UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH, AND LIFE SCIENCES

School of Medicine

Factors Influencing Osteogenic Differentiation in Fibrodysplasia Ossificans Progressiva

by

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ABSTRACT

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FACTORS INFLUENCING OSTEOGENIC DIFFERENTIATION IN FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

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Osteogenic differentiation can be influenced by modulating factors outside of normal signaling pathways. Two such factors are DNA methylation, a mechanism of epigenetic control, and cell surface heparan sulfate proteoglycans (HSPGs), known as glypicans and syndecans. To elucidate the involvement of these factors in bone morphogenetic protein (BMP) signaling pathways, studies were performed in C2C12 cells and lymphoblastoid cell lines (LCLs) from unaffected individuals and patients suffering from a rare genetic condition called fibrodysplasia ossificans progressiva (FOP), a disease of heterotopic bone formation and dysregulated BMP signaling.

C2C12 cells were used to investigate the effect of methylation and HSPGs on osteogenic differentiation. These studies demonstrated that DNA methylation can inhibit BMP-stimulated induction of alkaline phosphatase, a marker of osteogenic differentiation. In addition, removal of HSPG glycosaminoglycan (GAG) side chains reduced expression of alkaline phosphatase following BMP treatment, suggesting that HSPGs promote osteogenic differentiation in C2C12 cells.

Control and FOP LCLs were used to investigate the role of HSPGs in BMP signaling. Total and HSPG-specific GAG chain levels were increased on the surface of FOP cells compared to controls. Removal of the HSPG GAG chains reduced BMP signaling in control LCLs, but had no effect in FOP cells, suggesting that FOP cells are resistant to the modulatory effects of HSPGs. Profiling of all HSPGs demonstrated that glypican 1 and syndecan 4 are the most abundantly expressed on control and FOP LCLs, with increased levels on FOP cells, providing additional GAG binding sites. Downregulation of glypican 1 core proteins increased BMP signaling in control and FOP cells, suggesting an inhibitory role in BMP signaling. However, downregulation of syndecan 4 decreased BMP signaling in control cells, but not FOP cells, suggesting that FOP cells are resistant to the stimulatory effects of syndecan 4. The elevated BMP signaling observed in FOP LCLs may be at such a level that the cells are unaffected by HSPGs that promote signaling, but respond to HSPGs that inhibit signaling.

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Dedication

To my parents, without you I wouldn't be who I am today. You've always been there for me throughout both good times and bad. For, the support, guidance, encouragement, and love I have received from you throughout my life, I am eternally grateful. Thank you from the bottom of my heart.

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Finally, I wish to thank the entire FOP community, who suffer with this devastating disease everyday, and whose inspiration and unrelenting spirit has been the driving force of this work.

List of Abbreviations

^{35}S – Radioactive Sulfate

5-aza-d – 5-aza-2-deoxycytidine

5azaC – 5-azacytidine

°C – Degrees Celsius

ά-MEM – α – Modified Eagle's Media

ACVR1 – Activin Receptor 1

ACVR2 – Activin Receptor 2

ACVR2B – Activin Receptor 2B

AER – Apical Ectodermal Ridge

AHO – Albright hereditary osteodystrophy

Akt – Thymoma Viral Proto-Oncogene 1

ANOVA – Analysis of Variance

ATF – Activating Transcription Factor

β-catenin – Beta Catenin

bHLH – basic Helix-Loop-Helix

BAMBI – BMP and Activin Membrane Bound Inhibitor

BCR – B Cell Antigen Receptor

BMK1 – Big MAP kinase 1

BMP – Bone Morphogenetic Protein

BMPRs – Bone Morphogenetic Protein Receptors

BMPRIA – Bone Morphogenetic Protein Receptor IA

BMPRIB – Bone Morphogenetic Protein Receptor IB

BMPRII – Bone Morphogenetic Protein Receptor II

BRAM1 – Bone Morphogenetic Protein Receptor-Associated Molecule 1

BSA – Bovine Serum Albumin

C1 / C2 – Constant or Conserved Region

C2C12 – Mouse Premyoblast Cell Line

C3H – Myoblast Cell Line

C3H10T1/2 – Osteogenic Cell Line

C-6-S – chondroitin sulfate

cDNA –Complementary DNA

COX - cycloxygenase

CpG – Cytosine Guanine

CXCL1 – B Cell Chemokine (C-X-C motif) Ligand 1

DAPI - 4',6-Diamidino-2-phenylindole

DEPC – Diethyl Pyrocarbonate

dH_2O – Distilled Water

Dlx5 – Distal Less Homeobox 5

DMB - Dimethylmethylene Blue

DMEM – Dulbecco's Modified Eagle's Media

DNA – Deoxyribose Nucleic Acid

dpp – Decapentaplegic

DTT – Di-thio-threitol

EBV – Epstein Barr Virus

ECM – Extracellular Matrix

ELISA – Enzyme-Linked Immunosorbent Assay

ERK – Extracellular Signal-Regulated Kinase

ES – Embryonic Stem

EtOH – Ethanol

EXT1 – Exostosin 1

EXT2 – Exostosin 2

FACS – Fluorescence Activated Cell Sorting

FCS – Fetal Calf Serum

FGF – Fibroblast Growth Factor

FKBP12 – FK506 binding protein 12

FOP – Fibrodysplasia Ossificans Progressiva

FRS2 - Fibroblast Growth Factor Receptor Substrate 2

FSH – Follicle Stimulating Hormone

GAG – Glycosaminoglycan

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase

GDF – Growth and Differentiation Factor

GlcA – Glucoronic Acid

GlcNAc – N-acetylglucosamine

GM-CSF – Granular Macrophage – Colony Stimulating Factor

GNAS1 – G-s-alpha

GPI – Glycosyl Phosphatidyl Inositol

Grb2 – Growth Factor Receptor-Bound Protein 2

GS – Glycine Serine

GSK-3β – Glycogen Synthase Kinase 3 beta

GTPases – Guanosine Triphosphatases

HBSS – Hanks Balanced Salt Solution

HLH – Helix-Loop-Helix

HO – Heterotopic Ossification

HME – Hereditary Multiple Exostoses

HS – Heparan Sulfate

HSP27 – Small Heat Shock Protein 27

HSPG – Heparan Sulfate Proteoglycan

ID1 / Id1 – Inhibitor of DNA Binding and Differentiation 1

ID2 / Id2 – Inhibitor of DNA Binding and Differentiation 2

ID3 / Id3 – Inhibitor of DNA Binding and Differentiation 3

ID4 / Id4 – Inhibitor of DNA Binding and Differentiation 4

IGF-1 – Insulin-like growth factor 1

I-Smad – Inhibitory Smad

IU – International Units

JNK – C-Jun N-terminal Kinase

kDa – Kilo Dalton

LCLs – Lymphocyte Cell Line

LEF1 - Lymphoid Enhancer-Binding Factor 1

LiCl – Lithium Chloride

MAP – Mitogen Activating Protein

MAPK – Mitogen Activating Protein Kinase

MAPKAP or M2 – MAP-Kinase Activated Protein Kinase 2

MC3T3E1 – Osteoblastic Cell Line

MEF2C – Myocyte Enhance Factor 2C

MEK – MAP kinse-ERK kinase

MFI – Mean Fluorescent Intensity

mg – Milligram

MH1 – Mad Homology Domain-1

MH2 – Mad Homology Domain-2

MHE – Multiple Hereditary Exostoses

μCi - MicroCurie

MKK – MAP Kinase Kinase

ml – Millilitre

MMC – Mouse Marrow Cells

MMPs – Matrix Metalloproteinases

MNK1 – MAP Kinase Interaction Protein Kinase

mRNA – Messenger RNA

MSK – Mitogen and Stress Activating Kinase

MSX-2 – *msh* Homeobox Homolog 2

$Na_2{}^{35}SO_4$ – ^{35}S in the form of Sodium Sulfate

NaOH – Sodium Hydroxide

NSAIDS – non-steroidal anti-inflammatory drugs

Nse – neuron-specific enolase

ng – nanogram

nl - nanolitre

nmols – Nanomoles

NTC – No Template Control

OL – Oligodendrocyte

OLIG - Oligodendrocyte Transcription Factor Family

P1 – Passage 1

P2 – Passage 2

PAX – Paired Box Transcription Factor

PBS – Phosphate Buffered Saline

PBSB – 1% BSA in PBS

PCR – Polymerase Chain Reaction (PCR)

PIP2 – Phosphatidylinositol-4,5-Bisphosphate

PI-PLC – Phosphatidylinositol Phospholipase C

PKC – Protein Kinase C

PLC-γ – Phospholipase C-γ

PMSF – Phenylmethyl-Sulfonylfluoride

PNPP – p-Nitro-Phenol Phosphate

POH – Progressive Osseous Heteroplasia

PRAK – p38 Regulated/Activated Kinase (PRAK)

PRDC - Gremlin 2, Cysteine Knot Superfamily, Homolog

PTHrP – Parathyroid Related Protein

PTN – Pleiotrophin

Raf-1 – v-raf-1 Murine Leukemia Viral Oncogene Homolog 1

rhBMP – Recombinant Human Bone Morphogenetic Protein

RNA – Ribose Nucleic Acid

rpm – Revolutions per Minute

RPMI – Roswell Park Memorial Institute

R-Smad – Receptor Smad

RT-PCR – Reverse Transcriptase - Polymerase Chain Reaction

Runx2 / cbfa1 – Runt Related Transcription Factor 2

SARA – Smad Anchor for Receptor Activation

Sap1 – SRF Accessory Protein 1 (Sap1)

SA-HRP – Streptavidin Horseradish peroxidase

SA-PE – Streptavidin Phycoerythrin

SAPK – Stress Activated Protein Kinase

SEM – Standard Error of the Mean

Sfrp2 – Secreted Frizzled Related Protein 2

SGBS – Simpson-Golabi-Behmel syndrome

Shp-2 – Shatterproof 2

siRNA – Small Interfering RNA

Ski - V-Ski Sarcoma Viral Oncogene Homolog

siRNA – Small Interfering RNA

Smurf – Smad Ubiquitination Regulatory Factors

SRF – Serum Response Factor

TAB – TAK Binding Proteins

TAK1 – TGF-β Activated Kinase 1

TCF - Hepatocyte Nuclear Factor 4, Alpha

Tob – Transducer of ERBB2

T,S,P – Theronine, Serine, and Proline

TGF-β – Transforming Growth Factor beta

µg – microgram

µl – microlitre

V – Variable Region

VEGF – Vascular Endothelial Growth Factor

Wif1 – Wnt Inhibitory Factor 1

XIAP – X Linked Inhibitor of Apoptosis

Presentations

Portions of this thesis were presented at the following meetings:

HSPGs Core Proteins and Glycoaminoglycan side Chains are Elevated in the Pathological Condition Fibrodysplasia Ossificans Progressiva. British Orthopaedic Research Society (BORS). University of Southampton, Highfield, Southampton, S09 5NH, United Kingdom. July 2006.

Mechanisms of Bone Induction are Misregulated in Patients with Fibrodysplasia Ossificans Progressiva. The University of Southampton annual postgraduate conference. University of Southampton, Southampton, United Kingdom. June 2006.

Mechanisms of Bone Induction are Misregulated in Patients with Fibrodysplasia Ossificans Progressiva. The ARC annual fellows meeting, Loughborough University, United Kingdom. December 2005.

HSPG-Mediated BMP4 Signaling Is Disrupted in Fibrodysplasia Ossificans Progressiva Lymphoblastiod Cells. Presented at the Musculoskeletal Disorders symposium, Hospital of the University of Pennsylvania, Philadelphia, United States. October 2005.

HSPG-Mediated BMP4 Signaling Is Disrupted in Fibrodysplasia Ossificans Progressiva Lymphoblastiod Cells. Presented at the American Society for Bone and Mineral Research (ASBMR), 27th annual meeting, Nashville, TN, United States. September 2005.

Differences in BMP Mediated Osteoinduction in C2C12 Cells are Affected by DNA Methylation Status. Presented at the Gordon Research Conference (GRC), University of New England, Biddeford, Maine, United States. July 2005.

The Role of HSPGs in FOP. Grampian Fellowship for FOP research, McKay Laboratory for Orthopaedic Surgery Research, University of Pennsylvania School of Medicine, Philadelphia, United States. April 2005.

The Role of HSPGs in FOP. Roemex Fellowship for FOP research, McKay Laboratory for Orthopaedic Surgery Research, University of Pennsylvania School of Medicine, Philadelphia, United States. March 2005.

Chapter 1

Introduction

Chapter 1: Introduction

Skeletal Function and Bone Types

The skeleton is made up of a specialized form of connective tissue that is very durable due to mineralization. Weighing between 10-12kg in the average person, bone forms one of the largest tissue masses in the body. The gross appearance of bone gives the impression of a supporting structure, however the microenvironment is highly metabolically active, constantly performing complex alterations to the structure under the control of hormones and mechanical stresses. The five main functions of bone are: 1) protection of the vital organs, 2) sites of attachment for muscle, ligaments, and tendons giving the body structural support, 3) mineral homeostasis (calcium and phosphate) in the form of hydroxyapatite, 4) allowing movement by forming articulations, and 5) formation of blood cells from the bone marrow's hematopoietic tissue (Baron, 2003).

The structure of bone is readily observed by studying long bones such as the femur or humerus (Figure 1.1). A long bone consists of a diaphysis, the long central circular shaft of the bone, the epiphyses, the proximal and distal ends of the long bone, and the metaphyses, the region where the diaphysis joins the epiphyses. The metaphyses contains the growth plate (a layer of thin hyaline cartilage that allows the diaphysis to grow in length), a region where cartilage is replaced by bone. The epiphysis is covered by a layer of articular cartilage that provides shock absorption and reduction in friction at articulations (joints). The periosteum is a sheath of irregular connective tissue around the bone surface, which provides protection, assists in fracture repair, and serves as an

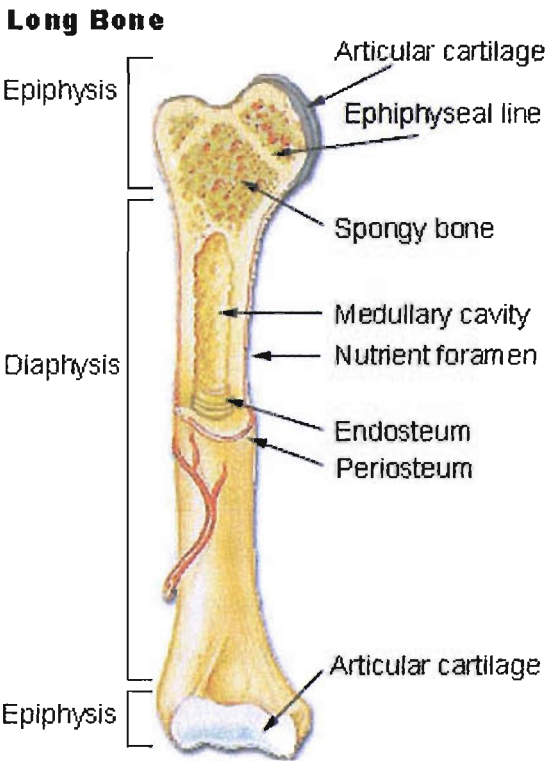


Figure 1.1: Structure of a long bone (Source – http://training.seer.cancer.gov/module_anatomy/unit3_4_bone_classification.html)

attachment point for ligaments and tendons. The periosteum also allows growth of the horizontal plane of the bone. The central core of bone is known as the marrow cavity or medullary cavity (Tortora and Grabowski, 2003).

There are two type of bone tissue: compact bone and spongy bone. Compact bone forms the external layer of all bones (Figure 1.2). It contains few spaces and makes up the majority of the diaphysis. Compact bone is responsible for relieving stresses caused by everyday movement and provides protection and support. The arrangement of compact bone is in units called Haversian systems or osteons that run longitudinally along the long bone, in the same direction as the stresses, resulting in a resistance to bending or fracturing. The compact bones' blood, lymph, and nerve supply comes from the periosteum through vessels known as Volkmann's or perforating canals, and joins with the Haversian systems and medullary cavity. Rings of calcified matrix known as concentric lamellae circle the central canal of the Haversian systems. Between these concentric lamellae, small spaces called lacunae are formed which contain osteocytes. Channels called canaliculi that are filled with extracellular fluid are formed between lacunae. The network formed by the canaliculi and central canals provide routes for the diffusion of nutrients, oxygen, and waste to and from osteocytes trapped in compact bone (Tortora and Grabowski, 2003).

The interior of compact bone is filled with spongy or cancellous bone that consists of thin strands that make up a mesh network. Despite the loose structure of this network, spongy bone provides most of the mechanical strength, and the spaces between provide storage for bone marrow (Solomon et al, 1996).

Bone Cells

Multipotent mesenchymal stem cells have the ability to differentiate into cells of the osteogenic, chondrogenic, myogenic, adipogenic, and fibroblastic lineages. As with hemopoietic differentiation, each of these final lineages is thought to arise from different progenitors. The differentiation of osteoblasts can be divided into four developmental stages: mesenchymal stem cells, osteoprogenitor cells, preosteoblasts, and osteoblasts. Stem cells, such as mesenchymal stem cells, have the potential to produce multiple cell lineages and also have the capacity for self-renewal (Bianco et al, 2001).

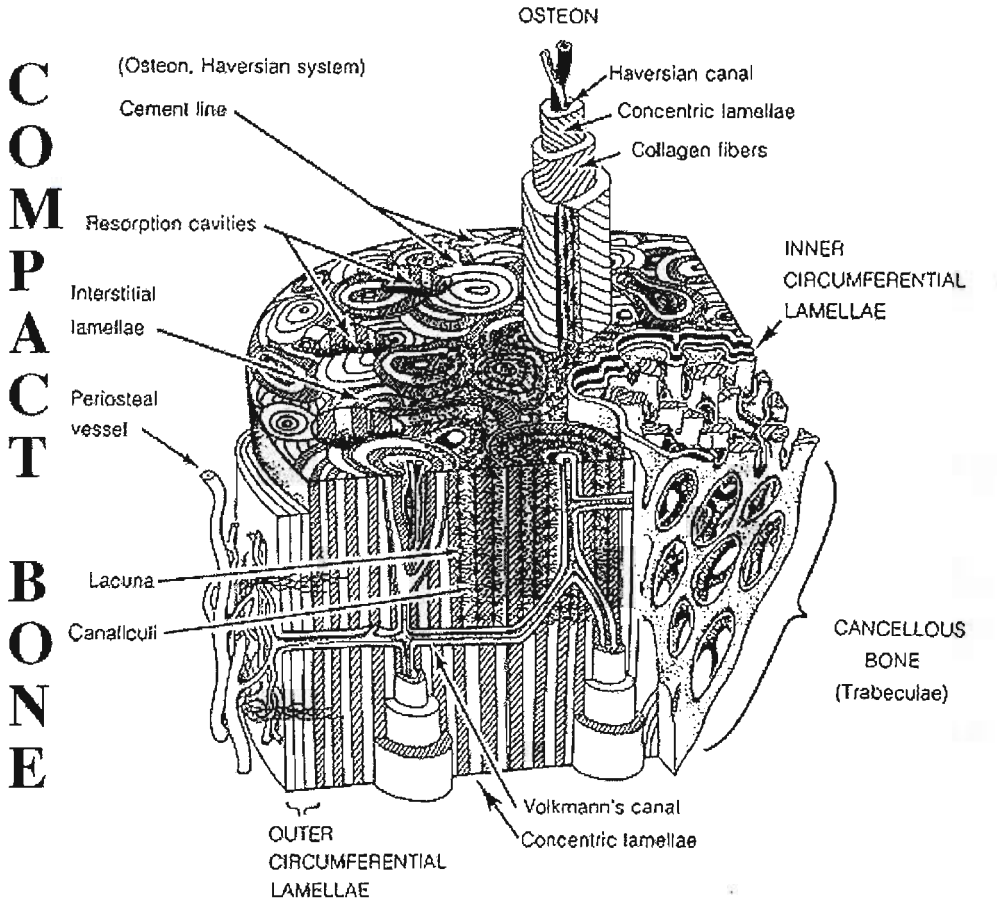


Figure 1.2: Drawing of compact bone
(Source:www.ithaca.edu/faculty/lahr/HistPath/HP_jpegs/connect_tis_jpegs/ct_diagrams/haersian_diag.jpg)

Osteoprogenitor cells lack the self-renewal capacity of stem cells, but are capable of generating cells of more than one lineage (e.g. adipocytes and chondrocytes). Preosteoblasts are representative of a transitional stage between the osteoprogenitor and the osteoblast. In addition, preosteoblasts express lower levels of osteogenic markers, such as alkaline phosphatase and osteopontin, compared to osteoblasts (Long, 2001).

The three major cell types involved in bone formation and turnover are: osteocytes, osteoblasts, and osteoclasts. Osteocytes are approximately ten times more abundant than osteoblasts in adult human bone, and osteoclasts represent only a small fraction of the number of osteoblasts (Marks and Hermey, 1996). Osteocytes are cells that have become trapped in the matrix secretions of osteoblasts. They are derived from osteoblasts and no longer secrete matrix, but are instead responsible for the maintenance of the bone tissue environment.

Osteoblasts are the bone building cells of the body and are responsible for the secretion of collagen fibers and organic components required for the building of bone matrix. Bone matrix is rich in inorganic mineral salts, primarily in the form of hydroxyapatite and calcium carbonate. There are also low levels of sulfates, magnesium hydroxide, and fluoride in the bone matrix. The collagen fibers of the matrix provide a site for these mineral salts to be deposited and crystallized. Crystallization results in the hardness of bone whereas the collagen fibers provide the tensile strength.

Osteoclasts are made from the fusion of up to 50 monocytes from the endosteum. They are large cells with a ruffled border at the plasma membrane which faces the bone. It is at this ruffled border that powerful acids (such as lactic, carbonic, and citric acid) and lysosomal enzymes are produced to degrade and digest the mineral and protein components of the underlying bone (Tortora and Grabowski, 2003).

In order for bone formation to occur, osteoblasts require a substrate to which the osteoid, the non-mineralized bone matrix, can be deposited for mineralization to proceed. That substrate is provided through a membrane (intramembraneous ossification), a cartilage intermediate stage (endochondral ossification), or bone matrix.

Intramembraneous Ossification

Intramembraneous ossification occurs during the development of flat bones, calvarium, the mandible, and the subperiosteal bone shafts of long bones. Cartilaginous anlage formation is not essential for this process to occur. During intramembraneous ossification, mesenchymal cells condense, followed by the vascularisation of the tissue, and finally differentiation into osteoid secreting cells (Mundlos and Olsen, 1997). Intramembranous ossification of the cranial vault results in the formation of ossification centers due to direct bone matrix deposits which form plates. These ossification centers expand during development but do not fuse with other cranial junctions, resulting in the formation of a suture. This structure allows expansion of the skull during growth (Ornitz and Marie, 2002).

Endochondral Ossification

Endochondral ossification involves a cartilage intermediary stage whereby condensed embryonic mesenchyme transforms into cartilage and eventually forms

bone. Bones that undergo endochondral ossification are typically those involved in the formation of joints and weight bearing responsibilities. Endochondral bone formation occurs when mesenchymal cells migrate to specific areas within the developing embryo and condense into tightly packed amalgams that secrete large amounts of extracellular matrix (ECM) components. These ECM components are characteristic of cartilage, such as cartilage specific proteoglycans and collagen type II. Fibroblast-like cells form around this area, creating a boundary between the developing cartilage and the surrounding tissue, which is followed by the infiltration of capillaries to provide a vascular supply. The cells in the middle of this cartilage mass undergo hypertrophy, the matrix mineralises, and phagocytic cells infiltrate and remove the dead cartilage, resulting in a cavity. Next, this cavity becomes infiltrated by osteoprogenitor cells that subsequently differentiate into bone producing cells (osteoblasts) under the control of osteoinductive factors (Rosen and Thies, 1992).

Diseases Associated with Heterotopic Bone Formation

The abnormal formation of bone within the connective tissues is known as heterotopic ossification (HO). The pathophysiology of HO is currently unclear, but histological studies have revealed that the bone being formed is metabolically active and the percentage of osteoblasts is typically twice as much as that of normal bone (Hastings and Graham, 1994). Bone morphogenetic proteins (BMPs) have been shown to induce HO when placed into the soft tissue of animal models, suggesting a role in the formation of HO (Urist et al, 1982).

The genetic disorders progressive osseous heteroplasia (POH), Albright hereditary osteodystrophy (AHO), and the devastating condition of ectopic bone formation known as fibrodysplasia ossificans progressiva (FOP) have all been described as diseases of HO. POH, a disease caused by paternally inherited inactivating GNAS1 mutations, results in HO formed primarily through intramembranous ossification. POH is characterized by dermal ossification during infancy, followed by progressive HO of the cutaneous, sub-cutaneous, and deep connective tissues (Kaplan and Shore, 2000; Shore et al, 2002). AHO is associated with a resistance to hormones such as parathyroid hormone and is characterised by distinct features such as short stature, brachydactyly, round fancies, and other skeletal abnormalities (Patten et al, 1990). The HO occurring in AHO is typically more superficial and milder than that of POH. FOP is the most severe form of progressive HO known, resulting in complete immobilization.

Fibrodysplasia Ossificans Progressiva (FOP)

The first description of fibrodysplasia ossificans progressiva (FOP) was made by Patin in 1692 (Cohen et al, 1993). Subsequently, over 700 cases have been reported to date (Mahboubi et al, 2001). It is one of the rarest known genetic disorders, with a prevalence of approximately 1 in 1.6 million (Connor and Evens, 1982; Kaplan et al, 2002). This disease has no racial, ethnic, sexual, or geographical predilections. The ectopic ossification associated with FOP occurs throughout life, with the initial onset of ossification occurring between the ages of three to five years (Nucci et al, 2000).

FOP is a disabling disease of connective tissue involving heterotopic ossification of skeletal muscle, ligaments, tendons, fasciae, aponeurosis, and skin (Figure 1.3). The diaphragm, larynx, heart, smooth muscle, and sphincter are spared (Baysal et al, 1998). It is an autosomal dominant genetic disorder, transmitted with complete penetrance and variable expression. FOP has three main distinguishing features: congenital malformations of the great toes, characteristic anatomical patterns of osteogenesis, and heterotopic ossification of soft connective tissues (Mahboubi et al, 2001). Patients with FOP have two skeletons: a normotopic skeleton formed during embryogenesis and a heterotopic skeleton formed after birth. The heterotopic skeleton is similar to the normotopic skeleton in terms of strength, function, and mineral storage, but appears in undesirable areas. The heterotopic skeleton appears to be formed independently, but under the same control mechanisms, as the normotopic skeleton. The sites of heterotopic ossification eventually form ridged synostoses with the normotopic skeleton, enhancing immobility. The gross appearance of the normotopic skeleton is essentially normal, with certain exceptions: there is shortening of the first metatarsals and proximal phalanx which results in malformations of the great toes, segmentation deficits of the cervical spine, and broad short femoral necks that are undetectable at birth (Mahboubi et al, 2001).

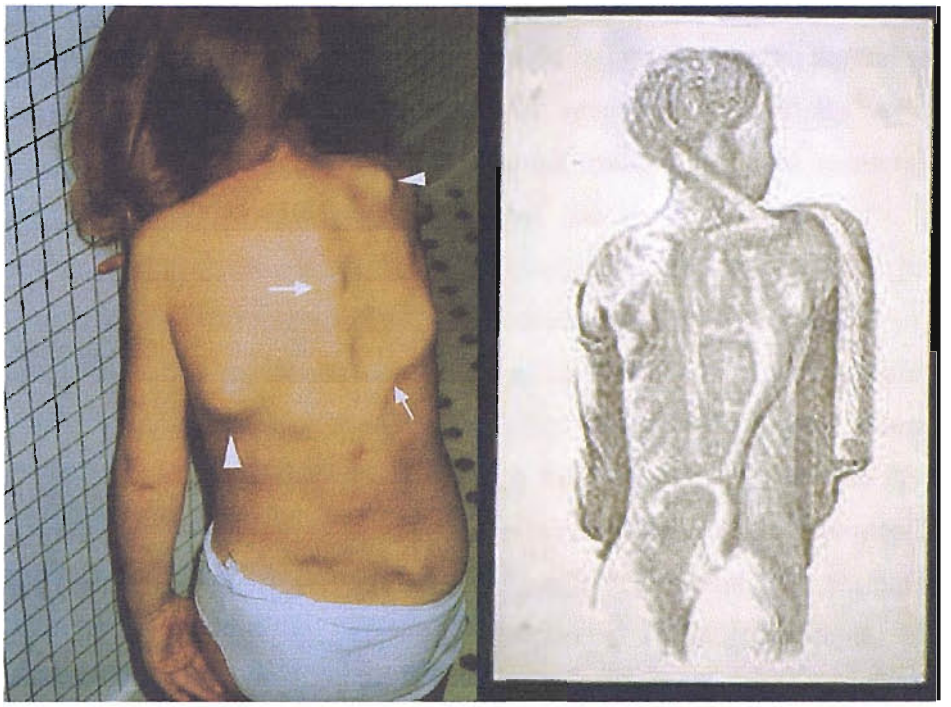


Figure 1.3: The effects of FOP. Left: ectopic ossification caused by FOP can be seen protruding through the skin (arrows). Right: an illustration of the effects of FOP.
(Images courtesy of F.S. Kaplan, Philadelphia)

The FOP Lesion

The appearance of preosseous fibrous nodules forming from the soft connective tissues occurs typically within the first decade of life. These nodules will eventually become new sites of heterotopic ossification. The initial development of these nodules occasionally regresses, but more often develops into permanent heterotopic bone. Severe scoliosis may occur due to the formation of asymmetric bars of heterotopic ossification connecting the rib cage to the pelvis. This can be joined by paravertebral muscle ossification, limiting thoracic kyphosis, and resulting in restrictive chest wall disease. Heterotopic ossification of the jaw in FOP can also result in complications, including starvation and the resulting death of patients (Mahboubi et al, 2001; Kaplan and Shore, 1998).

As previously mentioned, the initial signs of impending ossification are the appearance of lesions or nodules in the connective tissue. These lesions are described as painful swellings that histologically resemble aggressive juvenile fibromatosis (Gannon et al, 1997). Aggressive juvenile fibromatosis is characterized by the appearance of benign tumours exhibiting fibroblastic proliferation at any site of the fascia, but with a preference for muscle (Kaplan et al, 1996). FOP is distinguished from aggressive juvenile fibromatosis by the overexpression of BMP4 messenger

RNA and protein in lymphocytes and lesional tissue (Gannon et al, 1997). The initial lesions cause pain, erythema, tenderness, warmth, and swelling. After several weeks, the swelling subsides and the pain decreases (the intermediate lesion). By week 12, the formation of the late lesion will have occurred, resulting in a hard, non-tender mass of heterotopic ossification (Mahboubi et al, 2001).

The main sites for appearance of these lesions are the shoulder joints, jaw, and spine. Spinal involvement can lead to complete fusion of the vertebrae, mimicking the symptoms of ankylosing spondylitis, which include lumbar pain and stiffness that progresses to involve the entire spine (Kocyigit et al, 2001). Fibrous tissue replaces the discs and ligaments, making the spine rigid. Heterotopic ossification occurs through an endochondral process to form mature heterotopic bone that will eventually immobilise the joints of the axial and appendicular normotopic skeleton, rendering movement impossible (Kaplan et al, 1996). Heterotopic ossification occurs in characteristic patterns over time. Initial involvement is typically seen in the dorsal, proximal, cranial, and axial regions spreading as time progresses to the ventral, distal, caudal, and appendicular regions of the body (Mahboubi et al, 2001).

FOP Genetics

An autosomal dominant pattern of inheritance was confirmed by the transmission of the mutated gene from a father to three of his offspring (Kaplan et al, 1993). However, due to the low reproductive fitness of FOP patients, most cases are considered to be the result of spontaneous mutations (Smith et al, 1996). An examination of two half-sisters who both suffered from FOP and whose parents were unaffected by the disease suggested that the cause of the FOP may have been due to maternal gonadal mosaicism. The estimated chance of both sisters developing FOP from independent mutations is 3.6×10^{-13} (Janoff et al, 1996).

Due to the lack of multi-generational families with FOP, traditional linkage analysis and positional cloning to identify the mutated gene has been difficult. However, an initial genome-wide linkage analysis using four small multigenerational families supported linkage of the FOP gene to a 36cM region on chromosome 4q27-31 (Feldman et al, 2000). However, more recent investigation using eight multigenerational families suggests that the region on chromosome 4 is less strongly linked than previously described, and a region of 24 million base pairs on

chromosome 2q23-24 appears to have consistent linkage in all families examined (Shore et al, 2005).

Next, a candidate gene approach was taken to identify the mutated gene in FOP to aid in the linkage analysis studies. Based on the distinguishing features of FOP, a candidate gene would need to be functional both during embryogenesis, due to the congenital malformations seen at birth, and postnatally, due to the generalised heterotopic ossification of the connective tissues which develops throughout life. Genes that fit these criteria are the bone morphogenetic protein (BMP) and BMP pathway genes (Kaplan and Shore, 1998).

Recently, the primary genetic defect in FOP was identified to be a mutation in the BMP receptor, activin receptor 1 (ACVR1). The mutation is a heterozygous single nucleotide change at cDNA position 617 (617G→A), resulting in the replacement of an arginine with histidine in codon 206 (R206H) at the end of the highly conserved glycine-serine (GS) activation domain necessary for phosphorylation of the type I receptor by the type II receptor (Shore et al, 2006). Protein modeling studies have demonstrated that the R206H mutation may result in stabilization of ACVR1 and/or alter interactions between the GS domain and other signaling molecules. This may result in constitutive or promiscuous ACVR1 activity.

Treatment of FOP

FOP poses many uncertainties when considering experimental treatment due to severity, rarity, and fluctuating clinical course of the disease (Einhorn and Kaplan, 1994; Kaplan et al, 2003). The use of surgery to release joint contractures is unsuccessful, as is surgical intervention of heterotopic bone to mobilize joints since this results in exacerbation of the disease through formation of new heterotopic bone at and around the operative site. A joint may occasionally be repositioned surgically to improve the patients overall mobility, but spinal bracing has been found to be ineffective (Kaplan et al, 2005A).

Due to the extreme variability and rarity of FOP, double blind randomised trials are difficult to conduct. Treatment of FOP has been attempted using many different classifications and combinations of drugs including steroids, calcification blockers (EDTA), and mineral binding agents, all of which have demonstrated limited effectiveness. Hopefully, a better understanding of the pathophysiology of FOP will lead to improved treatment and therapies.

During the early stages of an FOP flare-up, immunosuppressant therapy is often used based on pathological evaluations revealing lymphocytic and mast cell infiltration (Kaplan et al, 1998; Kaplan et al, 2002; Kaplan et al, 2003; Gannon et al, 1998; Gannon et al, 2001). Corticosteroids are used during early symptomatic flare-ups affecting major joints, but have shown a more limited effectiveness towards flare-up of the trunk or back of the neck (Glaser and Kaplan, 2005). The use of mast cell stabilizers, leukotriene inhibitors, non-steroidal anti-inflammatory drugs (NSAIDS), antihistamines, and COX-2 inhibitors help reduce mast cells, lymphocytes, and their associated inflammatory mediators during the early stages of FOP flare-ups (Kaplan et al, 2002; Simmons, 2004; Glaser and Kaplan, 2005). The release of angiogenic and chemotactic factors may be inhibited using mast cell stabilizers and the downstream effects of released mediators may be inhibited by leukotriene inhibitors. Further, the anti-angiogenic activity of the NSAIDS and COX-2 inhibitors may be therapeutic during early stage lesion formation. Angiogenesis, aside from being required for the development of the skeleton and normal fracture healing, is also required for the formation of heterotopic bone. This provides a potential therapeutic target using NSAIDS, COX-2 inhibitors, vascular growth factor traps (a high affinity soluble decoy receptor for VEGF), and thalidomide, all of which are currently being considered (Kaplan et al, 2005B).

The aminobisphosphonates are also being considered as a potential therapy for FOP. Pamidronate and zoledronate may inhibit the proliferation of rapidly dividing populations of cells (Still et al, 2003) and they also have anti-angiogenic properties (Wood et al, 2002; Santini et al, 2002). However, their exact mechanism of action on the early FOP lesion remains unknown. Although all of the above mentioned agents may have therapeutic benefits, currently there remains no completely effective treatment or cure for FOP.

Animal Models

The generation of animal models with typical features of FOP provides a system in which to better understand the pathological events that occur and to test the effectiveness of emerging therapeutic treatments. The first animal model that provided a clue into the pathophysiology of FOP came from the fly. *Drosophila decapentaplegic (dpp)* mutants resulted in a disturbance of the body plan, similar to FOP (Kaplan et al, 1990). This model helped suggest a molecular basis for FOP and provided insight, before the availability of vertebrate animals, to link bone formation

with pattern abnormalities (Kaplan et al, 1990; Kaplan et al, 2005C). The BMP genes and *Drosophila dpp* are likely derived from a common ancestral gene due to the conservation of protein sequences across such a large evolutionary distance.

A sporadic FOP-like condition was described in six domestic house cats, but no surviving animals are available for further study. Radiographic examination revealed multiple areas of heterotopic ossification within affected muscle, but no congenital malformations of the distal limbs. Interestingly, perivascular infiltration of lymphocytes at the edge of the fibroproliferative lesions was seen, similar to human FOP, but the clinical course in the feline was rapid with the development of severe disability occurring within months, sometimes weeks (Valentine et al, 1992).

Progressive postnatal heterotopic chondrogenesis and osteogenesis, due in part to BMP4 signaling, was seen in mice exhibiting embryonic overexpression of the c-Fos proto-oncogene. The phenotypic features of this model are similar to those seen in children with FOP (Kaplan and Shore, 1996; Olmsted et al, 1998). However, the defect in FOP may be independent of the effects of c-Fos since early FOP lesions express abundant BMP4 without increased c-Fos mRNA. Comparisons of c-Fos embryonic stem cell chimeras with those of FOP may provide insight into the earliest events of genetically induced heterotopic chondrogenesis and osteogenesis (Kaplan et al, 2005C).

The importance of BMP signaling in heterotopic ossification and joint morphogenesis is demonstrated by several animal models. Murine homozygous deletions of the gene for Noggin, a BMP antagonist, are lethal and the animals die soon after birth due to multiple congenital defects. Noggin knockout mice exhibit failure to form joints, multiple synostosis, and ankylosis of the chest wall (Brunet et al, 1998). Homozygous deletions of the BMP receptor BMPRIB result in normal mice with the exception of digital malformations similar to those seen in brachydactyly type A2 and FOP (Lehmann et al, 2003; Yi et al, 2000).

Genetically manipulated mice that overexpress Bmp4 under the control of several different promoters have been reported, but the majority fail to induce postnatal heterotopic ossification. Neither transgenic mice overexpressing Bmp4 under control of the *keratin* promoter (Guha et al, 2002) or expressing Bmp4 using an expression vector based on the *cytokeratin IV* promoter develop heterotopic ossification (Blessing et al, 1993). However, mice that overexpress Bmp4 under the control of the *neuron-specific enolase (nse)* promoter develop postnatal heterotopic ossification similar to FOP (Kan et al, 2004). Mating of these mice with transgenics

overexpressing noggin under control of the same promoter completely rescues the animals from the development of heterotopic ossification. The ossification in the Nse-Bmp4 transgenic mice is progressive, but spares the diaphragm, tongue, and extraocular muscles, similar to FOP. Unlike FOP, heterotopic ossification begins subcutaneously rather than in deep connective tissue, the mice lack malformations of the digits and other joints, and the progressive anatomical patterns are different.

Interestingly, lymphoblastoid cells from unaffected patients, when implanted in matrigel and injected subcutaneously into mice, results in a small mass that persists, but shows little evidence of a fibrotic or angiogenic response. In contrast, cells implanted into mice from FOP patients results in a solid, fibrotic cellular mass (Billings et al, 2005). Further histological examination demonstrated that the FOP cells proliferate in the host and induce a response similar to that seen in early stage FOP lesion formation, but do not progress to the formation of heterotopic bone (Billings et al, 2005).

Similarly, when BMP4 is implanted in matrigel and subcutaneously injected into mice, heterotopic bone formation occurs that mimics the stages of bone formation in FOP (Glaser et al, 2003). Local delivery of Noggin or systemic delivery of a Noggin mutein (NogginΔB2), with the heparan sulfate proteoglycan binding site removed, prevented formation of heterotopic bone in these animals. This suggests that Noggin therapy may be an effective way to treat FOP.

B Cells and the LCL Cell System

As the use of Epstein - Barr virus (EBV) transformed lymphocytes are the current cell system used to study FOP, it is necessary to understand the biology of the immune system. There are two types of white blood cells involved in the immune response, all of which are present in blood samples obtained from patients: lymphocytes that consist of B cells, T cells, and natural killer cells, and phagocytes that consist of neutrophils and macrophages. *In vitro* transformation of B cells with EBV, a gamma herpes virus associated with specific lymphoid malignancies such as Burkitt's lymphoma, results in their immortalisation into continuously growing lymphoblastoid cell lines (LCLs) (Cesarman, 2002).

Millions of B cells produced in the bone marrow every day carry multiple receptors that bind to specific antigens resulting in the rapid division of the cell to form a clone. Most of these activated B cells then differentiate into plasma cells and secrete specific antibodies. Some become memory cells that continue to produce a

small amount of antibody that, when exposed to the antigen a second time, results in a much more rapid immune response. B cell activation is a complex process involving antigen presenting cells (macrophages) and T cell release of interleukins that stimulate B cells to proliferate and differentiate (Sompayrac, 2003).

The immune system is strongly implicated as playing a role in FOP through many pathological, clinical, and epidemiological features including: perivascular B and T cell infiltration and mast cell involvement in early FOP lesion (Glaser et al, 2003; Gannon et al, 1998; Gannon et al, 2001), massive soft tissue swelling of early flare-ups (Kaplan et al, 2002; Cohen et al, 1993; Moriatis et al, 1997), and lymphocyte associated skeletal muscle destruction (Glaser et al, 2003; Gannon et al, 1998; Gannon et al, 2001). Further immune implications include: flare-ups caused by immunization, viruses, and tissue trauma (Kaplan et al, 2002; Cohen et al, 1993; Lanchoney et al, 1995; Luchetti et al, 1996; Scarlett et al, 2004) and a clinical response to corticosteroids in the first 24 to 36 hours following a flare-up (Kaplan et al, 2002).

The use of LCLs to study FOP provides acquisition of a cell system that does not result in any contraindications to the patients. As retrieval of cells of connective tissue origin results in exacerbation of the disease, the LCLs are an attractive model to study both the role of the immune system and the developmental intracellular signaling pathways in FOP (Shafritz et al, 1996; Olmstead et al, 2003; Ahn et al, 2003; de la Pena et al, 2005; Fiori et al, 2006).

Developmental Signaling Pathways

There are five main signaling pathways associated with development. These are the fibroblast growth factor (FGF) pathway, the Wnt pathway, the hedgehog signaling pathway, the Notch pathway, and the transforming growth factor β (TGF-β) pathway that includes the bone morphogenetic protein (BMP) pathway. These pathways have different roles in the developing embryo and form a complex signaling network that runs from the extracellular environment at the level of ligand delivery to receptor, through the intracellular environment via signaling proteins, to the nucleus where gene transcription can be influenced. Most often, the end result of signaling is the "switching on" or "switching off" of target genes. However, each pathway is not exclusive to its own signaling network and a significant amount of crosstalk occurs between pathways. For example, when the BMP pathway is

activated, the genes responsible for the production of alkaline phosphatase are "switched on". These genes are under the control of the Wnt pathway.

The FGF Signaling Pathway

The FGF pathway has a critical role in mesoderm formation during embryonic development. Currently four FGF receptors have been identified, FGFR1-4, all of which interact with the 23 members of the FGF family. The receptors are membrane–spanning tyrosine kinases with multiple autophosphorylation sites (Mohammadi et al, 1996). Following ligand binding, receptor dimerization, and receptor phosphorylation, the FGF pathway leads to Crk binding, tyrosine kinase activity, and binding to Shb and phospholipase C-γ (PLC-γ) (Larsson et al, 1999; Mohammadi et al, 1991; Cross et al, 2002). PLC-γ binding results in generation of inositol 1,4,5-trisphosphate and diacylglycerol from hydrolysis of membrane phospholipids and subsequent activation of certain isoforms of protein kinase C (PKC) (Divecha and Irvine, 1995). PKC acts on a variety of substrates including the MARCKS proteins, vitamin D3 receptors, Raf kinases, and MAP kinases. FGF pathway activation also leads to phosphorylation of FRS2, followed by Grb2 and Shp-2, interactions required for Ras/Raf-1/MEK/ERK pathway activation (Kouhara et al, 1997; Hadari et al, 1998). The FGF signaling pathway has been implicated in embryonic development, vascularization, inflammation, tissue growth, tissue remodeling, and tumour growth (Powers et al, 2000).

The Wnt Signaling Pathway

BMP, Wnt, and Notch all play a central role in osteoblastic cell fate (Canalis et al, 2005). The Wnt proteins were initially implicated in viral carcinogenesis associated with mammary tumors. However, studies focusing on the Wnt pathway and their receptors (Frizzled) have advanced to demonstrate the critical roles of Wnts during embryonic cell patterning, differentiation, proliferation, and orientation (Li et al, 2006). The controlled regulation of proliferation and differentiation is essential for embryonic development. Downstream signal transduction proteins that modulate the effects of Wnt include: Dishevelled, beta-catenin, protein kinase C, Akt, intracellular calcium, and glycogen synthase kinase-3beta (Li et al, 2006). The armadillo repeat protein, beta-catenin, is important in both processes of proliferation and development, and is a key signaling molecule of the Wnt pathway (Brembeck et al, 2006). Beta-catenin activates crucial target genes, controls E-cadherin-mediated

plasma membrane cell adhesion, and mediates adheren junction molecules with the cytoskeleton (Brembeck et al, 2006). Wnt, in combination with BMPs, is also essential for osteoblastogenesis as demonstrated by the differentiation of mesenchymal cells towards mature osteoblasts and mutations of Wnt co-receptors which are associated with changes in bone mass (Canalis et al, 2005). Further, frizzled related protein 1 has been shown to regulate Wnt signaling following BMP2 induced chondrocyte differentiation (Gaur et al, 2006). Recently, loss and gain of function experiments in mice have demonstrated a role for Wnt in lymphopoiesis. Hematopoietic stem cells maintenance and self-renewal is regulated by Wnt. In the absence of LEF1/TCF, proteins involved in the regulation of gene expression by Wnt, differentiation of T cells and natural killer cells is blocked. Further, pro-B cell proliferation is regulated by Wnt signals (Timm and Grosschedl, 2005).

The Hedgehog Signaling Pathway

Hedgehog signaling plays a key role in specifying ventral cell fates throughout the neuroectoderm, demonstrating a crucial role in dorsoventral patterning of the neural tube which is critical in shaping the functional organisation of the central nervous system (Lupo et al, 2006). Hedgehog has also been shown to be important in mammalian gastrointestinal development, regulating normal axial patterning of the embryonic gut (Lees et al, 2005). Recently, hedgehog signaling has been demonstrated to be active in chondrosarcomas and benign cartilage tumors, regulating tumor cell proliferation (Tiet et al, 2006). Interestingly, Indian hedgehog (Ihh) has been shown to have a critical role in regulating endochondral ossification and, along with parathyroid related protein (PTHrP) and vascular endothelial growth factor (VEGF), potentially regulates tissue differentiation (Brouwers et al, 2005).

The Notch Signaling Pathway

Notch consists of a family of co-receptors that oppose Wnt signaling and inhibit osteoblastic differentiation (Canalis et al, 2005). Notch 1 overexpression inhibits osteoblastogenesis by suppressing the Wnt pathway, but not the BMP pathway (Deregowski et al, 2006). Notch 1 activity has been shown to be associated with the formation of the prostate buds, however this process is derepressed in BMP7 null urogenital epithelium (Grishina et al, 2005). Notch signaling is also essential for T-cell development with a link to c-Kit, a receptor tyrosine kinase that has crucial roles in lymphocyte development. Recent investigation has shown that following

Notch signaling, c-Kit expression was rapidly upregulated, whereas in the absence of Notch signaling, c-Kit expression remained low (Rodewald, 2006).

The TGF-β Signaling Pathway

TGF-β (transforming growth factor beta) is a potent growth inhibitor for a large variety of cell types. These include epithelial cells, hematopoietic cells, vascular endothelial cells, and immune lymphocytes (Miyazono et al, 1994; Blobe et al, 2000). The TGF-β superfamily of signaling molecules consist of nearly 30 members in humans, with orthologs in mouse, Xenopus, and other vertebrates (Hogan, 1996; Massague, 1998). The superfamily is divided into two main groups: the TGF-β/Activin/Nodal and the BMP/GDFs. Members of each family have diverse and often complementary roles, with some family members only being expressed for a limited time during development and other being present throughout embryogenesis and adult tissues (Massague et al, 2000). Signaling of these pathways occur through ligand binding to assembled receptor complexes of type I and type II receptors. The constitutively active type II receptor phosphorylates the type I receptor, propagating intracellular signaling (Massague, 1998; Takatsu et al, 2000; Engel et al, 1999).

Bone Morphogenetic Proteins (BMPs)

The discovery of osteoinductive factors followed the observation that demineralised bone matrix could induce bone formation if placed subcutaneously within rabbits, mice, rats, and guinea pigs (Urist, 1965). These osteoinductive factors were purified and called bone morphogenetic proteins (BMPs) (Table 1.1). BMPs belong to the TGF-β (transforming growth factor β) superfamily of cytokines, currently containing over 47 members. BMPs share a 20-92% homology with the TGF-β family members (Chubinskaya and Kuettner, 2003).

All members of the BMP family are synthesised as large precursor molecules, consisting of pro and mature domains. The pro domain is processed via proteolytic cleavage at a –R-X-X-R– site, leaving a mature monomer of 100 – 140 amino acids. There is a cysteine rich domain within each monomer consisting of 7 cysteine residues, 6 of which are linked by intrachain disulphide bonds causing a knot-like core structure in the mature domain of BMP. Two BMP monomers dimerize using

the 7th cysteine residue of each monomer, resulting in active BMP (Chubinskaya and Kuettner, 2003).

BMP	Function
BMP-2	Osteoinductive, osteoblast differentiation
BMP-3	Inhibits osteogenesis, most abundant in bone
BMP-4	Lung / eye development / osteoinductive
BMP-5	Chondrogenesis
BMP-6	Chondrogenesis, osteoblast differentiation
BMP-7	Kidney / eye development, osteoinductive
BMP-8	Osteoinductive
BMP-9	Hepatogenesis
BMP-10	Cardiac development
BMP-11	Mesodermal and neuronal tissue patterning
BMP-12	Tendon-iliac tissue formation
BMP-13	Tendon and ligament-like tissue formation
BMP-14	Enhances bone formation and chondrogenesis
BMP-15	Modifies follicle-stimulating hormone activity

Table 1.1: The BMP superfamily

Those BMPs having the greatest role in the bone induction are BMP-2, BMP-4, and BMP-7. Studies have shown that the bone marrow of rats and mice have increased osteoblastic parameters in the presence of recombinant human BMP-2 (rhBMP-2) and the bone marrow of humans has increased osteoblastic parameters in the presence of BMP-7 (Groeneveld and Burger, 2000). Human BMP-2 induces endochondral bone formation when injected into muscle (Clancy et al, 2003). Various animal studies have shown that rhBMP-2 and recombinant human BMP-4 (rhBMP-4), along with osteogenin (BMP-3), can repair large cavities in the bones of various species within three months. However, human studies have shown inconsistent results, suggesting that currently unknown factors can negatively affect bone induction cascades in humans (Groeneveld and Burger, 2000).

BMP Signaling

BMPs signals by binding to BMP-receptors (BMPRs), of which there are two types: type I and type II (Figure 1.4). Type I receptors include BMPR-IA, BMPR-IB and ACVR1. The type II receptors include BMPR-II, ACVR2, and ACVR2B. Upon binding of BMP ligand, the constitutively active type II receptor phosphorylates the type I receptor in the GS domain, resulting in activation (Kusanagi et al, 2000).

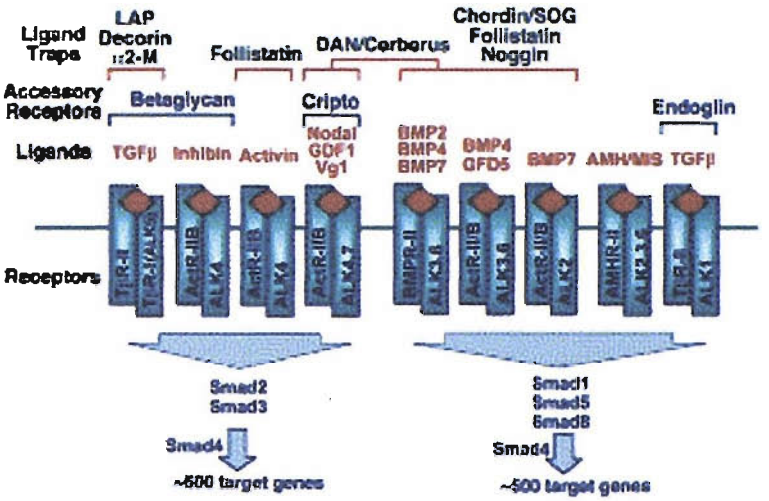


Figure 1.4: TGF-β family receptors
(Source: Shi and Massague, 2003)

The overall structure of type I and II receptors are similar. They have relatively short extracellular domains with conserved cysteine residues and one transmembrane domain. The intracellular domain has a serine / threonine region and the type I receptor contains a GS domain (repeats of serines and glycines) at the C-terminus. Not every BMP binds to all of these receptors or binds them with equal affinity (Kawabata et al, 1998). BMP-2, BMP-4, and BMP-7 bind to BMPR-II weakly in the absence of BMPR-I. However, there is increased binding affinity in the presence of BMPR-I, suggesting cooperation exists between the type I and type II receptors to optimize ligand binding (Kawabata et al, 1998).

BMP signaling can be affected by the conformation and composition of the type I and type II receptors. Ligand binding to preformed heteromeric complexes of type I and type II BMP receptors on the cell membrane induces signaling via the Smad pathway. However, heteromeric complexes formed after ligand binding, by the recruitment of the type II to type I receptors, signal via the p38 MAPK pathway (Canalis et al, 2003).

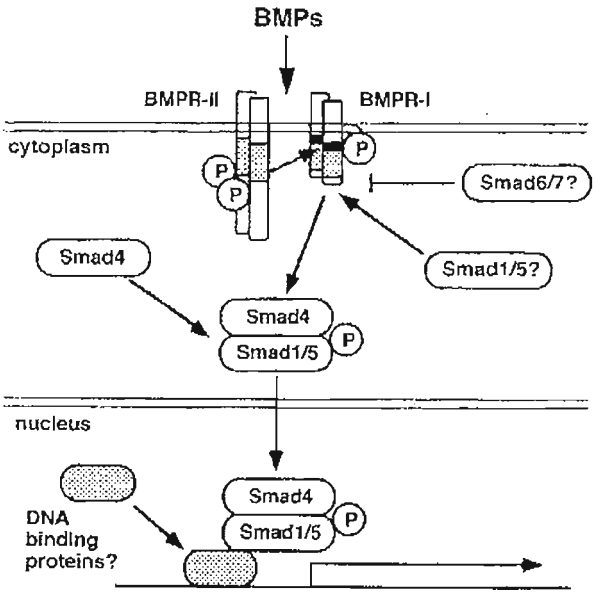


Figure 1.5: Intracellular Smad signaling
(Source: Kawabata et al, 1998)

The Smad Pathway

Smad proteins are classified into three groups: receptor regulated (R-Smads), a common signaling partner, Smad 4 (co-Smad), and inhibitory Smads (I-Smads). Eight Smad proteins have been identified to date, which make up the Smad family (Figure 1.5; Table 1.2). They are approximately 500 amino acids in length with structural differences that correlate with their function (Figure 1.6). The R-Smads and co-Smad all share a conserved structure known as mad homology domain-1 or MH1 located at the N-terminus and a MH2 domain located at the C-terminus. The MH1 domain consists of a β-hairpin loop stabilized by a zinc ion for DNA binding. The MH2 domain is involved in protein-protein interactions. The MH1 and MH2 domains are connected by a linker with proline rich regions of variable sequence and length that have binding sites for ubiquitin ligases and phosphorylation sites for protein kinases (Massague et al, 2005).

Smad	Type	Ligand
Smad 1	Receptor	BMP
Smad 2	Receptor	TGF-β
Smad 3	Receptor	TGF-β
Smad 4	Co-Smad	None
Smad 5	Receptor	BMP
Smad 6	Inhibitory	None
Smad 7	Inhibitory	None
Smad 8	Receptor	BMP

Table 1.2: Smad subtypes

Smads 2 and 3 are TGF-β-specific and Smads 1, 5, and 8 are BMP-specific. Smads 1, 5, and 8 directly interact with the type I receptor at a basic pocket, and subsequently the last two serines of their SSXS motif are phosphorylated (Kawabata et al, 1998). Once phosphorylated, the R-Smads form a heteromeric complex with the co-Smad, Smad4, that translocates to the nucleus to regulate gene transcription through direct DNA binding and association with transcriptional co-activators (Wan and Cao, 2005; Zawel et al, 1998; Yingling et al, 1997; Dennler et al, 1998). Unlike R-Smads, Smad 4 does not have the SSXS domain and is not a direct substrate for type I receptors. The nuclear translocation of Smad 4 is dependent on R-Smads that recruit transcriptional co-activators such as p300. This implies that Smad 4 interacts with transcriptional proteins via R-Smads, but is not directly involved in transactivation (Kusanagi et al, 2000).

Figure 1.6: A Ligand specific (R-Smad) intracellular Smad protein
(Adapted from Kawabata at al, 1998)

In the absence of ligand, the I-Smads (Smads 6 and 7) are predominantly located in the nucleus. Following ligand stimulation, the I-Smads are rapidly exported into the cytoplasm where they antagonize BMP signaling pathways by binding to the activated type I receptors, preventing R-Smad access. This subsequently interferes with the phosphorylation of the R-Smads (Reddi, 1998; Itoh

et al, 1998; Itoh et al, 2001; Nakayama et al, 1998; Zhu et al, 1999A). Smad 6 and 7 do not have SSXS motifs or MH1 domains, but do contain the MH2 domain for protein interactions (Kawabata et al, 1998). Smad 6 and 7 can inhibit the TGF-β and BMP pathways, but it is generally believed that Smad 6 preferentially inhibits BMP signaling. Following ligand stimulation, Smad 7 is induced in a rapid and transient manner, but Smad 6 expression is induced 3 hours after ligand stimulation and continues for more than 48 hours. These finding suggest that I-Smads are induced by R-Smads, and regulate signaling activity by forming a negative feedback loop (Miyazono, 1999).

Once nuclear translocation occurs, Smad proteins can directly bind to consensus DNA sequences, recruit transcriptional co-activators and co-repressors, and interact with other DNA binding proteins to regulate transcription of target genes (Miyazono, 2000). Within the nucleus, Smad 1 binds to multimerized copies of GCCG motifs and the binding affinity increases with increased copies, suggesting that the binding affinity for a single GCCG motif would be relatively low. Therefore R-Smads may require Smad 4 for DNA stabilisation (Kusanagi et al, 2000).

The p38 Pathway

Although BMP signaling is thought to occur primary through the canonical Smad pathway, receptor conformation and internalization mechanisms in different cell types can result in utilization of other pathways such as the mitogen activated protein kinase (MAPK) pathways (Canalis et al, 2003, de la Pena, 2005).

MAPK signal transduction is functional in numerous mammalian cells and occurs in response to a variety of growth factors and cytokines (Rudd, 2005). The MAPK signaling pathways consist of four distinct groups: the extracellular signal-regulated kinases (ERK), the ERK5/Big MAP kinase 1 (BMK1), the c-jun N-terminal or stress activated protein kinases (JNK/SAPK), and the p38 protein kinases (Figure 1.7) (Ono and Han, 2000). TGF-β has been shown to activate the Rho family guanosine triphophatases (GTPases), and TGF-β and BMP2 have the ability to activate all MAPK members though interactions with their upstream activators (Attisano and Wrana, 2002, Lai and Cheng, 2002). BMP2- dependent p38 signaling results in upregulation of alkaline phosphatase, osteopontin, osteocalcin, type I collagen, and fibronectin, whereas ERK signaling stimulates fibronectin and osteopontin expression in osteoblasts (Lai and Chenge, 2002).

While evidence exists that BMPs can activate the ERK and JNK pathways, most studies have focused on the effects of BMP on the p38 MAPK pathway. Signaling through p38 MAPK occurs via activation of upstream kinases. BMP stimulation results in the activation of TGF-β activated kinase 1 (TAK1) (Shibuya et al, 1998; Kimura et al, 2000). TAK1 activity is enhanced by TAK binding proteins (TAB1 and TAB2). TAB1 has been shown to directly bind to the catalytic domain of TAK1 and also to the X-linked inhibitor of apoptosis (XIAP), which is a positive regulator of BMP signaling. XIAP serves as a link between the activated BMP receptors and the TAK-TAB1 complex (Yamaguchi et al, 1999). Although the complete mechanism of BMP signal transduction through p38 remains unclear, it is plausible that the BMP-p38 MAPK pathway synergise with the BMP-Smad pathway to promote BMP target gene transcription (von Bubnoff and Cho, 2001).

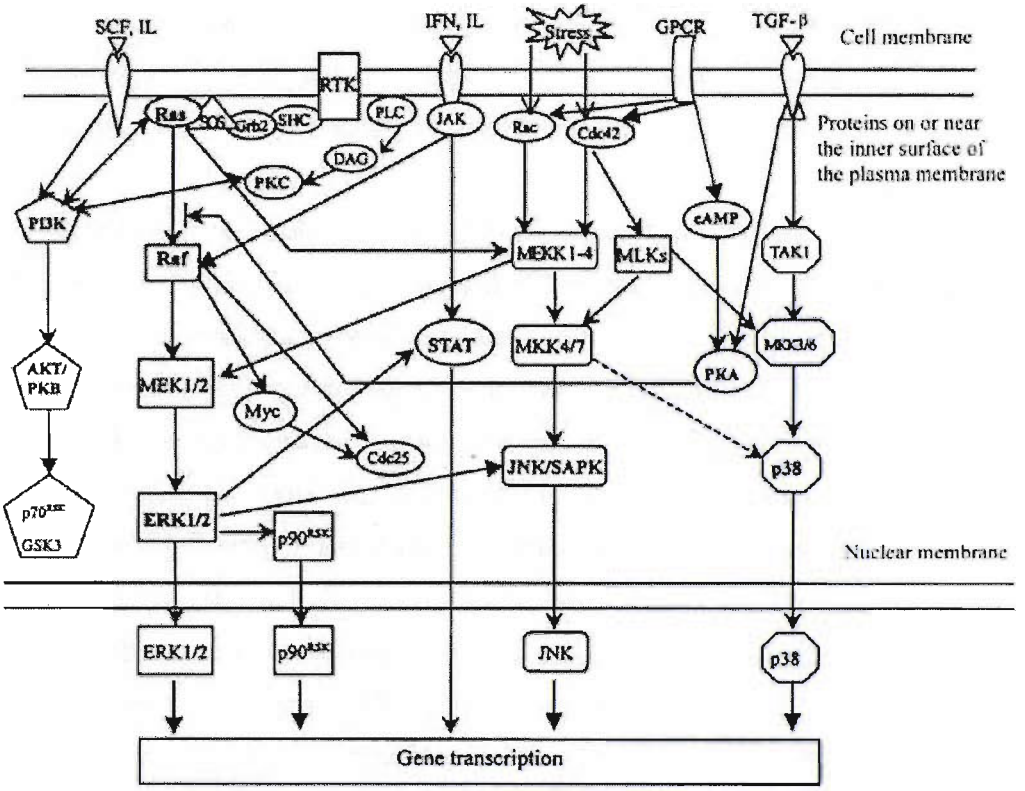


Figure 1.7: The MAP kinase signaling pathways
(Source: Zhang and Liu, 2002)

BMP Signaling in FOP

The lymphoblastoid cell system used to study FOP has been shown to overexpress the BMPR-IA and signal primarily through the BMP-p38 MAPK pathway (de la Pena et al, 2005; Fiori et al, 2006). Further, following BMP4

stimulation, elevated levels of the BMP-specific target genes, ID1 and ID3, were detected in FOP patient cell lines compared to unaffected individuals. Interestingly, these cells appear unable to signal through the BMP-Smad pathway since levels of Smad 1 protein and mRNA are very low. However, introduction of a Smad 1 construct into LCLs restored the ability of these cells to utilize the Smad pathway, as measured by MSX-2 mRNA expression (a BMP Smad-specific target gene). Further, transfection of both a Smad 1 and Smad 6 (inhibitory Smad) construct reduced MSX-2 expression, demonstrating LCLs are capable of Smad signal transduction if the required components are present (Fiori et al, 2006).

Transcriptional Targets of BMP Signaling

Following intracellular activation of BMP associated signaling molecules, transcription target genes are activated that promote or inhibit cellular differentiation. Further, epigenetic control mechanisms, such as DNA methylation, can affect the transcription of these target genes. Targets of the BMP pathway include: ID1, ID3, MSX-2, Runx2, alkaline phosphatase, and p38 specific target genes.

The Inhibitors of DNA Binding and Differentiation Gene Family

The inhibitors of DNA binding and differentiation gene family (IDs: human, Ids: mice) are four related proteins implicated in control of differentiation and cell cycle progression. ID proteins play a role in many physiological processes including neurogenesis, angiogenesis, tumour vascularization, control of cell growth, and cell signaling (Lyden et al, 1999, Maeda et al, 2004). In mice, disruption of Id1 and Id3 results in the expression of neural specific differentiation markers and premature withdrawal from the cell cycle of neuroblasts. Double knockout mice have an absence of sprouting of blood vessels into the neuroectoderm and vascular malformations in the forebrain. Offspring lacking Id1 or Id3 alleles in any combination are indistinguishable from wild type mice, however null alleles of both genes are embryonic lethal, suggesting a redundant function for Id1 and Id3 (Lyden et al, 1999).

Direct injection of BMP2 into the murine calvariae results in increased expression of Id1. Id1 expression is also increased in BMP2 treated MC3T3E1 osteoblastic cell lines (Maeda et al, 2004). Id1 expression is increased following BMP6 stimulation in endothelial cells, showing an important role for Id1 in the

induction of endothelial cell migration, tube formation, and angiogenesis (Valdimrsdottir et al, 2002).

The Msh Homeobox Homologue 2

Msh homeobox homologue 2 (MSX-2) is a member of a small family transcription factors related to the *Drosophila* gene muscle segment homeobox (msh) (Davidson, 1995). MSX-2 is a BMP Smad-specific target gene and is not regulated through p38 MAPK (Brugger et al, 2004). Studies have shown that MSX-2 null mice exhibit defective chondrogenesis and osteogenesis due to a decreased number of osteoprogenitor cells, as well as defects in skull ossification and endochondral bone formation (Satokata et al, 2000).

The Runt Related Transcription Factor 2

The runt related transcription factor 2 (Runx2, also known as cbfa1), is an osteogenic master transcription factor and one of the first osteogenic markers of differentiation. It was believed to play a role in BMP mediated Smad activation by interacting with Smad 1. However, more recent studies suggest that distal-less homeobox 5 (Dlx5), a BMP-2 induced osteoblast differentiation regulator, is present upstream of Runx2, but downstream of Smad 1. Dlx5 is the proximal target for BMP signaling and subsequent cooperation between Dlx5 and Runx2 results in the expression of bone marker genes for osteoblast differentiation (Lee et al, 2003). Runx2 is capable of osteogenic actions in the absence of Smad 1 or 5 (via Dlx5), but involvement of Smad proteins enhances the osteogenic capability of Runx2 (Nishimura et al, 2002).

Alkaline Phosphatase

Alkaline phosphatase is another osteogenic marker that is stimulated prior to bone formation. Alkaline phosphatase activity in C2C12 cells (a pre-myoblastic cell line that can be forced down the osteogenic pathway) is only weakly induced with adenovirus constructs expressing Smad 1 and 5. Alkaline phosphatase activity is enhanced slightly with Smad-4 co-expression. However, when the same cells are treated with BMP, alkaline phosphatase activity dramatically increases (Miyazono, 2000). Recombinant human BMP-2 increases alkaline phosphatase activity in C26 cells, a potent osteoblastic precursor cell line also capable of differentiating in

muscle cells, but not in C20 cells, a more mature osteoblastic cell line (Yamaguchi et al, 1991). This suggests that BMPs exert stronger effects on immature osteoblasts.

p38 Target Genes

The downstream targets of p38 include MAP-kinase activated protein kinase 2 (MAPKAP-K2 or M2), which subsequently phosphorylates a range of proteins including cAMP response element binding protein (Tan et al, 1996), small heat shock protein 27 (HSP27) (Stokoe et al, 1992), and tyrosine hydroxalase (Thomas et al, 1997). P38 also activates MAP kinase interaction protein kinase (MNK1), the stress activating protein p38 regulated/activated kinase (PRAK), and mitogen and stress activating kinase (MSK) (Ono and Han, 2000). Transcription factors activated by p38 include activating transcription factor (ATF) 1 and 2, p53, myocyte enhance factor 2C (MEF2C), and serum response factor (SRF) accessory protein 1 (Sap1) (Ono and Han, 2000).

The Effect of Epigenetics

Epigenetic factors, such as DNA methylation, influence cellular mechanisms of differentiation through control of gene expression. DNA methyltransferases catalyze the transfer of the methyl group from S-adenosyl-methionine to cytosine residues resulting in formation of 5-methylcytosine, a modified base found mostly at CpG islands throughout the genome (Momparler and Bovenzi, 2000). DNA methyltransferases re-methylate new DNA strands through enzymatic modification following replication if the mother strand was methylated. Methylation of CpG islands in gene promoter regions can interfere with transcription factor binding. Furthermore, evidence in tumor cell lines and early mammalian embryos suggests that active methylation can occur *in vivo*. If DNA methyltransferases are absent during replication, demethylation can occur. This process is also known as passive demethylation (Reik and Dean, 2001). The modified cytosine is believed to be responsible for the inhibition of transcription due to methylation. It has been reported *in vitro* that the DNA helixes major groove is the target for 5-methylcytosine and that transcription factors bind with less affinity to methylated target sequences (Momparler and Bovenzi, 2000).

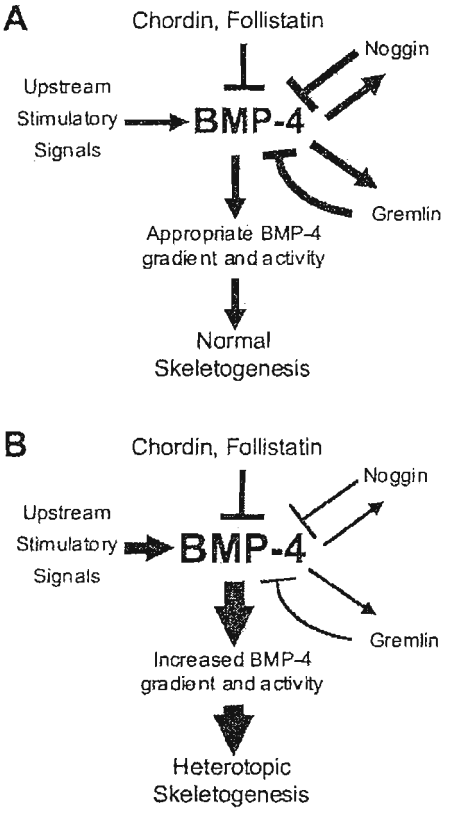
Modulators of BMP Signaling

External Antagonist

As the level of BMPs secreted from cells increases, so does the level of BMP antagonists. Secreted antagonists of BMP include Noggin, chordin, follistatin, and gremlin, all of which are involved in the regulation and balance of BMP induced bone formation (Figure 1.8). These antagonists are unique proteins but share the same functional properties of inhibiting BMP signaling. Inhibition is a consequence of antagonists binding extracellular BMPs and preventing them from receptor engagement (Ahn et al, 2003).

Noggin

Noggin was first isolated from *X. laevis* based upon its ability to rescue dorsal development in embryos ventralized by UV treatment (Smith and Harland, 1992). It is a 26-kDa protein (205 amino acids) with a signal sequence on its acidic hydrophobic amino-terminus (Zimmerman et al, 1996). The cysteine-rich carboxy-terminus contains seven cysteine residues (the molecule has nine overall) that contribute to the biological activity of molecule by the formation of disulphide bonds that hold the homo (noggin-noggin) and heterodimers (noggin-BMP) together (Semonin et al, 2001). Noggin directly binds to BMP2 and BMP4 with a higher affinity than BMP7. Crystal structures have shown that Noggin inhibits BMP signaling by blocking the receptor interfaces of BMP binding epitopes (Groppe et al, 2002). Mutations in Noggin are associated with human disorders characterised by joint fusion (Gong et al, 1999), and knockout mouse models have demonstrated noggin expression is essential for normal skeletal development (Brunet et al, 1998).



A: Normal conditions result in a balance of BMP signaling by the opposing effects of BMP synthesis verses BMP antagonist levels.

B: Increased upstream stimulatory signals resulting in an insufficient antagonist level causing increased BMP signaling.

Figure 1.8 Model of BMP / BMP antagonist balance

(Source: Ahn et al, 2003)

Chordin

Like Noggin, chordin binds directly to BMP2 and BMP4 in the extracellular environment, inhibiting receptor engagement (Piccolo et al, 1996). It plays a major role in formation of the dorsoventral axis and in neural tissue induction, differentiation, and maintenance during gastrulation (Sasai et al, 1994). In *Xenopus*, chordin aids in the development of dorsally derived tissues by interfering with BMP signaling (Miller-Bertoglio et al, 1997).

Follistatin

Unlike Noggin and chordin, follistatin inhibits BMP signaling by forming trimeric complexes with BMP receptors (Iemura et al, 1998). It is widely expressed in a variety of embryonic and adult tissues and was originally isolated from ovarian fluid due to its ability to suppress release of follicle stimulating hormone (FSH) from the pituitary gland (Patel, 1998). Follistatin preferentially binds to Activin, but also binds to BMPs with a lower affinity (Nakamura et al, 1990).

DAN/Cerberus

The members of the Dan/Cerberus family include DAN, cerberus, gremlin, Dante, and PRDC and are all structurally related to the TGF-β superfamily cysteine knot motif (Hsu et al, 1998; Pearce et al, 1999). The mechanism of BMP inhibition by DAN, cerberus, and gremlin is similar to that of Noggin and chordin. Direct physical binding prevents correct ligand/receptor interaction, thus inhibiting signaling (Hsu et al, 1998). Dante and PRDCs mechanism of action currently remains unknown (Balemans and Van Hul, 2002).

Sclerostin

Sclerostin contains six conserved cysteine residues that make up a cysteine knot, suggesting that it binds to BMPs in a similar manner to members of the DAN/Cerberus protein family, and therefore interferes with BMP binding to receptors (Balemans and Van Hul, 2002). However, recent studies have shown that sclerostin is expressed in osteocytes of mouse and human bone and inhibits bone formation by a mechanism different from that of other BMP antagonists (van Bezooijen et al, 2004; Poole et al, 2005). Further, the BMP antagonising capability of sclerostin is limited as it is unable to prevent all BMP responses (Kusu et al, 2003).

Cell Surface Associated Signaling Molecules

Aside from the established developmental signaling pathways and epigenetic factors that control cell signaling and gene expression, there are also cell surface associated signaling molecules capable of influencing signaling. These molecules act as enhancers, repressors, or inhibitors of signaling through interactions at both the cell surface and within the intracellular environment. Cell surface associated signaling molecules include Dragon, BAMBI, and the heparan sulfate proteoglycans (HSPGs).

Dragon

Dragon, a recently discovered BMP co-receptor, acts at the cell surface to enhance BMP signaling through direct interactions with BMP2 and BMP4, but not BMP7 (Samed et al, 2005). This interaction occurs in both the presence and absence of BMP receptors and is shown to be ligand dependent as the enhancing effects of Dragon can be competed away through the administration of Noggin, a BMP antagonist. Dragon has no transmembrane domain and is anchored to the cell

membrane via a glycophosphatidylinositol (GPI) link and therefore is unlikely to directly alter the intracellular kinase activity of the BMP receptors. Dragon exerts its effects at the membrane, possibly through the stabilization of ligand-receptor complexes (Samed et al , 2005).

BAMBI

The BMP and Activin membrane bound inhibitor (BAMBI) is a transmembrane protein that influences BMP signaling through receptor interaction. Its extracellular domain is structurally related to the type I serine/threonine kinase receptors, but it lacks an intracellular domain (Onichtchouck et al, 1999; Grotewold et al, 2001). The extracellular domain of BAMBI stably associates with the serine/threonine kinase receptors of the TGF-β, Activin, and BMP families, preventing the formation of active receptor complexes, and subsequently inhibiting signaling. It is co-expressed with BMP4, a requirement for its expression, suggesting the formation of a negative feedback loop to regulate BMPs (Mariani and Harland, 1998).

Heparan Sulfate Proteoglycans (HSPGs)

Heparan sulfate proteoglycans (HSPGs) are present on almost every type of cell. A major function is to bind extracellular ligands using their heparan sulfate side chains, facilitating protein interactions and downstream events, i.e. intracellular signaling caused by enhanced formation of ligand-receptor complexes. HSPGs regulate ligand turnover and immobilize ligands, if required, by cell surface shedding, resulting in reduced ligand availability at the cell surface. HSPGs can also sequester proteins within secretory vesicles and link extracellular matrix (ECM) proteins together.

Heparan sulfate chains are targets for many effector molecules. These include: cell-cell adhesion molecules, protease inhibitors, growth factors and their receptors, degradative enzymes, extracellular matrix proteins, etc (Zimmerman and David, 1999). The HSPGs that this study will focus on are the cell surface HSPGs: the syndecan family and glypican family.

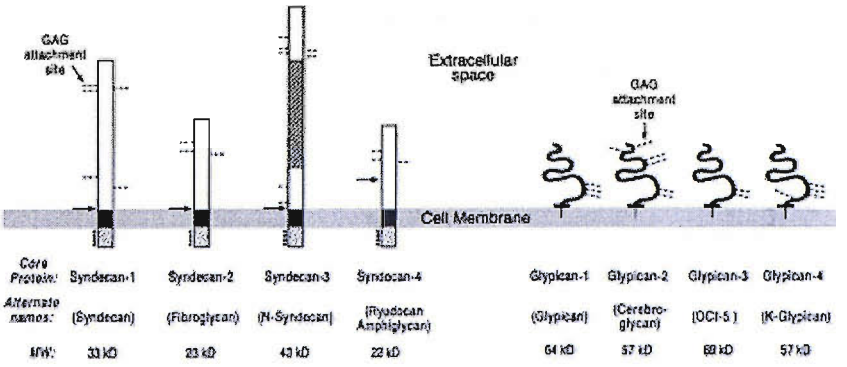


Figure 1.9: The cell surface HSPGs
(Source: Rosenberg et al, 1997)

The syndecans have a single transmembrane domain, whereas the glypicans are linked to the cell membrane via glycosylphosphoinositide molecules (Bernfield et al, 1999). The first syndecan discovered was named syndecan 1 and was identified as a type-1 transmembrane protein capable of binding components of the extracellular matrix to epithelial cells. Following this initial discovery, other structurally related syndecans were discovered as well as HSPGs that could bind directly to membrane phospholipids via glycosyl phosphatidyl inositol or GPI linkage (David et al, 1990). Due to the GPI linkage, these distinct HSPGs were named glypicans and like syndecans, other structurally related glypicans were soon discovered (Bernfield et al, 1999). Ligands bind to both syndecans and glypicans with high affinity (Bernfield et al, 1992).

Biosynthesis

The synthesis of HS (heparan sulfate) is carried out in three stages: 1) region formation resulting in an area of linkage for the HS chain to the protein, 2) polysaccharide chain generation, and 3) enzymatic modification of the chain resulting in saccharide sequences responsible for protein binding and structural organisation (Figure 1.10) (Bernfield et al, 1999). The syndecans and glypicans are known as full time proteoglycans. The occurrence of these molecules without their glycosaminoglycan (GAG) chains is only present in newly synthesised molecules to which the chain has not yet been added (Carey, 1997). The formation of the tetrasaccharide linkage region of the chain is the initial step. This linkage region is – GlcA-Gal-Gal-Xyl-Ser and is formed by xylose transfer from UDP-xylose to serine specific hydroxyl residues on the HSPGs core protein (Bernfield et al, 1999). The linkage region is identical in both heparin sulfate and chondroitin sulfate chains. The

main difference is that chondroitin sulfate chains have a higher net negative charge than the HS chains, but the HS chains have greater binding affinity for most extracellular ligands (Carey, 1997).

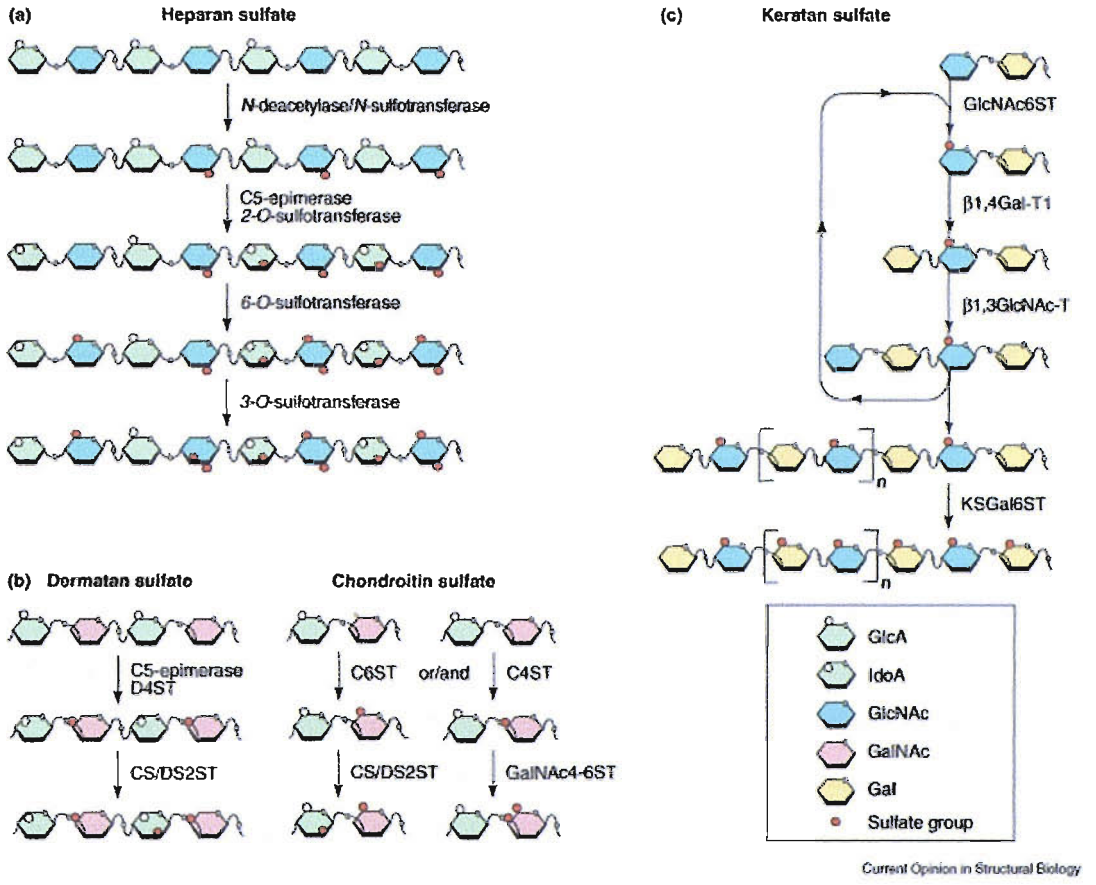


Figure 1.10: Heparan Sulfate Biosynthesis
(Source: Kusche-Gullberg and Kjellen, 2003)

Once attachment of the linkage region to the core protein has occurred, α-N-acetylglucosaminyltransferase I adds a GlcNAc (N-acetylglucosamine) moiety to the other end of the linkage region, committing the chain to HS synthesis. Chain elongation can then occur through the addition of alternating GlcNAc moieties and glucuronic acid molecules (Carey, 1997). The mechanism of chain termination or secession of growth is mostly unknown; however chain length varies according to cell type and core protein structure. The difference can be up to 10-fold, but once chain assembly is complete, it consists of 50 – 150 disaccharides, all subject to individual enzymatic modifications resulting in the product of one reaction becoming the substrate of another (Bernfield et al, 1999). Since not all of the substrate is modified, final chain structures can be quite diverse and of differing lengths. Initial modification is carried out by N-deacetylase / N-sulfotransferase replacing the GlcNAc N-acetyl group with sulfate groups on GlcNAc clusters, resulting in

modified and unmodified regions of GAG chains (Orellana et al, 1994; Aikawa and Esko, 1999). Following this modification, D-glucuronic acid residues are epimerized by glucuronyl C-5 epimerase to L-iduronic acid units and the modified disaccharides receive the bulk of the subsequent O-sulfations (Li et al, 1997; Bernfield et al, 1999).

The HSPG syndecans consist of GAG chains, a core protein containing a transmembrane domain, and a cytoplasmic domain (which contains sub-domains, C1, C2, etc). The GAG chains from different cell types that have the same core protein have structural differences, such as their O-sulfation pattern and variation in domain structure. The presence of GAG chains results in a large attractive force between the ligands and the sulfated domains that extend the entire length of the chains. The GAG chains also result in a spatial increase in the range of binding area (Carey, 1997). The core proteins that the GAG chains attach to on the cell surface are mainly the syndecans and glypicans. The proximity of GAG chains to the cell surface, expression of the GAG chains, and the GAG chain turnover rate is determined by the core proteins as the GAG chains bind to serine residues on the core proteins (Bernfield et al, 1999).

The Syndecans

The syndecans are type 1 transmembrane HSPGs, consisting of four members in vertebrates (Figure 1.9). Syndecan 1 is found mainly in epithelial cells, syndecan 2 in fibroblasts, syndecan 3 in neuronal cells, and syndecan 4 has widespread expression (Zimmerman and David, 1999). The extracellular domains of the core proteins have little activity and are divergent among the family members. Sites for shedding and surface binding have been identified on the extracellular domain (Rapraeger, 2001). The extracellular domain also contains a region for GAG chain attachment close to the N-terminus consisting of consecutive serine-glycine sequences (2-3) that are surrounded by acidic and hydrophobic residues. All syndecans can undergo proteolytic cleavage from the cell surface. Exogenous cleavage of syndecans by thrombin or plasmin occurs at a mono or dibasic sites, which are adjacent to the membrane. Endogenous cleavage of syndecans by the cell itself occurs within 5 amino acid residues of the plasma membrane (Bernfield et al, 1999).

The transmembrane domain and the cytoplasmic domain of the syndecans are highly homologous to one another, allowing them to be grouped into subfamilies (Carey, 1997; Zimmerman and David, 1999). Syndecan 2 and 4 form one subfamily,

and syndecan 1 and 3 are part of a second subfamily. The localization of membrane microdomains of syndecans may be a function performed by the transmembrane domain. This has been demonstrated in both syndecan 1 and syndecan 4, members of different subfamilies, where syndecan 1 polarizes to the basolateral epithelia cell surface and syndecan 4 localizes to focal adhesions (Rapraeger et al, 1986; Hayashi, 1987; Woods and Couchman, 1994; Bernfield et al, 1999).

The cytoplasmic region of syndecans contains three major domains denoted as C1, C2, and V. The C1 and C2 domains are two highly conserved regions that occur proximal and distal, respectively, to the membrane. These conserved regions are common to all syndecans and are separated by a V or variable region, unique to each syndecan (Zimmerman and David, 1999). The C1 region is rich in basic residues and in the syndecan 4 core protein, phosphorylation of the invariant serines of the C1 region occurs. The C1 region is homologous to the neurexin I/III C1 region. Neurexin is another type 1 membrane protein that, like the syndecans, has PDZ-binding motifs at the C-terminus and is associated with the cytoskeleton. The PDZ domains are protein – protein binding modules found in abundance among cytoskeletal and signaling proteins which bind to short carboxy-terminal motifs of 3 or 4 residues (Zimmerman and David, 1999). The homology between the C1 regions of syndecans and other type 1 membrane proteins suggests that this region is involved in cytoplasmic signaling complex formation. The V region of syndecans varies among different syndecans, but appears to have remarkable homology across species. For example, the syndecan 1 V region is identical in hamster, mouse, rat, and human, and the syndecan 2 V region is identical in *Xenopus,* human, and rat (Carey, 1997).

Functions of the Syndecans

The types of syndecans and their roles differ depending on syndecan location and the variety of effector molecules present. However, there are general features of extracellular ligand binding that must be considered. First, ligand binding syndecans is specific. Second, a large number of molecules bind to syndecans, including growth factors, cell-cell adhesion molecules, etc (Carey et al, 1997).

Syndecan 1 is involved in microfilament cross talk and cell behaviour. The changes in cell behaviour parallel the changes in syndecan 1 expression that occur during wound repair and development. In mouse mammary epithelial cells, syndecan 1 co-localizes at the basolateral surface, with intracellular microfilaments, and this

function is linked to the V region of syndecan 1 (Bernfield et al, 1999). Syndecan 1 and 4 are shed into human acute dermal wound fluid where they modify the proteolytic activity and balance of the fluid. The ectodomain of purified syndecan 1 prevents the inhibition of cathepsin G by α1-antichymotrypsin and the inhibition of elastase by α1-proteinase inhibitor (Kainulainen et al, 1998).

Syndecan 2 is involved in granular macrophage – colony stimulating factor (GM-CSF) signaling. GAG chains are bound by GM-CSF, resulting in signaling through α and β chains of a cytokine receptor. This activates kinases such as JAK2, but also results in receptor binding to syndecan 2, leading to tyrosine phosphorylation of the cytoplasmic domain. The impact of this syndecan phosphorylation remains unknown, but the formation of the receptor-ligand-syndecan complexes suggests a role for syndecan core proteins in the signaling pathway (Rapraeger, 2001).

Syndecan 3 (also known as N-Syndecan) is the largest syndecan molecule. It has a mass of 43 KDa consisting of a 405 amino acid core protein, a 33 amino acid cytoplasmic domain, and a 25 amino acid transmembrane domain. The extracellular domain is 347 amino acids with a secretory signal sequence domain at the N-terminus. HS GAG chains are potential sites for extracellular ligand interactions and the T,S,P (threonine, serine, and proline) domain, which is present only on syndecan 3, is a potential functional domain for cell-cell interactions. The T,S,P domain resembles domains present in mucin-like proteins that have O-oligosaccharides linked to the T and S residues (Figure 1.11) (Kosher, 1998).

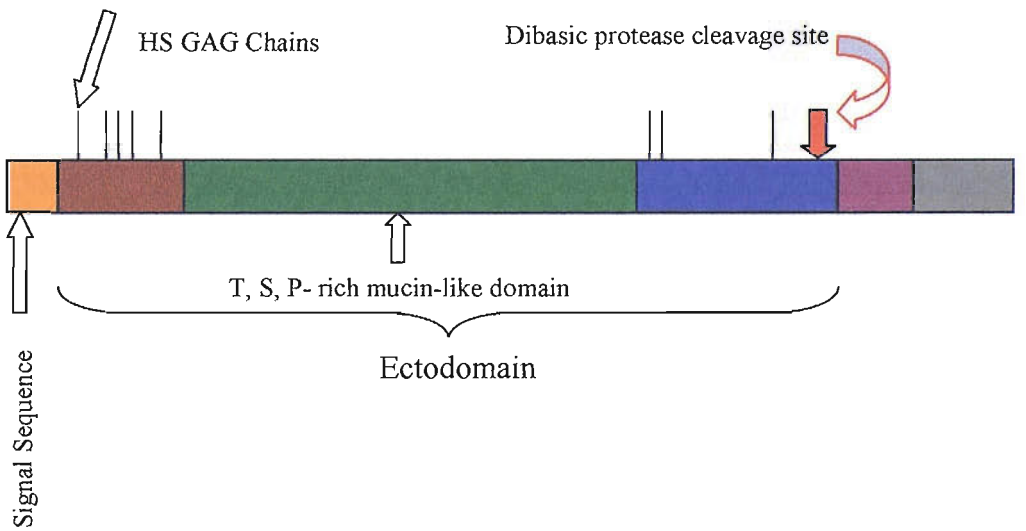


Figure 1.11: Syndecan 3
(Adapted from Kosher, 1998)

Syndecan 3 is highly expressed during the early stages of limb development. Expression is detected in distal mesenchymal cells, suggesting an interaction may occur between syndecan 3, the apical ectodermal ridge (AER), and the subridge mesoderm, all of which are involved in patterning of the limb bud. Syndecan 3 is also highly expressed during precartilage condensation of the skeletal elements, suggesting a mediatory role for syndecan 3 in cell-matrix and cell-cell interactions during the onset of chondrogenesis (Kosher, 1998). Syndecan 3 gene expression is associated with proliferating chondrocytes and the expression changes according to the topographical distribution of chondrocytes during continuous embryonic development (Kirsch et al, 2002). Increased expression of syndecan 3 at areas of pre-joint formation during limb development suggests a role for syndecan 3 in this process. Syndecan 3 is also highly expressed in proliferating chondrocytes, near the top of the growth plate, indicating a regulatory role during ossification (Kosher, 1998). It is possible that syndecan 3 plays a role in the modulation of heparin binding to signaling molecules involved in mediating the functions of the perichondrium. Signaling molecules with heparin binding domains at their N-terminus include BMP-2, 4, and 7, suggesting that HSPGs at the cell surface and in the extracellular matrix regulate BMP activity during skeletogenesis and chondrogenesis (Kosher, 1998).

Pleiotrophin (PTN) is an extracellular matrix heparin binding growth association molecule that binds to HSPG GAG chains. Syndecan 3, as well as pleiotrophin, is present at high levels in neuronal cells and both play a role in neurite outgrowth. However, PTN is also expressed in the bone matrix where it is believed to promote osteoblast recruitment and migration through interactions with syndecan 3 (Rapraeger, 2001).

Syndecan 4 is integrated into focal adhesions that contain integrins, suggesting a role in extracellular matrix adhesion signaling. It is also present in many developing organs, although most prominently in the kidneys and liver. Syndecan 4's V cytoplasmic region undergoes dimerization with PIP2 (phosphatidylinositol-4,5-bisphosphate), which allows the exposed side of the dimer to bind to the catalytic region of protein kinase Cα (Rapraeger, 2001).

The Glypicans

The glypicans are a family of GPI (glycosylphosphatidylinositol) anchored cell surface HSPGs (Table 1.3). They control growth and development by regulating

and/or modulating ligand receptor interactions at the cell surface (Fransson, 2003). The major differences between glypicans and syndecans is that glypicans do not have a transmembrane domain since they are GPI linked at the C-terminus and the GAG chains of glypicans reside much closer to the plasma membrane. Glypicans have 14 conserved cysteine residues located mainly near the N-terminus, with some in the central domain. GAG chains can be attached to the cysteine residues located in the central domain.

Glypican	Location (Embryo)	Location (Adult)
1 (Gpc1)	CNS	Ubiquitous
2 (Gpc2)	CNS	N/A
3 (Gpc3)	Ubiquitous	CNS (Reduced)
4 (Gpc4)	Neurons, Kidney	CNS (Reduced)
5 (Gpc5)	CNS, Limb Bud, Kidney	CNS
6 (Gpc6)	Liver, Heart, Kidney	Intestine, Ovary

Table 1.3: Glypican locations in embryo and adult
(Source: Adapted from Fransson, 2003)

All glypicans are 60-70 kDa in size and removal of the central domain results in a substitution of heparin sulfate to chondroitin sulfate (Chen and Lander, 2001; Fransson, 2001). There are six glypican HSPGs identified to date in mammals denoted as glypican 1-6, two glypicans in *Drosophila* known as Dally forms, and one in zebrafish called Knypek (Esko and Selleck, 2002).

The N-terminal sequence of glypicans is responsible for translocation to the endoplasmic reticulum and the C-terminal sequence is involved in initial membrane attachment and the subsequent GPI anchorage (Fransson, 2003). The GPI linkage area of the glypicans generally associates with ordered regions of the membrane. The GPI anchors mediate turnover of cell surface components by rapid endocytosis and degradation via lysosomes. However, in fibroblasts and endothelial cells, once internalisation has occurred, the proteoglycan and ligand can be recycled back to the cell surface (Bernfield et al, 1999). Figure 1.12 illustrates the GPI anchor.

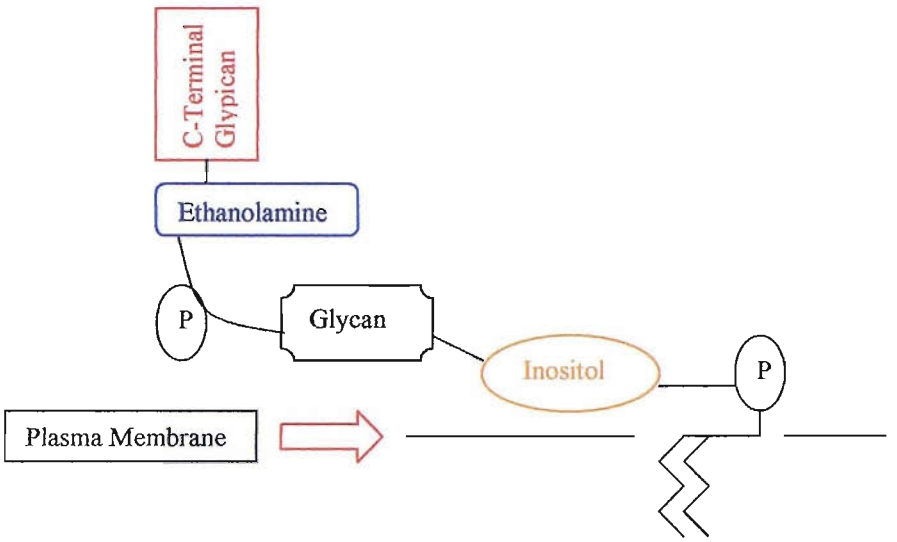


Figure 1.12: The GPI anchor of a glypican
(Source: Adapted from De Cat and David, 2001)

Functions of the Glypicans

Even though the systems in which the glypicans function are known, the underlying mechanism of performing those functions remains unknown. Glypican GAG chains can bind collagen, antithrombin, fibronectin, and fibroblast growth factor *in vitro* depending on which cell type the glypican resides on. Glypicans have large globular heads that may prevent big or insoluble molecules from accessing the GAG chains, which are present on the neck above the GPI anchor, suggesting that glypicans are capable of self regulation (De Cat and David, 2001) and perform a filtering mechanism for small molecules. It has been further suggested that glypicans are orphan receptors for dual docking ligands in which the GAG chains provide a molecular rope between ligand and receptor. Since glypicans have no direct interaction with the intracellular environment, signaling must be performed by transmembrane molecules associated with glypicans or by lipid raft clustering, resulting in endocytosis (Simons and Toomre, 2000; De Cat and David, 2001). The lipid raft clustering mechanism is still theoretical and the exact mechanism(s) by which glypicans signal remains unknown.

Syndecans Versus Glypicans

The fact that there are two distinct families of cell surface HSPGs implies that they carry out different functions. However, the establishment of distinct family specific functions remains unknown. Glypicans have cysteine rich ectodomains, which likely form globular proteins due to disulphide bond formation between cysteine residues. In contrast, syndecans have ectodomains rich in proline,

suggesting a more extended structure (Bernfield et al, 1999). The HSPG ectodomains undergo cell surface shedding by proteolytic cleavage of the core protein. The shedded syndecan ectodomains are present in body fluids, indicating a post shedding physiological role. Glypican ectodomains are found in cell culture media, but the physiological role of glypican shedding remains unclear (Subramanian et al, 1997; Penc et al, 1998; Kato et al, 1998; Bernfield et al, 1999).

The association of the GPI link of glypicans and the transmembrane domain of syndecans with the cell surface results in different roles for each HSPG on the same cell. Glypican associated internalization results in both ligand and receptor recycling, whereas syndecan internalization results in lysosomal degradation of both HSPGs and bound ligand. Similarly, signaling differences occur between the two HSPGs. Syndecans contain a cytoplasmic domain, resulting in association with signaling molecules, whereas glypicans require association proteins to signal on their behalf (Bernfield et al, 1999).

HSPGs and Disease

The importance of syndecan shedding in wound fluid has already been discussed, but HSPGs are also implicated in pathological conditions. Changes in cell morphology, differentiation, proliferation, and adhesion are all factors associated with tumorigenesis. Proteoglycans are associated with many of these processes and it has been suggested that syndecan 1 is a key factor. This is due to syndecan 1's ability, along with E-cadherin and integrins, to control cell differentiation.

The glypicans also play a role in tumorigenesis. Glypican 1 is present on pancreatic cancer cells and surrounding fibroblasts, but not present on normal pancreatic cells (Kleeff et al, 1998). The growth of cancer cells may be regulated by glypican 1 binding to growth factors such as fibroblast growth factor 2 (FGF 2).

Two tumour repressor genes, *EXT1* (Exostosin 1) and *EXT2* (Exostosin 2), are glycosyltransferases involved in HSPG biosynthesis, suggesting that GAG chains are important in maintaining normal physiological function. However, the precise role of the GAG chains in tumorogenesis remains unclear (Tumova et al, 2000). The EXT proteins are associated with HS biosynthesis, catalyzing GlcNAc and GlcA transfer to nascent chains (Zak et al, 2002). The *EXT* genes are mutated in the autosomal dominant disease hereditary multiple exostoses (HME), also known as multiple hereditary exostoses (MHE), resulting in osteochondromas (bony outgrowths with cartilaginous caps) at the growth plates of long bones. Most HME

patients have frameshift or missense mutations in *EXT1* or *EXT2,* resulting in truncated forms of the proteins. HME, along with fibrodysplasia ossificans progressiva (FOP), is on a short list of hereditary disorders involving osteochondromas, suggesting a similar pathway deficit could exist in both conditions.

Even though mutations in *EXT* genes results in HME, the exact changes that occur in HS synthesis leading to ectopic bone growth remain unknown. However, changes in HS expression around the area of exostoses could have profound effects on chondrocyte growth and differentiation. Since HSPGs act as co-receptors for growth factors such as BMP and FGF and affect signaling of Wnt and hedgehog proteins, the lack of HSPG GAG chains observed in HME could affect normal signaling, resulting in ectopic bony outgrowths (Zak et al, 2002).

Intracellular Mediators

Further modulators of BMP signaling are the intracellular mediators. These include the previously mentioned I-Smads that act through interacting with the type I receptors, preventing phosphorylation of R-Smads. Other intracellular mediators of BMP signaling include: SARA, Smurf, FKBP12, BRAM1, Ski, and Tob.

SARA

The adaptor protein smad anchor for receptor activation (SARA) facilitates the interaction of TGF-β R-Smads (Smad 2 and 3) with activated receptors by anchoring R-Smads to the early endosome and plasma membrane (Tsukazaki et al, 1998). Internalization of activated receptor complexes through clatherin coated pits results in formation of the early endosome, where SARA-bound Smads are easily accessible. This interaction can also take place prior to internalization, at the plasma membrane (Di Guglielmo et al, 2003). Although SARA does not directly interact with Smad 1, similarities between the TGF-β and BMP pathways suggests the existence of SARA-like molecules for BMP R-Smads is highly probable (Massague et al, 2005).

Smurf

Smurfs are Smad specific E3 ubiquitin ligases that interact with BMP receptor-associated Smads 1, 2, 3, and 5. Binding of smad ubiquitination regulatory

factors (Smurf1 and Smurf2) to R-Smads results in their inactivation by proteosomal degradation (Zhu et al, 1999B; Zhang et al, 2001).

FKBP12

FK506 binding protein 12 (FKBP12) was isolated in a yeast two hybrid screen to interact with the cytoplasmic domain of the type I TGF-β receptors (Wang and Donahoe, 2004). FKBP12 binds to ligand free type I receptors and is released following type II receptor mediated phosphorylation of the type I receptor, which is essential for the activation of signaling (Wang et al, 1996; Chen et al, 1997). Some studies have suggested that the inhibitory action of FKBP12 occurs through its interaction with a cytoplasmic inhibitor, such as calcineurin, rather than through direct binding to the type I receptor (Wang et al, 1996).

BRAM1

BRAM1 is a splice variant of the nuclear adenovirus E1A-associated protein BS69 (Hateboer et al, 1995). This cytoplasmic protein was identified using BMPRIA as bait in a yeast two-hybrid assay (Kurozumi et al, 1998). BRAM1 interacts with TAB1 and the cytoplasmic domain of BMPRIA, suggesting that these molecules may form a trimeric complex with BRAM1 mediating the interaction of TAB1 with BMPRIA (Kurozumi et al, 1998). No interactions of BRAM1 with BMPRII have been described. Whether BRAM1 acts as a positive or negative regulator of BMP signaling is currently unknown.

Ski

Ski is a Smad-dependent co-repressor of BMP, Activin, and TGF-β signaling. Ski directly interacts with the MH2 domain of Smads 1, 2, 3, 4, and 5, suppressing target gene activation (Berk et al, 1997; Luo et al, 1999; Wang et al, 2000). Ski's ability to inhibit BMP signaling is dependent on receptor activation, and association with R-Smads needs to occur prior to the R-Smad/co-Smad complex formation because Ski does not displace the complex (Canalis et al, 2003).

Tob

Tob has been suggested to be a negative regulator of BMP signaling in mouse osteoblasts since mice carrying Tob gene deletions have greater bone mass than wild type mice, resulting from enhanced BMP2-induced osteoblast differentiation and

proliferation (vob Bubnoff and Cho, 2001; Yoshida et al, 1997). Transcriptional activation of a BMP2 reporter gene with multiple Smad binding sites was blocked using Tob, demonstrating that Tob interacts with BMP R-Smads (Yoshida et al, 2000).

Aims and Objectives

The aim of this project was to investigate factors that affect and influence BMP and BMP-associated signaling pathways with reference to the mechanisms of bone induction in FOP. Based on the known functions of HSPGs in BMP, FGF, and hedgehog signaling, along with osteochondroma formation, the role of HSPGs in BMP signaling in FOP cells was investigated.

The objectives:

1. Assess HSPG gene expression in pre-myoblast, myoblast, and osteoblastic C2C12 cells.
2. Investigate how epigenetic factors such as hypermethylation influence BMP signaling in C2C12 cell culture using agents that affect the methylation status of the cell.
3. Examine the differences in cell surface GAG chain levels between unaffected control and FOP patient LCLs.
4. Determine the effects of soluble heparin on BMP ligands ability to induce signaling in LCLs.
5. Investigate if and how HSPGs and GPI linked cell surface proteins affect BMP signaling in control and FOP LCLs.
6. Examine HSPG mRNA and protein profiles in control and FOP LCLs and investigate how potential differences may contribute to signal dysregulation.

The overall goal of this study was to increase our understanding of association factors that can influence osteogenic differentiation, specifically DNA methylation and HSPG molecules.

Chapter 2

Effect of Demethylation and Heparinase Treatment on BMP Signaling in C2C12 Cells

Chapter 2: Effect of Demethylation and Heparinase Treatment on BMP Signaling in C2C12 Cells

Introduction

C2C12 cells are multipotent premyoblastic mouse cells that are a subclone of mouse C3H muscle myoblasts and are widely used in differentiation and signaling studies. Further, in FOP the connective tissues undergo heterotopic ossification, which includes the muscles. Therefore, C2C12 cell provide a good model to study the effects of osteoinductive factors on bone formation in muscle. In C2C12 cells, differentiation occurs rapidly, and cells will begin to form myotubes and produce muscle-specific proteins if left untreated in confluent monolayers. C2C12 cells are capable of differentiation down either the myogenic pathway (default), or in the presence of BMP2, the osteogenic pathway (Figure 2.1).

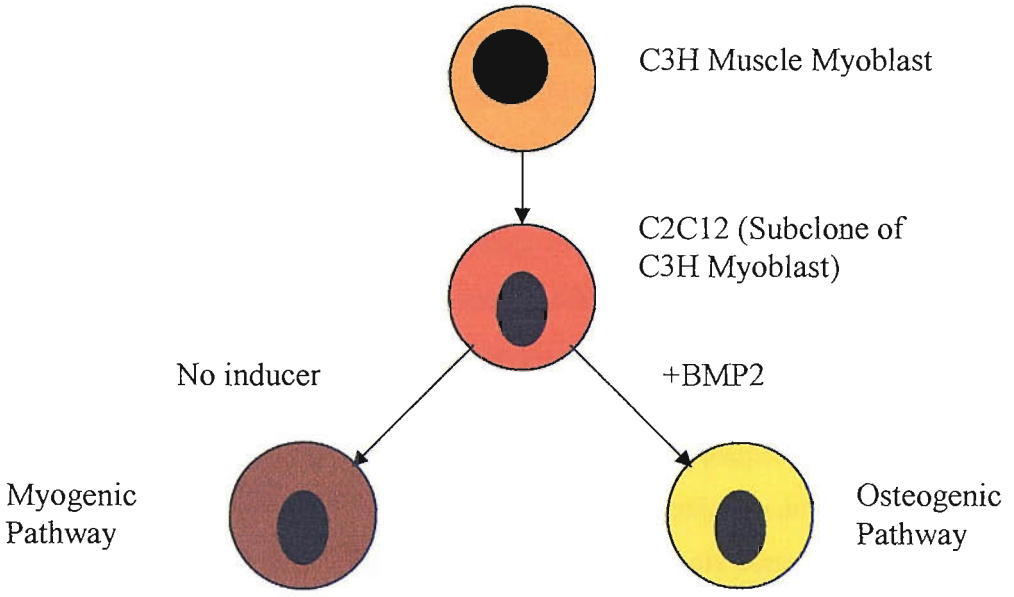


Figure 2.1: Lineage map of C2C12 cell and the effect of BMP2

Many studies have demonstrated that BMP2 treatment of C2C12 cells results in expression of osteogenic markers such as alkaline phosphatase (Hu et al, 2005; Kim et al, 2004; Rawadi et al, 2003). Stabilization of beta-catenin by the Wnt pathway results in increased alkaline phosphatase expression. However, the exact role of Wnt signaling during osteogenic differentiation remains unknown. Using microarray studies of C2C12 and MC3T3 cells following BMP2 treatment, the regulation of Wnt signaling components during late phase bone development was

investigated (Vaes et al, 2005). Expression of the Wnt antagonists, secreted frizzled related protein 2 (Sfrp2) and Wnt inhibitory factor 1 (Wif1), are upregulated during osteoblast differentiation. This indicates that Wnt expression results in a negative feedback loop essential for controlling osteoblast differentiation (Vaes et al, 2005), that Wnt is capable of pathway crosstalk with BMPs, and it provides a mechanism to downregulate early osteogenic signals.

The murine alkaline phosphatase promoter contains a Dlx-5 binding cis-acting element. Dlx-5, along with its target gene Runx2, binds to the alkaline phosphatase promoter. However, in Runx2 (-/-) cells, Dlx-5 is still capable of stimulating alkaline phosphatase activity, demonstrating that Dlx-5 functions independently of Runx2 (Kim et al, 2004). Interestingly, BMP2 stimulated alkaline phosphatase mRNA expression can be suppressed by overexpression of Msx-2, which competes with Dlx-5 for the cis-acting element in the alkaline phosphatase promoter (Kim et al, 2004). In C2C12 cells, the levels of Msx-2 are consistently high, but decrease following BMP2 treatment. This may explain why Dlx-5 is rapidly upregulated after BMP2 treatment, while alkaline phosphatase expression occurs much later. Dlx-5 may need to reach a threshold level to compete successfully for the cis-acting element.

The process of osteogenic differentiation can be influenced by a number of mechanisms. DNA methylation can influence cellular mechanisms of differentiation through control of gene expression by methylation of CpG islands in gene promoter regions. The DNA helixes major groove is the target for 5-methylcytosine (Momparler and Bovenzi, 2000) and this can interfere with transcription factor binding, a process believed to be caused by the modified cytosine.

Other factors that can influence osteogenic differentiation are the cell surface modulators heparan sulfate proteoglycans (HSPGs) which are present on most cells and are important in regulating cytokine and morphogen signaling (Cadigan, 2002; Guimond and Turnbull, 2004; Kreuger et al, 2004). HSPGs consist of a core protein with glycosaminoglycan (GAG) side chains. GAG chains interact with effector molecules including morphogens and their antagonists, cell surface adhesion molecules, protease inhibitors, growth factors, degradative enzymes, and extracellular matrix proteins (Zimmermann and David, 1999).

In this chapter, BMP signaling via Wnt was examined in C2C12 cells using alkaline phosphatase as an endpoint. First, a BMP2 dose response was performed to

determine the levels of alkaline phosphatase induction. Next, the effects of demethylation on BMP signaling in C2C12 cells that had lost their responsiveness to BMP2 were examined. Finally, a comparison of HSPG mRNA expression was performed and the effects of heparinase treatment on HSPG modulated BMP signaling was examined.

Materials and Methods

Cell Culture of C2C12s

C2C12 cell were obtained from the European Collection of Cell Cultures (ECACC). C2C12 cells were maintained at sub-confluent levels in DMEM, 10% fetal calf serum (FCS; Sigma). Cells were passaged twice a week or as needed.

C2C12 Dose Response

Cells were seeded in 24 well plates (Bibby Sterilin) in DMEM, 10% FCS at a density of $1x10^5$ cells per well until confluent (17 hours). Before treatment, cells were washed twice with PBS (Sigma) and placed in DMEM, 2% FCS. Cells were stimulated with 0-100ng/ml recombinant human BMP2. The cells were then grown for 48 hours, fixed with 95% ethanol (EtOH) for alkaline phosphatase staining, or placed in 0.05% Triton X (BDH) for alkaline phosphatase quantification.

C2C12 HSPG Gene Expression

C2C12 cells at approximately 90% confluency were left untreated (myogenic) or treated with 100ng/ml BMP2 (osteogenic) in DMEM, 2% FCS for 48 hours in a T-80 (Bibby Sterilin) tissue culture flask. After 48 hours, RNA was extracted using TRIzol (Invitrogen). RNA was also extracted from subconfluent, untreated C2C12 cells. As a positive control, mouse marrow cells (MMCs) were extracted through dissection of the femur and tibia. MMCs were cultured in ά-MEM, 10% FCS with 1% penicillin/streptomycin, 100nM dexamethasone, and 50μM ascorbic acid for 23 days with media changes at days 7, 13, and 19.

C2C12 5-aza-2-deoxycytidine Treatment

C2C12 cells that had lost their ability to differentiate following BMP2 treatment, possibly due to long term storage at -80°C or hypermethylation due to culture conditions, were treated for 48 hours with 1μM 5-aza-2-deoxycytidine (5-aza-d), a non specific demethylating agent, in DMEM, 10% FCS. Fresh media containing 5-aza-d was added after the first 24 hours. Cells were then re-seeded in 24 well plates in DMEM, 10% FCS at a density of $1x10^5$ cells per well and incubated overnight. The cells were washed twice with PBS, placed in DMEM, 2% FCS, and stimulated with 100ng/ml BMP2 for 48 hours. The cells were then fixed with 95% ethanol (EtOH) for alkaline phosphatase staining or placed in 0.05% Triton X100 for alkaline phosphatase quantification. Untreated C2C12s served as a negative control.

Long Term Effect of Demethylation

C2C12 cells were cultured in 5-aza-d as described above. However, when cells were seeded into a 24 well plate after 48 hours, some were also placed into a T-80 (P1) and grown in DMEM, 10% FCS for 6 days. The cells in the 24 well plate were immediately treated with 100ng/ml of BMP2 for 48 hours. The cells in the T80 (P1) were split into another flask (P2) to continue growing for 6 more days or placed into a 24 well plate to be treated with 100ng/ml BMP2 for 48 hours. The P2 cells were then seeded in a 24 well plate, grown overnight, placed in DMEM, 10% FCS, and stimulated with 100ng/ml BMP2 for 48 hours. The cells were then fixed with 95% ethanol (EtOH) for alkaline phosphatase staining or placed in 0.05% Triton X for alkaline phosphatase quantification.

Heparinase Treatment

C2C12 cells were seeded at a density of $1x10^5$ cells/well in DMEM, 10% FCS and incubated at 37°C, 5% CO_2 overnight (17 hours). The cells were washed twice with PBS and treated with 0.1-1 mU/ml of heparinase I, II, and III (Grampian Enzymes) in serum free DMEM for 45 minutes at 37°C. Next, cells were washed twice with PBS, placed in DMEM, 2% FCS, and stimulated with 100ng/ml BMP2 for 48 hours. The cells were then fixed with 95% ethanol (EtOH) for alkaline phosphatase staining or placed in 0.05% Triton X100 for alkaline phosphatase quantification.

Alkaline Phosphatase Stain and Assay

To stain for alkaline phosphatase, 1ml of a solution containing 9.6ml distilled water, 0.4ml Napthnol AS mix (Sigma), and 2.4µg of Fast violet B salts (Sigma) was added to C2C12 cells in 24 well plates for 1 hour at 37°C. For alkaline phosphatase quantification, cells in 0.05% Triton X100 underwent 3 freeze-thaw cycles to lyse the cell membranes and an alkaline phosphatase assay using PNPP as substrate was performed. Standards (10, 50, 100, and 200 nmol/ml) were made up using AP standard (Sigma) and assay buffer consisting of 10mls of 2-AMP alkaline buffer solution (Sigma), 20mls distilled water, and 60µl NP40 (an uncharged detergent). Using a 96 well plate, 10µl of sample, 10µl of standard, or 10µl of assay buffer (blank) were added to each well. 90µl of substrate was added to every well and the plate was incubated at 37°C for 1 hour. Next, 100µl of sodium hydroxide (NaOH)

was added to the samples and standards to terminate the reaction. The plate was then read on a spectrophotometer and DNA assays were performed. 5µl of sample in 95µl of Tris-EDTA (TE; Sigma) was added to a 96 well plate. Standards contained 0-200ng/ml DNA in TE. 100µl of PicoGreen (Molecular Probes) was added to each well, the plate was left to incubate in the dark for 3 minutes at room temperature, and it was read using a fluorometer (Bio-Tek Instruments). Alkaline phosphatase levels were then normalised to DNA content (PNPP/min/ngDNA).

RNA Extraction

RNA was extracted from C2C12 cells using the TRIzol method. 5mls of TRIzol (invitrogen) was added to a confluent T-80 tissue culture flask for 5 minutes at room temperature. The contents of the T-80 were transfered to a sterile 15ml conical tube and 0.2ml chloroform per ml of TRIzol was added. The samples were shaken vigorously for 15 seconds, left at room temperature for 2 minutes, and centrifuged at 10,000g for 15 minutes (4°C) using a Sorvall Legend RT (Kendro). The aqueous phase was transferred to a fresh sterile conical tube, precipitated with 2.5ml isopropanol, incubated at room temperature for 10 minutes, and then centrifuged at 10,000g for 15 minutes (4°C). The pellet was washed with 1ml 75% ethanol, transfered to a 1.5ml DEPC treated conical tube, centrifuged at 13,000rpm for 10 minutes in a Biofuge Fresco (Heraeus), air dried briefly, and resuspended in 100µl ultra pure water. Quantification of RNA was performed on a GeneQuant RNA/DNA calculator (Pharmacia Biotech) and RNA was diluted to 1µg/µl in ultra pure water.

RT-PCR

cDNA was made from 1µg RNA on a Eppendorf mastercycler epgradientS. 7µl ultra pure water, 1µl DNase buffer (Promega), 1µl RNA sample (1µg RNA), and 1µl DNase (Promega) was added to two 200µl PCR tubes (labelled + and -). The tubes were left at 37°C for 30 minutes and 1µl stop solution (Promega) was added to each tube. The tubes were heated to 65°C for 10 minutes, followed by cooling to 10°C. 1µl (100ng) poly-T primers (Promega) were then added, followed by heating to 70°C for 5 minutes and cooling to 10°C. 13µl of a master mix containing 1.5µl ultra pure water, 4µl 5X first-strand buffer (Invitrogen), 2µl 0.1M DTT (Di-thio-threitol, Invitrogen), 5µl 10mM dNTPs (Invitrogen), and 0.5µl RNAsin (Invitrogen) was added to each tube. 0.25µl of SuperScript II Reverse Transcriptase (Invitrogen)

was added to the + tube. The tubes were then heated to 42°C for 60 minutes, 95°C for 5 minutes, and cooled to 10°C. Primers were designed for GADPH, BMP4, Syndecan 1,2,3, and 4, and Glypican 1 using Primer select software (Table 2.1; DNAStar). The primers were then checked for thermodynamic melting temperatures (Tm), GC content, secondary structures, and palindromes using the computer software Gene Runner. Polymerase Chain Reaction (PCR) was performed to amplify the gene of interest and detect mRNA expression. The conditions for PCR were 95°C for 2 minutes, 35 cycles of 95°C for 15 seconds, varied temperatures for 15 seconds, 72°C for varied amounts of time (annealing and extension temperatures changed according to the properties of the primers), and finally 72°C for 5 minutes.

Primer	Annealing Temperature (°C)	Extension Time (seconds)
GAPDH F- ACGGCAAATTCAACGGCACAGTCA R- CATCACGCCACAGCTTTCCAGAGG	68	30
BMP4 F- CCAGGGAACCGGGCTTGAGTA R- TGGCGACGGCAGTTCTTATTCTT	63	40
GLYPICAN 1 F- GTGGCCGGAGGGTCAGCAAGAAGA R- CAGCGGGAAGAGGCAGGAGAGGAA	68	30
SYNDECAN 1 F- CCTCCCGCAAATTGTGGCTGTAAA R- CCCCGTGCGGATGAGATGTGAC	66	30
SYNDECAN 2 F- GCGGCAGCTCGGTTTCA R- GCAGGGCCCAGCTTCTCTTC	62	45
SYNDECAN 3 F- CTTGGACACAGAGGCCCCGACACC R- CGCCCACCACCCCACCCACGAT	68	30
SYNDECAN 4 F- AGCCCTCCCCGACGACGAAGAT R- ACGCCCGCCACCCACAACC	68	30

Table 2.1: Primer sequences, annealing temperatures, and extension times

Statistical Analysis

For all data, statistical analysis was performed using ANOVA and the values are considered to be significantly different at $p \leq 0.05$. Asterisks mark values at: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Results

C2C12 Cells Respond in a Dose Dependent Manner to BMP2 Treatment

To examine the response of C2C12 cells to BMP2 treatment, alkaline phosphatase activity was quantified and normalized to total DNA levels. C2C12 cells were stimulated with increasing amount of BMP2 for 48 hours and alkaline phosphatase activity was measured using p-nitro-phenol phosphate (PNPP) as a substrate. As expected, alkaline phosphatase activity increased with increasing amounts of BMP2 (Figure 2.2). A 3.5 fold induction in alkaline phosphatase activity was detected in the presence of 100ng/ml of BMP2 compared to the untreated control.

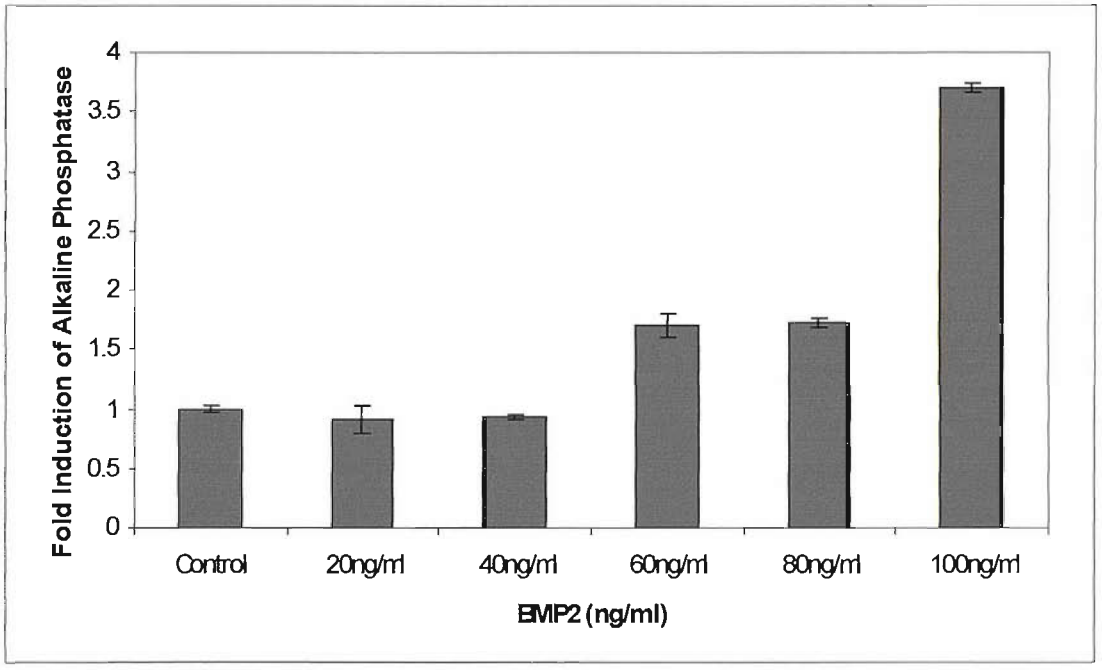


Figure 2.2: BMP2 dose response in C2C12 cells. Alkaline phosphatase induction in C2C12 cells following BMP2 treatment. Activity was determined by levels of p-nitro-phenol phosphate per minute per ng DNA (n=3, error bars-SEM).

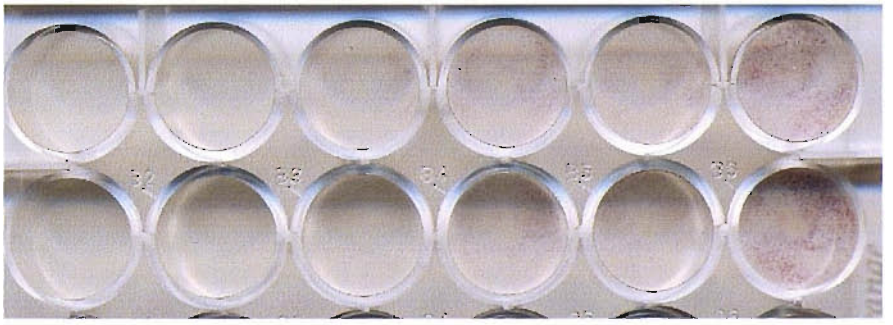


Figure 2.3: BMP2 dose response in C2C12 cells (staining). Alkaline phosphatase staining following 48 hours of BMP2 treatment (representative figure of n=3).

To confirm increased alkaline phosphatase in the presence of BMP2, C2C12 cells were treated with increasing amount of BMP2 and stained to visualize alkaline phosphatase. As observed with PNPP assays, alkaline phosphatase increased following BMP2 treatment and the levels were greatest at 100ng/ml of BMP2 (Figure 2.3).

Decreased Sensitivity of C2C12 cells to BMP2 is due to Methylation of the DNA

Our laboratory identified a C2C12 cell line that lost the ability to differentiate down the osteogenic lineage following BMP2 treatment (Figure 2.4). One hypothesis for this observation was that hypermethylation of the DNA prevented osteogenic transcription factor binding. Alkaline phosphatase activity is required for osteogenic differentiation and "silencing" of this gene due to hypermethylation could prevent differentiation. To address this, C2C12 cells were pre-treated with 5-aza-d, a non specific demethylating agent, for 24 or 48 hours, followed by BMP2 stimulation for 48 hours, and stained for alkaline phosphatase.

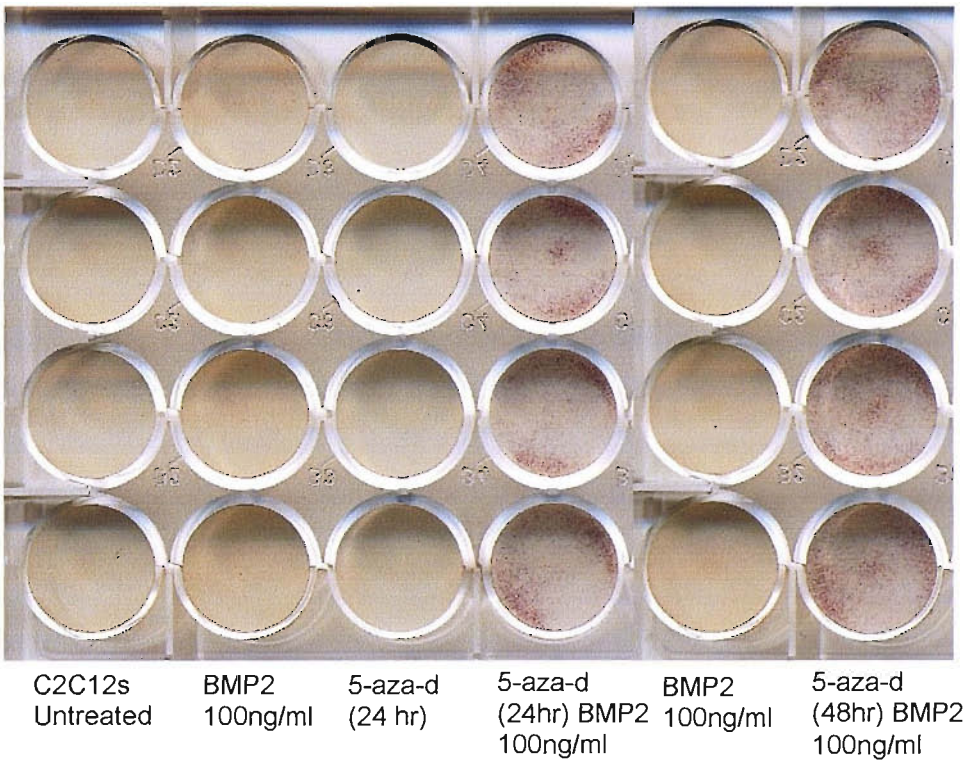
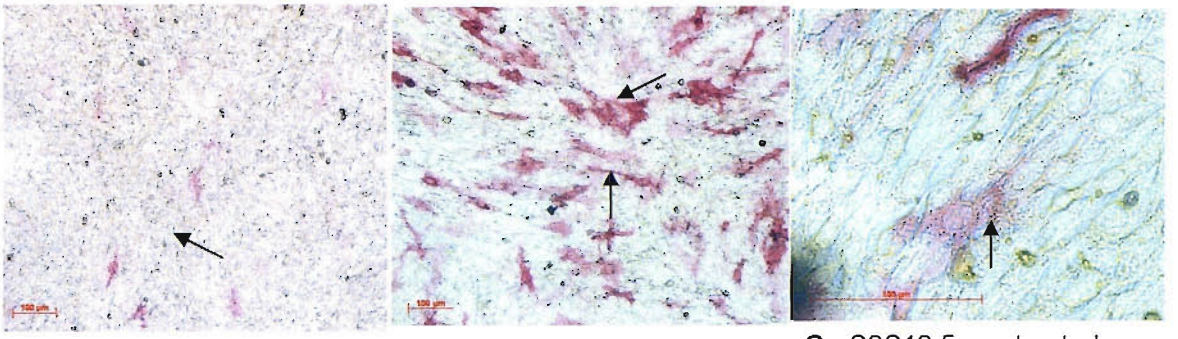


Figure 2.4: 5-aza-d treatment of C2C12 cells. C2C12 cells were treated with 5-aza-d for 24 or 48 hours, followed by BMP2 stimulation for 48 hours. Untreated cells and 5- aza-d alone served as controls (representative figure of n=3).

Alkaline phosphatase staining was greatly increased in cells treated with 5-aza-d and BMP2 compared to untreated cells, cells treated with BMP2 alone, and

cells treated with 5-aza-d alone (Figure 2.4). In addition, alkaline phosphatase staining was more intense after 48 hours of treatment with 5-aza-d, suggesting that the inability of these cells to differentiate down the osteogenic pathway may be due to DNA methylation of the promoter of alkaline phosphatase.



A - C2C12 BMP2 100ng/ml x10 objective magnification

B - C2C12 5-aza-d treated (24 hours) BMP2 100ng/ml x10 objective magnification

C - C2C12 5-aza treated BMP2 100ng/ml x40 objective magnification

Figure 2.5: Microscopic magnification of alkaline phosphatase stained 5-aza-d treated C2C12 cells. A)Magnification of C2C12 cells treated with BMP2. B) Magnification of C2C12 cells treated with 5-aza-d and BMP2. C) Magnification of C2C12 cells treated with 5-aza-d and BMP2.

Alkaline phosphatase staining was further examined using an inverted light microscope. Figure 2.5A has very little alkaline phosphatase staining, demonstrating that these C2C12 cells do not respond significantly to BMP2 treatment. Figure 2.5B and C show that 5-aza-d treated C2C12 cells have increased alkaline phosphatase activity.

To quantify the alkaline phosphatase activity of 5-aza-d treated cells, PNPP assays were performed and alkaline phosphatase levels were normalized to DNA content. Alkaline phosphatase activity was significantly increased (120-fold) in the 5-aza-d and BMP2 treated cells compared to cells treated with only BMP2. The level of alkaline phosphatase produced in cells treated with BMP2 was statistically significant compared to untreated controls (Figure 2.6).

To determine if 5-aza-d treatment is sustained following removal of the enzyme, a time course was performed. C2C12 cells were treated with 5-aza-d for 48 hours followed by BMP2 stimulation for 48 hours on days 0 (after 48 hours of 5-aza-d treatment), 6, and 12. Alkaline phosphatase activity was measured using a PNPP assay.

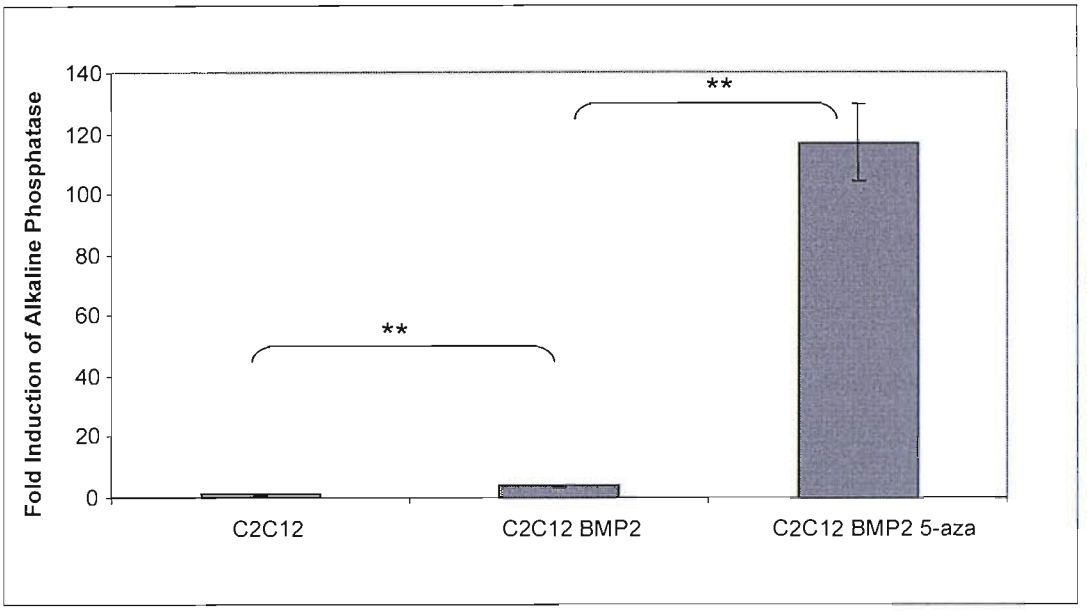


Figure 2.6: Quantification of alkaline phosphatase in 5-aza-d and BMP2 treated C2C12 cells. Cells were treated with 5-aza-d for 48 hours and subsequently treated with BMP2 (100ng/ml) for 48 hours. Alkaline phosphatase production was measured by levels of p-nitro-phenol phosphate per minute per ng DNA and normalized to the untreated control (n=4 error bars-SEM, ***p<0.001).

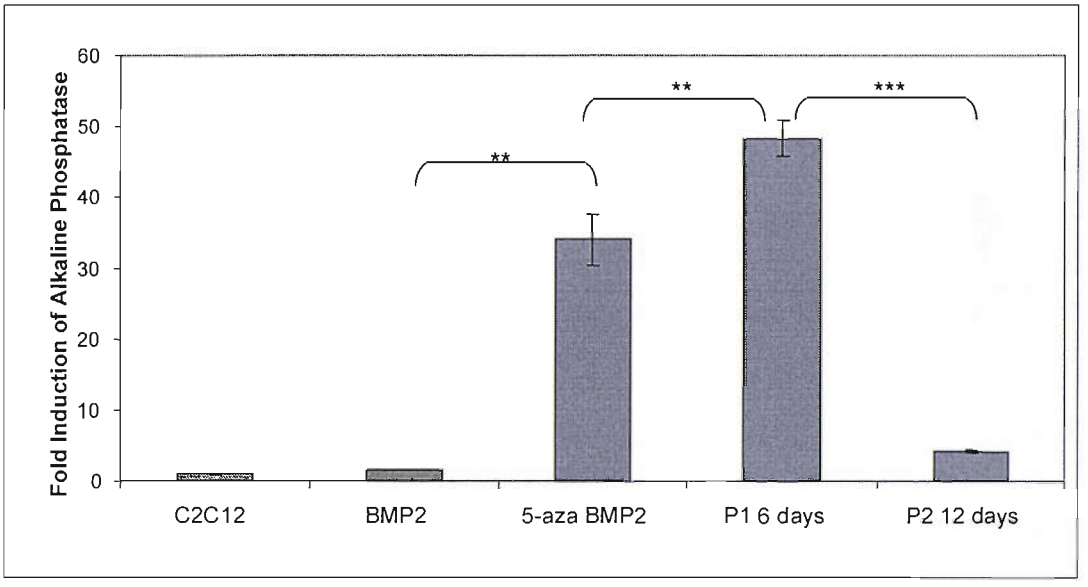


Figure 2.7: The lasting effects of 5-aza-d treatment on C2C12 cells. Cells were treated with 5-aza-d for 48 hours followed by BMP2 (100ng/ml) stimulation for 48 hours on days 0, 6, and 12. Alkaline phosphatase production was measured by levels of p-nitro-phenol phosphate per minute per ng DNA and normalized to the untreated control (n=4 error bars-SEM, *p<0.05, ***p<0.001).

Following 5-aza-d and BMP2 treatment, alkaline phosphatase was induced 35-fold over the untreated control (Figure 2.7). After 6 days of culture, in the absence of 5-aza-d, the induction of alkaline phosphatase was approximately 45-fold greater

than the untreated control. However, after 12 days in the absence of the enzyme, the induction was only 5-fold greater than the untreated control. This reduction was statistically significant compared to day 6. These results demonstrate that DNA is readily demethylated by 5-aza-d, and indicate once the enzyme is removed, it is remethylated after 12 days.

Removal of GAG Chains Decreases Alkaline Phosphatase

In addition to patterns of DNA methylation, osteogenic differentiation can be affected by cell signaling associated molecules. Heparan sulfate proteoglycans (HSPGs) are cell surface molecules that can affect BMP signaling through BMP binding sites on their glycosaminoglycan (GAG) side chains. To determine the role HSPGs play in osteogenic differentiation, GAG chain removal was performed by enzymatic digestion.

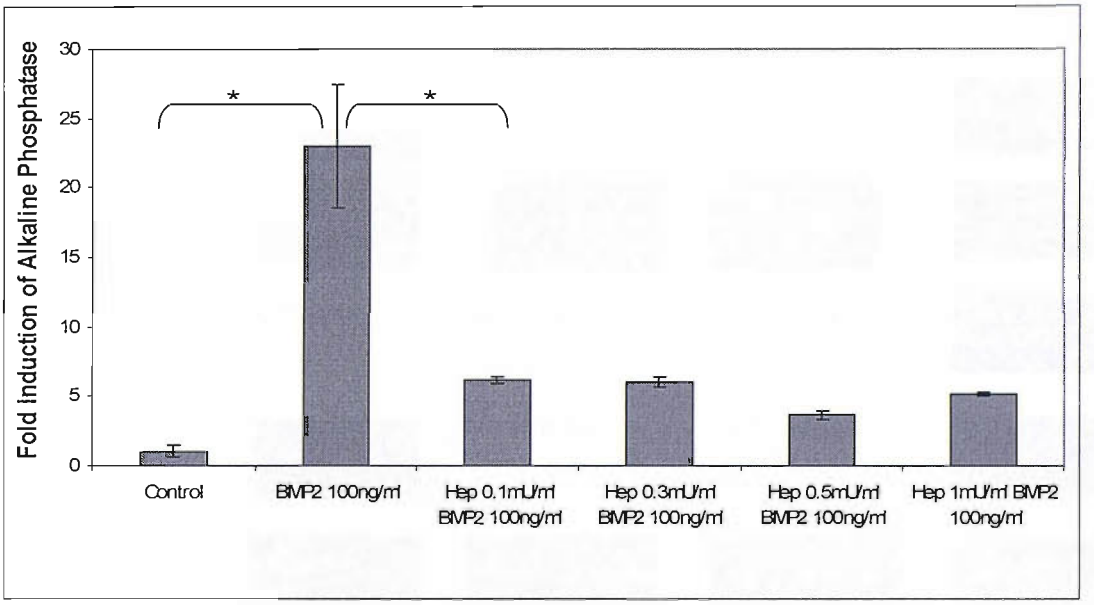


Figure 2.8: The effect of heparinase treatment on C2C12 cells. Cells were treated with increasing amounts of heparinase I, II, and III for 45 minutes and subsequently treated with BMP2 for 48 hours. Alkaline phosphatase was then measured by levels of p-nitro-phenol phosphate per minute per ng DNA and normalized to the untreated control (n=3 error bars-SEM, *$p<0.05$).

A cocktail of heparinase I, II, and III was used to digest the GAG chains of C2C12 cells. Heparinase I degrades highly sulfated regions while heparinase II and III degrade areas with little or no sulfation. Following heparinase treatment, C2C12 cells were treated with BMP2 for 48 hours and alkaline phosphatase levels were assessed by PNPP assays. Heparinase digestion resulted in a significant decrease in alkaline phosphatase production in BMP2 stimulated C2C12 cells (Figure 2.8),

suggesting that HSPG GAG chains play a critical role in BMP induction of alkaline phosphatase and osteogenic differentiation.

HSPG mRNA Expression Changes as C2C12 Cell Differentiate

Since HSPGs play a role in osteogenic differentiation in C2C12 cells, HSPG mRNAs were examined to determine whether HSPG gene expression changes at different stages of differentiation (Table 2.2). Total RNA was extracted and cDNA synthesized from undifferentiated (subconfluent), myogenic (48 hours post confluency), or osteogenic (48 hours of BMP2 stimulation) C2C12 cells. PCR was performed for BMP4, glypican 1, syndecan 2, syndecan 3, and syndecan 4 to demonstrate their presence.

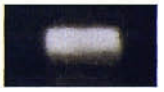
	MMC (+ control)	C2C12 Undifferentiated	C2C12 Myogenic	C2C12 Osteogenic
BMP4 633bp		Not Tested	Not Tested	
Glypican1 565bp				
Syndecan 2 729bp	Not Tested	No Product	No Product	
Syndecan 3 473bp		No Product	No Product	
Syndecan 4 531bp				
GAPDH				

Table 2.2: HSPG mRNA expression in C2C12 cells. Total RNA was extracted from undifferentiated, myogenic, or osteogenic C2C12 cells. cDNA was synthesized and PCR for BMP4 and various HSPGs was performed. Mouse marrow cells (MMC) served as positive controls.

Syndecan 2 and syndecan 3 mRNAs were upregulated in osteoblastic C2C12 cells, but not in undifferentiated or myogenic cells, suggesting that these HSPGs play a role in bone formation. Syndecan 4 and glypican 1 mRNAs were present in undifferentiated, myoblastic, and osteoblastic C2C12 cells, suggesting a ubiquitous role for these HSPGs.

Discussion

These studies demonstrated that C2C12 cells produce increased levels of alkaline phosphatase following BMP2 treatment in a dose-dependent manner. In C2C12 cells that had lost their responsiveness to BMP2, treatment with a non-specific demethylating agent (5-aza-d) restored BMP responsiveness, as measured by alkaline phosphatase induction. Further, the effect of 5-aza-d treatment was sustained over 12 days following removal of the enzyme. To further study factors that affect the osteogenic potential of C2C12 cells, HSPG GAG chains were removed by heparinase digestion. This resulted in decreased BMP2 induced alkaline phosphatase expression, suggesting that HSPG GAG chains are important for osteogenic differentiation. Further examination demonstrated that C2C12 cell surface HSPG expression profiles change as the cells begin to differentiate.

Alkaline phosphatase levels increase in BMP2 stimulated C2C12 cells following treatment with 5-aza-d, suggesting that osteogenesis can be inhibited by the methylation status of the DNA and that removal of abnormal hypermethylation may permit osteogenic differentiation. The alkaline phosphatase gene may be hypermethylated in C2C12 cells that lost their responsiveness to BMP2 due to culture conditions. Once 5-aza-d has been removed, the cells upregulate alkaline phosphatase activity for approximately 12 days, after which time they are capable of remethylation, restoring their inability to respond to BMP. Studies in MG63 cells (a human osteoblastic cell line) treated with 5-azacytidine (5azaC), another demethylating agent, demonstrated induced alkaline phosphatase expression that was augmented by the addition of glucocorticoids (Locklin et al, 1998). These studies demonstrated another cell line that requires demethylation for alkaline phosphatase induction and osteogenic differentiation. However, other cells, such as human bone marrow fibroblasts (osteogenic progenitor cells), do not require 5azaC for alkaline phosphatase expression. Glucocorticoid treatment alone stimulated osteogenic differentiation. Interestingly, osteocalcin expression was also unaffected by 5azaC treatment, suggesting that osteocalcin and alkaline phosphatase are unaffected by DNA methylation in these cells (Locklin et al, 1998).

In addition to osteogenic markers, methylation status also influences BMP expression. BMP6 overexpression is associated with prostate cancer and some evidence suggests that it is also associated with bone forming skeletal metastasis (Tamada et al, 2001; Wang et al, 2006). The Sp 1 site of the BMP6 promoter contains a CpG island and selective demethylation of that island results in increased

BMP6 expression. Our results demonstrate that BMP4 mRNA is expressed is osteogenic C2C12 cells at similar levels to control mouse marrow cells, however it is possible that BMP4 undergoes methylation in undifferentiated or myogenic C2C12 cells.

In addition to the methylation status of DNA, cell surface modulators of BMP signaling, such as HSPGs, affect osteogenic differentiation. The expression of HSPG mRNAs were characterized following treatment with BMP2 to observe any changes following differentiation of C2C12 cells. Interestingly, upregulation of syndecan 2 and syndecan 3 was observed in osteogenic C2C12 cells, with no differences in the expression of syndecan 4 or glypican 1. This is in agreement with the observation that BMP2 produces an augmentation in the synthesis of syndecan 2, syndecan 4, perlecan, and decorin in C2C12 cells (Gutierrez et al, 2006). Further, decorin expresses larger GAG chains in BMP2 stimulated cells compared to untreated cells. These results suggest that HSPG profiles change during differentiation, and the length of GAG chains may also vary. The extension of GAG chains may have significant physiological consequences since GAG chains play multiple roles in morphogen delivery and transport along the cell surface.

While these studies use alkaline phosphatase as an indicator of BMP pathway activity, alkaline phosphatase activity is mediated through the Wnt pathway. This has been demonstrated in studies using lithium chloride (LiCl) to stabilize β-catenin in C3H10T1/2 osteogenic cells (Bain et al, 2003). The addition of LiCl resulted in increased alkaline phosphatase expression, but not osteocalcin expression, which is observed with BMP2 treatment. LiCl acts as a GSK-3β inhibitor, preventing phosphorylation of β-catenin and allowing its translocation to the nucleus rather than degradation by ubiquitin. However, due to crosstalk between Wnt and BMP signaling cascades, alkaline phosphatase activity has been used to examine both pathways.

These studies have shown that both methylation status of osteogenic markers and the expression of cell surface heparan sulfate proteoglycans are important for alkaline phosphatase activity and osteogenic differentiation in C2C12 cells.

Chapter 3

Effect of Enzymatic Digestion of HSPGs on BMP Signaling in LCLs

Chapter 3: Effect of Enzymatic Digestion of HSPGs on BMP Signaling in LCLs

Introduction

ID proteins are a family of molecules that act as positive or negative regulators of cell proliferation and cell differentiation respectively. These proteins play a role in many different cellular processes including cell signaling, angiogenesis, neurogenesis, and tumor vascularization (Lyden et al, 1999; Maeda et al, 2004). Over-expression of ID proteins is associated with a block or stimulation (Norton et al, 1998; Ogata et al, 1993; Neuman et al, 1993; Voronova and Lee, 1994; Deed et al, 1998) of differentiation (Heemskerk et al, 1997; Martinsen and Bronnes-Fraser et al, 1998; Katagiri et al, 1994) in different cell contexts.

ID genes encode for helix-loop-helix (HLH) proteins that lack a basic region and do not bind DNA. However, they are capable of binding and sequestering other basic helix-loop-helix (bHLH) proteins by associating with ubiquitous E proteins, preventing their binding to DNA and therefore inhibiting transcription in a dominant negative manner (Katagiri et al, 2002). They also associate with the Rb family of tumor suppressor proteins and Ets transcription factors (Ouyang et al, 2002; Yates et al, 1999). Further, they are direct downstream targets of TGF-β and BMP signaling pathways (Ruzinova and Benezra, 2003). Interestingly, Twist and Snail, downstream targets of FGF signaling, can be inhibited by ID proteins, suggesting that FGF and BMP signaling can regulate development antagonistically. This method of regulation has been demonstrated in tooth and palate development (Rice et al, 2005). ID gene expression is induced in lymphoblastoid cells (LCLs) following BMP4 treatment and the levels are elevated in FOP cells compared to control cells (de la Pena et al, 2005; Fiori et al, 2006).

As previously mentioned, HSPGs are capable of influencing signaling and subsequently differentiation through interactions with their GAG side chains. HSPG GAG chains bind members of the BMP superfamily, including BMP2, 4, and 7, and the BMP antagonist, Noggin (Zimmermann and David, 1999; Paine-Saunders et al, 2002). Following HSPG binding, Noggin remains functionally active and capable of binding BMPs at the cell surface (Paine-Saunders et al, 2002). Binding of Noggin to the cell surface is highly selective for heparan sulfate, requiring specific structural motifs for this interaction (Viviano et al, 2004).

HSPGs have multiple roles in lymphocytes, specifically B cells. Activation of B cell antigen receptor (BCR) and CD40 results in a strong transient expression of HSPGs on human tonsillar B cells. Furthermore, HSPGs act as functional co-receptors, promoting cytokine signaling in B cells, suggesting a dynamic role for HSPGs in B cell differentiation (van der Voort et al, 2000). HSPGs have also been shown to influence B cell chemokine (C-X-C motif) ligand 1 (CXCL1), paired box transcription factor (PAX), and extracellular regulated kinase (ERK) signaling (Wang et al, 2003).

In this chapter, the kinetics of ID1 and ID3 gene expression in LCLs were examined. Confirmation of previously reported elevated ID gene expression in FOP cells was performed (de la Pena et al, 2005; Fiori et al, 2006). We investigated the effects of exogenous heparin on BMP signaling and the role of GAG chains on control and FOP LCLs following BMP4 stimulation. Further, the effects of GPI-linked protein removal on BMP signaling in control and FOP LCLs were also investigated.

Materials and Methods

EBV Transformation of Lymphocytes

Blood samples were obtained from FOP patients and unaffected individuals with informed consent and with Institutional Review Board approval in accordance with institutional guidelines. Lymphoblastoid cells lines (LCLs) were established from peripheral blood mononuclear cells by transformation using Epstein-Barr virus (EBV). Blood samples with diluted 1:1 in Roswell Park Memorial Institute 1640 (RPMI; Invitrogen) media and placed over a Ficoll (Amersham) Paque. Samples were centrifuged at 1600 rpm for 45 minutes at room temperature and the buffy coat (cell layer at the border of the ficoll and blood plasma) was collected. Next, the buffy coat was placed into a conical tube and the total volume was brought up to 15mls using Hanks balanced salt solution (HBSS; Invitrogen). Cells were washed twice with HBSS and pelleted at 1600 rpm for 10 minutes, resuspended in 3.5mls RPMI containing 20% FCS (HyCloan), 2% L-glutamine (Invitrogen), and 1% Antibiotic-Antimycotic (Invitrogen). The lymphocyte suspension was then added to 1ml of EBV stock (50mls of supernatant from cultured Marmocet cells and 50μg cyclosporine (Sigma)). The EBV cell suspension was placed into an upright T-25 and incubated for 3-4 weeks at 37°C, 5%CO_2. Media was replaced as necessary and transformation was considered complete when clumps of cells were visible.

Cell culture of LCLs

LCLs were passaged twice a week in RPMI, 15% FCS, and 1% Antibiotic-Antimycotic. Cells were split 1:4 and kept at 37°C, 5% CO_2. Prior to treatments, 2-4x10^6 cells were washed using either phosphate buffered saline (PBS; Invitrogen) or HBSS.

LCL Dose Response and Time Course

BMP dose response and time course experiments using control LCLs were performed. Cells were treated with 0-400ng/ml BMP4 (R&D) in RPMI, 1.5% FCS for 1.5 hours at 37°C, 5% CO_2. For the time course, cells were treated with 200ng/ml BMP4 in RPMI, 1.5% FCS for 0-240 minutes. RNA was extracted, cDNA synthesized, and ID gene expression was analyzed by real time PCR.

Heparin and Enzymatic Treatments

Heparin competition treatment was performed on control cells. LCLs were pre-treated with 0.1, 1, and 10μg/ml of heparin (Sigma) in RPMI, 1.5% FCS and incubated at 37°C, 5% CO_2 for 30 minutes. 200ng/ml of BMP4 was then added and samples were incubated for 1.5 hours at 37°C, 5% CO_2.

Heparinase III treatment was used to remove HSPG-specific GAG chains on control and FOP cells. LCLs were pre-treated with 5mu/ml (IU) heparinase III (Sigma) in serum free RPMI for 2 hours at 37°C, 5% CO_2. Next, cells were washed with PBS and treated with 200ng/ml BMP4 in RPMI, 1.5% for 1.5 hours 37°C, 5% CO_2.

Phosphatidylinositol phospholipase C (PI-PLC) treatment was performed to remove all GPI-linked protein on the cell surface. Control and FOP LCLs were treated with 1U/ml of PI-PLC (Sigma) in serum free RPMI for 2 hours. Cells were then washed with PBS and subsequently treated with 200ng/ml BMP4 for 1.5 hours at 37°C, 5% CO_2. For all treatments, RNA was extracted, cDNA synthesized and ID gene expression was analyzed by real time PCR.

RNA Extraction

For RNA extraction, cells were washed and pelleted at 1500rpm for 5 minutes. Total RNA was extracted using the TRIzol method. 1ml TRIzol (Invitrogen) was added to each sample followed by 200μl chloroform (Fisher). Samples were mixed and left at room temperature for 3 minutes. They were spun at 12,000g for 15 minutes at 4°C and the aqueous phase was transferred to a fresh tube. 1.5μl glycogen carrier and 500μl isopropanol (Fisher) was then added and samples were left at room temperature for 10 minutes. Samples were then spun at 12,000g for 10 minutes at 4°C, supernatant was removed, and the pellet was washed in 75% EtOH in DEPC treated water (Fisher). Next, samples were pelleted at 7,500g for 5 minutes at 4°C, the supernatant was removed, and pellets air dried for 5 minutes. The pellets were resuspended in DEPC treated water and heated to 55°C for 10 minutes. The total amount of RNA was calculated using a photospectometer. All samples were DNase (Invitrogen) treated prior to cDNA synthesis.

cDNA Synthesis and Real Time PCR

Briefly, 5μg of RNA in DEPC treated H_2O (Fisher), 2μl of random primers (Invitrogen), and 1μl of 0.1mM dNTPs (Invitrogen) were brought up to a total

volume of 12µl with DEPC treated H_2O. Samples were heated to 65°C for 5 minutes, then reduced to 4°C in a thermal cycler (MJ Research PTC-200). Next, 4µl of 5x first strand buffer (Invitrogen), 2µl of 0.1M DTT (Invitrogen), and 1µl of superscript reverse transcriptase II (Invitrogen) was added and the samples were heated on a thermal cycler to 25°C 10 minutes, 42°C 50 minutes, and returned to 4°C.

Primers were designed for human GAPDH, ID1, and ID3 on Primer Express (Table 3.1; Applied Biosystems). Gene expression was quantified using real time PCR and values were normalized to GAPDH mRNA. All PCR reactions were performed in triplicate. Reactions contained 12.5µl of SYBR green, 7µl of distilled water, 3µl of primers (1.5µl of each; final concentration 50 nmols), and 2.5µl of template cDNA (1:20 dilution of cDNA, made from 5µg RNA) in each well. For each gene, standard curves and no template controls (NTCs) were run in triplicate. An ABI Prism 7000 sequence detection system (Applied Biosystems) was used for 40 stage real time PCR. Samples were run at 50°C for 2 minutes, 95°C for 10 minutes to denature, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Values are expressed as mean fold induction +/- standards error of the mean (SEM) and are results obtained from at least three independent experiments.

Primer	Sequence
ID1	F – GGTGGAGATTCTCCAGCACG R – TCCAACTGAAGGTCCCTGATG
ID3	F – TTCCCATCCAGACAGCCG R – GCGTTCTGGAGGTGTCAGGA
GAPDH	F – AGATCATCAGCAATGCCTCC R – ATGGCATGGACTGTGGTCATG

Table 3.1: Primer sequences

Statistical Analysis

For all data, statistical analysis was performed using ANOVA and the values are considered to be significantly different at $p \leq 0.05$. Asterisks mark values at: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Results

Induction of ID1 and ID3 Occurs in a Dose Dependent Manner in LCLs

To confirm that lymphoblastiod cell lines (LCLs) respond to BMP4 by upregulating ID1 and ID3 mRNA, a BMP4 dose response was performed. Control cells were treated with increasing amounts of BMP4 and the BMP early response genes ID1 and ID3 were quantified by real time PCR. LCLs respond to BMP4 in a dose dependent manner, demonstrating a statistically significant (20-fold) induction of ID1 at 200ng/ml BMP4 compared with untreated controls (Figure 3.1).

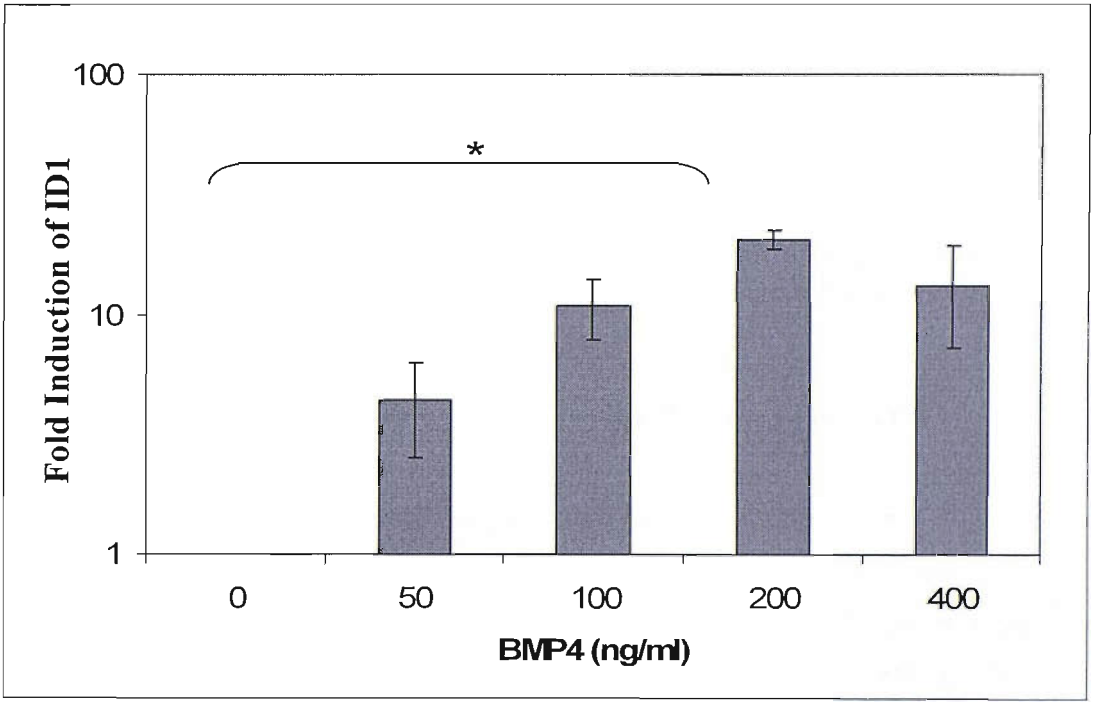


Figure 3.1: ID1 dose response in control LCLs. Fold induction of ID1 following stimulation with increasing amounts of BMP4 (1.5hrs). ID1 mRNA expression was quantified by real time PCR and normalized to levels of GAPDH mRNA. (n=3 error bars-SEM *p<0.05).

Similar patterns of induction were observed with ID3 mRNA levels in LCLs (Figure 3.2). Interestingly, the levels of induction of ID3 are lower than ID1, demonstrating that ID1 is a more inducible gene in this cell system. ID3 was upregulated 5-fold at 200ng/ml of BMP4 stimulation compared to untreated controls, however ID3 levels are significantly increased at 100ng/ml of BMP4 treatment.

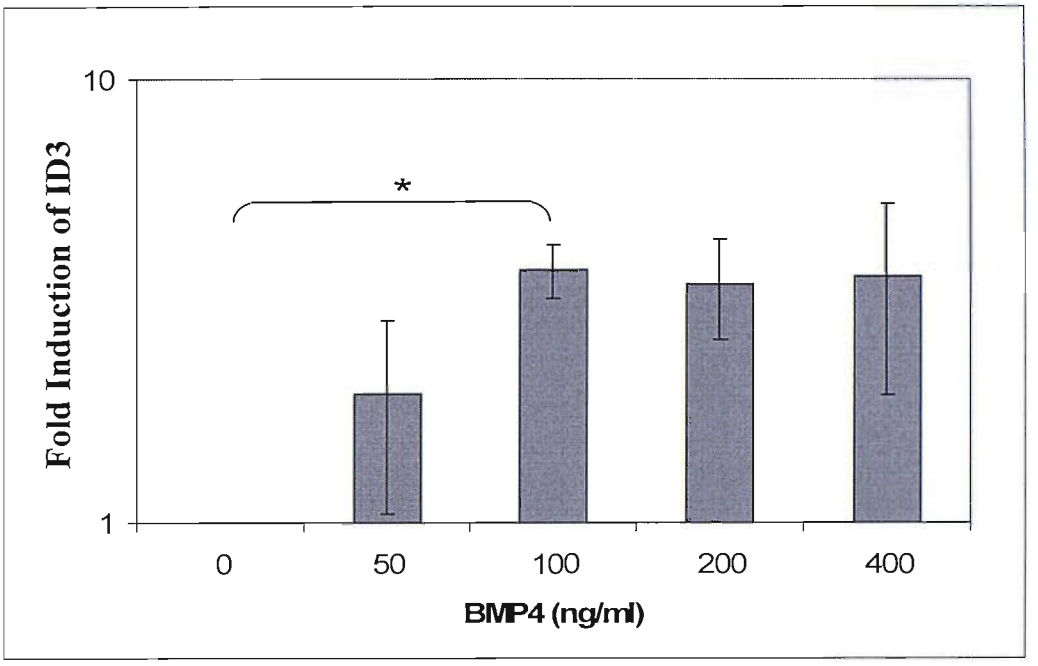


Figure 3.2: ID3 dose response in control LCLs. Fold induction of ID3 following stimulation with increasing amounts of BMP4 (1.5hrs). ID3 mRNA expression was quantified by real time PCR and normalized to levels of GAPDH mRNA. (n=3 error bars-SEM *$p<0.05$).

ID1 and ID3 are Upregulated in a Time Dependent Manner in Response to BMP4

Since ID1 and ID3 are BMP early response genes, their expression diminishes over time. To investigate the kinetics of ID1 and ID3 induction, time course experiments were performed. ID1 and ID3 induction is greatest between 1 and 2 hours of BMP4 treatment. A statistically significant decrease for both ID1 and ID3 can be seen following 4 hours of BMP4 treatment when compared to 2 hours (Figure 3.3). This suggests that ID1 and ID3 are only induced for a short period of time, reinforcing their roles as BMP early response genes in our cell system. Further, basal levels of ID1 are significantly lower than ID3 (0 time point), despite greater induction levels of ID1 than ID3 following BMP4 treatment (120 time point).

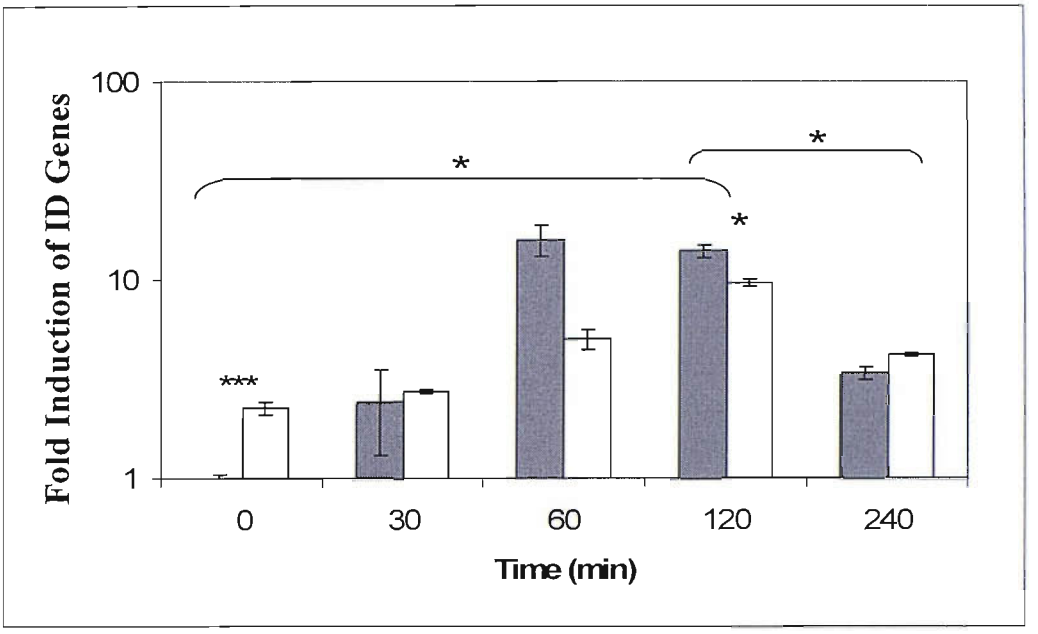


Figure 3.3: ID time course in control LCLs. Fold induction of ID1 and ID3 following BMP4 (200ng/ml) treatment over increasing periods of time. ID1 and ID3 mRNA expression was quantified by real time PCR and normalized to levels of GAPDH mRNA (n=3 error bars-SEM *$p<0.05$, ***$p<0.001$).

Heparin Suppresses ID Induction

In order to investigate the role of HSPG GAG chains in modulating BMP signaling, BMP ligands ability to interact with heparan was assessed. Heparin, a chemical analog of heparan, was used to demonstrate modulation of BMP4 induced ID1 and ID3 expression. Heparin competition assays were performed and ID gene expression was analyzed by real time PCR. Cells were treated with heparin for 30 minutes and subsequently stimulated with BMP4 for 1.5 hours. Both ID1 and ID3 induction was significantly reduced following heparin pre-treatment. The fold induction of ID1 was decreased by approximately 0.5-fold following treatment with 0.1μg/ml heparin compared to the BMP4 treated control (Figure 3.4). Similar results were observed for ID3, with an inhibition of approximately 0.5-fold (Figure 3.5). As heparin concentration increased, ID induction decreased, demonstrating a dose dependent inhibition of these genes. These data suggests that heparin interferes with BMP4 ligand-receptor interactions, possibly through competitive inhibition, and that ID1 and ID3 induction is dependent on receptor engagement.

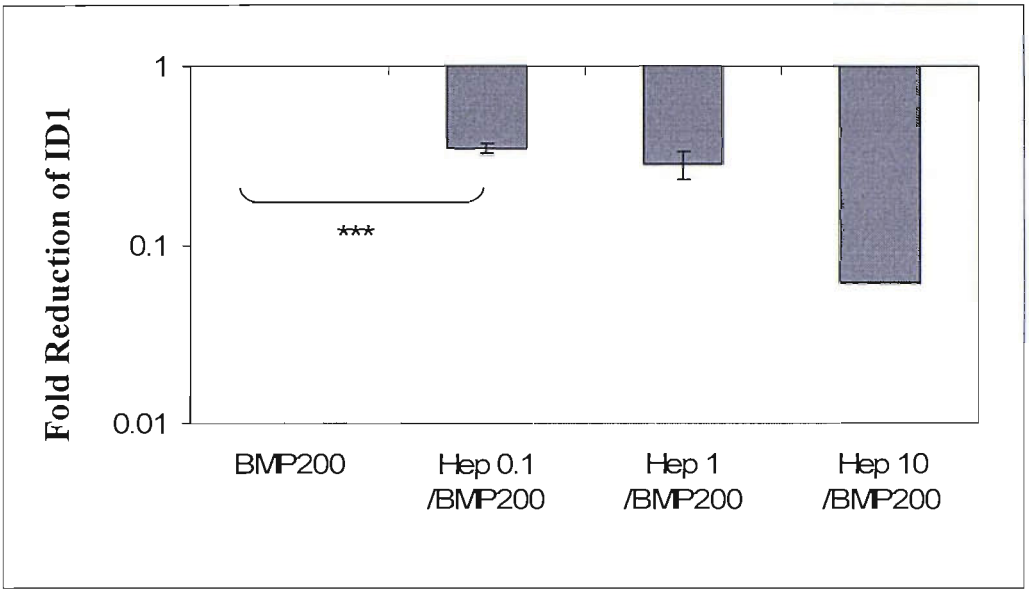


Figure 3.4: Effect of heparin on ID1 induction in control LCLs. Fold induction of ID1 following heparin (ug/ml, 30mins) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to levels of GAPDH mRNA (n=3 error bars-SEM ***p<0.001).

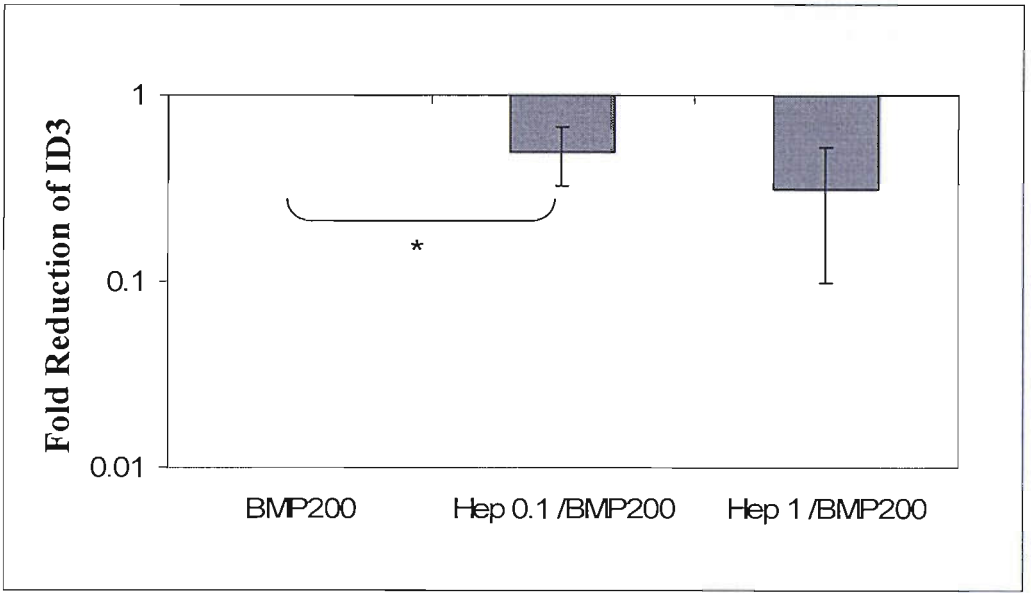


Figure 3.5: Effect of heparin on ID3 induction in control LCLs. Fold induction of ID3 following heparin (ug/ml, 30mins) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to levels of GAPDH mRNA (n=3 error bars-SEM *p<0.05).

FOP Cells Exhibit Increased ID1 and ID3 Expression

To quantify the levels of ID1 and ID3 induction in FOP cells compared to controls, both cell types were stimulated with BMP4 and ID gene expression was analyzed using real time PCR. Both control and FOP cells demonstrated an increase in ID1 induction following BMP4 treatment. However, in the presence of BMP4, FOP cells upregulated ID1 mRNA greater than control cells (Figure 3.6).

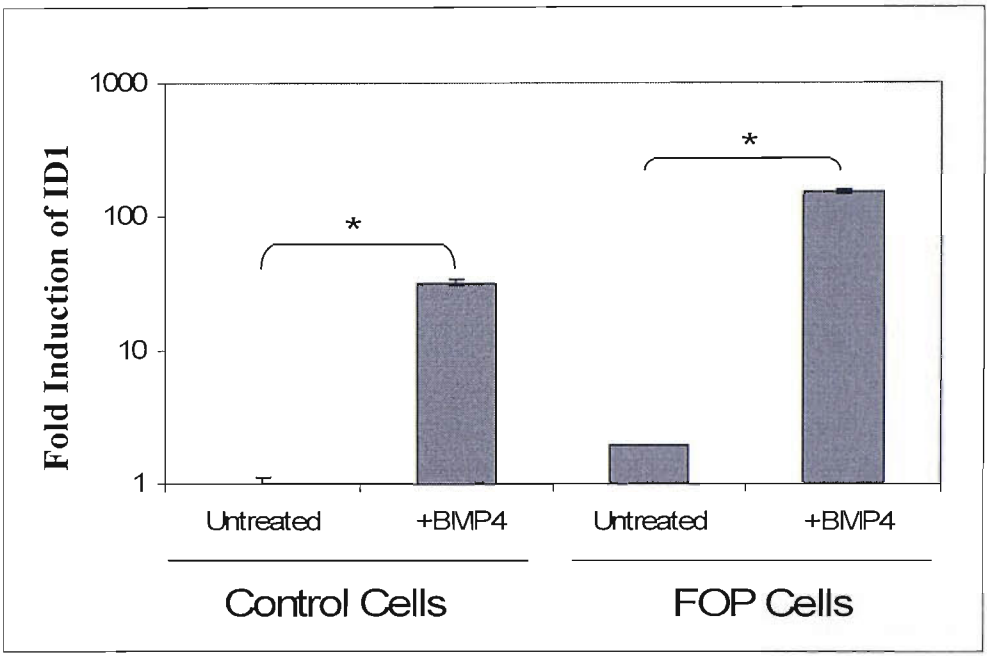


Figure 3.6: Induction levels of ID1 in control and FOP cells. Fold induction of ID1 following BMP4 (200ng/ml, 1.5hrs) treatment in control and FOP cells. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *$p<0.05$).

Similar results were observed for ID3 induction following BMP4 treatment of control and FOP cells (Figure 3.7). BMP4 stimulated ID3 expression was approximately 2-fold greater in FOP cell compared to controls. These results demonstrate that FOP cells are much more sensitive to BMP4-stimulated ID1 and ID3 induction, consistent with the observation that FOP is a disease of dysregulated BMP signaling (de la Pena et al, 2005; Fiori et al, 2006).

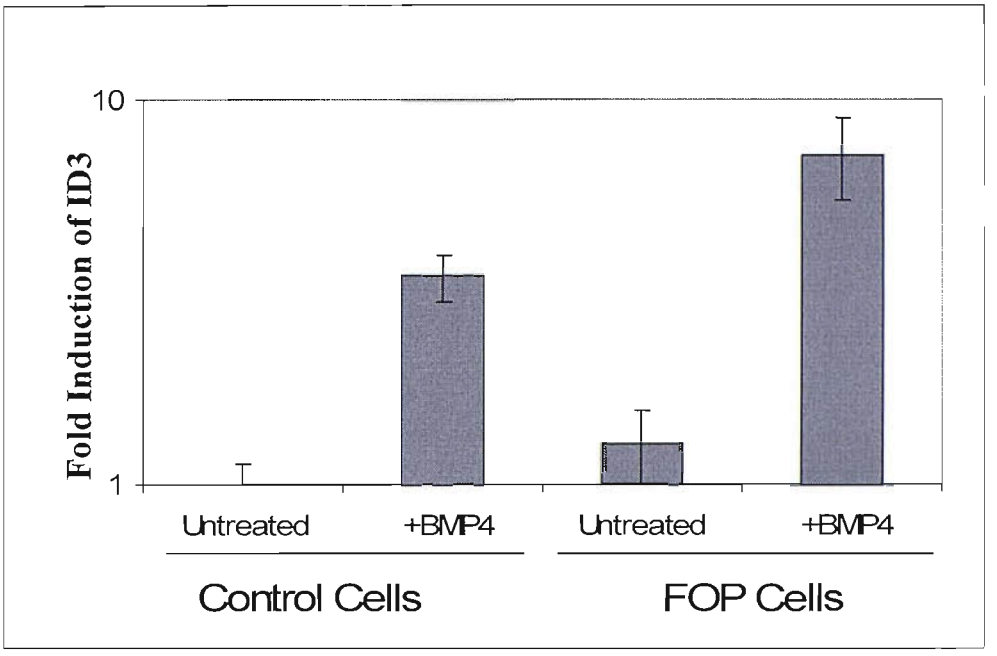


Figure 3.7: Induction levels of ID3 in control and FOP cells. Fold induction of ID3 following BMP4 (200ng/ml, 1.5hrs) treatment in control and FOP cells. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM).

Removal of HSPG GAG Chains Reduces BMP Signaling in Control, but not FOP Cells

Since BMPs are capable of binding to both HSPGs and BMP receptors, the role of HSPG GAG chains as modulators of BMP signaling was investigated. Enzymatic treatment to remove HSPG-specific GAG chains using heparinase III was performed prior to BMP4 treatment. The effect of this treatment on BMP signaling was measured by real time PCR analysis of ID1 and ID3 gene expression. In control cells, BMP4 induced ID1 expression 14-fold greater than the untreated cells (Figure 3.8). However, heparinase pre-treatment, followed by BMP4 stimulation, significantly reduced ID1 induction to near basal levels. Heparinase treatment alone had no effect on ID1 expression.

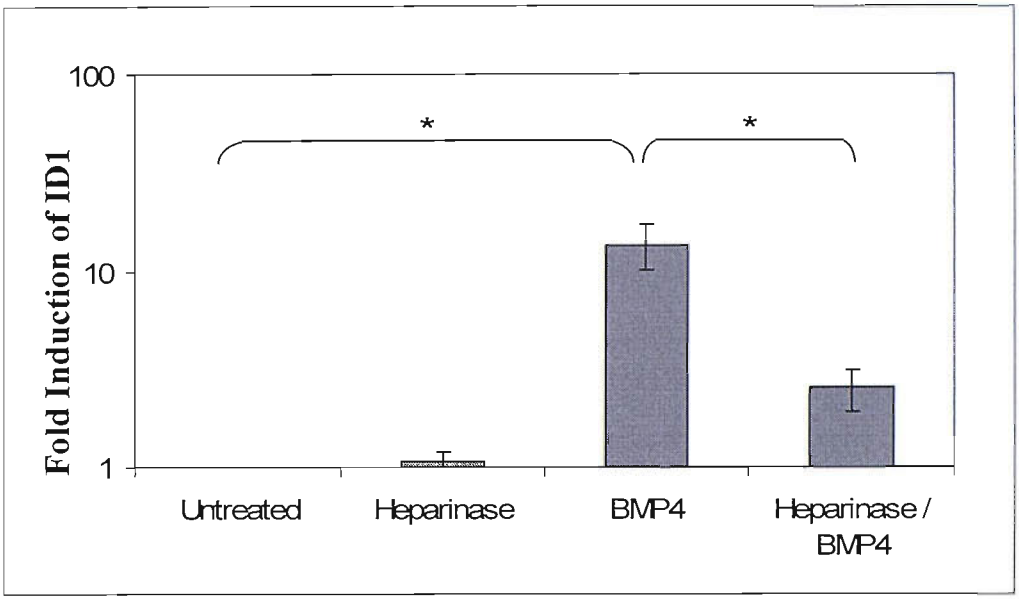


Figure 3.8: Effect of heparinase III treatment on ID1 induction in control LCLs. Fold induction of ID1 following heparinase III (5mU/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *p<0.05).

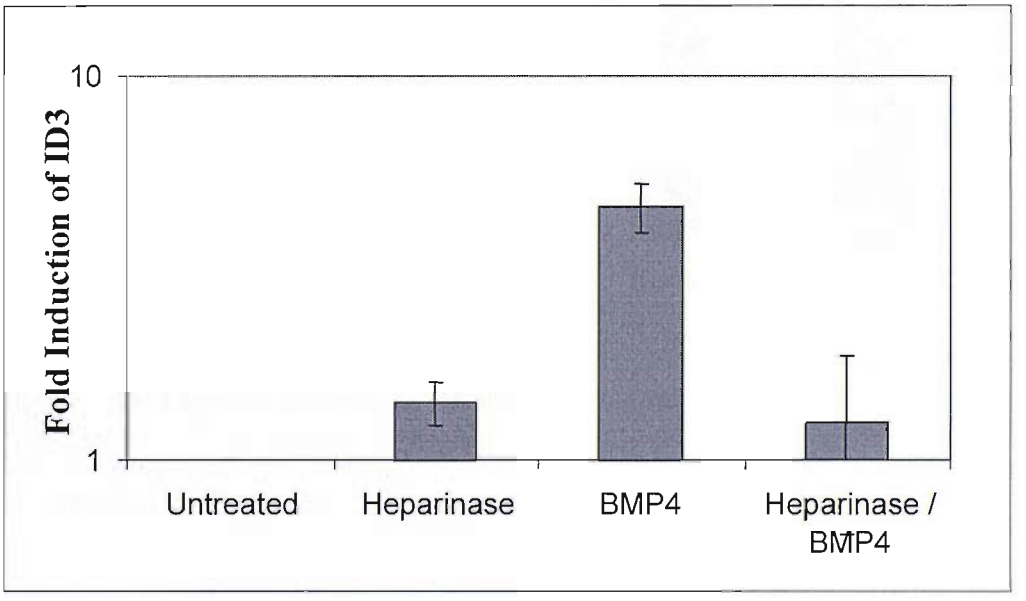


Figure 3.9: Effect of heparinase III treatment on ID3 induction in control LCLs. Fold induction of ID3 following heparinase III (5mU/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM).

Similar patterns of induction were seen with BMP4 stimulated ID3 induction in control cells (Figure 3.9). BMP4 induced ID3 expression was 6-fold greater in control cells compared to untreated cells. Heparinase pre-treatment reduced ID3 induction to near basal levels. Heparinase treatment alone had no effect on ID3 expression. These data suggest that in control LCLs, GAG chains stimulate BMP4 mediated induction of ID1 and ID3, since removal of GAG chains by heparinase treatment decreases signaling.

Interestingly, the same effect is not seen in FOP cells. FOP cells demonstrate an increase in induction of ID1 following BMP4 treatment (70-fold), but heparinase III pre-treatment did not result in any reduction of ID1 expression as observed in the control cells (Figure 3.10). Similar to control cells, heparinase III treatment alone had no effect on ID1 induction.

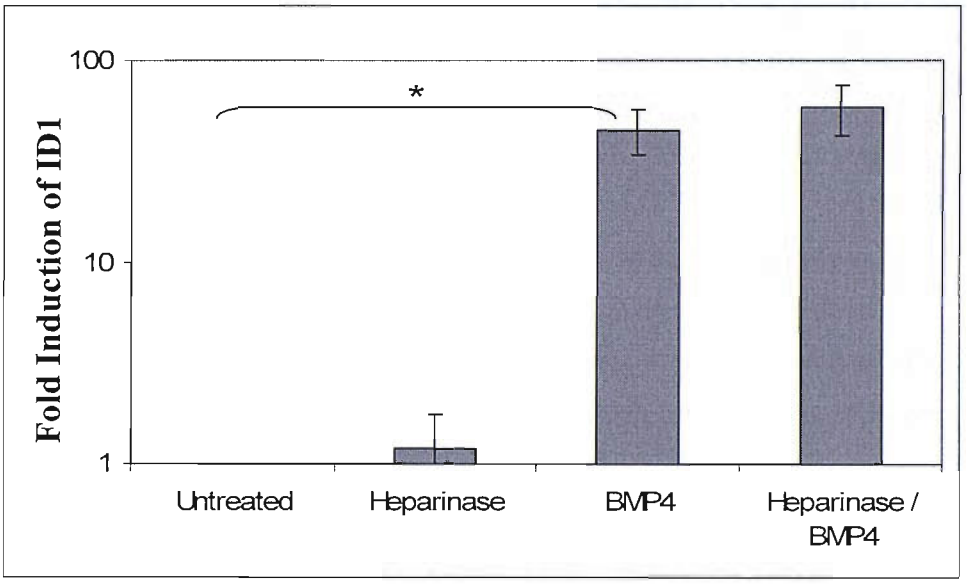


Figure 3.10: Effect of heparinase III treatment on ID1 induction in FOP LCLs. Fold induction of ID1 following heparinase III (5mU/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *$p<0.05$).

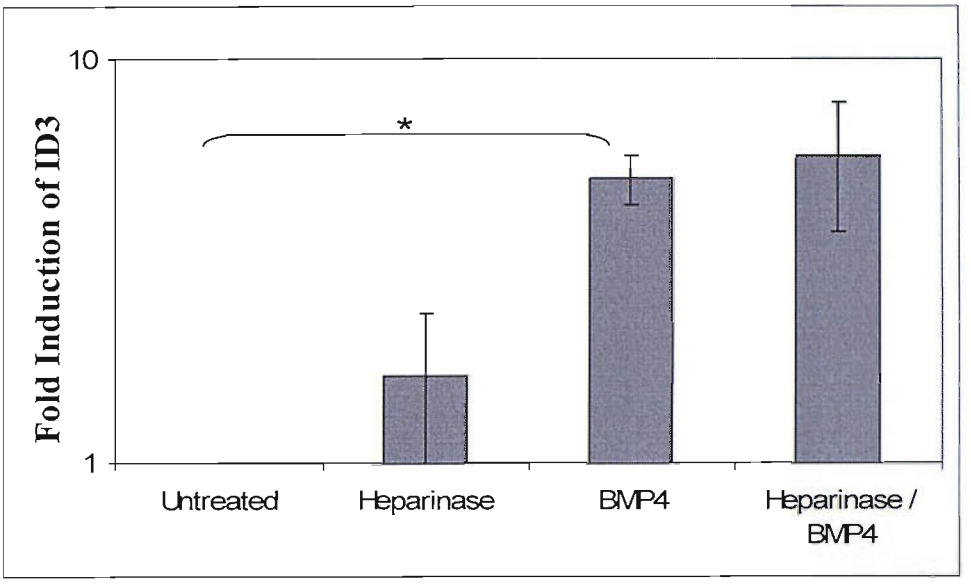


Figure 3.11: Effect of heparinase III treatment on ID3 induction in FOP LCLs. Fold induction of ID3 following heparinase III (5mU/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *$p<0.05$).

The same pattern of induction was seen for ID3 following heparinase III pre-treatment in FOP cells (Figure 3.11). BMP4 stimulated ID3 induction was 7-fold greater than untreated controls, but heparinase III pre-treatment did not result in any reduction in ID3 expression. These data suggest that FOP cells may exhibit a resistance to the modulating effects of the GAG chains, or that FOP cells are already signaling beyond the stimulatory capacity of GAG chains, possibly due to increased BMPRIA on the surface of FOP cells.

Removal of GPI-linked Proteins Increases BMP Signaling in Control, but not FOP Cells

To investigate if GPI-linked proteins play a modulatory role in BMP signaling in control and FOP cells, PI-PLC enzymatic treatment was performed. This treatment removes all GPI-linked proteins, including glypicans, but not syndecans. Control cells induced ID1 following BMP4 stimulation 50-fold compared to untreated controls (Figure 3.12). Interestingly, PI-PLC pre-treatment and subsequent BMP4 stimulation resulted in a statistically significant increase in ID1 induction compared to untreated controls (70-fold) and compared to cells treated with BMP4. PI-PLC treatment alone had no statistically significant effect on ID1 induction.

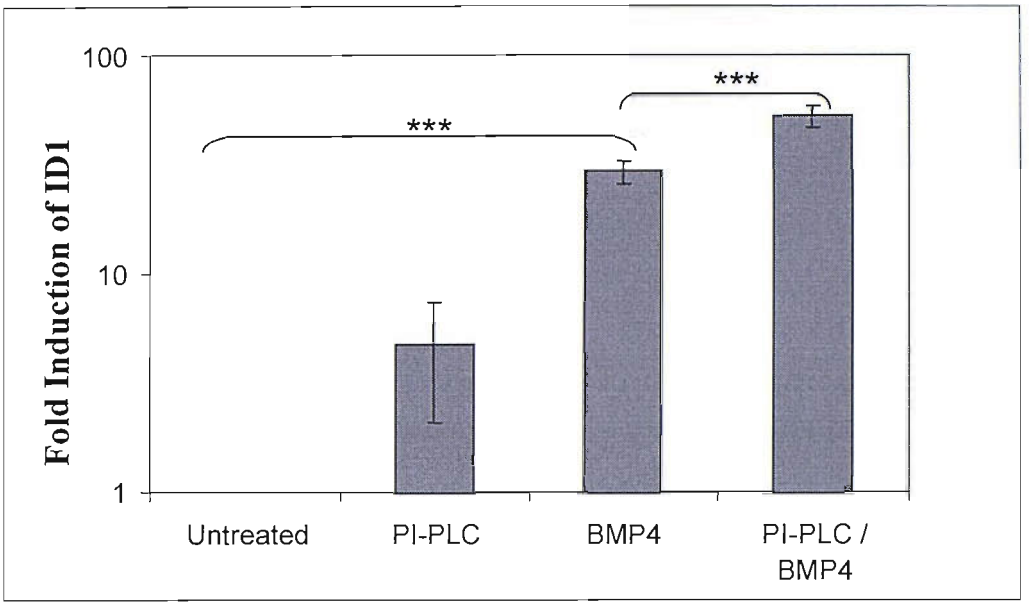


Figure 3.12: Effect of PI-PLC treatment on ID1 induction in control LCLs. Fold induction of ID3 following PI-PLC (1U/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM ***p<0.001).

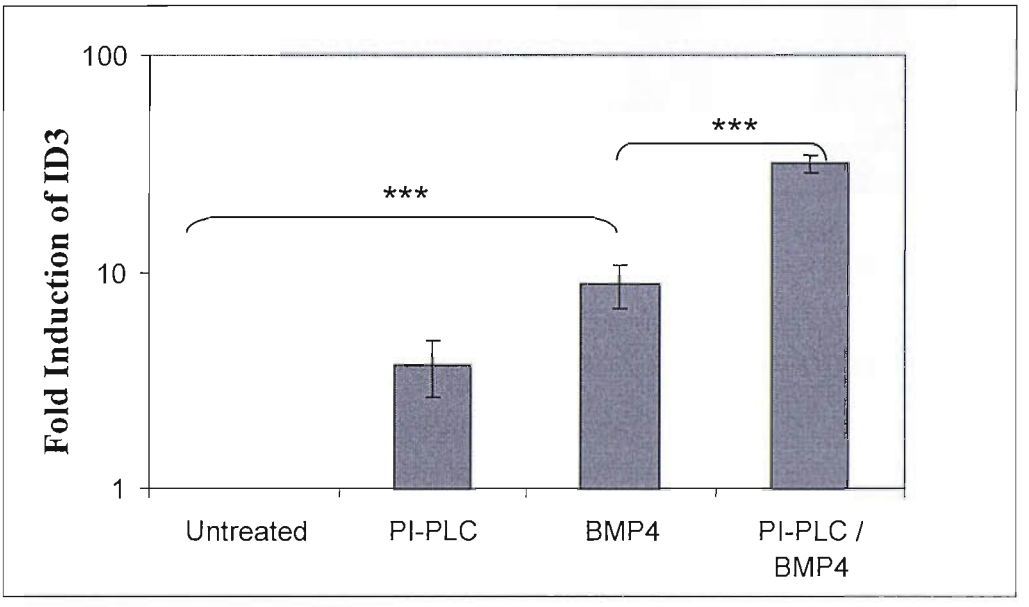


Figure 3.13: Effect of PI-PLC treatment on ID3 induction in control LCLs. Fold induction of ID3 following PI-PLC (1U/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM ***p<0.001).

Similar results were observed for ID3 induction (Figure 3.13). Following BMP4 stimulation, ID3 expression was induced to statistically significant levels (10-fold) compared to untreated controls. Following PI-PLC treatment and subsequent

BMP4 stimulation, ID3 levels were induced to significantly higher levels than with BMP4 treatment alone. PI-PLC treatment alone had no statistically significant effect on ID3 expression. These data suggest that GPI-linked proteins may play an inhibitory role in BMP signaling in control LCLs since removal of these proteins results in increased induction of ID1 and ID3.

Interestingly, the same results are not seen in FOP cells. BMP4 stimulated ID1 induction in FOP cells resulted in a statistically significant upregulation (70-fold) compared untreated controls (Figure 3.14). PI-PLC pre-treatment and subsequent BMP4 stimulation had no effect on the levels of ID1 induction when compared to BMP4 treated cells. PI-PLC treatment alone also had no effect on ID1 expression.

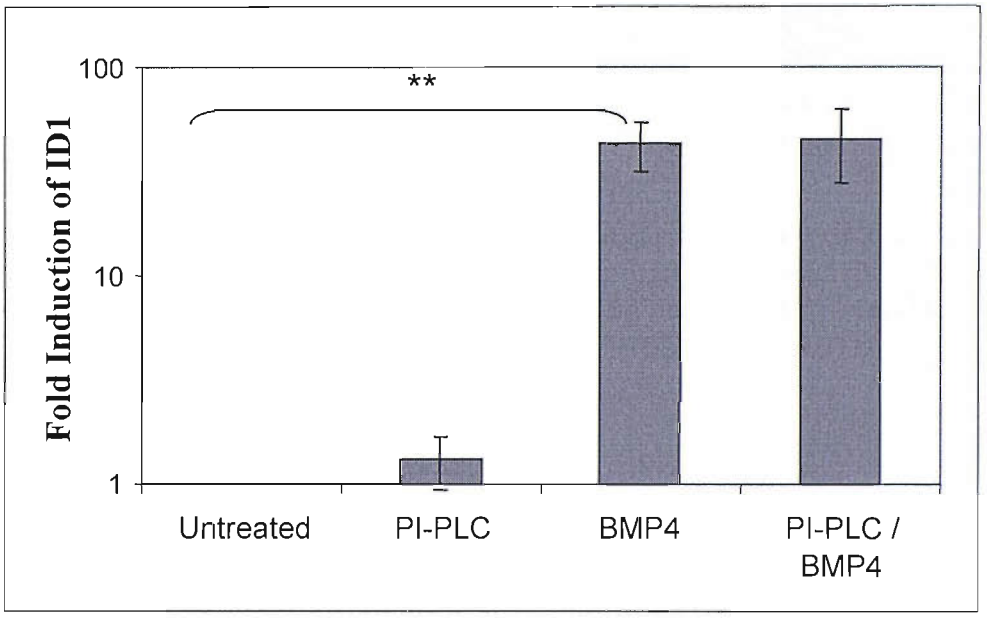


Figure 3.14: Effect of PI-PLC treatment on ID1 induction in FOP LCLs. Fold induction of ID1 following PI-PLC (1U/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM **$p<0.01$).

Similar patterns of induction were observed for ID3 expression (Figure 3.15). BMP4 stimulated ID3 induction in FOP cells resulted in a statistically significant upregulation (9-fold) compared to untreated controls. PI-PLC pre-treatment and subsequent BMP4 stimulation had no effect on the levels of ID3 induction when compared to the BMP4 treated cells. PI-PLC treatment alone also had no effect on ID3 expression. These data suggest that FOP LCLs may be resistant to the inhibitory effects of GPI-linked proteins observed in control cells. However, as PI-PLC

treatment removes all GPI-linked proteins, other modulating proteins important for BMP signaling may also be removed.

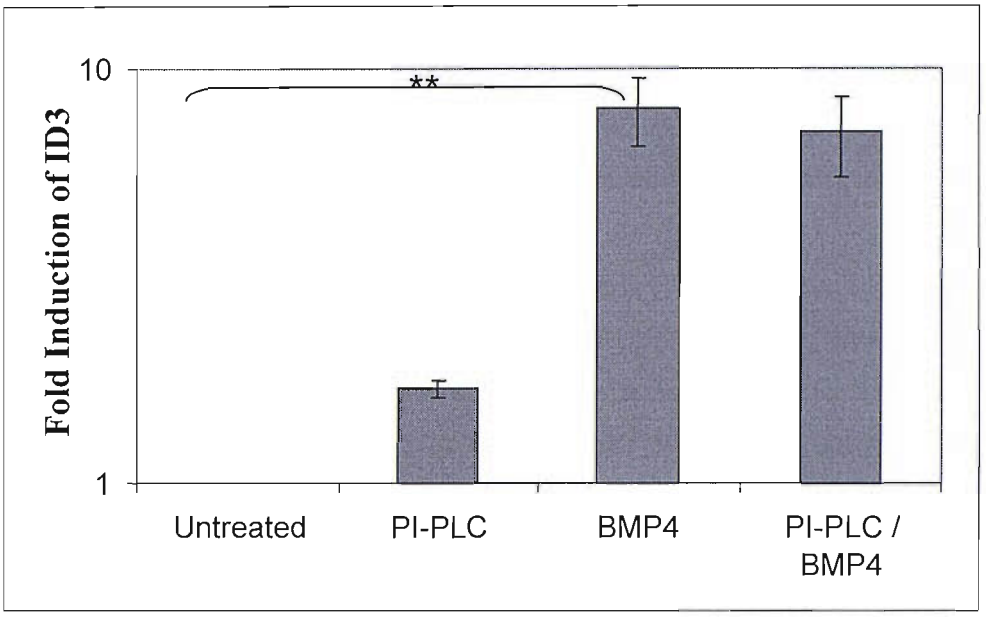


Figure 3.15: Effect of PI-PLC treatment on ID3 induction in FOP LCLs. Fold induction of ID3 following PI-PLC (1U/ml, 2hrs) pre-treatment and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM **$p<0.05$).

Discussion

These studies demonstrated the kinetics of ID1 and ID3 induction in control LCLs. ID1 is induced to much greater levels than ID3 in the presence of BMP4, however, the patterns of induction are the same for both genes. Maximal induction of ID1 and ID3 was achieved at 200ng/ml BMP4, with reduced expression at lower and higher concentrations. Further, time course experiments showed that ID mRNA are induced following BMP4 stimulation for approximately 2 hours, after which expression is reduced.

Studies using cultured mouse embryonic stem (ES) cells have shown that Id1, Id2, and Id3 are all downstream targets of BMP2 and BMP4, along with other genes such as Msx-1, Msx-2, and the proto-oncogene JunB (Hollnagel et al, 1999). In mouse fetal neuroepithelial cells, BMP2, BMP4, and BMP7 have equivalent potential to inhibit neurogenesis and concomitantly induce astrocytogenesis through activation of Id1. This results in the inhibition of neurogenic transcription factors such as Mash1 and neurogenin (Yanagisawa et al, 2001). Further, BMP4 has been shown to block oligodendrocyte (OL) lineage commitment by the upregulation of Ids 1-4, which subsequently interact with OLIG proteins that are required for OL generation. However, only overexpression of Id2 and Id4 in cultured progenitor cells resulted in the same effects (Samanta and Kessler, 2004). These results suggest that despite BMP4-mediated upregulation of Ids 1-4, the effects seen in different cell types are Id subtype specific.

The presence of several Id proteins suggests different activities or redundancy in function. The results presented here demonstrated an upregulation of both ID1 and ID3 following BMP4 treatment. This similar pattern of induction, both in a dose and time-dependent manner, suggests that both genes are performing similar roles in transcriptional regulation, as shown in mouse models where Id1 and Id3 have compensatory roles (Lyden et al, 1999). Another hypothesis is that ID1 and ID3 are inhibiting or promoting different transcription factors independently of one another to optimize BMP signaling.

In fact, the differing roles of Id proteins have been investigated in *Xenopus*. Comparison of Id4 mRNA expression with Id2 and Id3 lead to the observation of complementary patterns of expression during myogenesis, neurogenesis, kidney development, tailbud formation, and neural crest migration (Liu and Harland, 2003). Further investigation demonstrated different roles for the Id proteins: Id2 inhibited

MyoD and neuroD, Id3 inhibited only neuroD, and Id4 inhibited neurogenin, neuroD, and MyoD (Liu and Harland, 2003).

To investigate the BMP signaling pathway and its associated molecules, such as HSPGs, in control and FOP LCLs, a reliable endpoint must be used. The induction of ID1 and ID3 in LCLs provides a transcriptional target that is inducible within 2 hours and is BMP pathway specific, unlike other possible markers, such as alkaline phosphatase.

Depletion of cell surface HSPG GAG chains by heparinase III treatment reduced BMP signaling in control cells. These data are consistent with previous studies in other cell types showing that GAG chain removal decreased Smad phosphorylation, and therefore BMP signaling (Irie et al, 2003). The results presented here extend these earlier findings by demonstrating that HSPG depletion also reduces downstream transcriptional targets of BMP signaling. However, one recent study showed that the hpa gene that encodes endogenous heparinase is progressively expressed in murine bone marrow stromal cells undergoing osteoblastic differentiation (Kram et al, 2006). These results suggest that GAG chain removal promotes osteoblastic differentiation and taken together with our findings, heparinase cleavage of GAG chains may promote or inhibit BMP signaling depending on the cell type.

Interestingly, in FOP cells, GAG chain removal did not have any effect on BMP signaling. It is possible that GAG chains on other proteoglycans (non-HSPGs) that are unaffected by heparinase III treatment may compensate for HSPG specific GAG removal. Alternatively, the profound elevation of BMP signaling seen in FOP cells may negate any contributory effects of the HSPG GAGs.

The depletion of all GPI-linked proteins by PI-PLC, including glypicans, resulted in an increase in BMP4 stimulated ID1 and ID3 expression in control cells, but not in FOP cells. However, there are many GPI-linked molecules on the cell surface and depletion of all of them may remove proteins that are both stimulatory and inhibitory to BMP signaling. For example, the GPI-linked protein Dragon is a BMP co-receptor that acts at the cell surface to enhance BMP signaling through direct interactions with BMP2 and BMP4 in both the presence and absence of BMP receptors (Samed et al, 2005).

Our results demonstrate that in FOP cells, HSPG GAG chain removal or GPI-linked protein removal had no effect on BMP signaling. It is possible that removal of the HSPG GAG chains is being compensated for by other proteoglycan GAG chains

still on the surface of FOP cells (chondroitin sulfate, etc), increased levels of GAG chains and/or HSPG proteins on FOP cells that are not sufficiently removed following heparinase treatment, or enzymatic resistance due to GAG chain sulfation pattern differences in FOP cells.

These studies have shown that the BMP early response genes ID1 and ID3 are induced in a dose and time dependent manner in LCLs and induction of these genes can be inhibited by exogenous heparin. Further, removal of HSPG specific GAG chains can reduce BMP signaling in control, but not in FOP LCLs. These studies also demonstrated that removal of all GPI-linked protein increases BMP signaling in control, but not FOP LCLs.

Chapter 4

Elevated HSPG GAG Chains on FOP LCLs

Chapter 4: Elevated HSPG GAG Chains on FOP LCLs

Introduction

Glycosaminoglycans (GAG) are linear carbohydrates that participate in a variety of biological processes which include activation of chemokines, growth factors, and enzymes (Taylor and Gallo, 2006). GAG chains are present on many molecules including HSPG, chondroitin sulfate proteoglycans, dermatan sulfate proteoglycans, and keratin sulfate proteoglycans.

Heparan sulfate enables growth factor function and modifies enzyme / inhibitor function, such as pleiotrophin (Vacherot et al, 1999). Further, heparan sulfate interacts with cytokines and chemokines to regulate leukocyte selectin binding, promoting the recruitment of leukocytes (Luo et al, 2001; Netelenbos et al, 2003; Celie et al, 2005). Chondroitin and dermatan sulfate regulate growth factor activity and leukocyte migration by binding to selectins and CD44 (Luo et al, 2001; Kawashima et al, 2000), and dermatan sulfate also induces endothelial cell ICAM-1 expression (Penc et al, 1999).

CD44 is a cell surface HSPG that binds hyaluronic acid (HA), osteopontin, collagen, and matrix metalloproteinases (MMPs). HA has been shown to recruit leukocytes and activate macrophages and various inflammatory cells via CD44 dependent signaling (Higa et al, 2005; Khan et al, 2004; Murai et al, 2004). Further, hyaluronan signals through Toll-like receptor 4, resulting in dendritic cell maturation and cytokine release of both dendritic and endothelial cells (Termeer et al, 2002; Taylor et al, 2004).

HSPG GAG chains have been show to modulate BMP signaling by binding to BMP superfamily members, including BMP2, 4, and 7, and the BMP antagonist, Noggin (Zimmermann and David, 1999; Paine-Saunders et al, 2002). Noggin binding is highly selective for heparan sulphate (Viviano et al, 2004) and once bound, remains functionally active and capable of binding BMPs at the cell surface, suggesting a modulatory role for the GAG chains in BMP signaling.

It is clear that GAG side chains have multiple physiological roles in a variety of cell types, including lymphocytes. In this chapter, the total levels of GAG chains present on the cell surface and their sulfate incorporation at 24 hours was investigated on control and FOP LCLs. Further, HSPG-specific GAG levels were investigated using the BMP antagonist Noggin.

Materials and Methods

Dimethylmethylene blue (DMB) / GAG assay

Dimethylmethalene blue (DMB) binds to GAG chains that are present on the cell surface. Approximately $1x10^6$ LCLs were washed and resuspended in 100µl phosphate buffered saline (PBS; Invitrogen). 40µl of samples and a standard curve using 0-50µg/ml of chondroitin sulfate (C-6-S) was added to a 96 well plate. 125µl DMB was then added to all samples and standards, plates were then read at 520nm using a Bio-Tek Synergy HT spectrophotometer, and compared to the standard curve. Samples were normalised to control cells to demonstrate fold-difference.

Radioactive ^{35}S Treatment

In the presence of $Na_2{}^{35}SO_4$, cells will incorporate radioactive sulfate into newly synthesized chains. Control and FOP cells were treated with $Na_2{}^{35}SO_4$ (PerkinElmer) to allow incorporation of ^{35}S. Cells were washed in PBS, placed in DMEM (Invitrogen), 0.5% FCS in the presence of 100µCi/ml of $Na_2{}^{35}SO_4$ for 24 hours. Cells were then centrifuged (600 rcf, 5 minutes), washed and re-pelleted, and placed in 300µl RIPA buffer (PBS, 1% NP-40, 0.5% Na-deoxycholate) containing Protease Inhibitor cocktail (Sigma), 10µg/ml phenylmethyl-sulfonylfluoride (PMSF), 1mM Na-Orthovanadate, and 0.1mM NaF. RIPA samples were kept on ice and vortexed every 5 minutes for 30 minutes. Cells were then ethanol (EtOH) precipitated with 1ml of 100% EtOH and left on ice for 30 minutes. Samples were collected on a microfiber filter and washed with 100% EtOH. Next, the filters were placed in scintillation vials containing 3mls of scintillation fluid. All samples were then analyzed on a scintillation counter for ^{35}S and counts were normalized to protein levels, determined using a BCA protein assay (Pierce) with bovine serum albumin (BSA) as a standard.

Protein Assay

Protein levels were quantified by BCA protein assays (Pierce) using BSA as a standard. Briefly, using a 96 well plate, 25µl of dH_2O was added to each well. Next, 2µl of unknown was added to each well and a standard curve using 1µg/µl BSA in increasing amounts (0-20µg) was also added to the wells. To all wells, 200µl of substrate was added (10ml solution A, 200µl solution B). Samples were left at room temperature for 30 minutes to develop and absorbance was read at 550nm on a Bio-Rad 550 microplate reader.

Noggin Binding Assays

In the absence of ligand, Noggin will specifically bind to HSPG GAG chains. To determine Noggin binding levels, Noggin binding assays were performed. Control and FOP LCLs were pelleted (1500rpm, 5 minutes) and washed with PBS. Cells were placed in serum free media for 2 hours at 37°C, 5%CO_2 and subsequently treated with 900ng/ml of Noggin or NogginΔB2 (heparan binding domain removed; Regeneron) for 30 minute at room temperature. Next, cells were pelleted (1500rpm, 5 minutes) and washed with PBS. Cells were blocked on ice with 1% BSA in PBS (PBSB) for 30 minutes and then treated with 1μg/ml of anti-Noggin biotin (Regeneron) in 1% PBSB and incubated on ice for 2 hours. Cells were washed in PBS and incubated with 1:1000 dilution of either Streptavidin Phycoerythrin (SA-PE; BD Biosciences) or Streptavidin horseradish peroxidase (SA-HRP; Sigma) in 1% PBSB for 45 minutes. Next, cells were washed twice with PBS and fixed using 0.3% formalin for 10 minutes. Analysis was carried out by immunofluorescence, FACS or modified ELISA.

For immunofluorescence, SA-PE treated samples were transferred to Themo Shandon double cytoslides using a Shandon Cytospin 2. Cells were visualized under UV light with DAPI (360/470nm) and CY3/CY5 (546/600nm) wavelength filters using a Leica DMR microscope at the same exposure rate. Images were assembled using Adobe Photoshop. For FACS analysis, the cells treated with SA-PE secondary were resuspended in 1ml PBS and analyzed on a Becton Dickinson FACscan flow cytometer using 10,000 cells, gating only live cells. Data analysis used Cell Quest Pro to determine the mean fluorescent intensity (MFI). For modified ELISA, using a 96 well plate, 50μl of the SA-HRP treated sample was added to 50μl of substrate and left at room temperature to develop for 30 minutes. 50μl of 1:10N H_2SO_4 was added to terminate the reaction. The plate was then read at 440nm to determine absorbance. For all experiment no antibody and isotype controls were performed and demonstrated negative staining.

Statistical Analysis

For all data, statistical analysis was performed using ANOVA and the values are considered to be significantly different at $p \leq 0.05$. When only 2 data sets were used Student t-Test was performed. Asterisks mark values at: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Results

Increased Levels of GAG Chains are Present on FOP Cells.

Since FOP cells do not respond to GAG chain removal in the same manner as control cells (Chapter 3), we examined total GAG chain levels on LCLs. Using DMB, which binds to GAG chains present on the cell surface, the total levels of GAG chains on control and FOP cells were detected. FOP cells demonstrated approximately 2-fold greater levels of GAG chains when compared to controls (Figure 4.1).

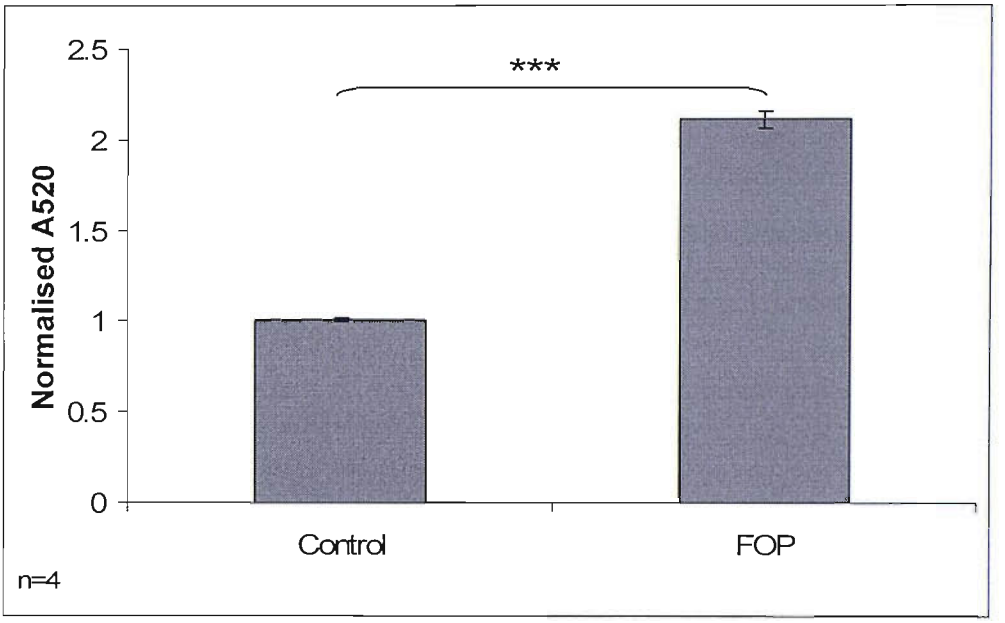


Figure 4.1: Total GAG chain levels detected using DMB binding assays. Cells were treated with dimethylmethylene blue (DMB) and read at a wavelength of 520nm (n=4, error bars-SEM, ***p<0.001).

To analyze incorporation ^{35}S into newly synthesized GAG chains, cells were treated with $Na_2{}^{35}SO_4$ for 24 hours. Following treatment, sulfate incorporation into GAG chains was detected using a scintillation counter and counts were normalized to protein levels. FOP cells have increased ^{35}S incorporation after 24 hours when compared to controls, suggesting that the rate of GAG chain synthesis is increased in FOP cells.

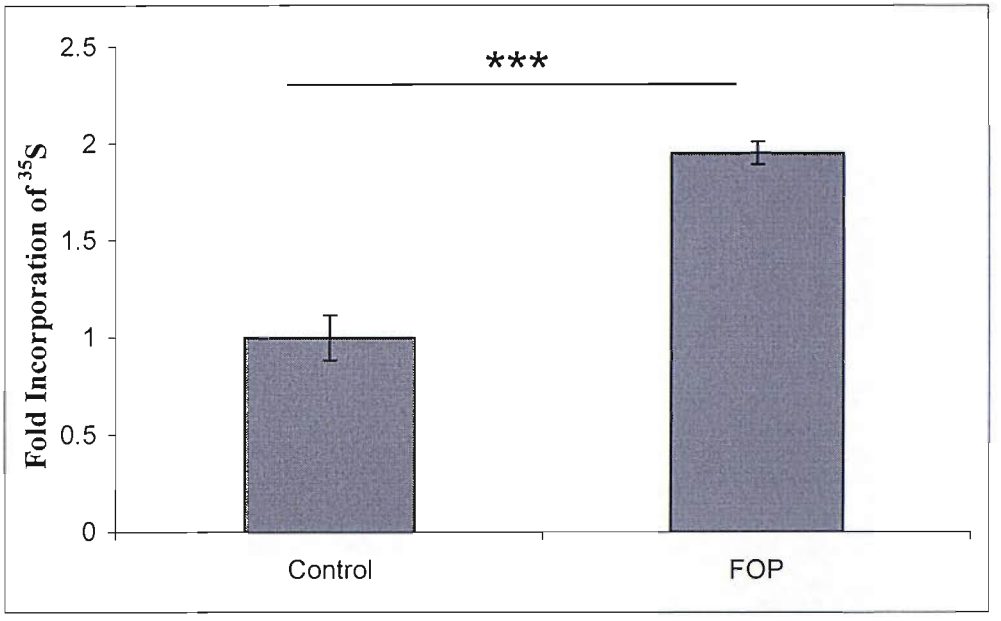


Figure 4.2: Total GAG chain levels detected using ^{35}S incorporation. Cells were treated with $Na_2{}^{35}SO_4$ for 24 hours and counts per minute (CPM) were detected on a scintillation counter. Counts were then normalised to μg protein (n=3, error bars-SEM, ***p<0.001) and calculated for fold incorporation.

HSPG Specific GAG Chains are Elevated on FOP Cells

Total levels of GAG chains have been shown to be elevated on FOP cell. To investigate if HSPG-specific GAG chains are also increased, Noggin binding was performed. Noggin protein has been shown to specifically bind to BMPs and HSPGs. Therefore in the absence of ligand, Noggin should only bind to HSPGs. To investigate Noggin binding to HSPG GAG chains on control and FOP cells, immunofluorescence was performed. Cells were pretreated with Noggin for 30 minutes and binding was detected using an anti-Noggin antibody and SA-PE. FOP cells demonstrated increased Noggin binding when compared to control cells, suggesting increased HSPG-specific GAG chains are present on FOP cells (Figure 4.3).

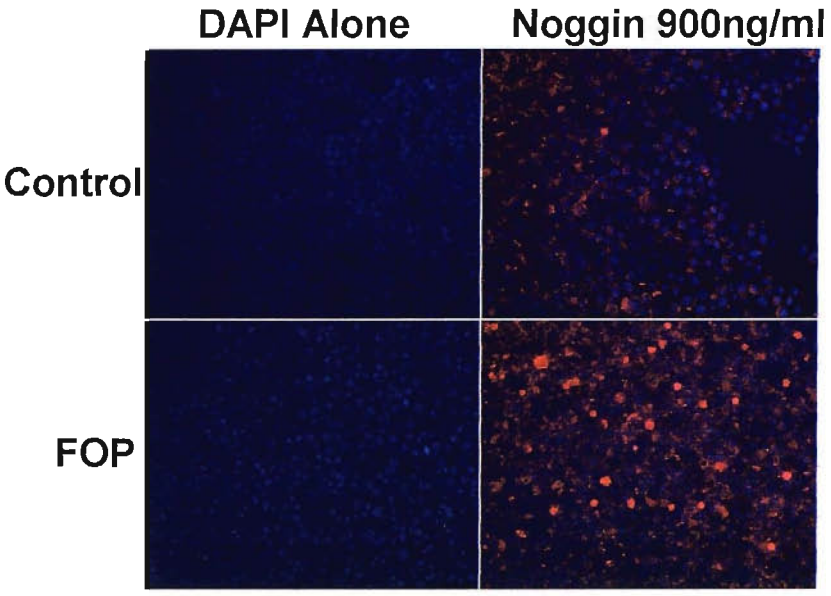


Figure 4.3: Noggin binding immunofluorescence. Control and FOP cell were treated with 900ng/ml Noggin and binding was detected using an anti-Noggin antibody and SA-PE. The blue staining is DAPI (nuclear) and red is SA-PE (indicative of Noggin binding). All images x20 objective mag.

To quantify the abundance of HSPG GAG chains on the surface of control and FOP cells, Noggin binding assays were performed using modified ELISA and FACS analysis.

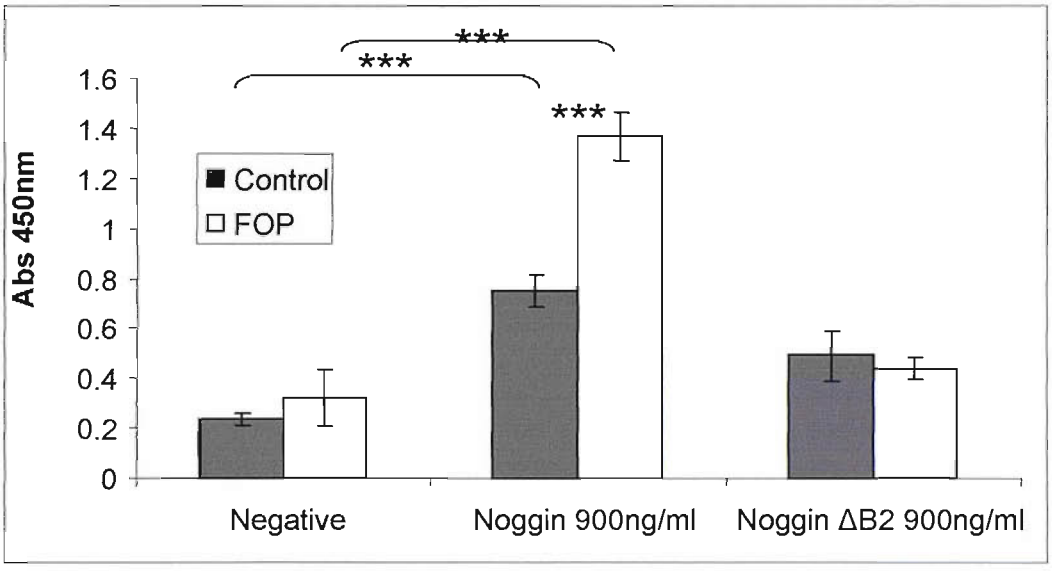


Figure 4.4: Noggin binding to HSPG specific GAG chains was quantified using modified ELISA. Noggin binding levels were detected using a modified ELISA. Cells were treated with Noggin, followed by an anti-Noggin biotin antibody and SA-HRP. Controls were untreated cells (Negative) and cells treated with modified Noggin protein (NogginΔB2). Samples were read at a wavelength of 450nM (n=3 error bars-SEM, ***$p<0.001$).

Noggin binding was detected at statistically significant levels on both control and FOP LCLs compared to untreated cells (Negative), however Noggin binding was greater on FOP cells, demonstrating increased levels of HSPG-specific GAG chains as well as increased total GAG levels (Figure 4.4). As a control, cells were also treated with a NogginΔB2, which has its heparan binding domain removed so it cannot bind to HSPG GAG chains. NogginΔB2 binding levels were indistinguishable from untreated cells. To confirm the results obtained using modified ELISAs, further quantification was performed by FACS analysis.

Noggin treatment resulted in a significant increase in mean fluorescent intensity (MFI) in both control and FOP cells compared to cells alone (Figure 4.5). As detected by modified ELISA, Noggin binding on FOP cells was statistically higher than control cells by FAC analysis, demonstrating elevated HSPG-specific GAG chains on FOP cells. Cells were also treated with NogginΔB2 as a control, resulting in significantly lower binding than observed with Noggin in both cell types. However, elevated binding was observed with NogginΔB2 compared to cells alone, which may be due to non-specific binding of the protein.

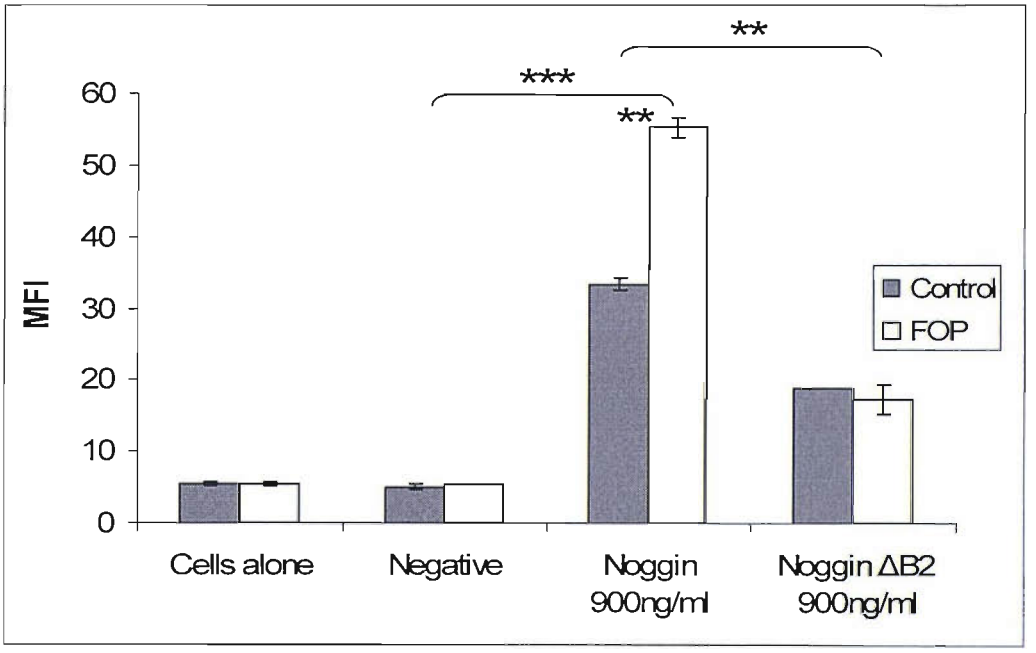


Figure 4.5: Noggin binding to HSPG specific GAG chains was quantified using FACS analysis. Noggin binding levels were quantified by FACS analysis. Cells were treated with Noggin, followed by an anti-noggin biotin antibody and SA-PE. Controls were untreated cells (cells alone), cells treated with only primary antibody (no Noggin), and cells treated with modified Noggin protein (NogginΔB2). (**MFI**: mean fluorescent intensity, n=3 error bars-SEM, **p<0.01, ***p<0.001).

Taken together, these results demonstrate that total GAG chains are increased on FOP cells compared to controls. Further, HSPG-specific GAG chains are also increased on FOP cells. Increased GAG chain levels may provide increased ligand binding sites for any molecule capable of interacting with GAG chains, including BMPs.

Discussion

These studies demonstrated increased sulfate incorporation and DMB binding in FOP cells, indicating increased levels of total GAG chains (HSPGs and non-HSPGs). More specifically, increased Noggin binding on the surface of FOP cells suggested elevated levels of HSPG-specific GAG chains. The BMP antagonist Noggin has HSPG binding sites (Paine-Saunders et al, 2002) and binding of Noggin to the cell surface is highly selective for HSPGs, requiring specific structural motifs for their interaction (Viviano et al, 2004). Therefore, in the absence of BMPs, Noggin should specifically bind to cell surface HSPGs.

Interestingly, another BMP antagonist, chordin, has been shown to interact with HSPGs. It was demonstrated that chordin binds heparin with a similar affinity as other factors that interact with HSPGs. Further, in mouse embryonic tissue, chordin binding was dependent upon interactions with cell surface HSPGs, but not with basement membrane associated HSPGs such as perlecan (Jasuja et al, 2004). These studies also demonstrated that chordin-HSPG interactions strongly potentate the antagonism of BMP signaling and are required for successful retention and uptake of chordin by the cell (Jasuja et al, 2004). BMP antagonists such as Noggin and chordin may interact with HSPG GAG chains and modulate the activity of BMP signaling. Therefore, an increase in GAG chain levels on FOP LCLs may alter the BMP modulating potential of the cells.

It is predicted that the mutation in the ACVR1 gene that causes FOP results in constitutive or leaky activation of the BMP receptor (Shore et al, 2006). Any effect that GAG chains have on BMP signaling would occur before or during ligand binding to receptors. Therefore, if ACVR1 is constitutively activated, elevated GAG chain levels would be unable to successfully modulate the intensity of BMP signaling through that receptor. This effect may explain why heparinase treatment had no effect on BMP signaling in FOP cells (Chapter 3). However, if the mutation results in leaky receptor activation, the GAG chains may be capable of some influence on BMP signal transduction. Further, BMPRIA is overexpressed (de la Pena et al, 2005) and HSPG GAG chains are elevated on FOP LCLs, which provides additional binding epitopes for BMP ligand. As a result, FOP LCLs may be primed for increased BMP signaling.

These studies have demonstrated that total and HSPG-specific GAG chains are elevated on FOP cells. The increased levels are caused by either elongated GAG chains on core proteins or an increase in the number of core proteins. Our results

suggest increased ligand binding sites on the surface of FOP cells, promoting BMP signaling.

Chapter 5

HSPG Profiling and Effect of HSPG siRNA on BMP Signaling

Chapter 5: HSPG Profiling and Effect of HSPG siRNA on BMP Signaling

Introduction

HSPGs are a specific type of proteoglycan that contain at least one GAG chain attached to the core protein via a serine residue (Forsten et al, 2000). Proteoglycans associated with the cell surface or extracellular matrix can dramatically influence the cellular response to heparin-binding cytokines (Hardingham and Fosang, 1992; Nugent and Iozzo, 2000). Further, growth factor kinetics, stability, transport, and cellular potency may be regulated through proteoglycan binding (Conrad, 1998).

Two families of cell surface HSPGs, glypicans and syndecans, have been well characterized. Glypicans have cysteine rich ectodomains, which likely form globular proteins. In contrast, syndecans have proline rich ectodomains, suggesting a more extended structure (Bernfield et al, 1999). Glypican core proteins are attached to cell membranes via a glycophosphatidylinositol (GPI) link, while syndecan core proteins have transmembrane and cytoplasmic domains, resulting in association with signaling molecules. (Bernfield et al, 1999). Since glypicans have no direct contact with the intracellular environment, any glypican mediated signaling must be performed by associated transmembrane molecules (De Cat and David, 2001). The association of the GPI link of glypicans and the transmembrane domain of syndecans with the cell surface results in different roles for each HSPG on the same cell. Internalization of glypican results in both ligand and receptor recycling, whereas internalization of syndecan results in lysosomal degradation of both HSPGs and bound ligand (Bernfield et al, 1999). This suggests that different HSPG may utilize different internalization mechanisms, such as clathrin coated pits or lipid rafts. The presence of two types of cell surface HSPGs implies that they have different roles in signaling, but establishment of distinct family-specific functions remains unknown.

In this chapter, HSPG mRNA and protein expression on control and FOP LCLs was investigated. Further, siRNA was performed for glypican 1 and syndecan 4, two of the most abundantly expressed HSPGs. The effect of HSPG-specific siRNA on BMP signaling was investigated.

Materials and Methods

RNA Extraction

For RNA extraction, cells were washed and pelleted at 1500rpm for 5 minutes. 1ml TRIzol (Invitrogen) was added to each sample followed by 200µl chloroform (Fisher) and samples were mixed and left at room temperature for 3 minutes. Samples were spun at 12,000g for 15 minutes at 4°C, the aqueous phase was transferred to a fresh tube, 1.5µl glycogen carrier and 500µl isopropanol (Fisher) was then added and samples were left at room temperature for 10 minutes. Samples were then spun at 12,000g for 10 minutes at 4°C and the pellet was washed in 75% EtOH in DEPC treated water (Fisher) following removal of the supernatant. Next, samples were pelleted at 7,500g for 5 minutes at 4°C, the supernatant was removed, and pellets air dried for 5 minutes. Pellets were resuspended in DEPC treated water, heated to 55°C for 10 minutes, and the total amount of RNA was calculated using a photospectometer. All samples were DNase (Invitrogen) treated prior to cDNA synthesis.

cDNA Synthesis and Real Time PCR

Briefly, 5µg of RNA in DEPC treated H_2O (Fisher), 1µl of 0.1mM dNTPs (Invitrogen), and 2µl of random primers (Invitrogen) were brought up to a total volume of 12µl with DEPC treated H_2O. Samples were heated to 65°C for 5 minutes and then reduced to 4°C in a thermal cycler (MJ Research PTC-200). Next, 4µl of 5x first strand buffer (Invitrogen), 2µl of 0.1M DTT (Invitrogen), and 1µl of superscript reverse transcriptase II (Invitrogen) was added and the samples were heated on a thermal cycler to 25°C 10 minutes, 42°C 50 minutes, and returned to 4°C.

Primers were designed for human GAPDH, ID1, ID3, syndecan 3, syndecan 4, glypican 1, glypican 3, and glypican 5 (Table 5.1). Gene expression was quantified using real time PCR and values were normalized to GAPDH mRNA. All PCR reactions were performed in triplicate. Reactions containing 12.5µl of SYBR green, 7µl of distilled water, 3µl of primers (1.5µl of each; final concentration 50 nmols), and 2.5µl of template cDNA (1:20 dilution of cDNA, made from 5µg RNA) were added to each well. For each gene, standard curves and no template controls (NTCs) were run in triplicate. Samples were run at 50°C for 2 minutes, 95°C for 10 minutes to denature, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute in an ABI Prism 7000 sequence detection system (Applied Biosystems). Values are

expressed as mean fold induction +/- standards error of the mean (SEM) and are results obtained from at least three independent experiments.

Primer	Sequence
GAPDH	F – AGATCATCAGCAATGCCTCC R – ATGGCATGGACTGTGGTCATG
ID1	F-GGTGGAGATTCTCCAGCACG R-GCGTTCTGGAGGTGTCAGGA
ID3	F-TTCCCATCCAGACAG CCG R-GCGTTCTGGAGGTGTCAGGA
Syndecan 3	F-GCTC AGACCCCAACTCCAGA R-TGGCTCATTCCGGATTGTG
Syndecan 4	F-AGGCCGATACTTCTCCGGA R-CATCCAGATCTCCAGAGCCAG
Glypican 1	commercially designed by SuperArray (proprietary sequence)
Glypican 3	F-GGTTTTCCAAGAGGCCTTTG R-CAAAAGCTTGTGGAGTCAGGCT
Glypican 5	F-TGAAGATCACAGAC TGGATGCC R-TCCTGCTCCTGTTGTGTC TAAAGT

Table 5.1: Primer sequences

Immunofluorescence

Control and FOP LCLs were pelleted at 1500rpm for 5 minutes and washed using PBS. Cells were blocked for non-specific binding using 1% BSA in PBS (PBSB) with DAPI (1:5000) for 30 minutes on ice. Cells were treated with primary antibodies, Glypican 1, Syndecan 3, and Syndecan 4 (1:100; Santa Cruz) in 1% PBSB and incubated for 2 hours on ice. Cells were then washed twice with PBS and biotinylated secondary antibodies (1:250; R&D) were added in 1% PBSB for 45 minutes on ice. Next, cells were washed in PBS and treated with SA-PE (1:1000) in 1% PBSB for 45 minutes on ice. Cells were washed twice with PBS and fixed using 0.3% formalin for 10 minutes. Finally, cells were washed again and spun onto Themo Shandon double cytoslides using a Shandon Cytospin 2. Cells were visualized under UV light with DAPI (360/470nm) and CY3/CY5 (546/600nm) wavelength filters using a Leica DMR microscope at the same exposure rate. No antibody and isotype controls were performed and demonstrated negative staining. Images were assembled using Adobe Photoshop.

FACS (Fluorescence Activated Cell Sorting)

Cells were washed with PBS, blocked in 1% PBSB for 30 minutes and incubated with the primary antibodies Glypican 1, Syndecan 3, and Syndecan 4

(1:100) for 2 hours on ice. Next, cells were washed, incubated with biotinylated secondary antibodies (1:250) in 1% PBSB for 45 minutes on ice, washed again, and incubated with Streptavidin Phycoerythrin (SA-PE) (1:1000) for 45 minutes on ice. Finally, cells were fixed in 0.3% formalin in PBS and resuspended in 1ml PBS for analysis on a Becton Dickinson FACscan flow cytometer using 10,000 cells (gating only live cells). Cell Quest Pro was used to determine the mean fluorescent intensity (MFI).

siRNA Transfection

Cells were transfected with *glypican 1, glypican 5, syndecan 3, syndecan 4,* or scrambled (control, non-targeting) siRNA duplexes (4 for each gene in the same master mix. Scrambled siRNA was a single siRNA with 4 mismatches across all known human, mouse, and rat genes, and had minimal, well defined , reproducible off target effects) commercially designed by Dharmacon. Cells were placed in medium (RPMI, 15% FCS) lacking antibiotics for 24 hours prior to transfection. The cells were washed and resuspended at $2x10^6$ cells in 5 mls Optimem medium (Invitrogen). Control and FOP cells were transfected with siRNA master mix containing 20 nmol of each duplex in Lipofectamine 2000 (1:250; Invitrogen) and incubated at 37°C, 5% CO_2. After 4 hours, 5 mls of RPMI, 30% FBS was added to cells, resulting in a final concentration of 1:1 Optimem:RPMI, 15% FCS. Cells were grown for 72 hours at 37°C, 5% CO_2, placed into six-well plates in RPMI, 1.5% FCS, treated with 200ng/ml of BMP4 for 1.5 hours, and ID target gene expression was determined by real time PCR.

Statistical Analysis

For all data, statistical analysis was performed using ANOVA and the values are considered to be significantly different at $p \leq 0.05$. Asterisks mark values at: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Results

Glypican 1 and Syndecan 4 are the Most Abundantly Expressed HSPGs on LCLs

Since cell surface HSPGs consist of the glypican and syndecan families, HSPG profiling of individual members was performed in LCLs. Previous data demonstrated the presence of only glypicans 1, 3, and 5, and syndecans 3 and 4 mRNA in LCLs by RT-PCR (personal communication – Dr. G. Deirmengian). To quantify the expression of these HSPGs, real time PCR was performed. The most abundantly expressed HSPG mRNAs were glypican 1 and syndecan 4, with much lower levels of glypican 3, glypican 5, and syndecan 3.

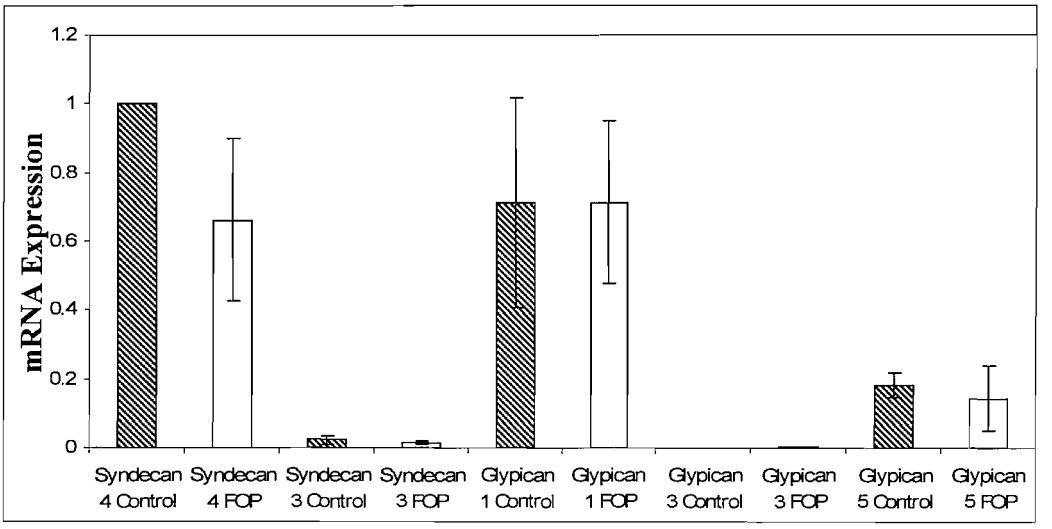


Figure 5.1: HSPG mRNA profiling in control and FOP cells. Expression of HSPG mRNA was quantified by real time PCR and normalized to levels of GAPDH mRNA (n=3 error bars-SEM). In order to examine expression levels, samples were then compared to control cells syndecan 4 levels (labeled as 1).

Glypican 1 and Syndecan 4 Core Proteins are Elevated on FOP Cells

Since glypican 1 and syndecan 4 mRNAs are abundantly expressed in LCLs, we examined the expression of core proteins on control and FOP cells using immunofluorescence (Figure 5.2). Control and FOP cells were treated with anti-glypican 1 or anti-syndecan 4 primary antibodies followed by a biotinylated secondary antibody and visualized using SA-PE. Glypican 1 and syndecan 4 core proteins were present on control and FOP cells.

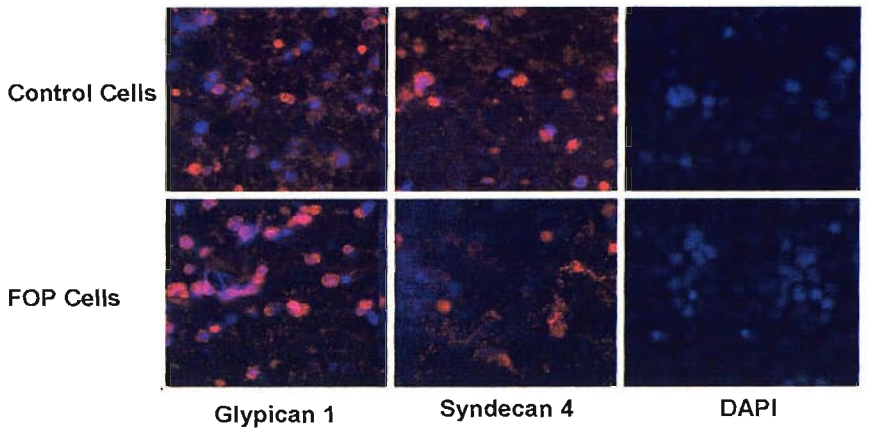


Figure 5.2: Immunofluorescence of glypican 1 and syndecan 4 protein expression on control and FOP cells. Control and FOP LCLs were treated with specific antibodies to glypican 1 and syndecan 4 core proteins, which were visualized following treatment with a biotinylated secondary antibody and SA-PE. DAPI staining served as a control. Blue = DAPI (nuclear), Red = SA-PE (core proteins). All images x20 objective magnification.

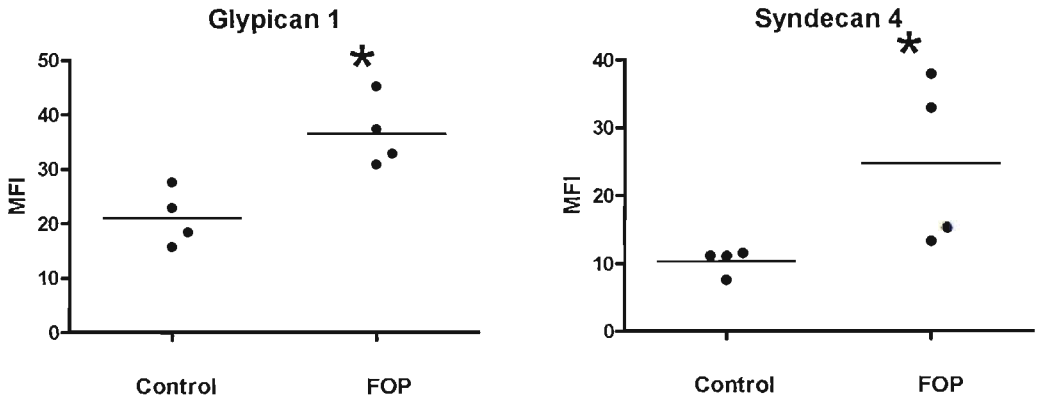


Figure 5.3: Quantification of glypican 1 and syndecan 4 core protein levels on control and FOP cells. Control and FOP LCLs were treated with specific antibodies to glypican 1 and syndecan 4 and levels were analyzed by FACS (MFI: mean fluorescent intensity, n=4 error bars: mean $\pm$ SEM, *p=<0.05).

To quantify the levels of HSPG core proteins, FACS analysis was performed. Significantly increased levels of glypican 1 and syndecan 4 (approximately 2-fold) were observed on FOP cells compared to control cells (Figure 5.3). Elevated core proteins result in an increase in the overall levels of GAG chains available on the cell surface (Chapter 4).

Glypican 1 Core Protein Down-Regulation Enhances BMP4 Induced ID Expression in LCLs

Since HSPG GAG chains have been shown to modulate BMP signaling and glypican 1 and syndecan 4 are abundantly expressed on LCLs, we used siRNA

targeted towards those HSPGs to downregulate expression and examine their roles in BMP signaling. Before transfection of HSPG-specific siRNAs, we determined if LCLs could be transfected using a fluorescent oligo (Figure 5.4). The transfection ability of control LCLs was high at 24 hours and even greater by 48 hours.

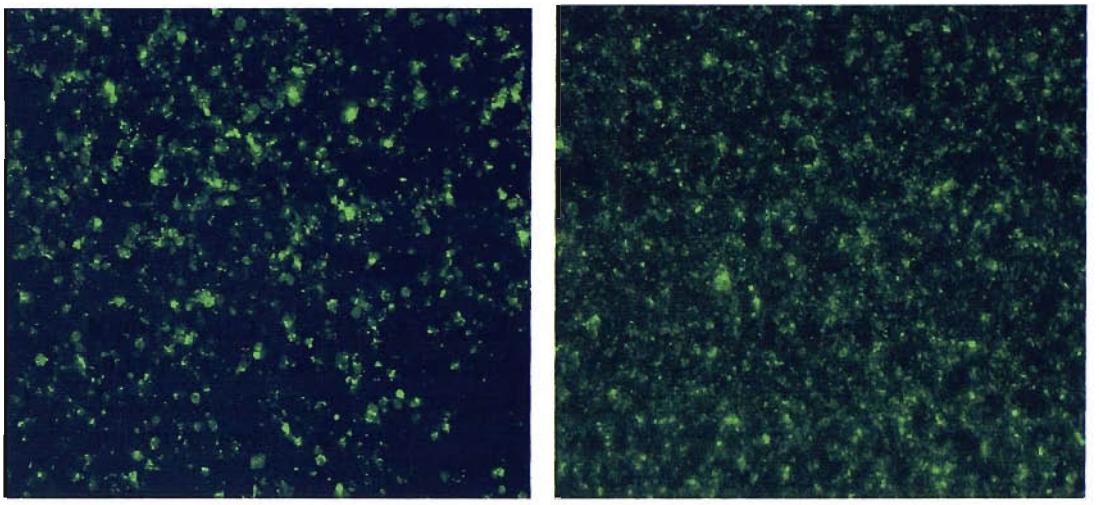


Figure 5.4: Transfection of LCLs. Control cells were seeded at $2x10^6$ cells/well and transfected with a fluorescent oligo using Lipofectamine 2000. The green fluorescence represents successful transfection at 24 and 48 hours. All images x20 objective magnification.

In order to determine to levels of gene silencing achieved through siRNA transfection, target gene expression was analyzed (Figure. 5.5). In control cells, glypican 1 siRNA resulted in a 50% reduction in gene expression, whereas syndecan 4 resulted in a 60% reduction. In FOP cells, both target genes were reduced 60%.

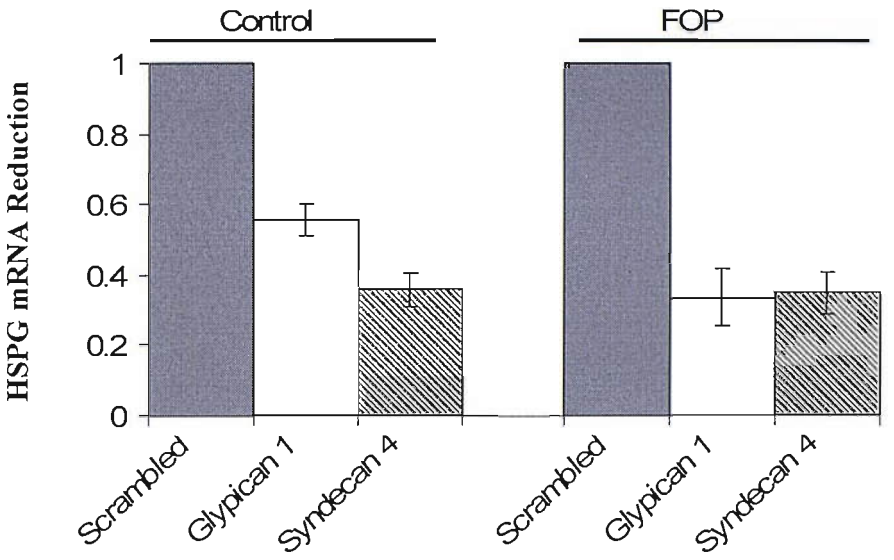


Figure 5.5: Effect of siRNA transfection on their target genes. Fold induction of glypican 1 and syndecan 4 in control and FOP cells following transfection. HSPG mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM). In order to examine expression levels, samples were then compared to scrambled siRNA levels (labeled as 1).

Control cells transfected with non-targeting (scrambled) siRNA and treated with BMP4 upregulated ID1 expression by 12-fold (Figure 5.6) and ID3 expression by 5-fold (Figure 5.7). Glypican 1 siRNA transfected cells that were treated with BMP4 induced ID1 expression approximately 50-fold and ID3 by 25-fold compared to cells treated with only glypican 1 siRNA. The statistically significant difference in ID expression between BMP4 stimulated cells transfected with non-targeting siRNA and glypican 1 siRNA, suggests that glypican 1 may act as an inhibitor of BMP signaling in control cells.

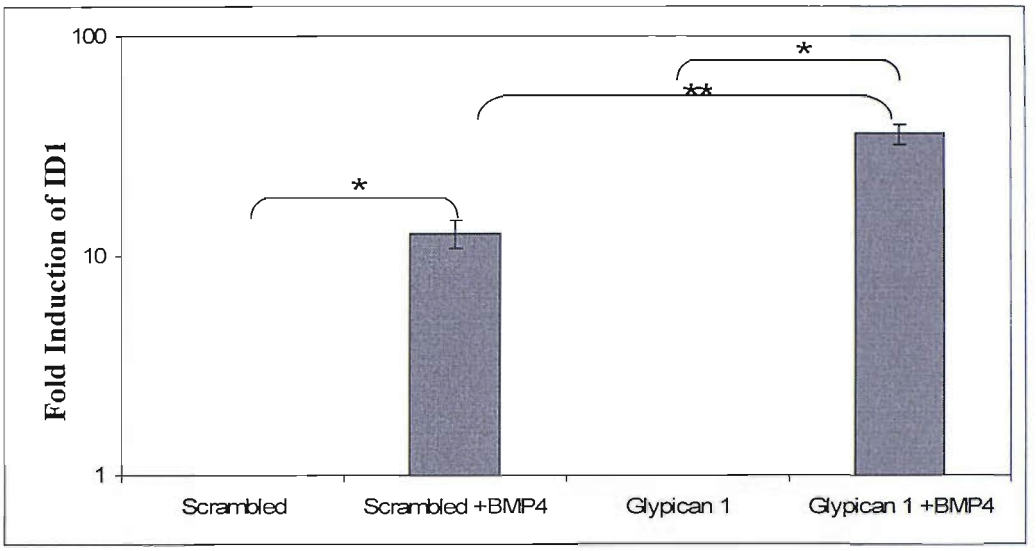


Figure 5.6: Effect of glypican 1 downregulation by siRNA on ID1 induction in control LCLs. Fold induction of ID1 in control cells following transfection of glypican 1 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *p<0.05, **p<0.01).

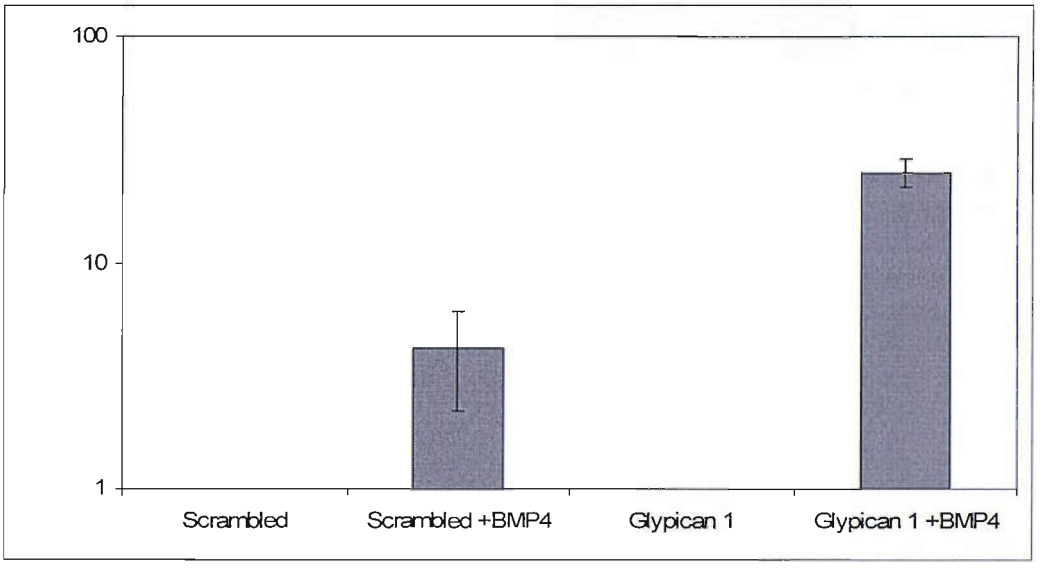


Figure 5.7: Effect of glypican 1 downregulation by siRNA on ID3 induction in control LCLs. Fold induction of ID3 in control cells following transfection of glypican 1 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM).

FOP cells transfected with non-targeting (scrambled) siRNA and treated with BMP4 upregulated ID1 expression by 10-fold (Figure 5.8) and ID3 expression by 4-fold (Figure 5.9). Glypican 1 siRNA transfected cells that were treated with BMP4 induced ID1 expression approximately 20-fold and ID3 by 6-fold compared to cells treated with only glypican 1 siRNA. Interestingly, BMP4 stimulated ID3 induction

resulted in similar levels of expression in both the scrambled and glypican 1 siRNA transfected cells. These results suggest that glypican 1 may act an inhibitor of BMP4-induced ID1, but not ID3 expression in FOP cells. This effect may be due to the observation that ID1 is the more inducible gene in this system (Chapter 3). FOP cells exhibit a dysregulation of BMP signaling, which may be affected by increased HSPG GAG chains (Chapter 3 and 4), resulting in FOP cells being more resistant to the modulatory effects of glypican 1.

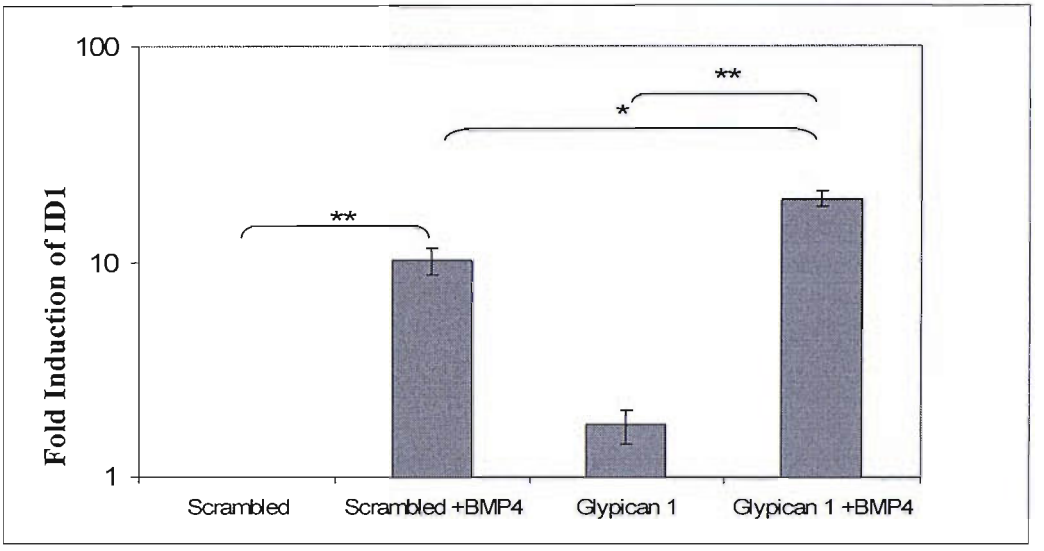


Figure 5.8: Effect of glypican 1 downregulation by siRNA on ID1 induction in FOP LCLs. Fold induction of ID1 in FOP cells following transfection of glypican 1 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM, *p<0.05, **p<0.01).

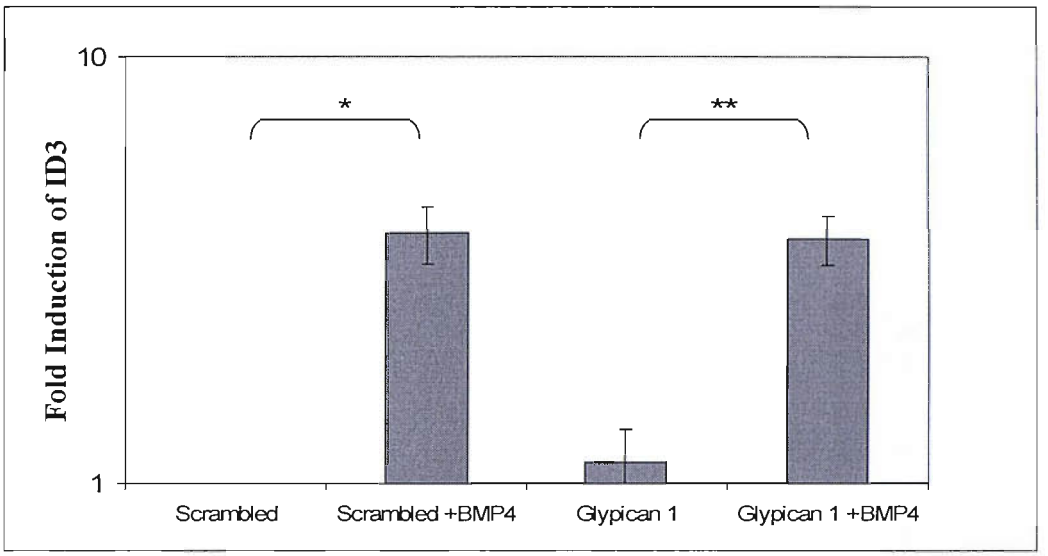


Figure 5.9: Effect of glypican 1 downregulation by siRNA on ID3 induction in FOP cells. Fold induction of ID3 in FOP cells following transfection of glypican 1 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM, *p<0.05, **p<0.01).

Syndecan 4 Core Protein Down-Regulation Reduces BMP4 Induced ID Expression in Control, but not FOP Cells

To investigate the effect of syndecan 4 down-regulation on BMP induced ID1 and ID3 expression, syndecan 4 siRNA was transfected into control and FOP cells. ID1 was induced 40-fold (Figure 5.10) and ID3 6-fold (Figure 5.11) following BMP stimulation in control cells transfected with scrambled siRNA. In control cells transfected with syndecan 4 siRNA, ID1 expression was only induced 20-fold and ID3 5-fold, a significant reduction when compared to BMP4 treated cells transfected with scrambled siRNA. These results suggest that syndecan 4 may enhance BMP signaling in control LCLs.

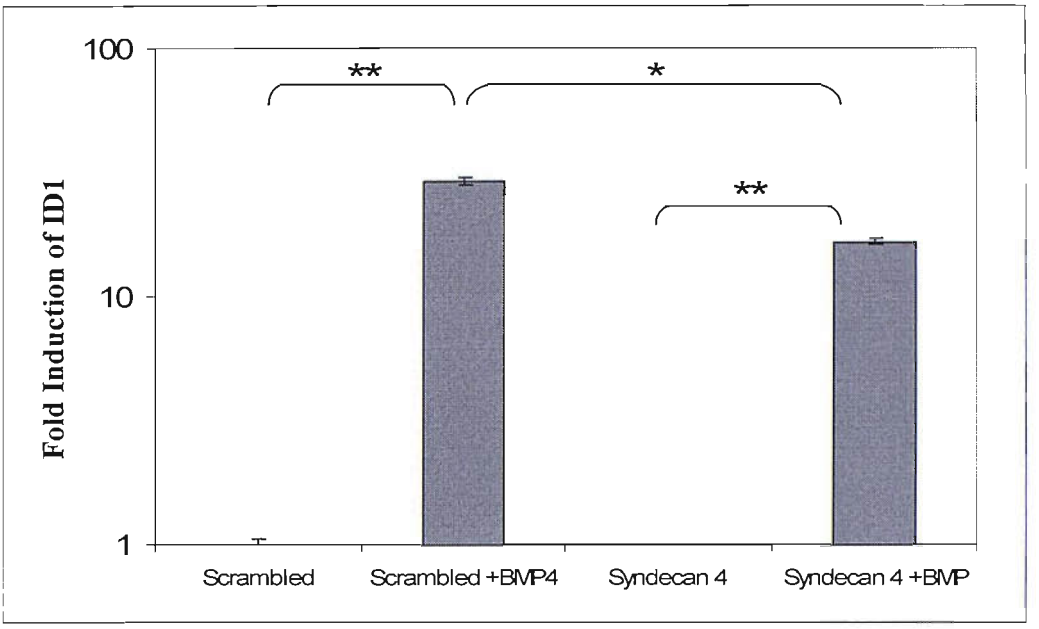


Figure 5.10: Effect of syndecan 4 downregulation by siRNA on ID1 induction in control LCLs. Fold induction of ID1 in control cells following transfection of syndecan 4 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *p<0.05, **p<0.01).

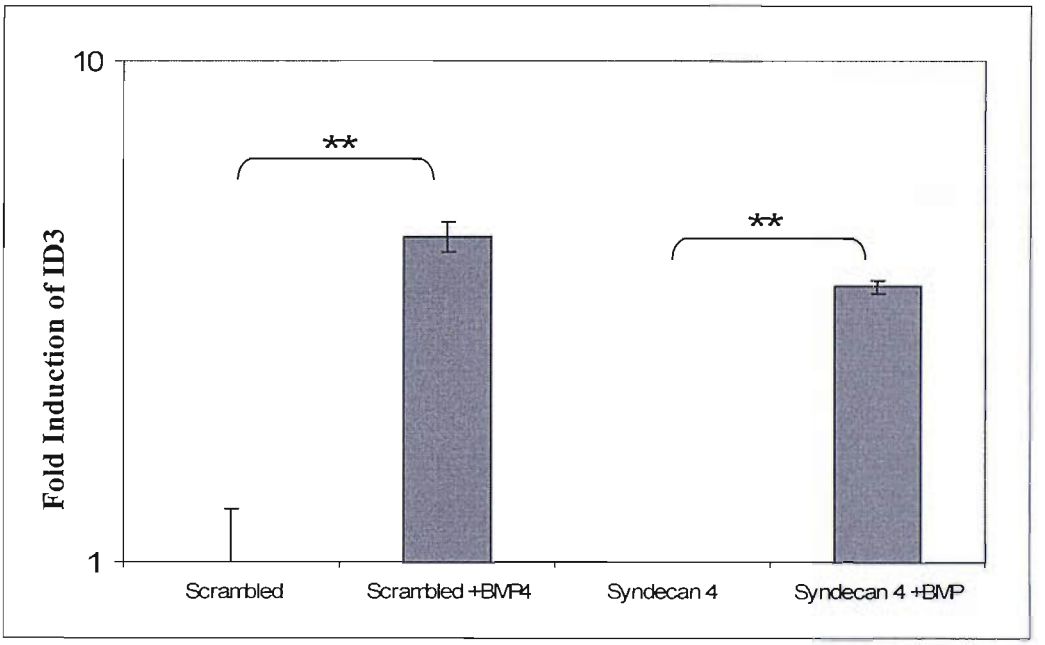


Figure 5.11: Effect on syndecan 4 downregulation by siRNA on ID3 induction in control LCLs. Fold induction of ID3 in control cells following transfection of syndecan 4 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM, **p<0.01).

Interestingly, no reduction in ID1 or ID3 expression was detected in FOP cells following siRNA transfection. FOP cells transfected with scrambled siRNA and stimulated with BMP4 induced ID1 expression approximately 40-fold (Figure 5.12) and ID3 6-fold (Figure 5.13). Following syndecan 4 siRNA transfection, BMP4 stimulated ID1 expression was induced approximately 40-fold, the same level as the scrambled siRNA induction. A similar effect was seen in FOP cells for ID3 induction (6-fold) following syndecan 4 siRNA and BMP4 stimulation. These results suggest that FOP LCLs may be resistant to the modulatory effects of syndecan 4.

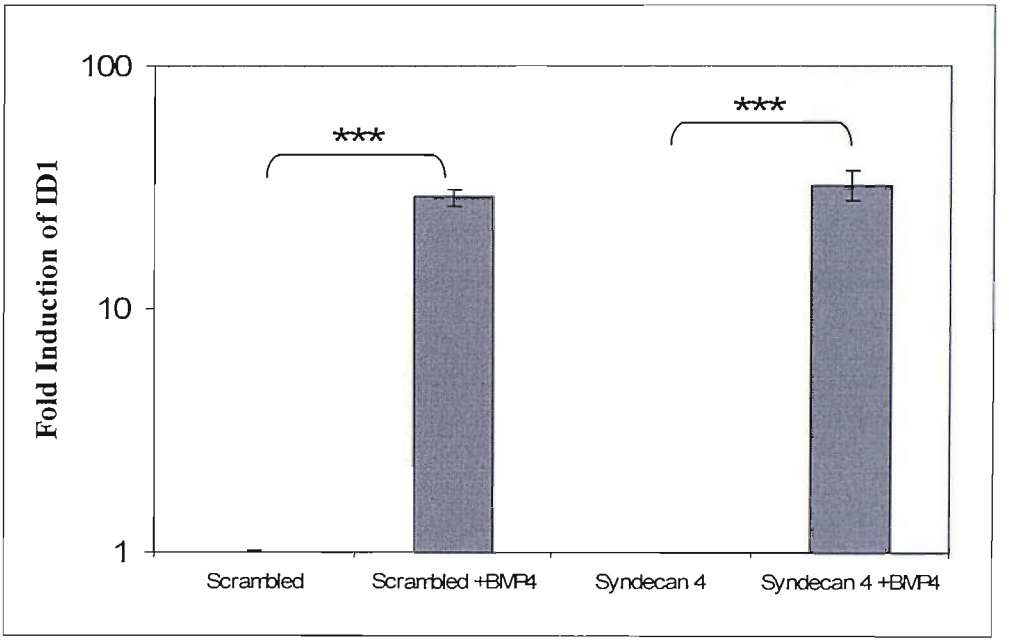


Figure 5.12: Effect of syndecan 4 downregulation by siRNA on ID1 induction in FOP LCLs. Fold induction of ID1 in FOP cells following transfection of syndecan 4 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID1 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *p<0.05, **p<0.01).

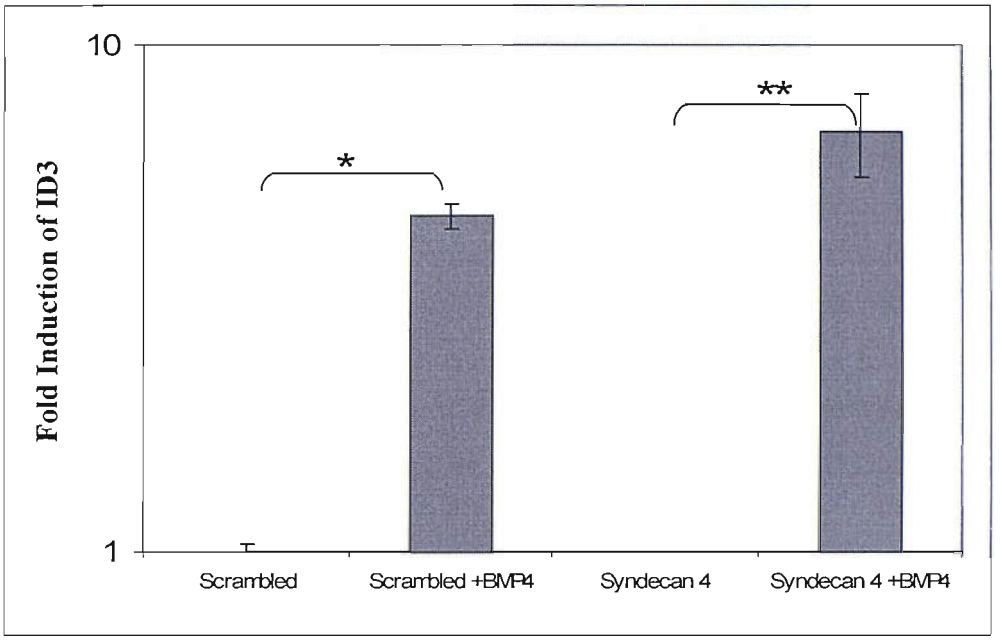


Figure 5.13: Effect of syndecan 4 downregulation by siRNA on ID3 induction in FOP LCLs. Fold induction of ID3 in FOP cells following transfection of syndecan 4 siRNA and BMP4 (200ng/ml, 1.5hrs) stimulation. ID3 mRNA expression was quantified by real time PCR and normalized to GAPDH mRNA (n=3 error bars-SEM *$p<0.05$, **$p<0.01$).

Discussion

Investigation of the presence of HSPGs by RT-PCR demonstrated that glypican 1, glypican 3, glypican 5, syndecan 3, and syndecan 4 are expressed on both control and FOP LCLs. However, using real time PCR, we demonstrated that the most abundantly expressed HSPGs are glypican 1 and syndecan 4, both of which exhibit elevated core protein levels on FOP cells. Reduction in core protein levels of glypican 1 and syndecan 4 by siRNA had opposing effects on ID1 and ID3 expression in control cells. Glypican 1 downregulation resulted in the same effects in FOP cells as control cells, however FOP cells demonstrated resistance to syndecan 4 mediated BMP signaling.

Currently very little is known about the role of syndecan 4 on BMP signaling in LCLs. However, studies have demonstrated that syndecan 4 is present on pre-B cells, acquired at a early stage of differentiation, and retained until mature B cell undergo isotype switching (Yamashita et al, 1999). Further, chemokine-stimulated migration of monocytes and lymphocytes can be inhibited by antithrombin, due to its interaction with syndecan 4, activating protein kinase C and Rho signaling (Kaneider et al, 2002). Therefore, syndecan 4 is capable of influencing cell signaling in lymphocytes.

Selective reduction of syndecan 4 core protein synthesis by siRNA decreased BMP signaling in control cells, but not in FOP cells, consistent with GAG chain removal (Chapter 3). A reduction in BMP-induced ID1 levels in control cells indicates that syndecan 4 normally enhances BMP signaling in LCLs. The enhancing effects of syndecan 4 may not be observed in FOP cells due to the already significantly increased levels of BMP signaling (Fiori et al, 2006).

In contrast to syndecans, glypicans have cysteine rich ectodomains capable of forming intramolecular disulphide bonds which result in the formation of large globular proteins that may prevent bulky molecules from interacting with GAG chains (Bernfield et al, 1999; Filmus and Selleck, 2001; De Cat and David, 2001). Selective reduction of glypican 1 by siRNA treatment increased BMP signaling in both control and FOP cells as measured by elevated ID1 mRNA levels. These data suggest that glypican 1 normally has an inhibitory role in BMP signaling in LCLs, which is unaffected by dysregulated BMP signaling in FOP cells.

Previous reports have shown that glypicans can be either stimulatory or inhibitory for different growth factors (Bonneh-Barkay et al, 1997; Iozzo and San

Antonio, 2001). For example, glypican 1 stimulated fibroblast growth factor 1 (FGF-1) activity while inhibiting FGF-7, presumably by acting as a competitive inhibitor (Berman et al, 1999). Glypicans may be responsible for the extracellular distribution of growth factors and morphogens, and removal of glypicans could disrupt these gradients, thus impairing signaling (Baeg et al, 2001). Interestingly, glypicans have also been shown to be associated with diseases of the skeleton such as Simpson-Golabi-Behmel (SGBS) syndrome. This is an X-linked condition characterized by overgrowth and skeletal anomalies resulting from glypican 3 mutations (Mariani et al, 2003).

Although siRNA is a very effective technique at reducing target gene expression, the controls used have brought about a lot of debate. In these studies the controls used were the transfection of scrambled non-targeting siRNA. Some reports have stated that the use of scrambled siRNA may be of limited value as a control. The scrambled sequence is too unrelated to the active probe to function as a truly informative control. However, the use of an antisense with a one or two base pair substitution in the middle can be a useful negative control. Unfortunately, this may convert an siRNA into an miRNA (micro RNA) that inhibits translation in a similar manner to siRNA (No authors listed, 2003). Further, the use of an empty transfection (transfection agent only) would provide an additional negative control for siRNA. This was not performed in these studies as a comparison of specific target siRNA with scrambled siRNA is more appropriate, and in each case the transfection reagent was at the same concentration. It has been further suggested that titration of the siRNA should be performed. Often, siRNA is extremely effective at minimal concentration (No authors listed, 2003). This was not performed in these studies as successful reduction of mRNA expression was achieved at the concentration used, however, titration may have provided a greater reduction in target gene expression. The most comprehensive control is to use ectopic expression of an siRNA insensitive copy of the target gene. This will in turn rescue the observed phenotype (Lehner et al, 2004). Multiplicity controls have also been proposed. The use of two or more siRNAs to different sites in the same target gene to reproduce the reduction in gene expression (No authors listed, 2003). Multiplicity controls were not performed in these experiments but the siRNA mastermix contained 4 duplex's targeted to different sites of the target gene. This results in an effective method to reduce the target gene but does not show a reduction from independent experiments.

This data presented allows us to propose a hypothetical schema of glypican and syndecan action on BMP signaling in LCLs. The combination of large globular heads on glypicans along with the proximity of covalently attached GAG chains allows them to act as inhibitors of BMP signaling. In contrast, the extended protein structure of syndecans and the relative position of their GAG chains may allow them to bind both ligand and receptor, enhancing signaling. In FOP LCLs, we observe the inhibitory action of glypican, however, the enhancing effect of syndecan may not be seen due to already elevated levels of BMP signaling.

These studies have demonstrated that FOP cells have elevated core protein levels of glypican 1 and syndecan 4. Further, FOP cells are resistant to the modulating effects of syndecan 4, but not glypican 1 on BMP signaling.

Chapter 6

Conclusions and Future Directions

Chapter 6: Conclusions and Future Directions

Summary of Major Findings

To further understand the role of methylation in osteogenic differentiation, alkaline phosphatase activity was examined in C2C12 cells that were unresponsive to BMP2. These studies showed that the alkaline phosphatase gene can be methylated, preventing osteogenic differentiation, and demonstrating an epigenetic control mechanism.

To examine other factors that can modulate BMP signaling and osteogenic differentiation, HSPG profiling and the effect of GAG chain removal was performed in C2C12 cells. HSPG mRNA expression changed during osteogenic differentiation and expression of alkaline phosphatase decreased following HSPG GAG chain removal. These results suggested that not only is alkaline phosphatase activity required for osteogenic differentiation, but so are HSPG GAG chains.

In order to understand the modulating activity of HSPGs on BMP signaling pathways, it is crucial to elucidate their functions under normal and pathological conditions. FOP is a devastating disease of ectopic bone formation and lymphoblastiod cell lines (LCLs) from these patients exhibit dysregulated BMP signaling. HSPGs are present on the surface of LCLs, providing a model to understand HSPG associated BMP signaling. Studies demonstrated that HSPGs play a critical role in enhancing and inhibiting BMP signaling under normal conditions, which is dysregulated in FOP. An elevation of total and HSPG-specific GAG chains and HSPG core proteins for GAG chain attachment provide increased BMP ligand binding sites that may contribute to the profound elevation of BMP signaling seen in FOP cells.

The goal of this final chapter is to discuss the results presented in earlier chapters in the wider context of epigenetics and HSPG modulatory effects on BMP signaling, and to discuss future research directions that can build upon these data, hopefully leading to therapeutic benefits for skeletal diseases, such as FOP.

Epigenetics in Osteogenic Differentiation

Epigenetic gene silencing by methylation has been shown to influence signaling pathways both positively and negatively. In desmoid-type fibromatosis with focal metaplastic bone formation, BMP signaling was increased at the ossifying focus, but not at the typical desmoid-type fibromatosis. The increase in BMP signaling was due to hypermethylation of the BAMBI promoter. This epigenetic event which led to increased BMP signaling may have played a crucial role in the formation of metaplastic bone (Kitazawa et al, 2005), similar to the elevation of BMP signaling in FOP which leads to the formation of heterotopic bone. Our data demonstrated that following non-specific demethylation, alkaline phosphatase activity increased in C2C12 cells in response to BMP2. This suggests that the methylation status of genes provides an epigenetic control mechanism for osteogenic differentiation. Methylation prevents chromatin unwinding and transcription factor binding, resulting in gene silencing and an inhibition of differentiation. However, gene transcription cannot be completely controlled by methylation since some genes, such as osteocalcin, are not methylated in all cell types (Locklin et al, 1998).

Recent studies have shown that culture conditions can affect the methylation status of DNA. Hypermethylation can occur in cultured rat hepatocyte cell lines, but this effect is not seen in primary cell lines (Asada et al, 2006). However, it has also been shown that colon cancer cell lines have a similar pattern of methylation to primary colorectal carcinomas (Lind et al, 2004). In Chapter 2, we examined the methylation status of the DNA in a C2C12 cell line that was unresponsive to BMP2 and therefore did not undergo osteogenic differentiation. Based on the aforementioned studies, it is possible that the alkaline phosphatase gene was hypermethylated in these cells, a result which may be an artifact of *in vitro* cell culture conditions. Therefore, this may not be representative of the *in vivo* DNA methylation status, however it provides a cell system in which to study the epigenetic effect of methylation in the control of osteogenic differentiation.

HSPGs and BMP Signaling

The diverse roles of HSPGs in signaling have been illustrated consistently in both the literature and throughout this thesis. One of the many roles of HSPGs include influencing and modulating different signaling pathways, such as transforming growth factor beta (including BMPs), Wnt, fibroblast growth factor (FGF), and hedgehog (Lin, 2004).

The roles in signaling include the following: HSPGs have been shown to modulate BMP activity during limb cartilage differentiation (Fisher et al, 2006); HSPGs are required for the successful cellular uptake of the BMP antagonist chordin, which is also potentiated by HSPGs (Jasuja et al, 2004); HSPGs are required for FGF2 and BMP7 signaling (Midorikawa et al, 2003); BMP and FGF signaling during renal branching morphogenesis is modulated by HSPGs, specifically glypican 3 (Grisaru et al, 2001); and Zebrafish Ext2 and Extl3, enzymes required for heparan sulfate biosynthesis, are necessary for FGF10 signaling during limb development (Norton et al, 2005).

The data presented in Chapters 2 and 3 demonstrated that removal of HSPG GAG chains resulted in a reduction in BMP signaling, measured by decreased expression of alkaline phosphatase in C2C12 cells and ID1 and ID3 in LCLs. These results are reinforced by other studies demonstrating that GAG chain removal results in decreased Smad phosphorylation, and therefore BMP signaling, following BMP treatment (Irie et al, 2003).

Alkaline phosphatase is under the control of the Wnt pathway, acting through beta-catenin. ID1 and ID3 are BMP pathway specific target genes. The ligands used in these studies were BMP2 and BMP4, therefore the reduction of ID1 and ID3 expression following HSPG GAG chain removal was due to decreased BMP signaling. The reduction in alkaline phosphatase activity following HSPG GAG chain removal and BMP2 treatment must also be an effect of reduced BMP signaling, and not Wnt signaling since BMP is not a ligand for the Wnt receptors. These results suggest that pathway crosstalk (BMPs / Wnts) can be influenced by the modulatory effects of the HSPG GAG chains.

Interestingly, removal of all GPI-linked proteins, which includes the HSPG family of glypicans, enhanced signaling under normal conditions. This may demonstrate a dual function for the HSPGs, with some subtypes acting as suppressors of signaling and others as enhancers of signaling. However, the effects of HSPG GAG chain removal and GPI-linked protein removal are not seen in cells from FOP patients. This suggests that due to the dysregulation of BMP signaling seen in FOP cells (de la Pena et al, 2005; Fiori et al, 2006), the modulatory roles of HSPGs may be negated.

Heparan sulfate proteoglycans are not the only proteoglycans that have GAG chains attached. Along with heparan, there are also dermatin, keratin, and chondroitin sulfate proteoglycans. Based on the elevated total GAG chain levels on

FOP cells demonstrated in Chapter 4, it is plausible that other proteoglycans may be compensating for the reduction in HSPG GAG chains by heparinase treatment and GPI-linked proteins by PI-PLC treatment, resulting in no effect on BMP signaling. In addition, the elevated levels of total GAG chains on FOP cells may be so abundant that the heparinase digestion is not sufficient to remove all GAG chains or structural differences, such as GAG chain sulfation patterns, may be altered in FOP, providing resistance to exogenous enzymatic cleavage.

Due to the diversity of HSPGs and their multiple roles, dissecting the function of individual HSPGs can be difficult. Following HSPG mRNA and protein profiling, glypican 1 and syndecan 4 were found to be the most abundantly expressed HSPGs in LCLs. However, increased levels of core proteins were found on the surface of FOP cells. This discovery reinforces the increased GAG chain levels seen on FOP cells since an increase in core protein levels will provide increased binding sites for GAG chain attachment. Selective downregulation of glypican 1 increased BMP signaling in both control and FOP cells, suggesting that glypican 1 can act as an inhibitor of signaling in LCLs. However, selective downregulation of syndecan 4 reduced BMP signaling in control, but not FOP cells. This suggests that syndecan 4 acts to enhance BMP signaling in control cells, but FOP cells are resistant to the stimulatory effects of syndecan 4. In addition, it is possible that the elevated BMP signaling seen in FOP cells is so high that the fine tuning stimulatory role of HSPGs (such as syndecan 4) may not be required, but the inhibitory functions are still present. This data reinforces the hypothesis of subtype specific roles for the cell surface HSPGs.

The data presented here demonstrate that HSPGs are involved in the dysregulation of BMP signaling, but are not the underlying cause of FOP. Although HSPGs, with few exceptions such as Simpson-Golabi-Behmel syndrome (SGBS), are not usually directly responsible for disease, they play role in the pathophysiology. It is clear that inactivating mutations in *EXT1* and *EXT2*, genes required for HSPG biosynthesis, result in multiple hereditary exostoses (MHE) (Duncan et al, 2001), but the molecular pathophysiology of osteochondroma formation remains unknown (Tumova et al, 2000). Most FOP patients present with osteochondromas, suggesting a role for HSPGs in this disease, as dissected, in part, in this report.

Implications for Treatments

Although the next stage in FOP research will be the development of transgenic mice with an identical mutation in the ACVR1 gene as FOP patients, effective preventions and treatments derived from the study of these mice will take many years. The more immediate potential for therapy provided through targeting HSPGs may provide benefits for patients both short and long term. Targeted downregulation of GAG chain synthesis to levels comparable to unaffected individuals may provide a reduction in BMP signaling that will reduce the formation of heterotopic bone. Reducing HSPG subtypes that are stimulatory or increasing HSPG subtypes that are inhibitory to BMP signaling may also reduce heterotopic bone formation. Insulin-like growth factor 1 (IGF-1) has been shown to stimulate the expression of cell surface HSPGs in breast cancer cells (Mitropoulou et al, 2004). However, modification of HSPGs expression could results in unwanted effects due to the complexity of their role in both signaling and development.

Since the mutation in ACVR1 results in a mutated BMP receptor, the treatment therapies may need to be targeted directly towards the receptor or downstream signaling pathways. ACVR1 is expressed in many tissues including skeletal muscle and chondrocytes, and constitutive activation of this receptor results in upregulation of BMP4, downregulation of BMP antagonists, and induction of alkaline phosphatase (Payne et al, 2001; Zhang et al, 2003). The ACVR1 mutation in FOP suggests leaky or constitutive activation of the receptor, which is consistent with increased BMP signaling seen in FOP cells (de la Pena et al, 2005; Fiori et al, 2006; Shore et al, 2006). An increase in BMP signaling through ACVR1 has been shown to increase Ihh signaling, which has subsequently been attributed to the formation of osteochondromas (Grimsrud et al, 2001; Minina et al, 2001; Zhang et al, 2003; Koziel et al, 2004). Therefore, the osteochondromas observed in FOP may be due to increased Ihh signaling downstream of elevated BMP signaling as a result of the mutation. Based on these data, the increased HSPG core proteins and GAG chains on FOP cells are likely to be a secondary effect of the primary mutation. The increased HSPGs may be unable to sufficiently modulate the promiscuous activity of ACVR1.

Future Directions

In order to further elucidate the roles of HSPGs on BMP signaling in FOP, continued investigation is required. Based on the known interactions of HSPGs with

BMPs, and the recently discovered mutation in ACVR1, an investigation into HSPGs interactions with ACVR1 is required. Similarly, an in-depth study of HSPG modulation of BMP signaling using different ligands (BMP2, BMP6, BMP7) must be carried out. Since heparinase cleaves sulphated regions of HSPG GAG chains, a detailed study of the sulfation patterns of GAG chains on control and FOP cells may explain why FOP cells do not respond in the same manner as control cells to heparinase treatment. Due to osteochondroma formation in FOP and MHE, an analysis and comparison of the enzymes involved in heparan sulfate biosynthesis in control and FOP cells needs to be performed.

While the majority of this work focused on the roles of HSPGs on BMP signaling, investigations into the effects of methylation on osteogenic differentiation were also performed. Further studies include the use of methylation specific restriction enzymes to determine if regions of the alkaline phosphatase gene are methylated. Examination of the methylation status of other osteogenic master transcription factors, such as Runx2, should also be performed in these C2C12 cells to determine whether methylation of other genes, in addition to alkaline phosphatase, prevents osteogenic differentiation.

Concluding Remarks

The recent discovery of the FOP gene (ACVR1) and the previously reported evidence of increased BMPRIA, along with the data presented here, demonstrate that although FOP is caused by a single nucleotide change, the effects on the signaling networks are substantial, affecting direct and indirect pathway modulation. The demonstration of increased levels of GAG chains, differences in HSPG subtype specific functions, and altered HSPG mediated downstream signaling provides additional evidence for dysregulated BMP signal transduction in FOP. Dissecting the molecular biology of the pathophysiology of FOP is the first step towards establishing the underlying causes of this disabling disorder and implementing effective treatment regimes and, ultimately, a cure.

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