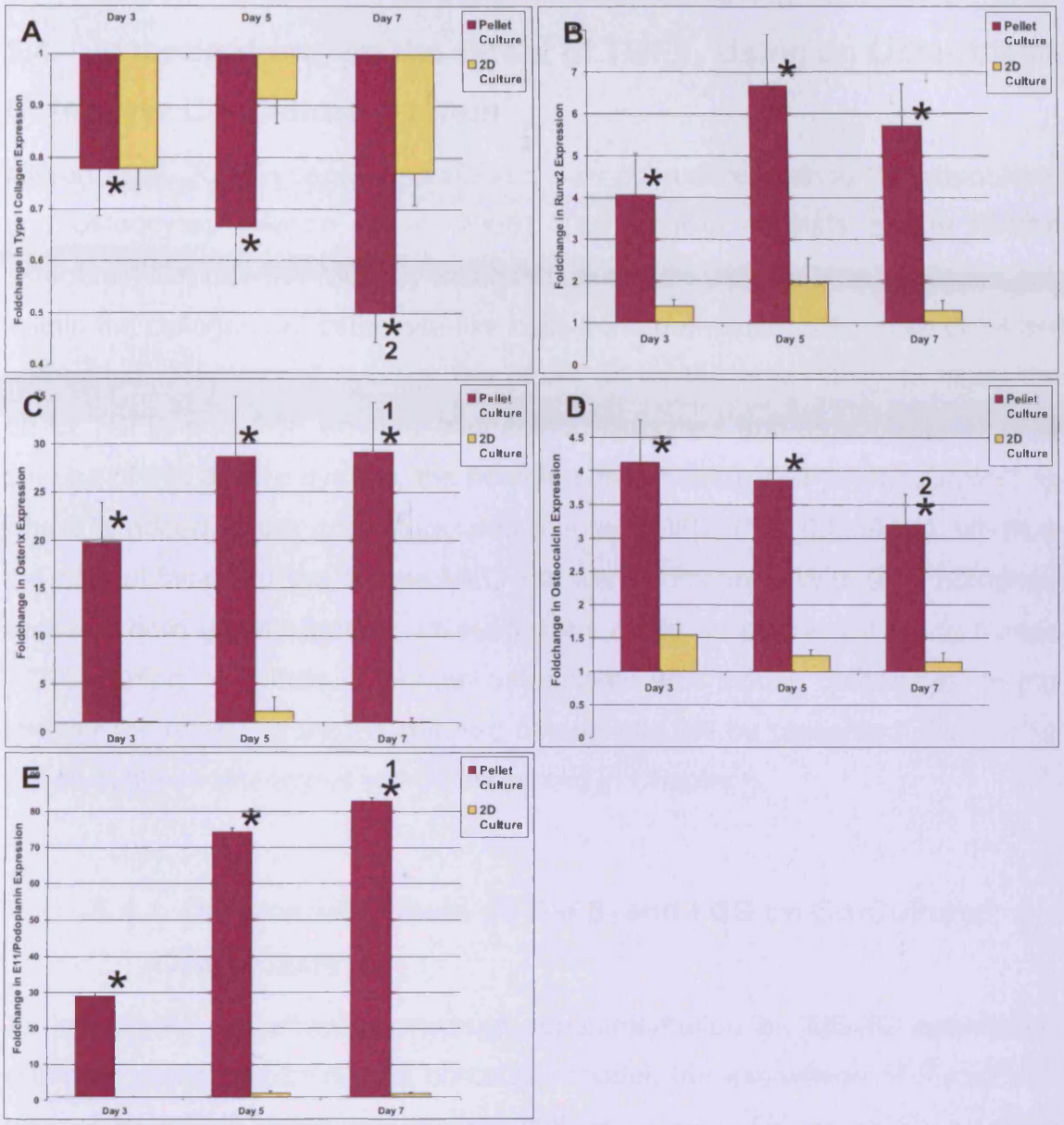

Fig. 5.27: Comparative qPCR of type I collagen (A), Runx2 (B), osterix (C), osteocalcin (D) and E11 (E) was performed during the 7 day culture period of osteoblasts within monolayer or pellet culture (♀ 53 years, ♀ 73 years, ♂ 49 years, ♂ 59 years; n=14). Diagrams show the mean fold-change in relative gene expression and the standard error of the mean. Gene expression levels were normalised to 18SrRNA, and were made relative to gene expression levels in monolayer at culture day 1. The dCT data was used for statistical analysis: type I collagen data was normally distributed and analysed with one-way ANOVA with Tukey, other data was not normally distributed and analysed with Mann-Whitney test and Bonferroni correction. Statistical significance was determined as $p \leq 0.05$; *: significant over all days compared to monolayer; 1: significantly increased from day 1 to day 7; 2: significantly decreased from day 3 to day 7.The expression of type I collagen was significantly lower during pellet culture compared to monolayer culture, while expression of the osteoblast differentiation markers Runx2, osterix, osteocalcin and E11 was significantly increased over all time points compared to monolayer culture.

5.4. Investigations on the Effect of TGFβ3 Using an Osteoblast-Osteocyte Co-Culture System

Mason *et al.* 2009 recently described a new co-culture method for osteoblasts and osteocytes (Mason *et al.* 2008). The system consists of the human osteoblast-like cell-line MG-63, which is cultured on top of a type I collagen gel. Within the collagen gel osteocyte-like cells from the mouse cell-line MLO-Y4 are embedded. This model system, therefore, offers the opportunity to study the effect TGFβ3 and FCS on inter-connected osteocytes and osteoblasts. Prior to the use of this culture system, the homologies of human TGFβ3 (NP_003230.1), which is added to the co-culture, and mouse TGFβ3 (NP_033394.1), which is the natural factor for the mouse MLO-Y4, were compared. With 98% homology between both growth factors, investigations could be performed using human TGFβ3 during co-culture of human osteoblasts with mouse osteocytes. In this chapter the results of the co-cultured osteoblasts will be presented. Results for the co-cultured osteocytes will be presented in Chapter 6.

5.4.1. Differential Effects of TGFβ3 and FCS on Co-Cultured Osteoblasts

To investigate the effect of medium supplementation on MG-63 osteoblasts within an osteoblast-osteocyte co-culture model, the expression of Runx2 and osteocalcin mRNA levels was investigated.

TGFβ3 did not influence osteocalcin mRNA expression in co-cultured osteoblasts [Fig. 5.28B, D], however, Runx2 expression was significantly decreased on day 4 in the presence of TGFβ3 and FCS compared to FCS alone ($p=0.008$) [Fig. 5.28A]. This effect was also present when the data over all culture days was combined ($p=0.002$) [Fig. 5.28C]. The TGFβ3-induced down-regulation of Runx2 expression was dependent on the presence of FCS, as TGFβ3 did not influence Runx2 expression in osteoblasts cultured in SF medium [Fig. 5.28A, C].

The relative expression of Runx2 and osteocalcin by co-cultured osteoblasts was significantly affected by the presence or absence of FCS. Runx2 mRNA expression was significantly decreased in SF medium compared to FCS-containing medium on day 4 and day 6, (day 4 p=0.005, day 6 p=0.002) [Fig. 5.28A]. The relative expression of osteocalcin mRNA significantly increased with time using either αMEM + FCS +/- TGFβ3 or αMEM SF medium (day 4 $p \leq 0.025$) [Fig. 5.28B]. Moreover, if data from all time points were pooled, αMEM SF significantly lowered expression of Runx2 ($p \leq 0.0001$) and increased expression of osteocalcin (p=0.002) in co-cultured osteoblasts compared to αMEM + FCS culture [Fig. 5.28C, D].

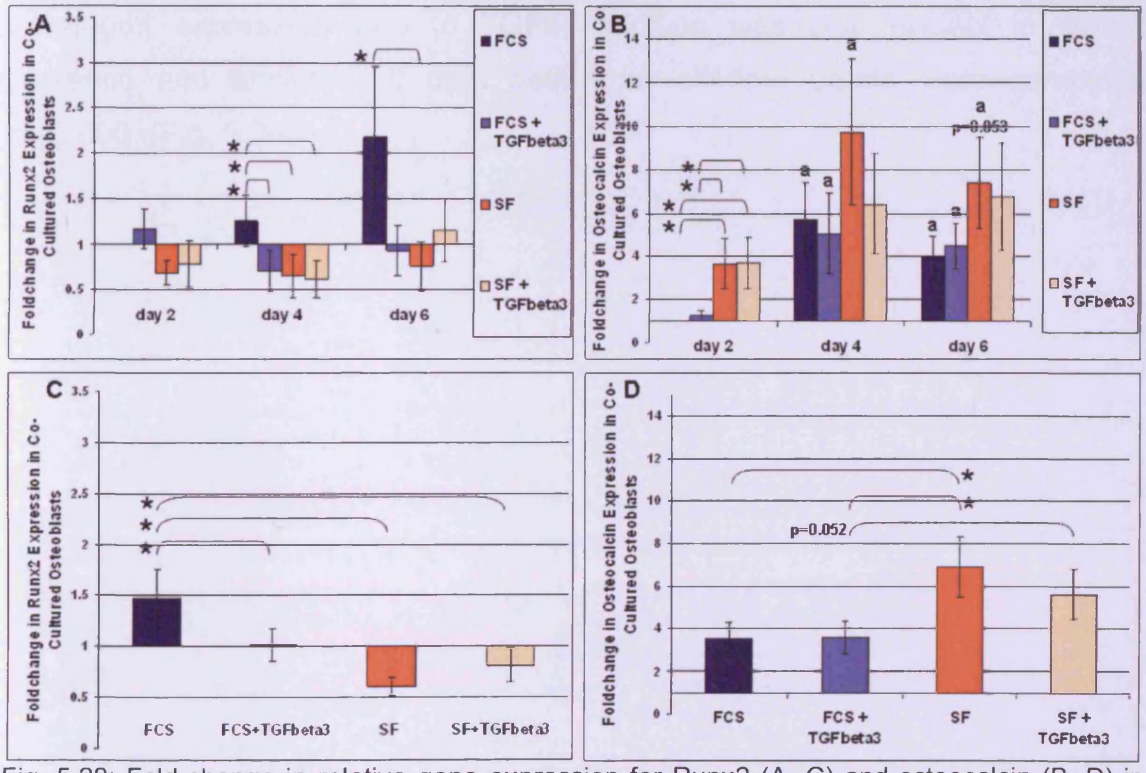

Fig. 5.28: Fold-change in relative gene expression for Runx2 (A, C) and osteocalcin (B, D) in co-cultured osteoblasts. Gene expression levels were normalised to 18SrRNA, and were was made relative to αMem + FCS on day 2. The dCT data was used for statistical analysis: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; *: significant on that day; 1: compared to same medium group on day 2. Bar charts show the average fold-change as well as the standard error of the mean. Runx2 expression was significantly higher using αMem + FCS ($p \leq 0.0001$). Osteocalcin expression was increased by the use of αMem SF (p=0.002). TGFβ3 down-regulated Runx2 expression only in the presence of FCS (p=0.008).

5.4.2. Type I Collagen Expression in Co-Cultured Osteoblasts – the Effect of TGFβ3 and FCS

Type I collagen expression was investigated at the mRNA level in co-cultured osteoblasts. Osteoblast type I collagen expression was significantly affected by the addition of TGFβ3. Co-culture of osteoblasts in the presence of TGFβ3 and FCS led to a significantly higher type I collagen expression on day 4 ($p=0.045$) than FCS alone [Fig. 5.29A]. Slightly increased type I collagen expression was also seen in SF medium by the addition of TGFβ3 ($p=0.057$) [Fig. 5.29A]. Yet, type I collagen mRNA expression in co-cultured osteoblasts in SF medium + TGFβ3 was only significantly increased compared to culture in FCS medium alone (day 2: $p=0.003$; day 6: $p=0.012$). Moreover, a significant increase in type I collagen expression due to TGFβ3 addition was also evident in serum presence and absence, if data sets from all time points were combined ($p \leq 0.003$) [Fig. 5.29B].

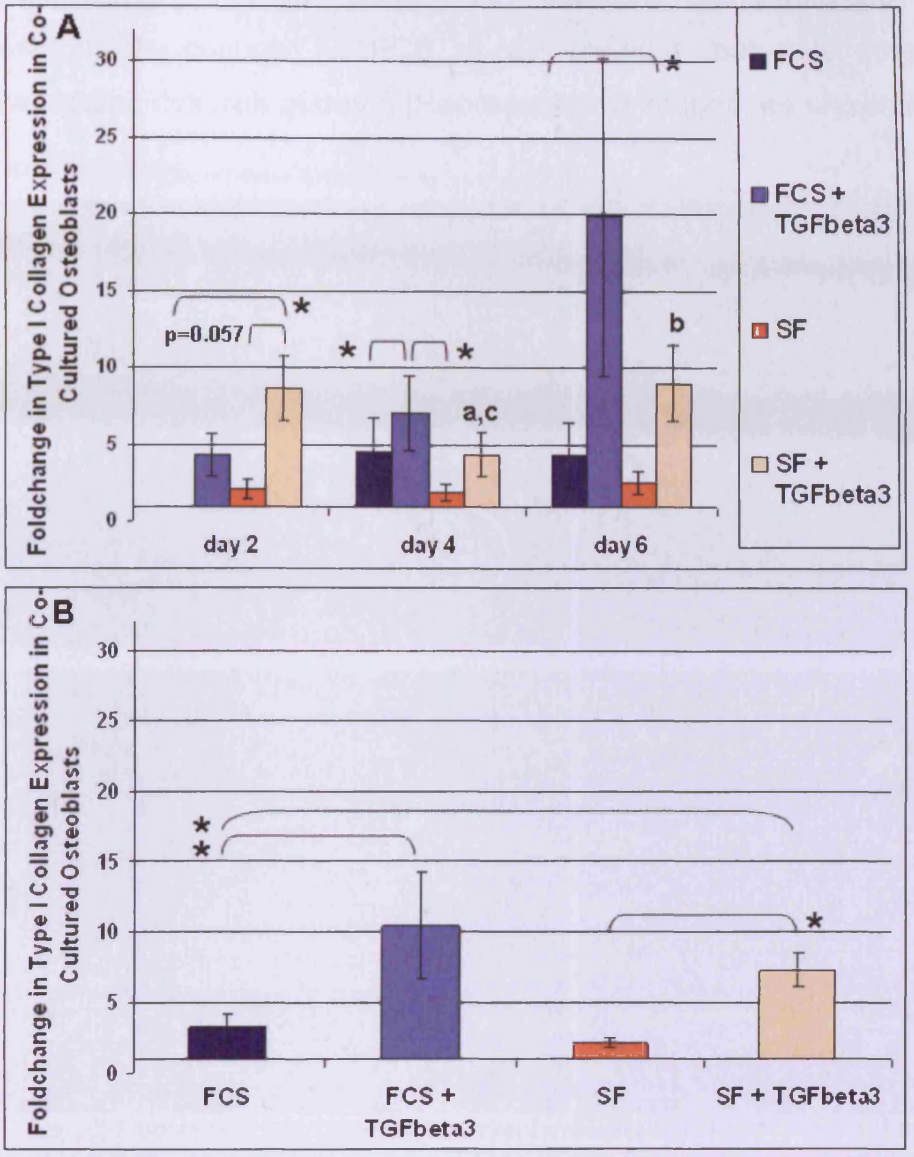

Fig. 5.29: Fold-change in relative gene expression of type I collagen in co-cultured osteoblasts. Gene expression levels were normalised to 18SrRNA, and were made relative to αMem + FCS on day 2. The dCT data was used for statistical analysis: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; *: significant on that day; 1: compared to same medium group on day 2; 2: compared to same medium group on day 4; 3: compared to same medium group on day 6. Bar charts show the average fold-change as well as the standard error of the mean. Type I collagen expression was positively regulated in osteoblasts by the addition of TGFβ3 (B: $p \leq 0.003$).

Immunocytochemical labelling of ProCI was used as qualitative analysis of type I collagen protein expression by co-cultured osteoblasts. The labelling revealed a difference in the onset of newly formed collagenous matrix deposition by the co-cultured osteoblasts depending upon TGFβ3 presence. However, even though type I collagen mRNA expression was increased by TGFβ3 addition; on a protein level type I collagen synthesis was delayed if TGFβ3 was added to

FCS or SF medium. ProCI was detected on culture day 2 on surface osteoblasts cultured in FCS or SF medium, but only detected in TGFβ$_3$ containing cultures at day 6 [Representative images are shown in figure 5.30].

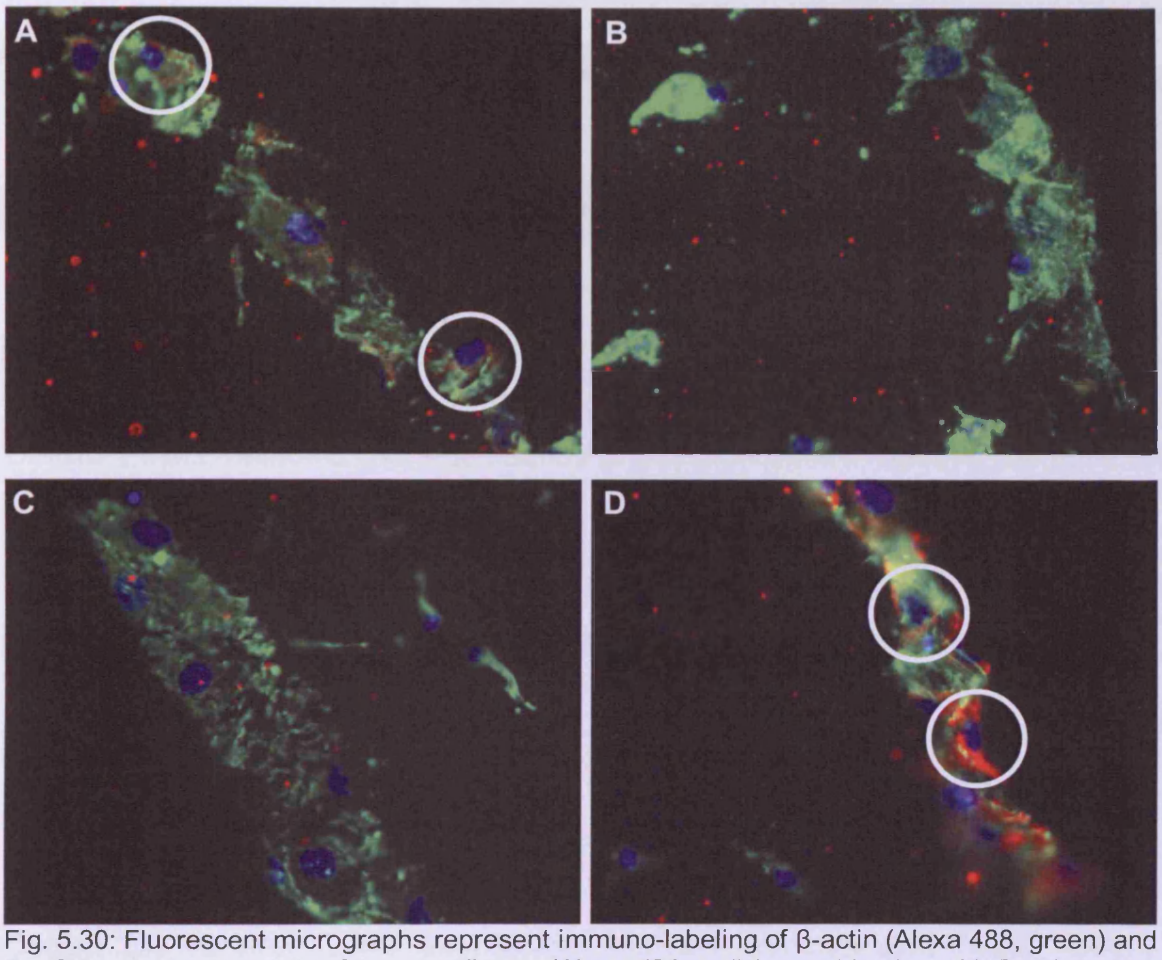

Fig. 5.30: Fluorescent micrographs represent immuno-labeling of β-actin (Alexa 488, green) and the C-terminal propeptide of type I collagen (Alexa 594, red) in combination with DAPI nuclear staining (blue). Shown are the micrographs for labelled surface osteoblasts on culture day 2 (A, C) and day 6 (B, D), either cultured in αMem SF (A, B) or αMem SF + TGFβ$_3$ (C, D). ProCI labelling in TGFβ$_3$-containing medium was delayed till day 6.

5.5. Discussion

Within this chapter the effects of TGFβ3 and FCS have been investigated during *in vitro* culture of osteoblast-lineage cells. Several culture models have been introduced for these evaluations.

Within the monolayer culture system, different osteoblast populations have been investigated. Early and late osteoblast isolated populations have been compared for their potential to respond to TGFβ3 treatment in the presence or absence of serum. Both cell isolations showed the typical increase in DNA content over time in monolayer culture (Lian & Stein 1992). Cell number was significantly higher by day 14 in early isolated cells than in late ones cultured in DMEM + FCS medium. The significance of this difference in relative cell number might be explained, due to a higher differentiation stage of later isolated osteoblasts compared to earlier ones, by which mean these cells would be less proliferative.

Further investigations were made to characterise both cell isolations. The relative cell activity evaluation of both cell isolations did not reveal significant differences. Yet, mineral deposition by later isolated osteoblasts cultured in DMEM + FCS was comparable to the high mineral deposition level achieved in DMEM SF culture. Gene expression analysis demonstrated higher type I collagen expression in later isolated osteoblasts. Yet, neither Runx 2 nor osterix expression differed between cell isolation populations cultured in DMEM + FCS. Runx2 expression under SF culture was, however, higher in later than earlier isolated osteoblasts. Therefore, it seems more likely that later isolated osteoblasts are of higher differentiation stage than earlier ones.

Compared to the monolayer culture systems for human primary osteoblasts, the exponential increase in DNA amount during culture of human primary osteoblast pellet cultures was not present, demonstrating that initial proliferation in cell pellets was absent and this is likely due to contact inhibition. Resulting from *in vitro* osteoblast differentiation studies (Gallagher 2003; Kartsogiannis &

Ng 2004; Lian & Stein 1992), we predicted that a reduction in cell proliferation, as seen during osteoblast pellet culture, would result in increased osteoblast differentiation. To test this hypothesis we first investigated the activity of the tissue-nonspecific alkaline phosphatase (TNSALP) an early indicator of osteoblast differentiation. Even though the precise role of TNSALP in bone is not completely understood, it is known that cellular matrix vesicles rich in TNSALP arise *in vivo* at the onset of ECM mineralisation (Ali *et al.* 1970). Within the pellet culture system, the activity of TNSALP was found to shift in location. During early osteoblast pellet culture, an outer ring of TNSALP-positive cells was visible, which seemed to expand to the central region of the pellets within the 7-culture days. It is hard to speculate what the exact reason for the shift in TNSALP activity in pellets is. It is for certain that TNSALP activity is needed for mineral deposition (Anderson 1969). Lian and Stein were able to show that ALP mRNA expression is up-regulated during matrix maturation, yet, ALP activity relative to amount of DNA peaks later during mineral deposition (Lian & Stein 1992). We hypothesise that during the early culture of osteoblast pellets (day 1-day 5) the outer population of ALP-active cells was characterised by a more differentiated state, but most-likely benefited from availability of nutrients in comparison to the central pellet population; which may result in a later onset of ALP activity in the pellet centre.

Calcium incorporation into the ECM of the pellets was investigated using Alizarin Red S staining. The quantification of this staining showed no significant differences between monolayer and pellet culture by the end of the 7-day culture period. However, contrary to monolayer culture, ARS per DNA in pellet culture demonstrated a significant increase during culture time, demonstrating, as we believe, increased cellular activity in the form of mineral deposition in pellets compared to monolayer culture. Also, for monolayer culture, an increase in ARS per relative cell number is reported after 7 days of culture (Poulsson 2007). Therefore, the pellet culture of primary osteoblasts seemed to show a more rapid mineral deposition increase than monolayer.

Active osteoblasts *in vivo* demonstrate a cuboidal cell morphology, while inactive osteoblasts or bone-lining cells are spindle-shaped and flat (Aubin & Liu

1996). Also, during osteoblast differentiation, cells undergo a dramatic change in cell morphology, resulting in the formation of stellar-shaped osteocytes which possess long slender-like cell processes to aid cell communication throughout the bone matrix (Nijweide *et al.* 2002). Human primary osteoblast cultured pellets possessed at least two different cell morphologies. While the surface cells appeared flat and spindle-shaped, the central cell population was cuboidal in shape, suggesting that the central cell population represents a later osteoblast differentiation state than the surface cells. This was further confirmed by the presence of long cell processes detected via β-actin and connexin 43 immunocytochemical double labelling, connecting cells of the central areas of cultured osteoblast pellets. Even though osteoblasts in pellets are in relatively close contact to one another, it seems that the late differentiation state led to the formation of cell processes by these cells. Osteocytes are known to use these processes within the cellular network to communicate throughout the borders of extracellular bone matrix (Aarden *et al.* 1994; Nijweide *et al.* 2002). Moreover, these processes are a distinctive marker for these terminally differentiated cells and important for mechanical transduction by these cells (Jiang *et al.* 2007).

Osteocytes are not just characterised by morphological differences to osteoblasts. They are furthermore distinguished from osteoblasts by high expression levels of osteocalcin, while demonstrating little activity of alkaline phosphatase activity (Aarden *et al.* 1994; Aarden *et al.* 1996). Within the 7-day culture period, attempts to quantify the amount of ALP activity per hOB cell pellet (3 pellets were pooled for each data point. The ALP activity in pellets by day 3 was so low (data not shown) that further quantification was not performed. Possibly, this result was partly caused by the difficulties to homogenise the osteoblast cell pellets for ALP quantification.

Relative gene expression of type I collagen was significantly decreased during osteoblast pellet culture compared to monolayer. Type I collagen is the main ECM protein found in bone. It serves as a scaffold for the forming bone matrix. Its peak mRNA expression is believed to be characteristic for osteoblasts prior to ECM maturation and mineralisation (Lian & Stein 1992). However, before and after the peak, type I collagen mRNA expression by younger / pre-osteoblasts as well as mature osteoblasts / osteocytes is reduced compared to osteoblasts in the proliferative phase (Aubin & Liu 1996; Lian & Stein 1992). If we would have to interpret the decrease in type I collagen mRNA production found in osteoblasts cultured in pellets compared to the expression found in monolayer, we would speculate that osteoblasts in pellets are of later differentiation state compared to ones in monolayer, which still have to increase their collagen production during the initial proliferation phase.

If the decrease in type I collagen production in osteoblast pellet cultures is taken as evidence for a later/more mature differentiation stage of osteoblasts, it seems questionable why later isolated osteoblasts cultured in monolayer can be determined as more mature osteoblasts even though the later isolation population exhibited increased type I collagen production compared to earlier isolated osteoblasts. We suggest that these later isolated osteoblasts are demonstrating increased type I collagen mRNA expression compared to earlier ones, as these cells are 'still on their way' to peak mRNA expression found in proliferating osteoblasts. We would speculate that the later isolated osteoblasts are caught during a later stage of their differentiation as the earlier isolated ones. However, it might also be possible that the influence of a heterogeneous cell population found in primary isolated cells plays an important role in the monolayer evaluation. The early isolated cell population might consist partly of osteoblasts and resting bone lining cells. These cells could diminish the outcome of generalised population results. The later osteoblast isolation might represent a mix of osteoblasts and osteocytes, demonstrating on one hand high type I collagen production by osteoblasts and higher expression of Runx2 and osterix by more differentiated osteoblasts/early osteocytes.

In the pellet cultures, the relative expression of all other investigated differentiation markers – Runx2, osterix, osteocalcin and E11 – was significantly increased compared to monolayers during the complete 7-day culture period. Osteocalcin is a small calcium-binding protein found mainly in the ECM of bone, but a small amount enters the blood (Calvo *et al.* 1996; Hauschka *et al.* 1989). Even though its function is still not completely understood, it is expressed by fully differentiated osteoblasts (Aarden *et al.* 1996; Hauschka 1986). The role of Runx2 during osteoblast differentiation is of crucial importance. Runx2 was the first transcription factor, identified through its binding site (OSE2) within the osteocalcin promoter (Ducy 2000). Its expression is up-regulated during mesenchymal condensation leading to the formation of pre-osteoblasts (Ducy *et al.* 1997). Therefore, Runx2 has been described as an early osteoblast differentiation marker. Nakashima *et al.* (2002) demonstrated that osterix -/- mice do not show signs of intramembranous or endochondral bone formation with the absence of bone markers such as osteocalcin, osteopontin, or osteonectin (Nakashima *et al.* 2002). However, Runx2 was expressed in osterix-null mice, suggesting Osterix acts downstream of Runx2. The results of mRNA expression analysis clearly showed the difference in differentiation level of human primary osteoblasts cultured in 2D or 3D. While, osteoblasts in monolayer culture are within an earlier stage of osteoblast differentiation, characterised firstly by a slight increase in Runx2 expression, osteoblasts in pellet culture are undergoing a later stage in cell differentiation with the increase in osterix, osteocalcin including increased osteocalcin protein production, and E11/podoplanin expression. Taken together, the human osteoblast pellet culture model showed faster differentiation compared to monolayer culture, as seen by the presence of a central highly differentiated cell population of cuboidal shape, which possessed long cell processes. The large up-regulation in E11/podoplanin mRNA within pellets suggests that the differentiation state of mature osteoblasts or possibly early osteocytes was achieved within a short time frame (3-7 days). We would, therefore, suggest this culture model to be used for studies aiming to use highly differentiated primary osteoblasts / osteocyte-like cells.

Further investigations in this chapter concerned the effects of FCS and TGFβ3 on osteoblast-lineage cells within different culture models. TGFβ3 is one of the members of the TGFβ superfamily. It has been shown to be a potent regulator of bone formation (Cox 1995). Ten Dijke *et al.* (1990) demonstrated, that the stimulatory effect of TGFβ3 on type I collagen and DNA synthesis in foetal rat osteoblast-enriched cultures was more potent in comparison to TGFβ1 (ten Dijke *et al.* 1990). The effect on type I collagen synthesis by TGFβ might be due to an increase in type I collagen mRNA stability, mRNA expression, or enhanced type I collagen synthesis (Centrella *et al.* 1994).

Monolayer culture of human primary osteoblasts demonstrated significant effects of TGFβ3 on the cells; moreover, these were dependent on the presence of serum. Type I collagen expression by early isolated osteoblasts cultured with TGFβ3 and FCS was increased compared to FCS alone. This effect was opposite to that with late isolated osteoblasts cultured SF, where TGFβ3 decreased type I collagen expression. These different results firstly demonstrate the dependence of a classical known TGFβ effect, to increase type I collagen synthesis (Noda 1989), on the presence of serum, as well as the differentiation stage of the cell type used. Another classical effect of TGFβ is to decrease mineral deposition into the ECM (Kato *et al.* 1988). However, in monolayer cultures this was not significantly demonstrated. If trends could be considered, a decrease in mineral deposition was only detectable, if FCS was present in the culture medium, while the opposite might be true for SF culture. Furthermore, no significant effect of TGFβ3 on Runx 2 expression could be determined in monolayer cultures. This surprising result might indicate that both isolations used are heterogeneous mixtures of cell types and possible effects could have compensated each other or were not visible due to the presence of 'non-responding' cells within the population. Osterix on the other hand, was significantly influenced by TGFβ3 in monolayer cultures. Osterix expression by osteoblasts cultured in the presence of FCS was increased by TGFβ3 addition. However, this was not the case for cells cultured SF.

Following on from the monolayer investigations, we were interested to test the effects of TGFβ3 and FCS on osteoblasts cultured in multilayer. For this cell culture model no exponential increase in relative cell number could be determined. However, a significant increase in DNA content within 6 culture days was detected. We speculate, that osteoblasts on top of the multilayer still proliferated, while osteoblasts in the centre of the multilayer were inhibited in their proliferation. Within this culture system, the effect of TGFβ3 was overall more similar regardless of whether the cells were cultured with or without FCS. Mineral deposition was increased on day 4 by TGFβ3 addition; however, this was only a trend in SF medium. On the contrary, mineral deposition was decreased on day 6 by TGFβ3 addition; yet, this result was only significant in SF medium. This interesting pattern would suggest that cell in multilayer undergoes immense and rapid differentiation, which influences dramatically the known effects caused by TGFβ3. In this model, contrary to monolayer culture, no significant effect on type I collagen production could be determined. Yet, osterix expression, as well as E11 expression was significantly increased by TGFβ3 independently of FCS presence. Osteocalcin expression, on the other hand was reduced by TGFβ3, which is a known effect of this growth factor (Noda 1989). Over all, the effects caused by TGFβ3 in multilayers were similar if FCS or SF culture was chosen. However, significant differences in the total amount of mineral deposition, as well as expression of osteoblast marker genes between SF and FCS culture were determined.

Within the co-culture system the differential effects of TGFβ3 and FCS were investigated in the presence of osteoblast-osteocyte interconnectivity. A significant up-regulation of type I collagen mRNA expression in osteoblasts in the presence of TGFβ3 was determined. Contrary to monolayer culture, this was also seen using SF culture. Type I collagen protein synthesis in co-cultures was largely prevented by TGFβ3 at days 2 and 4 and only detectable at day 6. Thus TGFβ3 seems to increase type I collagen mRNA expression but must also activate post-transcriptional or post-translational processes to reduce pro-collagen synthesis or enhance pro-collagen degradation. Moreover, within the osteoblast-osteocyte co-culture TGFβ3 significantly decreased Runx2

expression, but only in the presence of serum. Even though the effects of TGFβ are controversial during *in vitro* culture of osteoblast-like cells, accountable reasons seem to be the cell type used and the applied culture conditions (Centrella *et al.* 1987; Pfeilschifter *et al.* 1988; Tashjian, Jr. *et al.* 1985). It was shown by several authors, that TGFβ induces initial pre-osteoblast proliferation and differentiation, while inhibiting terminal differentiation (Alliston *et al.* 2001; Bonewald & Dallas 1994; Centrella *et al.* 1994). Alliston *et al.* demonstrated the down-regulation of Runx2 mRNA by TGFβ in mice calvarial-derived osteoblasts in the presence of serum (Alliston *et al.* 2001). Within in the co-culture model, we detected a down-regulation of Runx2 mRNA by osteoblasts also exclusively in the presence of serum. This could not be detected in monolayer or multilayer culture. Contrary to multilayer culture, as well as previous studies reporting the down-regulation of osteocalcin by TGFβ addition (Alliston *et al.* 2001; Noda 1989), osteocalcin mRNA expression in co-cultured osteoblasts was not reduced by $TGF\beta_3$ addition. Moreover, a slight increase in osteocalcin mRNA expression was detected over time if osteoblasts were co-cultured in the presence of $TGF\beta_3$ and FCS. MG-63 osteosarcoma cell line was used as model for the human osteoblast cell type in the co-culture. This cell line has been shown previously to present a number of features of a younger osteoblast phenotype, synthesising type I collagen and a basal level of ALP (Clover & Gowen 1994; Franceschi & Young 1990; Kartsogiannis & Ng 2004). The MG-63 osteocalcin expression however, is almost non-existing under normal culture conditions (Clover & Gowen 1994; Pautke *et al.* 2004). Therefore, MG-63 osteoblasts might be an inappropriate model to detect a down-regulation of osteocalcin mRNA by $TGF\beta_3$.

This chapter highlights the importance of defining appropriate culture models and culture conditions to investigate cellular effects to the addition of single factors. We would recommend that the pellet culture model, as well as the multilayer culture model, can be used for short term investigations aiming to evaluate cellular effects on more active and mature osteoblasts (possibly early osteocytes). In addition, we believe, that single factor effects need to be investigated more frequently using SF medium, to reduce possible interactions

of the various factors in FCS with the additionally added factor of interest. To overcome negative viability effects caused by SF culture, the culture models suggested could be most appropriate, as high osteoblast differentiation levels can be gained in a rapid culture time, even using primary cells.

Chapter 6: *In Vitro* Investigations on Osteocyte Survival – the Effect of TGFβ$_3$ and FCS

Osteocyte biology has become of major interest to the field of bone research. It is commonly accepted that osteocytes, as the most abundant cells in bone tissue, are playing an important role in the maintenance of bone matrix (Aarden *et al.* 1994; Knothe Tate 2003; Noble & Reeve 2000). Yet, very little is known about the role of osteocytes in bone remodelling. Osteocytes are terminally differentiated osteoblasts, which due to their location within the ECM of bone posses a different morphology, intra-cellular partitioning and, therefore, have to fulfil different functions than osteoblasts (Aubin & Liu 1996). Proposed roles for osteocytes range from calcium homeostasis, micro-damage repair, to mechanically adaptive control (Dunstan *et al.* 1993). Osteocyte death and the disturbance of the osteocyte-canalicular network are suspected to be responsible for 'loss of function' within bone diseases. Increased osteocyte cell death *in vivo* has been shown during i.e. estrogen withdrawal (Tomkinson *et al.* 1997), the pathogenesis of osteoarthritis (Wong *et al.* 1987), and aging (Frost 1960; Wong *et al.* 1985). The incidence of age-related bone loss will increase in future generations as the average life expectance continuously increases. Dunstan and co-workers (1993) verified that only a very limited number of osteocytes survive in the elderly (Dunstan *et al.* 1993). With just about 58% of viable osteocytes in human femoral heads present in the age range of 70-89 years, the osteocyte-canalicular network is greatly impaired. Moreover, the group was also able to demonstrate that hip fracture patients showed an increased variation in the number of viable osteocytes compared to the non-fracture group. Considering the exceptional location of osteocytes in bone and their proposed role in sensing and transmitting the mechanical stimuli, the maintenance of osteocyte survival is of significance importance to prospective bone loss treatment strategies.

The results in Chapter 4 demonstrated that the positive survival effect on *ex vivo* maintained osteocytes by TGFβ$_3$ is dependent on the presence serum proteins. Yet, during the *ex vivo* investigation of the cellular effects on osteocytes we recognised the challenging aspects of this task. As osteocytes are embedded within the dense and highly mineralised ECM of bone, their isolation is not trivial (Kartsogiannis & Ng 2004) and processing of the bone explant is required. However, cutting of the tissue is a major concern especially for viability investigations where false negative results will be produced. Cell viability methods for *ex vivo* cultured bone explants have been investigated extensively in previous experiments in our lab. Davies *et al.* (2005 and 2006) demonstrated the problem using fluorescent dyes to detect viable and dead cells in the environment of highly calcified bone matrix. Bone matrix is known for its strong auto-fluorescence, making it extremely hard to distinguish between labelled cells and the ECM itself (Davies 2005; Davies *et al.* 2006). A modified version of the LDH viability assay (Stoddart *et al.* 2006) was successfully used in a previous study to investigate the effect of TGFβ$_3$ and serum on osteocyte survival *ex vivo* (Simpson *et al.* 2009). Lactate dehydrogenase (LDH, EC 1.1.1.27) is an ubiquitinous cytoplasmic enzyme present in almost all living cells in a variety of organisms. The major advantage of this assay is the long stability of the LDH enzyme after cell death, up to 36 hours, eliminating any false negative results due the processing of the bone explant prior to analysis. The original LDH assay was established for the use on bone sections in 1982 by Wong *et al.*

Taking into account that the study of cellular effects *ex vivo* is not trivial, *in vitro* investigations seem more feasible. The results in Chapter 5 have shown that osteocyte generation by the *in vitro* differentiation of osteoblasts is potentially an alternative to obtain these cells, which are otherwise hidden in the ECM. Yet, the distinction between osteoblasts and osteocytes still remains challenging (Nijweide *et al.* 2002). Many characteristics are shared between osteoblasts and osteocytes, which does not ease their separation *in vitro*. Therefore, osteocyte isolation is considered problematic. The availability of osteocyte-specific antibodies for osteocyte isolation (Nijweide *et al.* 2010; van der Plas A.

& Nijweide 1992), and low number of osteocyte cell lines (Bodine *et al.* 1996; Kato *et al.* 1997; Kato *et al.* 2001; Zhao *et al.* 2002) is still limiting *in vitro* osteocyte research.

In this chapter we aim to understand the possible role of TGFβ$_3$ in osteocyte survival. Primary isolated cells, as well as the cell line MLO-Y4 were used for the investigations in this study. Moreover, the possible roles of serum and TGFβ$_3$ were investigated in cultured osteocytes.

6.1. The Effect of FCS and TGFβ$_3$ on the Viability of Human Primary Osteocyte-Like Cells

As it is of crucial importance to understand how osteocyte viability can be influenced, the effect of TGFβ$_3$ on human osteocyte survival was investigated *in vitro*. Primary osteocyte-like cells (♀ 80 years, ♂ 49 years, ♂ 59 years) were isolated as described in 2.2.12.2 and cultured in monolayer according to 2.2.16.4. Therefore, enzymatically isolated cells were seeded onto type I collagen-coated glass-chamber slides. Cells were then cultured up to 7 days either in the presence or absence of serum. Moreover, the effect of TGFβ$_3$, in the presence of absence of FCS, on osteocyte survival was investigated using the TUNEL assay.

Representative micrographs of isolated human osteocyte-like cells (♀ 80 years) cultured in monolayer for 3 days are shown in figure 6.1. Morphologically, the cell population demonstrated the presence of cells with a rather star-shaped morphology [Fig. 6.1A; see red arrows]. Possibly, the occurrence of long cell processes was higher in SF cultured osteocyte-like cells than in cultures in the presence of FCS [Fig. 6.1B, D; see yellow arrow]. The addition of TGFβ$_3$ to FCS-containing medium resulted in the generation of areas of increased cell density [Fig. 6.1C]. The culture in SF medium seemed to morphologically change the cells into more flat and spread-out cells [Fig. 6.1B, D]. Both effects described for TGFβ$_3$ addition in the presence of serum, or caused by serum removal, were also prominent during monolayer culture of early and late isolated osteoblasts (see Chapter 5).

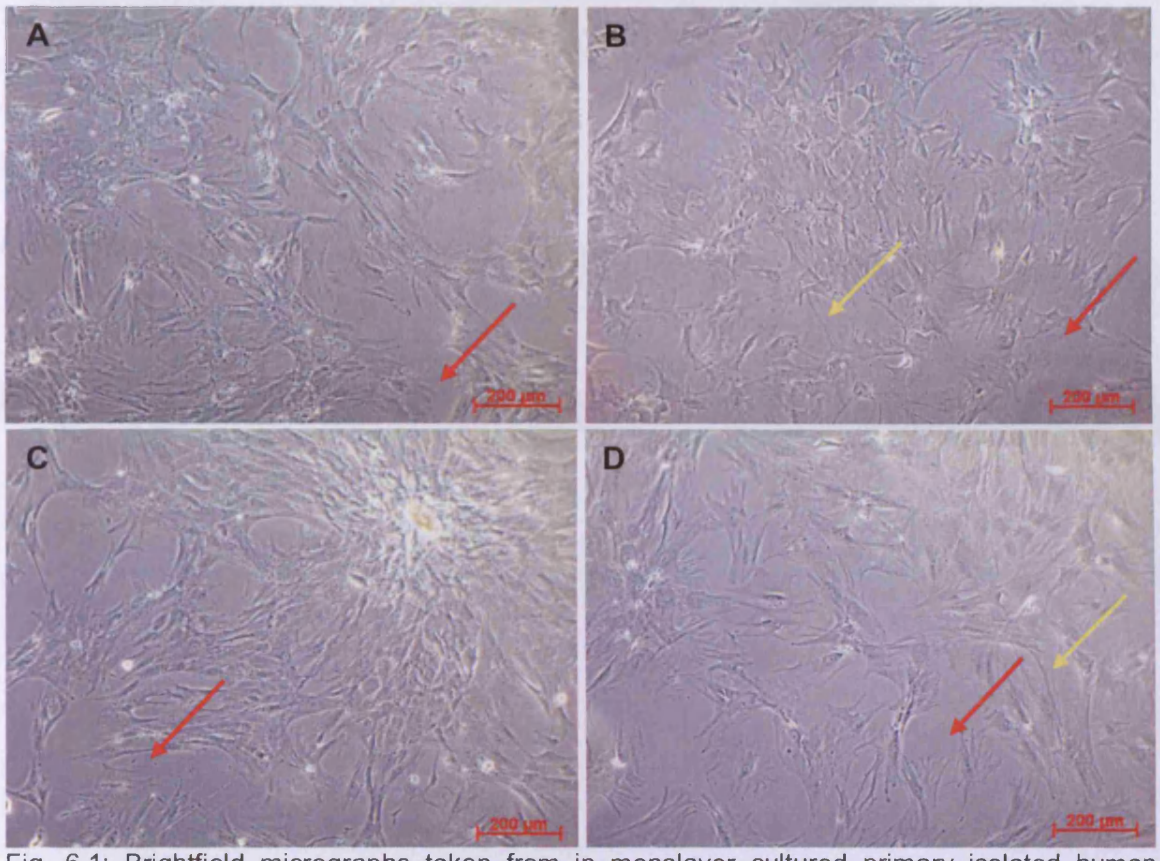

Fig. 6.1: Brightfield micrographs taken from in monolayer cultured primary isolated human osteocytes (♀ 80 years). A: DMEM+FCS; C: DMEM+FCS+5 ng/ml TGFβ$_3$; B: DMEM SF; D: DMEM SF+5 ng/ml TGFβ$_3$. The images show the presence of flat, star-shaped cells (red arrows), which are connected in some cases by long cell processes (yellow arrows).

The evaluation of osteocyte viability revealed that cell death was highest if cells were cultured SF in the presence of TGFβ$_3$ ($p \leq 0.006$) [Fig. 6.2]. On day 2, the culture in DMEM SF + TGFβ$_3$ led to a maximal cell death of 45.3% +/- 30.3%. The cell death occurring in the presence of TGFβ$_3$ was significantly higher than in SF medium without TGFβ$_3$ over all culture days ($p \leq 0.0001$), suggesting that the addition of TGFβ$_3$ to SF medium had a negative effect on osteocyte viability. The use of SF medium caused an average cell death of 25.5% +/- 23.2 over all days investigated, whereas serum-containing medium had much lower cell death ($p \leq 0.0001$). The use of TGFβ$_3$ within FCS-containing medium lead to a significant reduction of cell death from 5.9% +/- 6.2 to 3.8% +/- 4.8 over culture day 5 and 7 ($p=0.018$). Therefore, the osteocyte death was dependent upon TGFβ$_3$ addition, but this effect was also dependent upon serum presence.

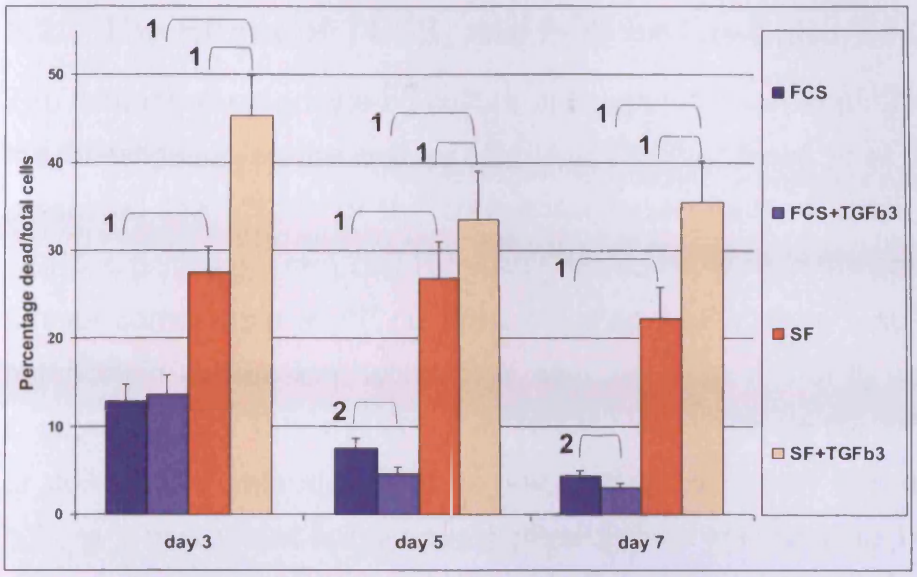

Fig. 6.2: Diagram illustrates the effect of medium supplementation on the viability of human primary osteocytes (♀ 80 years, ♂ 49 years, ♂ 59 years). Data was not normally distributed: Kruskal-Wallis, Mann-Whitney test and Bonferroni correction were used as statistical analysis. Statistical significance was defined as $p \leq 0.05$, 1: over all culture days, 2: day>5. Bar chart shows the average osteocyte death and standard error of the mean of osteocytes at different time points for the media supplementations: FCS or SF +/- TGFβ$_3$.

6.2. The Effect of TGFβ$_3$ and FCS on Co-Cultured Osteocytes

The osteoblast-osteocyte co-culture system by Mason *et al.* 2008 was used for the investigation on the cellular effects of TGFβ$_3$ (Mason *et al.* 2008). Chapter 5 presented the results of the co-cultured osteoblasts. It could be shown, that TGFβ$_3$ significantly reduced Runx2 mRNA expression in the presence of FCS to a level comparable to SF cultures. Moreover, SF culture resulted in increased osteocalcin expression, which was also the case if TGFβ$_3$ was present. This chapter will deal with the effect of TGFβ$_3$ and serum on the differentiation level of co-cultured osteocytes. Moreover, the influence on cell death during co-culture of osteocytes will be investigated and set in relation to TGFβ$_3$ and FCS.

Figure 3 shows representative images of co-cultured osteoblasts [Fig. 6.3A] and osteocytes [Fig. 6.3B-D]. Cells were cultured for 2 days [Fig. 6.3A, C, D] or 6 days [Fig. 6.2B] within the co-culture system. Immuno-labelling for β-actin (green) was performed; cell nuclei are labelled with DAPI (blue). While osteoblasts formed a cell layer on top of the type I collagen gel [Fig. 6.3A], osteocytes within the gel possessed a star-shaped morphology [Fig. 6.3B-D]. Moreover, osteocytes demonstrated the presence of long slender-like cell processes to connect to one another and the osteoblast cell layer.

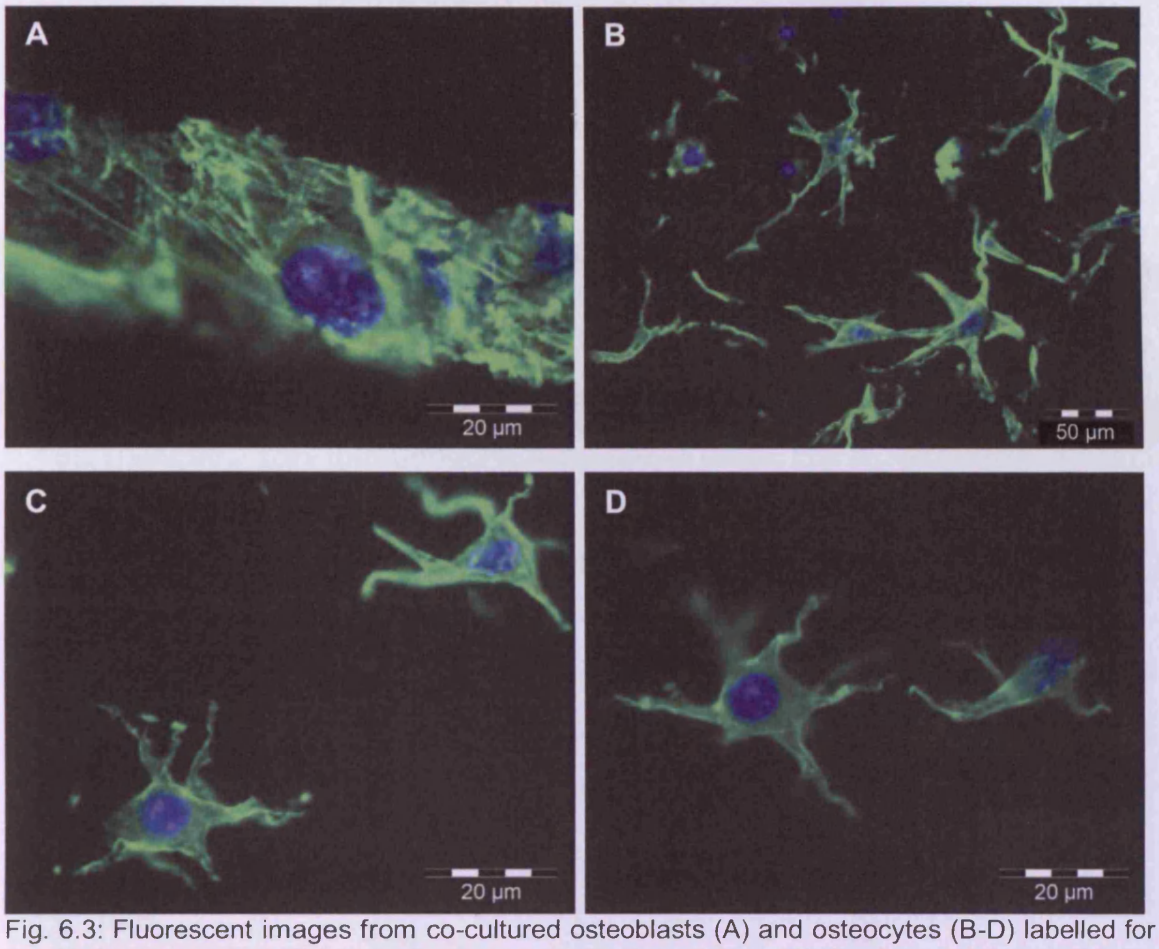

Fig. 6.3: Fluorescent images from co-cultured osteoblasts (A) and osteocytes (B-D) labelled for β-actin (green) and DAPI (blue) after 2 days (A, C, D) or 6 days (B) of culture. While osteoblasts (A) are cultured in a dense cell layer, osteocytes (B, C, D) are more loosely dispersed in the type I collagen gel. Osteocytes demonstrate a 3D-star-shaped morphology.

6.2.1. Co-Cultured Osteocytes

The effect of TGFβ$_3$ was investigated in co-cultured osteocytes. Messenger-RNA expression of the late osteoblast marker osteocalcin and the early osteocyte marker E11 were evaluated. No significant effect on osteocalcin expression of co-cultured osteocytes was found by the addition of the growth factor to FCS-containing or SF medium at individual time points investigated [Fig. 6.4A], as well as the combined data set for all time points [Fig. 6.4C]. The mRNA expression of E11 decreased significantly at day 4 and day 6 compared with day 2 in FCS-containing medium (day 4 p=0.001, day 6 p=0.000) [Fig. 6.4B], this was not apparent in osteocytes cultured in FCS medium with TGFβ$_3$ addition [Fig. 6.4B]. Moreover, the pooled time point data indicated that overall expression of E11 was higher in MLO-Y4 cultured in FCS + TGFβ$_3$ than all

other conditions, and that E11 expression was higher in the presence of FCS and TGFβ$_3$ than in all other conditions ($p \leq 0.02$) [Fig. 6.4D].

The effect of SF culture on the phenotype of co-cultured osteocytes showed significant differences in osteocalcin and E11 expression. Osteocalcin expression was maintained at a significantly higher level by osteocytes cultured SF compared to culture with FCS ($p=0.046$) if the data sets from different time points were combined [Fig. 6.4C]. E11 mRNA expression was significantly decreased in SF culture compared with FCS culture at day 2 ($p=0.0001$) [Fig. 6.4B]. This effect was independent of the presence of TGFβ$_3$. Moreover, looking at the combined data sets from all time points, the use of SF medium significantly decreased E11 expression ($p=0.002$) [Fig. 6.4D].

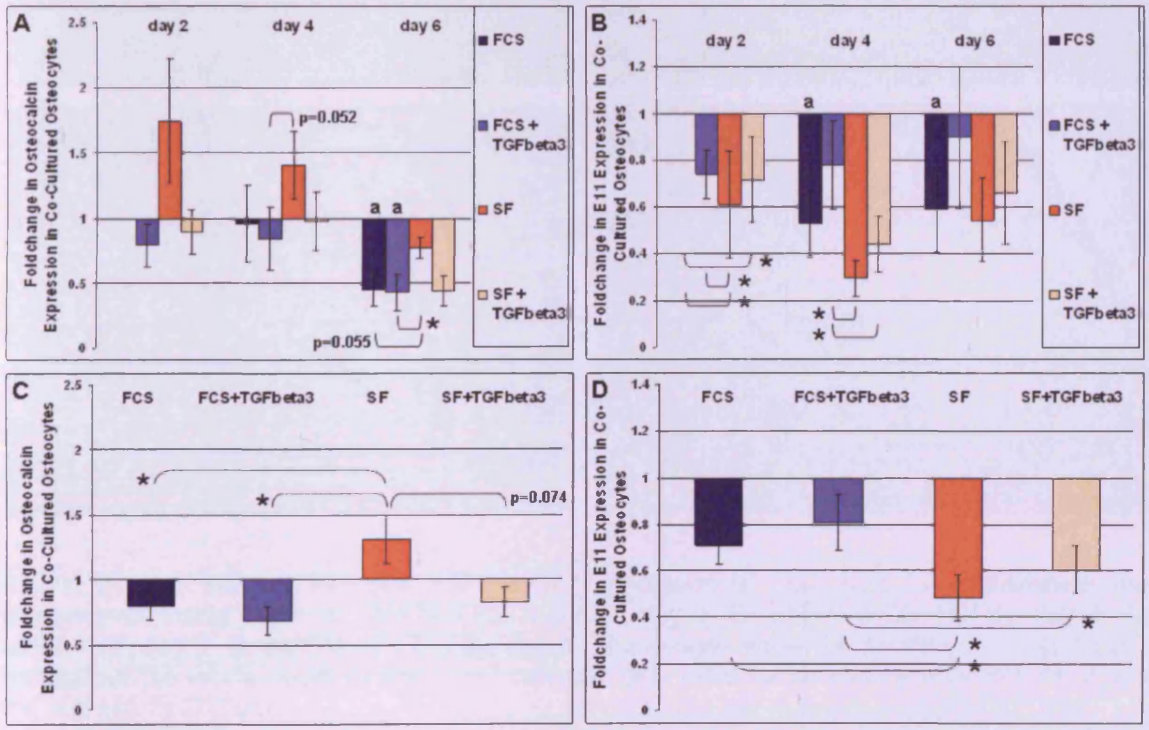

Fig. 6.4: Fold-change in relative gene expression for osteocalcin (A, C) and E11 (B, D) in co-cultured osteocytes. Gene expression levels were normalised to 18SrRNA, and made relative to αMem + FCS on day 2. ddCT data was used for statistical analysis: normally distributed, one-way ANOVA with Tukey. Statistical significance was determined as $p \leq 0.05$; *: significant on that day; 1: compared to same medium group on day 2. Bar charts show the average fold-change as well as the standard error of the mean. Osteocalcin expression was increased using SF medium ($p \leq 0.046$). E11 expression in osteocytes was positively regulated by the addition of FCS and TGFβ$_3$ to αMem ($p \leq 0.02$).

6.2.2. The Effect of TGFβ$_3$ and FCS on Osteocyte Cell Death in the Co-Culture Model

A qualitative LDH activity assay showed the presence of viable osteoblasts and osteocytes under all culture conditions. For a complete record, representative images are shown in figure 6.5. However the quality of the images is not good.

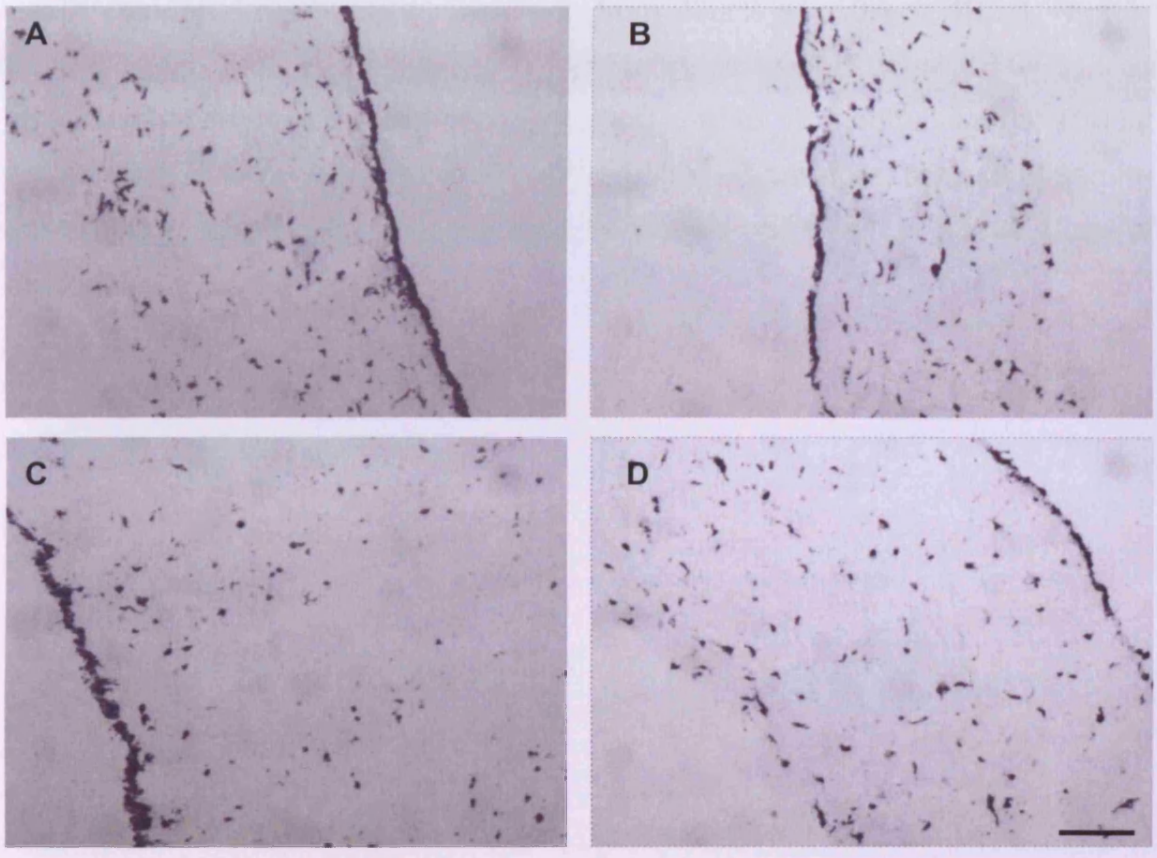

Fig. 6.5: LDH stained sections from osteoblast-osteocyte co-cultures demonstrating the presence of viable cells. A: αMEM+FCS medium; day 6. B: αMEM+FCS+TGFβ$_3$; day 4. C: αMEM SF; day 6. D: αMEM SF+TGFβ$_3$; day 4. The images show the presence of viable cells throughout the whole depth of the type I collagen gels used for co-culture experiments. Scale bar represents 200 µm.

Quantitative TUNEL assay was performed to evaluate the effect of TGFβ$_3$ and FCS-depletion on osteocyte cell death within an osteoblast-osteocyte co-culture system. No cell death could be detected in co-cultured osteocytes in the presence of FCS at day 2 [Fig. 6.6A]. All other treatments showed the presence of dead osteocytes already by day 2. Cell death on day 2 was significantly increased in SF medium independently of the addition of TGFβ$_3$, compared to

αMem + FCS ($p \leq 0.007$). At a later stage of the co-culture, osteocyte death was detectable under all culture conditions. The presence of serum and TGFβ$_3$ reduced the average osteocyte death compared to the culture in serum presence without the growth factor on day 4. This effect was near significance with a probability value of 0.052. If data sets from day 4 and day 6 were combined [Fig. 6B], FCS and TGFβ$_3$ presence slightly decreased osteocyte death compared to FCS addition alone from 23.2% +/- 10.1 to 12.3% +/- 8.3. FCS + TGFβ$_3$ ($p=0.096$). Co-culture of osteocytes using FCS and TGFβ$_3$ also significantly decreased osteocyte death compared to SF medium with or without the addition of the growth factor (day$\geq$4: $p \leq 0.006$). Therefore, the use of serum-containing medium with TGFβ$_3$ reduced osteocyte cell death at a later stage of co-culture.

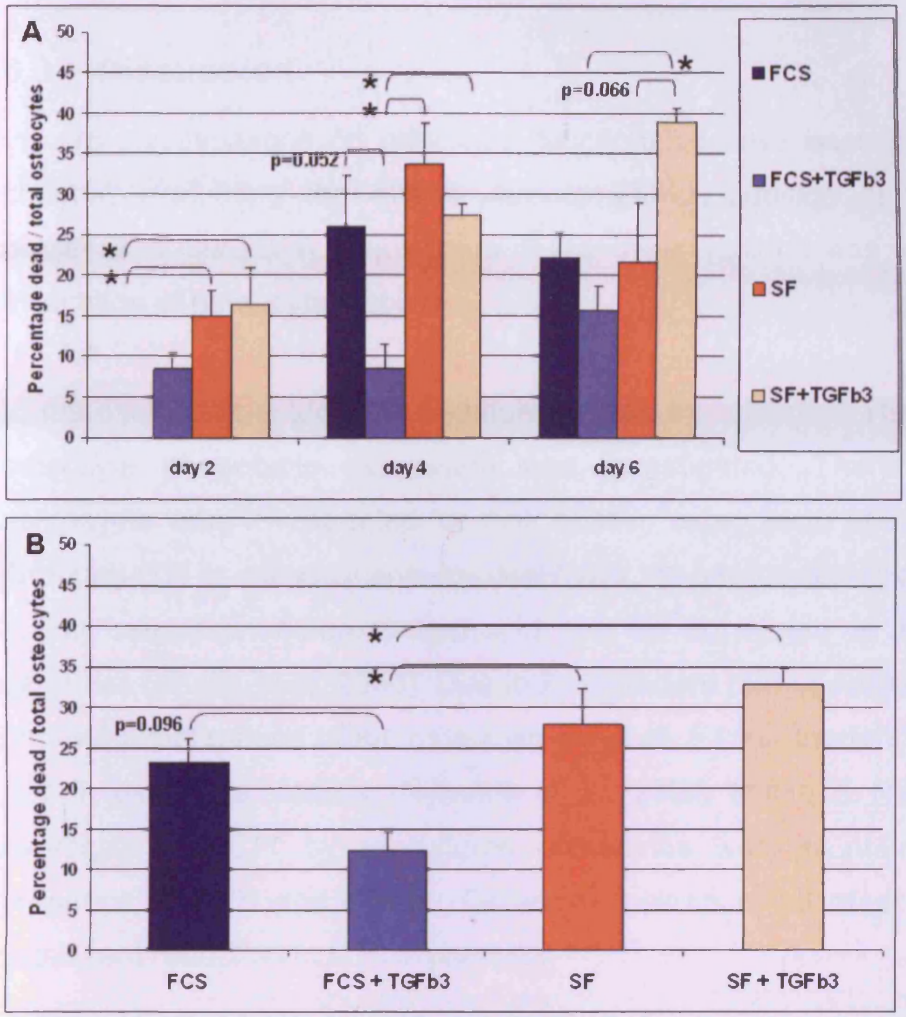

Fig. 6.6: Diagrams illustrate the effect of medium supplementation on the viability of co-cultured osteocytes. Data was normally distributed: one-way ANOVA and Tukey were used as statistical analysis. Statistical significance was defined as * $p \leq 0.05$. A: Bar chart shows the average osteocyte death and standard error of the mean within co-cultures at different time points for the media supplementations – FCS or SF +/- TGFβ$_3$. B: Bar chart shows the average osteocyte death and standard error of the mean within co-cultures at the later culture stage (combined data from day 4 and day 6) for different media supplementations. FCS and TGFβ$_3$ increased osteocyte survival.

6.3. Discussion

In vitro investigations on osteocyte functionality have been performed in this chapter, evaluating the effects due to TGFβ$_3$ addition in the absence or presence of serum. A major focus of the investigations was on placed on the evaluation of osteocyte viability.

In the osteoblast-osteocyte co-culture model, the effect of TGFβ$_3$ and FCS on osteocyte phenotypic expression was investigated. Therefore, co-cultured osteocytes were investigated for their relative osteocalcin and E11 expression. Although E11 is not exclusive to osteocytes, its expression is greatly increased during osteoblast differentiation and can be correlated to the formation of dendrites (Zhang *et al.* 2006). Due to its important role in osteocyte morphology and the maintenance of the osteocyte network, E11 is thought to play a crucial role in osteocyte viability (Nijweide *et al.* 2002; Noble & Reeve 2000). The expression of E11 by co-cultured osteocytes was maintained only in the presence of FCS and TGFβ$_3$. Osteocyte culture in all other media led to a significant reduction in E11 expression.

As for co-cultured osteoblasts (see Chapter 5), the highest level of osteocalcin expression in co-cultured osteocytes was found with SF culture. The use of FCS-containing medium led to a significantly lower osteocalcin mRNA level independently of the presence or absence of TGFβ$_3$. Generally, TGFβ is known to reduce the expression of this late osteoblast marker (Noda 1989). Taking into consideration, that macromolecular proteins, hormones, and growth factors such as TGFβ$_1$ are the major components of FCS (Barnes & Sato 1980), the effects of additionally added TGFβ$_3$ will likely depend dramatically on the amount of TGFβ already present within serum. TGFβ within FCS might alter the cellular response to the added TGFβ$_3$ (Barnes & Sato 1980; Meghji *et al.* 2001). We hypothesise that the effect of TGFβ within FCS was already sufficient to decrease osteocalcin mRNA from osteocytes to a basal level. Moreover, if the data set of day 6 was investigated excluding data from experiment 1, a significant decrease in osteocalcin mRNA was detected in by the addition of

TGFβ$_3$ to SF medium. This delayed and irregular response of osteocalcin expression to TGFβ$_3$ further demonstrates the dependence of the effect of TGFβ$_3$ on the presence of FCS even in this co-culture system.

It is known since Lian and Stein (Lian & Stein 1992) that the highest level of osteocalcin expression is seen at the stage of mineralisation *in vitro*. However, before and after this peak expression, the level of osteocalcin mRNA is lower. As a decrease in osteocalcin was detected in osteocytes during co-culture in TGFβ$_3$-containing medium, both a reduction as well as an increase in differentiation were conceivable. Taking into consideration, that only culture under TGFβ$_3$ and FCS presence maintained E11 expression in co-cultured osteocytes, the results on osteocyte phenotypic maintenance suggest that medium containing TGFβ$_3$ plus serum is the most appropriate culture medium to maintain the differentiation level of MLO-Y4 osteocytes in co-culture.

As presence of TGFβ$_3$ and serum proteins was found to maintain E11 gene expression of osteocytes during co-culture, the effect on osteocyte survival was further investigated in co-culture. During the late stage (day 4 - day 6) of osteoblast-osteocyte co-culture, the addition of TGFβ$_3$ to serum-containing medium slightly decreased *in vitro* cell death of osteocytes. This effect was not visible in serum depleted medium with TGFβ$_3$ addition. Moreover, the co-culture under serum-depletion demonstrated the highest level of osteocyte cell death in the co-culture model. Therefore, contrary to the *ex vivo* osteocyte survival, which greatly benefited from SF culture, the *in vitro* investigation within the co-culture system led to increased cell death working under serum depletion. However, the most likely candidate for increased *ex vivo* osteocyte survival working SF was the reduction of the non-physiological surface fibrous membrane due to culture without serum proteins. The reduction of the surface-fibrous membrane covering the bone explant during *ex vivo* culture would increase nutrient availability of the osteocytes and, therefore, increase their survival. Yet, this is of course not an issue for the *in vitro* culture models used.

Moreover, the monolayer culture of human primary osteocytes underlined the findings gained in the co-culture system as well as during *ex vivo* evaluations. TGFβ$_3$ significantly decreased osteocyte cell death in the presence of FCS. However, a significantly increased cell death was found during culture in SF medium with TGFβ$_3$ addition.

Karsdal and co-workers have studied extensively the effect of TGFβ$_1$ on osteoblast-like cell survival. The group was able to demonstrate that TGFβ$_1$ can rescue matrix metalloprotease-induced apoptosis of MC3T3-E1 cells (Karsdal *et al.* 2002). In 2001 the same group showed that TGFβ$_1$ was able to activate the p38 MAPK in osteoblasts. This MAP kinase pathway seems to be involved in anti-apoptotic effects of TGFβ$_1$ on osteoblasts (Karsdal *et al.* 2001). Moreover, MT1-MMP activation of latent TGFβ$_1$ is proposed to be responsible for the maintenance of osteoblast viability during trans-differentiation into osteocytes (Karsdal *et al.* 2004). The second member of the TGFβ family, TGFβ$_2$, has been also shown to possess a protective function in osteoblast apoptosis (Dufour *et al.* 2008). However, an influence of one of the TGFβ family members on osteocyte survival was only recently demonstrated in our group (Simpson *et al.* 2009).

Concerning the presence of serum proteins, to our knowledge, the studies by Karsdal, Dufour and Simpson were performed in the presence of serum proteins. Therefore, the osteocyte viability results in the presence of TGFβ$_3$ and serum go along with the previous reports that demonstrate that TGFβ seems to increase osteoblast-lineage cell survival. Yet, in our co-culture study we were able to show that if TGFβ$_3$ was added to a SF medium a positive effect on osteocyte survival was absent. This result which is contrary to a study from Jilka *et al.* where TGFβ$_1$ was able to rescue apoptotic MC3T3-E1 cells in a SF environment (Jilka *et al.* 1998). A variety of possible explanations for this contrary result can be given. In the study from Jilka, 0.5% BSA was used in the SF medium preparation, whereas in our study the SF medium contained only 0.01% BSA. However, we were not able to determine a great influence by serum depletion on the total amount of growth factor being available for the

cells. Moreover, different cell types were used in the evaluations, while we were using primary cells and an osteocyte cell line; the study from Jilka used an osteoblast cell line. Time frames of experiments were different; we investigated up the 14 days *ex vivo*, or up to 6 days *in vitro*, whereas the effects in the Jilka study were reported after 16-24 hours experimental time. Furthermore within the study by Jilka, the authors report about experiments using MG63 cells, where the survival effect of TGFβ was not consistent.

In conclusion from our study, all three culture systems - organ culture, monolayer, and co-culture - demonstrated the highest long-term osteocyte survival during culture under serum and TGFβ$_3$ presence. Yet, the effect of serum free culture, as well as TGFβ$_3$ addition to SF medium had inconsistent effects. While in the organ culture system, serum free culture had a positive effect on osteocyte survival compared to serum-containing culture, serum free culture in the co-culture model had a cell survival comparable to serum-containing culture, and serum depletion during monolayer culture of primary osteocytes decreased cell survival. Moreover, the effect of TGFβ$_3$ on osteocyte survival under SF conditions ranged from no effect in the *ex vivo* situation and co-culture system, to increased cell death in monolayer culture. We would speculate that the *in vitro* effect of TGFβ$_3$ in the absence of serum seem to depend partly on the dimensionality of the culture system used. In a 3D culture situation - *ex vivo* or co-culture - TGFβ$_3$ in a SF culture had no significant negative survival effect. In our opinion, this result demonstrates very clearly that 3D culture models for osteoblast-lineage cells are essential for relevant bone biology investigations. We believe that the 3D culture of osteocytes is more physiologically relevant than 2D culture. Therefore, we can speculate that TGFβ$_3$ is an *in vitro* osteocyte survival factor, at least in the presence of serum. However, further studies will have to clarify, which factor in foetal calf serum is needed for the positive cellular effect of TGFβ$_3$ on osteocytes, and most importantly, if this finding is relevant to an *in vivo* situation where TGFβ$_3$ protein in bone ECM is age dependently reduced.

Chapter 7: General Discussion

Within this study, the importance of relevant culture systems for osteoblast-lineage cells has been highlighted. This study had two main foci. Firstly, to evaluate different culture models for osteoblast-lineage cells. This evaluation ranged from *ex vivo* bone organ culture to *in vitro* culture methods. During the evaluation of the organ culture system, the importance of $TGF\beta_3$ as an osteocyte viability factor was highlighted. The second focus of this study, therefore, dealt with the use of the established *in vitro* cell culture systems for the investigation of the cellular effects by $TGF\beta_3$ on osteoblast-lineage cells.

Investigations under Micro-Gravity

One section of this study was dedicated to an exceptional opportunity - to perform the biological analysis of a micro-gravity experiment using an *ex vivo* organ culture system (see Chapter 3). The micro-gravity experiment was performed and planned by the Catholic University Leuven in collaboration with the ESA to investigate the effectiveness of high frequency, low amplitude mechanical loading on bone turnover of cancellous bone in weightlessness. The biological analysis of the micro-gravity experiment demonstrated the impact of short-term, near orbit space flights on the activity of bone cells. Type I collagen production was found to significantly increase only during culture at normal gravity. Moreover, the pH of the medium was significantly lower in cultures at micro-gravity compared to normal gravity cultures, this effect was, most likely, caused by the slightly higher bone resorption detected at micro-gravity, which was identified by the measurement of type I collagen breakdown products released into the culture medium.

It has been shown by several studies that micro-gravity missions leads to an uncoupling of bone remodelling processes and that astronauts reveal increased serum NTx and decreased levels of ProCI (LeBlanc *et al.* 2007). The novelty of our results is reflected by the use of an *ex vivo* bone explant culture system for

the study of bone biology at micro-gravity, which allows to test parameters acting on bone cells in a controlled *in vitro* environment without the influence of systemic factors. Our results partly confirm the *in vivo* findings, demonstrating as expected reduced bone formation, which was reflected by the absence of a significant increase in ProCI medium levels seen at normal gravity. Yet, the NTx release by culture in micro-gravity was not significantly higher compared to culture at normal gravity. Smith (2005) demonstrated increased NTx serum levels of astronauts and cosmonauts after 14 days at micro-gravity (Smith *et al.* 2005). This data demonstrates the rapid response of osteoclasts if exposed to micro-gravity.

It is open for speculation whether the choice of using a non-load-bearing bone was appropriate, as bone loss at micro-gravity is found predominantly at sites of load-bearing bones (Trappe *et al.* 2009). Also questionable is, if the source of osteoclast precursor cells in cultured bone explants is adequate to be able to detect significant influences on bone resorption, but ,moreover, if is a systemic stimulus like parathormone (Poole & Reeve 2005) is missing in the *ex vivo* situation that would potentially activate osteoclasts *in vivo* at micro-gravity.

A study from Rucci (2006) demonstrated increased levels of RANKL by mouse primary osteoblasts cultured at micro-gravity that had the potential to activate osteoclastogenesis *in vitro* (Rucci *et al.* 2007). Also the recent study by Tamma (2009) that was conducted on the same Foton M3 mission as the FreqBone experiment, demonstrated increased osteoclast maturation and activation at micro-gravity (Tamma *et al.* 2009). Therefore, it seems likely that the absence of osteoclast precursors that could be potentially activated in the *ex vivo* bone explant culture situation could explain the missing increase in bone resoption at micro-gravity in the FreqBone experiment.

The hypothesis that a dynamic, high frequency, low amplitude load applied daily on the cultured bone explants would result in higher bone formation, could not be proven at normal or micro-gravity. However, a trend towards an increase in bone volume in relation to total volume was detected at micro-gravity due to the application of this particular mechanical load. Therefore, further studies would be needed to clarify how big the impact of the high frequency, low amplitude mechanical loading on the prevention of bone loss during space missions really is, and if additional factors such as cosmic radiation or high gravitational forces during launch are responsible for diminished cell survival or responsiveness during space missions.

A clinical trial by Rubin (2004) demonstrated the effectiveness of high frequency, low amplitude loading on the prevention of bone loss in elderly women (Rubin *et al.* 2004). The potential of this very particular loading regime to be used as a non-invasive treatment for osteoporosis, however, was strongly dependent upon patient's compliance.

Comparing the successful clinical trial by Rubin (2004) with the FreqBone experiment, one main point needs to be considered. In the FreqBone study bone from a non-load-bearing bone, the bovine sternum, was exposed to low frequency, high amplitude loading, while Rubin's study (2004) resulted in effects only in load-bearing bones such as the lumbar spine and the distal femur (Rubin *et al.* 2004). The clinical trial was not able to detect any effect of the loading regime on the non-load-bearing bone radius. This observation in the non-load-bearing situation corroborates with the results of the FreqBone study. Therefore, it is strongly recommended to conduct a study similar to the FreqBone one with explants from a load-bearing bone. This could be performed possibly at a situation of simulated weightlessness.

Role of Culture Models and Serum

The evaluation of different culture models for osteoblast-lineage cells included an *ex vivo* bone explant culture system (see Chapter 4), similar to the one used for the micro-gravity experiment. Cancellous bone explants from adult human donors were cultured in this system for 14 days. Culture medium was perfused around the explants to increase nutrient availability during long-term culture. Moreover, a dynamic, compressive loading was applied daily to aid the maintenance of the cancellous bone explants. The reduced cell survival in the centre of bone explants, which had been cultured long-term, was determined as most critical issue. Attempts were undertaken to decrease cell death within this culture system. The use of an improved culture chamber with applied channels on the surface of baseplate and piston were evaluated. Even though the new chamber design increased medium distribution on the surface of cultured explants, as well as medium perfusion into the explants, this improvement had no influence on central osteocyte survival. Yet, further studies might investigate in more detail, if this lack of effect on cell survival is partly due to the use of a serum-containing culture medium, which led to increased occurrence of the formation of a surface fibrous membrane.

A comparison of SF and serum-containing culture medium during human cancellous bone explant culture demonstrated increased central osteocyte survival using SF culture. The most likely candidate for increased *ex vivo* cell survival, was the reduction of the non-physiological surface fibrous membrane due to SF culture. Clearly, working without foetal calf serum enables the culture of cells within a defined culture milieu and is, therefore, a benefit for all culture systems.

The availability of nutrients and oxygen has been considered as the main problem for mature bone organ culture and possibly prevents its more frequent use during *in vitro* investigations (Trowell 1961). As suggested by Trowell (1959), the use of a SF medium reduced the occurrence of a non-physiological surface fibrous membrane in our study. Moreover, we were able to show that this significant reduction in fibrous membrane in SF culture led to increased cell

survival during long-term bone explant culture. Yet, further studies will have to improve the cell survival *ex vivo*, as the induction of osteocyte death of up to 50% during 14 days culture will alter the outcome of investigations dramatically.

The absence of the *ex vivo* culture system in the second part of this study abolished any further bone explant culture improvements. Studies investigating the possible use of artificial blood substitutes during culture to increase oxygen availability, as well as inserting defined rest-periods within the loading cycle to create relaxation-contraction intervals, which could increase medium penetration in the explant centers, were intended. Yet, clearly the SF culture medium was a major benefit to the *ex vivo* culture of human cancellous bone explants.

Within a monolayer culture model for primary isolated human osteocytes (see Chapter 6), the comparison of serum-containing versus SF medium demonstrated the complete opposite than gained from the explant culture work. Human primary osteocyte isolation was attempted from cancellous bone explants using type II collagenase enzymatic digestion. Cells were then cultured in monolayer on type I collagen-coated glass slides. The use of SF medium in the monolayer culture system resulted in significantly increased cell death, approximately three- to four-times higher than cell death seen using a serum-containing medium.

Osteocyte survival was also decreased using SF compared to serum-containing medium in an osteoblast-osteocyte co-culture model (see Chapter 6). The co-cultured MLO-Y4 osteocytes, which were cultured entrapped in a type I collagen gel, demonstrated the overall highest cell death in SF medium. Yet, the increase in cell death caused by culture in SF medium compared to serum-containing culture was only approximately one- to two-times higher. The difference in cell death due to SF culture was more pronounced in the human primary osteocyte monolayer culture model than in the co-culture using the MLO-Y4 cells.

When only comparing the viability of serum-containing versus SF culture, we would suggest, that SF culture can be a favourable medium for the culture of 3D bone explants constructs, avoiding the non-physiological overgrowth with fibroblast-like cells that diminishes central cell survival. Yet, SF medium for *in vitro* culture systems still needs improvement in its composition to allow human primary osteoblast-lineage culture.

The SF culture approach should be considered most desired in the field of cell culture investigations as only the absence of serum allows for investigations in a truly defined culture environment. The commonly used FCS, however, contains an array of growth factors and hormones at varying and undefined concentrations (Barnes & Sato 1980). The problematic issue using a serum containing culture medium for *in vitro* investigations is that the effects of any additionally added growth factor might be dramatically altered by the variety of factors already present in serum containing medium (Barnes & Sato 1980; Meghji *et al.* 2001).

The optimal composition of a defined SF medium for a particular cell type is not trivial. Attempts to identify 'survival factors' within serum were undertaken in the past (Barnes & Sato 1980). Due to the small quantities of growth factors or homones present, it was/is difficult to extract enough for structural analyses.

Jilka (1998) demonstrated the influence of serum depletion on the occurrence of cell death in monolayer culture using the osteoblast-like cells MC3T3-E1 (Jilka *et al.* 1998). Within 24 hours, a significantly increased cell death in the absence of serum was detected.

Further investigations in the comparison of serum-containing versus SF medium were performed concerning the state of cell differentiation. Considering that SF culture misses a great variety of factors, such as fibroblast growth factor, or epidermal growth factor that would induce cell proliferation in cells cultured in serum containing medium. Therefore, SF medium does not induce the same high proliferation response than serum containing medium (Freshney 2000b).

As Owen's work (1990) demonstrated, the inhibition of osteoblast proliferation can lead to increased osteoblast differentiation (Owen *et al.* 1990). Therefore, we were expecting that SF culture increases osteoblast differentiation.

While the use of SF culture for human primary osteoblasts cultured in monolayer (see Chapter 5) decreased cellular proliferation and increased cell activity compared to serum-dependent culture, only the early osteoblast marker gene type I collagen was increased by SF culture compared to serum-containing culture. There was no difference in Runx2 and osterix expression found between SF and serum-containing culture of human primary osteoblasts in monolayer. Mineral deposition detected via Alizarin staining relative to DNA was increased in SF cultured compared to FCS-dependent culture.

Using a multilayer culture model for primary osteoblasts (see Chapter 5), the use of SF medium again decreased cell proliferation relative to the use of serum-dependent culture, even though the difference was not as pronounced in multilayer as in monolayer culture. Yet, there was a similar response in proliferation in monolayer and multilayer culture with or without FCS which suggests that contact inhibition, which enhances osteoblast differentiation during multilayer culture does not diminish the FCS-dependent osteoblast proliferation boost. The change of osteoblast culture model from monolayer to multilayer led to significant changes in gene expression. While SF culture in monolayer significantly increased type I collagen, this early osteoblast marker gene was not affected by SF culture in multilayer. We would speculate that due to contact inhibition, as seen during multilayer culture, the first transition point from initial proliferation to matrix maturation had lapsed and, therefore, the peak in type I collagen production was passed and could not be influenced by SF culture. However, later osteoblast marker genes, such as Runx2, osteocalcin and E11 were significantly up-regulated by SF culture of osteoblast multilayers. The Runx2 increase is contrary to monolayer culture results and might suggest, in combination with increased osteocalcin and E11 expression during SF culture of osteoblasts in multilayer, that cells were induced to a more mature

differentiation stage by SF culture in multilayer compared to SF culture in monolayer.

No significant difference in mineral deposition relative to total amount of DNA between SF and FCS-dependent culture of osteoblast multilayers was detected. Yet, the amount of calcium deposited in multilayers on day 2 was already comparable to the amount found in monolayers cultured with FCS on day 14. This suggests that in this case the culture system itself had a greater effect on calcium deposition than the medium used.

The use of SF medium during co-culture of MG-63 osteoblasts and MLO-Y4 osteocytes showed significant changes in marker gene expression. Co-cultured osteoblasts (see Chapter 5) in SF medium demonstrated decreased Runx2 expression, but increased osteocalcin expression. No significant change in type I collagen mRNA expression by SF culture could be detected. Again, this suggests that co-cultured osteoblasts cultured SF were maintained in a more mature osteoblast differentiation stage than their serum-dependent cultured companions. Co-cultured osteocytes (see Chapter 6) in SF medium also demonstrated an increased osteocalcin mRNA expression compared to culture in serum-containing medium. Yet, E11 mRNA expression was significantly lower in SF co-cultured osteocytes. Maybe, for each culture model – monolayer, multilayer and co-culture the use of SF medium instead of serum-containing medium leads to a very specific osteoblast differentiation stage. In general, SF medium led to more mature osteoblasts in all culture systems; yet, the system itself seemed to define the start point for this evolution.

Role of TGFβ$_3$ on Osteoblast-Lineage Cells

The second aim of this study was to evaluate the cellular effect of TGFβ$_3$ on cultured osteoblast-lineage cells. In the first instance, the positive osteocyte survival effect found during SF culture of human cancellous bone explants was proposed to be associated with the addition of TGFβ$_3$ to the SF medium (see Chapter 4). During serum-dependent culture of human cancellous bone

explants, *ex vivo* osteocyte survival was increased due to TGFβ$_3$ addition. However, this positive survival effect by the growth factor could not be proven under SF conditions.

Previous studies demonstrated a positive survival effect by TGFβ family members on cultured osteoblast-lineage cells (Dufour *et al.* 2008; Jilka *et al.* 1998; Karsdal *et al.* 2001; Karsdal *et al.* 2002; Karsdal *et al.* 2004; Simpson *et al.* 2009), yet, to our knowledge, all of the conducted studies in the presence of serum. The only study that investigated a possible survival effect by a TGFβ family member SF was done by Jilka (1998). The study demonstrated that TGFβ$_1$ without the presence of serum was able to rescue apoptotic MC3T3-E1 cells. Yet, in the results section of the Jilka (1998) manuscript the authors stated 'However, although TGFβ exerted antiapoptotic effects in some experiments, this effect was not reproducible (data not shown).' leading to the suspicion about the relevancy of the illustrated data.

The TGFβ$_3$ work was continued *in vitro* using different culture systems. As during the *ex vivo* work, the main finding was that the effect of TGFβ$_3$ on osteocyte survival is dependent upon the presence of serum. In detail, the death of human primary osteocytes cultured in monolayer (see Chapter 6) was significantly reduced by the presence of TGFβ$_3$ and serum compared to serum alone at a later stage of culture (day 5 till 7). Comparable results were gained for the MLO-Y4 osteocytes in the co-culture system (see Chapter 6). Therefore, as all three culture systems - organ culture, monolayer, and co-culture - demonstrated the highest long-term osteocyte survival during culture under serum and TGFβ$_3$ presence. Yet, the effect of TGFβ$_3$ addition to SF medium had different effects. The effect of TGFβ$_3$ on osteocyte survival under SF conditions ranged from no effect in the *ex vivo* situation and in the co-culture system, to increased cell death in the osteocyte monolayer culture. We would speculate that the effect of TGFβ$_3$ in the absence of serum depends on the dimensionality of the culture system. In a 3D culture situation - *ex vivo* or co-culture - TGFβ$_3$ in a SF culture had no effect. In our opinion, this result demonstrates very clearly that 3D culture models for osteoblast-lineage cells

are essential for relevant bone biology investigations. We believe that the 3D culture of osteocytes is more physiologically relevant than 2D culture. Therefore, we can speculate that TGFβ$_3$ is an *in vitro* osteocyte survival factor in the presence of serum. However, further studies will have to clarify, which factor/s in foetal calf serum is/are needed for the positive cellular effect of TGFβ$_3$ on osteocytes, and most importantly, if this finding is relevant to an *in vivo* situation where TGFβ$_3$ mRNA declines with age in bone, and TGFβ$_3$ protein declines in serum.

Furthermore the cellular effects of TGFβ$_3$ on the differentiation stage of osteoblast-lineage cells were investigated. The main effect of TGFβ family members is to induce osteoblast differentiation and, therefore, secretion of extracellular matrix (Bonewald 2002). However, the TGFβ peptides seem to act within a very specific osteoblast differentiation window. Even though type I collagen expression is stimulated by TGFβ, the expression of the later osteoblast differentiation marker osteocalcin is inhibited (Noda 1989).

It was of great interest for us to relate the effects caused by TGFβ$_3$ addition back to the differentiation stage induced due to culture of osteoblast-like cells in the different *in vitro* culture models. Moreover, the novelty of this study is the comparison of the effects by TGFβ$_3$ in the presence of serum to the effects caused by TGFβ$_3$ in SF medium. We were expecting to detect differences of the cellular responses to TGFβ$_3$ under serum presence or absence, as SF was suggested to induce osteoblast differentiation, while serum presence was proposed to induce more strongly initial proliferation prior to allow for cell differentiation.

During monolayer culture of human primary osteoblasts (see Chapter 5), the addition of TGFβ$_3$ increased cell proliferation in SF culture compared to SF culture alone. The same proliferation response to TGFβ$_3$ addition in SF culture was found in the multilayer culture system. Yet, in the multilayer culture system the addition of the growth factor to serum-containing medium significantly

reduced cell proliferation. This effect was not present in the monolayer culture system.

$TGF\beta_3$ addition led to significant differences in mRNA expression of osteoblast marker genes within the different culture systems. Type I collagen expression during osteoblast monolayer culture was increased by $TGF\beta_3$ addition to serum-containing medium, and decreased by $TGF\beta_3$ addition to SF medium. Yet, no effect by $TGF\beta_3$ addition on type I collagen expression in the multilayer culture system could be detected. Possibly, the same reason that was suggested to explain why SF culture in multilayer did not affect type I collagen expression whereas it did in monolayer culture, is the reason for the difference in type I collagen expression by $TGF\beta_3$ addition within different culture models. Within the multilayer culture system of human primary osteoblasts the first transition point of differentiation could have passed and, therefore, type I collagen expression might not be inducible by $TGF\beta_3$ in multilayer. However, $TGF\beta_3$ addition to the co-culture system led to increased type I collagen mRNA expression by MG-63 osteoblasts in comparison to co-culture without the growth factor. It is known, that MG-63 are considered as 'younger', earlier osteoblast phenotype. Also, co-cultured MG-63 are still cultured in monolayer on top of the 3D type I collagen gel. The differentiation stage of co-cultured MG-63 might still be earlier in comparison to a primary osteoblast cultured within a multilayer system. Therefore, the MG-63 was reacting similar to monolayer cultured primary osteoblasts and demonstrated increased type I collagen mRNA expression due to $TGF\beta_3$ addition.

In both monolayer and multilayer culture of human primary osteoblasts, osterix mRNA expression was significantly increased by $TGF\beta_3$ addition. Yet, whereas in multilayer this response was as early as day 2, the up-regulation of osterix mRNA in monolayer cultured osteoblasts by $TGF\beta_3$ addition was seen from day 8 onwards. In our opinion, this result could be explained by the fact that human primary osteoblasts differentiate during monolayer culture and, therefore, need more time in culture to represent a more mature osteoblast phenotype than at the start of monolayer culture. These differentiated osteoblasts in monolayer

then respond more similar to growth factor addition to osteoblasts during early multilayer culture. Also, this result confirms that multilayer culture, most likely due to contact inhibition, increased the state of osteoblast differentiation when compared to cells cultured in 50%-confluence monolayer.

For studies aiming to create a mature osteoblast differentiation stage more rapidly, an osteoblast pellet culture model for human primary osteoblasts was developed. This model system demonstrated the presence of cuboidal central cells connected via long cell processes. In comparison to monolayer culture of cells of the same origin, this system showed elevated levels of Runx2, osterix, osteocalcin and E11 mRNA, while reducing type I collagen mRNA expression and demonstrated a later differentiation stage of cultured osteoblasts. Also, the relative expression of the named late osteoblast marker genes was higher in pellet cultures than in multilayer cultures, suggesting that the culture model adds an additional positive differentiation stimulus than 3D multilayer culture. We would recommend the pellet culture model to be used for human primary osteoblasts for short-term growth factor or hormone response *in vitro* experiments.

Summary

From the presented results, it became obvious that striking differences between *in vitro* osteoblast culture systems are present. Monolayer culture might be appealing to be used in the context of cellular responsiveness to culture surface characteristics, and adaptations can be done i.e. using a later isolated cell type, which demonstrates a slightly later differentiation stage. Multilayer and pellet culture seem more appealing to be used during short-term tests of cellular growth factor or hormone responses. An *in vitro* co-culture system of osteoblasts and osteocytes might be an excellent alternative for equipment-rich *ex vivo* organ cultures, due to the presence of inter-cell-connectivity in combination with 3-dimensionality. It is proposed that the use of a 3D culture models should be encouraged for *in vitro* osteoblast investigations, due to the creation of possibly more relevant results. It's recommended that future investigations of osteoblast biology consider using 3D culture models and compare results of different models for their relevancy prior to designing the follow-up animal studies.

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Appendix I

Publications and Presentations from this Study

Publications

- A.E. Simpson, M.J. Stoddart, C.M. Davies, **K. Jähn**, P.I. Furlong, J.A. Gasser, D.B. Jones, B.S. Noble, R.G. Richards: TGFβ_3 and Loading Increases Osteocyte Survival in Human Cancellous Bone Cultured *Ex Vivo*. *Cell Biochem Funct* 27: 23–29 (2009).
- R.G. Richards, A.E. Simpson, **K. Jaehn**, P.I. Furlong and M.J. Stoddart: Establishing a 3D *Ex vivo* Culture System for Investigations of Bone Metabolism and Biomaterial Interactions. *ALTEX* 24:56-9 (2007).

- **K. Jähn**, V. Braunstein, P.I. Furlong, A.E. Simpson, R.G. Richards, M.J. Stoddart: A Rapid Method for the Generation of Uniform Acellular Bone Explants - A Technical Note. *Journal of Orthopaedic Surgery and Research* 5:32 (2010).
- **K. Jähn**, R.G. Richards, C.W. Archer, M.J. Stoddart: Pellet Culture Model for Human Primary Osteoblasts: In submission to *European Cells and Materials.*
- **K. Jähn**, D.J. Mason, J.R. Ralphs, B. Evans, C.W. Archers, R.G. Richards, M.J. Stoddart: The Effects of TGFβ_3 on Co-Cultured Osteoblasts and Osteocytes are Serum-Dependent. In preparation.

Book Chapter

- **K. Jähn**, M.J. Stoddart: Viability Assessment of Osteocytes using Histological Lactate Dehydrogenase Activity Staining on Human Cancellous Bone Sections. In submission to *Humana Press* as chapter in the book 'Methods in Molecular Biology – Mammalian Cell Viability Methods'.

Conference Presentations

- **K. Jähn**, J.R. Ralphs, B. Evans, R.G. Richards, C.W. Archer, M.J. Stoddart, D.J. Mason: The effects of TGFβ$_3$ and serum free culture on osteoblasts and osteocytes within a co-culture system (poster). ESB 2009
- **K. Jähn,** C.W. Archer, R.G. Richards, M.J. Stoddart: A 3D culture model for primary adult human osteoblasts (poster). IBMS Sydney 2009
- **K. Jähn,** M.J. Stoddart, P.I. Furlong, D.B. Jones, C.W. Archer, R.G. Richards: A mechanical loading 3D culture system for *ex vivo* human cancellous bone – potential for biomaterials investigations (poster). WBC 2008
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: *Ex vivo* cultured human cancellous bone explants and the effects of TGFβ$_3$ (oral presentation). SBMS 2008
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: Effects of TGFβ$_3$ on osteocytes of ex *vivo* human cancellous bone explants (poster). IBMS Davos Workshop 2008
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: Osteocyte viability and influence of culture conditions on *ex vivo* human cancellous bone (oral presentation). ESB 2007
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: Viability and procollagen-I expression of cultured human cancellous bone explants using a serum free medium containing TGFβ$_3$ (oral presentation). ECM 2007
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: Effect of a serum free medium containing TGFβ$_3$ on osteocyte viability of cultured human cancellous bone explants (poster). SSB 2007*
- **K. Jähn**, M.J. Stoddart, P.I. Furlong, C.W. Archer, R.G. Richards: Effect of serum free medium on osteocyte viability in *ex vivo* cultured human cancellous bone explants (oral presentation). SBMS 2007**

Awards: Best poster at SSB 2007*, best fundamental research presentation at SBMS 2007**, SSB Travel Award 2008, and SBMS Travel Award 2007.