UNIVERSITY OF SOUTHAMPTON

OSTEOBLAST-STIMULATING FACTOR-1 (OSF-1): A PEPTIDE WITH DIVERSE ROLES IN BONE DEVELOPMENT

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ABSTRACT

FACULTY OF HEALTH, MEDICINE & BIOLOGICAL SCIENCES

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Doctor of Philosophy

OSTEOBLAST-STIMULATING FACTOR-1 (OSF-1): A PEPTIDE WITH DIVERSE ROLES IN BONE DEVELOPMENT

by Rahul Shrikant Tare

In search for anabolic agents that enhance osteoblast activity, the role of a 136-amino acid cytokine, referred to as osteoblast-stimulating factor-1 (OSF-1), was examined in transgenic mice over-expressing the human *osf-1* gene, and its effects analysed *in vitro*. In keeping with earlier reports on the function of OSF-1 in stimulating new bone formation, the protein was found localised at sites of new periosteal and endochondral ossification in bones of control and transgenic mice. OSF-1 was synthesized by osteoblasts during early stages of osteogenic differentiation and secreted into the bone matrix. Although over-expression did not result in larger animals, calcium content/ unit dry bone weight in transgenic mice was 10% higher than in control mice.

A distinct effect of *osf-1* over-expression was the synthesis of the OSF-1 protein by growth plate and articular chondrocytes of only transgenic mice. Articular chondrocytes of transgenic mice were also found to synthesize Type I collagen, a bone-type protein. To confirm that over-expression of *osf-1* had provided the stimulus, cartilaginous explants were cultured with recombinant OSF-1, which in turn induced Type I collagen synthesis by chondrocytes *in vitro*.

Expression of *osf-1* was demonstrated by *in situ* hybridization in human and mouse bone marrow cell cultures. *In vitro*, recombinant OSF-1 enhanced osteogenic differentiation of mouse bone marrow cells at an appreciably low (10 pg/ml) concentration in comparison to recombinant bone morphogenetic protein-2 (rhBMP-2), which served as the positive control. However unlike BMP-2, OSF-1 was not osteoinductive as it failed to divert the multipotent, pre-myoblastic C2C12 cells along the osteogenic lineage. Interactions with BMP-2, studied using C2C12 cells, revealed OSF-1 to be a BMP-2 antagonist if added together with BMP-2. However, if added to the cultures after osteoinduction by BMP-2 had been achieved, OSF-1 (at pg/ml concentrations) enhanced the osteogenic phenotype of cell populations that had been primed to differentiate along the osteogenic lineage.

Thus functions of OSF-1 in bone development ranged from enhancing bone mineral content and osteogenic differentiation of bone marrow cells to, perhaps, stimulating differentiation of chondrocytes along the osteogenic lineage. Not only were the effects of OSF-1 dependent on its concentration, the timing of its presence also played a crucial role. The factor can be considered as an accessory signaling molecule, which modulates primary signals of growth factors like BMPs.

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ABBREVIATIONS

AA	Ascorbic acid-2-phosphate
AEC	3-amino, 9-ethylcarbazole
ALK	Orphan receptor tyrosine kinase anaplastic lymphoma kinase
ALP	Alkaline phosphatase
A/S	Alcian blue/ Sirius red staining
BCIP/ NBT	5-Bromo 4-Chloro 3-Indolyl phosphate/ Nitroblue tetrazolium
BDF-1 mice	F1 generation hybrid progeny of C57Bl/6/Ola/Hsd (black) and DBA/2/Ola/Hsd (grey) strains of mice
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
bp	Base pair
BSP	Bone sialoprotein
Ca^{+2}	Calcium ions
CAT	Chloramphenicol acetyltransferase
cbfa1	Core-binding factor 1
CFU-F	Colony forming unit-fibroblastic
cm	Centimeter
C-terminal	Carboxy terminal
DEPC-PBS	Diethyl Pyrocarbonate Phosphate-buffered saline
Dex	Dexamethasone
DIG	Digoxigenin
D-MEM	Dulbecco's Minimum Essential Medium
2% FCS/ D-MEM	Dulbecco's Minimum Essential Medium containing 2% fetal calf serum
DNA	Deoxyribonucleic acid
ds DNA	Double stranded deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
FCFC	Fibroblast colony-forming cell
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor-2

FOP	Fibrodysplasia ossificance progressiva
g	Gram
g/ dl	Gram/ decilitre
g/ L	Gram/ litre
Gly	Glycine
H_2O_2	Hydrogen peroxide
HARP	Heparin affin regulatory peptide
HB-GAM	Heparin-binding growth-associated molecule
HBGF-8	Heparin-binding growth factor-8
HBNF	Heparin-binding neurotrophic factor, Heparin-binding neurite outgrowth promoting factor
HCl	Hydrochloric acid
hr	Hour
KCl	Potassium chloride
k-Da	Kilo Dalton
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar
M-CSF	Macrophage colony-stimulating factor
α-MEM	Minimum Essential Medium Eagles (α modification)
15% MEM	Minimum Essential Medium Eagles (α modification) containing 15% fetal calf serum
mg	Milligram
mg/ dl	Milligram/ decilitre
mg/ L	Milligram/ litre
$MgCl_2$	Magnesium chloride
$MgSO_4.\ 7H_2O$	Magnesium sulphate
min	Minutes
MK	Midkine
ml	Millilitre
mm	Millimeter
mM	Millimolar
mRNA	Messenger ribonucleic acid

N/ n	Number of samples in a group
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
ng/ ml	Nanogram/ millilitre
nM	Nanomolar
nm	Nanometer
nmol NP/ hr/ ng DNA	Nanomoles of p-Nitrophenol/ hour/ nanogram DNA
NMR	Heteronuclear magnetic resonance spectroscopy
NP	p-Nitrophenol
NPP	p-Nitrophenyl phosphate
N-terminal	Amino terminal
osf-1	Osteoblast-stimulating factor-1 (the gene)
OSF-1	Osteoblast-stimulating factor-1 (the protein)
p18	18k-Da neurite outgrowth-promoting protein
pBSSK-	Plasmid Blue Script SK-
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pg/ ml	Picogram/ millilitre
PTN	Pleiotrophin
RANKL	Receptor for activation of nuclear factor kappa B ligand
rhBMP-2	Recombinant bone morphogenetic protein-2
rhOSF-1	Recombinant osteoblast-stimulating factor-1
rhPTN	Recombinant pleiotrophin
RIHB	Retinoic acid-induced heparin-binding protein
RPTP β/ζ	Receptor protein tyrosine phosphatase
SD	Standard deviation
SE	Standard error of mean
sec.	Seconds
SSC	Saline sodium citrate buffer
TE buffer	Tris-EDTA buffer
TGF-β	Transforming growth factor-β

TRAP	Tartrate-resistant acid phosphatase
Tris	Tris(hydroxymethyl)methylamine
TSR	Thrombospondin type I repeat
Type I	Type I collagen
UTP	Uridine triphosphate
UTRs	Untranslated regions
w/v	Weight/ volume
Wks	Weeks
μg	Microgram
μg/ ml	Microgram/ millilitre
μl	Microlitre
μm	Micrometer
μM	Micromolar
3-D	Three dimensional

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INTRODUCTION

1.1 The skeletal system

The skeletal system, a framework that supports and protects organs and allows movement, consists primarily of bone tissue, cartilage, red and yellow bone marrow, and the periosteum, the membrane around bones. The skeletal system performs several basic functions, including:

1. *Support*

   Serves as a framework for the body by supporting soft tissues and providing points of attachment for many skeletal muscles.

2. *Movement*

   Bones of the skeletal framework form a system of levers that multiply forces generated during skeletal muscle contraction and transform them into bodily movements.

3. *Protection*

   Protects many internal organs from injury eg. cranial bones protect the brain, vertebrae surround the spinal chord, the rib cage encloses the heart and lungs, and the hip bones guard internal reproductive organs.

4. *Mineral homeostasis*

   Bone matrix serves as a reservoir of several minerals, especially calcium and phosphorus, which are important in muscle contraction and nerve impulse transmission amongst other functions. Under a complex endocrine control, the stored mineral is released from bone into circulation to maintain critical mineral homeostasis and distribution to other parts of the body.

5. *Haematopoiesis*

   Within certain parts of bones is a connective tissue called red bone marrow, which produces red blood cells, white blood cells and platelets by the process of

haematopoiesis.

6. *Fat reservoir*

Lipids stored in adipocytes of the yellow bone marrow are an important chemical energy reserve.

Remarkably strong but light-weight, bone is a dynamic ever-changing tissue, understanding the structure and function of which, will offer novel insights into the pathophysiology of bone disorders.

1.2 Bone

Bone is a remarkable organ with unique self-organizational capability, by virtue of which, it remodels its shape and mass in accordance with the surrounding physical and chemical environs. Osteology (osteon – bone; logos – study) is the study of bone structure and applying the knowledge gained from it to the treatment of bone disorders. Bone is a specialized connective tissue composed of cells and the intercellular calcified material, the bone matrix.

1.2.1 Morphology of a long bone

The morphology of a bone may be studied by considering the parts of a long bone, one that has greater length than width.

a. Diaphysis

The shaft or long main portion of the bone.

b. Epiphyses

The distal and proximal extremities of the bone.

c. Metaphysis

The region in a mature bone where the diaphysis joins the epiphysis. In a growing bone, it is the region of the epiphyseal growth plate where cartilage is replaced by

bone. The epiphyseal growth plate is a layer of hyaline cartilage that allows the diaphysis of bone to grow in length.

d. Articular cartilage

A thin layer of hyaline cartilage that covers the epiphysis, where the bone forms an articulation (joint) with another bone. This cartilage absorbs shock and reduces friction at freely movable joints.

e. Periosteum

The periosteum is a membrane around the surface of bone not covered by articular cartilage. It consists of two layers, the outer fibrous layer of fibroblasts and collagen fibres containing blood vessels, lymphatics and nerves that pass into bone, and the inner more cellular layer of flattened, spindle-shaped osteoprogenitor cells with a potential to divide by mitosis and differentiate into osteoblasts. The periosteum is essential for bone growth in diameter, repair and nutrition. It also serves as a point of attachment for ligaments and tendons.

f. Marrow cavity

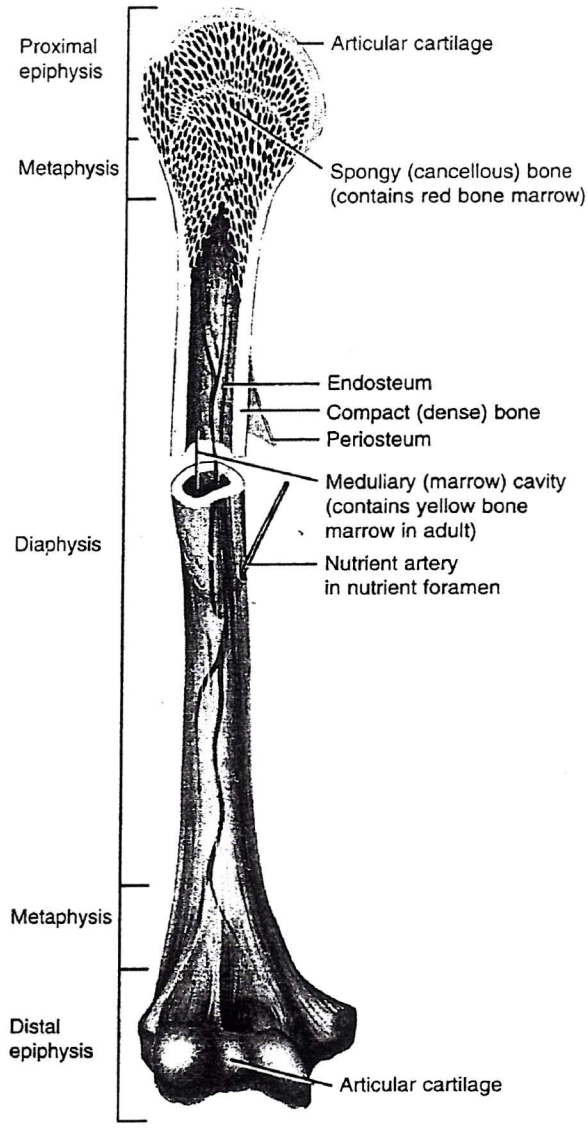
The marrow or medullary cavity is the space within the diaphysis containing the bone marrow. The cell population of the bone marrow consists of stem cells, the mesenchymal stem cells (Caplan, 1991) and the haematopoietic stem cells (Ogawa *et al.*, 1983). The mesenchymal stem cells are distinct from the haematopoietic stem cells as these give rise to those cells that form mesenchymal tissues. Mesenchymal stem cells can differentiate as osteoblasts (bone forming cells), chondrocytes (cartilage cells), myoblasts (muscle cells), adipocytes (fat cells), reticulocytes (reticular fibroblasts) or fibroblasts (Friedenstein, 1976; Owen and Friedenstein, 1988; Beresford, 1989), with an apparent degree of plasticity between the lineages (Bennett *et al.*, 1991; Beresford *et al.*, 1992; Oreffo *et al.*, 1997). The haematopoietic

stem cells give rise to cells of the haematopoietic lineage and the osteoclasts (bone resorbing cells) (Owen, 1985; Nijweide *et al.*, 1986; Osdoby *et al.*, 1987).

g. Endosteum

Lining the medullary or marrow cavity is the endosteum, a membrane that contains osteoprogenitor cells and a very small amount of connective tissue. The endosteum is therefore considerably thinner than the periosteum.

Figure 1. Structure of a long bone



(a) Partially sectioned humerus (arm bone)

1.2.2 Bone matrix

Bone matrix is made up of 25% water, 25% organic matter and 50% inorganic or mineral salts (Bourne, 1972).

a. Organic bone matrix

Almost 90% of the organic bone matrix is constituted by type I collagen, and the remainder by non collagenous glycoproteins and amorphous ground substance, the proteoglycan aggregates. Tissues other than bone, containing type I collagen, are not normally calcified. But then these tissues lack the specific non collagenous proteins, characteristic of bone tissue, which are implicated in the calcification process.

i) Type I collagen

Although more than 12 distinct types of collagen have been identified in mammalian tissues, bone tissue is particularly enriched in type I collagen. It has a typical triple helical structure composed of three polypeptide chains, each of which has approximately 1000 amino acid residues and is twisted into a helix. In turn, these three chains are wound into a super-helix to form a rod-like molecule. A striking characteristic of collagen is the occurrence of glycine residues at every third position in the polypeptide chains, it being the only amino acid small enough to be accommodated in the limited space of the central core of the triple helix. The repeating sequence of $(Gly\text{-}X\text{-}Y)_n$ is an absolute requirement for the triple helix formation. While X and Y can be any other amino acid, about 100 of the X positions are proline and 100 of the Y positions are hydroxyproline (Byers, 1989). Like reinforcing metal rods in concrete, the collagen fibres provide bone with tensile strength.

ii) Non collagenous proteins

About 10 % of the organic bone matrix is composed of non collagenous proteins, the major ones being: osteocalcin (also known as bone gla protein), osteonectin, osteopontin and bone sialoprotein. Since these proteins were identified in osteoblasts and mineralized bone matrix of humans (Bianco *et al.*, 1991), it was suggested that these proteins have a role in matrix mineralization. It is well established that the first mineral crystals form in the "gap region" of the bone collagen fibres, but collagen on its own is a very slow initiator of mineralization (Glimcher, 1989). It is therefore possible that one or more of these non collagenous proteins act as nucleators. A substance necessary for the initiation of mineralization should always be present at the "mineralization front" i.e. just ahead of the mineralized matrix. The distribution of osteopontin and bone sialoprotein not only corresponds to the area of mineralization, but extends slightly ahead of that region, to the sites within the osteoid where the initial mineral foci might be expected (Roach, 1994). In mineralizing cell cultures, bone sialoprotein is associated both with hydroxyapatite and the collagenous matrix (satisfying the criteria for a potential crystal nucleator), whereas osteopontin is associated with hydroxyapatite only (Kasugi *et al.*, 1991). Inhibiting the synthesis of osteopontin in rat cultures results in increased mineralization (Sodek *et al.*, 1992), suggesting that osteopontin may prevent the premature precipitation of calcium phosphate crystals that do not have the well-coordinated structure of hydroxyapatite (Roach, 1994). Osteocalcin and osteonectin are always confined to the very centre of the mineralized core (Roach, 1994). Excessive mineralization of growth plates in rats treated with warfarin to inhibit osteocalcin synthesis, suggests that osteocalcin is required for regulating the quantity of mineral (Price and Williamson, 1981). In cell-free in vitro systems, osteonectin inhibits de novo formation of apatite (Boskey, 1989)

and slows down crystal growth by blocking growth sites (Doi *et al.*, 1992). Therefore, osteonectin serves to slow down crystal growth and plays a role in regulating the size of hydroxyapatite crystals.

b. Inorganic mineral salts

The hardness of bone depends upon the inorganic mineral salts. Calcium and phosphorus are the major inorganic minerals, although bicarbonate, citrate, magnesium, potassium and sodium are also found. Calcium and phosphorus form crystals of hydroxyapatite $\{Ca_{10}(PO_4)_6(OH)_2\}$, appearing as plates alongside the collagen fibrils. Significant quantities of amorphous calcium phosphate are also present.

1.2.3 Bone cells

a. Osteoprogenitors

Progenitor populations, derived from mesenchymal stem cells, which undergo mitosis and differentiate into osteoblasts. These are found in the inner periosteal layer, the endosteum and within canals in bone that contain blood vessels, where they are referred to as stromal osteoprogenitor cells.

b. Osteoblasts

Osteoblasts synthesize organic bone matrix and are exclusively located adjoining the surfaces of bone tissue in a palisade that resembles simple epithelium. When they are actively engaged in matrix synthesis, osteoblasts have a cuboidal to columnar shape and a basophilic cytoplasm. During matrix synthesis, osteoblasts have the ultrastructure of cells actively synthesizing proteins for export. Osteoblasts are polarized cells and secretion of matrix components occurs at the cell surface in contact with older bone matrix, resulting in a layer of new (but not yet calcified) matrix called osteoid.

Stages in the differentiation of mesenchymal stem cells along the osteogenic lineage (and resultant formation of mineralized bone matrix) are characterized by the temporal expression of specific osteogenic markers. Expression of these osteogenic markers is regulated in a defined manner along the differentiation route to a terminally differentiated osteoblast phenotype. Core-binding factor 1 (*cbfa1*), an osteoblast-specific transcription factor, is the earliest and most specific marker of the osteogenic lineage (Ducy *et al.*, 1997). During embryonic development, *cbfa1* expression is initiated in a cell type that has characteristics of both, a future osteoblast and a chondrocyte, but later on its expression is restricted to osteoblast progenitors and differentiated osteoblasts (Karsenty, 2000). Binding sites for *cbfa1* have been identified in the promoter regions of the genes for bone sialoprotein and osteocalcin, both of which are markers of terminally differentiated osteoblasts (Ducy *et al.* 1997). Alkaline phosphatase expression is high during early stages of osteogenic differentiation, the enzyme being synthesized by early osteoblasts together with the collagenous bone matrix, into which it is then extruded prior to matrix mineralization (Bernard, 1978; Bronckers *et al.*, 1987; Strauss *et al.*, 1990). Type I collagen is another marker which is upregulated during initial stages of osteoblast differentiation (Sandberg and Vuorio, 1987; Strauss *et al.*, 1990). Non collagenous proteins like bone sialoprotein, osteonectin, osteopontin are secreted by more differentiated osteoblasts, while terminally differentiated osteoblasts secrete osteocalcin, the only known bone-specific protein (Lian and Gundberg, 1988).

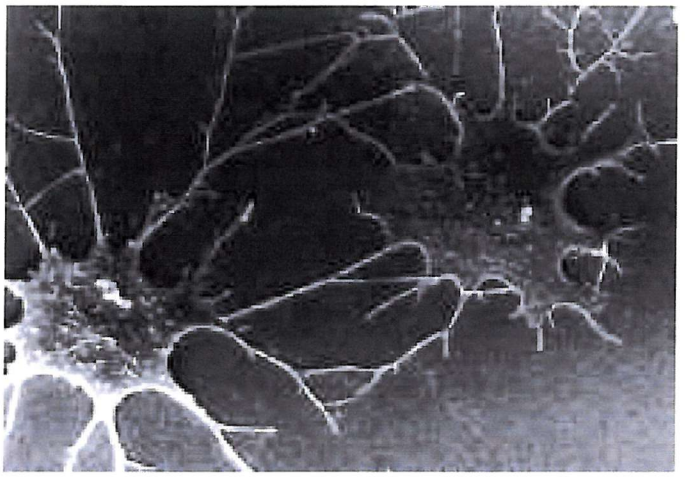
Figure 2. Osteoblasts

c. Osteocytes

Osteocytes are the most numerous of bone cells present in all living bones. Once embedded in the bone matrix, the osteoblast is referred to as an osteocyte. Osteoblasts have cytoplasmic processes that bring them into contact with neighbouring osteoblasts. These processes are more evident when the cell begins to surround itself with matrix and becomes an osteocyte. Lacunae and canaliculi appear because the matrix is formed around a cell and its cytoplasmic extensions. Osteocytes lie in lacunae situated between the lamellae of bone matrix, one osteocyte in each lacuna, and the canaliculi house cytoplasmic processes of the osteocytes. Processes of adjacent cells make contact via gap junctions, and molecules are passed via these projections from cell to cell. Some molecular exchange is believed to occur between osteocytes and blood vessels through the small amount of extracellular substance located between the osteocytes (and their processes) and the bone matrix. When compared with the osteoblasts, the flat, almond-shaped osteocytes exhibit a significantly reduced rough endoplasmic reticulum and Golgi complex, and a more condensed nuclear chromatin. Osteocytes serve as mechanoreceptors, the sensory

cells that detect changes in mechanical forces. These signals are then transmitted by osteocytes to the effector cells, the osteoblasts and osteoclasts, which form or take away the bone matrix depending upon the mechanical load.

Figure 3. Osteocytes



d. Bone lining cells

Once active bone formation ceases at a particular site, osteoblasts flatten out and become bone lining cells, which cover all endosteal bone surfaces. Although relatively inactive, these cells have an important function in protecting bone surfaces from resorption.

e. Osteoclasts

Osteoclasts are large, motile, multinuclear cells that resorb mineralized bone matrix. These are specialized macrophage polykaryons, formed by the fusion of blood-derived mononuclear progenitors of the monocyte/ macrophage family. Macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL), two molecules expressed by osteoblasts are essential for osteoclastogenesis (Shalhoub *et al.*, 2000). In areas of bone undergoing resorption, osteoclasts are found to lie within enzymatically etched depressions in the matrix

known as Howship's lacunae. Osteoclasts usually have acidophilic cytoplasm, and active cells are with their bone matrix-facing surface folded into irregular, subdivided projections constituting a ruffled border. Surrounding the ruffled border is a cytoplasmic zone, the clear zone, devoid of organelles, yet rich in actin filaments (microfilaments). This zone is an adhesion site for the osteoclast to bone matrix and creates a microenvironment for bone resorption. Some rough endoplasmic reticulum, abundant mitochondria and a well-developed Golgi complex are found in addition to a great number of lysosomes within the cell.

Figure 4. Osteoclasts

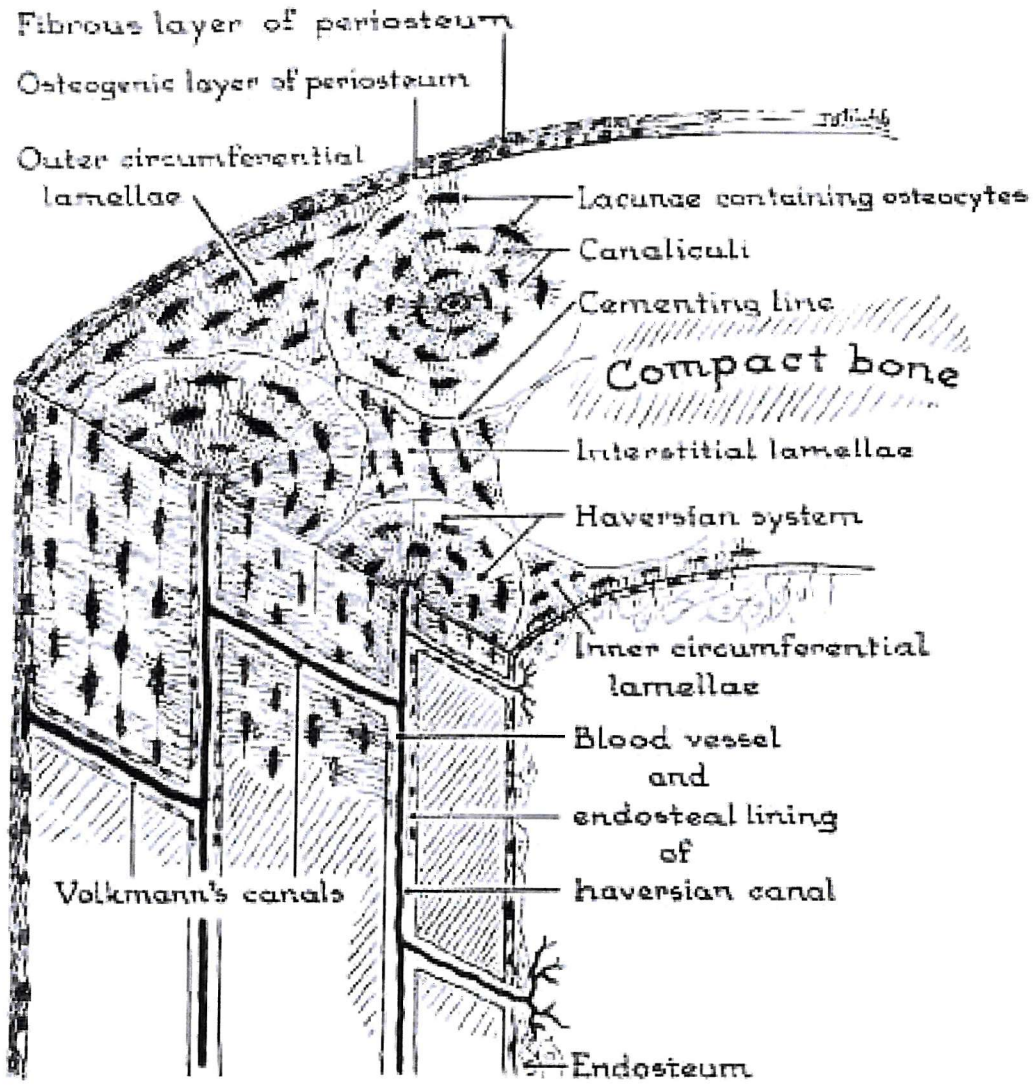
1.2.4 Types of bone

a. Compact bone

Compact (dense/ lamellar) bone forms the external layer of all bones and the bulk of the diaphyses of long bones. Compact bone tissue provides protection and support, and helps long bones resist the stress of weight placed on them. Adult compact bone has a concentric-ring structure. Blood vessels, lymphatic vessels and nerves from the periosteum penetrate the compact bone through perforating canals called Volkmanns'

canals. There are central canals running longitudinally through the bone called Haversian canals, around which are concentric rings of hard, calcified matrix or the concentric lamellae. Between the lamellae are small spaces called lacunae containing osteocytes. Radiating in all directions from the lacunae are tiny canals called canaliculi filled with extracellular fluid and slender finger-like processes of the osteocytes. The canaliculi connect lacunae with one another and eventually with the central canals. As materials cannot diffuse adequately through the calcified matrix, the branching canaliculi provide many routes for nutrients and oxygen to reach the osteocytes, and wastes to diffuse away. Osteocytes from neighbouring lacunae also form gap junctions with each other, facilitating movement of materials from cell to cell. Thus, there exists an intricate, miniature canal system throughout the bone. Each central canal, with its surrounding lamellae, lacunae, osteocytes and canaliculi, forms an osteon or Haversian system. Adult compact bone tissue is the only connective tissue which has a basic structural unit associated with it, the osteon. The areas between osteons contain interstitial lamellae. These also have osteocytes and canaliculi. Interstitial lamellae are fragments of older osteons, which have been partially destroyed during bone rebuilding or growth. Individual osteons are aligned parallel to the long axis of the diaphysis on a slightly spiral course. It is likely that the structure confers an evolutionary advantage. One hypothesis is that any microcracks will be deflected around the outer edge of the osteon, rather than propagating through the whole bone.

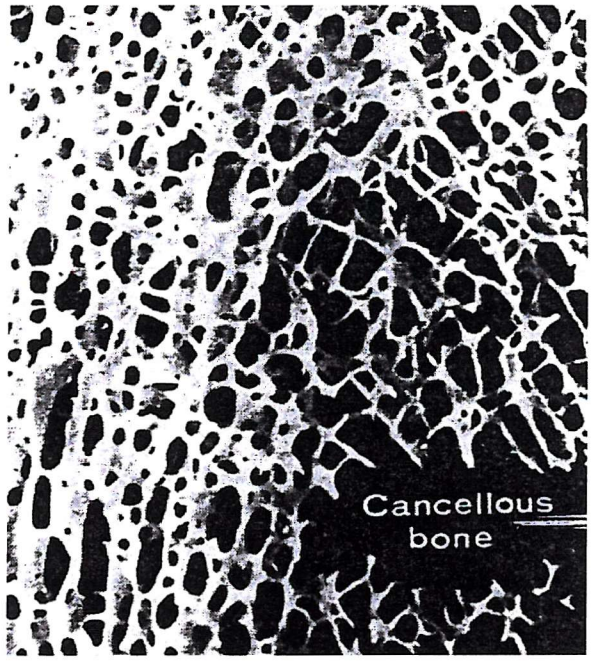
Figure 5. Structure of compact bone



b. Spongy bone

Spongy (cancellous/ trabecular) bone consists of lamellae arranged in an irregular lattice-work of thin plates called trabeculae. The macroscopic spaces between the trabeculae of some bones are filled with red bone marrow. Within the trabeculae are osteocytes that lie in lacunae, with radiating canaliculi. Osteocytes in the trabeculae receive nourishment directly from blood circulating through marrow cavities. Osteons are therefore not necessary in spongy bone as osteocytes are not deeply buried (as in compact bone) and have access to nutrients directly from blood. Spongy bone makes up most of the bone tissue of short, flat and irregularly shaped bones, and most of the epiphyses of long bones.

Figure 6. Spongy bone



1.3 Cartilage

Cartilage can be looked upon as a permeable, hydrated gel, the main function of which is to resist compression. Cartilage is particularly rich in extracellular matrix, which makes up almost 90% of the dry weight, while only 5% is constituted by chondrocytes or cartilage cells (Hardingham and Fosang, 1992). The cartilage matrix is primarily composed of proteoglycans, constituting around 60 % of the total matrix; while the remaining 40% is constituted by collagen. These macromolecules play an important role in the maintenance of a variety of functions including tissue strength, architecture and cell to cell interactions. Abnormal molecules in the cartilage matrix can compromise its functional integrity by disrupting the organized arrangement of chondrocytes (cartilage cells), their closely regulated proliferation and biosynthesis. Such abnormalities are called chondrodysplasias.

The main structural component of the cartilage matrix is proteoglycan. Proteoglycans are proteins with one or more attached glycosaminoglycan side chains. The main proteoglycan is aggrecan, a macromolecule composed of approximately 90% chondroitin sulfate chains. Synthesis of aggrecan is a specific marker of the chondrocyte phenotype (Doege *et al.*, 1990). Type II collagen is the most abundant of collagens, and since it is found almost exclusively in cartilage, it is a specific phenotype marker for chondrocytes (Dessau *et al.*, 1980). Type X collagen has a restricted tissue distribution within the hypertrophic calcifying region of the cartilage, where it makes up 45% of total collagen (Leboy *et al.*, 1989; Kirch and Von der Mark, 1992). It is proposed that type X collagen may have a role in regulating mineralization of hypertrophic cartilage. Another important matrix component is alkaline phosphatase, which is synthesized by hypertrophic chondrocytes and exported to the extracellular matrix, where the enzyme is associated with matrix

vesicles, the sites of initial calcification (Register *et al.*, 1986; deBernard *et al.*, 1986; Harrison *et al.*, 1995).

When bone tissue at the secondary ossification centre occupies the epiphysis, cartilage is restricted to two places: articular cartilage (at the end of the epiphysis where the bone forms an articulation/ joint with another bone) and the epiphyseal cartilage or epiphyseal growth plate, connecting the epiphysis to the diaphysis. The articular cartilage, which persists throughout adult life, does not contribute to bone formation. As the cartilage of the epiphyseal plate grows, it is continuously replaced by newly formed bone matrix. No further longitudinal growth of the bone takes place after growth of the epiphyseal plate ceases.

1.3.1 The growth plate

Growth plate chondrocytes are organized into different zones, with each cell population being a part of a different maturation stage in the endochondral sequence (Brighton, 1978).

a. Germinal/ Reserve/ Resting zone

Cells exist singly or in pairs separated by an abundant extracellular matrix. Chondrocytes in the germinal layer divide less frequently than the cells in the proliferative zone and are considered to be the stem cells or progenitors of epiphyseal chondrocytes (Kember, 1960; Ohlsson *et al.*, 1992). However, Price *et al.* (1994) do not agree with the term 'germinal zone' to describe this region as the cells here do not transcribe type II A collagen, the marker of pre-chondrocytic cells (Sandell *et al.*, 1991). These cells have already differentiated into chondrocytes i.e. this is not the germinal layer of "mother cartilage cells". The matrix consists of randomly oriented fibrils of type II collagen and proteoglycans. The type II collagen inhibits calcification and acts as a barrier to the advancing front of the secondary ossification centre in the

epiphysis (Robertson, 1990). Since these cells have a high lipid and vacuole content, it has led to the suggestion that this zone functions for storage of nutrients (Brighton, 1978). This is the zone with highest ratio of matrix volume to cell volume.

b. Proliferative zone

The chondrocytes in the proliferative zone take on a flattened appearance and are organized in columns parallel to the longitudinal axis of the bone. The function of the proliferative zone is matrix production and cell division that results in longitudinal growth. Some believe that this zone is the true germinal layer of the growth plate, with actively dividing cells (Kember, 1978). Type II collagen synthesis and its mRNA expression increases in this zone, as does that of aggrecan (Kosher *et al.*, 1986). The ratio of matrix volume to cell volume is lower in this zone as compared to the reserve zone. The type II collagen fibrils are longitudinally oriented and surround the columns of chondrocytes.

c. Upper hypertrophic zone/ Maturing zone

The cell size abruptly increases in the upper hypertrophic zone and the columnar arrangement is less regular. Although, not proliferating, hypertrophic zone cells retain the full complement of cytoplasmic components, and light microscopy reveals increasing vacuolation of these cells. Hypertrophic chondrocytes are metabolically active cells, with overall matrix synthesis per cell increased approximately three-fold compared to the proliferative zone (Hunziker *et al.*, 1987). The main matrix components synthesized are type II, type X collagen and aggrecan.

Lower hypertrophic zone

This is the zone of terminal chondrocytes. The end of this zone is marked by the last intact transverse cartilage septum. Matrix calcification occurs in the longitudinal septae between the columns of chondrocytes, and this calcified matrix becomes the

scaffolding for bone deposition in the metaphysis. The hypertrophic zone contains the highest levels of alkaline phosphatase, assisting in matrix calcification. The traditional view was that these cells were metabolically very inactive, and increasing vacuolation indicated death by hypoxia. However, these cells are clearly actively involved in synthesis of type X and type II collagen (Kirch and Von der Mark, 1992). This fully viable cell then dies by apoptosis, a distinct biological form of cell death, lasting approximately 18% of the terminal chondrocyte's life span (Farnum and Wilsman, 1989). A detailed study of the ultrastructure of terminal chondrocytes suggests a different mechanism of programmed cell death in these cells, clearly distinct from classical apoptosis (Roach and Clarke, 2000). These condensed chondrocytes have a convoluted nucleus with patchy chromatin condensations, while the cytoplasm is dark with excessive amounts of endoplasmic reticulum. Such a terminal chondrocyte is termed as 'dark chondrocyte'. It is possible that confinement of chondrocytes within their lacunae, which would prevent phagocytosis of apoptotic bodies, necessitates mechanisms of elimination different from classical apoptosis.

At the vascular front, where the transition from cartilage to bone occurs, chondrocyte lysis is evident from the empty lacunae invaded by vascular endothelial loops. The struts of longitudinal calcified cartilaginous septa serve as scaffolding onto which the osteoblasts lay down bone. Metaphyseal bone formation is associated with type I procollagen mRNA expression in the empty lacunae and osteoid (Reichenberger *et al.*, 1991), while types X and II collagen have restricted localization to the calcified cartilage trabecular remnants. Newly formed woven metaphyseal bone is gradually replaced by lamellar bone following osteoclastic degradation of bony matrix and chondroclastic removal of the remaining cartilage trabeculae.

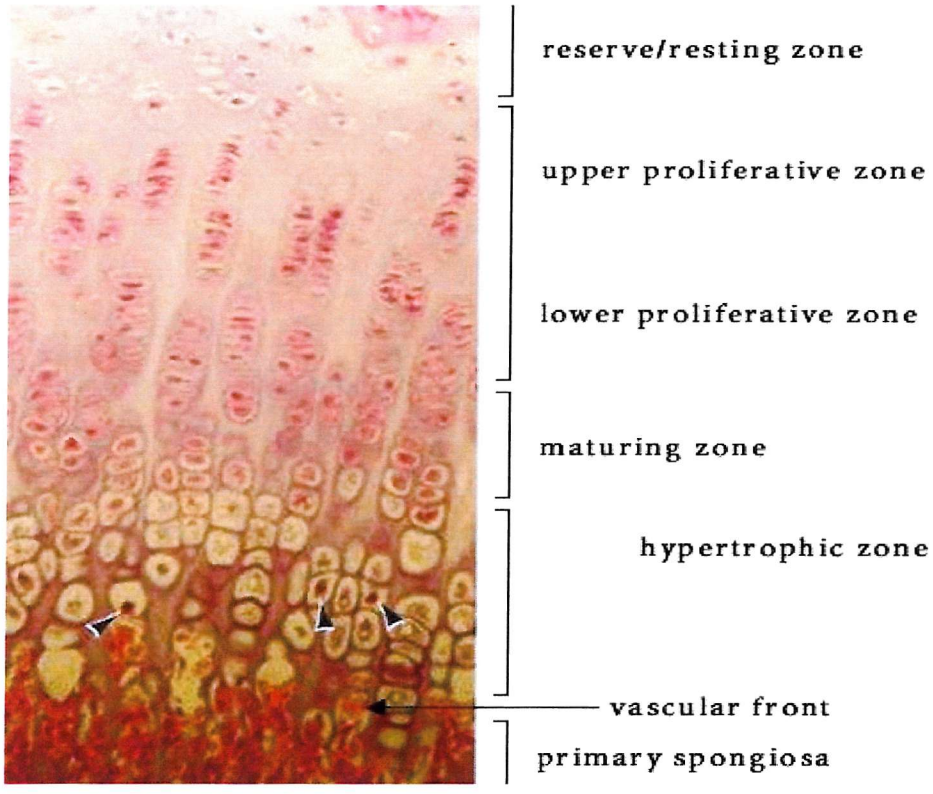


Figure 7. Cellular organization of the mammalian growth plate (Courtesy of Dr. Toshimi Aizawa, Tohoku University, Sendai, Japan). *Tibial growth plate from 5-week-old rat, stained with H/E, exhibiting the various zones. Arrowheads indicate condensed chondrocytes in the hypertrophic zone.*

1.4 Bone development

1.4.1 Bone formation

Bone formation/ ossification can occur along two distinct pathways, endochondral ossification and intramembranous ossification.

a. Endochondral ossification

Endochondral ossification is principally responsible for the formation of short and long bones. This form of ossification utilizes a cartilaginous scaffold (such as the growth plate) and consists of two phases. The first phase involves hypertrophy and elimination, largely by apoptosis, of the growth plate chondrocytes/ cartilage cells, thereby leaving behind empty lacunae. During course of the second phase, blood capillaries penetrate the spaces left by the degenerating chondrocytes. Transverse cartilaginous septa, separating these empty lacunae, are then resorbed by the vascular mononuclear resorbing cells, leaving behind unresorbed longitudinal septa of calcified cartilage matrix. Osteoprogenitors/ osteoblasts, brought in by the invading blood vessels, deposit bone matrix onto the unresorbed longitudinal cartilaginous septa, which serve as templates/ supports for the beginning of ossification.

b. Intramembranous ossification

Intramembranous ossification, the source of most flat bones, does not rely on a cartilaginous template. The mesenchymal progenitor cells differentiate directly into osteoblasts synthesizing the bone matrix. Mandibles, maxillae, parts of the occipital and temporal bones and the clavariae/ flat skull bones are formed by intramembranous ossification. This process also contributes to the growth of short bones and thickening of long bones. In the mesenchymal condensation layer, the starting point for ossification is called a primary ossification centre. The process begins when groups of cells differentiate into osteoblasts. New bone matrix is formed and calcification

follows, resulting in the encapsulation of some osteoblasts, which then become osteocytes. These islands of developing bone are known as spicules, which arise almost simultaneously at the ossification centre and fuse to give the bone a spongy structure. Several ossification centres grow radially and finally fuse together, replacing the original connective tissue. The fontanelles of new-born infants are soft areas in the skull that correspond to parts of connective tissue not yet ossified. The portion of the connective tissue layer that does not undergo ossification gives rise to the endosteum and periosteum of intramembranous bone.

1.4.2 Bone resorption

Osteoclasts are attracted to mineralized surfaces that are not covered by bone lining cells. The resorption of bone by osteoclasts is initiated when these attach to the mineralized bone surface via the ruffled border, which by a tight sealing creates an area of low pH needed for dissolution of the mineral. Osteoclasts secrete acid phosphatase, collagenase and other proteolytic enzymes that attack bone matrix, liberate the calcified ground substance, and are actively engaged in elimination of debris formed during bone resorption. The role of tartrate-resistant acid phosphatase (TRAP) in osteoclastic bone resorption is well known (Zaidi *et al.*, 1989). TRAP also plays a regulatory role in osteoclastic bone resorption, as it partially dephosphorylates bone matrix phosphoproteins like osteopontin and bone sialoprotein, which are then incapable of supporting osteoclast attachment to the bone surface (Ek-Rylander *et al.*, 1994). Cathepsin K is a lysosomal cysteine protease expressed selectively and at high levels in osteoclasts, with enzymatic properties suited for degrading type I collagen (Bossard *et al.*, 1999). Additional cysteine proteases, also active at low pH, are important for the digestion of non-collagenous proteins. Matrix metalloproteinases

exert their activity when the pH has increased slightly. Osteoclasts move along the bone, creating resorption pits until they are eliminated by apoptosis.

1.4.3 Bone remodeling

Bone remodeling is a dynamic physiological process that continues throughout life with losses from osteoclast resorption made good by osteoblast bone formation. The human skeleton is completely replaced by the remodeling process every 10 years. Remodeling of bone throughout life represents a major advantage for animals that have a long life-span, because

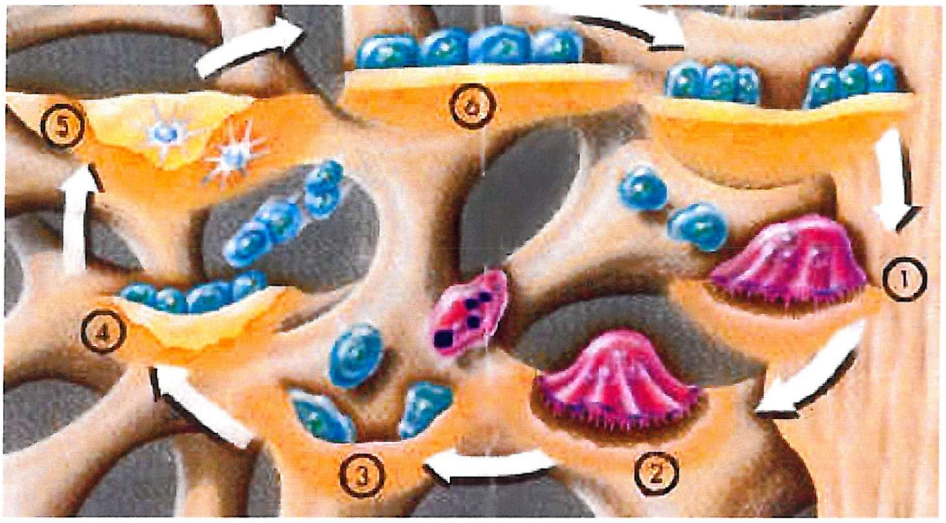
1. it enables adaptation to changes in mechanical load
2. it provides a mechanism to renew bone matrix continually and thus prevent or reduce 'bone fatigue'
3. it permits shape of the bone to be maintained while it grows
4. it plays a role in fracture healing

Cellular mechanism of remodeling:

1. Osteoclast precursors are attracted by chemotaxis to sites where osteoblasts or bone lining cells have moved away from the bone surface. The precursors fuse to become osteoclasts and attach to bone with their ruffled border.
2. Osteoclasts proceed to resorb packets of bone. After having received signals to cease resorption, osteoclasts undergo apoptosis and lift off.
3. Osteoprogenitor cells are attracted to the site vacated by osteoclasts, differentiate into osteoblasts, which then start to synthesize new bone matrix.
4. Normally, osteoblasts continue to lay down bone matrix until the resorption pit created by osteoclasts is filled in.
5. Osteoblasts get embedded in the bone matrix synthesized by them and are referred to as osteocytes.

6. Bone lining cells cover the inactive bone surfaces, and those osteoblasts that did not become either osteocytes or bone lining cells, undergo apoptosis.

Figure 8. The bone remodeling process



Bone remodeling, under physiological conditions, is characterized by an equilibrium between osteoclast bone resorption and osteoblast bone formation. This balanced process is described as coupling (Frost, 1964), and it is the uncoupling of resorption from formation that leads to skeletal diseases like osteoporosis. The reduction in bone mass and deterioration in bone architecture after the age of 40, which is characteristic of osteoporosis, results in an increase in the fragility of bone and its susceptibility to fractures. The net loss of bone, characteristic of osteoporosis, is a consequence of either too much resorption by osteoclasts or too early apoptosis of osteoblasts. The most common cause of osteoporosis in women is the decrease in estrogen that accompanies menopause. Estrogen inhibits bone resorption by suppressing the production of cytokines that promote osteoclast differentiation. Thus, most successful therapies in the anti-osteoporosis regimen have targeted osteoclasts. A class of

compounds termed 'bisphosphonates' reduce bone resorption, lower bone turnover and restore the equilibrium between bone resorption and formation, by inhibiting osteoclast activity and compromising osteoclast survival. Alendronate, a nitrogen-containing bisphosphonate, has been the drug of choice in the treatment of postmenopausal osteoporosis. However, there is a need for non-toxic anabolic agents, which will substantially increase bone formation in people who have already suffered significant bone loss. Although, results do looking promising with a class of compounds termed 'statins' (Mundy *et al.*, 1999), there are no currently approved drugs that significantly augment bone mass due to their stimulatory effects on osteoblasts. In the search for anabolic agents that increase osteoblast activity, the present study examined the role of a novel peptide termed **osteoblast-stimulating factor-1 (OSF-1)**.

1.5 Osteoblast-stimulating factor-1 (OSF-1)

An 18k-Da neurite outgrowth-promoting protein (p18), expression of which peaked in the brain tissue during the perinatal stage of rapid axon growth and synapse formation, was isolated from perinatal rat brain (Rauvala, 1989). Although the protein closely resembled fibroblast growth factors in its molecular mass and its high affinity to bind heparin, it was found to have a distinct structure and was shown to be immunochemically distinct from the basic fibroblast growth factor. Molecular cloning and sequencing of p18 confirmed that it had a novel sequence, and the protein was named "heparin-binding growth-associated molecule" (HB-GAM) due to its high affinity for binding heparin and its association with axon growth (Merenmies and Rauvala, 1990).

Deuel and co-workers also isolated a 17k-Da heparin-binding growth factor (HBGF-8) from bovine uterus, which was shown to be mitogenic for fibroblastic cells (Milner, Deuel *et al.*, 1989). Cloning of this mitogenic protein revealed the same sequence as HB-GAM, thereby confirming that the two proteins were identical (Li, Deuel *et al.*, 1990). The protein was also found to possess a neurite outgrowth activity and its expression was developmentally regulated. To reflect the diversity of its expression during early development, the protein was designated "pleiotrophin" (PTN) (Li, Deuel *et al.*, 1990).

Attempts by Tezuka and co-workers, in the search for possible factors implicated in the growth and differentiation of skeletal tissues, involved cloning cDNAs which were specifically expressed by osteoblasts (Tezuka *et al.*, 1990). Using a differential hybridization screening technique between the mouse osteoblastic cell line, MC3T3-E1 and fibroblastic cell line, NIH3T3, a new cDNA clone was reported. It encoded a lysine-rich basic protein termed "osteoblast-stimulating/ specific factor-1 (OSF-1)"

and was expressed by osteoblastic, but not fibroblastic cells. Interestingly, OSF-1 had the same sequence as HB-GAM.

Thus, it has now been accepted that heparin-binding growth-associated molecule, pleiotrophin and osteoblast-stimulating factor-1, are synonyms designating the same protein, which has also been referred to as heparin-binding neurotrophic factor (HBNF; Kovesdi *et al.*, 1990), heparin affin regulatory peptide (HARP; Courty *et al.*, 1991) and heparin-binding neurite outgrowth promoting factor (HBNF; Kretschmer *et al.*, 1991).

1.5.1 Developmentally-regulated osf-1 gene expression

The human *osf-1* gene was identified on chromosome 7, band q33 (Li *et al.*, 1992), while the mouse *osf-1* was found to be located on chromosome 6 (Katoh et al., 1992). The human *osf-1* gene was found to be expressed during mid to late gestation, highest levels being detected at birth in many tissues; while post-natal expression declined rapidly in many tissues, maximal expression was observed in adult brain (Kretschmer *et al.*, 1991). A high-level expression of the *osf-1* gene was detected by *in situ* hybridization in the spinal chord, brain, choroid plexus and bones of developing mouse embryos (Li *et al.*, 1992). In adult mice, expression of the *osf-1* gene was detected by Northern blotting analysis in calvarial osteoblasts and brain tissues, but not in thymus, spleen, kidney, lung, liver, testis or heart (Tezuka *et al.*, 1990). On the other hand, expression of *osf-1* mRNA in adult mice was shown to be ubiquitous, although maximal expression was observed in brain and bone tissues, such as the calvariae (Sato *et al.*, 1997).

Expression of the *osf-1* gene was found to be regulated by two promoters in humans (Lai *et al.*, 1992; Lai *et al.*, 1995) and mice (Katoh *et al.*, 1992), namely, the proximal (promoter I) and distal (promoter II) promoters. Sato and co-workers (Sato *et al.*,

1997) demonstrated that not only did the usage of promoter I predominate during embryonic development in mice, but it was also the predominant promoter regulating the expression of the *osf-1* gene in adult tissues. The usage of promoter II was found to be rare in murine development, and only a few tissues including brain (embryonic as well as adult) and calvariae (during early development - till 0.5 months of age and not later on) exhibited relatively high degree of expression of the promoter II-specific transcript.

1.5.2 OSF-1 protein structure

Human, bovine, rat, mouse and chick *osf-1 cDNAs*, which were cloned and sequenced (Li *et al.*, 1990; Merenmies and Rauvala, 1990), encoded a 168-amino acid OSF-1 protein (inclusive of a 32-amino acid signal recognition sequence). The signal recognition sequence was cleaved upon rapid secretion of the protein from cells (Raulo *et al.*, 1992), yielding a 136 amino-acid mature OSF-1 protein. The protein was made up of abundant (24%) cationic residues, mainly lysine and cysteine residues, and five intra-chain disulphide bonds (Merenmies and Rauvala, 1990).

A homology of more than 90% was reported among the OSF-1 amino acid sequences of human, bovine, rat, mouse and chick (Merenmies and Rauvala, 1990). This degree of sequence conservation for OSF-1 among species was found to be highest among any of the known cytokines (Deuel *et al.*, 2002). The OSF-1 sequence was clearly distinct from fibroblast growth factors, but it shared a 50 % homology with the sequences of the mouse midkine protein (MK) (Kadomatsu *et al.*, 1988) and the MK-type chicken protein, retinoic acid-induced heparin-binding protein (RIHB) (Vigny *et al.*, 1989). Thus, OSF-1, MK and RIHB constitute a new family of secreted, heparin-binding proteins, which are structurally unrelated to any other growth factor family.

Structural studies carried out using heteronuclear magnetic resonance spectroscopy (NMR), demonstrated that OSF-1 was made up of two heparin-binding β-sheet domains connected by a flexible linker (Kilpelainen *et al.*, 2000). The two domains were relatively independent and free to move with respect to each other, suggesting that the linker region between the domains was quite flexible. Three antiparallel β-strands constituted each domain. Search of sequence databases showed that the β-sheet domains of OSF-1 were homologous to the thrombospondin type I repeat (TSR) motif, which was found to mediate cell surface binding in a number of extracellular matrix and cell surface proteins (Klar *et al.*, 1992; Iwasaki *et al.*, 1997). TSR repeats have been shown to bind heparin and heparan sulfate (Guo *et al.*, 1992), and the finding that β-sheet domains of OSF-1 mediated heparin binding agreed with the view that these domains corresponded to the TSR sequence motifs. The main conserved features in TSR sequences were the cysteines, closely spaced tryptophans and a cluster of basic residues, which were also reflected in the β-sheet domains of OSF-1. The arrangement of intradomain cysteine bridges/ disulphide bonds in OSF-1 was consistent with that predicted for the TSR motif. In addition to this domain structure, OSF-1 contained the N-terminal and C-terminal polylysine-type sequences that lacked a detectable structure and formed random coils. The polylysine tails did not display heparin-binding activity and remained unstructured. Thus, OSF-1 was composed of two TSR domains, connected by a short linker, and short N and C-terminal polylysine tails.

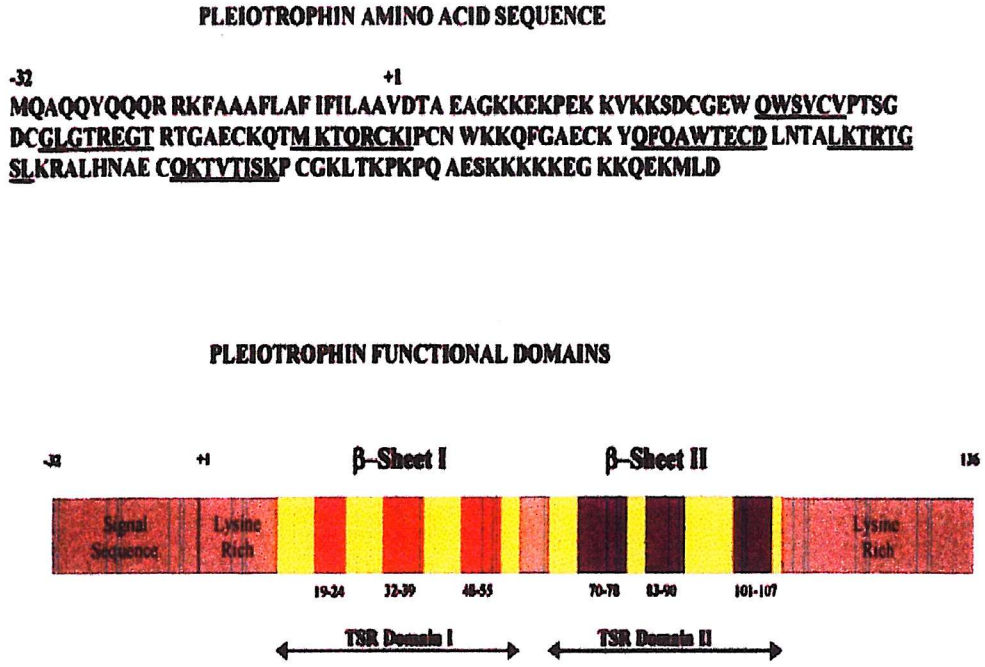


Figure 9. Amino acid sequence and schematic structure of OSF-1/ Pleiotrophin (Adapted from Deuel et al., 2001). *The predicted amino acid sequence of human OSF-1 protein illustrating underlined sequences representing the thrombospondin type I repeat domains (A). Schematic structure of OSF-1 illustrating the signal recognition sequence, the two β-sheet structures with thrombospondin type I repeat domains and the lysine-rich N- and C-terminals, in reference to the amino acid sequence.*

1.5.3 Localization of the OSF-1 protein and its mRNA expression in bone and cartilage

Relatively large amounts (3.5 mg protein/ kg wet weight of bone) of OSF-1 were isolated from bovine adult tibial bone (Zhou *et al.*, 1992), indicating that OSF-1, like other bone growth factors, was stored in the bone matrix. On the other hand, almost 15 mg OSF-1/ kg wet weight of cartilage was isolated from dissociative extracts of

bovine **fetal** epiphyseal and nasal cartilage, but only trace amounts detected in **adult** articular cartilage (Neame *et al.*, 1993). Serum levels of OSF-1 in healthy adult humans were in the range of 16-41 pg/ml, with an average value of 27 pg/ml (Souttou *et al.*, 1998).

Immunohistochemical analysis of OSF-1 distribution in fetal epiphysis (Neame *et al.*, 1993) indicated that it was associated with the chondrocytes. Immunohistochemical and *in situ* hybridization studies on the distribution of the OSF-1 protein and its mRNA expression respectively, in chick developing leg demonstrated the presence of OSF-1 in osteoblast/ osteoid layer (Dreyfus *et al.*, 1998).

In fetal rat bones, abundant expression of the OSF-1 protein and its mRNA was detected in the developing cartilage, which served as a template for future endochondral ossification (Imai *et al.*, 1998). In the same study, immunostaining for OSF-1 was observed in the secondary ossification centre, growth plate and periosteum of 1-week-old rat humeral head during the course of post-natal bone development.

In bones of mice (2-12 months), immunostaining for OSF-1 was observed in the cytoplasm and the pericellular matrix of osteoblasts that lined the longitudinal mineralized septae of the epiphyseal growth plate (Petersen and Raifi, 2001). The same study localized the OSF-1 protein within the pericellular matrix of hypertrophic chondrocytes, and within the opened lacunae of apoptotic hypertrophic chondrocytes. The resting zone and proliferative zone chondrocytes were negative for the presence of OSF-1.

1.5.4 Receptors for OSF-1

A number of receptors have been identified for OSF-1 in different tissues where the protein is expressed. N-syndecan, a heparan sulfate proteoglycan, has been identified as the receptor for OSF-1 in osteoblasts/ osteoprogenitors (Imai *et al.*, 1998) and

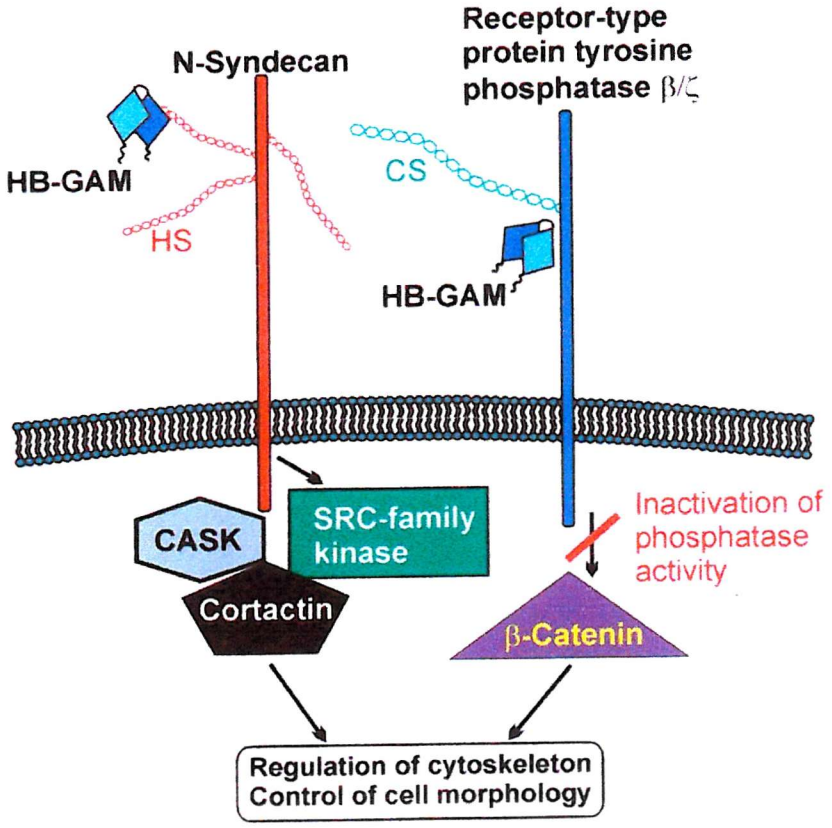
nervous tissue (Raulo *et al.*, 1994). In addition to heparan sulfate proteoglycans, a specific heparan sulfate-independent receptor was discovered for OSF-1 on human osteoblast-like osteosarcoma cell lines: HOS (TE85) and MG-63 (Gieffers *et al.*, 1993). Receptor protein tyrosine phosphatase (RPTP β/ζ), a chondroitin sulfate proteoglycan, has also been identified as one of the receptors for OSF-1 in neuronal cells (Maeda *et al.*, 1996; Maeda and Noda, 1998). The orphan receptor tyrosine kinase anaplastic lymphoma kinase (ALK) was immunolocalized in the central nervous system, especially in some neurons, glial cells and endothelial cells (Pulford *et al.*, 1997), and in a number of fibroblastic, endothelial and carcinoma cell lines (Stoica *et al.*, 2001). OSF-1 served as a ligand for the ALK receptor in the nervous system and in the endothelial cell line (Stoica *et al.*, 2001).

N-syndecan, a member of the syndecan gene family, was originally cloned from rat Schwann cell cDNA library (Carey *et al.*, 1992). Syndecans are sequence-related transmembrane heparan sulfate proteoglycans, expressed in cell- and development-specific patterns (Kim *et al.*, 1994). The four syndecans (syndecan -1, -2, -3, -4) have structurally variable extracellular domains, but highly conserved cytoplasmic domains (Bernfield *et al.*, 1992). The heparan sulfate structure of N-syndecan (syndecan 3) is exceptionally heparin-like, with respect to a high content of 2-O-sulfo-iduronic acid residues in the extracellular domain. The ectodomain of N-syndecan binds to matrix-associated OSF-1. The iduronic acid-2-O-sulfate residues of the heparan sulfate chains characterizing the extracellular domain of N-syndecan are implicated in this binding (Kinnunen *et al.*, 1996). The conserved cytosolic domain of N-syndecan binds to a protein complex containing src-family tyrosine kinases and the src-substrate cortactin (Kinnunen *et al.*, 1998). Binding of OSF-1 to N-syndecan leads to phosphorylation and activation of src, followed by phosphorylation of cortactin, the

src-substrate. Phosphorylation of cortactin is known to modulate its F-actin cross-linking activity (Hung *et al.*, 1997), leading to cytoskeletal reorganization pertinent to cell motility.

On the other hand, binding of OSF-1 to RPTP β/ ζ results in inactivation of the phosphatase activity of the receptor, thereby, changing the balance of phosphorylation/ dephosphorylation in the cell (Meng *et al.*, 2000). This results in β-catenin, the downstream substrate of RPTP β/ ζ, being maintained in the phosphorylated state and resultant cytoskeletal reorganization eliciting a migratory response in the cell.

Figure 10. Receptor - OSF-1: Signal transduction (Adapted from Rauvala et al., 2000)



1.5.5 Functions of OSF-1

In brain

OSF-1 has been shown to enhance neurite outgrowth in a number of studies (Rauvala, 1989; Kuo *et al.*, 1990; Kretschmer *et al.*, 1991; Hampton *et al.*, 1992). The recombinant protein, expressed in *E. coli*, was also found to promote neuronal development in cultures (Takamatsu *et al.*, 1992). Presence of OSF-1 was demonstrated in extracellular tracts that line the paths of growing neurites in developing brain, where it was found to enhance migratory responses in neurons (Rauvala *et al.*, 1994). Expression of *osf-1* peaked in fibre pathways of brain tissue during the perinatal stage of rapid axon growth and synapse formation i.e. a stage where neuronal connections developed rapidly (Rauvala, 1989), which was in keeping with the role for OSF-1 in development and/ or plasticity of neuronal connections (Rauvala *et al.*, 1994).

In bone

Transgenic mice over-expressing the human *osf-1* gene were found to have a high femoral bone mineral content compared to wild-type controls (Masuda *et al.*, 1997). Since these transgenic mice started off having a high inorganic bone mass, it compensated for the bone loss observed in them after an ovariectomy. Although an increase in osteoclast activity due to estrogen deficiency was evident in these transgenic mice, appositional bone formation due to transgene over-expression continued thereafter and compensated for the overall bone loss.

A chemotactic role was attributed to OSF-1 as it stimulated the migration of rat and human osteoblast-type cells in haptotactic transfilter and migration assays (Imai *et al.*, 1998). The same study suggested that OSF-1, which was immobilized in cartilage matrices that served as scaffolds for endochondral ossification, served to recruit

osteoblasts and osteoprogenitors to the site of new bone formation via cell-matrix interactions/chemotactic signaling.

OSF-1 was also demonstrated to be chemotactic for osteoprogenitors from human bone marrow (Yang *et al.*, in press).

In *in vitro* cultures of bovine mature articular chondrocytes supplemented with recombinant OSF-1, although chondrocyte proliferation was arrested, OSF-1 enhanced synthesis of glycosaminoglycans and expression of matrix proteoglycans (biglycan) and collagen type II (Tapp *et al.*, 1999).

In tumorogenesis

The *osf-1* gene is a proto-oncogene. Constitutive expression of the *osf-1* gene under the control of a heterologous promoter was found to transform NIH 3T3 fibroblastic cells and when clonally selected cells with constitutive *osf-1* gene expression were implanted into flanks of nude mice, highly vascularized tumours were formed (Chauhan *et al.*, 1993). In the 136-amino acid sequence of OSF-1, amino acid residues 1-40 constitute the N-terminal domain and those between 69-136 constitute the C-terminal domain. Amino acids 41-64, designated as the transforming domain, together with either the N-terminal domain or C-terminal domain were required for PTN-dependent transformation of NIH 3T3 cells (Zhang and Deuel, 1999; Zhang *et al.*, 1999). The strong net positive charges associated with the lysine-rich N- and C-terminal domains, enabled these domains to associate OSF-1 with a docking protein similar to N-syndecan (Zhang and Deuel, 1999; Zhang *et al.*, 1999). Amino acid residues 41-45 and 64-68, the internal repeats, functioned as important regulatory domains as they were found to suppress the transforming potential of OSF-1 (Zhang and Deuel, 1999).

In angiogenesis

OSF-1 residues 69-136 (the C-terminal domain) were found to contain domain/s, referred to as 'angiogenesis domain' (Zhang *et al.*, 1999). Expression of the angiogenesis domain greatly enhanced tumour angiogenesis in the already transformed SW 13 cells, a cell line derived from "low grade" human adrenal carcinoma (Zhang *et al.*, 1999), but on its own was not tumorogenic (Zhang and Deuel, 1999). Angiogenic activity of OSF-1 was confirmed in a number of *in vitro* and *in vivo* models, as OSF-1 stimulated endothelial cell migration and tube formation on matrigel, collagen and fibrin gels, and enhanced angiogenesis in the chicken embryo chorioallantoic membrane assay (Papadimitriou *et al.*, 2001). However in this study, both N and C-terminal domains were found to play an important role in angiogenesis. The study also explained that interaction of OSF-1 with endothelial cells might involve glycosaminoglycans (like heparan and chondroitin sulfate) and/ or a transmembrane specific receptor, or OSF-1 might have an indirect effect in potentiating the angiogenic effect of other growth factors, or releasing growth factors sequestrated in the ECM such as FGF-2, by inducing stimulation of proteases (Kojima *et al.*, 1995).

In cell proliferation

OSF-1 served as a mitogen for endothelial cells (Courty *et al.*, 1991; Fang *et al.*, 1992; Yeh *et al.*, 1998), epithelial cells (Fang *et al.*, 1992; Delbe *et al.*, 1995) and different fibroblast cell lines (Milner *et al.*, 1989; Fang *et al.*, 1992). However, its role as a mitogen has been challenged by some researchers (Hampton *et al.*, 1992; Raulo *et al.*, 1992; Szabat and Rauvala, 1996). Other investigators have reported that recombinant OSF-1 expressed in insect cells (baculovirus) or bacteria yielded a protein which exhibited neurite outgrowth, but lacked/ had limited mitogenic activity

(Raulo *et al.*, 1992; Seddon *et al.*, 1994). A likely explanation for this was that incorrect folding or incomplete processing of the recombinant OSF-1 polypeptide compromised its ability to stimulate proliferation.

The stimulatory effect of bFGF on cell proliferation was inhibited by OSF-1 (Szabat and Rauvala, 1996). Members of the FGF family depend upon specific heparin-type carbohydrate epitopes for exerting their proliferation-enhancing activity (Yayon *et al.*, 1991; Olwin and Rapraeger, 1992; Nurcombe *et al.*, 1993; Mason, 1994). OSF-1 has been known to bind N-syndecan, a heparan sulfate proteoglycan (Raulo *et al.*, 1994; Imai *et al.*, 1998). OSF-1 and bFGF have been shown to compete for binding the heparan sulfate chains of N-syndecan (Raulo *et al.*, 1994). This suggested that OSF-1 probably interacted with and consequently blocked the bFGF receptor, thereby inhibiting bFGF-stimulated cell proliferation (Szabat and Rauvala, 1996).

Recently it has been shown that the mitogenic activity of OSF-1 resides in amino acid residues 111-136 of the C-terminal domain (Bernard-Pierrot *et al.*, 2001).

1.6 Hypothesis and Aims

Although *osf-1* was expressed in a number of tissues during fetal development, maximal post-natal expression was observed in the bone and brain. Its functions in neuronal development have been delineated, but remain to be studied in-depth during bone development. Previous work suggested that OSF-1 might be one of the important bone mass determinants, which positively controlled appositional bone formation by osteoblast activation and recruitment (Masuda *et al.*, 1997; Imai *et al.*, 1998). This study is based on the hypothesis that OSF-1 plays an important role in post-natal bone development, which is distinct from other growth factors like bone morphogenetic proteins (BMPs).

The present work was aimed at examining the role of OSF-1 in post-natal bone development by,

a. - determining the stage of osteogenic differentiation at which this factor was expressed in osteoblasts

b. - investigating effects of over-expression of the human *osf-1* gene on bone development in transgenic mice

c. - studying the distribution of the protein in bones of transgenic and control mice

d. - examining the effects of exogenous OSF-1 on chondrocyte development in organ culture systems

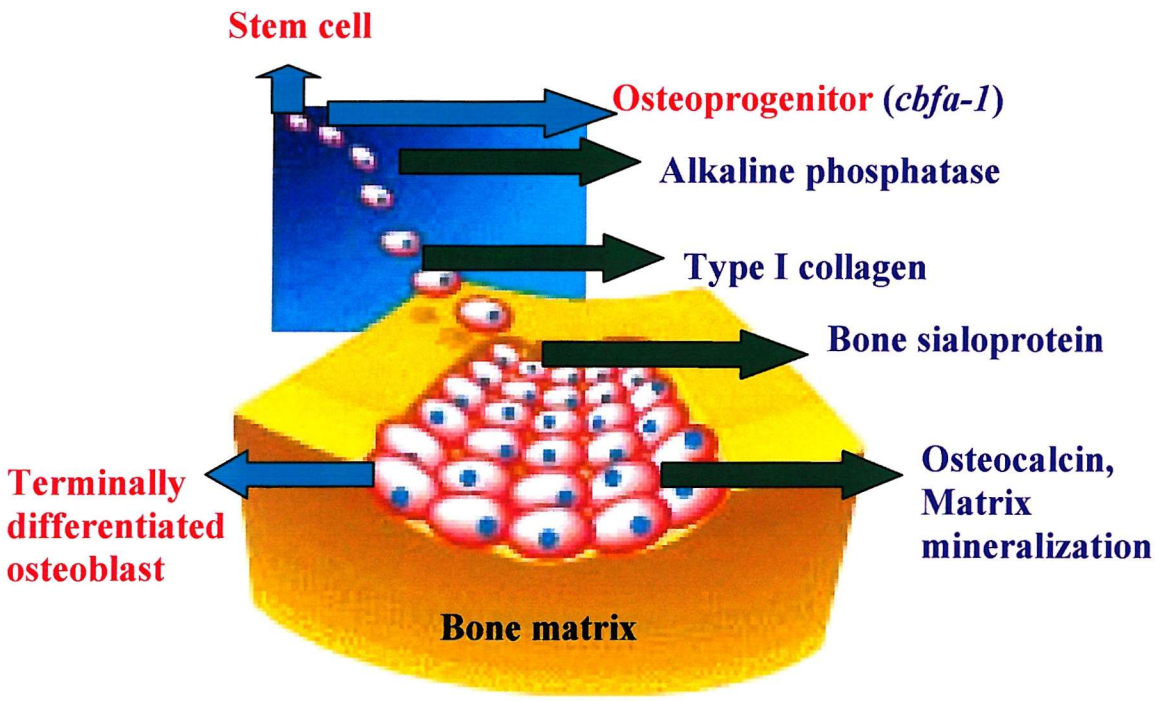
e. - exploring the osteogenic potential of OSF-1 with respect to its effect on osteogenic differentiation of bone marrow-derived cells, its possible role in initiating osteogenic differentiation and its interactions with BMPs.

OSF-1 PROTEIN EXPRESSION IN EMBRYONIC FEMUR

2.1 Introduction

The process of osteogenic differentiation is controlled by a cascade of molecular events involving a combination of genetic programming and gene regulation by various hormones, cytokines and growth factors. Significant advances have been made in recent years in identifying regulatory genes and molecular markers that define specific stages of osteoblast development (Fig. 11).

Figure 11. Temporal expression of osteogenic markers during the course of osteoblast differentiation (Adapted from Ostex information sheet)



Previous studies have demonstrated the synthesis of OSF-1 by osteoblasts and its deposition in the bone matrix (Tezuka *et al.*, 1990; Dreyfus *et al.*, 1998; Imai *et al.*, 1998; Petersen and Raifi, 2001). The present study was aimed at identifying the stage of osteoblast differentiation, in relation to the known osteogenic markers, at which OSF-1 was expressed.

2.2 Materials and methods

Reagents used in the study were of analytical grade and purchased from Sigma (Poole, UK) and BDH/ Merck Ltd. (Poole, UK), unless otherwise stated.

2.2.1 Histological analyses

a. Sample fixation, processing, paraffin wax embedding and section cutting

Fertilized chicken eggs (*Gallus domesticus*) were purchased from a registered supplier and incubated at 37^0C in an incubator (humidity controlled and with rotating trays, *Multihatch Automatic Incubator, Congresbury, England*) for 10 days. Femurs (n = 2) were dissected from 10-day-old chicken embryos, fixed overnight in 85% ethanol and processed immediately without decalcification. Samples were processed through graded alcohol (90%, 100% ethanol), twice in each concentration for an hour on the roller at room temperature. They were then put into the tissue processor (*Shandon Citadel 2000*) that carried them through chloroform (twice, an hour in each) and paraffin wax (twice, an hour in each). Samples were embedded in paraffin wax using the *Raymond Lamb Blockmaster III*. 6 μm thick sections were cut on the microtome (*Microm Hiedelberg, HM 330*).

b. Alcian blue/ Sirius red staining (Lison, 1954)

This staining technique was used to distinguish between cartilage and bone matrices, as Alcian blue stained the proteoglycan-rich cartilage matrix and Sirius red stained collagen matrix of bone.

Reagents

1. Weigert's haematoxylin
2. Acid alcohol
3. Alcian blue
4. Molybdophosphoric acid

5. Sirius red

Procedure

Sections were de-paraffinized using histoclear (twice, 7 min. in each), followed by rehydration in graded alcohols (100%, 90% and 50% methanol - 2 min. in each) and taken to water. Following nuclear staining with Weigert's haematoxylin (10 min.), sections were washed in water bath thoroughly for 10 min., de-differentiated in acid-alcohol, washed again and stained with Alcian blue for 10 min. Following a brief wash, sections were incubated in freshly prepared Molybdophosphoric acid for 20 min, washed and stained with Sirius red for an hour. Sections were washed, dehydrated through graded alcohols (50%, 90% and 100% methanol), taken through histoclear and mounted in DPX.

c. Alkaline phosphatase staining (Modified from Bancroft and Stevens, 1982 and described in Roach and Shearer, 1989)

The hydrolysis of a suitable substituted naphthol substrate by alkaline phosphatase produces an insoluble naphthol derivative, which then couples with a diazonium salt, like fast red TR, to produce a red coloured insoluble azo dye at the site of enzyme activity.

Reagents

1. Activating buffer (pH 7.4)
2. AS-B1 Phosphate stock solution
3. Fast red TR solution (0.1% in AS-B1 Phosphate stock soln., freshly prepared)
4. Alcian blue (0.5% in distilled water)
5. Methyl green (0.5% in 0.2 M Acetate buffer, pH 4.1)

Procedure

After de-paraffinizing the sections in histoclear, they were rehydrated through graded alcohols, taken to water and incubated in activating buffer over-night at room temperature. Freshly prepared Fast red TR solution in Naphthol AS-B1 Phosphate was filtered onto the sections, followed by an hour's incubation at 37^0C. Slides were washed in running water, counterstained with Methyl green (10 -15 min.) and Alcian blue (45 sec.), washed briefly and mounted in glycerol jelly.

d. Von Kossa method, counterstained with Van Gieson (Bancroft and Stevens, 1982)

This staining technique identified osteoid from mineralized bone matrix, which was stained brown-black.

Reagents

1% Silver nitrate

2.5% Sodium thiosulphate

Van Gieson stain

Procedure

After de-paraffinizing the sections in histoclear, they were rehydrated through graded alcohols, taken to water and incubated with 1% silver nitrate (1ml per slide) under ultraviolet light for 20 min. The sections were washed thoroughly in running water for 5 min. and incubated with 2.5% sodium thiosulphate for 8 min. After washing the sections thoroughly in running water for 5 min., they were stained with Alcian blue for a minute. and with the Van Gieson stain (filtered onto the sections) for 5 min. Excess stain was blotted using filter papers. Sections were dehydrated through 90% and 100% methanol, taken through histoclear and mounted in DPX.

e. Immunostaining

Reagents

1. 3% H_2O_2
2. 1% Phosphate-buffered bovine serum albumin
3. High salt wash buffer, pH 8.5
4. Low salt wash buffer, pH 8.5
5. 0.1 M Tris buffer, pH 8.5
6. H_2O_2, AEC soln.
7. Acetate buffer, pH 5
8. Primary antisera:

Rabbit polyclonal antibodies

i. Anti-type I collagen (LF-67, à human - raised against the human C-terminal propeptide of the α-1 chain and capable of detecting pro-α-1 chain as well as fully processed α-1 chain, cross-reacts with mouse, 1:300 dilution). A gift from Dr. Larry Fisher, NIH, USA.

ii. Anti-bone sialoprotein (à porcine, cross-reacts with mouse, 1:100 dilution, details in Chen *et al.*, 1991). A gift from Dr. J. Sodek, Toronto.

iii. Anti-OSF-1 (raised against the recombinant HB-GAM protein synthesized using the baculovirus expression system – Raulo *et al.*, 1992, 1:500 dilution). A gift from Dr. Heiki Rauvala, Finland.

9. Secondary antisera:

i. Goat anti-rabbit (biotin-linked, Dako E 0432, 1:300 dilution)

10. Extravidin peroxidase (Sigma E 2886, 1:50 dilution)
11. Light green
12. Alcian blue

Procedure

Sections were de-paraffinized, rehydrated through graded alcohols, taken to water and endogenous peroxidase activity was quenched by incubating the sections for 5 min. with 3% H_2O_2. Following a 5-min. incubation with 1% phosphate-buffered bovine serum albumin, sections were incubated with the relevant primary antiserum overnight at 4^0C. At the end of the incubation period, sections were washed thoroughly in the three wash buffers (high salt, low salt and Tris), and incubated for 30 min. at room temperature with the appropriate biotin-linked secondary antibody. After the routine washes in the three wash buffers, sections were incubated with Extravidin peroxidase for 30 min. at room temperature. The immune complex was visualized using hydrogen peroxide and 3-amino, 9-ethylcarbazole (AEC) in acetate buffer, yielding a brown reaction product. Sections were counterstained with Light green and Alcian blue, and mounted in glycerol jelly. Negative controls (omission of the primary antibody) were included every time. In addition, to confirm specificity of the anti-OSF-1 antibody, negative controls included blocking the antibody with a ten-fold higher concentration of the recombinant OSF-1 peptide (a gift from Dr. H. Rauvala, details in Raulo *et al.*, 1992). This abolished OSF-1 immunostaining, thereby confirming specificity.

2.3 Results

2.3.1 Stage of osteogenic differentiation associated with expression of the OSF-1 protein in osteoblasts

Femurs from 10-day-old chicken embryos were selected to determine the stage at which OSF-1 was expressed during *in vivo* osteogenic differentiation. The bones were predominantly cartilaginous at this stage of early development, with the primary ossification centre just beginning to develop in the diaphysis. In the section that was stained with the Von Kossa technique, mineralized bone matrix could be distinguished from the osteoid (unmineralized bone matrix), as mineralized bone matrix stained black-brown (Fig. 12 A). A region of osteoid was selected (marked by the rectangle in Fig. 12 B), in which it was possible to distinguish the outer fibroblastic (fl) and inner osteogenic (ol) layers of the periosteum above the osteoid (os) by Alcian blue/ Sirius red staining (Fig. 12 C). Sequential parallel sections were stained for alkaline phosphatase (ALP) and type I collagen, which served as markers of early osteogenic differentiation, and for bone sialoprotein (BSP) and matrix mineralization (by the Von Kossa technique), which served as late markers of osteogenic differentiation. Presence of ALP (Fig. 12 D) and type I collagen (Fig. 12 E), and absence of BSP (Fig. 12 F) and mineral (Fig. 12 G) in the bone matrix suggested an early stage of osteogenic differentiation. At this early stage of osteogenic differentiation, it was possible to immunolocalize OSF-1 in the osteoid (Fig. 12 H), suggesting that osteoblasts synthesized the OSF-1 protein during early stages of osteogenic differentiation.

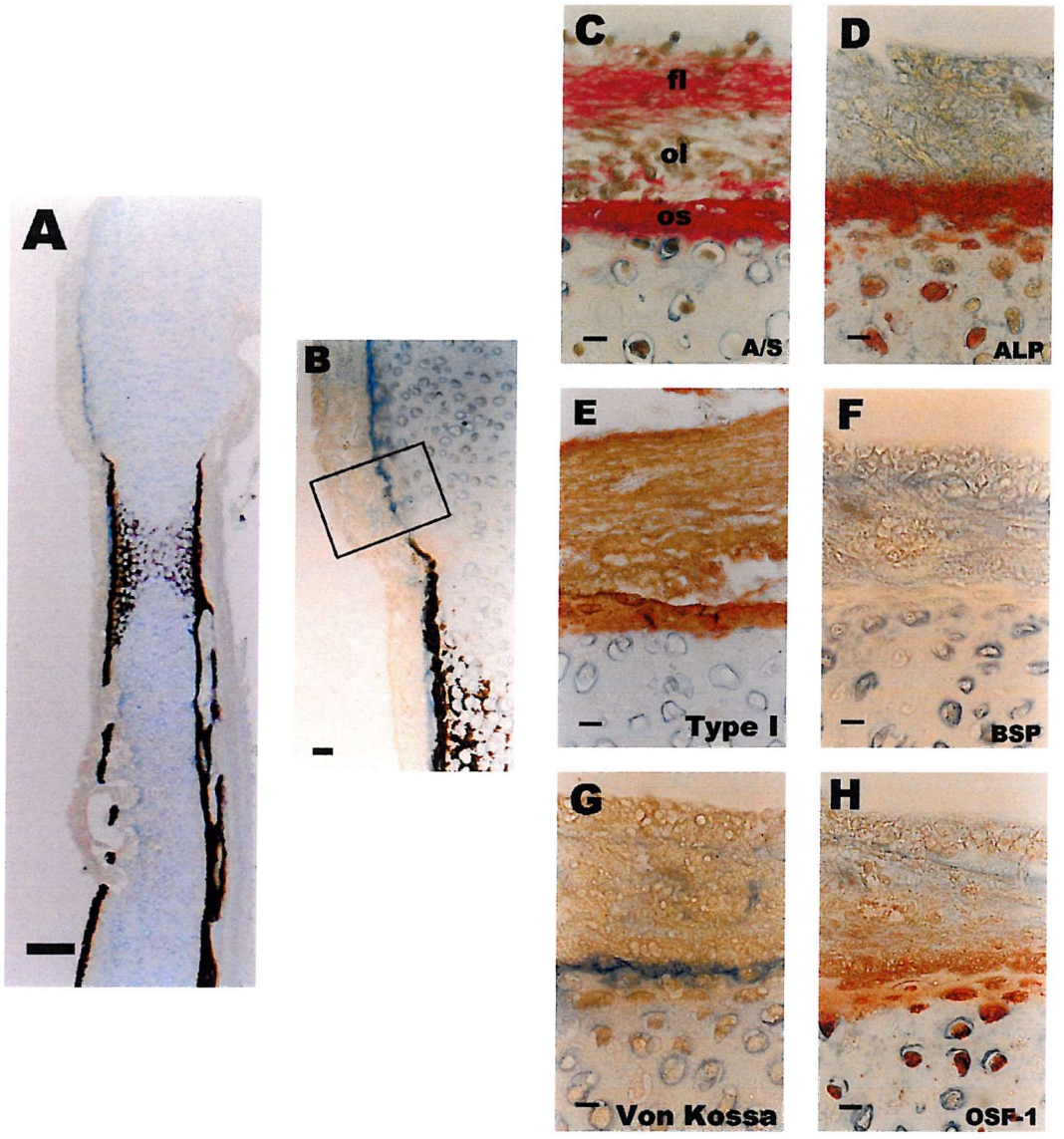


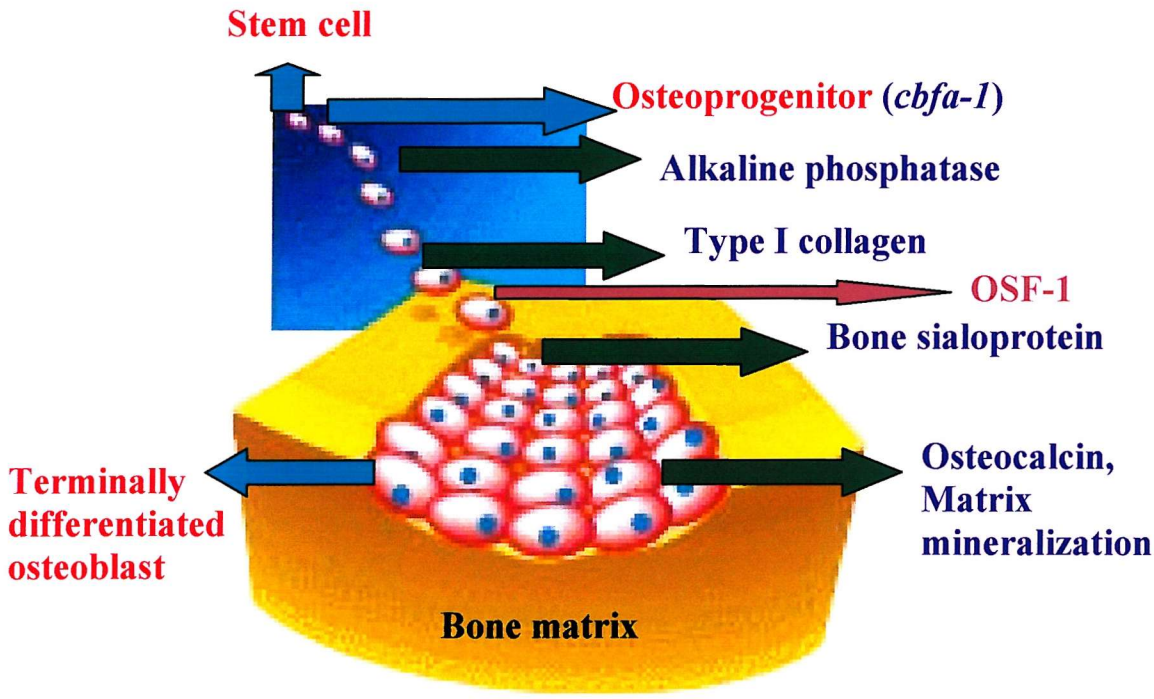
Figure 12. Sequential longitudinal sections of the region of new bone formation in a 10-day-old embryonic chick femur. *Von Kossa-stained overviews (A, B), illustrating the location of the region seen in C-H. Fibroblastic (fl) and osteogenic (ol) layers of the periosteum above the osteoid (os), illustrated in the section stained with Alcian blue/ Sirius red (C). Staining for alkaline phosphatase (ALP) was observed in the osteoid and few hypertrophoic chondrocytes (D). Type I collagen co-localized with the osteoid, and also extended as thin fibres throughout the periosteum (E). Bone sialoprotein (BSP) was absent (F), nor had the bone matrix mineralized, indicated by absence of staining with the Von Kossa technique (G). However, OSF-1 was present in the osteoid, the osteogenic layer of the periosteum and some hypertrophic chondrocytes (H). Scale bars = 250 μm for A; 50 μm for B; 10 μm for C-H.*

2.4 Discussion

Stages in the differentiation of mesenchymal stem cells along the osteoblast lineage (and resultant formation of mineralized bone matrix) are characterized by the temporal expression of specific osteogenic markers. Expression of these osteogenic markers is regulated in a defined manner along the differentiation route to a terminally differentiated osteoblast phenotype. Core-binding factor 1 (*cbfa1*), an osteoblast-specific transcription factor, is the earliest and most specific marker of the osteogenic lineage (Ducy *et al.*, 1997). Alkaline phosphatase expression has been shown to be high during early stages of osteogenic differentiation, the enzyme being synthesized by early osteoblasts together with the collagenous bone matrix, into which it is then extruded prior to matrix mineralization (Bernard, 1978; Bronckers *et al.*, 1987; Strauss *et al.*, 1990). Type I collagen is another marker which has shown to be upregulated during initial stages of osteoblast differentiation (Sandberg and Vuorio, 1987; Strauss *et al.*, 1990). Non collagenous proteins like bone sialoprotein, osteonectin, osteopontin are secreted by more differentiated osteoblasts, while terminally differentiated osteoblasts secrete osteocalcin, the only known bone-specific protein (Lian and Gundberg, 1988). Bone sialoprotein, expression of which is restricted to differentiated osteoblasts and cannot be detected in early osteoblasts (Bianco *et al.*, 1991), is a crystal nucleator whose presence in the osteoid heralds subsequent mineralization (Zhang *et al.*, 1990; Roach, 1994). In the present study, alkaline phosphatase, type I collagen and OSF-1 were immunolocalized in the osteoblast/ osteoid region in sequential sections of a 10-day-old embryonic chick femur. However, parallel sections did not stain for bone sialoprotein or for the presence of mineral by Von Kossa staining. This suggested that synthesis of OSF-1 by osteoblasts occurred during early stages of osteogenic differentiation, characterized by

the presence of alkaline phosphatase and type I collagen, and prior to the expression of bone sialoprotein and subsequent osteoid mineralization (Fig. 13). This finding was in agreement with the immunolocalization studies of Dreyfus and co-workers, who demonstrated the presence of OSF-1 in the osteoblast/ osteoid layer of developing chick tibia and suggested an involvement of the protein during early stages of osteogenesis (Dreyfus *et al.*, 1998).

Figure 13. Stage of osteoblast differentiation at which OSF-1 is expressed in relation to the osteogenic markers (Adapted from Ostex information sheet)



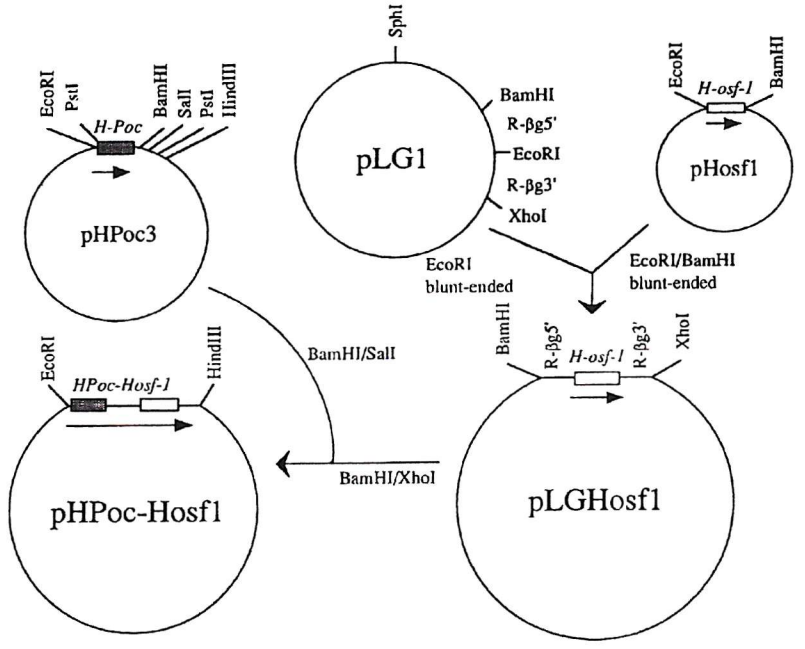
TRANSGENIC MICE OVER-EXPRESSING *osf-1*

3.1 Introduction

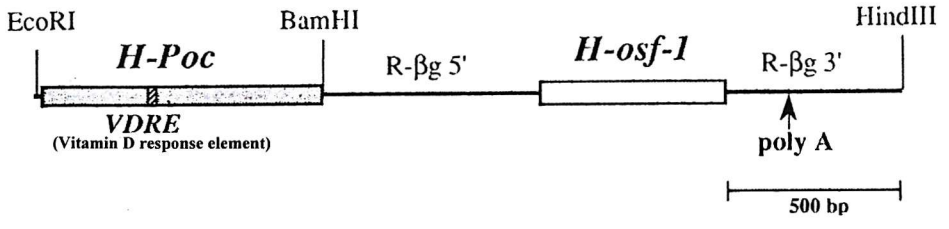
Previous studies have investigated the *in vivo* function of OSF-1 in mammalian bone metabolism by examining mice over-expressing the *osf-1* transgene. Masuda *et al.* (1997) generated transgenic mice over-expressing the human *osf-1* gene under the control of the human osteocalcin promoter, while Imai *et al.* (1998) generated transgenic mice over-expressing the rat *osf-1* gene under the control of the human β-actin promoter.

3.1.1 Transgenic mice over-expressing the human osf-1 gene (Masuda et al., 1997)

Figure 14. Construction strategy and structure of the transgenic human (H-) osf-1 gene (Adapted from Masuda et al., 1997)



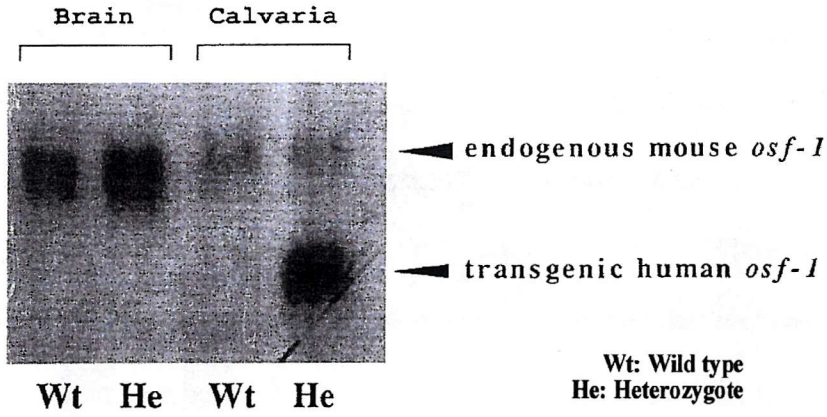
Arrows inside plasmid circles indicate transcriptional direction
R-β g5' and R- β g3' indicate the 5' and 3'-UTRs



1. Coding region for human *osf-1* cDNA (*H-osf-1* cDNA) was cloned between *EcoRI* and *BamHI* sites on plasmid pKF4, yielding pHosf1.
2. pHosf1 was excised, blunt-ended and recloned at the blunt-ended *EcoRI* site flanked by the 5'- and 3'-untranslated regions (UTRs) of the rabbit β-globin gene on pLG1, pLGHosf1.
3. The *BamHI* and *XhoI* fragment containing the *H-osf-1* coding region flanked by the rabbit 5'- and 3'-UTRs was excised and placed under the control of the human osteocalcin promoter (*H-Poc*) between the *BamHI* and *SalI* sites on pKF18k, pHPoc-Hosf1.
4. The *H-Poc-H-osf-1* transgene DNA fragment was excised by *EcoRI* and *HindIII* digestion, purified and microinjected into fertilized eggs of BDF-1 mice.

Three founder mice (F_0) harbouring the *H-osf-1* transgene were identified by Southern blot analysis of tail DNA samples and the F_1 heterozygotes were analysed for mRNA at 8 weeks of age.

Figure 15. Northern blot analysis of total RNA from brain and calvaria of osf-1 heterozygote and wild-type mice (Adapted from Masuda et al., 1997)



In two of the three lines, the transgene mRNA was detected specifically in the calvaria, whereas endogenous mouse *osf-1* transcripts were detected only in the brain and calvaria. One of the two lines, HPoc-Hosf1-6, carrying 6 transgene copies per haploid cell, was used for further studies.

3.1.2 Transgenic mice over-expressing the rat HB-GAM/ osf-1 gene (Imai et al., 1998)

HB-GAM cDNA, synthesized from purified mRNA isolated from 6-day-old postnatal rat brain (Merenmies and Rauvala, 1990), was cloned into the pHBApr-1-neo vector, which contained the promoter and first intron from human β-actin gene and the SV-40 polyadenylation signal. Western blot analysis of different neonatal tissues (postnatal day 1) from a transgene-positive mouse and its transgene-negative littermate, revealed intense protein expression in the heart and skeletal muscle. HB-GAM expression, detected by Western blot analysis in tissues of 1-yr-old transgenic mice, was highest in the periosteum.

3.1.3 Transgenic mouse model used in the present study

Transgenic mice, generated by Masuda, Hashimoto-Gotoh *et al.* (1997), were obtained from Japan. These mice were generated by inserting 6 extra copies of the human *osf-1* gene, under the control of the human osteocalcin promoter, in the BDF-1 strain of mice. In a separate study by Kesterson *et al.* (1993), the human osteocalcin promoter (used to drive the expression of *osf-1* in transgenic mice) was shown to direct expression of the chloramphenicol acetyltransferase (CAT) fusion gene to bone, namely osteoblasts and maturing chondrocytes, and unexpectedly to the brain. Osteocalcin is the most osteoblast-specific marker and expression of the osteocalcin gene is restricted to mature osteoblasts, although a very low-level expression (one hundredth of that observed for mature osteoblasts) can be detected in some growth plate chondrocytes of the maturing and

hypertrophic zones (Aizawa *et al.*, 1998; Lian *et al.*, 1993). Thus, over-expression of the *osf-1* transgene in these mice was targeted to skeletal tissues and brain.

In the HB-GAM transgenic mice generated by Imai *et al.* (1998), since the transgene was placed under the control of the β-actin promoter (expressed in a wide-range of tissues), over-expression of the *osf-1* transgene was not restricted to skeletal tissues and likely to occur in a wide variety of cells, including endothelial cells. Taking into consideration the angiogenic role of OSF-1 (Laaroubi *et al.*, 1994; Papadimitriou *et al.*, 2001) and the likelihood of *osf-1* over-expression in endothelial cells, enhanced angiogenesis could most likely make an indirect contribution to the bone formation process in these transgenic mice.

Thus, mice over-expressing the *osf-1* transgene under the control of the osteocalcin promoter served as better models in defining the direct effects of *osf-1* over-expression on the skeletal system, than mice over-expressing the transgene under the control of the β-actin promoter. Since the osteocalcin promoter was capable of directing over-expression of the *osf-1* transgene only to skeletal tissues and brain, any indirect effects of enhanced angiogenesis on bone formation could be eliminated.

3.2 Materials and methods

A total of 300 transgenic mice and 250 control mice were used in the study. BDF-1 mice, representing the F_1 generation hybrid progeny of C57 Bl/6/Ola/Hsd (black) and DBA/2/Ola/Hsd (grey) strains of mice, served as the control group in the present study. OSF-1 transgenic and BDF-1 control mice were bred under licence from the Home Office, in accordance with the Animals (Scientific Procedures) Act (1986). All mice were bred and maintained at the University of Southampton Biomedical Research Facility, and housed in appropriate environments maintained at 22^0 C with 12h: 12h light: dark cycle. The mice had free access to water and were fed *ad libitum*.

Bones from control and transgenic mice were analyzed for differences in organic and inorganic content, and examined histologically. Biochemical reagents used in the study were of analytical grade and purchased from Sigma (Poole, UK) and BDH/ Merck Ltd. (Poole, UK), unless stated otherwise.

3.2.1 Analytical measurements

Analytical measurements were carried out on femurs dissected from transgenic and control mice at 5-week intervals between 5 and 30 weeks.

a. Dry weight of femurs

Femurs were dried at 70^0C in a warming cupboard (*Raymond Lamb*) for 3-4 weeks. They were then weighed on a semi-microbalance (Sartorius), which had a precision of 0.01 mg. To ensure accurate measurement of dry weight, femurs were weighed at regular intervals during the 3-4 week period and the dry weight was noted only when the readings varied by less than 1%. Mean femoral dry weights, during the growth period between 5 and 30 weeks, were used for comparing bone growth trajectories between control and transgenic mice.

Once the dry weight had been noted, femurs were hydrolyzed overnight in 6N HCl (2 ml HCL/ femur) at 105^0C in a heating block. The hydrolysates were then used for estimation of hydroxyproline and calcium content.

b. Hydroxyproline assay (Modified from Roach et al., 1985)

Principle

When hydroxyproline is partially oxidized by chloramine T, it loses its hydroxyl group and is converted to pyrrole-2-carboxylic acid, which inturn reacts with Ehrlich's reagent to give a stable chromophore with a wavelength peak at 560 nm.

Reagents

1. Citrate buffer
2. Chloramine T (1.4 g/dl)
3. Ehrlich's reagent
4. Hydroxyproline stock std. Soln. (5mM)

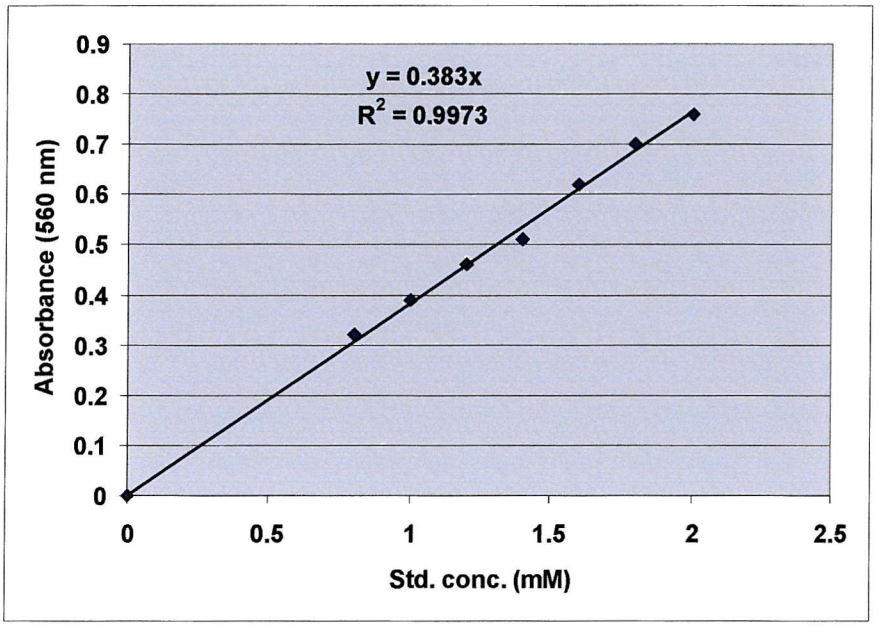
   Working std. Soln. ---- 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mM

Procedure

20 µl aliquots of the femoral hydrolysates were dried down by overnight evaporation at 105^0C. 1 ml citrate buffer was added to the residue left behind, shaking the tubes continuously during this addition. The tubes were incubated for 5-30 min. 250 µl chloramine T reagent was then added and the tubes were incubated for exactly 15 min. after shaking them well. Further oxidation by chloramine T was inhibited by decreasing temperature of the reaction mixture, by placing the tubes on ice. 2 ml of Ehrlich's reagent was added to each of the tubes, which were shaken well and incubated on ice overnight. Absorbance was read on the spectrophotmeter at 560 nm the following day. Standards were

treated similarly and a linear standard curve plotted. Concentrations of samples were extrapolated from the standard curve.

Figure 16. Standard plot for hydroxyproline estimation



c. Calcium assay (Diagnostic kit from Sigma)

Principle

Calcium reacts with arsenazo III in an acidic medium yielding the calcium-arsenazo III complex, with a wavelength peak at 600 nm.

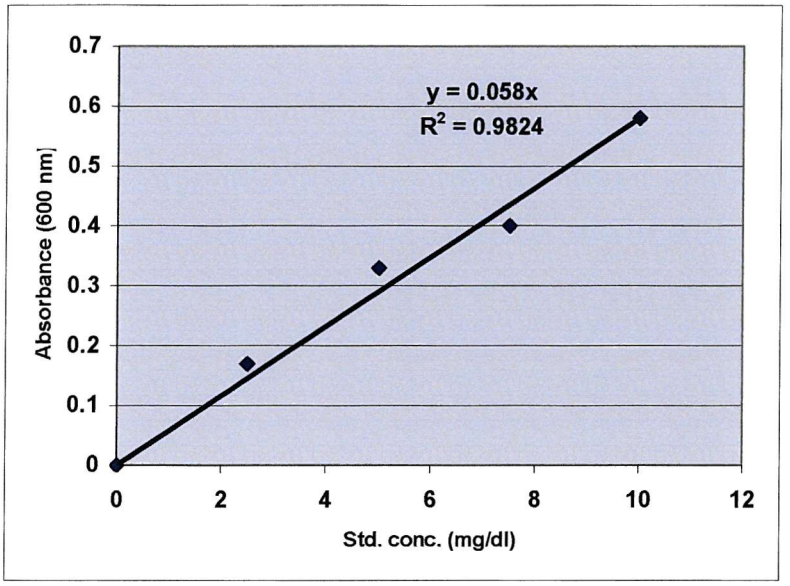
Reagents

1. Calcium reagent (Arsenazo III), Sigma Cat. No. 588-3P
2. Calcium stock std. 10 mg/dl, Sigma Cat. No. 360-5
3. Working std. solutions ---- 2.5, 5, 7.5 mg/dl

Procedure

Sample hydrosylates were diluted 1 in 20 and 10 µl of the diluted hydrolysate was used for the assay.1 ml of the arsenazo III reagent was then added to the diluted hydrolysate, mixed well, and absorbance measured at 600 nm.1 ml of the arsenazo III reagent was added to 10 µl of each working std. soln. and the absorbances used to plot a linear std. curve. Concentrations of samples were extrapolated from the std. curve.

Figure 17. Standard plot for calcium estimation



To compensate for differences in femoral dry weights between control and transgenic mice, and to determine the concentration of hydroxyproline/ calcium in 1 mg (equivalent to 1 unit) of dry bone, both measurements were expressed per mg dry femoral weight.

3.2.2 Histological analyses

Femurs and tibiae from 70 mice, aged 2.5 weeks to 30 weeks, were used for histological studies, with an average of 9 (range: 7-14) mice for each age group. In addition, calvarial bones were dissected from 6-day-old mice.

a. Sample fixation and decalcification

The long bones were fixed overnight either in 4% phosphate-buffered paraformaldehyde, pH 7.4 or in 85% ethanol, the latter fixative being essential for immunostaining with the anti-OSF-1 antibody. Paraformaldehyde-fixed samples were decalcified at 4^0C over a period of 3-5 weeks in 5% EDTA in 0.1M Tris-HCl buffer, pH 7.2. Alcohol-fixed samples were processed immediately without decalcification.

Each calvaria, dissected from 6-day-old transgenic and control mice, was cut into two halves along the sagittal suture. They were then fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) overnight and decalcified in 5% EDTA in 0.1M Tris-HCl buffer (pH 7.2) over a period of 7 days.

b. Sample processing, paraffin wax embedding and section cutting

Samples were processed through graded alcohols (50%, 90%, 100% ethanol), twice in each concentration for a hour on the roller at room temperature. They were then put into the tissue processor (*Shandon Citadel 2000*) that carried them through chloroform (twice, an hour in each) and paraffin wax (twice, an hour in each). Samples were then embedded in paraffin wax using the *Raymond Lamb Blockmaster III*. 6 μm thick sections were cut on the microtome (*Microm Hiedelberg, HM 330*).

c. Alcian blue/ Sirius red staining

(Refer to ***section 2.2.1 b*** *for details of method)*

d. Immunostaining

(Refer to ***section 2.2.1 e*** *for details of method)*

Primary antisera:

Rabbit polyclonal antibodies

i. Anti-type I collagen (LF-67, à human – raised against human C-terminal propeptide of the α-1 chain and capable of detecting pro-α-1 chain as well as the fully processed α-1 chain, cross-reacts with mouse, 1:300 dilution)

ii. Anti-osteonectin (LF-8, à chick, cross-reacts with mouse, 1:300 dilution)

Both antisera were gifts from Dr. Larry Fisher, NIH, USA.

iii. Anti-bone sialoprotein (à porcine, cross-reacts with mouse, 1:100 dilution, details in Chen *et al.*, 1991) was a gift from Dr. J. Sodek, Toronto.

iv. Anti-osteocalcin (à bovine, cross-reacts with mouse, 1:200 dilution) was a gift from Dr. Simon Robins, Aberdeen, UK.

v. Anti-OSF-1 (raised against the synthetic N-terminal peptide of HB-GAM isolated from perinatal rat brain, details in Rauvala, 1989, 1:500 dilution) was a gift from Dr. Heiki Rauvala, Finland.

Secondary antisera:

Goat anti-rabbit (biotin-linked, Dako E 0432, 1:300 dilution)

e. Measurement of calvarial thickness

Sections of calvariae were stained with Alcian blue/ Sirius red. Representative sections from 5 animals in each group were selected, photographed and calvarial width/ thickness

was measured in three different areas along the length of each calvaria. The mean value of the three readings was taken as the calvarial thickness for each sample.

Statistics.

Samples were run in triplicate for all assays. Statistical analysis was performed using the GraphPad InStat Software (GraphPad Software Inc., San Diego, California, USA), applying the unpaired student's t-test.

3.3 Results

3.3.1 Differences in bone growth and composition

To investigate whether *osf-1* over-expression affected overall bone growth and composition, femurs of transgenic and control mice were analyzed at 5-week intervals during the growth period between 5 and 30 weeks. Femoral dry weights were determined as an indication of overall bone weight. Collagen content was measured by assaying hydroxyproline to determine the amount of organic bone matrix, and calcium was assayed to determine bone mineral content.

Table 1. Dry weights of femurs in control and transgenic mice

The mean dry weights are given in mg (± SD). N = number of femurs. Significance levels, using the unpaired student's t-test are: *** = $p < 0.0001$; ** = $p < 0.001$; * = $p < 0.05$

Age groups (Weeks)	Control Females	Transgenic Females	Control Males	Transgenic Males
5	26.1 (1.3) N = 8	25.2 (0.8) N = 6	26 (0.4) N = 8	25.4 (0.5) N = 8
10	37.8 (1.8) N = 10	36.6 (3.0) N = 20	44.3 (3.0) N = 10	43.8 (5.0) N = 16
15	51.1 (3.0) N = 25	44.1 (4.0) *** N = 24	63.3 (2.9) N = 18	51.5 (5.8) *** N = 30
20	51.8 (5.3) N = 22	49.1 (4.3) N = 34	62.5 (3.5) N = 16	56.7 (6.6) ** N = 38
25	51.5 (3.6) N = 25	51.1 (2.5) N = 26	65.0 (4.1) N = 27	60.4 (3.3) * N = 16
30	56.4 (2.8) N = 8	52.5 (5.0) * N = 14	64.9 (3.1) N = 10	62.2 (6.6) N = 11

a. Bone weight:

At 5 weeks, there were no differences in the mean femoral dry weights of control and transgenic mice, neither were there differences between males and females with respect to their femoral dry weights. By 10 weeks, the more rapid growth rates of males had become apparent, but no differences were observed in the femoral dry weights between controls and transgenics. From 10 weeks onwards, the bones of females always weighed less than the bones of males. At 15 weeks, the femoral dry weights of transgenic mice were significantly *lower* than those of controls in both males and females. At 20-25 weeks, the magnitude of this difference decreased, and was only significant in males. By 30 weeks, the transgenic mice had caught up with the controls and dry weights were again similar.

b. Bone growth:

To compare bone growth trajectories between control and transgenic mice (Fig. 18), mean femoral dry weight at 5 weeks was taken as baseline to calculate the % increases in bone weights of both females and males between 5 and 30 weeks. The rates of bone growth were more or less uniform between 5 and 10 weeks in control and transgenic mice. In control mice, bone growth was remarkably rapid between 10 and 15 weeks, which represented the pubertal growth spurt. However, after 15 weeks the growth trajectories were found to plateau in both males and females.

In transgenic mice, overall rate of bone growth was slower in comparison to that in control mice. The pubertal growth spurt between 10 and 15 weeks was not marked. However, bone growth in transgenic mice continued for a longer duration of time, until 20-25 weeks, opposed to the 15 weeks for control mice.

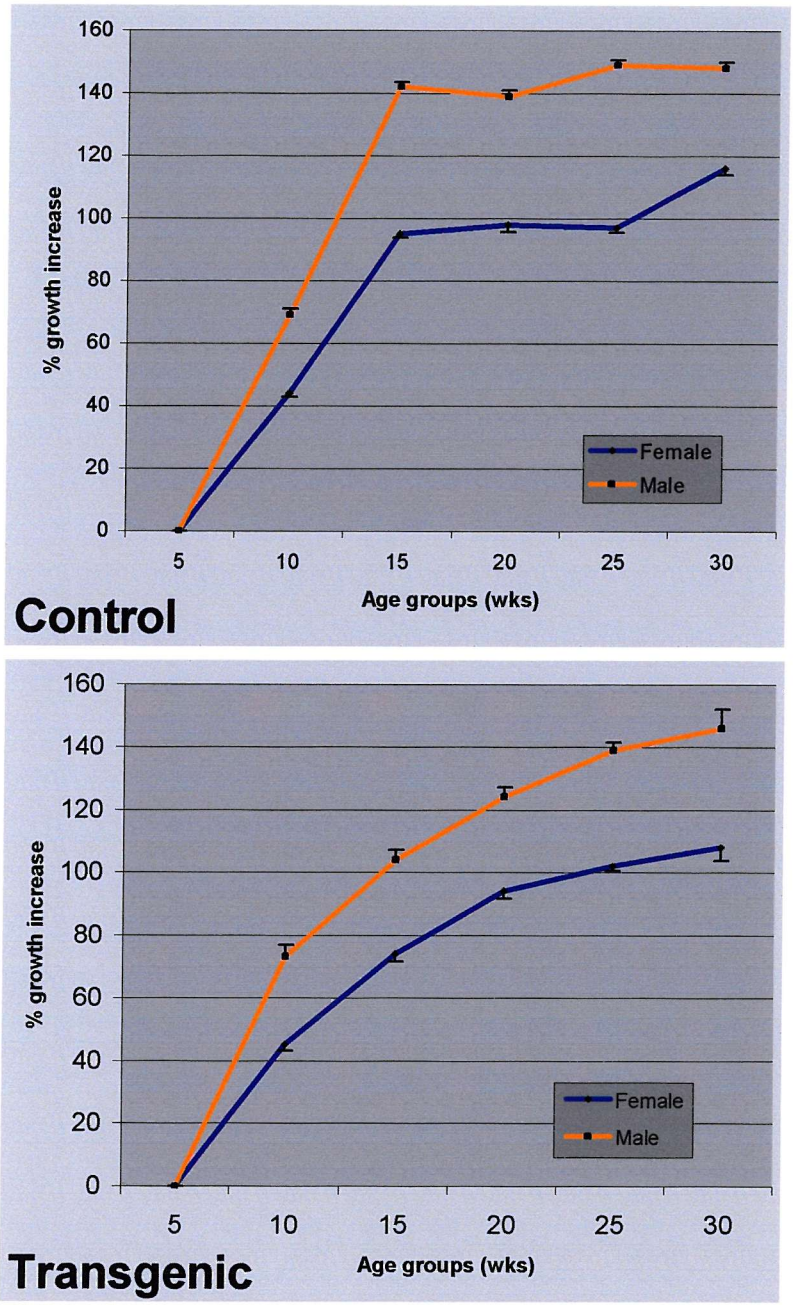


Figure 18. Bone growth trajectories in control and transgenic mice. *Dry weights of femurs between 5 and 30 weeks were used to plot bone growth curves, with mean femoral dry weight at 5 weeks taken as baseline. Bone growth was remarkably rapid in control mice between 10 and 15 weeks, after which their bone growth curves were found to plateau. Overall rate of bone growth was slower in transgenic mice, but bone growth continued for longer, until 20-25 weeks, in comparison to 15 weeks for control mice.*

Table 2. Hydroxyproline content per unit dry femoral weight

Results are expressed as μg/ mg bone, mean (± SD). N = 8 for each group.

Age groups (Weeks)	Control Females	Transgenic Females	Control Males	Transgenic Males
10	61 (4)	62 (6)	73 (2)	70 (3)
15	52 (3)	50 (2)	60 (3)	56 (2)
20	78 (6)	72 (7)	71 (9)	71 (10)
25	72 (9)	66 (10)	63 (6)	61 (5)
30	62 (3)	60 (7)	62 (5)	64 (2)

c. Organic bone matrix

Organic bone matrix was determined by assaying hydroxyproline as a measure of bone collagen. Hydroxyproline content was expressed per mg dry bone weight in order to compensate for the differences in femoral dry weights of control and transgenic mice (Table 2). There were no significant differences between control and transgenic mice with respect to their femoral hydroxyproline content/ unit dry femoral weight.

d. Bone mineral content

Calcium content was assayed as an index of bone mineral. It was also expressed per mg dry bone weight in order to compensate for the differences in femoral dry weights between control and transgenic mice (Fig. 19). The calcium content/ mg dry bone was found to be ~ 10 % higher in male transgenic mice compared to their male control counterparts at 15, 25 and 30 weeks. However, a difference was not observed at 20 weeks. The reason for this is not clear and maybe attributed to the biological variability observed within the hybrid strain of BDF-1 mice. A similar trend was observed for female mice, however the differences were not statistically significant.

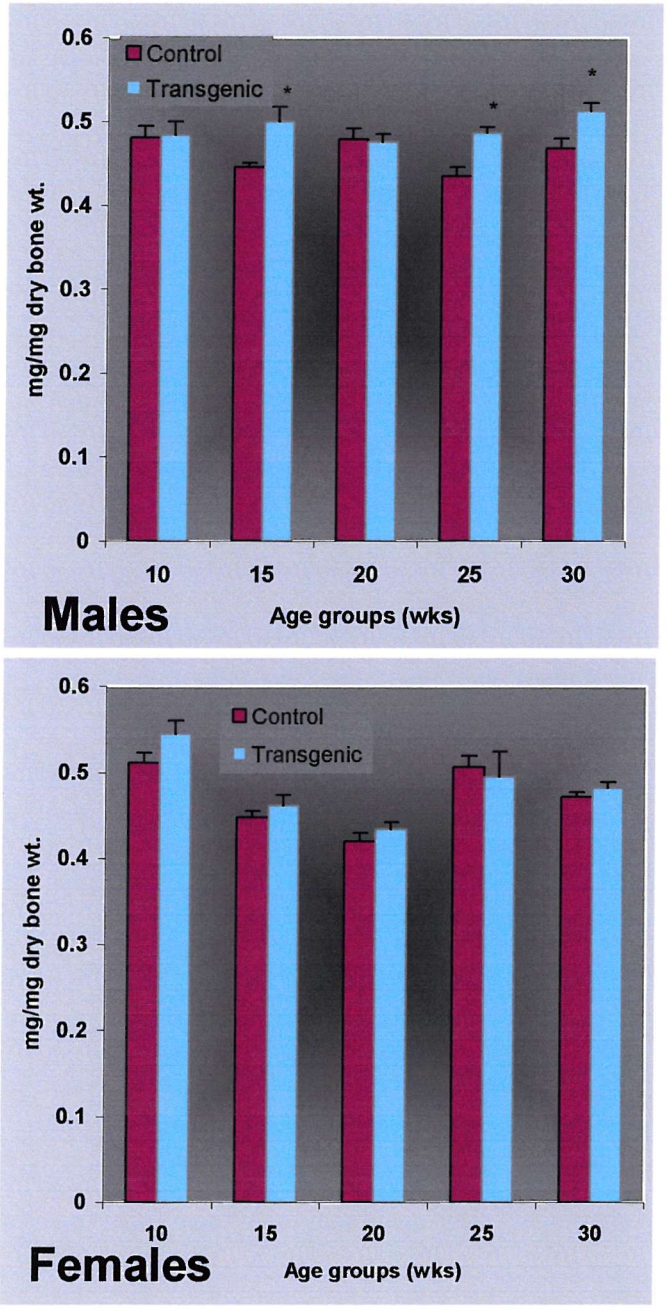


Figure 19. Calcium content per unit femoral dry weight (expressed as mg calcium/ mg femoral dry weight) in control and transgenic mice. *Male transgenic mice had a significantly higher calcium content/ mg femoral dry weight at 15, 25 and 30 weeks in comparison to their male control counterparts. Differences in calcium content/ mg femoral dry weight were not statistically significant for female mice. Results are expressed as mean* $\pm SE$, * = $p < 0.05$, $N = 8$.

3.3.2 Differences in bone and cartilage structure

Sections of femurs, tibiae and calvariae from control and transgenic mice, between 6 days (postnatal) to 15 weeks, were examined histologically for differences in bone and cartilage morphology.

a. Long bones

Overview of distal ends of femurs from 15-week-old male control and transgenic mice illustrated that femurs of transgenic mice (Fig. 20 B) were smaller than those of control mice (Fig. 20 A). This was in keeping with their low dry weights at 15 weeks. However, there were no differences in the height of the growth plate or thickness of the bone shaft (Fig. 20 A and B). Neither was there a difference between control and transgenic mice in the amount of spongiosa (Fig. 20 C and D). The same was reflected by the quantitative data of femoral hydroxyproline content (a measure of organic bone matrix), which was identical for control and transgenic mice at 15 weeks.

b. Calvarial (flat bones)

Over-expression of the *osf-1* transgene had a pronounced effect on the growth of calvariae/ flat skull bones of 6-day-old neonatal mice. When the width of calvarial bone was measured, mean (± SD) width of calvarial bone in transgenic mice was 46.4 ± 3.5 μm (n = 5), in comparison to 24.3 ± 4.6 μm (n = 5) as the mean (± SD) width of calvarial bone in control mice. Thus, the width of calvarial bone in transgenic mice (Fig. 21 C) was almost twice that in control mice (Fig. 21 A), suggesting that calvariae of transgenic mice were thicker than those of control mice. There was also a pronounced irregular appearance to the calvarial bone of transgenic mice (Fig. 21 D), when compared to that of control mice (Fig. 21 B).

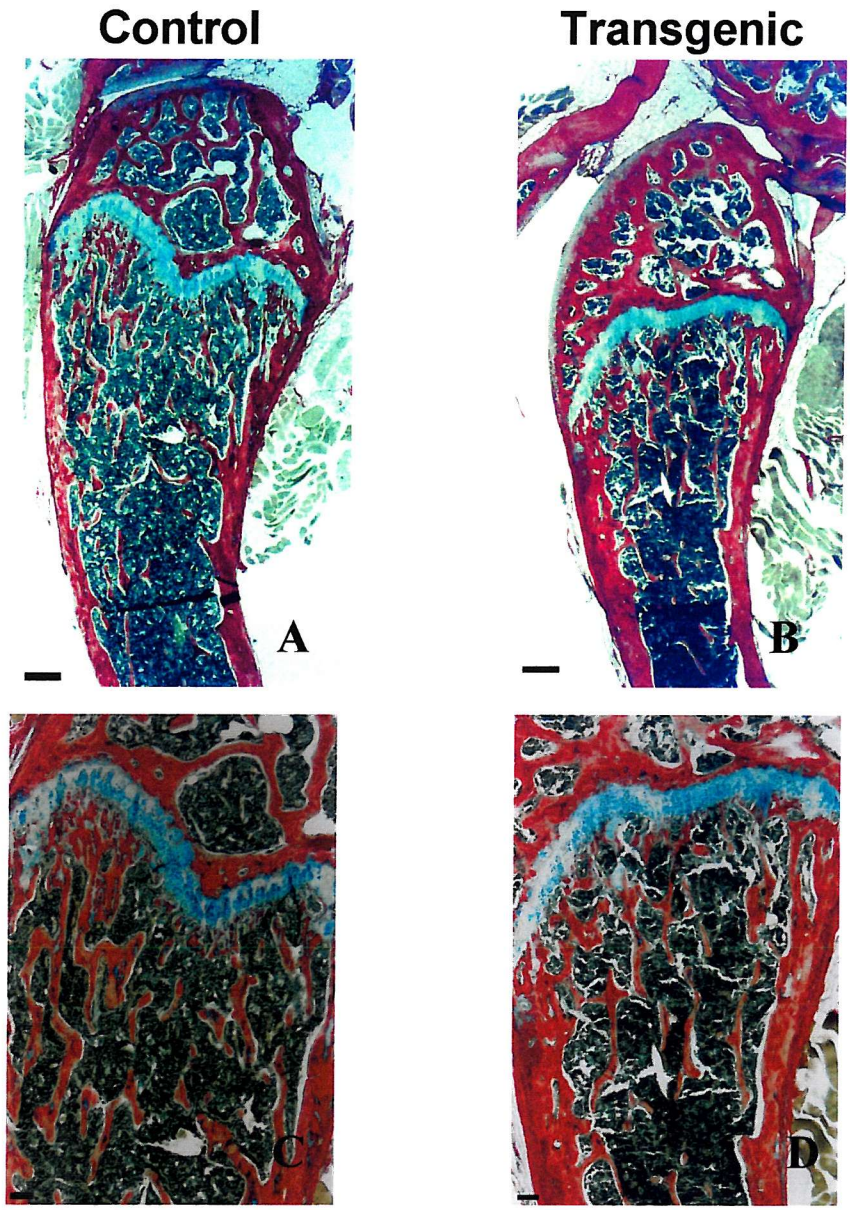


Figure 20.Overview of distal ends of femora from 15-week-old male control and transgenic mice, stained with Alcian blue/ Sirius red*. Femurs of transgenic mice (B) were smaller than those of control mice (A), however, there were no differences between control and transgenic mice in the height of the growth plate, thickness of the bone shaft or the amount of spongiosa (C and D). Scale bars = 200 μm for A and B; 100 μm for C and D.*

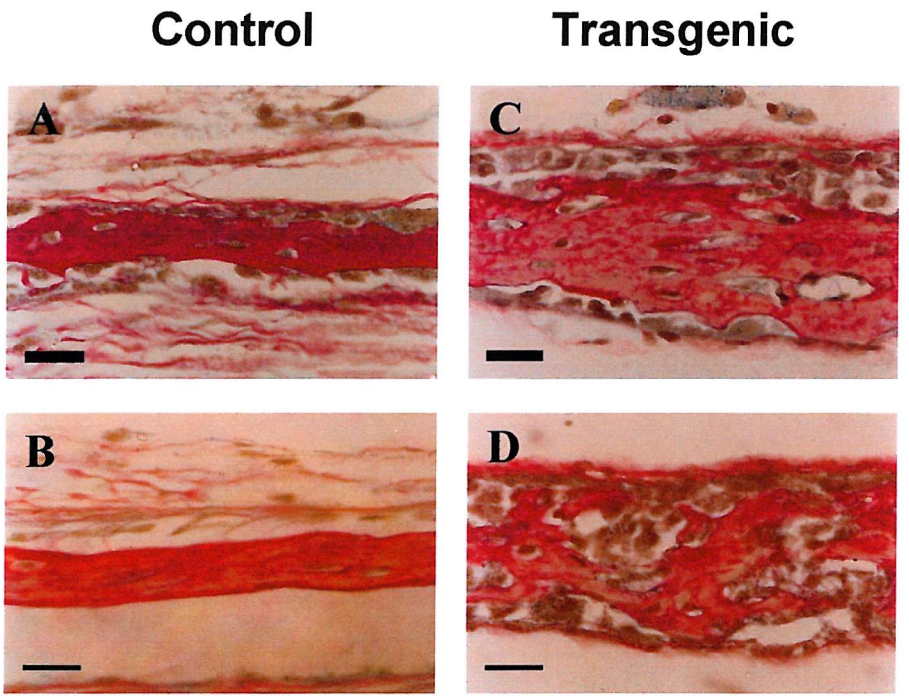


Figure 21. Cross-sections through calvariae of 6-day-old neonatal control and transgenic mice, stained with Alcian blue/ Sirius red and photographed at the same magnification. *Width of calvarial bone, stained with Sirius red, in transgenic mice (C) was almost twice the width of that in control mice (A). In some regions, calvarial bone in transgenic mice had a pronounced irregular appearance (D) in comparison to that in control mice (B). Scale bars = 20 μm.*

c. Articular cartilage

Over-expression of *osf-1* was found to alter the chondrogenic phenotype of some articular chondrocytes of transgenic mice. It was possible to demonstrate by immunostaining the synthesis of type I collagen, a bone matrix protein, by some articular chondrocytes of 15- (in 3 mice out of 8) and 20 -week-old (in 2 mice out of 8) transgenic mice (Fig. 22 F). Chondrocytes of control mice were never immunopositive for type I collagen (Fig. 22 E). To determine whether synthesis of type I collagen by articular chondrocytes of transgenic mice was indicative of early osteogenic differentiation, expression of other osteogenic markers was studied in articular chondrocytes of 15-week-old mice. These osteogenic markers included the non-collagenous proteins, bone sialoprotein, osteonectin and osteocalcin. Expression of bone sialoprotein was observed in articular chondrocytes of transgenic (Fig. 22 B) as well as control (Fig. 22 A) mice. Similarly, osteonectin was found to be present in articular chondrocytes of both transgenic (Fig. 22 D) and control (Fig. 22 C) mice. Since articular chondrocytes of control and transgenic mice were expressing osteogenic markers like bone sialoprotein and osteonectin, they could be considered to be some way down the osteogenic differentiation pathway. However, it was only in transgenic mice that over-expression of *osf-1* was found to induce the synthesis of type I collagen by some articular chondrocytes. Synthesis of osteocalcin, a marker of terminally differentiated osteoblasts, was however not observed in articular chondrocytes of transgenic mice. Encroachment of underlying subchondral bone (identified in the section by the presence of type I collagen) into the region of the articular cartilage was observed in some 15- (1 mouse out of 8) and 20-week-old (3 mice out of 8) transgenic mice (Fig. 23 A). On closer

inspection, distinct islands of articular chondrocytes entrapped in type I collagen-rich matrix were identified in the zone of encroachment (Fig. 23 B). The synthesis and secretion of this type I collagen-rich matrix could be attributed to the articular chondrocytes, since it was not possible to detect any vascular channels in the vicinity of these islands.

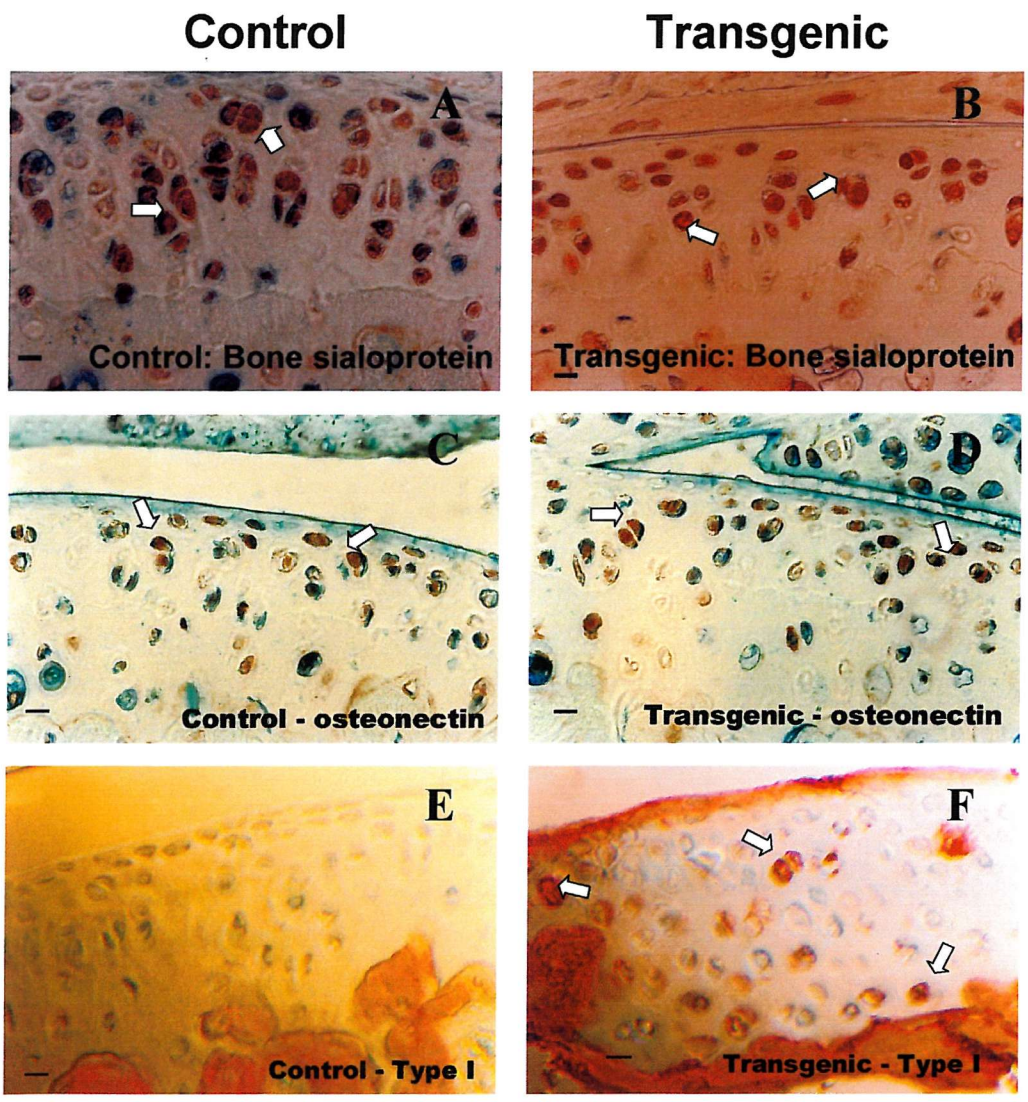


Figure 22. Articular cartilage surfaces at distal ends of femurs of 15-week-old control and transgenic mice, immunostained for bone sialoprotein, osteonectin, type I collagen and counterstained with Alcian blue/ Light green. *Positive immunostaining for the antigens*

examined was indicated by the presence of a brown reaction product in chondrocytes (arrowheads). Presence of bone sialoprotein was detected in articular chondrocytes of control (A) and transgenic (B) mice. Similarly, osteonectin was immunolocalized in articular chondrocytes of both control (C) and transgenic mice (D). Presence of type I collagen was not detected in any articular chondrocytes of control mice (E). However, it was only in transgenic mice that some articular chondrocytes were found to synthesize type I collagen (F). Scale bars = 10 μm.

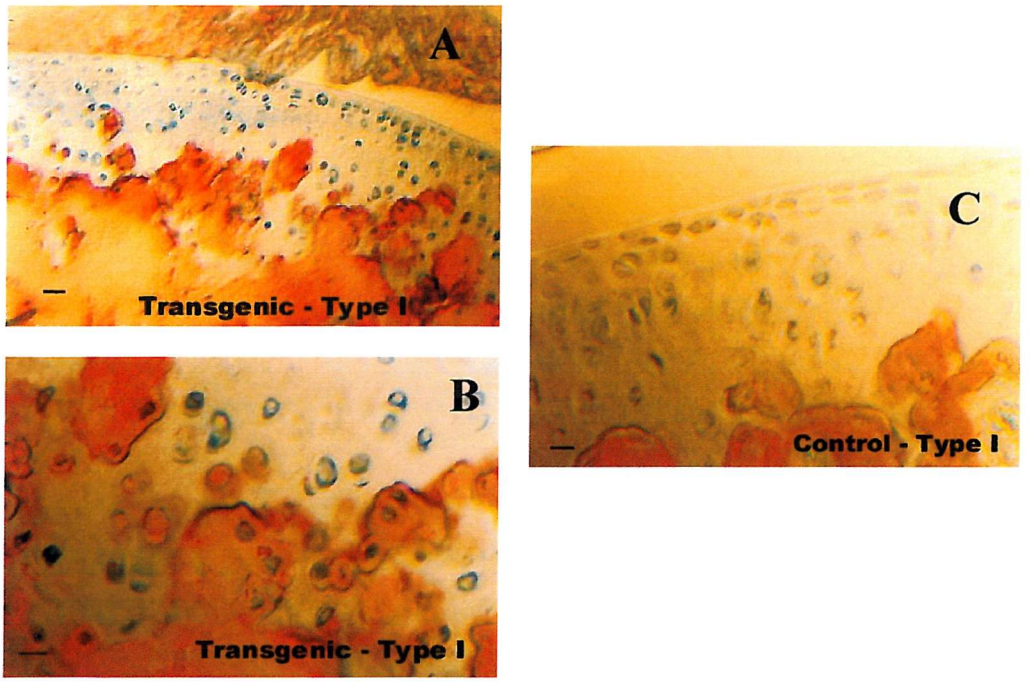


Figure 23. Articular cartilage surfaces and underlying subchondral bone at distal ends of femora of 15-week-old transgenic and control mice, immunostained for type I collagen. *Subchondral bone, characterized by the presence of type I collagen, was found to encroach upon the region of articular cartilage in transgenic mice (A). Distinct islands of articular chondrocytes entrapped in a type I collagen-rich matrix, were observed in the zone of encroachment (B). Encroachment of subchondral bone, stained for type I collagen, into the articular cartilage was not observed in control mice (C). Scale bars = 25 μm for A; 10 μm for B and C.*

3.3.3 Immunolocalization of OSF-1 in bone and cartilage

Results for OSF-1 immunostaining are with an antibody that was raised against the synthetic N-terminal peptide of OSF-1 isolated from perinatal rat brain (Rauvala, 1989). Antigenicity was observed only in ethanol-fixed sections.

a. Bone

There were no differences between control and transgenic mice with respect to the localization of OSF-1 in bone tissue.

Using the femur of a 2.5-week-old transgenic mouse as an example, sites of OSF-1 localization in bone were identified by immunostaining. Presence of OSF-1 was detected in the 'ossification groove of Ranvier' (Fig. 24 A) and the periosteum, especially in the inner layer of osteogenic cells (Fig. 24 B). Immunostaining for OSF-1 was observed in the palisade of osteoblasts, among the bone trabeculae of the spongiosa (Fig. 24 C). The protein was also found to be present in the new bone matrix laid down by osteoblasts on the unresorbed struts of growth plate cartilage during the process of endochondral ossification (Fig. 24 D), and in the bone matrix of the primary spongiosa (Fig. 24 E). However, mineral deposits overlaying the bone matrix were found to mask the OSF-1 epitope and staining for OSF-1 in the bone matrix was only evident when this overlaying mineral was removed by incubating the sections with EDTA (5 % EDTA in 0.1M Tris-HCl buffer, pH 7.2) for 5-8 hours prior to immunostaining. It was also possible to detect an occasional osteocyte that was immunopositive for OSF-1, suggesting continued expression of the protein by osteoblasts even at the osteocyte stage (Fig. 24 F).

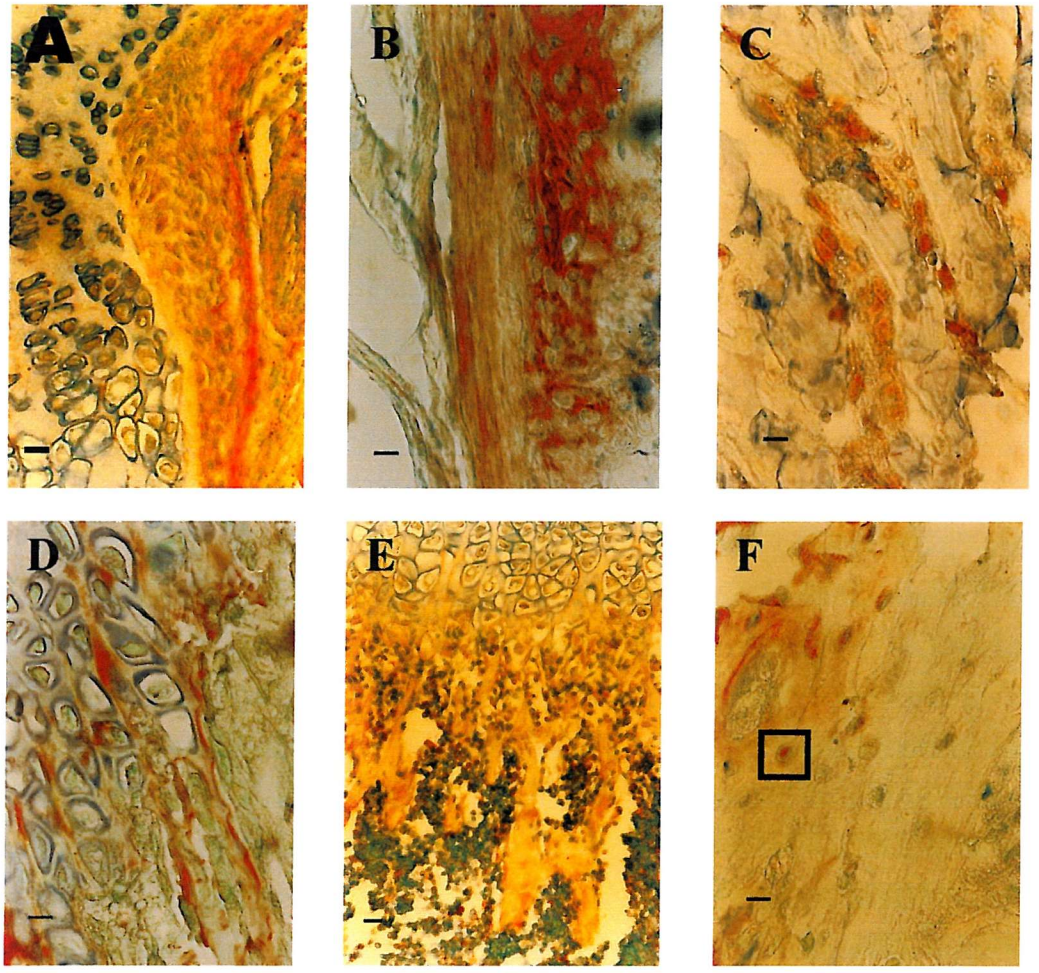


Figure 24. Regions in the femur of a 2.5-week-old transgenic mouse exhibiting immunostaining for OSF-1. *Immunostaining for OSF-1 was observed in the ossification groove of Ranvier (A), the periosteum, especially in the inner layer of osteogenic cells (B), the palisade of osteoblasts (C) and occasionally in osteocytes (F). Presence of OSF-1 in bone matrix was detected only when the overlaying mineral was removed by incubating sections with EDTA prior to immunostaining. OSF-1 was present in the newly deposited endochondral bone matrix at the vascular front of the growth plate (D) and in the primary spongiosa (E). Scale bars = 10 μm for A, B, C, D and F; 20 μm for E.*

b. Cartilage

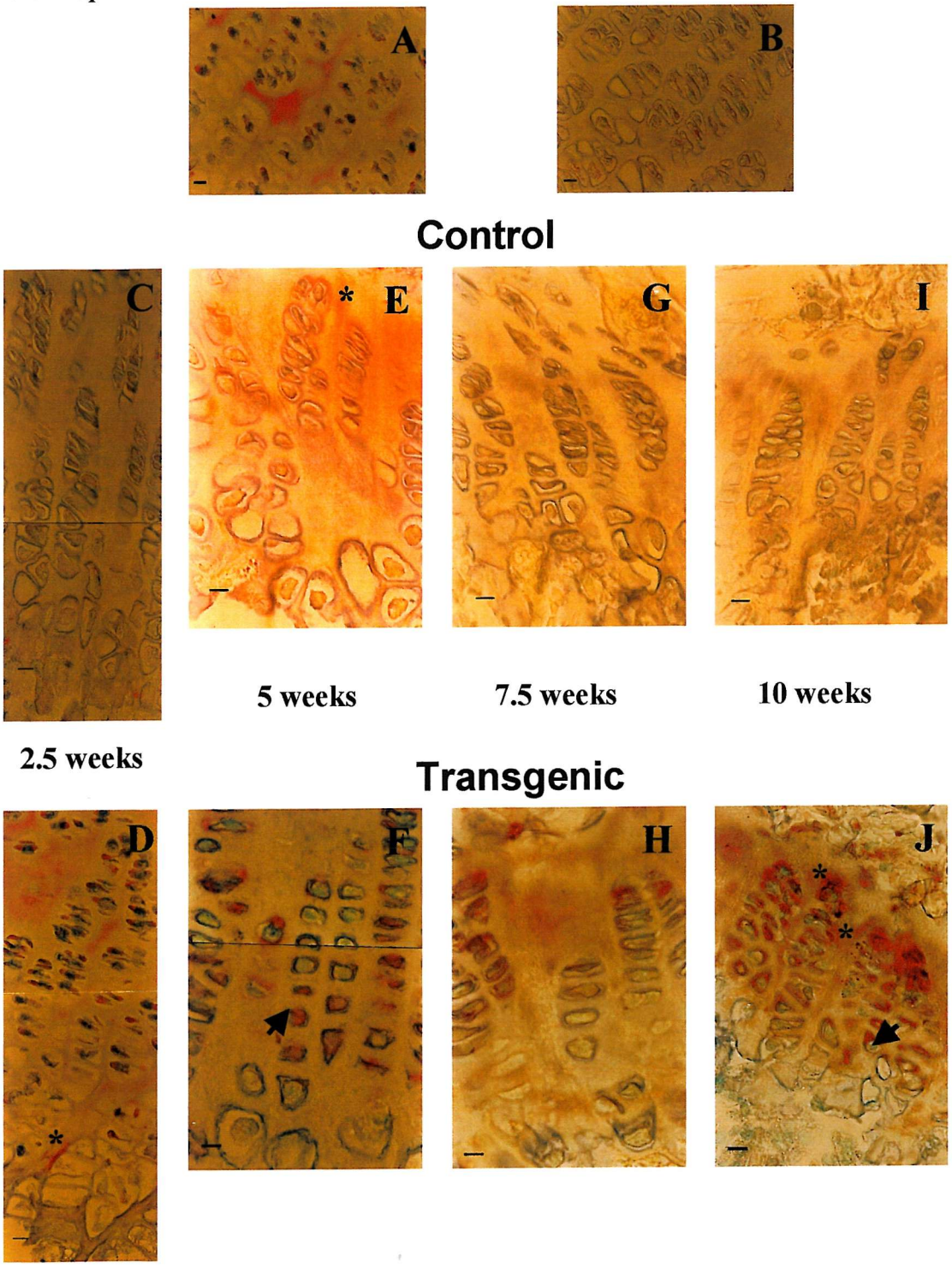
It was only in transgenic mice that immunostaining for OSF-1 was observed in cartilage, growth plate as well as articular. Since immunostaining for OSF-1 could only be demonstrated in ethanol-fixed, non-decalcified sections, which were impossible to obtain from older mice due to the higher degree of calcification, results for OSF-1 immunolocalization in this study were from bones of mice between 2.5 and 15 weeks of age.

Staining was observed in growth plate chondrocytes and matrix (Fig. 25 A), which was validated by blocking the antibody using a ten-fold higher concentration of the recombinant OSF-1 protein. This abolished staining from growth plate chondrocytes and matrix (Fig. 25 B). In control mice, between 2.5 and 10 weeks, occasionally faint immunostaining was observed in the inter-territorial growth plate matrix (Fig. 25 - asterix in E), however never in growth plate chondrocytes. On the contrary, intense immunostaining was observed in growth plates of transgenic mice (aged 2.5 weeks to 10 weeks), particularly in chondrocytes (Fig. 25 - arrow-head in F), and in the intra-territorial (Fig. 25 - arrowhead in J) and inter-territorial (Fig. 25 - asterix in J) matrix. Matrix staining was rather precise and localized to the transverse and longitudinal cartilaginous septa separating columns of growth plate chondrocytes (Fig. 25 J). Staining was also observed in the unresorbed longitudinal struts of cartilage at the vascular front, onto which osteoblasts are recruited during endochondral ossification (Fig. 25 - asterix in D).

Similarly, OSF-1 was not detected in articular chondrocytes of control mice (Fig. 25 K), in any of the specimens examined. However, in transgenic mice up to at least 15 weeks of age,

some 30-50% of the articular chondrocytes were found to exhibit presence of OSF-1 (Fig. 25 L).

Growth plates:



Articular cartilage:

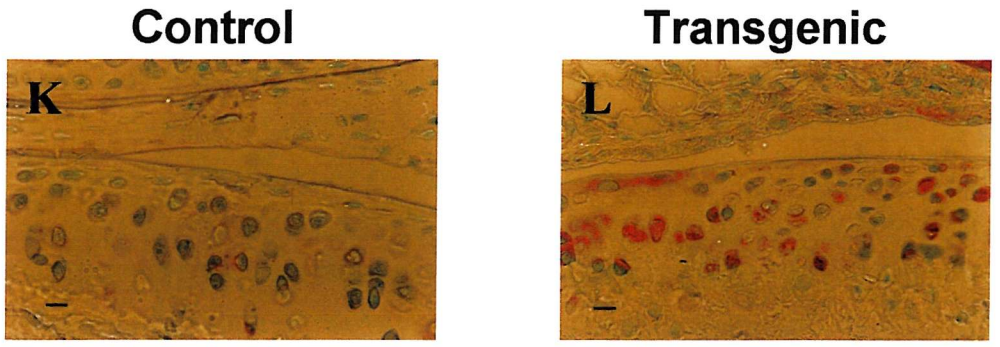


Figure 25. Growth plate and articular cartilage of control and transgenic mice, between 2.5 and 15 weeks of age, immunostained for OSF-1. *Immunostaining in growth plate chondrocytes and matrix of transgenic mice (A) was confirmed by blocking the anti-OSF-1 antibody with a ten-fold higher concentration of rhOSF-1, which abolished OSF-1 immunostaining in a parallel section of the growth plate (B). Overall, immunostaining for OSF-1 was not observed in growth plates of control mice(C, E, G, I), though faint matrix staining was evident occasionally (asterix in E). Intense staining for OSF-1was observed in the growth plates of transgenic mice (D, F, H and J), prominently in growth plate chondrocytes (arrow-head in F) and in the intra- (arrow-head in J) and inter- (asterix in J and D) territorial matrix. Staining for OSF-1 was not observed in articular chondrocytes of control mice (K). However, presence of OSF-1 was detected in around 30-50% of articular chondrocytes of transgenic mice (L) up to15 weeks of age. Scale bars = 10 μm.*

3.4 Discussion

The present study examined effects of *osf-1* over-expression, under the control of the human osteocalcin promoter, on post-natal bone development in transgenic mice. Since the osteocalcin promoter was capable of directing over-expression of the *osf-1* transgene only to skeletal tissues and brain (Kesterson *et al.*, 1993), these mice served as better models in defining the direct effects of *osf-1* over-expression on the skeletal system, than mice over-expressing the transgene under the control of the β-actin promoter (Imai *et al.*, 1998). The β-actin promoter was likely to target over-expression of the transgene to a variety of cell types, including endothelial cells. Taking into consideration the angiogenic role of OSF-1 (Laaroubi *et al.*, 1994; Zhang *et al.*, 1999; Zhang and Deuel, 1999; Papadimitriou *et al.*, 2001) and the likelihood of *osf-1* over-expression in endothelial cells, enhanced angiogenesis could most likely make an indirect contribution to the bone formation process in these transgenic mice. However, over-expression of the *osf-1* transgene under the osteocalcin promoter avoided confounding influences of enhanced angiogenesis on bone formation, by way of restricted targeting of the *osf-1* transgene to the osteoblasts and maturing chondrocytes. Hence the effects on bone development observed in the present study were most likely a consequence of increased *osf-1* expression in osteogenic cells and chondrocytes.

3.4.1 Sites of OSF-1 localization in bone

Since OSF-1 had originally been identified as a factor uniquely expressed by an osteoblastic cell line (Tezuka *et al.*, 1990), we sought to confirm synthesis of OSF-1 by osteoblasts *in vivo*. Presence of the protein was detected in osteoblasts, occasionally in osteocytes and in the bone matrix of the primary spongiosa. However, matrix staining was only evident when

the bone sections were pre-treated with EDTA, a compound that chelates calcium and removes overlaying mineral from the bone matrix. Mineral formation therefore masked the OSF-1 epitope and made it inaccessible to the antibody. Thus, OSF-1 was localized both in osteogenic cells and the bone matrix, suggesting export of the protein from osteoblasts into the bone matrix. This confirmed the findings of the OSF-1 immunolocalization studies in developing chick tibiae (Dreyfus *et al.*, 1998), wherein OSF-1 was localized to the osteoblast/ osteoid layer.

Considering that OSF-1 had been attributed with an important role in stimulating new bone formation, it was investigated whether the protein was localized at sites of new bone formation. The present study immunolocalized OSF-1 at sites of new bone formation, namely, the 'ossification groove of Ranvier', the osteogenic layer of the perisoteum and the newly deposited endochondral bone matrix of the primary spongiosa.

The ossification groove of Ranvier, a circumferential groove at the periphery of the epiphyseal cartilage, comprises of i) osteoprogenitor cells, ii) undifferentiated mesenchymal cells, some of which are chondrocyte precursors, iii) fibroblasts and fibrocytes among sheets of highly organized collagen fibres that form a fibrous layer which is continuous with the outer fibrous layer of the perisoteum (Shapiro *et al.*, 1977). During bone development, the group of densely packed osteoprogenitor cells located deep within the groove differentiate into osteoblasts and are responsible for the formation of the first bone bark/ periosteal bone collar, a cuff of bone surrounding the epiphyseal growth plate region and the adjacent part of the metaphysis. Thus, the ossification groove of Ranvier represents a site of first bone formation. Presence of OSF-1 was observed at this site of first bone formation.

Although most flat bones are formed by intramembranous ossification, it is also the primary process by virtue of which new bone is added onto the periosteal or outer surfaces of long bones, resulting in an increase in bone thickness/ diameter. The inner periosteal layer comprising of osteogenic cells plays an important role in deposition of new bone at this site of intramembranous ossification. Presence of OSF-1 was demonstrated in this osteogenic layer of the periosteum. The finding is in keeping with that by Imai *et al.* (1998), where intense expression of OSF-1 in the periosteum was demonstrated by immunostaining in a 1-week-old rat humeral head.

Presence of OSF-1 was also detected in the new bone matrix laid down by osteoblasts on the unresorbed struts of growth plate cartilage at the vascular front during the course of endochondral ossification. Thus, OSF-1 was immunolocalized at sites of new intramembranous and endochondral bone formation.

3.4.2 Effects of osf-1 over-expression on bone development

Since OSF-1 was localized at sites of new bone formation in the present study and previous studies had attributed it with a role in stimulating new bone formation (Masuda *et al.*, 1997; Imai *et al.*, 1998), enhanced bone formation would be expected at the sites of *de novo* bone formation. Calvariae in mice develop rapidly between 2-6 days post-natally, and the mode of ossification in calvariae is entirely intramembranous. Being flat structures, the extent of bone formation in calvariae is very well represented by the thickness/ width of bone layer. Width of calvarial bone was compared between control and transgenic mice aged 6 days, as these calvarial bones served as prime examples of new intramembranous bone formation. In neonatal transgenic mice, width of calvarial bone was almost twice that in neonatal control mice. However, it is not known whether

this increased thickness was maintained in later life, as it was not possible to examine calvariae from older animals due to their unavailability. Nevertheless, the difference between neonatal calvarial bones was remarkable and consistent with an autocrine effect of OSF-1 on osteoblasts, resulting in an overall increase in bone formation at 6 days of age. Not only were the calvarial bones from transgenic mice thicker, but they also had a very irregular and pronounced trabecular appearance. Bone development in calvariae is initiated as a network of bone trabeculae and expansion of flat bones like calvariae, to accommodate growth of the head, utilizes the process of remodeling wherein, new bone trabeculae are laid down on the outer perimeter while bone is resorbed on the inner perimeter. In absence of adequate evidence, at this stage, it is not possible to comment whether the pronounced trabecular appearance of calvariae in transgenic mice was a result of enhanced remodeling.

The remarkable effect of *osf-1* over-expression on neonatal calvariae contrasted the relatively modest long-term effects of *osf-1* over-expression on development of long bones. Transgenics were similar or slightly smaller in size than controls, as also found by Masuda *et al.* (1997). As against the ivory-like solid appearance of bones from mice over-expressing the *osf-1* transgene under the control of the β-actin promoter (Imai *et al.*, 1997), bones from transgenic mice in the present study and that of Masuda *et al.* (1997) did not have any distinct morphological characteristics. Transgenic mice also did not have large bones, contrary to initial expectations. Until 10 weeks of age, there were no differences between control and transgenic mice with respect to their femoral dry weights. At 15 weeks however, femoral dry weights of control mice were higher than those of transgenic mice. At 20 weeks, the magnitude of this difference decreased and

was only significant between males. By 30 weeks, transgenic males caught up with their male control counterparts and femoral dry weights were similar again.

However, long-term over-expression of *osf-1* influenced bone growth trajectories in transgenic mice. The growth spurt, which occurred in control mice between 10 and 15 weeks, did not take place in transgenic mice. Instead, a continuous increase in growth was observed until 25 weeks, suggesting that growth plates of transgenic mice remained active for longer. Since the skeletal growth spurt coincided with the pubertal surge in sex hormones, it is possible that *osf-1* over-expression interacted with the sex hormones and or growth hormone via an, as yet unknown, mechanism.

Bone is made up of 50% inorganic mineral, constituted largely by calcium and phosphorous, 25% organic matrix, 90-95% of which is Type I collagen, and the remaining 25% being water (Bourne, 1972). Once the water component is removed, dry weight of bone is constituted by inorganic mineral and the organic bone matrix. Transgene over-expression resulted in an increase in calcium content/ unit dry femoral weight (by ~10-11%) in male transgenic mice at 15, 25 and 30 weeks of age. The reason for not observing an increase in calcium content at 20 weeks is not clear and maybe attributable to the biological variability in the hybrid strain of control mice. This increase in bone calcium concentration was not a consequence of higher organic matrix content, as there was no difference between control and transgenic mice with respect to their femoral collagen content. Histological analysis of femurs from male control and transgenic mice served to confirm the same, as there was no difference in the apparent thickness of the bone shaft or in the amount of bone spongiosa. The mechanism by which *osf-1* over-

expression enhanced mineral concentration per unit bone is not clear, but may be related to the slower rate of growth, which perhaps allowed more time for mineral deposition. Masuda *et al.* (1997) reported an increase in bone mineral content in female OSF-1 transgenic mice at 30 weeks of age. Femoral mineral content, in terms of ash weights, was 43% for homozygous and 40.5% for heterozygous transgenics compared to 39.5% for wild-type controls. The study also suggested a possible role for OSF-1 in the treatment of osteoporosis, as the higher bone mineral content due to *osf-1* over-expression in these transgenic female mice compensated for the bone loss after an ovariectomy. Although the data from the present study, on bone calcium content, confirmed findings of Masuda and co-workers, significant results were observed in male transgenic mice at 15, 25 and 30 weeks unlike female transgenic mice at only 30 weeks in the latter study. This difference might be due to the different assay methods used in assessing mineral content, or due to the natural biological variability within the BDF-1 strain of mice used as the control group in the present study.

Studies by Imai *et al.* (1998) revealed that cortical bone was significantly thicker in HB-GAM transgenic mice, an increase in thickness of up to 147.4% for the humerus and 124.2% for the femur by 1 year of age. Histomorphometric analyses demonstrated that cortical and cancellous bone volumes were also significantly increased in the transgenic mice. Although bone marrow area, a good indicator of osteoclast function, was not altered, osteoblast surface, an indicator of the number of osteoblasts covering the bone surface, was increased in transgenic mice. However, the study did not analyze bones for their organic and inorganic content and it was therefore difficult to comment whether expression of the transgene under the control of the β-actin promoter had any effect on the above parameters.

3.4.3 Differences in the immunolocalization of OSF-1 in cartilage

In contrast to bone, there were major differences between control and transgenic mice in the immunolocalization of OSF-1 in cartilage. In embryonic and fetal cartilage, previous work (Imai *et al.*, 1998) had demonstrated expression of *osf-1* and also presence of the protein in chondro-epiphyses of long bones by *in situ* hybridization and immunocytochemistry respectively. In addition, up to 15 mg/g wet weight OSF-1 was extracted from fetal bovine epiphyseal and nasal cartilage (Neame *et al.*, 1993), confirming that fetal cartilage contained large amounts of OSF-1.

In the present study, faint immunostaining for OSF-1 was observed in growth plates of post-natal control mice (up to 5 weeks of age), but not in the cartilage of mature control mice. This contrasted the results of Petersen and Raifi (2001), who demonstrated immunostaining for OSF-1 in terminal hypertrophic growth plate chondrocytes and within opened lacunae of apoptotic chondrocytes of mice up to 20 weeks. However, fixation methods (36% neutral formalin in 80% ethanol, followed by decalcification) and the antibody (from R & D Systems, Minneapolis, USA) used in the study by Petersen and Raifi were different from those in the present study. This suggested that low levels of OSF-1 were probably detectable in the growth plates of older mice, but the present study was unable to demonstrate them by immunostaining. However, synthesis of OSF-1 by growth plate chondrocytes was maintained at high levels in post-natal and mature transgenic mice, as indicated by a strong immunostaining for OSF-1 in the cells and matrices of the growth plates until at least 15 weeks of age.

OSF-1 was absent from the articular cartilage of mature control mice, in agreement with the findings in mature bovine articular cartilage (Neame *et al.*, 1993). However, OSF-1

was clearly present in about 30-50% of the articular chondrocytes in mature transgenics (10 and 15-week old).

The following conclusions regarding OSF-1 protein expression in cartilage can be drawn on the basis of the above studies,

1. OSF-1 protein was abundantly expressed in fetal cartilage under physiological conditions (Imai *et al.*, 1998; Neame *et al.*, 1993)
2. A low-level expression of the protein was observed under physiological conditions in growth plate cartilage of postnatal (present study) and mature mice (Petersen and Raifi, 2001).
3. Under physiological conditions in post-natal life, the protein was either present in trace amounts (Neame *et al.*, 1993) or completely absent (present study) in articular cartilage.

Taking into account these observations, over-expression of the transgene enhanced appreciably the low-level OSF-1 protein expression in growth plates of postnatal transgenic mice, and also stimulated post-natal synthesis of the protein by articular chondrocytes.

The transgene was expressed under the control of the human osteocalcin promoter, which was able to target *osf-1* over-expression in bone to osteoblasts and maturing chondrocytes (Kesterson *et al.*, 1993). A low-level expression of the osteocalcin gene (one hundredth of that observed in osteoblasts) has also been reported in chondrocytes under physiological conditions (Aizawa *et al.*, 1998; Lian *et al.*, 1993). Theoretically, in transgenic mice, any stimulus influencing expression of the osteocalcin promoter in chondrocytes would result in the expression of the 6 *osf-1* transgene copies under its control. As a consequence, a 6-fold amplification in the synthesis of the OSF-1 protein by chondrocytes was likely to occur,

thereby making its detection by immunocytochemistry possible. Thus, six copies of the *osf-1* transgene considerably amplified a normally low-level OSF-1 protein expression in growth plate and articular chondrocytes.

3.4.4 Over-expression of osf-1 altered the chondrogenic phenotype in transgenic mice

Synthesis of OSF-1 by articular chondrocytes of transgenic mice coincided with the synthesis of type I collagen, i.e. a collagen type that is fairly specific for osteoblasts and not chondrocytes. In addition, 'islands' of bone-like, type I collagen-rich matrix had formed within the articular cartilage of transgenic mice. Since no vascular channels were seen in this region (and hence no source of osteogenic cells), the type I collagen-rich matrix appeared to have been synthesized by former chondrocytes. Non-collagenous matrix proteins like bone sialoprotein and osteonectin, which also serve as markers of osteogenic differentiation, were found synthesized by these articular chondrocytes, but this was the case for articular chondrocytes of control mice as well. There is considerable evidence for the synthesis of non-collagenous matrix proteins like bone sialoprotein (Bianco *et al.*, 1991) and osteonectin (Pacific *et al.*, 1990) by hypertrophic chondrocytes under physiological conditions *in vivo*, but synthesis of type I collagen by chondrocytes has been rarely reported.

Numerous lines of evidence suggest that chondrocytes, especially hypertrophic chondrocytes, have the potential *in vitro* for either further differentiation (Galotto *et al.*, 1994; Cancedda *et al.*, 1992; Ishizeki *et al.*, 1996 and 1997) or trans-differentiation (Roach *et al.*, 1992 and 1995) to bone-forming cells, and initiate the synthesis of type I collagen. However, this potential is not usually realized *in vivo*, the immediate microenvironment in the vicinity of chondrocytes and the presence of adequate osteogenic stimuli being the two deciding factors determining the osteogenic fate of chondrocytes *in vivo* (Riminucci *et al.*,

1998). Since synthesis of type I collagen was observed only in some articular chondrocytes of transgenic mice, in which over-expression of the transgene was evident by the synthesis of the OSF-1 protein, it can be suggested that over-expression of *osf-1* may have provided adequate osteogenic stimulus for the synthesis of bone-type proteins by some articular chondrocytes.

However, some caveats should be borne in mind. For a complete osteogenic differentiation to have occurred, former chondrocytes should also express markers of late osteogenic differentiation like osteocalcin. Presence of osteocalcin was not observed in articular chondrocytes of transgenic mice, suggesting that a complete osteogenic differentiation had not occurred. Synthesis of type I collagen by articular chondrocytes did not occur in every transgenic mouse, nor was it feasible to carry out a detailed 3-D study of the vascularity arising from the sub-chondral bone. Hence it was difficult to prove that the apparent islands of type I collagen around chondrocytes were true "islands" i.e. not connected to bone around the vascular channels. Nevertheless, over-expression of *osf-1* played a role in altering the chondrogenic phenotype of some articular chondrocytes, and imparting them with some osteogenic characteristics.

Thus, the overall effects of *osf-1* over-expression on long-term bone development were relatively modest. However, some marked changes were observed in the cartilage as a result of the transgene over-expression, especially, the post-natal synthesis of the OSF-1 protein by articular chondrocytes coinciding with the synthesis of the bone-type protein, type I collagen.

ORGAN CULTURES OF CARTILAGINOUS EXPLANTS

4.1 Introduction

Chondrocytes, especially hypertrophic chondrocytes, express proteins characteristic of the 'osteoblast phenotype' *in vivo*. These include membrane-associated alkaline phosphatase (Register *et al.*, 1986; Roach, 1999) and the bone-enriched non collagenous proteins like bone sialoprotein (Bianco *et al.*, 1991), osteonectin (Pacific *et al.*, 1990) and osteopontin (Mark *et al.*, 1988). All these proteins have a role to play in matrix mineralization (Roach, 1994) and hence, are synthesized by hypertrophic chondrocytes under physiological conditions. However, synthesis of type I collagen (a bone matrix protein) and osteocalcin (an osteoblast-specific marker) by chondrocytes is rarely observed *in vivo*, but has been reported in pathological conditions like fracture repair (Scammell and Roach, 1996) and in *in vitro* organ cultures of cartilage pieces of different origins (chick long bones - Roach and Shearer, 1989; Roach *et al.*, 1995; mouse metatarsal bones - Thesingh *et al.*, 1991; condylar cartilage of rat mandible – Yoshioka and Yagi, 1988). In these studies, synthesis of type I collagen-rich bone matrix by chondrocytes represented either a rapid mechanism by which fracture callus increased its mechanical strength (Scammell and Roach, 1996), or a contribution by the chondrocytes towards synthesis of the osteoid during endochondral ossification (Roach and Shearer, 1989; Yoshioka and Yagi, 1988), or an abnormal response of chondrocytes to some specific stimuli (Roach *et al.*, 1995; Thesingh *et al.*, 1991). Transdifferentiation (Roach *et al.*, 1995) or a further differentiation of chondrocytes along the osteogenic pathway (Cancedda *et al.*, 1992; Galotto *et al.*, 1994), were suggested as the possible mechanisms for alterations in the chondrogenic phenotype.

Thus, the developmental potential of chondrocytes, especially hypertrophic chondrocytes, along the osteogenic pathway has always been recognized.

Chondrogenic and osteogenic cells are known to share a common ancestor (Manduca *et al.*, 1992; Owen, 1989). Once a chondrocyte has reached the stage of early hypertrophy, it begins to express phenotypic traits of the osteogenic lineage (Riminucci *et al.*, 1998), evident by way of synthesis of alkaline phosphatase and the bone enriched non-collagenous proteins like bone sialoprotein, osteonectin and osteopontin. This osteogenic potential may not or may be realized (evident by the synthesis of type I collagen and osteocalcin) depending upon whether the chondrocyte receives appropriate signals/ osteogenic stimuli from its immediate microenvironment.

In the present study (Chapter 3), over-expression of *osf-1* induced the synthesis of type I collagen in some articular chondrocytes of mature transgenic mice. This coincided with the synthesis of the OSF-1 protein by articular chondrocytes, once again, in only transgenic mice. Since the osteogenic stimuli in the immediate microenvironment of chondrocytes played an important role in determining their osteogenic fate (Riminucci *et al.*, 1998), we hypothesized that an enhanced osteogenic stimulus exerted by over-expression of *osf-1* induced synthesis of the bone-type protein, type I collagen, by the articular chondrocytes.

To validate this hypothesis and to determine whether exogenous addition of OSF-1 had a similar influence on chondrocytes *in vitro* (i.e. provided adequate osteogenic stimulus for the synthesis of type I collagen by chondrocytes *in vitro)*, cartilaginous explants were cultured with recombinant OSF-1.

4.2 Materials and methods

Biochemical reagents used in the study were of analytical grade and purchased from Sigma (Poole, UK) and BDH/ Merck Ltd. (Poole, UK), unless stated otherwise.

4.2.1 Organ cultures

The serum-free organ culture system was based on the one developed by Roach (1990) for the culture of embryonic chick bones. Cartilaginous explants, in the form of growth plates from 2-week-old Wistar rats and pieces of nasal cartilage from 14-day-old chick embryos, were cultured in a modification of Fitton-Jackson's BGJ medium (*Refer to Chapter 8. Appendix – Reagents & Solutions*). This modification involved altering the concentrations of non essential amino acids to optimize collagen synthesis *in vitro* (Roach, 1986). Alanine was omitted, concentrations of other non essential amino acids were adjusted as follows - arginine (75 mg/L), aspartic acid (50 mg/L), glycine (400 mg/L), serine (100 mg/L) and proline (20 mg/L). The medium contained 1.8 mM Ca^{+2}, 2.13 mM phosphate (NaH_2PO_4 - 150 mg/L) and was buffered with sodium carbonate (3.7 g/L). The culture medium did not contain any fetal calf serum. Ascorbic acid (50 μg/ml) was added to the culture medium afresh every 24 hours.

Wistar rat pups were sacrificed at 2 weeks of age, their tibiae dissected out taking care to remove the adherent muscles and ligaments, and each tibia was cut longitudinally into two halves. The cartilaginous disc between the primary spongiosa of the diaphysis and the secondary ossification centre at the epiphysis was excised with the bony collar on either side left attached for support. This excised region contained the growth plate as well as some epiphyseal cartilage.

Fertilized eggs (*Gallus domesticus*) were purchased from a registered supplier and incubated at 37^0C in an incubator (humidity controlled and with rotating trays, *Multihatch Automatic Incubator, Congresbury, England*) for 14 days. Pieces of nasal cartilage were dissected from the region above the beak of these 14-day-old chick embryos.

On dissection, the cartilaginous explants were placed in 'holding medium' to prevent them from drying. The 'holding medium' contained the same components as the BGJ culture medium except that the bicarbonate concentrations had been adjusted to 1.7 g/L to maintain a pH of 7.4 under air. Excess medium was removed from the explants by placing them on a filter paper, and they were then transferred to 3-chambered petri-dishes (90 mm diameter). Two explants were cultured in each chamber of the petri-dish containing 3.5 ml of the BGJ culture medium. Each explant was placed on a Millipore filter resting on a stainless steel grid at the interface between the medium and gas phase. Small strips (4 mm^2) of absorbent nylon membranes (Cat. No. 1209299, Boehringer Mannheim, Heidelberg, Germany) soaked in a 50 ng/ml solution of recombinant human OSF-1 (rhOSF-1 - Cat. No. 450-15, PeproTech EC Ltd., London, UK) were then placed on these explants.

The petri-dishes were placed in a CO_2 incubator under 95% air-5% CO_2 conditions. The explants were transferred to fresh culture medium, supplemented with freshly prepared ascorbic acid (50 μg/ml), every 24 hours. This regime was shown to provide sufficient ascorbic acid for maximal collagen synthesis despite rapid degradation of the vitamin under culture conditions, and also served for elimination of wastes generated during the 24-hr culture period. Strips of nylon membranes, soaked in OSF-1, were also applied to the explants afresh every 24 hours.

Growth plates from 2-week-old rats were cultured for 11 days, while pieces of chick nasal cartilage were cultured for 4 days. Control cultures included growth plates/ pieces of nasal cartilage cultured for 11 and 4 days respectively under the same culture conditions, but with strips of nylon membrane soaked in PBS. In addition, 2 growth plates and 2 pieces of nasal cartilage were fixed immediately as pre-culture controls upon dissection. At the end of the culture period, explants were fixed in 85% ethanol overnight since alkaline phosphatase staining and OSF-1 immunostaining were best demonstrated using this fixative. Samples were processed immediately without decalcification through 90% and 100% (twice in each) ethanol, followed by chloroform (twice) and embedded in paraffin wax. 6 μm sections were cut on the microtome for histological examination.

4.2.2 Histological analyses

Sections were stained with Alcian blue/ Sirius red (for overall histology, details in ***Section 2.2.1 b***) and for presence of alkaline phosphatase (details in ***Section 2.2.1 c***). Sections were also immunostained (details in ***Section 2.2.1 e***) using the anti- type I collagen/ osteonectin/ OSF-1 antibodies. A double immunostaining technique using two primary antibodies, anti-type I collagen and anti-OSF-1, was also performed.

a. Double immunostaining

The double immunostaining technique involved detection of two separate antigens with two primary antibodies directed against them. The first primary antibody was visualized as a brown reaction product in the avidin-biotin method linked to peroxidase and AEC. The second antibody was visualized using the alkaline phosphatase anti-alkaline phosphatase method with fast blue as the substrate, resulting in a blue reaction product (Cordell *et al.*, 1984).

Reagents

1. 3% H_2O_2
2. 1% Phosphate-buffered bovine serum albumin
3. 1% Tris-buffered bovine serum albumin
4. High salt wash buffer, pH 8.5
5. Low salt wash buffer, pH 8.5
6. 0.1 M Tris buffer, pH 8.5
7. H_2O_2, AEC soln.
8. Acetate buffer, pH 5
9. Fast blue BB solution (freshly prepared)
10. Primary antisera:

Rabbit polyclonal antibodies

i. Anti-type I collagen (LF-67, à human - raised against the human C-terminal propeptide of the α-1 chain and capable of detecting pro-α-1 chain as well as fully processed α-1 chain, cross-reacts with rat and chick), 1:300 dilution, gift from Dr. Larry Fisher, NIH, USA.

ii. Anti-OSF-1 (raised against the recombinant HB-GAM protein synthesized using the baculovirus expression system – Raulo *et al.*, 1992), 1:500 dilution, a gift from Dr. Heiki Rauvala, Finland.

11. Secondary antisera:

i. Goat anti-rabbit (biotin-linked, Dako E 0432, 1:300 dilution)

ii. Goat anti-rabbit IgG (whole molecule, Sigma R 2004, 1:50 dilution)

12. Extravidin peroxidase (Sigma E 2886, 1:50 dilution)
13. Rabbit anti-alkaline phosphatase/ alkaline phosphatase complex (Sigma A 9811, 1:50 dilution)

Procedure

Sections were de-paraffinized in histoclear, rehydrated through graded alcohols, taken to water and endogenous peroxidase activity was quenched by incubating the sections for 5 min. with 3% H_2O_2. Following a 5-min. incubation with 1% phosphate-buffered bovine serum albumin, sections were incubated with the first primary antiserum for 6 hours at 4^0C. At the end of the incubation period, sections were washed thoroughly in running water, followed by 5-min. washes in the three wash buffers (high salt, low salt and Tris) and then incubated for 30 min. at room temperature with the appropriate biotin-linked secondary antibody. After the routine washes in the three wash buffers, sections were incubated with Extravidin peroxidase for 30 min. at room temperature. The immune complex was visualized using hydrogen peroxide and 3-amino, 9-ethylcarbazole (AEC) in acetate buffer, yielding a brown reaction product. Sections were washed in running water and the three wash buffers, and incubated overnight with the second primary antiserum at 4^0C. 1% Tris-buffered bovine serum albumin was used for diluting antisera etc. and the use of 1% Phosphate-buffered bovine serum albumin strictly avoided. After the three routine washes, sections were incubated with the goat anti-rabbit IgG for an hour and a half at room temperature. Sections were then incubated with rabbit anti-alkaline phosphatase/ alkaline phosphatase for an hour and a half at room temperature. Freshly prepared Fast blue BB solution was filtered onto the sections, followed by an hour's incubation at 37^0C. The immune complex with the second primary antibody was visualized as a blue reaction product. Sections were washed and mounted in glycerol jelly. Negative controls (omission of the primary antibodies) were included every time.

4.3 Results

4.3.1 Expression of bone-matrix proteins by chondrocytes of cultured rat growth plates

Osteonectin:

The cartilaginous region excised between the secondary ossification centre and the primary spongiosa of a 2-week rat tibia, comprised of the growth plate and a part of the epiphyseal cartilage. It was possible to distinguish clearly the reserve, proliferative and hypertrophic zones in pre-culture growth plates (Fig. 26 A). Due to the absence of a vascular supply and decreased survival of chondroclasts/ osteoclasts during the course of culture, resorption of the growth plate was not possible. Hypertrophic chondrocytes of cultured growth plates thus remained locked in their lacunae and were gradually found to shrink in size (Fig. 26 C and E).

Although presence of osteonectin was observed occasionally in some hypertrophic chondrocytes (Fig. 26 asterix in B), overall, most pre-culture growth plate chondrocytes were not immunopositive for osteonectin. Following an 11-day culture period, an appreciable increase was observed in the number of growth plate chondrocytes exhibiting the presence of osteonectin (Fig. 26 C and E). In control cultures, most chondrocytes in the reserve and proliferative zones, and a few in the hypertrophic zone of the growth plate were immunopositive for osteonectin (Fig. 26 D). In growth plates cultured with OSF-1, not only were most chondrocytes in the reserve and proliferative zones immunopositive for osteonectin, a remarkably large proportion of hypertrophic chondrocytes were also found to exhibit the presence of osteonectin (Fig. 26 F).

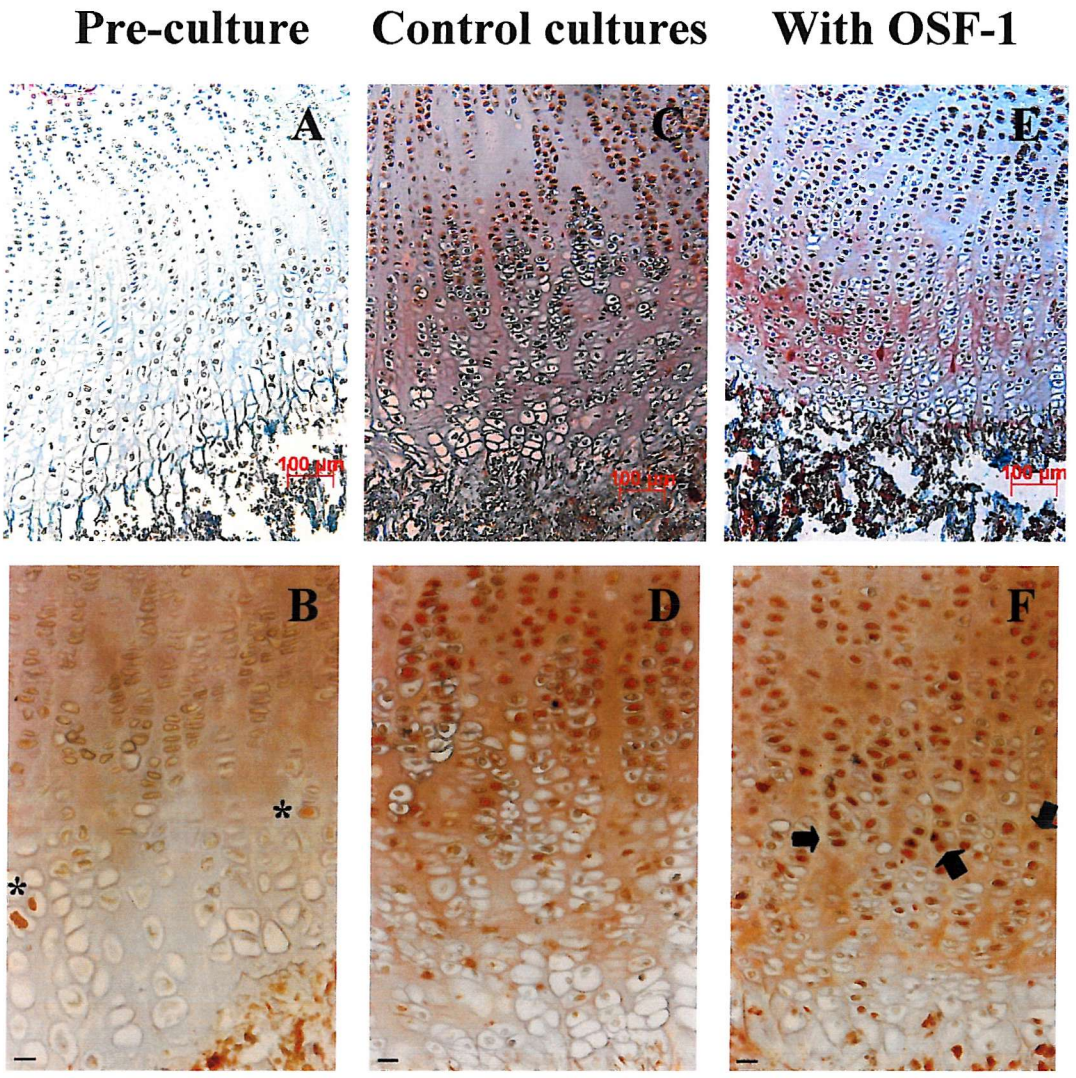


Figure 26. Growth plates from 2-week-old rats, before culture and cultured for 11 days without (control) and with 50 ng/ml rhOSF-1, immunostained for osteonectin. *Staining for osteonectin was not observed in most growth plate chondrocytes before culture (A), except in occasional hypertrophic chondrocytes (B). Number of osteonectin positive chondrocytes increased appreciably after the 11-day culture period (C and E). However, a large proportion of hypertrophic chondrocytes (indicated by arrowheads) in growth plates cultured with OSF-1 exhibited the presence of osteonectin (F), in comparison to control growth plate cultures (D). Scale bars = 100 μm for A, C and E; 40 μm for B, D and F.*

Type I collagen:

Presence of type I collagen, one of the early markers of an osteogenic phenotype, was not observed in growth plate chondrocytes of 2-week-old rats (Fig. 27 A). Neither did the culture conditions influence synthesis of this protein in chondrocytes of growth plates (n =3) cultured for 11 days under control conditions (Fig. 27 B and C). However, synthesis of type I collagen was observed within the lacunae of some hypertrophic chondrocytes in growth plates (in 3 growth plate samples out of 5) cultured for 11 days with 50 ng/ml rhOSF-1 (Fig. 27 D and E).

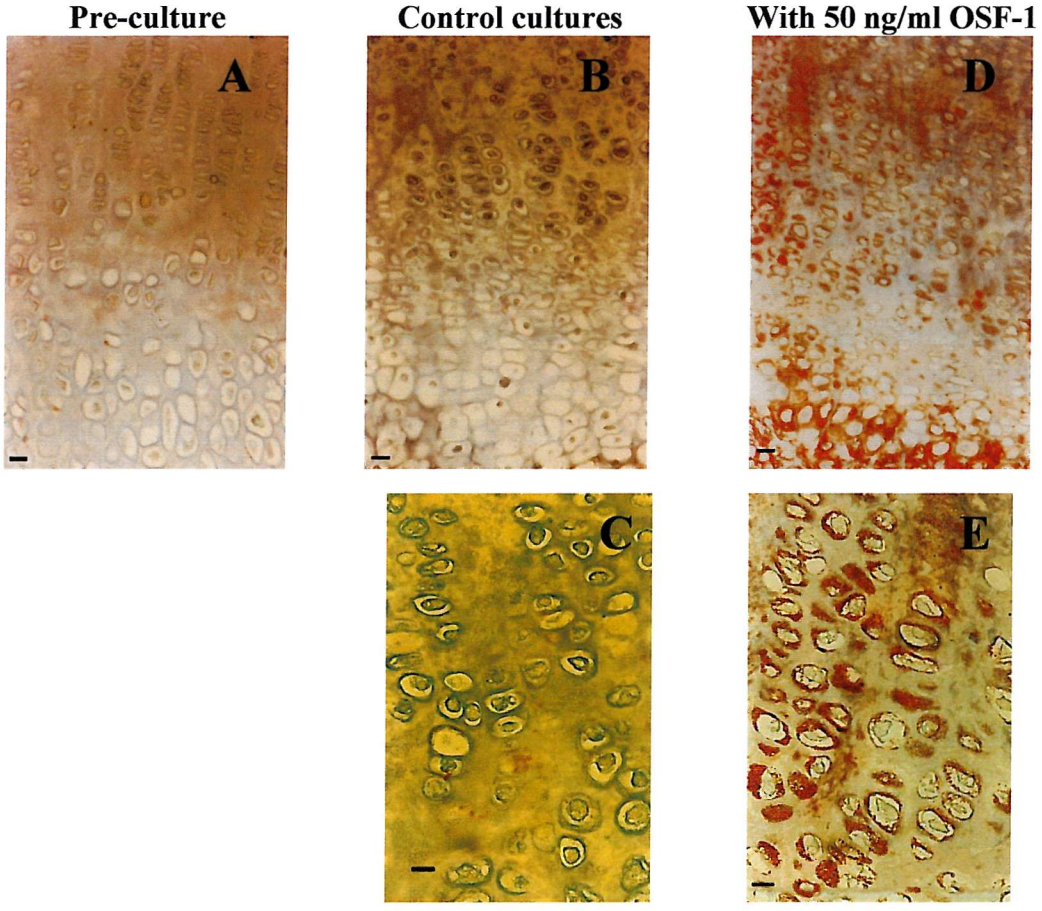


Figure 27. Growth plates from 2-week-old rats, before culture and cultured for 11 days without (control) and with 50 ng/ml rhOSF-1, immunostained for type I collagen. *Presence of type I collagen was not observed in growth plate chondrocytes before culture (A), or after the 11-day culture period under control conditions (B and C). However, synthesis of type I collagen was observed within the lacunae of some hypertrophic chondrocytes in growth plates cultured for 11 days with 50 ng/ml rhOSF-1 (D and E). Scale bars = 40 μm for A, B and D;10 μm for C and E.*

4.3.2 Synthesis of type I collagen by chondrocytes of cultured chick nasal cartilage

Overview of nasal cartilage from 14-day-old chick embryo, stained with Alcian blue/ Sirius red, illustrating the region in which changes in chondrocytes were observed on culture (Fig. 28 A). Before culture, most chondrocytes of embryonic nasal cartilage were found to express the OSF-1 protein (Fig. 28 B) and this expression was maintained in chondrocytes after the 4-day culture period (blue staining within chondrocytes in Fig. G). Pre-culture chondrocytes were also found to synthesize alkaline phosphatase (ALP), which was then secreted into the extracellular matrix (Fig. 28 C). Since ALP staining was not observed within the lacunae of these chondrocytes, it represented the physiological alkaline phosphatase activity of chondrocytes rather than an osteogenic differentiation. Synthesis of type I collagen was not observed in any chondrocytes of control nasal cartilage (n =3) after the 4-day culture period (Fig. 28 D), however some chondrocytes of nasal cartilage cultured for 4 days with 50 ng/ml rhOSF-1 (in 2 samples out of 3) were immunopositive for type I collagen (Fig. 28 E). Double immunostaining served to co-localize type I collagen (brown reaction product) with OSF-1 (blue reaction product) in chondrocytes of nasal cartilage cultured with rhOSF-1. Synthesis of type I collagen was observed within the lacunae of some chondrocytes, which were also found to express the endogenous OSF-1 protein (Fig. 28 F). Although most chondrocytes were found to maintain expression of the OSF-1 protein even after the 4-day culture period, synthesis of type I collagen was not observed in all chondrocytes (Fig. 28 G). This suggested that endogenous OSF-1, on its own, was not sufficient to bring about the synthesis of type I collagen in these chondrocytes, and exogenous addition of rhOSF-1 probably provided the stimulus in some chondrocytes for induction of type I collagen synthesis.

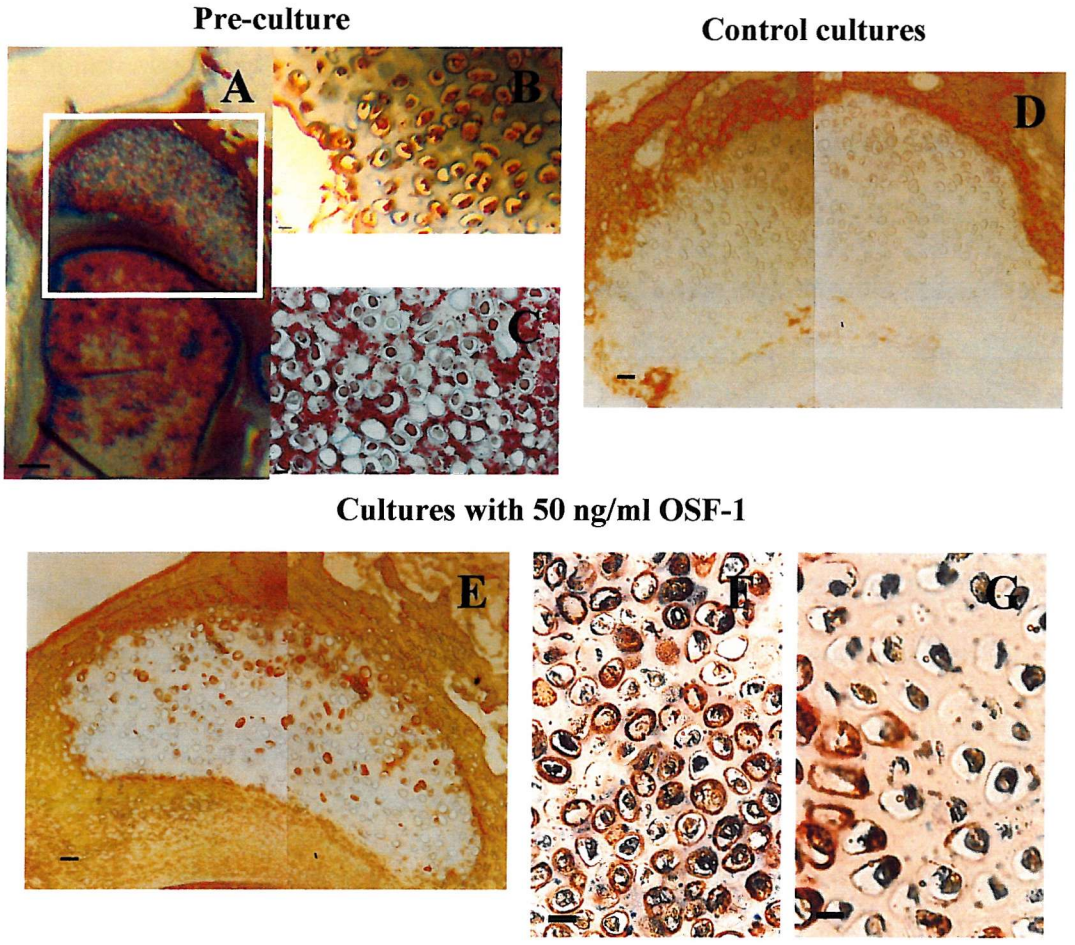


Figure 28. Nasal cartilage from 14-day chick embryo, before culture and cultured for 4 days without (control) and with 50 ng/ml rhOSF-1. *Overview of 14-day embryonic nasal cartilage, stained with Alcian blue/ Sirius red, exhibiting the highlighted region in which changes were observed (A). Chondrocytes of nasal cartilage were found to be immunopositive for OSF-1 (B) before culture, and also expressed alkaline phosphatase, staining for which was observed in the matrix as well (C). Immunostaining for type I collagen was not observed in any of the chondrocytes of control nasal cartilage after the 4-day culture period without rhOSF-1 (D). Synthesis of type I collagen was observed in some chondrocytes of nasal cartilage after the 4-day culture period with 50 ng/ml rhOSF-1 (E). Double immunostaining demonstrated presence of the OSF-1 protein (blue reaction product in G) in most chondrocytes of nasal cartilage after the 4-day culture period with rhOSF-1, however, it was possible to co-localize intra-lacunar type I collagen (brown reaction product) with OSF-1 (blue reaction product) in only some chondrocytes (F). Scale bars = 100 μm for A; 20 μm for B and C; 50 μm for D and E; 10 μm for F and G.*

4.4 Discussion

Chondrocytes secrete different types of collagen during the maturational events that lead eventually to the terminal hypertrophic stage. Among these types of collagen, type II and type X are the major collagenous proteins in the cartilage matrix. Type II collagen is secreted by maturing chondrocytes as a cartilage-specific collagen in the extracellular matrix (Dessau *et al.*, 1980; Yu *et al.*, 1991), whereas type X collagen is synthesized by hypertrophic chondrocytes as a main component of the extracellular matrix at the later stage of cartilage development (Schmid and Linsenmayer, 1985; Adams *et al.*, 1989). Synthesis of type I collagen, a bone matrix protein, by chondrocytes has been seldom reported. It has been generally assumed that hypertrophic chondrocytes are end cells, which ultimately degenerate, in spite of observations (Galotto *et al.*, 1994) indicating that these cells have a developmental potential in the direction of osteoblasts. Previous studies that immunolocalized type I collagen (a bone matrix protein) within the lacunae of hypertrophic chondrocytes, had also remarked on the osteogenic developmental potential of hypertrophic chondrocytes (von der Mark K. and von der Mark H., 1977; Yasui *et al.*, 1984). During the course of endochondral ossification in human growth plate cartilage (Yasui *et al.*, 1984) and embryonic chick tibia (von der Mark K. and von der Mark H., 1977), the territorial matrix of hypertrophic chondrocytes (generally last hypertrophic chondrocytes) was found to contain type I collagen within intact chondrocytic lacunae, thereby indicating that these chondrocytes were capable of contributing to the bone formation process by initiating the synthesis of type I collagen.

Synthesis of type I collagen by chondrocytes has been reported in *in vitro* cell cultures of isolated chondrocytes (Dessau *et al.*, 1978; Benya and Shaffer, 1982) and organ cultures of cartilaginous explants (Roach and Shearer, 1989; Thesingh *et al.*, 1991;

Yoshioka *et al.*, 1988). However, synthesis of type I collagen by cultured chondrocytes does not necessarily imply osteogenic differentiation, and a possible de-differentiation to fibroblasts (which also express type I collagen) cannot be ruled out. Isolated chondrocytes cultured in an adherent monolayer lost the ability to synthesize type II collagen and synthesized type I collagen, however that represented a de-differentiation to fibroblasts rather than osteogenic differentiation (Dessau *et al.*, 1978). The chondrogenic phenotype was preserved and synthesis of type II collagen was maintained *in vitro* when isolated chondrocytes were cultured within artificial matrices like collagen or agarose gels (Benya and Shaffer, 1982; Kimura *et al.*, 1984). This cell culture system assisted chondrocytes in maintaining a spherical morphology of anchorage-independent culture, in turn facilitating the synthesis and deposition of type II collagen and proteoglycans during culture (Benya and Shaffer, 1982). Alternatively, this cell culture system provided chondrocytes with a suitable environment in which to accumulate synthesized matrix macromolecules and preserve their chondrogenic phenotype (Kimura *et al.*, 1984).

Thus, organ cultures of cartilaginous explants were chosen in the present study over adherent monolayer cultures of isolated chondrocytes. In these cartilaginous explants, chondrocytes were maintained within their native extracellular matrices and this served to preserve their chondrogenic phenotype. At least under these conditions, synthesis of type I collagen by chondrocytes would not suggest a de-differentiation. Cultures of neonatal rat growth plates and embryonic chick nasal cartilage were chosen as experimental models because mouse growth plates and articular cartilage were too small to dissect.

After the 11-day culture period, immunostaining for type I collagen was observed within intact lacunae of some hypertrophic chondrocytes in 2-week rat growth plates

cultured with 50 ng/ml rhOSF-1. However, immunostaining for type I collagen was not detected in chondrocytes of pre-culture growth plates, neither did the culture conditions favour synthesis of this protein by chondrocytes in control growth plate cultures. Since deposition of type I collagen matrix was observed within intact lacunae, the chondrocytes contained within those lacunae could be attributed with the synthesis of this matrix following exposure to OSF-1. In the absence of a vascular supply and decreased survival of chondro/osteoclasts under culture conditions, there was hardly any possibility for growth plate resorption and deposition of type I collagen matrix by osteogenic cells brought in by the vascular supply. Although the bony collar (perichondrium) was retained, it is inconceivable that osteogenic cells derived from it would have entered and contributed to the synthesis of type I collagen in every single chondrocyte.

To resolve the question of whether type I collagen synthesis by chondrocytes on exposure to OSF-1 was indicative of osteogenic differentiation, we examined if other markers of osteogenic differentiation co-localized with type I collagen in these chondrocytes. The synthesis of one such marker, osteonectin, was observed in a comparatively large proportion of hypertrophic chondrocytes after the 11-day culture period with OSF-1. However, the protein was also immunolocalized in few hypertrophic chondrocytes of control growth plates and in isolated hypertrophic chondrocytes of pre-culture growth plates. Hypertrophic chondrocytes express several markers of the osteogenic phenotype (Lian *et al.* 1993), and when exposed to the appropriate microenvironment/ osteogenic stimuli, these can differentiate further to osteoblast-like cells (Bianco *et al.*, 1998). In relation to type I collagen synthesis by growth plate chondrocytes on exposure to OSF-1 in the present study, it can be suggested that OSF-1 acted on cells which were some way down the osteogenic

pathway, so that the additional osteogenic stimulus exerted by OSF-1 might have "pushed" these chondrocytes over a threshold towards the osteoblastic phenotype. Previous studies (Neame *et al.*, 1993; Imai *et al.*, 1998) have reported a prolific expression of OSF-1 in embryonic and fetal cartilage, particularly, 15 mg OSF-1/ kg wet weight of cartilage was isolated from dissociative extracts of bovine fetal epiphyseal and nasal cartilage. The present study demonstrated expression of the OSF-1 protein by immunostaining in most chondrocytes of 14-day embryonic chick nasal cartilage. Normal expression of alkaline phosphatase was also observed in these chondrocytes and matrix staining for the same suggested secretion of the enzyme by chondrocytes into the inter-territorial matrix. None of the chondrocytes in these pre-culture nasal cartilage samples were found to synthesize type I collagen. Thus, endogenous OSF-1 synthesized by these chondrocytes under physiological conditions was incapable of stimulating type I collagen synthesis. Neither did the culture conditions induce synthesis of type I collagen in chondrocytes of control explants after the 4-day culture period.

However, synthesis of type I collagen was observed in some chondrocytes of nasal cartilage cultured for 4 days with 50 ng/ml rhOSF-1. Although it was possible to co-localize type I collagen within the lacunae of chondrocytes also expressing the OSF-1 protein, type I collagen synthesis was not observed in every single chondrocyte expressing OSF-1. There were quite a few chondrocytes which were immunopositive only for OSF-1, indicating that endogenous expression of OSF-1 was maintained in the chondrocytes of these nasal cartilage explants after the 4-day culture period. However, endogenous OSF-1 was not sufficient to stimulate synthesis of type I collagen in these chondrocytes. On the other hand, synthesis of type I collagen was induced in some of these chondrocytes only on exposure to extra exogenous OSF-1.

Thus, exogenous OSF-1 induced the synthesis of type I collagen in some chondrocytes of cultured neonatal rat growth plates and embryonic chick nasal cartilage. Since type I collagen is one of the early markers of osteogenic differentiation, we examined whether any of these altered chondrocytes expressed late markers of the osteoblast phenotype. Presence of osteocalcin, the most osteoblast-specific marker, was not observed in these chondrocytes. Under these circumstances, it was therefore not possible to determine whether the synthesis of type I collagen by chondrocytes, under the influence of exogenous OSF-1, represented early stages of osteogenic differentiation or, perhaps, an abnormal response to excess OSF-1.

CELL CULTURES: BONE MARROW-DERIVED CELLS AND C2C12 CELL LINE

5.1 Introduction

The functional life span of the two cell types responsible for the formation and resorption of bone, osteoblasts and osteoclasts respectively, is limited (Parfitt, 1984; Nijweide *et al.*, 1986). The continued ability of the skeleton to respond appropriately to injury and to prevent accumulation of microdamage in postnatal life must therefore depend upon the precursor cell populations, that have the capacity to differentiate into fully functional osteoblasts and osteoclasts. The size of these precursor pools must in turn be maintained by the proliferative ability of undifferentiated cells that retain an extensive capacity for self-renewal throughout postnatal life, the stem cells. The stromal system of bone has been defined as those connective tissue elements within the marrow cavity that provide structural and functional support for haematopoiesis (Bentley, 1982; Dexter, 1982). A number of studies have shown the pluripotentiality of stem cells derived from bone marrow stroma and their ability to differentiate into fibroblastic, osteogenic, adipogenic, myogenic and reticular cells (Friedenstein, 1976; Owen and Friedenstein, 1988; Beresford, 1989) with an apparent degree of plasticity or interconversion (Bennett *et al.*, 1991; Beresford *et al.*, 1992; Oreffo *et al.*, 1997). Bone marrow is a tissue that can be obtained relatively easily and when disrupted/ disaggregated mechanically or enzymatically, serves as a good source of proliferating progenitor cells. The observation that the osteogenic potential of progenitors from marrow stroma is retained even when bone marrow is disrupted/ disaggregated to form single-cell suspensions, demonstrated the existence of determined precursors within bone marrow, which have a capacity for differentiation in an osteogenic direction that is not dependent upon the maintenance of any cell-cell and/ or cell-matrix interactions (Friedenstein *et al.*, 1966; Friedenstein *et al.*, 1982).

Bone morphogenetic proteins (BMPs) were originally identified in demineralized bone matrix as factors that induced ectopic bone formation when implanted into rat muscular tissue (Urist, 1965). More than 15 BMPs have been identified and they are highly conserved members of the transforming growth factor-β (TGF-β) superfamily (Celeste *et al.*, 1990; Centrella *et al.*, 1994; Groenveld and Burger, 2000). Among these genes, BMP-2, -4 and -7 have been shown to be potent bone inducing agents (Wang *et al.*, 1990; Sampath *et al.*, 1992; Mayer *et al.*, 1996). *In vitro* studies have shown that BMP-2 not only stimulated the maturation of osteoblastic progenitor cells (Yamaguchi *et al.*, 1991), but also induced differentiation of pluripotent C3H10T1/2 fibroblastic cells (Katagiri *et al.*, 1990) or W-20-17 stromal cells (Thies *et al.*, 1992) or C2C12 murine pre-myoblastic cells (Katagiri *et al.*, 1994; Lee *et al.*, 1999) into cells of the osteoblastic lineage.

Although, it is know that *osf-1*/ OSF-1 is expressed by osteoprogenitors and osteoblasts (Dreyfus *et al.*, 1998), and it has a role in stimulating new bone formation by the chemotactic recruitment of osteoprogenitors and osteoblasts (Imai *et al.*, 1999; Yang *et al.*, in press), the actions of OSF-1 on osteogenic differentiation of bone marrow-derived progenitors have not been studied. This chapter deals with the effects of OSF-1 on colony formation, osteogenic differentiation and proliferation of murine bone marrow-derived cells *in vitro*. Since the BMP-2-induced conversion of C2C12 premyoblasts to osteoblastic cells provides an excellent model for investigating mechanisms regulating commitment to an osteoblast phenotype, the same approach has been used to delineate the role of OSF-1 in osteogenic differentiation.

5.2 Materials and methods

Biochemical reagents used in the study were of analytical grade and purchased from Sigma (Poole, UK) and BDH/ Merck Ltd. (Poole, UK). Cell culture reagents, namely, Minimum Essential Medium Eagles (α modification)/ α-MEM (Cat. No. M0644), ascorbic acid-2-phosphate (Cat. No. A8960) and dexamethasone (Cat. No. D8893) were purchased from Sigma (Poole, UK). Dulbecco's Minimum Essential Medium/ D-MEM with Glutamax I (Cat. No. 61965-026) was purchased from Gibco/ Life Technologies. Fetal calf serum/ FCS (Cat. No. F553) was purchased from Meldrum Ltd. PicoGreen reagent (Cat. No. P7581) for quantitation of double stranded DNA was purchased from Molecular Probes (Leiden, The Netherlands). Recombinant human OSF-1 (rhOSF-1/ rhPTN, Cat. No. 450-15) was purchased from PeproTech (London, England). Recombinant human BMP-2/ rhBMP-2 was a generous gift from Prof. Walter Sebald, University of Wurzburg, Germany.

5.2.1 Cultures of bone marrow-derived cells

a. Human bone marrow-derived cells

Bone marrow samples were obtained from haematologically normal patients undergoing routine total hip replacement surgery (50-83 years of age). Only tissue, which would have been discarded, was used with the approval of the Southampton General Hospital Ethics Committee. Primary cultures of bone marrow cells were established as described previously by Oreffo and co-workers (1997). Marrow cells were harvested using α modification of Minimum Essential Medium (α-MEM) from trabecular bone marrow samples. The cell suspension was filtered through a 70-micron nylon mesh and pelleted by centrifugation at 500g for 5 min. at 4 ^{0}C. The supernatant was discarded and the cell pellet was resuspended in 10 ml α-MEM. Samples were diluted with 0.5% (w/v) trypan blue in 0.16 M ammonium chloride and

0.1% acetic acid, and the number and viability of nucleated cells determined. Cells were seeded at a density of 1 x 10^3 cells/ chamber in chamber slides (4-chamber) and cultured for 12 days in osteogenic medium (α-MEM supplemented with 10% fetal calf serum/ FCS, 100 μM ascorbic acid -2- phosphate and 10 nM dexamethasone). Medium was changed after 6 days of culture. On completion of the culture period (12 days), the cells were examined for *osf-1* gene expression by *in situ* hybridization.

b. Mouse bone marrow-derived cells

Primary cultures of bone marrow-derived cells from BDF-1 mice, aged 20-30 weeks, were established using a modification of the protocol for culturing human bone marrow cells (Oreffo *et al.*, 1997).

Femurs and tibiae were dissected from BDF-1 mice and after cutting off the proximal and distal epiphyses, the bone marrow contained within the cylindrical diaphysis was flushed out using a 5-ml syringe, filled with α-MEM and fitted with a 21/ 23 gauge needle. Marrow harvested from the femurs and tibiae of 2-3 mice was pooled and dispersed by flushing the marrow suspension with the syringe a couple of times, thereby yielding a homogeneous cell suspension. The cell suspension was filtered through a 70-micron nylon mesh and pelleted by centrifugation at 500g for 5 min. at 4 ^{0}C. The supernatant was discarded and the cell pellet was resuspended in 10 ml α-MEM. Samples were diluted with 0.5% (w/v) trypan blue in 0.16 M ammonium chloride and 0.1% acetic acid, and the number and viability of nucleated cells determined. Cells were seeded at a density of 2 x 10^6 cells/ well in 6-well plates and cultured for 12 days in basal (α-MEM supplemented with 15 % fetal calf serum/ FCS) and osteogenic (α-MEM supplemented with 15% FCS, 100 μM ascorbic acid -2-phosphate and 10 nM dexamethasone) media, to which rhOSF-1 was added in concentrations ranging from ng/ml to pg/ml. Cells cultured for 12 days in basal

medium supplemented with 50 ng/ml rhBMP-2 served as a positive control. Media were changed after 6 days of culture. On completion of the culture period (12 days), the media were removed, cells were washed twice with phosphate-buffered saline, air-dried and the cell layer used for alkaline phosphatase and DNA estimations. Alternatively, the cells were fixed in 95% ethanol after 9 days in culture, the colonies were stained for alkaline phosphatase and the number of alkaline phosphatase +ve colonies counted by eye using the colony counter (*Anderman CO Ltd, Kingston-on-Thames, UK*).

For examining OSF-1 protein and gene expression, cells were seeded at a density of 1 x 10^3 cells/ chamber in chamber slides (4-chamber) and cultured for 12 days in osteogenic medium. Medium was changed after 6 days of culture. After 12 days, cells were examined for *osf-1* gene expression by *in situ* hybridization. Protein expression was detected in 12-day cultures (fixed in 95% ethanol) by immunostaining with the anti-OSF-1 antibody.

5.2.2 C2C12 cell cultures

C2C12 cells (a murine pre-myoblastic cell line) were seeded at a density of 1 x 10^5 cells/ well in 24-well plates and cultured overnight in 10% FCS in D-MEM to reach 80% confluence. The cells were then cultured for a further two days in 2% FCS in D-MEM, to which 100 ng/ml rhBMP-2 and rhOSF-1 (0.05 pg/ml to 100ng/ml) were added either individually or together. In another set of experiments, after the overnight culture in 10% FCS in D-MEM, cells were cultured for two days in 2% FCS in D-MEM containing 100 ng/ml rhBMP-2. Thereafter, the cells were supplemented with fresh 2% FCS in D-MEM containing rhOSF-1 (5 pg/ml, 10 pg/ml and 50 ng/ml) and cultured for an additional 4 days. At the end of the culture period, medium was removed, cells were washed twice with phosphate-buffered saline, air-

dried and the cell layer was used for alkaline phosphatase and DNA estimations. Alternatively, the cells were fixed in 95% ethanol and stained for alkaline phosphatase.

5.2.3 Biochemical assays

For biochemical assays, cells (stored in the freezer) were thawed by incubating the culture plates at 37 ^{0}C for 10 min. Depending upon the degree of confluence, appropriate volumes (300 µl - 600 µl) of Triton X-100 (0.01%) were added to each well and the cells scraped off the tissue culture plastic using a cell scraper. The detached cells were then lysed by repeated (atleast 3) freeze-thaw cycles and the lysate used for the biochemical assays.

a. Alkaline phosphatase

Principle

Alkaline phosphatase activity was determined using a spectrophotometric method involving the cleavage of p-Nitrophenyl phosphate (NPP) by alkaline phosphatase to p-Nitrophenol (NP) and inorganic phosphate under alkaline conditions. The amount of p-Nitrophenol released was determined by comparing the colour intensity, at 410 nm, with a range of p-Nitrophenol standards treated similarly.

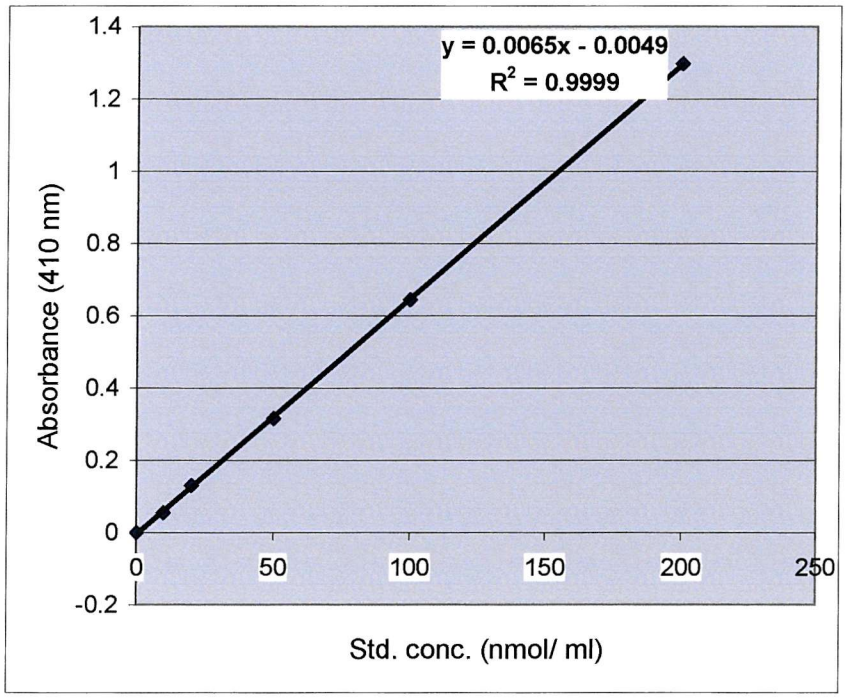
Reagents

1. Alkaline buffer (2-amino-2-methyl-1-propanol buffer, 1.5 mol/L, pH 10.3, Sigma Cat. No. 221)
2. p-Nitrophenol std. stock soln. (10 µmol/ ml, Sigma Cat. No. 104-1)
   Working std. soln. - 10, 20, 50, 100, 200 nmol/ ml
3. p-Nitrophenyl phosphate (Sigma Cat. No. 104-0, working soln. - 1.3 g/L)
4. 1M NaOH soln.

Procedure

50 µl of the cell lysate was incubated with 950 µl p-nitrophenyl phosphate substrate at 37 ^{0}C for 20-60 min. Once the desired colour was developed, the reaction was terminated by adding 1 ml NaOH to the reaction mixture and the incubation time for the reaction noted. 1 ml NaOH was added to 1 ml of each working std. soln., and the blank consisted of 50 µl assay buffer, 950 µl substrate and 1 ml NaOH. Samples, standards and blank were read on the spectrophotometer at 410 nm. Concentrations of samples were extrapolated from the standard curve.

Figure 29. Standard plot for alkaline phosphatase estimation



b. DNA

Double stranded DNA (ds DNA) in solution was quantitated using the ultrasensitive fluorescent nucleic acid stain, Picogreen.

Reagents

1. 1X Tris-EDTA (TE) buffer, pH 7.5

2. Stock std. soln. of ds DNA - 2 µg/ ml in 1X TE

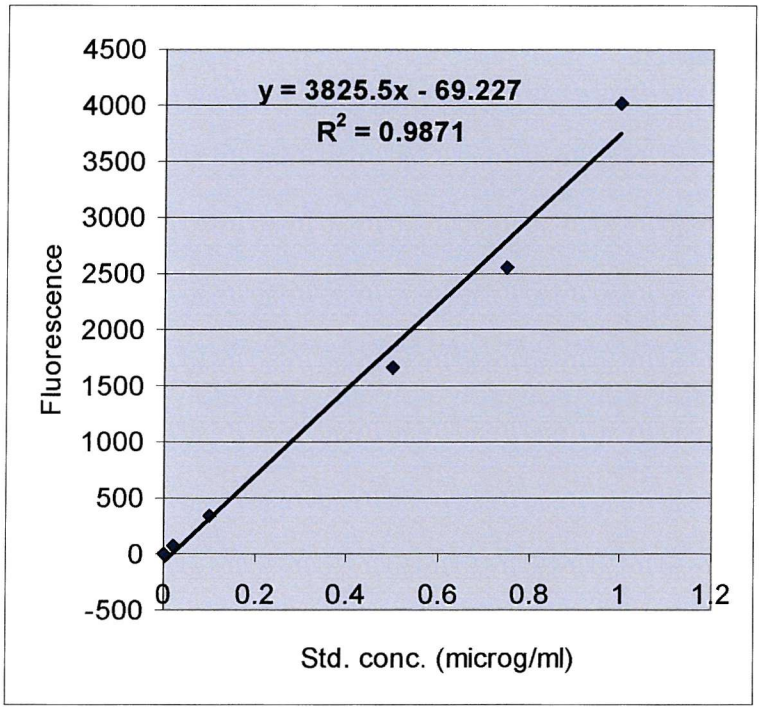
Working std. soln. - 0.02, 0.1, 0.5, 0.75 and 1 µg/ ml

3. PicoGreen reagent - Working soln. is 200-fold diluted in TE (prepare afresh)

Procedure

The assay was performed in 96-well low fluorescence CytoFluor microplates. Each sample was diluted 1:1 in 1X TE and to 100 µl of the diluted sample, 100 µl of PicoGreen working solution was added. Similarly, 100 µl of the PicoGreen working solution was added to 100 µl of each working std. The fluorescence was measured using a fluorescence microplate reader (Cytofluor II) using excitation at 485 ± 4.5 nm and emission detection at 525 ± 4.5 nm. Concentrations of the samples were extrapolated from the standard curve.

Figure 30. Standard plot for DNA estimation



5.2.4 Staining techniques

a. Alkaline phosphatase

The cleavage of a suitable substituted naphthol substrate by alkaline phosphatase produces an insoluble naphthol derivative, which then couples with a diazonium salt, like fast violet B salt, to produce a violet coloured insoluble azo dye at the site of enzyme activity.

Reagents

1. Naphthol AS-MX phosphate alkaline soln. (Sigma, Cat. No. 85-5)
2. Fast violet B salt (Sigma, Cat. No. F-1631)

Procedure

Naphthol AS-MX phosphate alkaline solution was diluted 1 in 25 in distilled water and the same used to prepare a 24 mg% solution of fast violet B salt. 1.5 ml of this solution was added to each well and the plates incubated at 37^0C in dark for colour development. Once staining was evident, the solution was discarded and the cells were washed with water twice.

b. Immunostaining

Cells were immunostained for the presence of OSF-1 using the anti-OSF-1 antibody (raised against the synthetic N-terminal peptide of HB-GAM isolated from perinatal rat brain, details in Rauvala, 1989), diluted 1:500, a gift from Dr. Heiki Rauvala, Finland. After quenching endogenous peroxidase activity with 3% H_2O_2, cells were incubated with the primary antiserum at 4 ^{0}C for 3 hours. Visualization of the primary antibody involved the avidin-biotin method linked to peroxidase and 3-amino, 9-ethylcarbazole (AEC). Negative controls (omission of the primary antibody) were included everytime. (*Details of the technique in* ***section 2.2.1 e***).

5.2.5 In situ hybridization studies

a. Amplification of a region of human osf-1 cDNA by PCR

A 315 base pair (bp) fragment of the human *osf-1* coding region (EMBL accession number D90226, nucleotides 363-677) was amplified by PCR using primers designed to create *Sac*I and *Sal*I restriction sites.

Forward primer: 5' TGAAGACCCAGAGCTCTAAGATCCCCTGCA 3'

Reverse primer: 5' CGGATCCTGTTTGTCGACGTCCTTTTTATG 3'

PCR conditions: 20 cycles for 'denaturation' at 94^0C, 20 cycles for 'primer annealing' at 55^0C, 30 cycles of 'primer extension' at 72^0C

The PCR product was purified using the QIAquick PCR purification kit protocol (Cat. No. 28104, Qiagen, Germany).

b. Sub-cloning of the osf-1 fragment (Sambrook, Fritsch and Maniatis, 1989)

This involved cloning of the *osf-1* insert from the plasmid p1822 (a gift from Prof. Hashimoto-Gotoh, Japan) into the *Sac*I and *Sal*I sites of pBluescript SK- (pBSSK-) (Stratagene, Amsterdam, Netherlands) (Fig. 31).

The pBluescript vector was digested with *Sal*I (Cat. No. R605A, Promega) and *Sac*I (Cat. No. R 606I, Promega) and, after digestion, the large fragment of the vector was purified from the small remnant of the polycloning site by agarose gel electrophoresis. A segment of the *osf-1* insert/ cDNA (284 bp) was excised from p1822 with *Sal*I and *Sac*I, and purified from the remainder vector by agarose gel electrophoresis. The Bluescript vector was then ligated to the excised segment of *osf-1* DNA, which contained the cohesive termini compatible with those generated by *Sal*I and *Sac*I digestion of the Bluescript vector. The resulting recombinant was then used to transform *E. coli* (TOP10 strain, Invitrogen) to ampicillin resistance. Due to lack of complementarity between the *Sal*I and *Sac*I protruding ends, the digested vector

fragment was incapable of circularizing effectively and therefore incapable of transforming bacteria to ampicillin resistance efficiently. Thus, most of the bacterial cells resistant to ampicillin contained the Bluescript vector with the *osf-1* insert, as it formed a bridge between the *Sal*I and *Sac*I sites. The recombinant plasmid was purified from the bacterial cells using the Qiagen Midiprep Kit (Cat. No. 12143, Qiagen, Germany). Sequence of the Bluescript vector containing the *osf-1* insert was validated by DNA sequencing (Oswel DNA Sequencing, University of Southampton, UK).

c. Synthesis of digoxigenin-labelled riboprobes

Sense and antisense riboprobes were generated using T 7 (Cat. No. 1175025) and T 3 (Cat. No. 1031163) RNA polymerases respectively, and labelled with digoxigenin – UTP according to manufacturer's instructions (Roche, Welwyn Garden City, UK). Although the riboprobes were designed using the human *osf-1* cDNA sequence, 85% homology was observed with the mouse *osf-1* cDNA sequence (EMBL accession number D90225).

d. Hybridization with riboprobes (Hesketh et al., 1994)

Cultures of bone marrow-derived cells were washed three times with DEPC-PBS and fixed in 4% paraformaldehyde (PFA) in DEPC-PBS. Endogenous alkaline phosphatase activity was quenched by incubating the cells with 0.2N HCl for 5 min. and cell membranes were permeabilized using 0.2% Triton X100 in 4% PFA, DEPC-PBS. Cells were incubated overnight at 55^0C with sense and antisense riboprobes, diluted 1:9 with the *in situ* hybridization mixture (Cat. No. H7782, SIGMA, Poole, UK). Cells were washed with 5X saline sodium citrate buffer (SSC) and 50% formamide, 2X SSC. Unhybridized riboprobes were digested by incubating the cells with 20 μg/ml RNAse (Cat. No. R4642, SIGMA, Poole, UK) for 30 min. at 37^0C.

e. Digoxigenin (DIG) detection

Anti-DIG-Alkaline phosphatase-Fab fragments (1:100 dilution, Cat. No. 1093274, Roche, Welwyn Garden City, UK) from sheep were used for digoxigenin detection. Following removal of excess unbound Ab-conjugate, colour development was facilitated by incubating with the BCIP/ NBT liquid substrate system (Cat. No. B1911, SIGMA, Poole, UK) in dark for 15 min. Cells were mounted in Aquamount.

Statistics:

Samples were run in triplicate for all assays. Statistical analysis was performed using the GraphPad InStat Software (GraphPad Software Inc., San Diego, California, USA), applying the unpaired student's t-test.

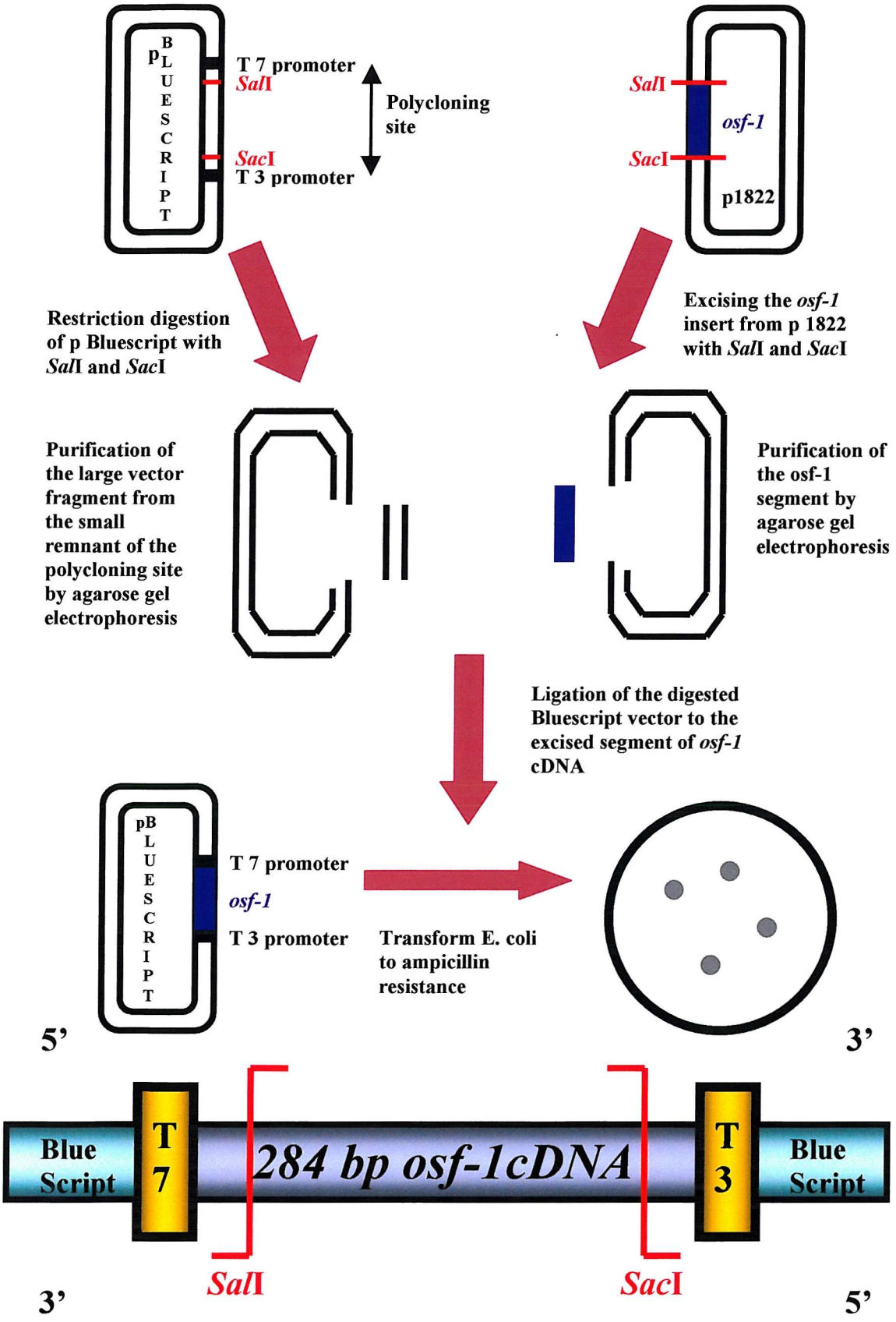


Figure 31. Schematic diagram - Sub-cloning of human osf-1 cDNA (284 bp segment)

5.3 Results

5.3.1 Expression of osf-1 mRNA and protein in osteoprogenitors from human and mouse bone marrow cell cultures

Primed osteoprogenitors from 12-day cultures of human bone marrow-derived cells, in osteogenic medium, were found to express the *osf-1* gene (Fig. 32 A and B). Expression of the *osf-1* gene (Fig. 32 D and E) coincided with the synthesis of the OSF-1 protein (Fig. 32 G) in osteoprogenitors from 12-day cultures of mouse bone marrow-derived cells in osteogenic medium.

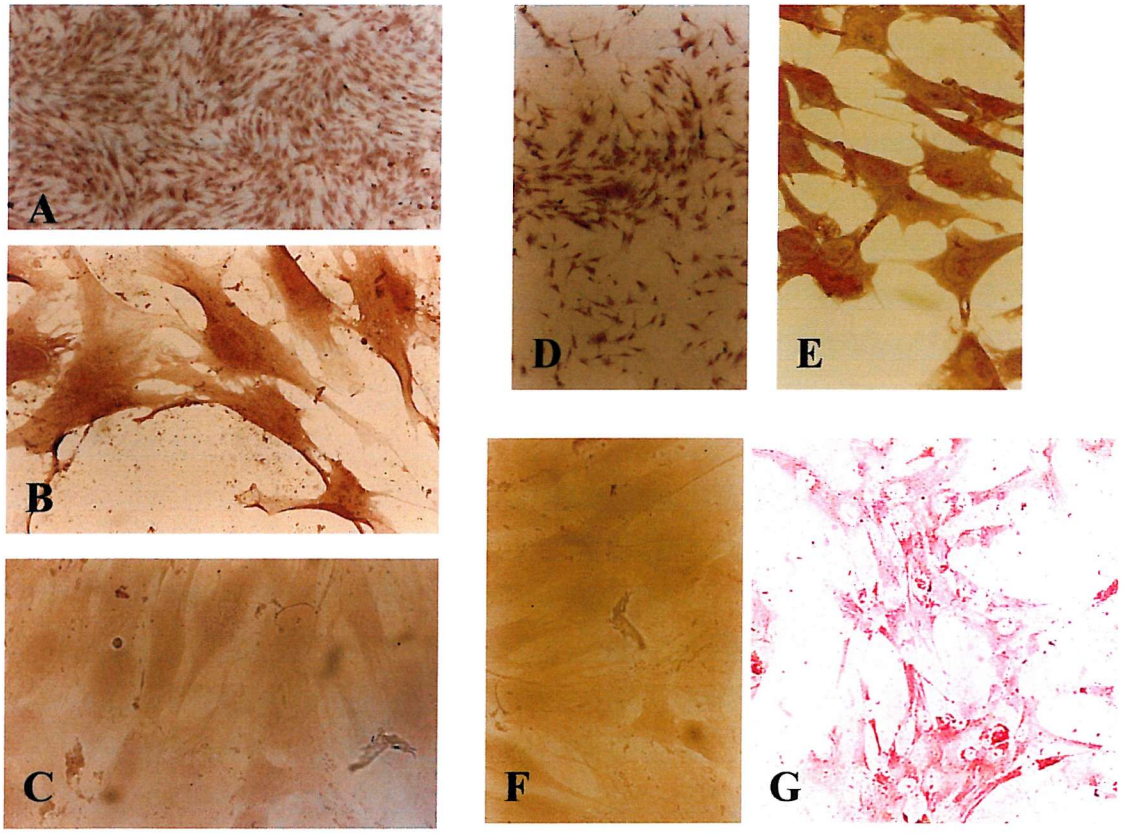


Figure 32. Osf-1 gene and protein expression in osteoprogenitors from bone marrow. *Hybridization to the antisense riboprobes demonstrated osf-1 gene expression throughout the cultures of human (A and B – day 12) and mouse (D and E – day 12) bone marrow-derived cells in osteogenic media. It was not possible to detect a signal with the sense riboprobes in human (C – day 12) and mouse (F – day 12) bone marrow cultures. The OSF-1 protein was also immunolocalized in osteoprogenitors from 12-day cultures of mouse bone marrow-derived cells in osteogenic medium (G).*

5.3.2 Effect of OSF-1 on osteogenic differentiation of mouse bone marrow cells

a. Dose-dependent effect of OSF-1 on alkaline phosphatase activity

To determine whether OSF-1 influenced alkaline phosphatase activity in a dose-dependent manner, murine bone marrow cells were cultured for 12 days in basal and osteogenic media to which rhOSF-1 was added in concentrations ranging from ng/ml to pg/ml (Fig. 33). At the end of the culture period, alkaline phosphatase (ALP) activity was measured in these cells as it served as one of the earliest markers of osteogenic differentiation. Results for the same were expressed as 'ALP specific activity' (by dividing total ALP activity of cells in a well with the total DNA content of that well) as it represented ALP activity per cell, and served to compensate for any effects of OSF-1 on cell proliferation, which would consequently alter the total ALP activity. It was possible to detect a baseline level ALP activity in control cultures under basal conditions. In basal media, ALP specific activity of cells remained unaltered when cultured with ng/ml concentrations of rhOSF-1, however, a 27% increase in ALP specific activity above the baseline level was observed when cells were cultured were with 10 pg/ml rhOSF-1 (Fig. 33 A). As expected, the basal level of ALP specific activity was 57% higher in cells cultured under osteogenic conditions in comparison to that in cells cultured under basal conditions. A further 15% increase in ALP specific activity above the baseline was observed when cells were cultured in osteogenic media supplemented with 10 pg/ml rhOSF-1 (Fig. 33 B). Once again, ng/ml concentrations of rhOSF-1 did not influence the ALP specific activity of cells cultured under osteogenic conditions (Fig. 33 B). Thus, OSF-1 was able to stimulate alkaline phosphatase activity of mouse bone marrow cells in a dose-dependent manner. While ng/ml concentrations of OSF-1 were incapable of exerting any

significant effect, a significant effect on alkaline phosphatase activity was observed with appreciably lower concentrations in the pg/ml range.

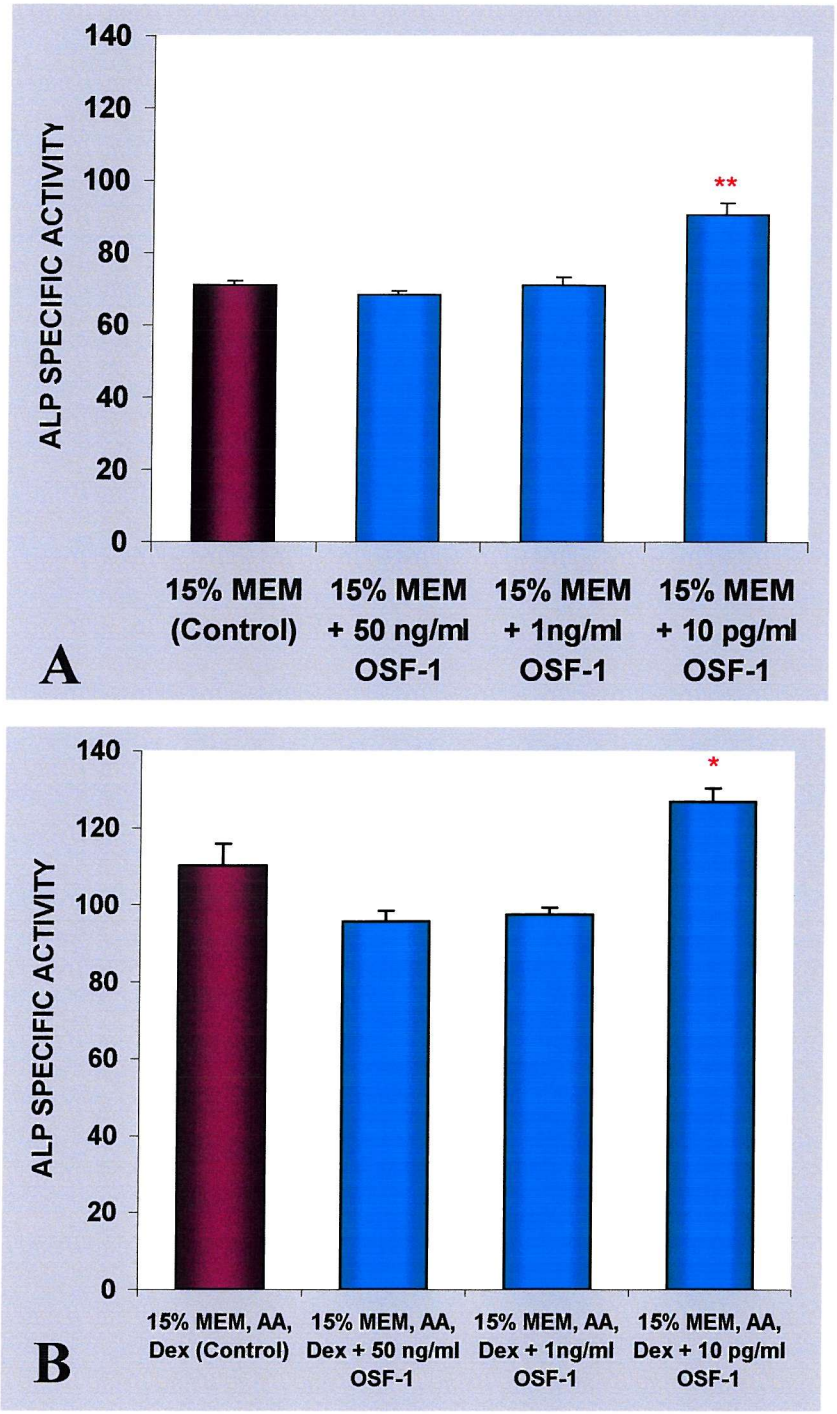


Figure 33. Dose-dependent effect of rhOSF-1 on alkaline phosphatase activity of mouse bone marrow-derived cells cultured for 12 days under basal (A) and osteogenic (B) conditions. *ALP specific activity, expressed as nmol NP/ hr/ μg DNA, served as an index of osteogenic differentiation. At ng/ml concentrations, namely 50*

*ng/ml and 1 ng/ml, rhOSF-1 did not have any effect on alkaline phosphatase activity of mouse bone marrow cells. However, rhOSF-1 significantly enhanced alkaline phosphatase activity in basal as well as osteogenic media at an appreciably lower concentration of 10 pg/ml. Results are expressed as mean ± SE; * $p < 0.05$, ** $p < 0.01$; $N = 6$.*

b. Comparison between the potential of BMP-2 and OSF-1 to stimulate alkaline phosphatase activity

Bone morphogenetic proteins (BMPs) are potent stimulators of osteogenic differentiation. Thus, mouse bone marrow cells cultured for 12 days in basal medium supplemented with 50 ng/ml rhBMP-2 served as a positive control. A concentration of 50 ng/ml rhBMP-2 was chosen since previous experiments with human bone marrow cells in our lab had demonstrated that it was the lowest concentration at which a stimulatory effect on osteogenic differentiation could be observed. In basal medium, 50 ng/ml rhBMP-2 was found to enhance ALP specific activity of mouse bone marrow cells by 70 % above the baseline level, in comparison to the 27% increase in ALP specific activity with 10 pg/ml rhOSF-1 (Fig. 34). Although the potential of BMP-2 to stimulate alkaline phosphatase activity of mouse bone marrow cells was appreciably greater than OSF-1, this was at a 5000-fold higher concentration of rhBMP-2 in comparison to rhOSF-1.

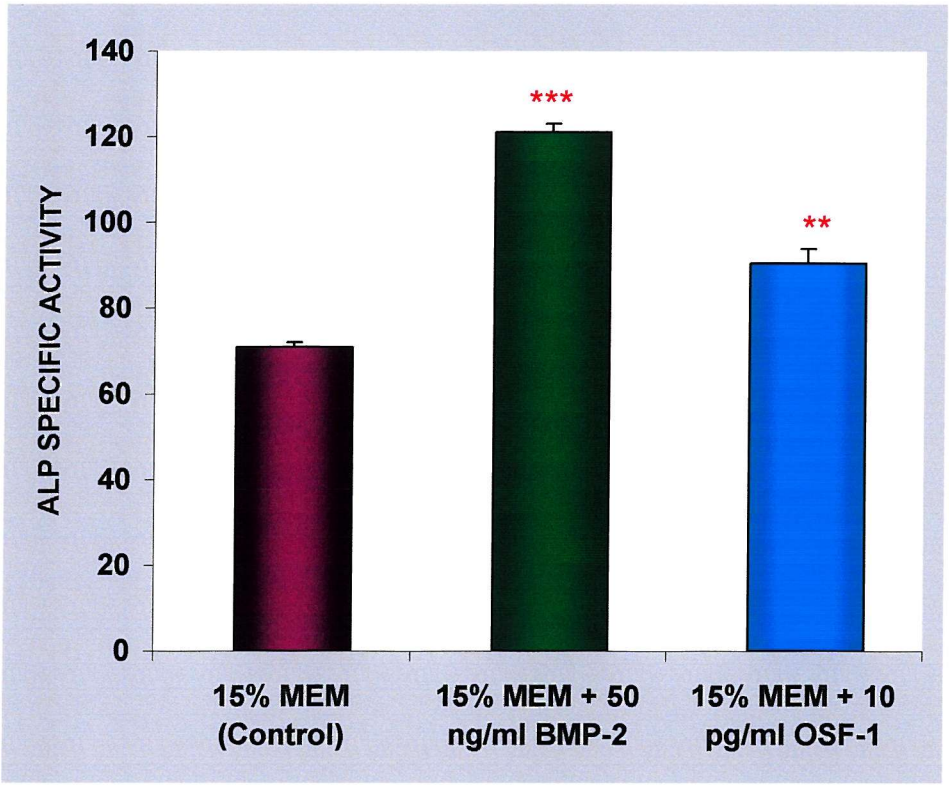


Figure 34. Effect of BMP-2 and OSF-1 on alkaline phosphatase activity of mouse bone marrow cells cultured for 12 days in basal medium. *ALP specific activity, expressed as nmol NP/ hr/ µg DNA, served as an index of osteogenic differentiation. A significant increase in ALP specific activity above the baseline level was observed in the presence of both, 50 ng/ml rhBMP-2 and 10 pg/ml rhOSF-1. Although the increase in ALP specific activity was greater in mouse bone marrow cells cultured with 50 ng/ml rhBMP-2, compared to that in cells cultured with 10 pg/ml rhOSF-1, a 5000-fold higher concentration of rhBMP-2 was required to elicit this response. Results are expressed as mean ± SE; ** $p < 0.01$, *** $p < 0.001$; $N = 6$.*

c. Effect of OSF-1 on formation of ALP +ve colony forming unit-fibroblastic (CFU-F)

Fibroblast colonies, each derived from a single precursor cell and termed colony forming unit-fibroblastic (CFU-F), can be detected as early as 'day 3/ 4' of mouse bone marrow cell culture. Being multipotent, cells of the CFU-F can be stimulated to differentiate along diverse lineages by culturing them under appropriate conditions. Culture under basal conditions maintains CFU-F in a relatively undifferentiated state, while culture under osteogenic conditions causes their differentiation along the osteogenic pathway. Osteogenic differentiation can be inferred by staining the colonies for alkaline phosphatase (ALP).

Mouse bone marrow cells were cultured for 9 days with 10 pg/ml rhOSF-1 under basal and osteogenic conditions, and the effect of pg/ml quantity of OSF-1 on stimulating ALP expression in the CFU-F was studied by counting the number of ALP +ve colonies. 10 pg/ml rhOSF-1 enhanced the number of ALP +ve CFU-F under basal conditions, an increase of almost 30% being observed in the number of ALP +ve colonies (Fig. 35). The number and size of alkaline phosphatase +ve CFU-F were significantly increased in osteogenic medium, in comparison to basal medium. However, 10 pg/ml rhOSF-1 was unable to influence the number of ALP +ve CFU-F under osteogenic culture conditions (Fig. 35).

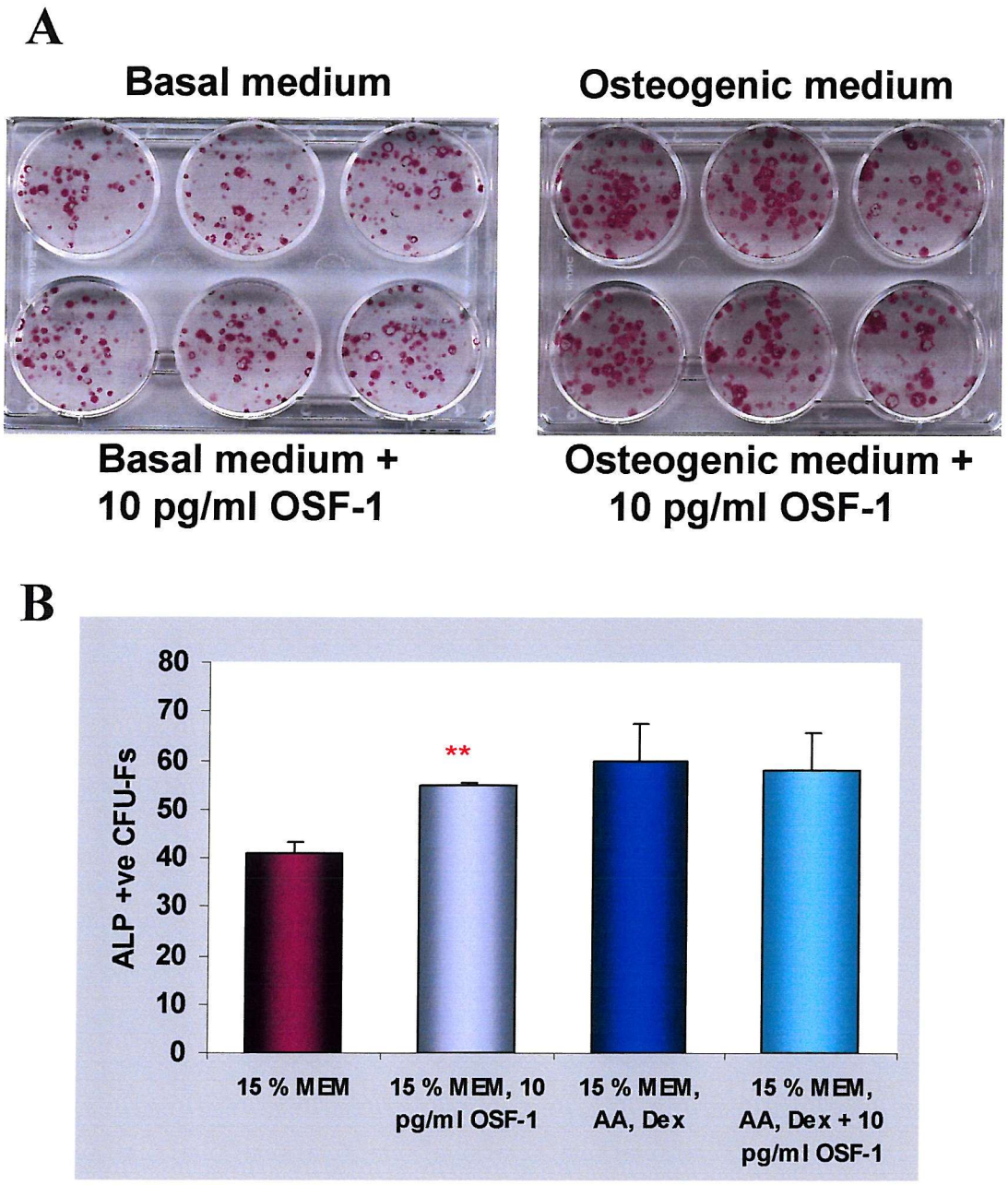


Figure 35. Effect of OSF-1 on the number of ALP +ve colony forming unit-fibroblastic/ CFU-F, derived from mouse bone marrow cells cultured for 9 days under basal and osteogenic conditions. *CFU-F exhibiting staining for ALP (A). Although a significant increase in the number of ALP +ve CFU-F was observed when mouse bone marrow cells were cultured with 10 pg/ml rhOSF-1 for 9 days in basal medium, 10 pg/ml rhOSF-1 was unable to influence the number of ALP +ve CFU-F under osteogenic culture conditions (B). Results are expressed as mean ± SE; ** $p < 0.01$; $N = 3$.*

5.3.3 Effect of OSF-1 on cell proliferation in cultures of mouse bone marrow cells

To determine the effect of OSF-1 on cell proliferation, DNA content/ well was determined in 12-day cultures of mouse bone marrow cells in basal and osteogenic media to which 10 pg/ml rhOSF-1 was added. OSF-1 had a modest, but significant, effect on cell proliferation, as indicated by a 13% increase in DNA content in basal medium and a 7% increase in DNA content under osteogenic culture conditions (Fig. 36).

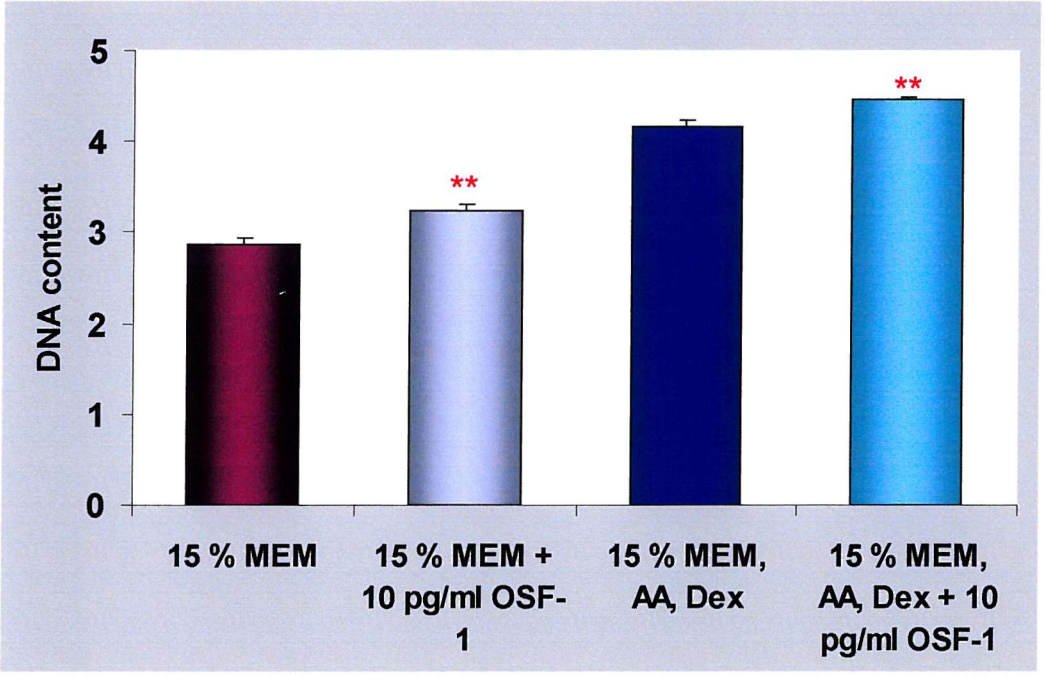


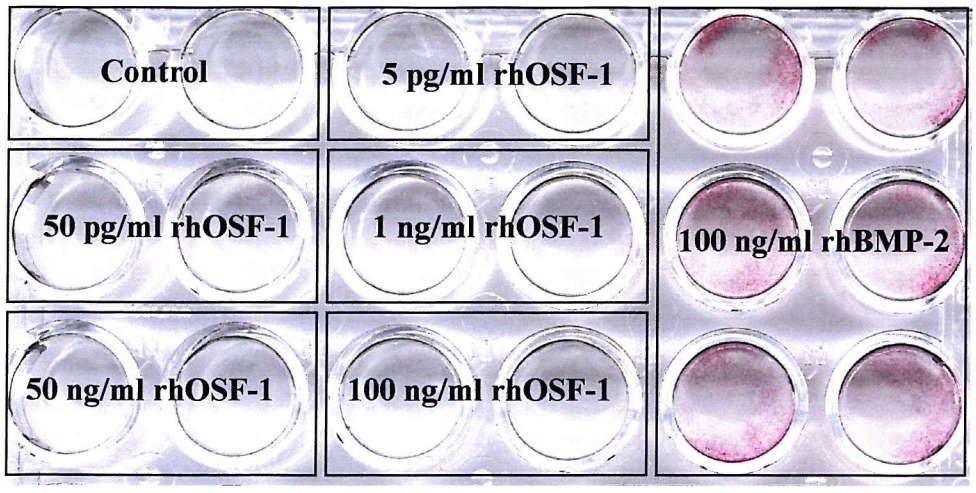
Figure 36. Effect of OSF-1 on proliferation of mouse bone marrow cells cultured for 12 days under basal and osteogenic conditions. *Cell proliferation was deduced in terms of DNA content, expressed as μg/ well. 10 pg/ml rhOSF-1 enhanced proliferation of bone marrow cells in basal as well as osteogenic media. Results are expressed as mean ± SE; ** $p < 0.01$; $N = 6$.*

5.3.4 Effects of exogenous OSF-1 on C2C12 cell cultures

Following the finding that OSF-1 stimulated osteogenic differentiation of bone marrow cells at appreciably low concentrations, the study further explored whether OSF-1 had osteoinductive capabilities similar to those of BMPs, and what its interactions were with BMPs. Towards this end, cells of the C2C12 cell line (murine pre-myoblastic cell line) were utilized, bearing in mind their multipotency and ability to differentiate along the osteogenic pathway.

a. Initiation of osteogenic differentiation in C2C12 cells

C2C12 cells are characterized by their exquisite sensitivity to BMPs, which can induce them to differentiate along the osteogenic pathway. Addition of 100 ng/ml rhBMP-2 to cultures of C2C12 cells for two days induced osteogenic differentiation, as indicated by the appearance of numerous alkaline phosphatase +ve cells (Fig. 37 B). However, addition of rhOSF-1 to cultures of C2C12 at concentrations between 5 pg/ml and 100 ng/ml for 2 days failed to induce osteogenic differentiation, evident by the absence of alkaline phosphatase staining (Fig. 37 C). Thus, OSF-1, on its own at the concentrations examined, lacked the osteoinductive potential of BMP-2 and failed to convert the multipotent pre-myoblastic C2C12 cells to the osteogenic phenotype.



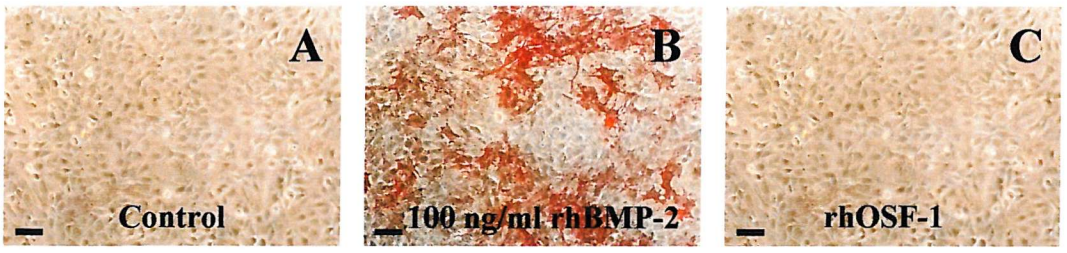


Figure 37. Effect of BMP-2 and OSF-1 on initiation of osteogenic differentiation in C2C12 cells. *Overview of plate exhibiting staining for ALP in C2C12 cells cultured for 2 days with 100 ng/ml rhBMP-2 and rhOSF-1 in concentrations ranging from 5pg/ml to 100 ng/ml. Staining for ALP was not observed in 2-day control cultures of C2C12 cells in 2% FCS-D-MEM (A). Numerous cells stained for ALP in 2-day cultures of C2C12 cells with 100 ng/ml rhBMP-2 (B). Absence of ALP staining in C2C12 cells cultured for 2 days with rhOSF-1 (C). Figure C is representative of a range of rhOSF-1 concentrations from 5 pg/ml to 100 ng/ml. Scale bars = 10 μm.*

b. Interactions between OSF-1 and BMP-2

Having observed that OSF-1 lacked the osteoinductive potential of BMP-2, interactions between OSF-1 and BMP-2 were studied to examine whether OSF-1 influenced BMP-2-induced osteogenic differentiation.

When C2C12 cells were cultured for two days with 100 ng/ml rhBMP-2, together with rhOSF-1 in concentrations of 0.05 pg/ml, 5 pg/ml and 100 ng/ml, OSF-1 inhibited BMP-2-stimulated osteoinduction. This was evident by way of a 65-75% decrease in ALP specific activity in cultures with BMP-2 and OSF-1 together, in comparison to cultures with BMP-2 alone (Fig. 38). The inhibitory effect of OSF-1 was apparent even at concentrations as low as 0.05 pg/ml. Thus, presence of OSF-1 during the osteoinductive phase almost abolished the osteogenic differentiation induced by BMP-2.

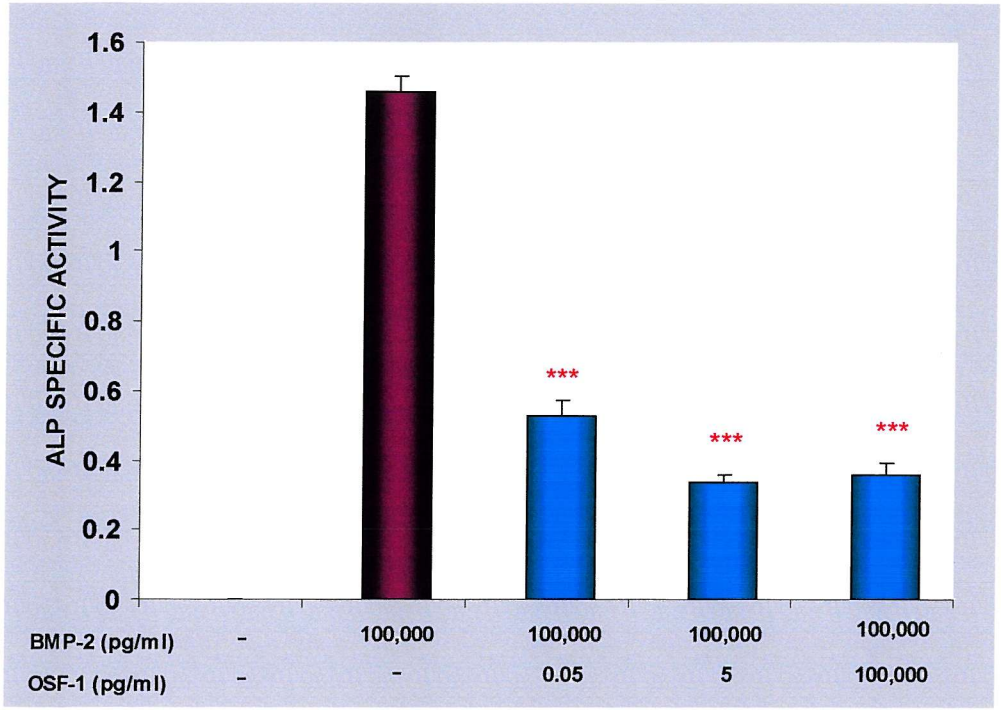


Figure 38. Effect of OSF-1 on BMP-2-stimulated osteoinduction in 2-day cultures of C2C12 cells. *Osteogenic differentiation, inferred as ALP specific activity (nmol NP/hr/ng DNA), of C2C12 cells induced by 100 ng/ml rhBMP-2, was inhibited by rhOSF-1 in concentrations of 0.05 pg/ml, 5 pg/ml and 100 ng/ml. Results are expressed as mean ± SE; *** $p < 0.001$; $N = 4$.*

To resolve the paradoxical effects of OSF-1 by way of enhancing osteogenic differentiation on one hand, while inhibiting BMP-2 osteoinduction on the other, a two-phase treatment regime was tested. A 2-day culture of C2C12 cells with 100 ng/ml rhBMP-2 alone (the osteoinductive phase) was followed by a 4-day culture period with rhOSF-1 (in concentrations of 5 pg/ml, 10 pg/ml and 50 ng/ml) alone. In this case, OSF-1 enhanced ALP specific activity by 40% (at a concentration of 10 pg/ml) and 80% (at a concentration of 5 pg/ml), in comparison with the ALP specific activity of cultures maintained in 2% FCS in D-MEM medium after the initial 2-day osteoinduction phase (Fig. 39). OSF-1 at a higher concentration of 50 ng/ml had no

effect. Thus, OSF-1 was found to enhance osteogenic differentiation of a cell population following osteoinduction by BMP.

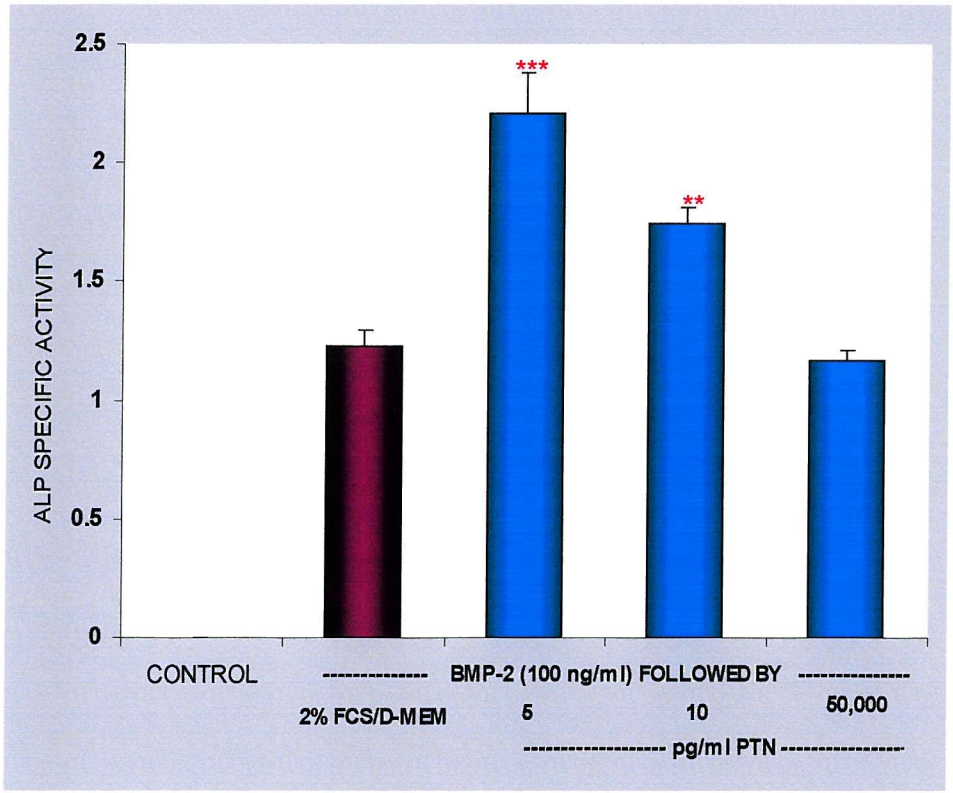


Figure 39. Effect of OSF-1 on osteogenic differentiation of C2C12 cells, in which osteoinduction had been accomplished with BMP-2. *When added to the culture after the initial 2-day osteoinductive phase with 100 ng/ml rhBMP-2, pg/ml concentrations of rhOSF-1 enhanced osteogenic differentiation (inferred as an increase in ALP specific activity – nmol NP/hr/ng DNA) of C2C12 cells, which had been primed to differentiate along the osteogenic lineage. Results are expressed as mean ± SE; ** $p < 0.01$, ** $p < 0.001$; $N = 4$.*

5.4 Discussion

The enormous capacity of bone tissue in post-natal life to regenerate and remodel, reflects the extent of stem cell involvement during bone development. Bone marrow contains multipotential stromal fibroblastic stem cells, which can differentiate along the fibroblastic, osteogenic, adipogenic, myogenic and reticular lineages (Friedenstein, 1976; Owen and Friedenstein, 1988; Beresford, 1989). Furthermore, these stem cells generate progenitors committed to one or more cell lineages, with an apparent degree of plasticity or interconversion (Bennett *et al.*, 1991; Beresford *et al.*, 1992; Oreffo *et al.*, 1997). Bone marrow is a tissue that can be obtained relatively easily and when disrupted/ disaggregated mechanically or enzymatically, serves as a good source of proliferating progenitor cells.

It is possible to culture an adherent population from the marrow cell suspension in appropriate tissue culture medium containing fetal calf serum. Initially, the cultures comprise a mixed population of stromal fibroblasts and haematopoietic cells. However, haematopoietic cells do not survive, float off and are removed during the medium changes. Thus, marrow cells, prepared as a single cell suspension and cultured *in vitro* with fetal calf serum, form fibroblastic colonies, each of which is derived from a single cell. The bone marrow cell type responsible for the initiation of colony formation *in vitro* was originally referred to as fibroblast colony-forming cell/ FCFC by Friedenstein (1976). More recently, however, the acronym colony-forming unit-fibroblastic/ CFU-F has gained widespread acceptance (Castro-Malaspina *et al.*, 1984; Lim *et al.*, 1986; Owen, 1989).

The colonies are first apparent 3 to 4 days postexplantation, the size but not the number of colonies increases between days 5 and 12 such that by day 10 individual colonies may be 0.5 to 0.8 cm in diameter and comprise 5×10^3 to 10^4 cells

(Friedenstein, 1976; Castro-Malaspina *et al.*, 1984). A number of experimental observations support the conclusion that the colonies formed from marrow suspensions *in vitro* are clonal in origin (Friedenstein, 1980; Latzinik *et al.*, 1986; Owen *et al.*, 1987). Culture under basal conditions (with fetal calf serum) maintains CFU-F in a relatively undifferentiated state, while culture under osteogenic conditions (with ascorbic acid and dexamethasone) causes differentiation along the osteogenic pathway. Dexamethasone is found to increase total colony number as well as the number of colonies expressing alkaline phosphatase (Maniatopoulos *et al.*, 1988; Oreffo *et al.*, 1997).

The present study demonstrated expression of the *osf-1* gene and OSF-1 protein in primed human and mouse osteoprogenitors from bone marrow cell cultures by *in situ* hybridization and immunolocalization respectively. This confirmed the findings of Dreyfus and co-workers, who demonstrated *osf-1* mRNA expression in committed osteoprogenitor cells of embryonic chick tibiae (Dreyfus *et al.*, 1998). Since N-syndecan, one of the receptors for OSF-1, is also expressed by the osteoprogenitor populations (Imai *et al.*, 1998), OSF-1 may have a role in influencing the activity of these osteoprogenitor populations in an autocrine manner.

CFU-F have been used extensively as a model system for studies on the effects of a number of biological agents on progenitor cells and also to measure their proliferation and differentiation potentials. The effect of OSF-1 on stimulation of alkaline phosphatase expression by CFU-F under basal and osteogenic culture conditions was examined in the present study. At an appreciably low concentration of 10 pg/ml, OSF-1 stimulated the number of alkaline phosphatase +ve colonies in basal culture. This was in support of a separate study by our group on the effects of OSF-1 on human bone marrow-derived cells (Yang *et al.*, in press), wherein 5 and 10 pg/ml

concentrations of OSF-1 were found to enhance the number of alkaline phosphatase +ve CFU-F in basal and osteogenic media, while higher concentrations did not exert a stimulatory effect.

Furthermore, OSF-1 was found to stimulate alkaline phosphatase activity in basal conditions by 27% and by 15% in osteogenic conditions. This stimulatory effect of OSF-1 was concentration-dependent, pg/ml concentrations (10 pg/ml) being more effective than ng/ml concentrations, thereby suggesting that OSF-1 exerted a stimulatory effect on osteogenic differentiation at appreciably lower (pg/ml) concentrations and higher concentrations (in the ng/ml range) did not elicit an osteogenic response from mouse bone marrow cells. This, once again, was in agreement with the findings of our group (Yang *et al.*, in press) that OSF-1 stimulated alkaline phosphatase activity of human bone marrow cells in basal and osteogenic media at pg/ml concentrations, while ng/ml concentrations were not stimulatory. Although, the present study only investigated the effect of OSF-1 on stimulation of ALP activity, an early marker of osteogenic differentiation, the study by Yang *et al.* confirmed that pg/ml concentrations of OSF-1 were able to maintain further osteogenic differentiation and promote mineralization in human bone marrow-derived cell cultures.

Bone formation involves the directed differentiation of mesenchymal cells into osteogenic cells, a process subject to regulation by a variety of hormones and factors (Bianco *et al.*, 2001; Bianco and Robey, 2001). BMPs, originally identified as proteins which could induce new cartilage and bone formation in non-bony tissues, are key osteoinductive factors pivotal in the recruitment, commitment and differentiation of osteoprogenitors (Wozney and Rosen, 1998). Native BMPs are 100-1000 fold more effective in inducing bone formation than individual recombinant

BMPs (De Groot, 1998). Despite the availability of recombinant BMPs, which particular recombinant BMP or which combination of recombinant BMPs will be most efficacious and cost-effective for bone induction and regeneration in clinical practice remains unclear.

The present study demonstrated that the concentration of recombinant OSF-1 required to stimulate osteogenic differentiation of bone marrow-derived cells was extremely low. Although the magnitude of osteogenic stimulation achieved by recombinant BMP-2 was greater than that by recombinant OSF-1, the concentration of recombinant BMP-2 (50 ng/ml) required to elicit this osteogenic response from bone marrow cells was 5000-fold higher than OSF-1. Given the relatively high levels (almost 3.5 mg/kg wet wt.) of OSF-1 in the bone matrix (Zhou *et al.*, 1992), the current findings further strengthen the proposed role of OSF-1 in maintaining osteoblastic activity of recruited populations at sites of new bone formation (Imai *et al.*, 1998).

The present study also demonstrated a modest, but significant, mitogenic effect of OSF-1 on murine mesenchymal cell populations. There have been reports for and against a potential mitogenic role for OSF-1. OSF-1 was originally purified as a weak mitogen from bovine uterus by Deuel's group (Milner, Deuel *et al.*, 1989), and the same group attributed OSF-1 with a mitogenic and neurite outgrowth- inducing activity (Li, Deuel *et al.*, 1990). Over-expression of bovine *osf-1* cDNA in NIH 3T3 cells resulted in enhanced cell number, anchorage dependent growth and tumour formation in nude mice (Chauhan *et al.*, 1993). In contrast, studies using Swiss mouse 3T3 and NIH 3T3 cells demonstrated little, if any, mitogenic activity for OSF-1 (Kuo et al., 1990). OSF-1 was not shown to be mitogenic for Balb/3T3 cells or human umbilical vein endothelial cells, though when presented as a substrate to the chick

embryo cerebral cortical derived neurons, neurite extension activity was observed (Hampton *et al.*, 1992). Furthermore, OSF-1 was suggested to have a role in contact-dependent proliferation arrest and cited as a proliferation arrest gene (Rauvala and Peng, 1997).

Following the finding that OSF-1 stimulated osteogenic differentiation of bone marrow cells, this study investigated whether OSF-1 had an osteoinductive potential similar to BMPs. To this end, the C2C12 cell line was utilized as it can be driven along the osteogenic pathway by BMP-2. Addition of 100 ng/ml rhBMP-2 to cultures of C2C12 cells for 2 days induced osteogenic differentiation, as indicated by the appearance of numerous alkaline phosphatase +ve cells. BMP-2 is not only a potent inducer of osteogenesis, but can block the differentiation pathway of C2C12 pre-myoblasts into fully-differentiated muscle cells by suppressing the master control genes for myoblast differentiation (Katagiri *et al.*, 1994). Subsequently, expression of typical osteoblast markers are induced in continued BMP-2-treated C2C12 cells, such as alkaline phosphatase activity, competency for parathyroid hormone responsiveness and osteocalcin production (Katagiri *et al.*, 1994). It has also been shown that inhibition of myoblastic differentiation and induction of osteoblastic differentiation in C2C12 cells by BMP-2 involves the BMP receptor-1A, and Smad1 and Smad5 transducers (Namiki *et al.*, 1997; Yamamoto *et al.*, 1997; Nishimura *et al.*, 1998). However, addition of rhOSF-1 at concentrations between 5 pg/ml and 100 ng/ml for 2 days failed to induce alkaline phosphatase activity, indicating that OSF-1 on its own was not osteoinductive at the concentrations examined.

It was then examined whether OSF-1 could influence BMP-2-induced osteogenic differentiation. When present with BMP-2 during the first two days of culture, OSF-1 inhibited BMP-2-induced osteogenic differentiation of C2C12 cells, indicated by a

significant decrease in alkaline phosphatase specific activity. Thus, presence of OSF-1 during the osteoinductive phase almost abolished BMP-2-induced conversion of C2C12 cells to the osteogenic phenotype, the inhibitory effect of OSF-1 being apparent at concentrations as low as 0.05 pg/ml in presence of 100 ng/ml BMP-2. OSF-1 therefore functioned as a BMP-2 antagonist during the initial osteoinductive phase of culture. The known antagonists of BMPs, such as Noggin (Zimmerman *et al.*, 1996; Aspenberg *et al.*, 2001), Chordin (Piccolo *et al.*, 1996; Zhang *et al.*, 2002), Cerebrus (Piccolo *et al.*, 1999), Follistatin (Iemura *et al.*, 1998) and Twisted (Chang *et al.*, 2001), inhibit BMP action by binding to the protein and thereby preventing BMP/receptor interactions. Although, the inhibition of BMP-2 by OSF-1 in the present study, even at appreciably low concentrations, was remarkable, at present the exact mechanisms of the antagonistic effects of OSF-1 are not known. However, they are unlikely to involve the direct binding of OSF-1 to BMP-2.

To resolve the paradoxical effects of OSF-1 of enhancing osteogenic differentiation on one hand, while inhibiting BMP-2 osteoinduction on the other, a two-phase treatment was tested. This involved an initial two-day osteoinductive phase with BMP-2, followed by a four-day culture period with OSF-1. When OSF-1 was added after osteoinduction had been achieved, the osteogenic phenotype of cell populations that had been primed to differentiate along the osteogenic pathway was enhanced and maintained better by OSF-1, in comparison to cell populations cultured in basal medium after the osteoinduction phase. Thus, OSF-1 was found to enhance the osteogenic phenotype of cell populations that had been primed to differentiate along the osteogenic pathway. Once again, this enhancing effect was with pg/ml concentrations of OSF-1, while ng/ml concentrations were ineffective.

Taken together, these data suggested that the timing and prevailing concentrations of OSF-1 were of utmost importance. Excessive OSF-1 was not beneficial, OSF-1 on its own was not osteoinductive, its presence too early during osteogenic differentiation antagonized BMP-stimulated osteoinduction and it enhanced the osteogenic phenotype of cell populations primed to differentiate along the osteogenic pathway by agents like BMPs.

DISCUSSION

6. Discussion

Bone is a self-organizational organ that remodels its shape and mass according to the physical and chemical environs around it. Osteoporosis is characterized by an imbalance in the remodeling process, culminating in a low bone mass condition with a high risk of bone fracture. Most anti-osteoporosis therapies are with compounds that target osteoclasts and alleviate the condition by decreasing bone resorption. However, there is a need for anabolic agents, which will substantially enhance osteoblast activity in individuals who have already suffered considerable bone loss. In the search for such anabolic agents, the present study investigated the role of a novel cytokine, osteoblast-stimulating factor-1, in bone development.

Since OSF-1 had originally been identified as a factor uniquely expressed by an osteoblastic cell line (Tezuka *et al.*, 1990), we sought to confirm synthesis of OSF-1 by osteoblasts *in vivo*. In bones of transgenic and BDF-1 mice, immunostaining for OSF-1 was observed in the palisade of osteoblasts lining the mineralized cartilaginous septa at the vascular front of the growth plate, as well as in osteoblasts lining the trabeculae of the primary spongiosa. These results were in agreement with the results of a previous study (Petersen and Raifi, 2001) that immunolocalized OSF-1 to the cytoplasm and pericellular matrix of osteoblasts, located amongst the mineralized septa of the growth plates of 20-week-old mice. The present study also demonstrated that synthesis of the OSF-1 protein occurred at an early stage of osteoblast differentiation, characterized by the presence of alkaline phosphatase and type I collagen, and prior to the synthesis of bone sialoprotein and osteoid mineralization. Expression of the *osf-1* gene was demonstrated by *in situ* hybridization in osteoprogenitors from 12-day cultures of mouse and human bone marrow-derived cells. These findings were in agreement with the studies of Dreyfus and co-workers

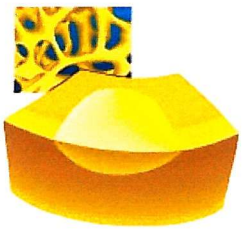
(Dreyfus *et al.*, 1998), who immunolocalized OSF-1 in the osteoblast/ osteoid layer of developing chick tibiae, and suggested a possible role for the protein in early stages of osteogenesis. The same study also demonstrated *osf-1* mRNA expression in committed osteoprogenitor cells of embryonic chick tibiae, but not in fully differentiated osteoblasts. The current study and previous work by Imai and co-workers (Imai *et al.*, 1998) indicated that, following synthesis by osteoblasts, OSF-1 was secreted in the bone matrix, where it was presumably stored for future use, analogous to stored BMPs (Urist, 1997) and TGFβ (Bonewald and Dallas, 1994). The present study immunolocalized OSF-1 at sites of new periosteal and endochondral bone formation, in keeping with its role in stimulating new bone formation, based on its function as a chemoattractant for osteoblast-like cells (Imai *et al.*, 1998) and osteoprogenitors from human bone marrow (Yang *et al.*, in press). Enhanced bone formation would therefore be expected at those sites of *de novo* bone formation. Calvariae in mice develop rapidly between 2-6 days post-natally, and are prime examples of new intramembranous bone formation. In the present study, the width of calvarial bone in 6-day-old neonatal transgenic mice was almost twice that in control mice. However, it is not known whether this increased thickness was maintained in later life, as it was not possible to examine calvariae from older animals due to their unavailability. Nevertheless, the difference between neonatal calvarial bones was remarkable and consistent with an autocrine effect of OSF-1 on osteoblasts, resulting in an overall increase in bone formation at 6 days of age. Bone development in calvariae is initiated as a network of trabeculae and expansion of flat bones like calvariae, to accommodate growth of the head, utilizes the process of remodeling. This involves laying down of new bone trabeculae on the outer perimeter, while bone is resorbed on the inner perimeter. Calvariae from 6-day

neonatal transgenic mice also had a pronounced irregular and trabecular appearance. In the absence of adequate evidence, it is not possible to comment whether pronounced trabecular appearance of calvariae in transgenic mice was a result of enhanced remodeling.

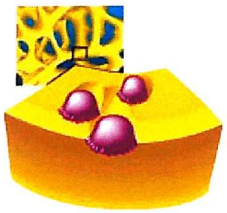
The function of matrix-stored OSF-1 in bone remodeling can only be presumed. Two known properties of OSF-1 are pertinent for its proposed role during bone remodeling: its role as a chemoattractant for human osteosarcoma cells (Gieffers *et al.*, 1993), osteoblastic cell lines (Imai *et al.*, 1998) and osteoprogenitors from human bone marrow (Yang *et al.*, in press), and its role in the enhancement of cell attachment (Gieffers *et al.*, 1993). It therefore seems feasible that matrix-bound OSF-1, released during the resorptive phase of bone remodeling, could play a role in stimulating new bone formation by the chemotactic recruitment of osteoprogenitors and osteoblasts, and promoting their attachment to the matrix (Fig. 40).

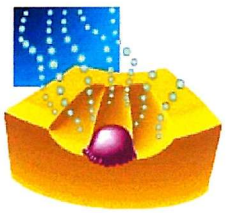
Figure 40. Possible role for OSF-1 in bone remodeling (Adapted from Ostex information sheet)

- OSF-1 is synthesized by osteoblasts
- Stored in the bone matrix for future use

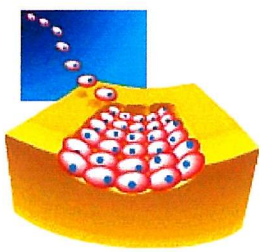


- OSF-1 is probably released from bone matrix upon resorption by osteoclasts





- Released OSF-1 serves for the chemotactic recruitment and attachment of osteoblasts for new bone formation



The remarkable effects of *osf-1* over-expression on stimulating calvarial bone development in neonatal transgenic mice contrasted with its relatively modest long-term effects on the growth of long bones of transgenic mice. Clues to resolving the apparent paradox of increased initial intramembranous bone formation in calvariae on one hand and modest effects on development of long bones (bone growth as well as bone mass) on the other, came from the results of *in vitro* experiments. When OSF-1 was present together with BMP-2 during the osteoinductive phase of C2C12 cell culture, BMP-mediated osteoinduction was inhibited by 70-80%, even in the presence of 0.05 pg/ml concentrations of OSF-1. On the other hand, when OSF-1 was added *after* osteogenic differentiation was initiated by BMP-2, further osteogenic differentiation was enhanced by low (5 and 10 pg/ml) concentrations of OSF-1. Similarly, low (10 pg/ml) concentrations of OSF-1 enhanced osteogenic differentiation in cultures of both mouse (present study) and human (Yang *et al.*, in press) bone marrow-derived cells, and when OSF-1 was added with BMP-2 in a carrier and implanted subcutaneously, BMP-induced ectopic osteogenesis was enhanced in presence of low OSF-1 concentrations (Sato *et al.*, 2002). It is important to note that the stimulatory effects of OSF-1 were found only at low concentrations of OSF-1, while higher concentrations were ineffective.

The studies therefore suggested that OSF-1 had multiple effects on bone formation, which were dependent on its prevailing concentrations and the timing of its presence at the sites of bone formation. OSF-1 was inhibitory for BMP-mediated osteoinduction, while stimulatory for osteogenic differentiation of BMP-primed cell populations/ late-stage osteoprogenitors from bone marrow, provided it was present at low concentrations. *In vivo*, bone matrix contained large amounts of OSF-1, of the order of 3.5 mg/ kg bovine bone (Zhou *et al.*, 1992), some of which would be

presumably released during remodeling. Circulating serum levels of OSF-1 in adult healthy humans ranged between 16-41 pg/ml with a value averaging at 27 pg/ml (Souttou *et al.*, 1998), which was in the same order of magnitude as the concentrations required to stimulate osteogenic differentiation *in vitro*. Thus, osteoblasts resided in an environment associated with high levels of OSF-1 and were probably responding optimally to the physiologic concentrations of circulating OSF-1. Physiological levels of OSF-1 provided the right balance between the enhancing and inhibitory effects on bone development *in vivo*.

In the case of early calvarial growth, bone was formed within a membrane, in which initially no OSF-1-containing bone matrix was present. Under those circumstances, the stimulatory effects of OSF-1 on osteo-differentiation presumably out-weighed its inhibitory effect on osteoinduction. By contrast, the slower rate of bone growth in transgenic mice during the growth spurt period could have resulted from a reduction in osteoinduction that was not counterbalanced by stimulation of osteo-differentiation, because levels of OSF-1 in *osf-1* over-expressing mice were above the physiologic optimum. Alternatively, since the skeletal growth spurt coincided with a pubertal surge in sex hormones, it was possible that in transgenic mice OSF-1 interacted with the sex hormones and/ or growth hormone via an, as yet unknown, mechanism. An enhancing effect on organic bone mass was not observed in transgenic mice, once again, because levels of OSF-1 were above the physiological optimum and any enhancing effects on osteoblasts could be observed only in the presence of low concentrations.

Although, the transgene over-expression did not enhance organic bone content, an increase of 10-11 % in inorganic calcium content/ mg bone dry weight was observed in male transgenic mice. This data contrasted that of Masuda and co-workers (Masuda

et al., 1997), who reported an increased bone mineral content in female, but not male, transgenic mice at 30 weeks of age. This difference might be due to the different assay methods used to assess mineral content i.e. direct measurements of calcium in the present study vs. ash weights, or due to the natural biological variability in the hybrid strain of BDF-1 mice used in the present study. The mechanisms by which OSF-1 enhanced mineral content are not clear, but may be related to the slower rate of bone growth, which perhaps allowed more time for mineral deposition.

Results of another study (Yang *et al.*, in press) from our laboratory demonstrated that OSF-1, at pg/ml concentrations, was able to stimulate colony formation (including alkaline phosphatase positive CFU-F number) in cultures of human bone marrow-derived cells, in basal as well as osteogenic media, when added during the later phase of cell culture. Time-course studies demonstrated that OSF-1 (10 pg/ml) stimulated osteogenic differentiation when added to cultures of human bone marrow-derived cells during the later phase (days 6- 9 in osteogenic media and days 7-12 in basal media), while additions during the initial phase (days 0-6 in osteogenic media and days 0-7 in basal media) were not stimulatory. These effects of OSF-1 on colony formation and osteogenic differentiation indicated an effect on late osteoprogenitors rather than early osteoprogenitor populations from bone marrow. In support of this, the same study further demonstrated that OSF-1 promoted mineralization and bone formation in primed osteoprogenitor preparations as assessed by *in vitro* culture, and subcutaneous and diffusion chamber *in vivo* assays. Thus, with respect to progenitors from bone marrow, OSF-1 enhanced osteogenic differentiation of late-stage osteoprogenitors i.e. the progenitor population that had been primed to differentiate along the osteogenic pathway.

In contrast to bone, there were major differences between control and transgenic mice in the immunolocalization of OSF-1 in cartilage. In embryonic and fetal cartilage, previous work had demonstrated expression of the OSF-1 protein and its mRNA throughout the chondro-epiphyses of long bones (Imai *et al.*, 1998). In addition, up to 15 mg/g wet weight OSF-1 had been extracted from fetal bovine epiphyseal and nasal cartilage (Neame *et al.*, 1993), confirming that fetal cartilage contained large amounts of OSF-1.

The present study concentrated on the immunolocalization of OSF-1 in cartilage of post-natal control and transgenic mice. Immunostaining for OSF-1 in growth plates of control mice was weak in post-natal mice (until 5 weeks) and absent thereafter. This contrasted with results of Petersen and Raifi (Petersen and Raifi, 2001), who were able to demonstrate immunostaining for OSF-1 in terminal hypertrophic growth plate chondrocytes and within opened lacunae of apoptotic growth plate chondrocytes in mice up to 20 weeks, using different specimen fixation methods and a different antibody. This suggested that a low-level expression of OSF-1 was maintained in the growth plate cartilage of normal mice after birth. However, synthesis of OSF-1 by growth plate chondrocytes was maintained at a much higher level in post-natal transgenic mice compared to control mice, as indicated by the strong immunostaining for OSF-1 in growth plate chondrocytes and matrices of transgenic mice until at least 15 weeks of age.

Based on the roles of OSF-1 as a mitogen for endothelial cells and as an angiogenic factor (Fang *et al.*, 1992; Laaroubi *et al.*, 1994; Yeh *et al.*, 1998), the study by Peterson and Raifi (2001) suggested that OSF-1, synthesized by hypertrophic growth plate chondrocytes, played a role in the migration of vascular endothelial cells within the growth plate. Thus, OSF-1 might be one of the many angiogenic factors

synthesized by growth plate chondrocytes that increase the susceptibility of the growth plate cartilage to vascular invasion during the process of endochondral ossification.

With respect to articular cartilage, no/ trace amounts of OSF-1 could be isolated from mature bovine articular cartilage (Neame *et al.*, 1993). The present study demonstrated the absence of OSF-1 in articular cartilage of postnatal control mice, however, OSF-1 was clearly present in the articular chondrocytes of postnatal transgenic mice until at least 15 weeks of age. This suggested that transgene over-expression considerably amplified a normally low degree of OSF-1 protein synthesis by chondrocytes. The transgene was expressed under the control of the human osteocalcin promoter, which was able to target *osf-1* over-expression in bone to osteoblasts and maturing chondrocytes (Kesterson *et al.*, 1993). A low-level expression of the osteocalcin gene (one hundredth of that observed in osteoblasts) has been reported in chondrocytes (Lian *et al.*, 1993; Aizawa *et al.*, 1998). Theoretically, in transgenic mice, any stimulus influencing expression of the osteocalcin promoter in chondrocytes would result in the expression of the 6 *osf-1* transgene copies under its control. As a consequence, a 6-fold amplification in the synthesis of the OSF-1 protein by chondrocytes was likely to occur, thereby making its detection by immunocytochemistry possible.

The increased expression of OSF-1 by articular chondrocytes of transgenic mice was associated with the synthesis of type I collagen, a collagen type typical for osteoblasts, but not chondrocytes. In addition, 'islands' of bone-like matrix had formed within the articular cartilage of transgenic mice. Since no vascular channels could be seen in this region, and hence no source of osteogenic cells, this bone matrix appeared to have been synthesized by former chondrocytes. Other markers of the osteoblastic

phenotype (eg. bone sialoprotein and osteonectin) were also synthesized by the articular chondrocytes, but this was the case for both control and transgenic mice. Previous studies have demonstrated the synthesis of non-collagenous matrix proteins like bone sialoprotein (Bianco *et al.*, 1991) and osteonectin (Pacific *et al.*, 1990) by hypertrophic chondrocytes *in vivo* under physiological conditions, but synthesis of type I collagen by chondrocytes has been rarely reported.

Numerous lines of evidence suggested that chondrocytes, especially hypertrophic chondrocytes, possessed the potential for either further differentiation (Galotto *et al.*, 1994; Cancedda *et al.*, 1992; Ishizeki *et al.*, 1996 and 1997) or trans-differentiation (Roach *et al.*, 1992 and 1995) to bone-forming cells, and initiated the synthesis of type I collagen *in vitro*. However, this potential was not usually realized *in vivo*, the immediate microenvironment in the vicinity of chondrocytes and the presence of adequate osteogenic stimuli being the two deciding factors that determined the osteogenic fate of chondrocytes *in vivo* (Riminucci *et al.*, 1998). Since synthesis of type I collagen was observed only in some articular chondrocytes of transgenic mice, in which over-expression of the transgene was evident by the synthesis of the OSF-1 protein, it is possible that over-expression of *osf-1* may have provided adequate osteogenic stimulus for the synthesis of bone-type proteins by some articular chondrocytes.

However, few caveats should be borne in mind. For a complete osteogenic differentiation to have occurred, former chondrocytes should also express markers of late osteogenic differentiation like osteocalcin. Presence of osteocalcin was not observed in articular chondrocytes of transgenic mice, suggesting that a complete osteogenic differentiation had not occurred. Synthesis of type I collagen by articular chondrocytes did not occur in every transgenic mouse, nor was it feasible to carry out

a detailed 3-D study of the vascularity arising from the sub-chondral bone. Hence it cannot be ruled out completely that the apparent islands of type I collagen around the articular chondrocytes were connected to bone formed around the vascular channels. Nevertheless, the results were consistent with the theory of Cancedda and co-workers (Riminucci *et al.*, 1998) that an osteogenic stimulus in the immediate vicinity of chondrocytes, in the form of over-expression of *osf-1* in articular chondrocytes of transgenic mice, altered the chondrogenic phenotype and imparted the articular chondrocytes with some osteogenic characteristics.

To confirm the same, cartilaginous explants in the form of embryonic chicken nasal cartilage and neonatal rat growth plates were cultured with exogenous OSF-1. At end of the culture periods, chondrocytes in explants cultured with recombinant OSF-1 (50 ng/ml) were found to synthesize type I collagen. It was possible to immunolocalize other markers of the osteoblastic phenotype (alkaline phosphatase, osteonectin) in the chondrocytes of these explants before culture, suggesting that OSF-1 acted on chondrocytes that were already some way down the osteogenic pathway. Thus, the osteogenic stimulus exerted by OSF-1 may have "pushed" these chondrocytes over a threshold towards the osteoblastic phenotype. It was noteworthy that endogenous OSF-1 (synthesized by the chondrocytes) was incapable of stimulating synthesis of type I collagen, which was accomplished only by exogenous OSF-1, added at an appreciably high concentration of 50 ng/ml. Once again, osteocalcin, a marker of terminally differentiated osteoblasts, was not found in any of the chondrocytes of explants cultured with recombinant OSF-1.

Thus, high concentrations of OSF-1, either due to over-expression of the *osf-1* transgene or exogenous addition of the recombinant protein, stimulated synthesis of type I collagen by chondrocytes. Since the presence of late markers of osteogenic

differentiation could not be detected in these altered chondrocytes, it was not possible to determine whether synthesis of type I collagen by chondrocytes represented early stages of osteogenic differentiation or, perhaps, an abnormal response to excessive OSF-1.

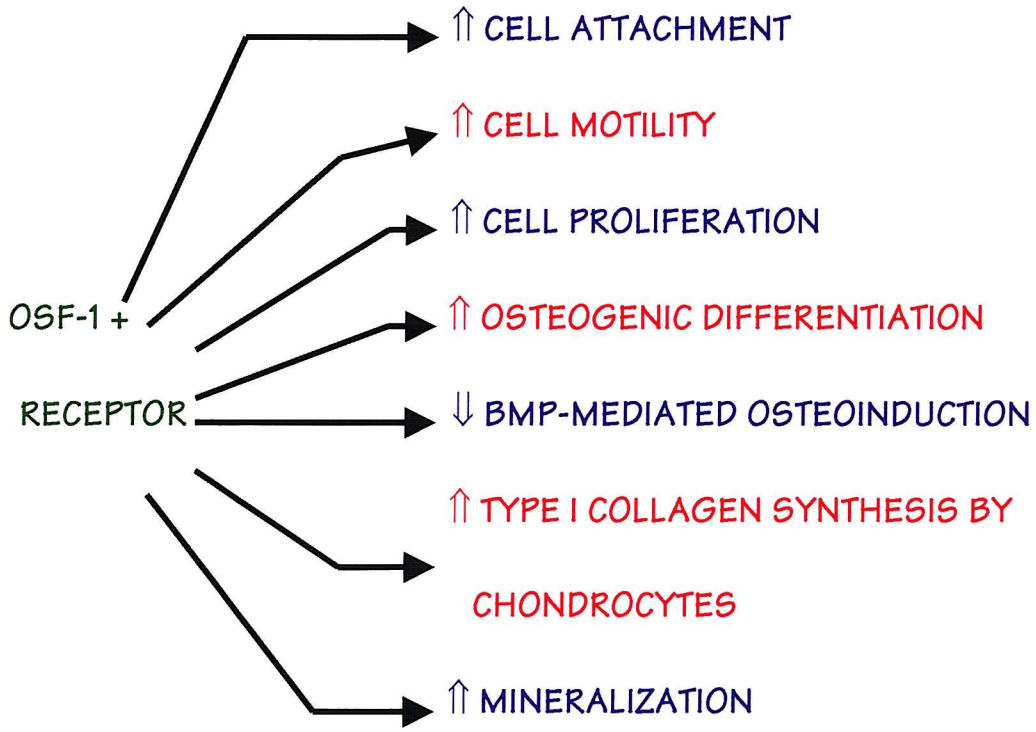
A previous study by Tapp and co-workers (Tapp *et al.*, 1999) involving *in vitro* cultures of bovine mature articular chondrocytes supplemented with recombinant OSF-1 demonstrated that, although chondrocyte proliferation was arrested, synthesis of glycosaminoglycans and expression of matrix proteoglycans (biglycan) and collagen type II were enhanced in these chondrocytic cultures by OSF-1. The study therefore suggested that OSF-1 may act directly or indirectly to regulate chondrocytic growth and proteoglycan synthesis in the developing cartilage.

Summary

Taking into account the results of this study and those of previous studies, OSF-1 is not a factor with a single role during post-natal bone development, but can fulfil a range of diverse functions (Fig. 41). The peptide can be considered as an accessory signaling molecule, that modulates primary signals of growth factors like BMPs (as elucidated in the present study) and bFGF (Szabat and Rauvala, 1996). The lack of an obvious bone phenotype in knockout mice (Amet *et al.*, 2001) suggested that other factors were able to fulfil the roles of OSF-1 in bone development. Modest effects of *osf-1* over-expression on bone development in transgenic mice could be explained on basis of the results of *in vitro* experiments, where enhancing effects of OSF-1 were only seen at appreciably low concentrations. Not only were the effects of OSF-1 dependent on its concentration, the timing of its presence also played a crucial role. When present together with BMP-2, OSF-1 inhibited BMP-mediated osteinduction, however, once osteogenic differentiation had been initiated by BMP-2, OSF-1

enhanced further osteogenic differentiation of the primed cell populations. The study also suggested that normal physiological levels of OSF-1 provided the right balance between its enhancing and inhibitory effects, which was optimal for bone development. Whether OSF-1 might have therapeutic benefits in pathological situations like osteoporosis, remains to be investigated. However, results of another study (Yang *et al.*, in press) from our laboratory have indicated that this factor has potential for bone tissue engineering.

Figure 41. Diverse roles of OSF-1 in bone development



Future work

The inhibition of BMP-2 by OSF-1, observed in the present study, was unexpected and warrants further work. Future work will involve identification of molecular mechanisms, by which OSF-1 antagonizes BMP-mediated osteoinduction in C2C12 cells. Taking into account that the C2C12 cell line is a pre-myoblastic cell line, it will

be worthwhile to determine whether OSF-1 is one of the factors that inhibits ectopic bone formation in muscle *in vivo*, if so, what possible mechanisms are involved in this inhibition. Answers to the above questions will be of relevance to 'fibrodysplasia ossificance progressiva' (FOP), a rare devastating disease, in which major striated muscles, tendons, ligaments and other connective tissues progressively turn into bone, especially after trauma.

Limitations of the study

1. Although the effect of *osf-1* over-expression on stimulating calvarial bone development in neonatal transgenic mice was remarkable, the exact mechanism involved in this could not be explained by the present study. Calvariae of neonatal transgenic mice also had a pronounced trabecular appearance, suggestive of a possible effect of OSF-1 on bone remodeling, which again could not be confirmed by the present study. It was also not possible to determine whether these differences in early calvarial development between transgenic and control mice were maintained in adult life.
2. Since antigenicity with the anti-OSF-1 antibody, used in the present study, could only be observed in ethanol-fixed, non-decalcified paraffin sections, which were impossible to obtain from older mice due to the degree of calcification, it was not possible to determine whether expression of OSF-1 was maintained in chondrocytes of mice beyond 15 weeks.
3. Effect of *osf-1* over-expression on osteogenic differentiation of progenitor populations from bone marrow of transgenic mice and possible differences with control mice were not examined by the present study.

Questions raised in 1. and 3. were unanswered in the present study due to loss of the colony of *osf-1* over-expressing transgenic mice to a hepatitis infection.

4. The unpaired students T test for analyzing quantitative data in Chapter 3 dealing with transgenic mice is not the gold standard statistical test to be used for this type of data, and its use may have over exaggerated the degree of statistical significance.
5. The results in chapter 2, dealing with OSF-1 protein expression in embryonic chick femur, were based on 2 femurs from two 10-day-old chick embryos.

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APPENDIX
REAGENTS & SOLUTIONS

Chemicals used in this study were of analytical grade and purchased from SIGMA (Poole, UK) and BDH/ Merck Ltd. (Poole, UK), unless otherwise stated.

Decalcification:

1. 5% EDTA in 0.1M Tris-HCl buffer, pH 7.2
   1.21 g Tris
   100 ml distilled water
   Adjust pH to 7.2 using HCl
   5 g EDTA

Staining techniques:

1. Alcian blue/ Sirius red staining

1. Weigert's haematoxylin: Equal volumes of Weigert's haematoxylin A and B
   Weigert's haematoxylin A - 10 g haematoxylin/ 1 L methanol, leave for 4 weeks to ripen.
   Weigert's haematoxylin B - 6 g Ferric chloride
   500 ml distilled water
   5 ml conc. HCl

2. Acid alcohol: 20 ml conc. HCl
   2 L 50% methanol

3. Alcian blue: 1.5 g Alcian blue 8GX
   300 ml distilled water
   3 ml acetic acid

4. Molybdophosphoric acid: 3 g Molybdophosphoric acid
   300 ml distilled water

5. Sirius red: 300 mg Sirius red F3B
   100 ml saturated picric acid
   200 ml distilled water

2. Alkaline phosphatase

1. Activating buffer (pH 7.4):
   Stock A (Tris Maleate): 12.1 g Tris
   11.6 g Maleic acid
   500 ml distilled water
   Stock B: 4 g Sodium hydroxide
   500 ml distilled water
   Buffer:
   75 ml Stock A + 25 ml distilled water
   81 ml Stock B + 19 ml distilled water
   + 100 ml distilled water
   + 600 mg Magnesium chloride

2. AS-B1 Phosphate stock solution:
Tris-HCl buffer: 4.36 g Tris in 180 ml distilled water, adjust pH to 8.3 with HCl
Solution B: 25 mg Naphthol AS-B1 Phosphate
10 ml Dimethylformamide
10 ml distilled water
Adjust pH to 8 with Sodium carbonate
20 ml Solution B + 180 ml Tris-HCl buffer + 300 ml distilled water

3. Fast red TR solution (0.1% in AS-B1 Phosphate stock soln., freshly prepared):
70 mg Fast red TR
70 ml AS-B1 Phosphate stock solution

4. Alcian blue (0.5% in distilled water):
1.5 g Alcian blue 8 GX
300 ml distilled water
3 ml Acetic acid

5. Methyl green (0.5% in 0.2 M Acetate buffer, pH 4.1):
1.5 g Methyl green
300 ml Acetate buffer (pH 4.1)
Filter
Acetate buffer: 246 ml Stock A + 54 ml Stock B, pH 4.1
Stock A/ 0.2 M Acetic acid: 6 ml Glacial acetic acid
500 ml distilled water
Stock B/ 0.2 M Sodium acetate: 3.28 g Anhydrous sodium acetate
200 ml distilled water

3. Von Kossa method, counterstained with Van Gieson

1. 1% Silver nitrate
500 mg Silver nitrate
50 ml distilled water

2. 2.5% Sodium thiosulphate
7.5 g sodium thiosulphate
300 ml distilled water

3. Van Gieson stain
180 mg Acid Fuchsin
100 ml sat. Picric acid
100 ml distilled water

4. Alcian blue (0.5% in distilled water):
1.5 g Alcian blue 8 GX
300 ml distilled water
3 ml Acetic acid

4. Immunostaining/ Double immunostaining

1. 3% H_2O_2:
   3 ml 30% H_2O_2
   27 ml distilled water

2. 1% Phosphate-buffered bovine serum albumin:
   1 g bovine serum albumin
   100 ml 0.01 M PBS
   (0.01M PBS – 1 tablet {Sigma PBS tablets, Cat. No. P4417} in 200 ml dist. water)

3. 1% Tris-buffered bovine serum albumin:
   1 g bovine serum albumin
   100 ml TBS
   (TBS – 300 mg Tris + 4.05 g Sodium chloride + 500 ml dist. water, adjust pH to 7.6 using 0.1 N HCl)

4. High salt wash buffer, pH 8.5: 46.7 g sodium chloride
   12.1 g Tris
   1 ml 0.05% Tween
   2 L distilled water

5. Low salt wash buffer, pH 8.5: 17.4 g sodium chloride
   12.1 g Tris
   1 ml 0.05% Tween
   2 L distilled water

6. 0.1 M Tris buffer, pH 8.5: 24.2 g Tris
   2 ml Tween
   2 L distilled water

7. H_2O_2, AEC soln.
   0.5 ml stock AEC soln. (10 mg 3-amino, 9-ethylcarbazole + 1.25 ml Dimethylformamide)
   5 µl 30% H_2O_2
   9.5 ml Acetate buffer

8. Acetate buffer, pH 5:
   16 ml acetic acid + 500 ml dist. water -A
   13.6 g sodium acetate + 500 ml dist. water - B
   9 ml soln. A + 25 ml soln. B and make up volume to 70 ml with dist. water

9. Fast blue BB solution (freshly prepared):
   24 mg Fast blue BB + 30 ml soln. A + 1.3 ml soln. B
   Solution A : 30 ml 0.1 M Tris soln. + 10 mg Magnesium chloride, adjust pH to 9 with 0.1 N HCl
   Solution B: 9 mg Naphthol-AS-TR-Phosphate + 1.3 ml N, N-dimethylformamide

10. Light green: 600 mg Light green
300 ml distilled water
0.6 ml acetic acid

Organ culture medium:

1. BGJ culture medium

NaH_2PO_4	90 mg/L
$MgSO_4.\ 7H_2O$	200 mg/L
KCl	400 mg/L
KH_2PO_4	160 mg/L
NaCl	5300 mg/L
Calcium lactate	555 mg/L
Glucose	5000 mg/L
Phenol red	20 mg/L
Sodium acetate	50 mg/L
L-arginine	175 mg/L
L-aspartic acid	50 mg/L
L-cysteine	90 mg/L
L-glutamine	200 mg/L
L-glycine	400 mg/L
L-histidine	150 mg/L
L-isoleucine	30 mg/L
L-lysine	240 mg/L
L-methionine	50 mg/L
L-phenylalanine	50 mg/L
L-proline	20 mg/L
L-serine	100 mg/L
L-threonine	75 mg/L
L-tryptophan	40 mg/L
L-tyrosine	40 mg/L
L-valine	65 mg/L
α-Tocopherol phosphate	1 mg/L (1 drop)
Biotin	0.2 mg/L
Calcium pantothenate	0.2 mg/L
Choline chloride	50 mg/L
Folic acid	0.2 mg/L
m-Inositol	0.2 mg/L
Nicotinamide	20 mg/L
p-aminobenzoic acid	2 mg/L
Pyridoxal phosphate	0.2 mg/L
Riboflavin	0.2 mg/L
Thiamine HCL	4 mg/L
Vitamin B 12	0.04 mg/L
Penicillin	120 mg/L
Streptomycin	100 mg/L
Ascorbic acid	50 mg/L (fresh each day)
$NaHCO_3$	4000 mg/L (culture medium) or
$NaHCO_3$	1700 mg/L (holding medium)

Analytical measurements:

1. Hydroxyproline assay

1. Citrate buffer
   Stock solution: 14.7 g Tri-sodium citrate
   (store at 4^0C) 9.2 g Citric acid
   500 ml distilled water
   Working solution (prepare fresh): 1 volume of stock + 5 volumes of propan-2-ol

2. Chloramine T
   Buffer for preparing Chloramine T: 28.44 g Sodium acetate
   (store at room temp.) 18.82 g Trisodium citrate
   2.52 g Citric acid
   191 ml distilled water
   309 ml propan-2-ol
   Chloramine T – 1.4 g/dl (working solution): 700 mg Chloramine T
   (prepare fresh) 10 ml distilled water
   40 ml buffer

3. Ehrlich's reagent: 20 g p-dimethylaminobenzaldehyde
   (prepare fresh) 33 ml conc. HCl
   267 ml propan-2-ol

4. Hydroxyproline stock std. soln. (5mM): 65.5 mg hydroxyproline in 100 ml 6N HCl

2. Alkaline phosphatase assay

1. p-Nitrophenyl phosphate substrate (1.3 g/L)
   40 mg p-Nitrophenyl phosphate
   10 ml alkaline buffer (1.5 mol/L, pH 10.3)
   20 ml distilled water

2. Assay buffer
   10 ml alkaline buffer (1.5 mol/L, pH 10.3)
   20 ml distilled water
   60 µl NP40 (0.2%)/ NONIDET

3. NaOH (1M)
   4 g NaOH
   100 ml distilled water

3. DNA assay

1. 1X TE buffer (10mM Tris-HCl, 1mM EDTA pH 7.5)
   20X TE buffer:
   0.788 g Tris-HCl
   0.186 g EDTA
   500 ml distilled water
   Adjust pH to 7.5
   1X TE buffer:
   10 ml 20X TE buffer
   190 ml DEPC water

2. DEPC water
   250 µl DEPC (Diethyl carbonate)
   500 ml distilled water
   Mix well for atleast 2 hours
   Autoclave

In situ hybridization:

1. 0.01 M Phosphate buffered saline (PBS), pH 7.4
   1 PBS tablet (Cat. No. P4417, SIGMA) in 200 ml distilled water

2. DEPC-PBS
   50 µl DEPC (Diethyl Pyrocarbonate)
   100 ml PBS
   Mix well for at least 2 hours
   Autoclave

3. 4% Paraformaldehyde in DEPC-PBS
   4 g Paraformaldehyde
   100 ml DEPC-PBS
   Mix well with heating

4. 0.2% Triton X100 in 4% Paraformaldehyde in DEPC-PBS
   200 µl 100% Triton X100
   100 ml 4% Paraformaldehyde in DEPC-PBS

5. 70% Ethanol
   70 ml Absolute alcohol (100%)
   30 ml DEPC-water

6. 50% Formamide, 2X SSC
   50 ml Formamide
   10 ml 20X SSC (Cat. No. S6639, SIGMA)
   40 ml DEPC water

7. 100 mM Tris pH 7.5, 150 mM NaCl
   2.42 g Tris
   200 ml dist. water
   Adjust pH to 7.5
   1.75 g NaCl
   Autoclave

8. 100 mM Tris pH 7.5, 150 mM NaCl containing 1% blocking reagent
   1 g blocking reagent (Cat. No. 1175041, Boehringer Mannheim)
   100 ml 100 mM Tris pH 7.5, 150 mM NaCl
   Mix well with heating

9. 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$
   1.21 g Tris
   100 ml dist. water
   Adjust pH to 9.5
   0.584 g NaCl
   0.101 g $MgCl_2$
   Autoclave

10. 10 mM Tris pH 8, 1mM EDTA
    0.121 g Tris
    100 ml dist. water
    Adjust pH to 8
    0.37 g EDTA (disodium salt)
    Autoclave

11. 0.4M NaCl, 10mM Tris pH 7.5, 5mM EDTA
    0.121 g Tris
    100 ml dist. water
    Adjust pH to 7.5
    2.33 g NaCl
    0.186 g EDTA (disodium salt)
    Autoclave

LIST OF PUBLICATIONS & ABSTRACTS

PUBLICATIONS

1. *Pleiotrophin/ Osteoblast-stimulating factor-1: Dissecting its diverse functions in bone formation*
   Tare R.S., Oreffo R. O. C., Clarke N. M. P., Roach H. I.
   Journal of Bone and Mineral Research, In press.

2. *Induction of human osteoprogenitor chemotaxis, proliferation, differentiation and bone formation by osteoblast-stimulating factor-1: Osteoinductive biomimetic scaffolds for tissue engineering*
   Yang X. B., Tare R. S., Partridge K.A., Roach H. I., Clarke N. M. P., Howdle S. M., Shakesheff K. M., Oreffo R. O. C.
   Journal of Bone and Mineral Research, In press.

ABSTRACTS
(14 abstracts: 8 oral presentations, 6 poster presentations)

1. *The effects of over-expressing the osteoblast-stimulating factor-1 (OSF-1)*
   Tare R. S., Hashimoto-Gotoh T., Clarke N. M. P., Roach H. I.
   J. Bone Joint Surg. [Br.] 2000; 82-B: Supp III.

2. *The long-term effects of over-expressing the osteoblast-stimulating factor-1 (OSF-1)*
   Tare R. S., Hashimoto-Gotoh T., Clarke N. M. P., Roach H. I.
   Calcified Tissue International 2000; 66 (1): S 59.

3. *The effects of over-expressing the osteoblast-stimulating factor-1 (OSF-1)*
   Roach H. I., Tare R. S., Hashimoto-Gotoh T., Oreffo R. O. C., Clarke N. M. P.
   Abstracts from the 50th Anniversary Meeting of the Bone and Tooth Society [Br.] 2000: O 20.

4. *Over-expression of the osteoblast-stimulating factor-1 (OSF-1) increases bone mineral content, but also affects growth plate and articular cartilage*
   Roach H. I., Tare R. S., Oreffo R. O. C., Clarke N. M. P.
   Transactions of the 47th Annual Meeting (Paper No. 341), Orthopaedic Research Society (Feb. 25-28, 2001), San Francisco, California.

5. *The roles of osteoblast-stimulating factor-1 (OSF-1) during endochondral ossification*
   Tare R.S., Rauvala H., Hashimoto-Gotoh T., Oreffo R. O. C., Clarke N. M. P., Roach H. I.
   Abstracts from the Bone and Tooth Society Annual Meeting [Br.] 2001: OC 18.

6. *Immunolocalization of osteoblast-stimulating factor-1 (OSF-1) in bone and cartilage: Novel insights into its function*
   Tare R. S., Oreffo R. O. C., Rauvala H., Hashimoto-Gotoh T., Clarke N. M. P., Roach H. I.
   British Orthopaedic Research Society (BORS) Conference (23rd -24th April, 2001), Belfast, Northern Ireland.

7. *Osteoblast-stimulating factor-1 (OSF-1): Insights into its diverse functions during osteogenesis*
   Tare R. S., Oreffo R. O.C., Clarke N. M. P., Roach H. I.
   Gordon Research Conference on Bones and Teeth (19^{th}-24^{th} Aug., 2001), Meriden, USA.

8. *Osteoblast-stimulating factor-1 (OSF-1) has multiple roles during post-natal bone development*
   Tare R. S., Oreffo R. O. C., Clarke N. M. P., Roach H. I.
   Bone 2002; 30 (3): C 50.

9. *The multiple functions of osteoblast-stimulating factor-1 (OSF-1) in bone development*
   Roach H. I., Tare R. S., Oreffo R. O. C., Clarke N. M. P.
   European Calcified Tissue Society Meeting (25^{th}-29^{th} May, 2002), Zagreb, Croatia.

10. *The importance of osteoblast-stimulating factor-1 (OSF-1) during bone development*
    Tare R. S., Oreffo R. O. C., Clarke N. M. P., Roach H. I.
    Journal of Bone and Mineral Research 2002; 17 (5): OC 31.

11. *Osteoblast-stimulating factor-1 enhances osteogenic differentiation of bone marrow stromal cells, but is not osteoinductive*
    Tare R. S., Partridge K. A., Yang X. B., Clarke N. M. P., Oreffo R. O. C., Roach H. I.
    British Bone and Tooth Society Annual Meeting (24^{th}-26^{th} June, 2002), Cardiff, UK. Adjudged as the **best poster discussion paper.**

12. *Pleiotrophin inhibits BMP-mediated osteoinduction in C2C12 pre-myoblastic cells while enhancing osteogenic differentiation*
    Tare R. S., Yang X., Clarke N. M. P., Oreffo R. O. C., Roach H. I.
    International Symposium on Ectopic Ossification with particular reference to Fibrodysplasia Ossificance Progressiva (FOP) (20^{th} -22^{nd} June, 2002), The Royal College of Surgeons of England, London.

13. *Pleiotrophin inhibits BMP-mediated osteoinduction: possible relevance to FOP?*
    Tare R. S., Yang X., Clarke N. M. P., Oreffo R. O. C., Roach H. I.
    British Orthopaedic Research Society Autumn Meeting (16^{th} -17^{th} Sep., 2002), Cardiff, UK.

14. *Osteoblast-stimulating factor-1 enhances osteogenic differentiation of bone marrow stromal cells, but is not osteoinductive*
    Tare R. S., Partridge K. A., Yang X. B., Clarke N. M. P., Oreffo R. O. C., Roach H. I.
    Tissue and Cell Engineering Society (19^{th}-20^{th} Sept., 2002), Glasgow, UK.