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THE ROLE OF SECRETED PHOSPHOPROTEIN-24 IN OSTEOLAST  
DIFFERENTIATION AND MATRIX MINERALIZATION

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

by

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## LIST OF ABBREVIATIONS

ActR	Activin-like Receptor
AHO-like	Albright's hereditary osteodystrophy-like
ALK	Activin-like Kinase
Alp	Alkaline Phosphatase
APC	Adenomatosis Polyposis coli
ATP	Adenosine triphosphate
BAMBI	BMP and Activin membrane bound inhibitor
BBP	BMP-binding protein
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
CaCN	Calcineurin
CamKII	Calcium/calmodulin-dependent protein kinase-II
CBFA1	Core Binding Factor A1
CDMP	Cartilage-Derived Matrix Protein
C/EBP $\beta$	CCAAT/enhancer binding protein $\beta$
CMV	Cytomegalovirus
coSmad	co-mediator Smad
COL1a1	Collagen type I alpha I
COL4a3	Collagen type IV alpha III
COL5a3	Collagen type V alpha III
COMP	Cartilage Oligomeric Matrix Protein
CR	Cysteine-Rich
DD	Differential Display
DEPC	Diethyl Pyrocarbonate
DLL3	Delta-like ligand 3
DNA	Deoxyribonucleic acid
DMEM	Dulbeco's modified eagle medium
DSH	Dishevelled
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factors
EST	Expressed Sequence Tag
FACIT	Fibril Associated Collagens with Interrupted Triple helices
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
Fgfr3	fibroblast growth factor receptor 3
FMC	Fetuin Mineral Complex
FRP	Frizzled-related protein

GDF	Growth Differentiation Factor
GPI	Glycosylphosphatidylinositol
GSK-3 $\beta$	Glycogen Synthase Kinase 3 $\beta$
HC	Hypercalcemic
HECT	Homology to E6Ap Carboxyl Terminus
H&E	Hemotoxylin and Eosin
HGF	Hepatocyte Growth Factor
HOXA11	Homeobox A11
IGF	Insulin-like Growth Factor
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
Ihh	Indian Hedgehog
I-Smad	Inhibitory Smad
JNK	c-Jun N-terminal kinase
kDa	Kilodaltons
LB Media	Luria-Bertani Media
LC	Hypocalcemic
LRP	Low-density Lipoprotein receptor-related protein
Mad	Mothers against decapentaplegic
MAP kinase	Mitogen-activated protein kinase
M-CSF	Macrophage Colony Stimulating Factor
MGP	Matrix Gla Protein
MMP	Matrix Metalloproteinase
MOI	Multiplicity of Infection
mRNA	messenger RNA
MSCs	Mesenchymal Stem Cells
NC	Normal calcium
NCP/BMP	Non-Collagenous Protein/Bone Morphogenetic Protein
OP	Osteogenic Protein
OPG	Osteoprotegrin
OPN	Osteopontin
OSX	Osterix
PDGF	Platelet-Derived Growth Factor
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PKA	Protein kinase A
PKC	Protein kinase C
PPAR $\gamma$	Peroxisome proliferator-activated receptor- $\gamma$
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
Q-PCR	Quantitative Polymerase Chain Reaction
RANK	Receptor Activator of NF $\kappa$ B

RANKL	Receptor Activator of NFκB Ligand
RGM	Repulsive Guidance Molecule
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonulceic Acid
R-Smad	Receptor activated Smad
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RUNX2	Runt related transcription factor 2
SARA	Smad Anchor for Receptor Activation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIBLING	Small Integrin-Binding Ligand, N-linked Glycoprotein
SOST	Sclerotisin
SOX	SRY-box Containing Gene
Spp18	Secreted Phosphoprotein- 18
Spp24	Secreted Phosphoprotein-24
TBS	Tris-Buffered Saline
TGFβ	Transforming Growth Factor β
TGFβR	Transforming Growth Factor β Receptor
TGFβRII	Transforming Growth Factor β Receptor Type II
Trb3	Tribbles-like protein 3
VEGF	Vascular Endothelial Growth Factor
Vgr	Vegetal related
VCU	Virginia Commonwealth University
WIF	Wnt Inhibitory Factor
1,25(OH) <sub>2</sub> D <sub>3</sub>	1,25 dihydroxyvitamin D <sub>3</sub>

## Abstract

### THE ROLE OF SECRETED PHOSPHOPROTEIN-24 IN OSTEOBLAST DIFFERENTIATION AND MATRIX MINERALIZATION

By Samuel C. Ramage, PhD.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2007

Major Director: Matthew J. Beckman, PhD.  
Assistant Professor, Department of Biochemistry and Molecular Biology

Secreted Phosphoprotein-24 (Spp24) was initially isolated and characterized as a component of bovine cortical bone matrix. Subsequent characterization has shown it is multiply phosphorylated and homologous to cystatin and TGF- $\beta$  receptor type II. Spp24 is a minor component of the serum fetuin mineral complex that binds calcium-phosphate minerals and prevents their deposition. The TGF- $\beta$  receptor homology domain binds BMP-2 weakly *in vitro* and enhances BMP-2's osteogenic effects *in vivo*. The ability of Spp24 to

affect BMP activity suggests an important role for Spp24 as a native, bioactive component of bone that regulates bone development.

Spp24 was highly up-regulated in rat cortical kidneys following a low calcium diet regime. Tissue distribution of both Spp24 protein and RNA showed that while Spp24 accumulates in bone, a majority is produced at distant sites, namely the liver and kidney. Additionally, Spp24 was present in more tissues than previously believed. Spp24 migrates to a number of different molecular weights, suggesting multiple, alternative post-translational modifications may generate subtly different forms of the protein. The expression of Spp24 in the kidney may be regulated to counteract changes in serum mineral levels. Additionally, homology in the Spp24 sequence suggests that it, like other bone and dentine matrix proteins, may interact with mineral as an important influencer of mineral calcification.

Utilizing microarray analysis of primary bone marrow-derived mesenchymal stem cells transduced with Spp24 and control viruses we examined changes elicited by the overexpression of Spp24. A change in overall morphology was observed for cells transduced with the Spp24 similar to changes described in cells undergoing osteoblastic differentiation. Nodule formation was also seen in the Spp24 transduced cells. Microarray results showed key markers of osteoblast differentiation, CBFA1/RUNX2 and osterix (OSX), were not up-regulated although there were distinguishable changes in the gene expression profile of mesenchymal stem cells. The cells appeared to be blocked from differentiation into a number of mesenchymal lineages: adipocytes, myocytes and chondrocytes. The changes appeared to prime cells for signals that activate osteoblast

differentiation by blocking other pathways and altering internal signaling response pathways to those signals.

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# **Chapter One**

## **General Introduction**

Bone is an important organ with myriad functions in the body ranging from mechanical support of the body to the protection of internal organs; it is also a critical metabolic tissue used as a reservoir for vital minerals. Bone is a specialized extracellular matrix laden with calcium phosphate crystals in the form of hydroxyapatite,  $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ . Bone is not a static mechanical support but rather is in a constant state of flux with bone producing cells, called osteoblasts, and bone destroying cells, called osteoclasts, in constant juxtaposition. Osteoblasts are responsible for the creation of the complex extracellular matrix that provides the framework for cell attachment and mineral deposition that enhances the mechanical strength of the bone while osteoclasts are uniquely specialized to dissolve the mineral and then degrade the extracellular matrix of bone. The continuous activity of these adversarial processes results in the turnover of the entire skeleton roughly every 10 years (Parfitt 1998); the coupled processes of bone formation and destruction are crucial to the strength of healthy bone and the repair of bone injuries and fractures. Disruption of the delicate balance of these two opposing functions causing



an excess of either formation or destruction can lead to serious pathophysiological consequences.

Bone development is a highly synchronous process with a multitude of cytokines, growth factors, and other signaling molecules carefully orchestrating the recruitment, differentiation, development, and function of osteoblasts. Particularly important are wnt family proteins and members of the transforming growth factor- $\beta$  (TGF- $\beta$ ) family of cytokines including bone morphogenetic proteins (BMPs). Similarly, a variety of signals control osteoclastic bone resorption by regulating osteoclast differentiation and action.

This introduction will cover bone remodeling including the role of osteoblast and osteoclasts; the basics of skeletal development; the differentiation of osteoblasts; and the role of BMPs in osteoblast function. The focus will then shift to convey the current understanding of Spp24 and its relation to several known bone matrix proteins.

## **Cellular Composition of Bone**

### *Osteoblasts*

Osteoblasts have specific molecular and morphological adaptations making them particularly well suited for the task of producing the bone matrix. Osteoblasts are mesenchymally derived cells that produce osteoid. They make prodigious amounts of collagen type I, the principal bone ECM component. Smaller amounts of type III and V collagen (Ashhurst 1990; Wood, Ashhurst et al. 1991) are present as part of the bone matrix as well as bone sialoprotein and other non-collagenous matrix proteins. Several of the FACIT (fibril associated collagens with interrupted triple helices) collagens, which are

non-fibril/non-triple helical collagens that associate with fibril collagens, have been found in bone and articular cartilage including collagen type IX, XII, and XIV (Walchli, Koch et al. 1994; Dharmavaram, Huynh et al. 1998; Opolka, Ratzinger et al. 2007). Osteoblasts have a large rough endoplasmic reticulum and Golgi apparatus (Baud 1968; Cameron 1968; Mandi, Gyarmati et al. 1974); the large volume of proteins that are produced and eventually directed to the ECM requires this greater than normal amount of intracellular machinery for the production and export of these proteins.

Terminally differentiated osteoblasts take on a cuboidal shape while the proliferating osteoblast precursors maintain a more spindle-shaped morphology (Nijweide, van der Plas et al. 1988; Cheng, Yang et al. 1994). Osteoblast in *in vitro* culture cluster together forming closely associated nodules (Nefussi, Boy-Lefevre et al. 1985; Bhargava, Bar-Lev et al. 1988). Osteoblasts are in close contact with each other in bone; N-cadherin and cadherin-11 play a major role in forming the junctions between osteoblasts in addition to several integrins (Cheng, Lecanda et al. 1998; Ferrari, Traianedes et al. 2000; Lecanda, Cheng et al. 2000). Terminal differentiation is also marked by increased expression of several mineral-interacting non-collagenous proteins such as osteopontin, osteocalcin, and bone sialoprotein (Huq, Cross et al. 2005). Extracellular matrix vesicles are deposited by osteoblasts, and chondrocytes, acting to accumulate sufficient concentrations of component ions of hydroxyapatite crystals to aid in mineralization of the matrix (Anderson 1989; Wiesmann, Meyer et al. 2005). It is thought that a number of constituents of the extracellular matrix vesicles, such as phospholipids and mineral ions, can act to form a

nucleation core for the growing crystal, aiding in the formation of the initial crystal – the most energy consuming step in the process of mineral formation (Wuthier 1989).

An oft utilized characteristic of these cells is their production of alkaline phosphatase. Many histological examinations of bone utilize staining to detect alkaline phosphatase as a marker of osteoblasts in tissue or osteoblast character in differentiating cells. Alkaline phosphatase's role in mineralization was debated for some time with different proposed roles for alkaline phosphatase such as degrading inhibitors of mineralization; however the hypothesis of Robison that alkaline phosphatase creates a local increase in inorganic phosphate concentration by hydrolyzing phosphate esters (Robison 1923) appears to have been borne out.

### *Osteocytes*

As the functional osteoblasts create bone matrix they are surrounded within their own organic matrix; this matrix eventually becomes mineralized, trapping the osteoblast inside the bone. These trapped osteoblasts extend long processes and decrease their size and abundance of organelles as they become less metabolically active. Transformed osteoblasts embedded within the bone interior are called osteocytes. Osteocytes are the most numerous cells in the bone, largely occupying the interior volume of the bone while most other cells such as osteoblasts and osteoclasts are present only on or near the bone surface. Osteocytes produce far less extracellular matrix than the more active osteoblasts (Nefussi, Sautier et al. 1991). Osteocytes are important regulators of bone metabolism and communicators of mechanical stresses upon the bone. Osteocytes have a large number of

long cellular projections that connect to other osteocytes through small tunnels in the bone. The small tunnels, or canaliculi, form an extensive network that allow interaction of osteocytes and connect internal cells to the bone surface. Deformation of the osteocyte due to strains on the bone and fluid flow through the canaliculi have both been shown to act through osteocytes to create changes in bone metabolism (Klein-Nulend, Helfrich et al. 1998; Plotkin, Mathov et al. 2005). A large number of different mechanisms have been explored in the detection and communication of mechanical stress. Membrane ion channels that are activated by the mechanical stress have been found in bone cells (McDonald and Houston 1992; Ypey, Weidema et al. 1992; Rawlinson, Pitsillides et al. 1996). In addition, integrins that act both as extracellular adhesion molecules and transmembrane receptors (Hughes, Salter et al. 1993); the plasma membrane and its constituent proteins (Rubin, Rubin et al. 2006); and cytoskeletal structures and their associated proteins all likely play a role in the mechanotransduction in bone (Rubin, Rubin et al. 2006). One interesting anabolic effect on bone in response to stress is the rapid upregulation of the canonical wnt signaling in response to loading (Robinson, Chatterjee-Kishore et al. 2006; Armstrong, Muzylak et al. 2007). Osteocytes, though not directly involved in the formation and destruction at the bone surface, are critical components of healthy bones and the maintenance of bone metabolism, and they sense and relay stresses to the cellular components of bone.

### *Osteoclasts*

Mature, multi-nucleated osteoclasts are formed by the fusion of mono-nuclear osteoclast precursors. Activation occurs as the non-resorbing or inactive osteoclast reaches and adheres to the bone surface. Morphological changes allow the creation of a contained compartment between the osteoclast and the bone called the resorption lacuna that is the site of resorption. The degraded bone fragments are then removed and secreted away from the bone surface. The specialized morphology of the osteoclast makes it extremely efficient at absorbing bone.

Active osteoclasts are highly polarized cells with specific features characterizing the surface adjacent to the bone. After recruitment, receptors on the plasma membrane interact with the bone matrix to form the sealing zone, a tight interaction that creates a sealed extracellular compartment between the osteoclast and bone. A portion of the cell membrane, called the ruffled border, encircled by the sealing zone undergoes changes resulting in significant endosomal character and finger-like projections of the membrane towards the bone surface. Activation of the osteoclast triggers intracellular vesicles to fuse with the membrane contained within the sealing zone. Fusion releases acid ( $H^+$ ) and other vesicular contents into the resorption lacuna (Blair, Teitelbaum et al. 1989; Vaananen, Karhukorpi et al. 1990). Fusion also increases the amount of membrane encompassed by the sealing zone, leading to the

development of the folds and extensions of the membrane into the resorption lacuna. Within the ruffled border a high concentration of vacuolar ATPase were found (Vaananen, Zhao et al. 2000). Osteoclasts use the many vacuolar ATPases to pump  $H^+$  into and further acidify the resorption lacuna. Vesicle fusion and the activity of vacuolar ATPases are key functions of the ruffled border that create an environment conducive to bone resorption within the resorption lacuna. Degradation products from the organic bone matrix are endocytosed by the osteoclast and trafficked to the apical region of the cell, called the functional secretory domain, where they are secreted into the extracellular space.

Following osteoclast activation the resorption lacuna becomes highly acidic as fusion of intracellular acidic vesicles with the ruffled border releases acid into the area of active resorption and vacuolar ATPases pump protons across the membrane into the resorption lacuna. The low pH achieved is necessary to dissolve the hydroxyapatite crystals surrounding the organic matrix of the bone. Mineral crystals in bone must be dissolved for the degradative enzymes secreted by the osteoclast to access the organic matrix. Hydroxyapatite crystals in the bone store a majority of the calcium in the body, and osteoclastic dissolution of the mineral component of bone is an important mechanism for maintaining serum levels of calcium and other ions contained in bone such as phosphate. Accordingly, the activity of osteoclasts is regulated in response to conditions such as hypocalcemia to maintain suitable concentration ranges of those ions.

Following demineralization the osteoclast secretes enzymes that further degrade the bone. The extracellular matrix made by osteoblasts and osteocytes is principally composed

of collagen but contains other non-collagenous extracellular matrix proteins as well. The matrix is principally degraded by two types of proteases, cathepsins and metalloproteinases (MMPs). Cathepsins are lysosomal proteases active at acidic pH. Their activity at low pH shows the importance of the osteoclast machinery responsible for lowering the pH of the resorption lacuna in providing a suitably acidic environment for cathepsins and other acidic proteases in addition to dissolving the hydroxyapatite. Osteoclasts produce cathepsin B, C, D, E, G, L, and K (D and E are aspartatic proteases while the others are cysteine proteases) (Goto, Yamaza et al. 2003). Cathepsin K, a lysosomal cysteine protease, has high enzymatic activity towards type I collagen, the main component of bone, as well as type II collagen (Bromme, Okamoto et al. 1996; Kafienah, Bromme et al. 1998). Cystatin C, a secreted protein produced by osteoclasts, osteoblasts, as well as cells in the liver and kidney (Yamaza, Tsuji et al. 2001), is an inhibitor of cysteine proteases. *In vitro* assays have shown the addition of cystatin C decreases bone resorption (Lerner, Johansson et al. 1997).

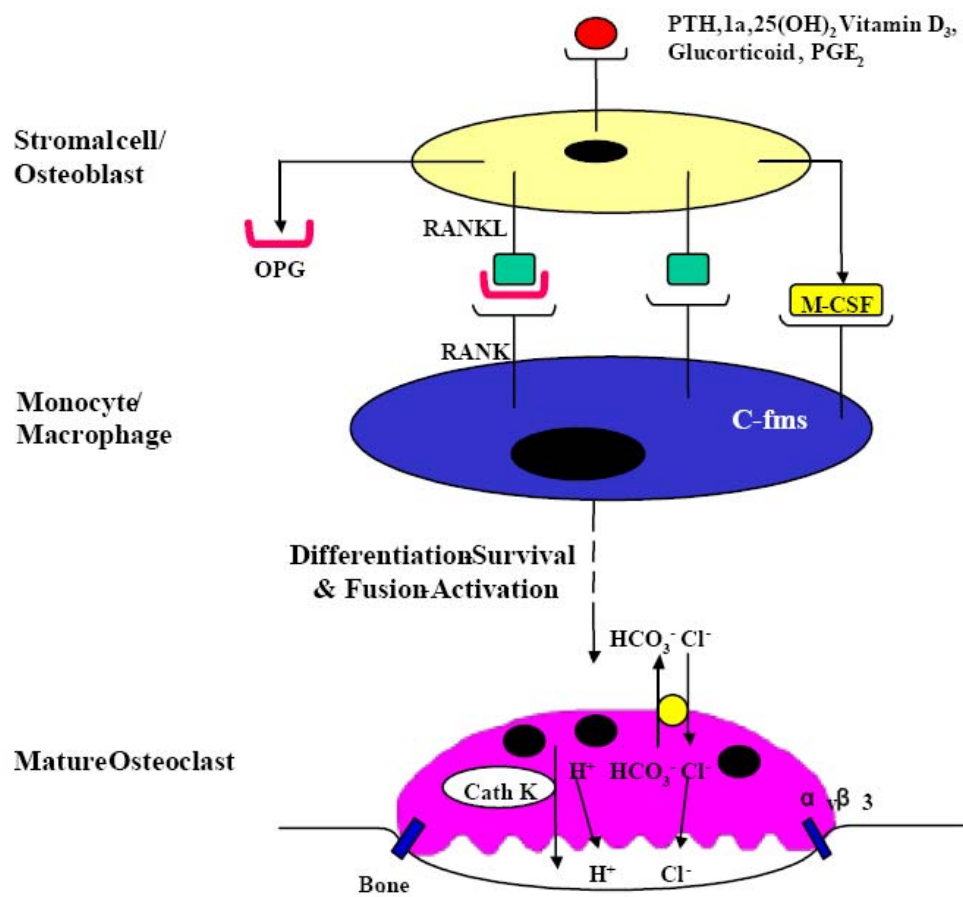
MMPs are zinc-dependent proteases produced by a wide variety of cells in the body. In bone osteoblasts, osteocytes, osteoclasts, and cells adjacent to and associated with osteoclasts at the bone surface are all capable of producing MMPs. Many of the MMPs present in bone show degradative activity against collagen. While most overlap in the ability to degrade collagen and gelatin in some manner their specificity betrays their native substrate. Several MMPs are made by osteoclasts including MMP-2, 9, 12, and 13. MMP-13 is a collagenase capable of degrading triple helical collagen fibers. MMP-2 and -9 are both gelatinases capable of degrading gelatin, byproducts of collagen hydrolysis. MMP-13

is also known as macrophage metalloelastase as it has high specificity for hydrolyzing elastin in addition to other ECM proteins. MMPs function in bone matrix degradation as well as being important in allowing osteoclast migration. MMP-9 and -14 are critically important, though distinctly so, in allowing osteoclast migration in bone (Delaisse, Andersen et al. 2003). MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs). Treating bone resorptive pathologies by targeting MMPs has proven an important and successful pharmacological methodology. Although the administration of bisphosphonates is used to inhibit and downregulate MMPs in bone resorption and other diseases such as the metastases of cancer (Teronen, Heikkila et al. 1999), long term consequences of these therapies are proving to be serious; numerous reviews detail these effects including the greatly reducing bone turnover rate as well as the conferring a greater risk of developing osteonecrosis of the jaw (Liberman 2006; Ponte Fernandez, Estefania Fresco et al. 2006; Woo, Hellstein et al. 2006).



**Figure 1: Osteoclast Differentiation and Bone Resorption**

Extensive research has showed that the accessory cells (osteoblasts and stromal cells) express two key molecules that are essential and sufficient to promote osteoclastogenesis: M-CSF and RANKL. Osteoclasts are multinucleated giant cells deriving from hematopoietic progenitors of the monocyte–macrophage lineage. Stromal cell/osteoblast cells express RANKL, OPG, and M-CSF and their expression is regulated by factors such as  $1,25(\text{OH})_2\text{D}_3$ , PTH,  $\text{PGE}_2$ , and glucocorticoids. RANKL and M-CSF will interact with the cell surface receptors on mono/macrophage cells. These will trigger the osteoclastogenesis process. These postmitotic committed precursors fuse to form multinucleated osteoclasts, which are then activated to resorb bone. Osteoblasts or bone marrow stromal cells are required as supporting cells for the in vitro differentiation of osteoclasts from their progenitor cells. Source: Adapted from American Association for the Advancement of Science (Teitelbaum 2000); Springer (Quinn, Neale et al. 1998); and International and American Association for Dental Research (Reddy and Roodman 1998).



## **Bone Remodeling**

The extracellular matrix of bone is continually undergoing both resorption and formation by cells of the bone. This activity, termed bone remodeling, is important in maintaining the mechanical properties of bone necessary to support the weight of the entire body and in releasing important minerals and growth factors embedded in the bone matrix. Early in bone development, at embryonic and nascent stages, the bone matrix is a more porous, disordered mat of collagen fibers known as woven bone (Weiner and Wagner 1998). As development progresses, bone remodeling removes and replaces the woven bone with the mature lamellar bone that is easily identified by its parallel collagen fibers that endow the bone with greater mechanical strength (Martin, Lau et al. 1996). Bone remodeling is also critical in the maintenance of mineral homeostasis within the body. Calcium and phosphate, the primary components of hydroxyapatite crystals, are liberated from bone in response to signals that counteract dropping serum concentrations of these minerals. Likewise, growth factors that have effects outside the bone can also be freed from the bone matrix.

Bone continuously undergoes the process of bone remodeling where the both bone formation and resorption are essential to the health of the bone. The loss of balance between osteoblastic formation and osteoclastic degradation of bone is a key feature of bone resorptive pathologies such as rheumatoid arthritis, osteopetrosis, osteoporosis, periprosthetic osteolysis, and osteolytic malignant cancers among others (Nakashima, Wada et al. 2003).

## **Embryonic Skeletal Development**

In the embryo, condensations of mesenchymal cells give rise to the mature skeletal features. Cells from the neural crest, paraxial mesoderm, and the lateral plate mesoderm are the ultimate sources of the mesenchymal cells that pattern skeletal development.

Mesenchymal precursor cells are pluripotent stem cells and the source of osteoblasts in the skeleton. Mesenchymal stem cells (MSCs) have the capacity to become numerous types of cells such as osteoblasts, chondrocytes, adipocytes, and myoblasts (Pittenger, Mackay et al. 1999). The composition of the extracellular milieu of cytokines and growth factors drives the differentiation of MSCs by signaling through pathways that eventually up- and down-regulate a variety of transcription factors in a manner characteristic of the cells' eventual fate.

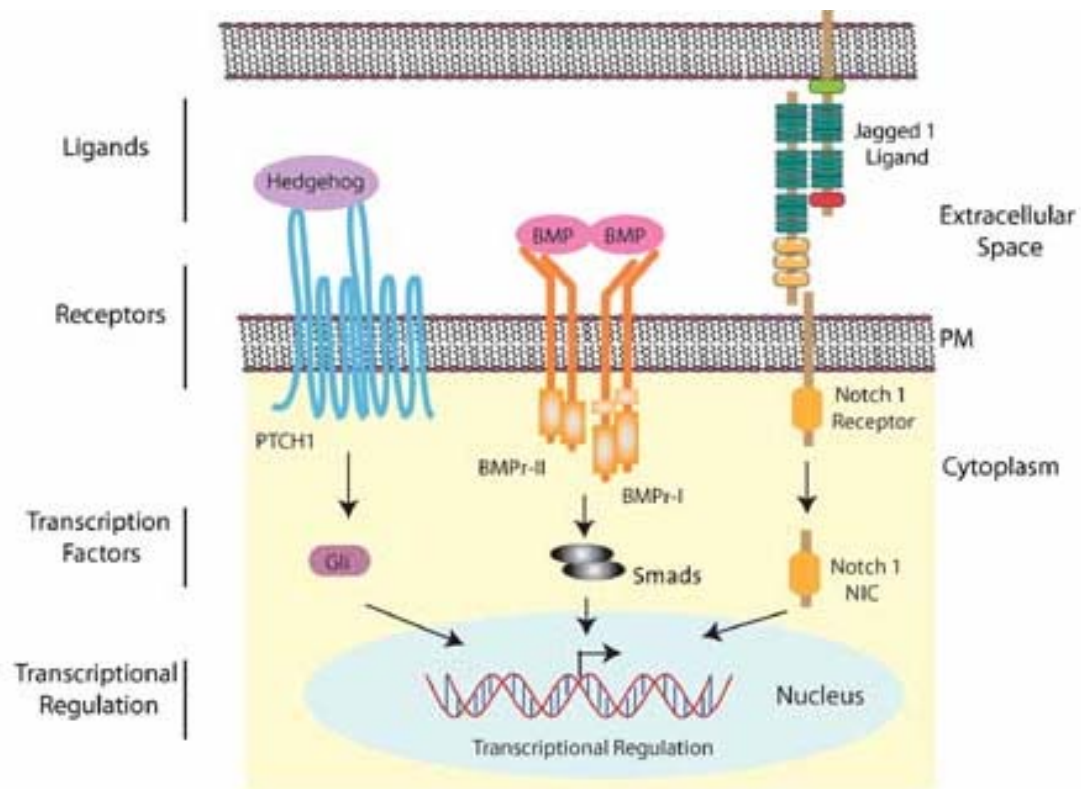
Following MSC condensation there are two mechanisms for the creation of the skeletal bones: membranous or endochondral ossification. Membranous ossification is the process that begets many of the cranial and facial bones; MSCs differentiate into osteoblasts and begin synthesizing bone matrix in the place of the prior mesenchymal condensations. The differentiation of MSCs into osteoblasts occurs through the activity of *wnt* family cytokines (Rawadi, Vayssiere et al. 2003). These extracellular ligands bind to receptors on mesenchymal cells resulting in elevated  $\beta$ -catenin levels (Akiyama 2000) that promote osteoblast differentiation (Bain, Muller et al. 2003; Westendorf, Kahler et al. 2004) and inhibit chondrocytic (Hill, Spater et al. 2005) and adipogenic (Kennell and MacDougald 2005) differentiation. Endochondral ossification is a multi-step process in which a cartilaginous matrix is produced by chondrocytes (in a low  $\beta$ -catenin environment)

as a pre-cursor to the eventual bone. In a process requiring  $\beta$ -catenin, the chondrocytes will cease to proliferate and transition to hypertrophy. The hypertrophic chondrocytes and their matrix will eventually be replaced by osteoblasts and bone matrix.

During development, mesodermal cells form segmented structures on either side of the dorsal midline of the embryo called somites. Cells in the somites will eventually migrate nearer the dorsal midline and become the sclerotomes that gives rise to the spine and ribs. Cell-cell interactions are particularly important in the patterning and transition of cells that ultimately comprise the skeleton. Crucial to the patterning of cells in the somites are interactions between the cell surface receptor notch, of which there are four types Notch1 through Notch4, and the ligands for those receptors such as Delta-like ligands including  $\delta$ -like 3 (DLL3) and Jagged family ligands like Jagged-1 (Artavanis-Tsakonas, Rand et al. 1999; Rida, Le Minh et al. 2004); significant skeletal defects are observed when these interactions are disrupted (Li, Krantz et al. 1997; Oda, Elkahoul et al. 1997; Saga, Hata et al. 1997; Wong, Zheng et al. 1997; Bulman, Kusumi et al. 2000). Notch ligand proteins are single-pass transmembrane proteins and as such the notch/delta/jagged signaling system is a two-cell paradigm requiring the presence of cells expressing the ligand on their surface and cells expressing the receptor in order for signaling to occur. Another protein, Sonic hedgehog, has been shown to also play a critical role in early patterning that eventually yields skeletal development (Chiang, Litingtung et al. 1996). This secreted protein produced in the adjacent notochord, another mesodermally derived structure, regulates the differentiation of cells in the sclerotome.

**Figure 2. Notch Signaling**

Notch signaling requires the presence of two cells; one with a notch ligand while the other presents notch receptor so that the two molecules may interact in the extracellular space with the receptor transducing the signal. Hedgehog signaling is also shown with the transcription factor Gli acting as the effector of hedgehog signaling. Source: (Bailey, Singh et al. 2007)



## **Limb Development**

Similar to embryonic bone development, the formation and growth of the adult skeleton is under comparable regulation in the limbs. The long bones of the limbs, with the exception of a subperiosteal layer of bone, are created through endochondral ossification. Mesenchymal progenitor cells in the limb begin the differentiation to chondrocytes due to the signaling of fibroblast growth factors, sonic hedgehog, TGF- $\beta$  family cytokines and others. As the development of the nascent limb progresses chondrocytes create a cartilaginous model of the skeletal features. These cartilaginous pre-patterned skeletal features, often called anlagen, lengthen the limb in the proximal to distal direction.

Sonic hedgehog and Indian hedgehog are also important regulators of the transition of cells through stages of chondrocytic differentiation, increasing the proliferation of chondrocytes and playing a role in regulating the rate of hypertrophic transition (Stott and Chuong 1997; St-Jacques, Hammerschmidt et al. 1999). Indian Hedgehog (Ihh) also enhances osteoblast differentiation during the process of endochondral bone formation (Krishnan, Ma et al. 2001). Hedgehog signaling acts via the expression of PTH related peptide (PTHrP). PTHrP limits chondrocyte differentiation to hypertrophy, and PTHrP signaling through the PTHrP receptor has been shown to limit Indian hedgehog expression. The interplay of hedgehog proteins and PTHrP generates an axis of signaling where Ihh signaling maintains proliferative chondrocytes at the ends of bone while PTHrP blocks Ihh expression allowing the transition to hypertrophy in more proximal portions of the limb (Shum and Nuckolls 2002). The development of cartilage from mesenchymal condensations occurs in areas of low  $\beta$ -catenin (Hill, Spater et al. 2005). The low  $\beta$ -catenin



results in the up-regulation of SOX-9, a critical transcription factor for chondrogenic differentiation (Akiyama, Lyons et al. 2004; Jin, Lee et al. 2006). This development contrasts MSC differentiation to osteoblasts which requires the presence of extracellular Wnt ligands that signal to increase  $\beta$ -catenin, leading to increased activity of osteoblast-specific transcription factors, among them core binding factor A1 (CBFA1/Runx2) and osterix (Osx) (Kato, Patel et al. 2002).

As chondrocytes continue producing anlagen there is a change at the apical end that transforms the chondrocytes. Chondrocytes at the leading edge continue to proliferate in the developing bone while those behind the leading edge begin to differentiate into non-proliferative, hypertrophic chondrocytes. This process of appositional growth through the formation of growth plates lengthens the forming bones. In the growth plates, proliferative chondrocytes continue to divide resulting in bone lengthening. Behind the proliferative chondrocytes the hypertrophic chondrocytes undergo subtle changes in preparation for the beginning of bone formation. Eventually hypertrophic chondrocytes will die and be replaced by osteoblasts as the bone lengthens. The matrix produced by the chondrocytes is different from that of osteoblasts; there is even a great variance between the matrix made by hypertrophic and proliferative chondrocytes. Proliferative chondrocytes produce a collagenous matrix principally composed of type II collagen (Sandberg and Vuorio 1987). Hypertrophic chondrocytes also produce type II collagen, however they are distinct in their production of type X collagen and, as such, type X production is used as an indicator of hypertrophic character (Hjelle and Gibson 1979; Schmid and Conrad 1982; Schmid and Linsenmayer 1983; Capasso, Tajana et al. 1984; Solursh, Jensen et al. 1986; Bashey,

Iannotti et al. 1991). As chondrocytes transition to hypertrophy they slow their proliferation and also begin to express VEGF-A, an important angiogenic protein that increases vascularization in preparation of osteoblast invasion and activity (Hall, Westwood et al. 2006). The changes in the cartilaginous matrix of the hypertrophic region create the necessary vasculature to allow osteogenesis to occur (Hall, Westwood et al. 2006). Mesenchymal cells recruited to the hypertrophic region that are exposed to Wnt signaling and high vascularity begin the process of commitment to the osteoblast rather than chondrocyte lineage and begin forming osteoid.

As endochondral ossification progresses more changes take place that lay the groundwork for the subsequent osteoblast activity. FGF-18 is thought to play an important role in dampening chondrocyte proliferation as well as initiating vascularization through the up-regulation of VEGF and playing a role the transition to hypertrophy (Liu, Lavine et al. 2007). Among the changes that occur during the differentiation of chondrocytes to the hypertrophic stage is the increased expression of the transcription factor CBFA1/RUNX2 that results in a variety of changes including increased vascularization (Zelzer, Glotzer et al. 2001). Vascularization is a necessary step to allow the pre-osteoblast cells to begin invading the hypertrophic region (Zelzer, Mamluk et al. 2004). The osteoblasts begin to finally lay down the bone matrix while proteases from chondrocytes and other cells act to break down the cartilaginous matrix made in earlier stages of endochondral development (Wu, Mwale et al. 2001; Chung 2004). Osteoblasts then proliferate from the center of the bone to the ends following the proliferating and hypertrophic chondrocytes in sequence. After the initial bone is formed, bone elongation occurs via the appositional growth

through the epiphyseal growth plate with proliferative chondrocytes expanding the border of growth plate and then transitioning to hypertrophic chondrocytes before dying.

Osteoblasts follow the hypertrophic chondrocytes and lengthen the bone through the process of endochondral ossification as the growth plate moves distally.

### **Osteoblast Differentiation**

Osteoblast differentiation is a key step in the generation of a normal, healthy skeleton. Several steps and differentiating signals have been mentioned in previous sections; however there is an abundance of other documented cytokines and growth factors that influence osteoblast differentiation and activity. The extensive body of research on osteoblastogenesis attests to its importance in skeletal development as does the number of studies demonstrating both human and analogous pathologies in other species when there is a disruption of the process.

In the differentiation of mesenchymal precursors to either osteoblasts or chondroblasts canonical wnt signaling is the first major signaling point. Wnts are extracellular ligands that show homology to the drosophila gene wg (wingless), and vertebrate Int-1 genes; the combination of the two names yields “wnt” (Li, Chong et al. 2005). Wnt cytokines bind to their cognate extracellular receptors, members of the frizzled family, and Lrp 5/6, a co-receptor, to transmit their signal to intracellular effectors (Bodine and Komm 2006). Wnt signaling raises intracellular  $\beta$ -catenin levels by activating a protein called disheveled (DSH) that blocks the proteasomal degradation of  $\beta$ -catenin in the cells (Lee, Ishimoto et al. 1999; Seidensticker and Behrens 2000; Zhong, Gersch et al. 2006).

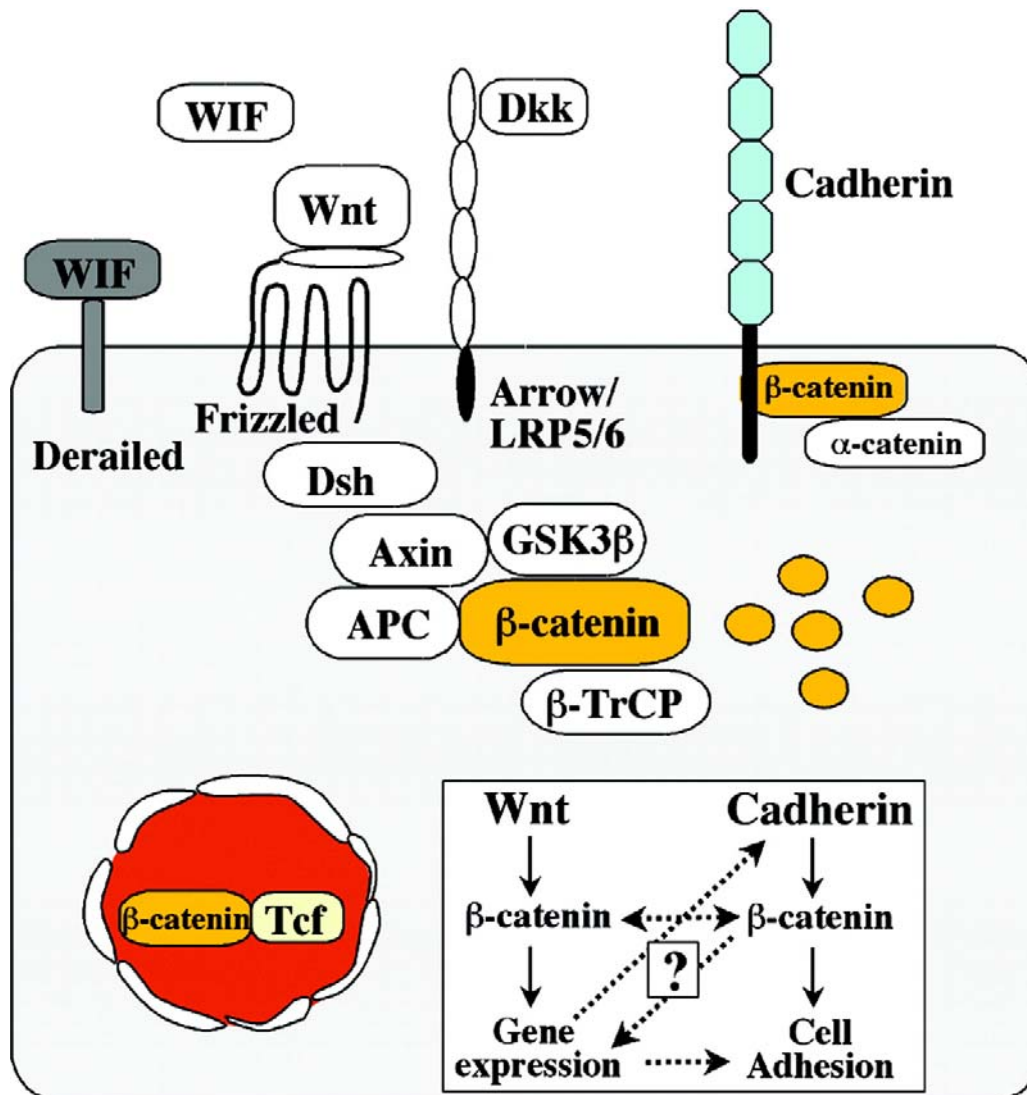
Inhibition of proteasomal degradation allows the accumulation of  $\beta$ -catenin; elevated  $\beta$ -catenin levels in the cell are responsible for stimulating osteoblastic genes and inhibiting chondrocytic genes (Day, Guo et al. 2005). Conversely, cells fated to become chondrocytes are low in  $\beta$ -catenin allowing the up-regulation of transcription factors of the SOX family, specifically SOX-9, that stimulate chondrocyte-specific genes (Hill, Spater et al. 2005; Yano, Kugimiya et al. 2005). In cells along the pathway to osteoblasts, the elevated  $\beta$ -catenin levels lead to up-regulation of other transcription factors (Komori and Kishimoto 1998; Gaur, Lengner et al. 2005), chief among them being CBFA1/RUNX2, a critical transcription factor in osteoblast development (Komori, Yagi et al. 1997; Otto, Thornell et al. 1997; Komori and Kishimoto 1998). CBFA1 also up-regulates another transcription-factor OSX that is critical to osteoblast development (Nakashima, Zhou et al. 2002). Wnt signaling has also been shown to regulate OSX expression in osteoblast differentiation of mesenchymal progenitor cells (Day, Guo et al. 2005). CBFA1-null mice do not express *Osx* while in *Osx* null mice no bone formation occurs showing that it is a transcription factor acting downstream of CBFA1 in osteoblast differentiation (Nakashima, Zhou et al. 2002).

There is an alternate pathway of Wnt signaling called the non-canonical  $\beta$ -catenin signaling pathway that utilizes the same frizzled receptor to bind and transduce Wnt signaling however it acts independent of  $\beta$ -catenin levels and signaling. Alternately called the Wnt/Calcium or Wnt/JNK pathways, these signaling mechanisms lead to an intracellular release of calcium following Wnt binding to frizzled and result in activation of calcium sensitive enzymes such as calcium-calmodulin dependent kinase II (CamKII) and

protein kinase C (PKC) or calcineurin (CaCN)(Kuhl, Sheldahl et al. 2000; Sheldahl, Slusarski et al. 2003). Wnt/Calcium signaling has been shown to affect *Xenopus* development (Kinoshita, Iioka et al. 2003) as well as skeletal muscle formation in mice (Steelman, Recknor et al. 2006). While not fully understood this alternate signaling pathway for Wnts may be critically important in development and given the prevalence of Wnt signals in bone its role in skeletal development needs further exploration.

**Figure 3. The Canonical Wnt Signaling Pathway**

$\beta$ -Catenin exists in a cadherin-bound form that regulates adhesion; in a complex with axin, APC, and GSK-3 $\beta$ , where it is phosphorylated and targeted for degradation by  $\beta$ -TrCP; or in the nucleus. Wnt signaling, proceeding through Frizzled and Arrow–LRP-5/6, activates Dishevelled (Dsh), which results in uncoupling  $\beta$ -catenin from the degradation pathway and its entry into the nucleus, where it acts to control transcription. The Wnt pathway is also subject to extensive regulation and feedback control by extracellular factors that bind Wnt [Wnt inhibitory factor (WIF) and Frizzled-related protein (FRP)] or the coreceptor LRP (Dickkopf). Source: (Nelson and Nusse 2004)



In addition to the key roles of wnt/canonical  $\beta$ -catenin signaling and the characteristic osteoblast transcription factors CBFA1 and Osx, there are a variety of growth factors and cytokines that affect osteoblast differentiation and activity. Perhaps the most widely known group of bone anabolic factors is the bone morphogenetic protein family or BMPs that will be discussed in detail in the following section. Among the many other cytokines affecting osteoblast differentiation are insulin-like growth factors (IGFs), epidermal growth factors (EGFs), hepatocyte growth factor (HGF), vitamin D, TGF- $\beta$ , parathyroid hormone (PTH), platelet derived growth factor (PDGF) and fibroblast growth factors (FGFs). TGF- $\beta$  cytokines have been shown to have dual effects; at early stage osteoblast differentiation they can act to promote differentiation while they suppress terminal differentiation of mesenchymal cells to osteoblasts (Moses and Serra 1996; Janssens, ten Dijke et al. 2005). PDGF, IGFs, EGFs, and HGF have all been shown to work to maintain osteoblast progenitors in proliferating stage and prevent terminal differentiation (Stephan, Renjen et al. 2000; Rasubala, Yoshikawa et al. 2003). These are important factors that increase osteoblast number as bone grows to keep pace with the growing skeleton. Vitamin D, through its active metabolite  $1\alpha, 25$ -dihydroxyvitamin D<sub>3</sub> ( $1,25(\text{OH})_2\text{D}_3$ ), has been shown to increase differentiation of osteoblast progenitors as seen in increased alkaline phosphatase activity and osteocalcin expression (van Driel, Koedam et al. 2006; van Driel, Koedam et al. 2006; Bosetti, Boccafroschi et al. 2007). Interestingly, a recent experiment coupled both HGF and  $1,25(\text{OH})_2\text{D}_3$  and saw an increase in osteoblast number and characteristics including mineralization which was not seen with  $1,25(\text{OH})_2\text{D}_3$



treatment in the absence of HGF (D'Ippolito, Schiller et al. 2002). This illustrates the complex interconnections of the cadre of cytokines acting to promote osteoblast development in the skeleton and the synergistic manner in which they can act.

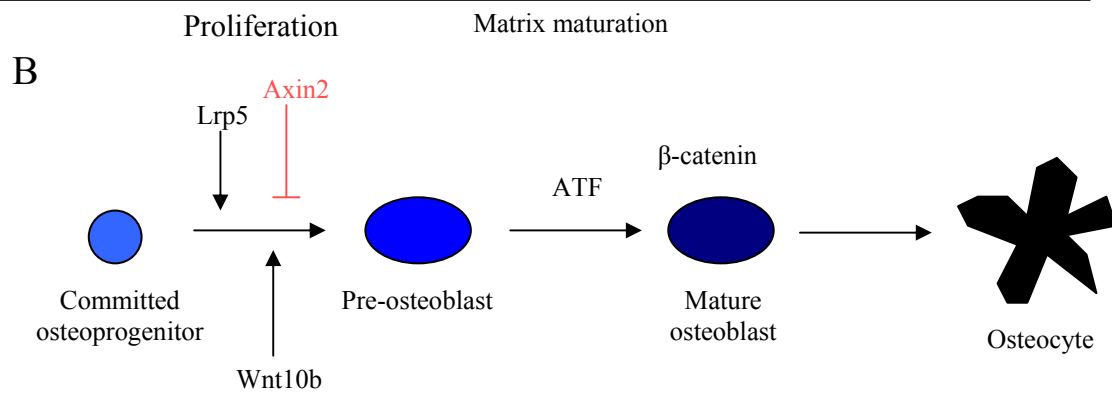
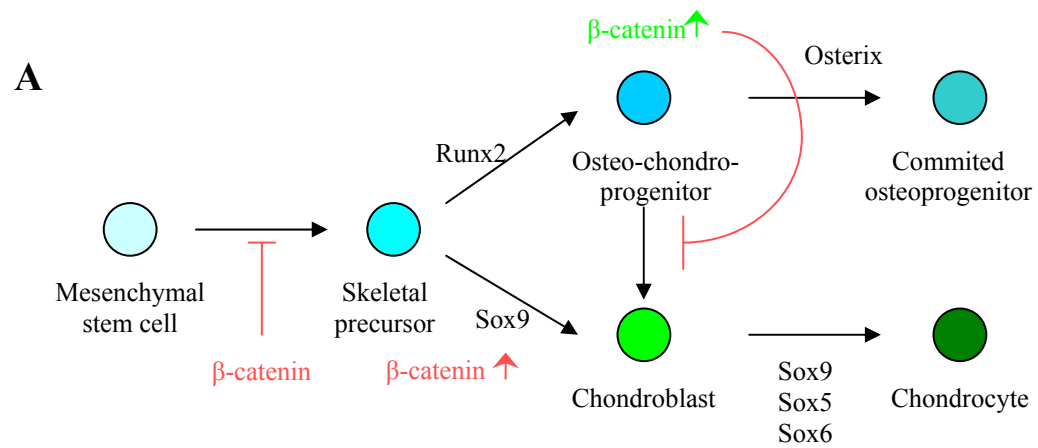
PTH is an important hormone in bone biology with diverse anabolic effects in bone as well as regulation of osteoclast function. Consistent administration of PTH increases osteoclast number and activity while intermittent administration has anabolic effects on bone (Dobnig and Turner 1995; Schmidt, Dobnig et al. 1995; Lotinun, Sibonga et al. 2002). PTH related protein (PTHrP) is produced and acts locally, instead of being produced in the parathyroid gland and acting systemically as does PTH. Both PTH and PTHrP bind to and activate the PTH/PTHrP receptor (Lanske, Divieti et al. 1998).

FGFs play an important role in osteoblast biology by decreasing proliferation and up-regulating osteoblast genes. FGF-2 promotes osteoblast differentiation and responsiveness of mesenchymal cells in bone marrow to BMPs (Hurley, Tetradis et al. 1999; Montero, Okada et al. 2000; Marie, Debais et al. 2002). FGF-2 can also activate CBFA1 by phosphorylation in osteoblasts (Xiao, Jiang et al. 2002; Franceschi and Xiao 2003). A further effect of FGF-2 is to protect osteoblasts from apoptosis (Chaudhary and Hruska 2001). FGF-18 is expressed in bone and cartilage and seems to play an important role in the progression of limb development as mice lacking the gene for FGF-18 show delayed ossification and increased chondrocytes at their joints (Ohbayashi, Shibayama et al. 2002). Similarly, mutations of FGF receptors cause serious skeletal defects; mutations in the gene for FGF receptor-3 (*fgfr3*) cause achondroplasia resulting in skeletal dwarfism (Rousseau, Bonaventure et al. 1994; Shiang, Thompson et al. 1994; Ikegawa, Fukushima et

al. 1995; Horton and Lunstrum 2002). There are a wide variety of other diseases that result from mutations in any of the four FGF receptors (Ornitz 2005).

**Figure 4: Overview of Osteoblastogenesis**

Figure 4 provides a pictorial overview of the process of osteoblastogenesis. Osteoblastogenesis is the result of many different signals being received by stem cells that ultimately result in the formation of new osteoblasts. As can be seen, osteogenic and chondrogenic cells arise from very similar cells. Adapted from Trends in Cell Biology (Hartmann 2006).



## **Bone Morphogenetic Proteins**

Marshall Urist described the isolation of non-collagenous proteins from decalcified bone matrix and how their implantation into muscle induced ectopic bone formation (Urist 1965). This seminal discovery paved the way for the exploration of bone morphogenetic proteins, their signaling mechanisms and effects. While the discovery of BMPs was a watershed moment in orthopaedic discovery there were important studies that preceded Urist's. While using decalcified bone as a mechanism to deliver iodoform as an antiseptic treatment for osteomyelitis, Senn discovered the ability of the decalcified bone matrix he implanted to create new bone at the site of the osteomyelitic defect (Senn 1889; Senn 1889). Similar discoveries were made in subsequent decades using alcohol-extracted (Levander 1934; Levander 1938) and EDTA-extracted (Ray and Holloway 1957; Sharrard and Collins 1961) bone matrix showing bone formation in muscle tissue and juvenile spinal fusion, respectively. These discoveries occurred over a long time period, with publication dates ranging from 1889 for Senn (Senn 1889) to 1961 for Sharrard and Collins (Sharrard and Collins 1961). Progress was initially slow in identifying the component of bone matrix responsible as results were not easily reproducible and there were technical hindrances to fully isolating and identifying the osteogenic component. Additionally, there existed no functional assay to identify the transforming component found in the bone matrix.

Marshall Urist's discovery in 1965 of a substance he described as non-collagenous proteins/bone morphogenetic proteins (NCP/BMPs) (Urist 1965) occurred while he was investigating the mineralization of bone. His primary research and funding at the time was

concerned with investigating the possibility of using strontium and tetracycline as therapies in the treatment of osteogenic sarcoma; mineralization of bone was, at the time, a side interest (Reddi 2003). Urist showed that demineralized bone matrices implanted in animals as controls induced bone formation leading to the theory of autoinduction (Reddi 2003). That discovery was the cornerstone in a brilliant career that saw Urist oversee the journal *Clinical Orthopaedics and Related Research* for 27 years as editor as well as significant research contributions in areas including: estrogen effects on bone, for which he won a kappa delta award; the development of total hip replacements using metal components; and bone induction.

Urist and others earnestly worked on isolating the bone inductive factor found in the demineralized bone matrices. The work of Urist and other investigators eventually resulted in the isolation and cloning of several BMPs, and in the decades since their initial discovery, over thirty BMPs have been identified including a number in species other than humans (Ducy and Karsenty 2000; Chen, Zhao et al. 2004). Many BMPs were cloned in early stages of research in the field, including BMP-2 through BMP-7; however Urist's original BMP was never completely isolated to homogeneity. Several key characteristics of Urist's original substance were relayed in several papers (Urist, Chang et al. 1987; Urist, Huo et al. 1987): An N-terminal sequence of 14 amino acids was obtained (Urist, Huo et al. 1987); repeated attempts at more complete sequencing were hindered by a blocking of the N-terminus (Urist, Huo et al. 1987); The protein was estimated to be 18.5 kDa; reduction abrogated the osteoinductive effect of the substance (Urist, Chang et al. 1987); limited proteolysis with pepsin or trypsin resulted in smaller fragments of the BMP with

increased osteoinductive activity (Urist called the digested, more active form BMP-p for BMP polypeptides) (Urist, Chang et al. 1987); and finally, Urist related in a personal communication to collaborators that purification approaching homogeneity resulted in a loss of osteogenic activity and that rescue of that activity was possible by combination with other fractions (Behnam, Phillips et al. 2005). Despite all of the work and the great many things that were known Urist's BMP was never fully isolated, although Wozney, *et al.* cloned a protein that co-eluted with other BMPs in their isolations that encoded a structurally distinct protein from the other BMPs and named this protein BMP-1. Unlike other BMPs which are TGF- $\beta$  family cytokines, BMP-1 is a secreted metalloprotease that contains a proteolytic domain, a protein-protein interaction domain, and an epidermal growth factor-like domain (Bond and Beynon 1995; Zhang, Ge et al. 2006; Hopkins, Keles et al. 2007). Despite the fact that the name BMP-1 suggests it is one in the same with Urist's original osteoinductive factor, Wozney's isolation procedure differed significantly in later steps and "distinguish it from any previously identified factor" (Wozney, Rosen et al. 1988). Consequently, the identity of the protein component of non-collagenous bone matrix that has the described characteristics that Urist assigned had yet to be identified.

BMPs are members of the TGF- $\beta$  family of cytokines. The TGF- $\beta$  family is comprised of over forty known members linked structurally by the presence of a cysteine knot found on one side of the protein (McDonald and Hendrickson 1993; Murray-Rust, McDonald et al. 1993; Kingsley 1994; Griffith, Keck et al. 1996); among the known family members are TGF- $\beta$ s, BMPs, activins, nodal, and mullerian inhibiting substances. BMPs, though named for their remarkable effects on bone generation, have been found to

be important signaling molecules with multiple signaling functions throughout the body. BMPs play a particularly important role in spatial patterning of the developing embryo.

Based on their osteogenic properties, BMPs have been explored as potential therapies for treating bone injuries and defects that require significant healing and regeneration (Chen, Zhao et al. 2004). Currently two BMPs, BMP-2 and -7, are approved for clinical use (De Biase and Capanna 2005), and multiple delivery mechanisms are being studied. Gels, cements, and organic polymer matrices are all being studied to determine the best mode of delivery for BMPs to induce bone regeneration (Geiger, Li et al. 2003; Hu, Zhang et al. 2003; Seeherman and Wozney 2005). The retention of BMPs is a critical aspect of their clinical efficacy (Uludag, Gao et al. 2001). There are several areas that have been seen as problematic in utilizing BMPs for treating bone defects. First, the cost to use a clinically effective dose is extremely high (Carlisle and Fischgrund 2005), and secondly, diffusion away from the site of administration lowers the efficacy of the BMP treatment. Additionally, there is some worry that BMP activity in tissue neighboring the application site could induce calcification in unwanted areas (Carlisle and Fischgrund 2005). New delivery devices for the use of BMPs in orthopaedic injuries will have to achieve the necessary retention to be clinically effective.



**Table 1: Members of the BMP Family**

The table provides the different members of the BMP family, the subfamilies they are classified into, and some of their alternative names (Termaat, Den Boer et al. 2005).

<b>BMP Subfamily</b>	<b>BMP Molecules</b>	<b>Synonym</b>
BMP-2/4	BMP-2 BMP-4	BMP-2A BMP-2B
BMP-3	BMP-3 BMP-3B	Osteogenin GDF-10
BMP-7	BMP-5 BMP-6 BMP-7 BMP-8 BMP-8B	Vgr-1 OP-1 OP-2 OP-3
CDMP/GDF	BMP-12 BMP-13 BMP-14	CDMP-3 or GDF-7 CDMP-2 or GDF-6 CDMP-1 or GDF-5
Miscellaneous	BMP-9 BMP-10 BMP-11 BMP-15 BMP-16	GDF-2  GDF-11
GDF = growth differentiation factor; Vgr = vegetal-related; OP = osteogenic protein; and CDMP = cartilage-derived morphogenetic protein.		

## BMP Signaling

BMPs signal through type I and II serine-threonine kinase receptors spanning the membrane of target cells. Signal transduction in these receptors has been most widely studied for TGF- $\beta$  receptors however it is thought to be very similar to BMP receptor activity (Yamashita, Ten Dijke et al. 1996). BMP binding to the receptors leads to their activation. Activated receptors are capable of turning on intracellular signaling cascades involving Smad proteins; Smad proteins are named for their homology to the drosophila family of proteins Mothers against decapentaplegic (Mad) and the *C. elegans* gene Sma. Following translocation to the nucleus, Smads alter transcriptional activity of target genes. There exist a number of regulatory checkpoints for BMP signaling with both extracellular inhibitors of BMP signaling and intracellular modulators of BMP signal transduction playing important roles in the process.

BMPs, a sub-family of TGF- $\beta$  family cytokines, can be subdivided into several distinct classes based upon their amino acid sequences and homology. BMP-2 and -4 are members of one group; BMP-5 through -8 form a second group; also a group of BMP-3 and BMP-3b, also known as growth and differentiation factor-10 (GDF10) (Cunningham, Jenkins et al. 1995). BMP-5, BMP-6, BMP-7 (OP-1) and BMP-8 (OP-2) are classified as the A60 group because of their similarity to the *Drosophila* A60 protein (Kingsley 1994). As referenced above, several BMPs have multiple names due to particular properties or aspects of their discovery; a number of BMPs are alternately known as osteogenic proteins (OP), cartilage derived morphogenetic proteins (CDMP), and growth and differentiation factors (GDF). The synonyms of a number of known BMPs are listed in Table 1 BMP

ligands activate their receptors as dimers (Kirsch, Sebald et al. 2000); crystallographic studies have shown that BMP-2 homodimers are held together by a single intermolecular disulfide bond and other non-covalent interactions (Scheufler, Sebald et al. 1999).

Dimerization is crucial to the formation of a ligand complex capable of eliciting signaling from its cognate receptors. Formation of specific heterodimers, namely BMP-2/4 and BMP-2/7, have been shown to be more efficacious in generating BMP signaling than corresponding homodimers (Israel, Nove et al. 1996).

BMP ligands bind to type-I and type-II BMP receptors. There exist three type-I BMP receptors: type-IA (BMPR-IA) also known as activin-like kinase receptor-3 [ALK-3]); BMP receptor type-IB (BMPR-IB) or activin like kinase-6 (ALK-6); and binding has also been shown to activin type-I receptors (ActR-I or ALK-2). Three type-II receptors that bind BMPs have also been characterized: BMP receptor type-II (BMPR-II) and activin type-II and -IIB receptors (ActR-II and ActR-IIB, respectively). Studies have shown the binding of type I receptors to BMPs in the absence of type-II receptors shows significantly lower affinity than when paired with type-II receptors (Koenig, Cook et al. 1994; ten Dijke, Yamashita et al. 1994); likewise, the same was shown of BMP binding to type-II receptors (Kawabata, Chytil et al. 1995; Nohno, Ishikawa et al. 1995; Rosenzweig, Imamura et al. 1995). Each BMP ligand binds to one type-I and one type-II receptor, as a result the dimeric ligand will bind to two of each of the receptors forming a heterotetrameric receptor complex (Wrana, Attisano et al. 1992; Mathews and Vale 1993; Yamashita, ten Dijke et al. 1994; Luo and Lodish 1996; Weis-Garcia and Massague 1996). Multiple permutations of heteromeric receptor complexes can be formed from the various

type-I and type-II receptors, each with varying affinities for the different BMP ligands (Yamashita, Ten Dijke et al. 1996).

The binding of a BMP ligand to the heteromeric receptor complex is the initial step in the activation of BMP signaling. The binding of the ligand brings the type-I receptor in such proximity to the type-II receptor that the constitutively active, intracellular kinase domain of the type-II receptor can phosphorylate the type-I receptor (Wrana, Attisano et al. 1994; Attisano, Wrana et al. 1996). Phosphorylation of the type-I receptor activates its own kinase domain which is responsible for the initiation of intracellular BMP signaling (Wrana, Carcamo et al. 1992; Wieser, Attisano et al. 1993; Wrana, Attisano et al. 1994). The type-I receptor's kinase domain phosphorylates and activates Smad proteins that are the effectors of BMP signaling inside the cell. There are three subsets of Smad proteins: receptor regulated Smads (R-Smads), common mediator Smads (Co-Smads), and inhibitory Smads (I-Smads). Smad1, Smad5, and Smad8 are R-Smads that are phosphorylated by BMP type I receptors (Kretschmar, Liu et al. 1997; Nishimura, Kato et al. 1998; Kawai, Faucheu et al. 2000). Smad2 and Smad3 are also R-Smads, but they are phosphorylated by TGF- $\beta$  type-I receptors, not BMP receptors (Ferguson, Schwarz et al. 2000). The phosphorylated R-Smads then bind to Smad4, a Co-Smad (Kretschmar, Liu et al. 1997; Nishimura, Kato et al. 1998; Kawai, Faucheu et al. 2000). The Co-Smad/R-Smad will migrate to the nucleus where they regulate transcriptional activity of target genes. I-Smads, Smad6 and Smad7, disrupt this signaling in three ways: they can compete with R-Smads for binding to activated type-I receptors (Hata, Lagna et al. 1998; Zhang, Fei et al. 2007), they can target type-I receptors for proteasomal degradation by the recruitment of

ubiquitin ligases (Moren, Hellman et al. 2003; Moren, Imamura et al. 2005), and they can act in the nucleus to inhibit transcription either as co-repressors (Bai, Shi et al. 2000) or via the modification of histone acetylation states (Bai and Cao 2002).

In addition to I-Smads there are other intracellular modulators of BMP signaling that act upon the Smad signaling cascade. Smad ubiquitin regulatory factors (Smurfs) are E3 ubiquitin ligases that regulate the activity of numerous Smads. Smurfs, in their role as HECT (homology to E6Ap carboxyl terminus) E3 ubiquitin ligases, can accept an ubiquitin from an E2 conjugating enzyme and transfer it to a Smad protein (Ogunjimi, Briant et al. 2005). The ubiquitination of the Smads can either target the Smad for proteolytic degradation by the proteasome or in certain cases cytoplasmic retention (preventing the nuclear translocation necessary for activated Smads to affect their signaling function).

A recent report has delineated a novel mechanism regulating BMP signaling via protein interaction with the cytoplasmic “tail” domain of the BMPRII; Tribbles-like protein 3 (Trb3) is a BMPRII “tail” domain-interacting protein that dissociates upon BMP ligand binding (Chan, Nguyen et al. 2007). The release of Trb3 causes the degradation of Smurf1, resulting in increased stability of R-Smads and increased BMP signal transduction through Smads. Conversely, downregulation of Trb3 leads to increased Smurf1 levels and greater repression of BMP signaling.

In addition to the intracellular regulation, a number of other molecules act to modulate BMP signaling extracellularly. Several proteins are found in the extracellular space that inhibit BMP signaling by binding to BMP ligands preventing their interaction

with receptors found on the cell surface. Follistatin, follistatin-related protein (FSRP), noggin, chordin, DAN/Cerberus family proteins, Decorin, Gremlin, Lefty, LTBP1, THBS1, and sclerostin (SOST) are all among the proteins currently identified to inhibit BMP binding in this manner. Bone morphogenetic protein-binding endothelial cell precursor-derived regulator (BMPER) also plays a key role in patterning through the regulation of BMP-4 signaling in embryonic patterning (Moser, Binder et al. 2003), likely through the presence of cysteine-rich (CR) von Willebrand C-like domains as has been shown for Chordin (Larrain, Bachiller et al. 2000). This protein is also a secreted BMP antagonist shown to bind BMP-2, -4, and -6, affecting the BMP-4 driven differentiation of endothelial cells in the embryo. These proteins play many roles in regulating BMP signaling, but principal among those may be the ability to inhibit BMP signaling and create gradients of BMP action which are crucial in establishing the patterning of the developing embryo. The differing affinities and focalized expression allows the generation of gradients for different BMPs in numerous spatial regions. The crucial role for these proteins in embryonic development is seen in both the abnormally patterned and deformed animals and the numerous inviable embryos when knockout animals for these BMP antagonists are created, as reviewed by Balemans and van Hul (Balemans and Van Hul 2002).

A fairly recent addition to the known regulators of TGF- $\beta$  cytokine signaling is the pseudoreceptor BMP and activin membrane bound inhibitor (BAMBI) identified in *Xenopus* and its mammalian homologue Nma. BAMBI is highly similar to the type-I serine threonine kinase receptors except it lacks the intracellular signaling domain (Onichtchouk, Chen et al. 1999; Grotewold, Plum et al. 2001). The stable association of BAMBI with

other serine threonine receptors prevents the initiation of signaling once ligand binding occurs (Onichtchouk, Chen et al. 1999). In *Xenopus*, frogs, and mice BAMBI was expressed with BMP-4 during embryonic development, but despite the findings in these model organisms the role of this antagonist of TGF- $\beta$  signaling is not fully understood (Onichtchouk, Chen et al. 1999; Grotewold, Plum et al. 2001; Knight, Simmons et al. 2001).

Another family of proteins that regulates the intracellular signaling of TGF- $\beta$  family cytokines by affecting the cellular localization of R-Smads has been identified. Zinc finger FYVE domain-containing protein-9, also known as Smad anchor for receptor activation (SARA), is a membrane bound anchor that binds to R-Smads (Smads 2 and 3) as well as TGF- $\beta$  receptor type I (Tsukazaki, Chiang et al. 1998). SARA also is suggested to play an additional role in enhancing multiple phosphorylation of Smads by binding to monophosphorylated Smads and retaining them to increase the likelihood of multiple phosphorylation (Ottesen, Huse et al. 2004). An analogous protein to zinc finger FYVE domain-containing protein-9 for TGF- $\beta$  signaling, called endorphin for its endosomal localization, has been identified for BMP signaling that acts on Smad-4 (Seet and Hong 2001; Chen, Wang et al. 2007; Shi, Chang et al. 2007). These families of proteins appear to play important roles in the facilitation of R-Smad phosphorylation however the ultimate purpose of the regulation is not fully established.

While the other aforementioned regulators of BMP and TGF- $\beta$  signaling are antagonists there are reports in the literature of potentiators of BMP signaling. DRAGON, a glycosylphosphatidylinositol (GPI)-anchored membrane bound protein, has been shown



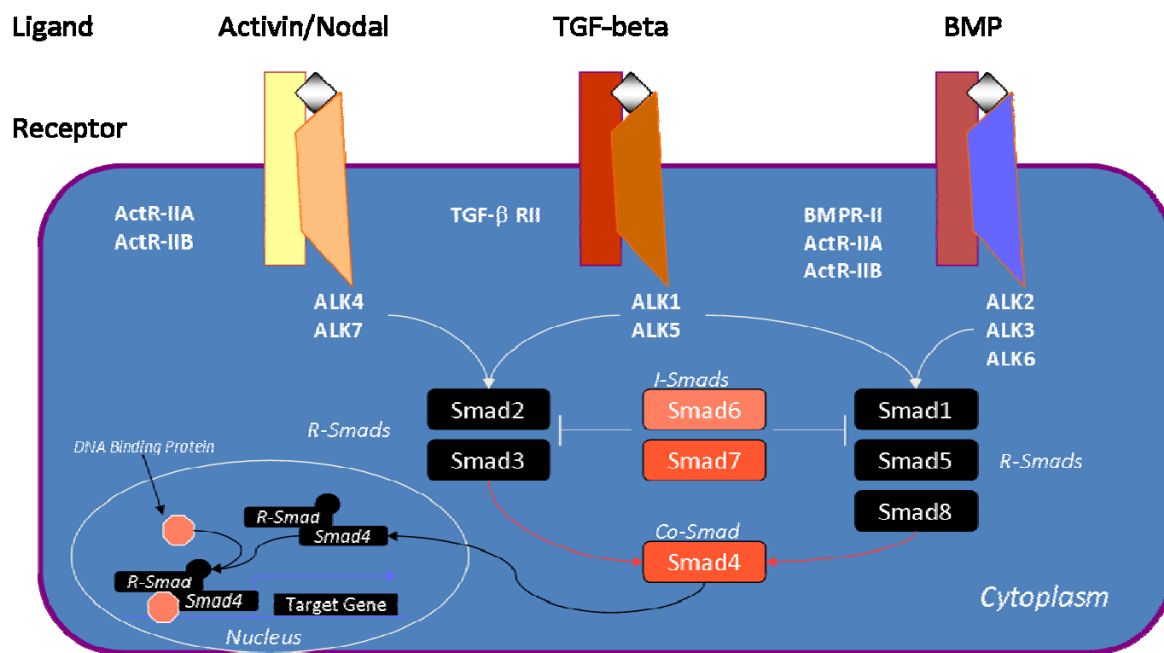
to specifically enhance the signaling of certain BMPs while not showing the same effect for TGF- $\beta$  ligands. DRAGON (RGMb) is a member of the repulsive guidance molecule (RGM) family. DRAGON binds to BMP-2 and -4 but not BMP-7. It also binds to type-I and type-II BMP receptors and enhances BMP signaling. Recently, hemojuvelin, another repulsive guidance molecule (RGM) family member was also found to mediate BMP signaling as a co-receptor (Babitt, Huang et al. 2006). Hemojuvelin plays a specialized role in regulating cellular uptake of iron in the liver (Papanikolaou, Samuels et al. 2004; Niederkofler, Salie et al. 2005).

Interestingly, another GPI-anchored protein, Cripto, enhances TGF- $\beta$  ligand binding to activin receptors while blocking activin binding (Yan, Liu et al. 2002; Gray, Shani et al. 2006). Cripto can act either as a soluble or membrane bound receptor/co-receptor depending upon the species examined (Rosa 2002). Cripto binds to nodal (another class of TGF- $\beta$  ligand) and to activin-like kinase-4 (ALK4) which is a TGF- $\beta$  type I receptor (Yeo and Whitman 2001). Cripto can also potentiate the signaling of nodal through ALK7 (Reissmann, Jornvall et al. 2001). Cripto appears to possess an ability to modulate the differential use of these receptors as their native affinities vary greatly; in the absence of cripto ALK4 has a high affinity for and robust activation by nodal whereas ALK7 is only weakly activated (Rosa 2002). Cripto also binds to activins in concert with Activin type II receptors to block signaling (Gray, Harrison et al. 2003), an opposing function to that seen in the binding of cripto to nodal ligands.

**Figure 5. TGF- $\beta$  Family Cytokine Signaling**

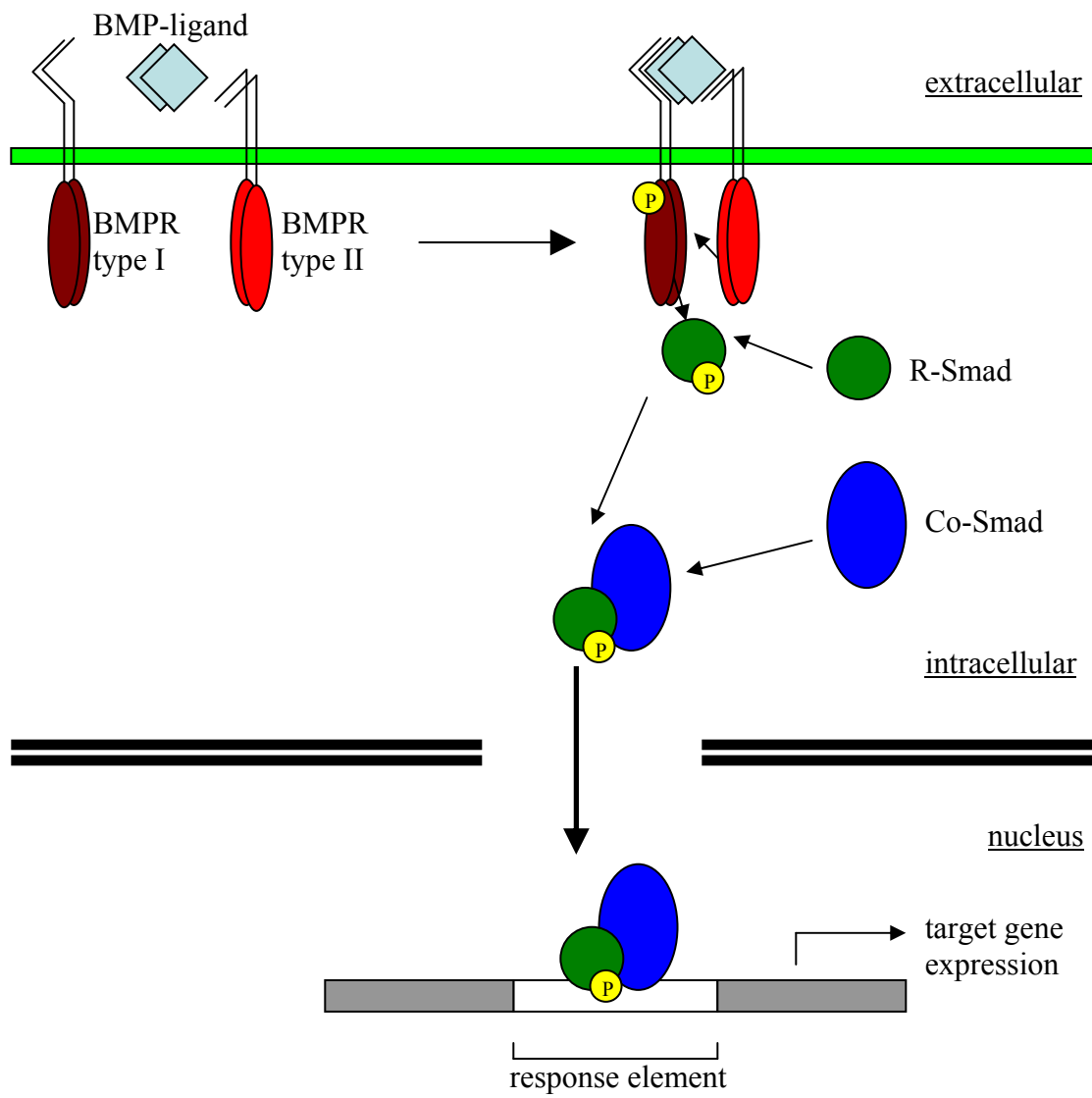
TGF- $\beta$  family signaling can be grouped by the three types of ligands: activins/nodal, TGF- $\beta$ , and BMPs. The figure depicts these signaling ligands and their cognate receptors and their Smad signaling partners.

# TGF $\beta$ Superfamily Thematic Pathways



**Figure 6. The BMP Signaling Pathway.**

Figure 6 shows the BMP signaling pathway, from the cell membrane to the nucleus. In mammals, the R-Smads are Smads-1, -5, and -8, and the lone Co-Smad is Smad-4. Though they are not shown in figure 6, there are two inhibitory Smads (I-Smads), also called Smads-6 and -7. Source: (Balemans and Van Hul 2002))



### **Secreted Phosphoprotein-24**

Secreted Phosphoprotein-24 (Spp24) was initially isolated as a non-collagenous bovine bone matrix protein (Hu, Coulson et al. 1995) in an attempt to isolate and characterize potentially novel, bioactive bone matrix proteins. Although this was the first characterization to purity there are reports in the literature of isolations of bone matrix proteins with strikingly similar characteristics to those described for Spp24. Sen, *et al.* described their “primary osteogenic factor” as a 23 kDa protein isolated from bovine bone (Sen, Walker et al. 1987). Their intent was to isolate large amounts of homogenous bone matrix proteins. They looked within the range of 10 to 30 kDa as Urist’s (Urist 1965; Urist, Huo et al. 1984; Urist, Chang et al. 1987; Urist, Huo et al. 1987) and Reddi’s (Sampath, DeSimone et al. 1982) groups had noted the presence of an osteogenic molecule in this range. Reddi and collaborators also showed the presence of a 22 kDa protein that they believed possessed the ability to induce mitogenic activity in mesenchymal cells similar to that observed in the early stages of ossification-inducing experiments that implant demineralized bone matrix (Sampath, DeSimone et al. 1982). Reddi’s report suggests there may be a closely associated factor in bone matrix that controls mesenchymal cell proliferation and differentiation but it is limited in providing identifiable characteristics of the factor. Sen, *et al.*, in seeking to establish a protocol that would provide adequate amounts of bone matrix isolated BMPs, found their “primary osteogenic protein” (Sen, Walker et al. 1987). The sequencing of a small portion of this protein is nearly identical to the N-terminal sequence of bovine Spp24 (Figure 7) while the overall amino acid composition was highly similar to the composition of Spp24, suggesting that the primary

osteogenic factor and Spp24 are one and the same. Their isolation of Spp24 yielded a 23 kDa protein that they found, upon implantation into animals to induce bone formation. Despite these early works that appear to have found Spp24 is a bioactive molecule, little research was performed subsequently to elaborate on these discoveries and until quite recently there was still little known about Spp24.

**Figure 7: Sequence Comparison of Sen, *et al.*'s Primary Osteogenic Factor with the Bovine Spp24 Sequence**

The N-terminal sequence (upper sequence) reported by Sen et al in 19?? of their “primary osteogenic factor” isolated via a protocol that was based on the work of Urist and modified slightly. They reported significant osteogenic properties of this protein. Sen et al also report the primary osteogenic factor as a 23kDa protein. The sequence of the N-terminal portion of Spp24 is shown (lower sequence) for comparison to illustrate that Sen’s primary osteogenic factor was most likely Spp24.



Primary Osteogenic Factor	F P V Y D Y S P A R L K E A 
Bovine Spp24	F P V Y D Y D P A S L K E A

Hu, *et al.* found that Spp24 was a 24 kDa protein that was phosphorylated on multiple serines that conformed to the recognition motif for secretory pathway protein kinase (Meggio, Boulton *et al.* 1988). Additionally, Hu, *et al.* showed Spp24 contains a cystatin homology domain near its N-terminus. Spp24 was seen in a Northern blot of a variety of bovine tissues to be expressed in the bone periosteum and to a greater extent in the liver (Hu, Coulson *et al.* 1995). Expression of Spp24 in the liver was also seen in microarray of hepatocarcinoma cells (Tackels-Horne, Goodman *et al.* 2001). While Hu, *et al.* did not see expression in the kidney in their bovine tissues, microarray of genes regulated in response to salt-sensitive hypertension did show expression of an expressed sequence tag (EST) similar to Spp24 in the renal medulla of the kidney (Liang, Yuan *et al.* 2002).

Isolation and analysis of the fetuin mineral complex (FMC) in Etidronate treated rats showed that Spp24 was a minor fraction of the complex (Price, Nguyen *et al.* 2003). The FMC is a serum protein-mineral complex that acts to prevent the precipitation and growth of mineral in bone (Price, Caputo *et al.* 2002; Price and Lim 2003). The FMC was shown to be primarily composed of fetuin and minor amounts of matrix Gla protein, Spp24, serum amyloid P component, and prothrombin (Price, Nguyen *et al.* 2003). The complex is thought to form as local concentrations of calcium rise and nucleation of mineral crystals occurs; soluble fetuin then forms the complex with these mineral crystals arresting their growth (Price, Nguyen *et al.* 2003).

A detailed analysis of the sequence of Spp24 in nine species showed the presence of several critical conserved features that added to the understanding of Spp24's function

(Bennett, Khorram Khorshid et al. 2004). The gene encoding Spp24 (*SPP2*) is comprised of eight exons with the first seven containing the coding sequence for the peptide and the final exon containing the 3' untranslated region of the messenger RNA (Bennett, Khorram Khorshid et al. 2004). The organization of the gene, with the stop codon present in the penultimate exon, is rare and highly indicative of secreted proteins (Nagy and Maquat 1998; Bennett, Khorram Khorshid et al. 2004). The human sequence of Spp24 contains a 29 amino acid signal peptide allowing secretion of the mature peptide to the extracellular space. The N-terminal portion of the mature peptide was shown to have a cystatin homology domain. Two such regions can be found in the protein fetuin (Demetriou, Binkert et al. 1996). Cystatins are a family of cysteine protease inhibitors (Brzin, Popovic et al. 1984; Goto, Yamaza et al. 2003). Proteins with cystatin homology have been shown to possess the ability to inhibit cysteine protease activity (Lerner, Johansson et al. 1997). This can greatly affect bone degradation by limiting cathepsins, cysteine proteases involved in proteolytic break down of bone. At the C-terminal end of the cystatin domains lies a TGF- $\beta$ -receptor II homology domain. As mentioned previously the C-terminal portion of the mature peptide contains a number of phosphorylated serines.

Spp24 was localized to the 2q37 chromosome by *in situ* hybridization (Swallow, Merrison et al. 1997). Interestingly, some deletions in this region have been shown to be related to the Albright hereditary osteodystrophy-like (AHO-like) syndrome. AHO-like syndrome symptoms include brachydactyly and craniofacial defects (Chassaing, De Mas et al. 2004; Shrimpton, Braddock et al. 2004; Chaabouni, Le Merrer et al. 2006). While most cases are related to lowered levels of Gs alpha protein signaling, there are reports of cases

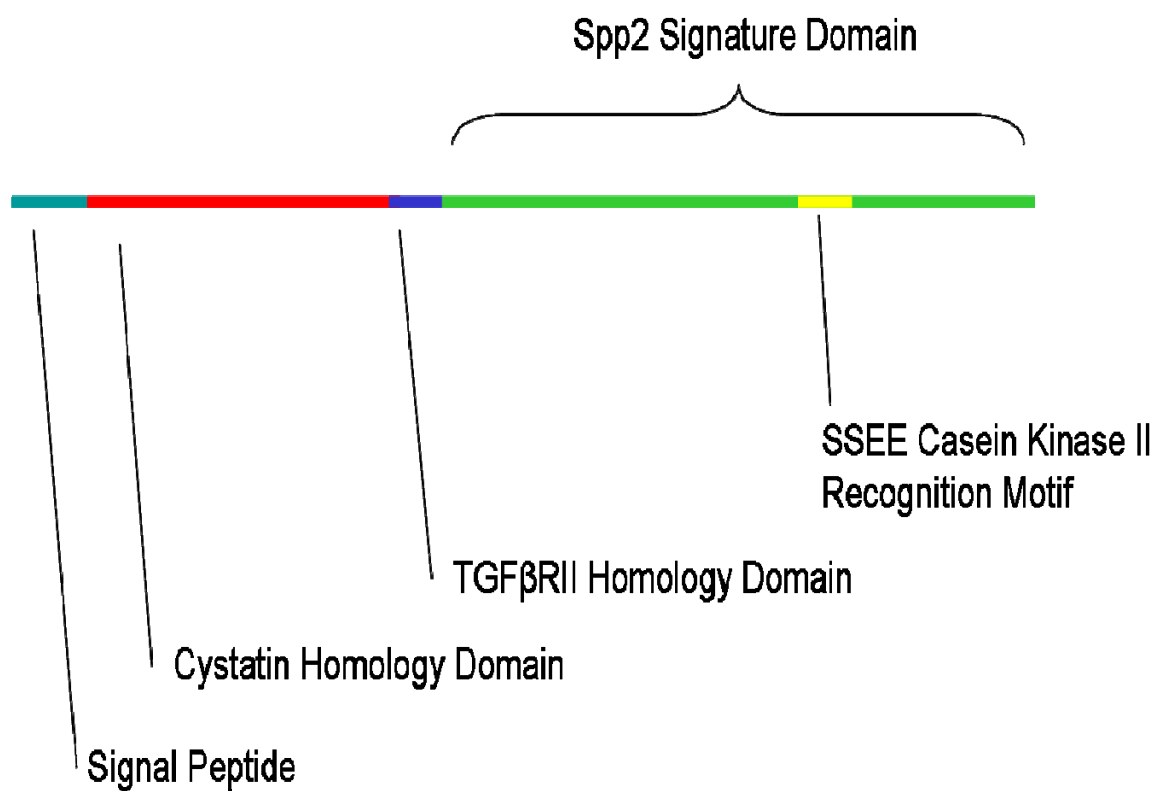
with cryptic deletions where normal activity of the Gs alpha persists (Chassaing, De Mas et al. 2004). If this deletion is related to Spp24, it would suggest the importance of Spp24 in bone development and morphology.

The early work on Spp24 has shown a striking similarity of several aspects of Spp24 biology with the protein fetuin. Fetuin and its human homolog,  $\alpha$ 2-HS glycoprotein, are serum glycoproteins that, like Spp24, accumulate in bone (Yang, Chen et al. 1992; Demetriou, Binkert et al. 1996; Binkert, Demetriou et al. 1999; Price and Lim 2003; Price, Nguyen et al. 2003). Fetuin has been shown to have two cystatin domains similar to the one present in Spp24, and like Spp24 there is a TGF- $\beta$  receptor homology domain in the second cystatin domain of fetuin (Demetriou, Binkert et al. 1996). *In vitro* assays have shown the ability of the full length fetuin to bind to TGF- $\beta$  family members including BMPs (Demetriou, Binkert et al. 1996). Fetuin is the primary component of the fetuin mineral complex, described earlier to play a role in preventing mineral deposition by binding calcium (Schinke, Amendt et al. 1996; Schafer, Heiss et al. 2003).

**Figure 8: Schematic Depiction of the Domains Found in Spp24**

The signal peptide at the N-terminal end of the peptide is essential for the proper secretion of the protein product and is cleaved upon secretion. The mature peptide, after signal peptide cleavage, has a cystatin homology domain at its N-terminus. Contained within the cystatin domain is the TGF $\beta$  receptor type II homology domain. The C-terminal portion of the protein is a Spp24 signature domain conserved in Spp24 across numerous species, mostly vertebrates. Within the Spp24 signature domain are numerous phosphorylated residues; Hu, *et al.* (Hu, Coulson et al. 1995) found that numerous serines were phosphorylated and were found in sequences conforming to the secretory pathway protein kinase. Additionally there serines in a SSEE sequence that is a recognition sequence for casein kinase II and in SIBLING family proteins is important in hydroxyapatite binding (Veis, Sfeir et al. 1997; Huq, Cross et al. 2005).

## Spp2 Domain Structure



Additionally, a blast search using the amino acid sequence of Spp24 revealed that there is also homology to cathelicidin. Cathelicidins are members of a group of anti-microbial peptides expressed in a large number of tissues such as skin, vaginal mucosa, airway, bone marrow and the digestive tract. Additionally, cathelicidins have been shown to play a role in the response to viral infection and to modulate immune and inflammatory responses (Bals and Wilson 2003; Braff, Hawkins et al. 2005; Gordon, Huang et al. 2005; Durr, Sudheendra et al. 2006). At present the relation of Spp24, a bone matrix protein also found at low levels circulating in serum as part of the FMC, to an antimicrobial peptide is unclear. Homology between antimicrobial peptides, namely battenectin and cathelicidin, and cystatins and cystatin homologous proteins has been documented previously.

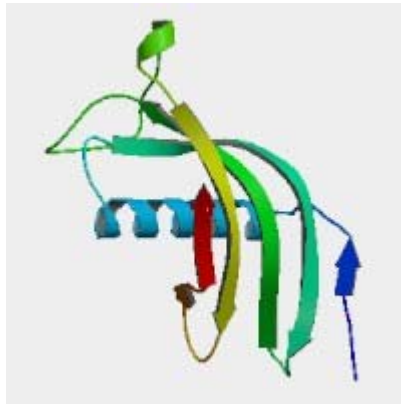
Cathelicidins contain two domains: the N-terminal prosequence (sometimes called “cathelin-like”) and the C-terminal antimicrobial domain (in the lone human cathelicidin, hCAP18/LL-37, this domain is called LL-37). The N-terminal cathelin-like fragment that is proteolytically cleaved to liberate the LL-37 domain has been shown to have anti-microbial activity (Zaiou, Nizet et al. 2003). This domain also is similar to cystatins and can inhibit cysteine proteases (Zaiou, Nizet et al. 2003). Spp24 is more closely related to cystatin and kininogen (which also contains a cystatin homology domain) than to antimicrobial peptides. Due to its relation to both cystatins and antimicrobial peptides Hu, *et al.* have suggested that Spp24 may be an evolutionary bridge between these cystatins and antimicrobial peptides (Hu, Coulson et al. 1995). It is unclear what effect this may

have in bone biology and whether there is a functional antimicrobial effect seen with Spp24 that matches with the sequence homology observed.

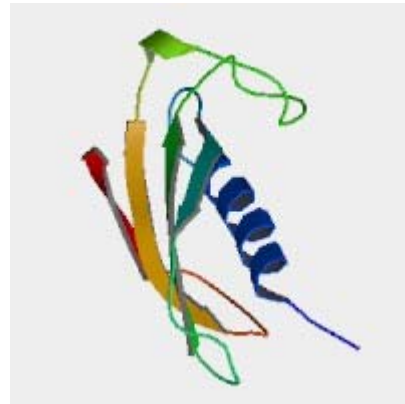


**Figure 9. Structural Similarity of Spp24 and Cathelicidin-like Peptide**

Ribbon structures show the similarities between Spp24 and Cathelicidin-like peptide. The anti-parallel  $\beta$ -sheets and the  $\alpha$ -helix are positioned similarly in both molecules as are the loop connecting the two (green).



Rat Spp24



Cathelicidin Like Peptide

In 2005, Behnam, *et al.*, based on Urist's original isolation procedure, were able to isolate an 18.5 kDa protein that upon analysis was revealed to be a fragment of Spp24 (Behnam, Phillips et al. 2005). This fragment was similar to the protein fetuin, containing a cystatin homology domain. In addition, the 18.5 kDa peptide had a TGF- $\beta$ -receptor II homology region. Behnam, *et al.* then made a synthetic peptide containing the region suspected of being responsible for the BMP-like properties, a 19 amino acid region containing the TGF- $\beta$ -receptor homology domain. The synthetic peptide, which they called BMP binding peptide (BBP), contained cysteines at either end that, based on comparison with fetuin, were likely responsible for a disulfide bond and were used to create a cyclic BBP. Implantation of BBP into muscle pouches of mice induced ectopic calcification. When BMP-2 was implanted alone or with the BBP there was greater calcification seen with the combination of BBP and BMP-2 showing the ability of BBP to enhance BMP activity. Based on the activity of the BBP, Spp24 appears to enhance BMP signaling through its TGF- $\beta$ -receptor homology domain.

Spp24 has also been identified as shown to be highly up-regulated in articular cartilage in a model of osteoarthritis. This limited study did not elaborate on any role of Spp24 either as causative or counteractive to the progression of the osteoarthritis. The proposed biology of Spp24 to act to modulate BMP signaling through binding of TGF- $\beta$  ligands to the TGF- $\beta$  receptor II homology domain could be an important factor as TGF- $\beta$ s play an important role in chondrocyte biology in addition to BMPs.

Recent work by Dalgleish and his collaborators (personal communication) has found the sequence of the Spp24 gene in 22 vertebrate species including the cartilaginous

dogfish. A number of aspects of the sequence were found to be conserved throughout all species implying that these conserved amino acids may be critical to the native function of Spp24. Two pairs of cysteines were found to be conserved throughout all species and these were shown by Bennett, *et al.* to be involved in disulfide bridges, with the second pair involved in the formation of the disulfide bridge flanking the TGF- $\beta$  receptor II homology domain structure (Demetriou, Binkert et al. 1996; Bennett, Khorram Khorshid et al. 2004). A number of serines were additionally shown to be highly conserved. Some of the conserved serines were shown by Hu, *et al.* to be phosphorylated and correspond to the recognition motif of SXE/S(P)-specific secretory pathway kinase (Hu, Coulson et al. 1995). While Hu, *et al.* have shown that a number of serines were phosphorylated, it is unclear whether all of the conserved serines and threonines are modified in this manner (Hu, Coulson et al. 1995). Also unknown is the function of an Asparagine conserved through all 21 species examined (N52 in the human sequence).

Another conserved aspect of the sequences was the presence of a four amino acid stretch of SSEE. This sequence is recognized by the casein kinase-II and highly indicative of a class of proteins called the SIBLING family of proteins. SIBLING proteins are a family of ECM proteins found to associate with hydroxyapatite crystals. These proteins are primarily found in bone and dentine ECM. SIBLING family proteins often contain a consensus sequence (SSEE) for phosphorylation by casein kinase II (Veis, Sfeir et al. 1997; Huq, Cross et al. 2005). The presence of Spp24 within the bone matrix and the association of Spp24 with mineral crystals as a portion of the fetuin mineral complex may suggest that in bone Spp24 acts similar to SIBLING proteins as a stabilizer of

hydroxyapatite interaction with the bone matrix (Huq, Cross et al. 2005). SIBLING proteins undergo numerous post-translational modifications. Though made as a full-length proteins they are often found in the extracellular space as proteolytically cleaved forms (Qin, Baba et al. 2004). The enzyme PHEX (Qin, Baba et al. 2004) and several MMPs (Ogbureke and Fisher 2004; Ogbureke and Fisher 2005) have been shown to be co-expressed with SIBLINGS. As Behnam, *et al.* have identified a bioactive fragment of Spp24 that is ~18.5 kDa (Behnam, Phillips et al. 2005) the relation to SIBLING proteins and their proteolytic activation may be noteworthy similarity that could provide clues to how Spp24 is cleaved to generate the bioactive form Interestingly, in the sequence comparison performed by collaborators (Dalglish and co-workers) the SSEE sequence was conserved even in the cartilaginous dogfish (figure 10). The presence of this feature in a cartilaginous fish would seem to belay the role of the SSEE consensus sequence in calcification, but upon closer examination it may still play the same vital role in the dogfish. The dogfish, though called a cartilaginous fish, still contains mineralized skeletal features. The dogfish contains a cartilaginous skeleton that is produced by chondrocytes, however a portion of the skeleton is mineralized with three distinct types of mineral formed depending on the matrix features (Dean and Summers 2006). Also there exist, in the vertebrae, areas that contain a mineralized “bone” that contains osteoblasts, osteocytes as well as a matrix comprised primarily of type-I collagen (Peignoux-Deville, Lallier et al. 1982). The presence of mineralized cartilage and bone suggests that the SSEE domain could be an important feature of Spp24 that enhances calcification in the extracellular matrix even in the primitive skeleton of the dogfish.

**Figure 10. Alignment of Spp24 sequences from 22 Species**

Alignment of Spp24 amino acid sequences from 22 species of vertebrates including the cartilaginous dogfish. The degree to which residues are conserved across species is indicated by the bar graph below each position. Residues conserved throughout are denoted with an asterisk (\*). Residues with similar amino acids are noted by one (·) (moderately conserved) or two dots (:)(highly conserved) and Source: Dalglish



## **CHAPTER 2**

### **Characterization of Secreted Phosphoprotein-24 Expression: Relation to Kidney and Bone Cell Models**

#### **Abstract**

Secreted phosphoprotein-24 (Spp24) is a non-collagenous bone extracellular matrix molecule comprised of a cystatin-like domain at its N-terminal end and a signature Spp24 homology domain found only in vertebrate species. Spp24 has previously been demonstrated to associate with the serum fetuin mineral complex and localize to bone as a 24 kDa protein. It also has been demonstrated to play a role in facilitating the ectopic bone forming action of bone morphogenetic protein-2. The purpose of this study was to examine Spp24 gene expression and protein localization in multiple tissues, particularly in the rat. Assessment of the tissue distribution of Spp24 gene expression in rat showed transcript detection in only the liver and kidney tissues. Spp24 was found to be expressed in kidney tissue of rats at a level of one third the expression in liver. The protein form of Spp24 also was studied in multiple human tissues and human/mouse kidney and bone cells and



discovered to be ubiquitous and migrate at multiple molecular weight sizes, indicating extensive potential for post-translational processing. Applying the techniques of differential display RT-PCR and GeneChip microarray to rat kidney cortical RNA samples from in vivo models of hypo- and hypercalcemia, we identified the differential up-regulation of Spp24 in the hypocalcemic model. Renal Spp24 mRNA up-regulation in the low calcium condition was confirmed by Real-Time Q-PCR as being 2.5 fold up-regulated in hypocalcemic kidneys compared to its gene expression in kidneys obtained from normal diet fed control rats. Detailed analysis of Spp24 mRNA and protein in histological sections of rat kidney revealed that Spp24 is expressed in the epithelial cell layer of convoluted tubules in kidney cortex. In conclusion, Spp24 predominantly originates from source tissue outside of bone including kidney. Also the tissue distribution is more widely spread than previously appreciated, indicating that Spp24 could regulate renal and extrarenal functions as well as those presently assigned to bone.

## **Introduction**

Skeletal development is characterized by a continuous turnover of the bone extracellular matrix (ECM) and mineral components. In this process there is first an increase in resorption by osteoclasts followed by reactive bone formation mediated by the recruitment of osteoblasts derived from mesenchymal stem cell progenitors. In the adult skeleton the bone remodeling process occurs simultaneously at millions of discrete foci called bone remodeling units. The process ensures the mechanical integrity of the skeleton throughout life and plays an important role in calcium homeostasis. Factors that disrupt the

intimate balance between bone resorption and bone formation result in offsetting metabolic pathologies such as osteoporosis.

Secreted phosphoprotein 24 (Spp24), so named for its 24 kDa size in bovine cortical bone, was isolated from partially purified bovine cortical bone. The sequence of the isolated Spp24 was 200 residues; the first 20 residues contained the signal peptide with the remaining 180-residues being translated into the mature Spp24 protein (Hu, Coulson et al. 1995). The gene encoding Spp24 was later assigned to the human chromosome band 2q37 by fluorescence *in situ* hybridization (Swallow, Merrison et al. 1997). The 24 kDa protein resulting from the Spp24 gene contains an N-terminal sequence of 107 amino acid residues similar in sequence to cystatin; this similarity gives Spp24 structural and possibly functional similarity to bone related cystatins (Brage, Lie et al. 2004; Brage, Abrahamson et al. 2005; Danjo, Yamaza et al. 2007). Sequences related to cystatin are thiol protease inhibitors. Given that Spp24 is found in bone, it could function to modulate the thiol protease activities known to be involved in bone turnover. The cystatin homology region in Spp24 overlaps a transforming growth factor- $\beta$ -receptor II (T $\beta$ RII) homology domain that was suggested by Benham, *et al.* to affect BMP signaling in bone based on its ability to bind bone morphogenetic proteins (BMPs) and other transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily cytokines (Behnam, Phillips et al. 2005). The C-terminal half of the protein also contains several phosphorylated serines (Hu, Coulson et al. 1995). Within Spp24 there is a region of approximately 140 amino acid residues that represents the Spp24 homology domain that is conserved in vertebrates.

Spp24 was also found to be a minor fraction of the fetuin-mineral complex, a serum complex important in inhibiting mineralization and comprised primarily of fetuin, 46% by mass (Price, Nguyen et al. 2003). The fetuin-mineral complex has been shown to participate in resisting bone mineral crystallization and may be important in maintaining homeostasis with respect to mineralization (Price, Caputo et al. 2002; Price and Lim 2003). Other proteins such as matrix Gla protein (MGP) were found in smaller proportions (Price, Nguyen et al. 2003), and the remainder of the complex is composed of calcium-phosphate minerals. The fetuin-mineral complex is proposed to bind calcium via acidic residues of its constituent proteins, preventing the precipitation of calcium-phosphate ions liberated by osteoclasts during bone resorption. Disruption of normal fetuin mineral complex function, either by blocking acidic residues or by targeted mutations of individual components of the complex such as MGP or fetuin, result in decreased management of extraskeletal calcification (Schinke, Amendt et al. 1996; Jahnke-Dechent, Schinke et al. 1997; Munroe, Olgunturk et al. 1999).

Like Spp24, both Fetuin and MGP accumulate in bone and contain several phosphoserine residues potentially important to their function in bone (Price, Nguyen et al. 2003). Spp24 and fetuin also share cystatin homology domains in their N-terminal protein sequences (Hu, Coulson et al. 1995) categorizing them both among the cystatin super-family of cysteine protease inhibitors. The phosphorylation sites of MGP and fetuin are present near their respective C-termini. Similarly, the bovine Spp24 was also found to be multiply phosphorylated at serine residues that conform to the Ser-X-Glu/Ser(P) sequence, a recognition motif for phosphorylation by the secretory pathway protein kinase (Meggio,

Boulton et al. 1988). Spp24 also contains the conserved sequence for casein kinase II phosphorylation (SSEE) (Veis, Sfeir et al. 1997; Huq, Cross et al. 2005) across multiple species. This consensus sequence is found to be phosphorylated in small integrin-binding ligand N-linked glycoprotein (SIBLING) family proteins such as bone sialoprotein, dentin and osteopontin. The phosphorylated serines in these bone and dentine extracellular matrix (ECM) proteins have been hypothesized to play an important part in the controlling interaction of the ECM with hydroxyapatite and mediating mineralization of these tissues (Narayanan, Ramachandran et al. 2003; Qin, Baba et al. 2004; Toyosawa, Kanatani et al. 2004; Gordon, Tye et al. 2007). Osteopontin (OPN) is another secreted phosphoprotein found in bone. Phosphorylated residues of osteopontin have also been shown to play a role in binding calcium mineral (Gericke, Qin et al. 2005).

The work of Behnam, *et al.* has shown that Spp24 possesses osteoinductive qualities based on the ability of the T $\beta$ R II homology domain to interact with BMPs (Behnam, Phillips et al. 2005). The ability of Spp24's T $\beta$ R II domain to enhance the effects of BMPs in addition to the putative ability of the phosphoserine residues to modulate mineralization suggest that Spp24 may be an important bone matrix protein in regulating bone mineralization.

This study details our finding of the up-regulation of Spp24 in the kidney under low calcium conditions in the rat. Serum and extracellular calcium is an important regulator of neuromuscular function and skeletal mineralization. The increased synthesis of renal Spp24 during hypocalcemic conditions may relate to a regulatory mechanism for controlling extracellular calcium. The absolutely conserved SSEE region within the Spp24

homology domain of multiple vertebrate species predicts that Spp24 can adhere to hydroxyapatite and affect an aspect of mineralization.

## **Materials and Methods**

### *Animals*

Hypocalcemic (LC), hypercalcemic (HC), and normal calcium (NC) groups of rats were generated by following the diet regime of Beckman and DeLuca (Beckman and DeLuca 2002). Briefly, rats were fed either low calcium or normal calcium diets. To generate the LC group, animals on the low calcium diet were supplemented with vitamin D. The HC group was created by feeding animals the normal calcium diet and administering two injections of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>] at 16 hrs and at 4 hrs prior to sacrifice. The NC group was fed the normal calcium diet without additional supplements.

### *Cell Culture*

All cells were kept at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. MG-63 and SAOS-2 cells were maintained in DMEM:F12 with 10% and 15% FBS, respectively. Kidney Cells, mouse proximal (MPCT) and distal (DKC-8) tubule epithelial cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin.

### *Isolation of RNA*

Total RNA was isolated from tissues and cells using the Trizol reagent (Invitrogen, Carlsbad, CA) as described in our previous work (Bajwa, Horst et al. 2005). The resulting

RNA pellet was re-dissolved in RNase-free water. DNA was removed using a MessageClean Kit (GenHunter Inc., Nashville, TN) as per the manufacturer's instructions.

#### *Quantitative RT-PCR*

Specific TaqMan® probes were generated to the rat Spp24 and 18s rRNA genes. Real-time RT-PCR (TaqMan) was performed in a PE Biosystems Model 7700 instrument at the VCU Nucleic Acids Research Core facility (NARF). Primers were designed using Primer Express software (PE Biosystems, Foster City, CA) from gene sequences obtained through GenBank. Gene transcript levels were normalized to 18s rRNA.

#### *Cell and Tissue Distribution Western Blots*

For the determination of Spp24 protein regulation, monolayers of cells were grown in 60mm culture dishes up to 80-90% confluent. Cells were washed with ice cold PBS and then lysed in MPER lysis buffer (Pierce, Rockford, IL) containing 10 µl/ml of Protease Inhibitor Cocktail (Pierce). Cell lysates were centrifuged at 14,000g to separate cell protein from cell debris. The soluble protein content was measured by a Bradford assay (Bio-Rad Inc, Hercules, CA) and frozen at -70°C. After thawing, the cell lysates were combined with an equal volume of Laemmli sample buffer (Bio-Rad) heated at 95°C, and separated using a 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad). After transfer, the membranes were blocked with 5% instant nonfat milk in TBST (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20) pH 8.0 for 1 hr at room temperature.

Spp24 was detected in mouse and human cell extracts by immunoblotting using an overnight incubation with 1:1,000 dilution of rabbit polyclonal antiserum specific for

bovine Spp24 (provided by Dr. Samuel Murray). The blots were incubated in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) overnight at 4°C. Blots were washed 4x for 10 minutes each in TBST. Blots were then incubated in solution containing Odyssey anti-mouse IgG secondary antibody Alexa Fluor-IR800 nm (LI-COR) diluted 1:5000 in Odyssey blocking buffer for 1 hr and 30 minutes. Blots were finally washed 4x in TBST and read by the Odyssey Infrared Imaging System (LI-COR).

A membrane representing a human multiple tissue protein blot was obtained from EMD Biosciences (Calbiochem, San Diego, CA). According to the manufacturer, the proteins on this blot were isolated from various tissues by preparing tissue homogenates in the presence of protease inhibitors. Proteins samples (75 µg) from each tissue were solubilized in SDS-lysis buffer and electrophoresed in a 4-20% SDS-polyacrylamide gradient gel, followed by electroblotting on PVDF membrane. This membrane was washed, blocked and then hybridized with a rabbit anti-human Spp24 polyclonal antibody at a dilution of 1:500. The membrane was washed with TBS containing 0.05% Tween-20 (TBST) and then a fluorescent-conjugated (800nm) secondary antibody was added to the membrane at a dilution of 1:10,000. Following wash steps, the blot was visualized using an Odyssey Infrared imaging system (LI-COR). Alpha-tubulin was used as a housekeeping protein control.

#### *Differential Display RT-PCR*

Differential Display RT-PCR (DD) was performed using the RNAspectra kit (GenHunter), containing three one-base-anchored oligo-dT primers to subdivide the mRNA population, except they were labeled with 5'-fluorescein. The use of three one-

base-anchored primers reduced the redundancy and potential under-representation of the sub-population of mRNAs encountered by using either eight arbitrary 13mers or three degenerate two-base-anchored oligo-dT primers. With built-in restriction sites at the 5' ends of both anchored and arbitrary primers, the longer primer pairs produce highly selective and reproducible cDNA patterns. To promote high-efficiency reactions, amplicons were selected in the 50-150 base pair range.

Briefly, samples were run in duplicate to reduce the number of false positives as a result of high signal to noise ratio often associated with the DD technique. An aliquot of 200ng of total RNA was mixed with an anchor primer (anchor primers: 5'-AAGCTTTTTTTTTTTTG, 5'-AAGCTTTTTTTTTTTTA, 5'-AAGCTTTTTTTTTTTTC) and heated to 65°C for 5 minutes. The sample was transferred to a thermocycler and maintained at 37°C. Reverse transcriptase was added after 10 minutes and the reactions were incubated at 37°C for an hour. The generated cDNAs were then used in PCR reactions. The eight arbitrary primers (arbitrary primers: 5'-AAGCTTGATTGCC, AAGCTTCGACTGT, AAGCTTTGGTCAG, AAGCTTCTCAACG, AAGCTTAGTAGGC, AAGCTTGCACCAT, AAGCTTAACGAGG, AAGCTTTTACCGC) were used in combination with each of the anchor primers. PCR cycling was performed and the cycle conditions were: 40 cycles of 94°C for 30 seconds, 40°C for 2 minutes, and 72°C for 1 minute.

A 6% Accugel polyacrylamide gel (National Diagnostics USA, Atlanta, GA) was loaded and run for approximately 45 minutes at 60 watts. Banding patterns were analyzed using a Typhoon AutoImager (Molecular Dynamics Inc., Sunnyvale, CA). Differentially



regulated bands were isolated from the gel and reamplified using the same combination of initial primers. The PCR fragments were T-A cloned into pGEM T-easy vector (Promega Corp., Madison, WI), isolated and sequenced. NCBI blast searches were used to analyze the obtained sequences.

### *Histology*

Rat kidneys were frozen in OCT embedding medium and sectioned. Confocal immunofluorescence was performed as previously described (Ramage, Urban et al. 2007) using the anti-Spp24 antibody and corresponding fluorescent-conjugated secondary antibody. H&E staining was performed on rat kidneys embedded in paraffin at the VCU Medical Center Pathology lab.

### *Oligonucleotide Microarray*

Microarray was performed on the same RNA samples as were used in the DD experiment. LC and HC RNA samples were exposed to the Affymetrix Inc. (Santa Clara, CA), Rat Genome U34A GeneChip® Set, which probed for 7,000 known rat gene sequences plus 1,000 EST gene cluster sequences. The U34A GeneChips were hybridized in the presence of fluorescently labeled RNA from each treatment. The GeneChips were read and analyzed using an Affymetrix GeneChip Reader at the VCU Nucleic Acids Research facility.

### *Hydroxyapatite Binding*

Media from cells transduced with a CMV promoter controlled Spp24 viral construct was collected after 6 days. 10 mls of media was placed onto a hydroxyapatite column and allowed to enter the column by gravity flow using a modified version of the

protocol of Weckslar and Norman (Weckslar and Norman 1979). The media flowed through the column and was collected. The column was washed until the  $A_{280}$  of the wash coming off the column dropped to zero. The bound protein was eluted and collected by running 6M urea through the column. 10ul aliquots of the media, flow through, and eluate fractions were saved for Western blotting using an anti-Spp24 antibody as described above.

### *Statistical Analysis*

Where it applies, results are expressed as the mean  $\pm$  SE. Significance was determined by analysis with an unpaired student's t-test for two-group comparison;  $p < 0.05$  were considered significant.

## **Results**

### *Expression of Spp24 in Tissues and Cell Models*

Spp24 gene expression had previously been demonstrated in bovine cortical bone and liver tissues, but no expression studies of Spp24 have been done in smaller lab animals. Using the annotated rat Spp24 cDNA sequence, we designed a TaqMan probe with the Applied Biosystems Primer Express v2.0 software package. Multiple tissues were harvested from three individual rats, which included; heart, kidney, spleen, stomach, bone smooth muscle, lung, thymus, small intestine, liver testes and the thyroparathyroid unit. In the case of bone tissue, we used whole femur, and we followed up this work by also analyzing epiphyseal ends of the bone and the central diaphyses of each bone. In the multiple tissue analysis, Spp24 gene expression was only detected in the liver, and

surprisingly, the kidney (figure 11A). No gene expression of Spp24 was detected in whole bone (figure 11A), femur ends or midshafts (data not shown). The relative gene expression of Spp24 from kidney was 1/3 the amount compared to liver (figure 11B). The lower limit of detection was 80 pg of RNA. It is possible that a low Spp24 gene expression level in bone tissue was undetectable by this measurement technique.

Given the small number of tissues that express Spp24 mRNA and the fact that Spp24 is a secreted protein, it is reasonable to predict that the protein localization is more widespread than its gene expression. This was tested with a multi-tissue blot of human protein source using a polyclonal anti-bovine Spp24 antibody that can cross-react with human Spp24 (figure 11C). Detection of Spp24 was nearly ubiquitous in all tissues and was observed as multiple size bands that were predominantly 55 kDa for most tissues. Brain tissue had a predominant protein band at approximately 45 kDa, while kidney had weak detection of a band at 55 kDa and a band strongly detected at 90 kDa. Various kidney and bone cell models also were utilized for Western blot analysis of Spp24 (figure 11D). Cellular protein was extracted in two ways. First, lysis buffer was added directly to the culture well following removal of the medium. This procedure allows for the inclusion of adhered extracellular matrix proteins with cellular protein content. In the second procedure, cells were lifted from the well by trypsin, washed with PBS and pelleted to remove the medium. Then the pellets were lysed for protein extraction. This second procedure was done to enrich the protein fraction with cellular protein content as opposed to extracellular protein content. Protein from both procedures was used in Western blots. Use of 30µg protein from the whole well lysis (first panel of figure 11D) resulted in

detection of 45 kDa sized bands in murine kidney epithelial cell models, MPCT and DKC-8. A similar size band was also detected in human MG-63 osteoblast-like cells whereas in human SaOS2 osteosarcoma cells the detected band size was 55 kDa. No band was observed in the lane representing the pluripotent murine C2C12 myoblast cells. These myoblast cells can convert to osteoblasts under the correct conditions (Katagiri, Yamaguchi et al. 1994). In contrast, Western blot detection of Spp24 in MG-63 and C2C12 revealed a 24 kDa protein band when the trypsin lysis procedure was used instead of whole cell lysis (second panel of figure 11D). This demonstrates that MG-63 and C2C12 cells make at least a small amount of unprocessed Spp24. Presumably, secretion results in higher molecular weight moieties of Spp24.

#### *Regulation of Spp24 in the Rat Kidney*

Figure 12A depicts the three main protein components of the fetuin mineral complex, which include fetuin, matrix-gla-protein and Spp24 (Price, Caputo et al. 2002). Similar to fetuin, Spp24 contains a cystatin homology domain at its N-terminal side. Fetuin contains two such domains. Q-PCR of RNA isolated from rat kidneys under normal calcium (NC) and low calcium (LC) showed the up-regulation of Spp24 transcripts under LC (figure 12B). The RNA isolated from the kidney cortex of LC (hypocalcemic) and HC (hypercalcemic) rats was tested for differentially expressed genes involved in the regulation of calcium homeostasis by differential display (DD) RT-PCR (figure 12C). The DNA fragments correlating to several negatively and positively regulated mRNA transcripts were recovered, amplified, and sequenced. The band differentially up-regulated under LC conditions denoted by the arrow in figure 12C corresponds to Spp24. This

fragment yielded a partial sequence of Spp24 exactly matching 167 bases at the C-terminus when compared to the annotated rat Spp24 sequence previously published in GenBank.

RNA from rat kidneys was also subjected to cDNA oligonucleotide microarray to identify differentially regulated genes in the LC versus the HC. Microarray showed a 5.6-fold increase in Spp24 gene expression in the LC group (figure 12D) as compared to the HC group. In contrast to Spp24, OPN was down-regulated by hypocalcemia in the rat kidney (figure 12D). In our model several other  $1,25(\text{OH})_2\text{D}_3$  dependent genes were also repressed as expected by the hypocalcemic condition in the LC group such as 24-hydroxylase, Vitamin D receptor, calbindin, and Ca-ATPase. Therefore, an increase of Spp24 in the LC condition indicates a lack of regulation by  $1,25(\text{OH})_2\text{D}_3$  on Spp24 gene expression in the kidney.

#### *cDNA Sequencing and Protein Structure of the Rat for Spp24*

Using RNA isolated from rat liver we cloned out the rat cDNA for Spp24 using primers to the annotated sequence. The cDNA was sequenced revealing three additional nucleotides (figure 13A & C) not in the published sequence in GenBank. The additional nucleotides corresponded to an Arginine in the c-terminal portion of the protein (figure 13B).

#### *Interspecies Post-Translational Modification Mapping of Spp24*

Figure 14 depicts rat and mouse species of Spp24 both contain consensus sites for N-glycosylation just proximal to their SSEE domains. Most phosphorylation domains among all species analyzed (rat, bovine, human and mouse) appear to be via casein kinase II, followed by protein kinase C. Human and bovine Spp24 additionally have consensus

sites for phosphorylation by protein kinase A. Finally, the bovine Spp24 contains one site for possible tyrosine kinase phosphorylation, and both bovine and mouse forms of Spp24 have consensus myristylation sites in their C-terminal domains.

#### *Localization of Spp24 in the Kidney*

Transverse sections of the rat kidney illustrates cortex and medulla areas (figure 15 A and D). Spp24 was detected only in the epithelial cells of the convoluted tubules of the cortex layer (figure 15 D). Staining was also absent in the collecting ducts (figure 15 E) and in the glomeruli of cortex sections (figure 15 F).

#### *Hydroxyapatite Binding*

Western blot of conditioned media (lane 2), column flow through (lane 3), and eluate fractions (lanes 3-5) is shown in figure 16. The large amount of Spp24 seen in the blot entered the column and a majority bound though some is seen in the flow through (perhaps exceeding the binding capacity of the column). In the eluted material a band recognized by the anti-Spp24 antibody is seen in early fractions. The large volume of elution fractions accounts for the considerably smaller band in the elution than that seen in the conditioned media (lane 2).

### **Discussion**

To date, as few as five papers directly relate to Spp24 and provide only minimal information about its functional purpose. From these studies Spp24 is described as a thiol protease inhibitor that affects an aspect of bone remodeling. In the present study, we make use of the first available antibody raised against bovine Spp24 to examine the tissue

distribution and protein regulation of Spp24 in several species. The antibody cross-reacts well with human, rat and mouse forms of Spp24. We also document that renal tissue along with liver are the predominant sites of Spp24 gene expression, but low amounts of protein were detected in renal and bone cell models. Intact 24kDa protein can be detected in myoblast precursors (C2C12 cells) and osteoblasts (MG-63 cells) if the cells are harvested free of the extracellular matrix component. Secreted Spp24 undergoes post-translational processing and is detected as higher molecular weight products in bone and kidney cell models and in a host of tissues. The predominant sizes of Spp24 protein, examined in mouse and human sample were 45-55kDa. One exception was forms of 55 and 90kDa in human kidney. The stage of differentiation may also be a factor since we observed a 45kDa protein in terminally differentiated MG-63 osteoblast like cells and a 55kDa protein in less differentiated SaOS-2 osteosarcoma cells.

There is extensive tissue distribution of Spp24 in most if not all peripheral tissues. Based on this finding, we speculate that Spp24 has a broader role in vivo than previously understood. Spp24 associates with fetuin, matrix Gla protein (MGP) and osteopontin (OPN) in serum and is thought to participate in regulation of calcium homeostasis along with these proteins. Targeted mutation of either fetuin, MGP or OPN leads to complications involving heterotopic calcification of soft tissue organs (Jono, Ikari et al. 2002; Schafer, Heiss et al. 2003; Jono, Ikari et al. 2004; Speer, Chien et al. 2005; Westenfeld, Schafer et al. 2007). However, Spp24 appears to be involved in processes that positively regulate bone ossification by cooperating with BMPs. We tested this concept by examining the regulation of Spp24 in kidney tissue exposed to extremes in blood calcium

concentration. Since OPN is vitamin D dependent for its expression and undergoes down-regulation during hypocalcemic conditions we reasoned that Spp24 would follow a similar pattern of expression. In contrast, gene expression of the two related phosphoproteins were oppositely regulated with Spp24 being markedly increased in hypocalcemia. We additionally found Spp24 expression to be localized to the tubules of the kidney cortex and not in the medulla.

Isolation of RNA from several rat tissues showed Spp24 expression mainly in the liver and kidney. The metabolic tissues are important producers of proteins that are released into the serum. Spp24 was not seen in bone however expression in bone has been previously reported in bovine tissue studies (Hu, Coulson et al. 1995). Although we did not see it in the rat, we saw human osteoblast cells in culture showed expression via Western blot. Accumulation of Spp24 protein in bone (Hu, Coulson et al. 1995), regardless of expression levels, is an important factor in bone development as reported by others (Behnam, Phillips et al. 2005). Western blot of human tissue using an anti-human Spp24 antibody shows Spp24 is present in a myriad of tissues with a variety of molecular weights. It seems clear that there are numerous post-translational modifications that result in the variable sizes detected in the immunoblot.

We have established that there is an additional previously unreported amino acid present in the rat Spp24 sequence. The importance of the additional arginine, if any, is unknown at this time. It is worth noting that the difference in published sequence may be due to the use of Sprague-Dawley rats whereas the GenBank sequence is published from Norway rats.

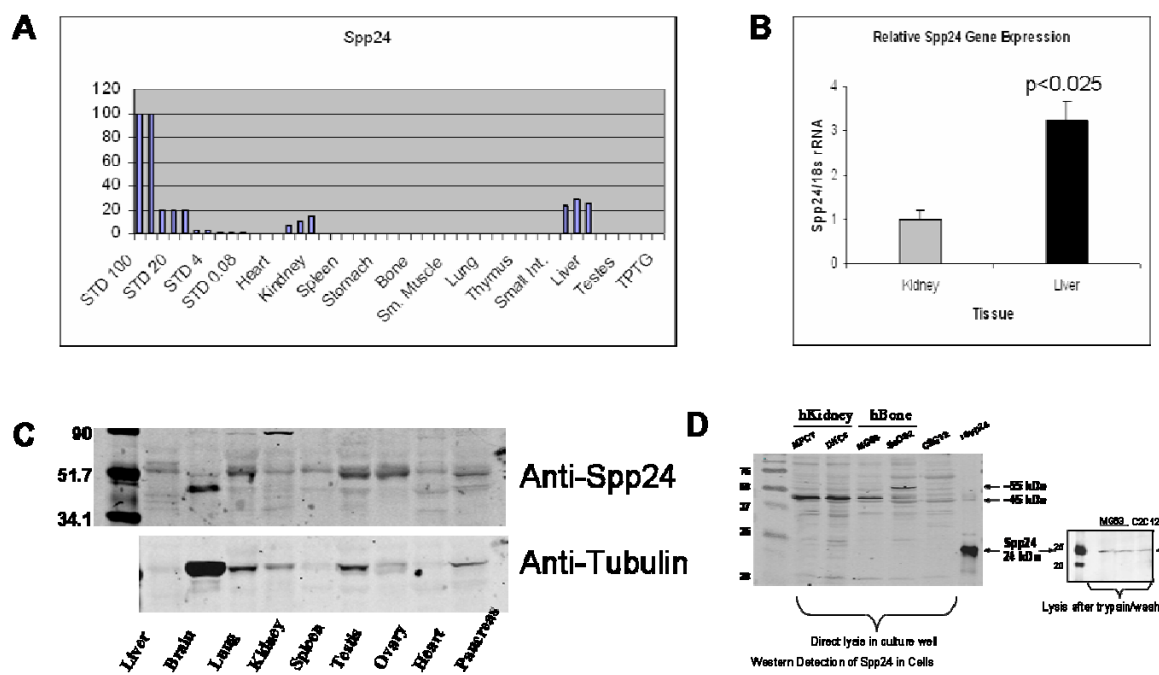


Post-translational modifications of extracellular matrix proteins can play important roles in the proteins' function. We suspect the multiple sizes of Spp24 seen in our Western blots results from post-translational modification. SIBLING family proteins, known to bind mineral in calcified tissue, are notably post-translationally modified and are related to Spp24 by the presence of a SSEE consensus sequence. Similar to SIBLING proteins' interactions with mineral we found that Spp24 bound to hydroxyapatite. Binding is often associated with anionic residues perhaps relating a modification such as phosphorylation to the mineral binding abilities of Spp24 and suggesting a role of Spp24 in matrix calcification (Veis, Sfeir et al. 1997; Huq, Cross et al. 2005).

This study provides evidence for multiple molecular weight forms of Spp24 (principally 45-55kDa) that were detected in all tissues examined including liver, brain, lung, kidney, spleen, reproductive organs, heart and pancreas. The source gene expression of Spp24 was confirmed to be predominantly from the liver, but at least in rats, kidney is also a major organ for Spp24 expression and the level of expression increases in during hypocalcemia. The relationship of extracellular Spp24 to bone was reemphasized by the minimal detection of Spp24 protein in several bone, as well as kidney, cell models and the ability of Spp24 to bind hydroxyapatite. In conclusion, Spp24 is a multiply phosphorylated circulating molecule with potentially wide ranging effects on calcium homeostasis.

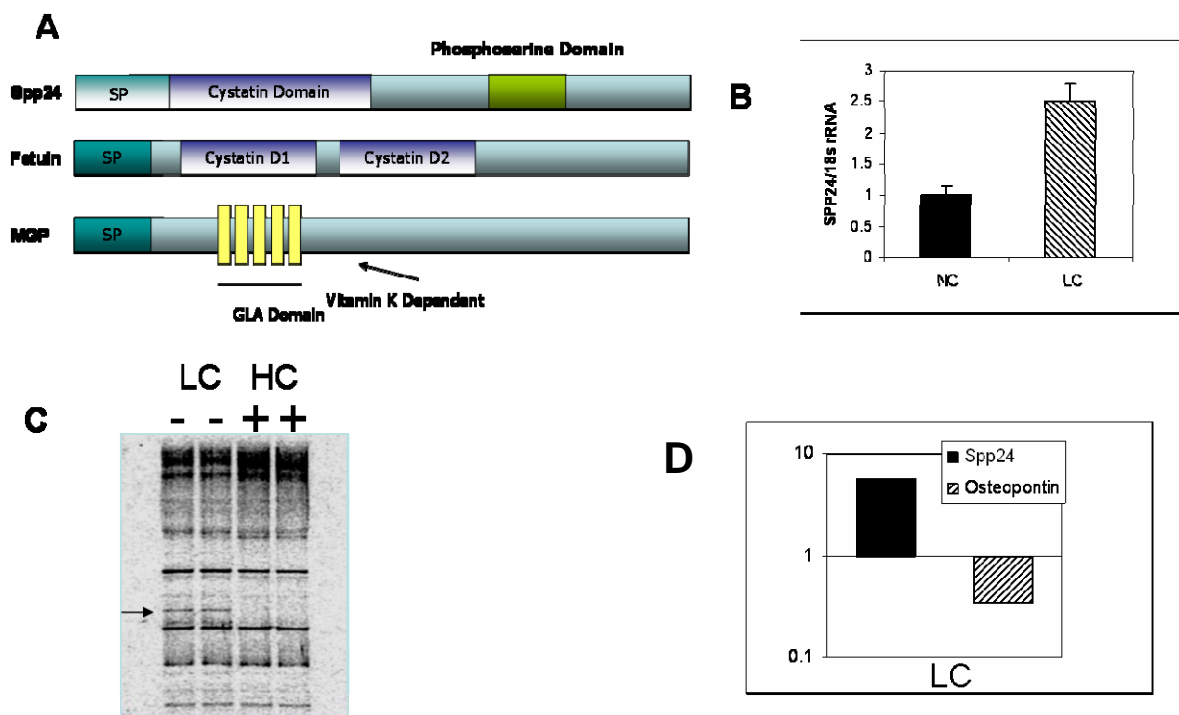
**Figure 11. Tissue Distribution of Spp24**

Q-PCR of rat liver RNA shows the tissues expression Spp24 (A). Greater expression levels were seen in the liver (B). A Western blot of human tissue protein showed Spp24 was present in numerous tissues and migrated to several different sizes (C). Western blots of immortalized cell lines protein extracts showed the presence of Spp24 in kidney epithelial cells, bone cells and myoblasts (D). The protein extracted from immortalized cells resulted in different size Spp24 bands based on extraction method; lysis after trypsin treatment of cells resulted in a sample enriched in intracellular, not extracellular, protein and the subsequent protein was 24 kDa. rSpp24 in figure D is a recombinant Spp24 protein used as a positive control.



**Figure 12. Regulation of Spp24 Expression by Serum Calcium Levels**

(A) Homology of Spp24 to other bone-related secreted phosphoproteins is depicted showing the presence of cystatin domains in Spp24 and osteopontin (OPN). Phosphoserine residues in Spp24 may interact with calcium while MGP interacts with calcium via Gla domains. (B) Q-PCR of RNA from rat cortical kidney shows Spp24 is up-regulated by low serum calcium (LC) conditions compared to normal serum calcium (NC) levels. (C) A gel of differential display PCR from cortical kidney shows the differentially regulated band (arrow) corresponding to Spp24 under low calcium conditions (LC) as compared to high serum calcium (HC). (D) An inverse regulation of Spp24 and OPN under low calcium conditions was found in microarray studies.



**Figure 13. Sequence of Rat Spp24**

A cDNA from Rat liver RNA was generated by reverse transcription PCR and sequenced. The sequence showed the presence of an additional amino acid in the sequence that was not seen in the annotated sequence available from GenBank. Fig A is our complete cDNA sequence with the nucleotides encoding the additional amino acid in the box. Fig B shows the corresponding amino acid sequence with the additional amino acid indicated by an arrow. Fig C shows the DNA sequence chromatograph with the additional nucleotides underlined.

**A**

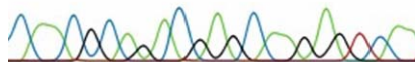
Nucleotide	10	20	30	40	50
0	ATGGAGCTGG	CAACGATGAA	GACGCTGGTT	ATGTTGGTGC	TGGGAATGCA
50	CTACTGGTGT	GCCTCAGGTT	TCCCGGTGTA	CGACTACGAC	CCTTCCTCTC
100	TGCAAGAAGC	TCTCAGTGCC	TCAGTGGCTA	AGGTGAACTC	GCAATCCCTG
150	AGTCCTTACC	TGTTTCGGGC	GACGCGGAGC	TCCCTGAAGA	GAGTTAATGT
200	CCTGGATGAA	GACACACTGG	TCATGAACTT	AGAGTTCACC	GTTTCAGGAAA
250	CTACATGCCT	AAGAGAGTCT	GGTGATCCCT	CCACCTGCGC	CTTCCAAAGG
300	GGCTACTCTG	TGCCAACAGC	TGCTTGCAGG	AGCACAGTGC	AGATGTCCAA
350	GGGACAGGTG	AAGGATGTGT	GGGCTCACTG	CCGCTGGGCG	TCCACATCCG
400	AGTCCAACAG	CAGTGAGGAG	ATGATTTTGT	GGGACATGGC	AAGATCCCAC
450	CGACGAAGAA	ATGATTATCT	ACTTGGCTTT	CTTTATGATG	AACCCAAAGG
500	TGAGCAGTTC	TATGACCGGT	CGATTGAGAT	CACGAGGAGG	GGACATCCTC
550	CTGCCCATAG	AAGGTTCCCTG	AACCTCCAAC	GCAGACGAGC	AAGAGTCAAT
600	TCTGGCTTTG	AGTGA			

**B**

Protein	10	20	30	40	50
0	MELATMKTIV	MLVLGMHYWC	ASGFPVYDYD	PSSLQEALSA	SVAKVNSQSL
50	SPYLFRAIRS	SLKRVNVLDE	DTLVMNLEFT	VQETTCCLRES	GDPSTCAFQR
100	GYSVPTAACR	STVQMSKGQV	KDVWAHCRWA	STSESNSSEE	MIFGDMARSH
150	RRRNDYLLGF	LYDEPKGEQF	YDRSIEITRR	GHPPAHRRL	NLQRRARVN
200	SGFE!				

**C**

CAACGCAGACGAGCAAGAGTCAA



**Figure 14. Post-Translational Modification Sites of Spp24**

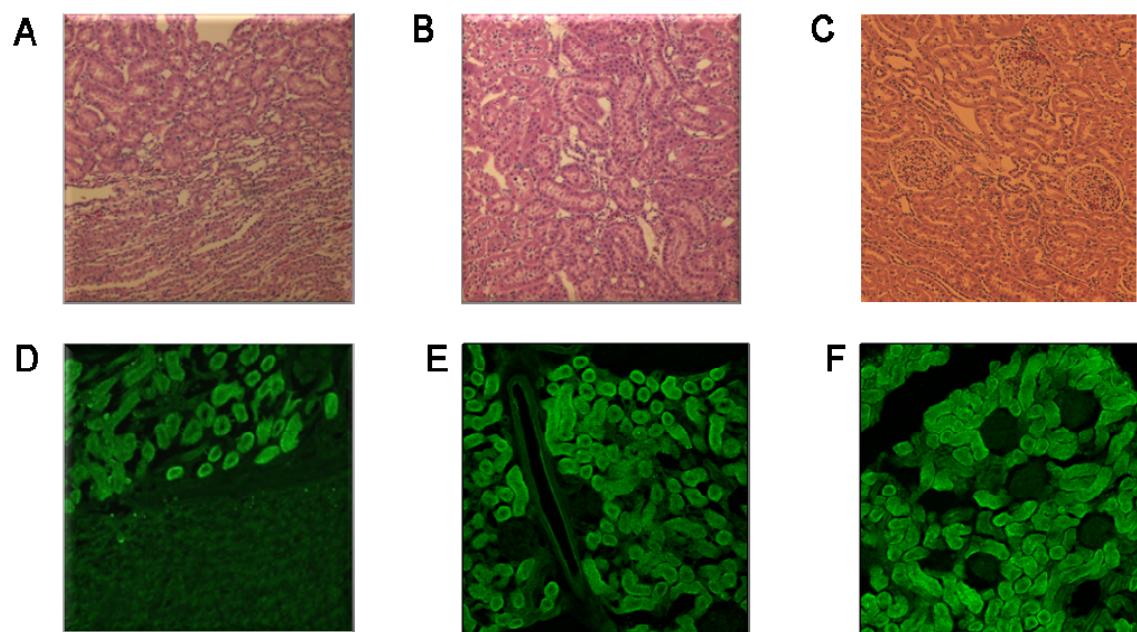
Post-translational modification sites of the rat, bovine, human and mouse Spp24 were determined using FindMod and GlycoMod software available on ExPASy.com. Glycosylation, PKC-dependent phosphorylation, PKA-dependent phosphorylation, casein kinase-II phosphorylation, and myristylation sites are all shown with the corresponding amino acid numbers of the computed sites.



	<b>N-Glycosylation</b>	<b>PKC- Dependent</b>	<b>PKA-Dependent</b>	<b>Casein Kinase II</b>	<b>Myristylation</b>
<b>Rat</b>	136 - 139: NSSE	5 - 7: TmK 81 - 83: SIK 149 - 151: ShR 178 - 180: TrR		33 - 36: SlqE 80 - 83: TvqE 131 - 134: StsE 137 - 140: SseE 200 - 203: SgfE	
<b>Bovine</b>		30 - 32: SIK 58 - 60: SVK 82 - 84: TcR 110 - 112: TvR	84 - 87: RReS 184 - 187: RRyS	30 - 33: SlkE 87 - 90: SeaD 134 - 137: SssE 135 - 138: SseE 163 - 166: SrgE	132 - 137: GSesSE 148 - 151: GSstSR <u>Tyrosine Kinase</u> 162 - 169: RsrqEpl.Y
<b>Human</b>		39 - 41: SiR 87 - 89: SIK 88 - 88: SiR 91 - 93: TcR 119 - 121: TvK 157 - 159: ShK	93 - 96: RKdS	3 - 6: SrmE 39 - 42: SlrD 86 - 89: SirE 96 - 99: SgeD 139 - 142: StsE 145 - 148: SseE 173 - 176: SisE 208 - 211: TdfE	
<b>Mouse</b>	136 - 139: NSSE	81 - 83: SIK 149 - 151: ShR		33 - 36: SlqE 80 - 83: SvqE 131 - 134: SssE 137 - 140: SseE 165 - 168: SrsE 200 - 203: SgfE	181 - 186: GQppAH

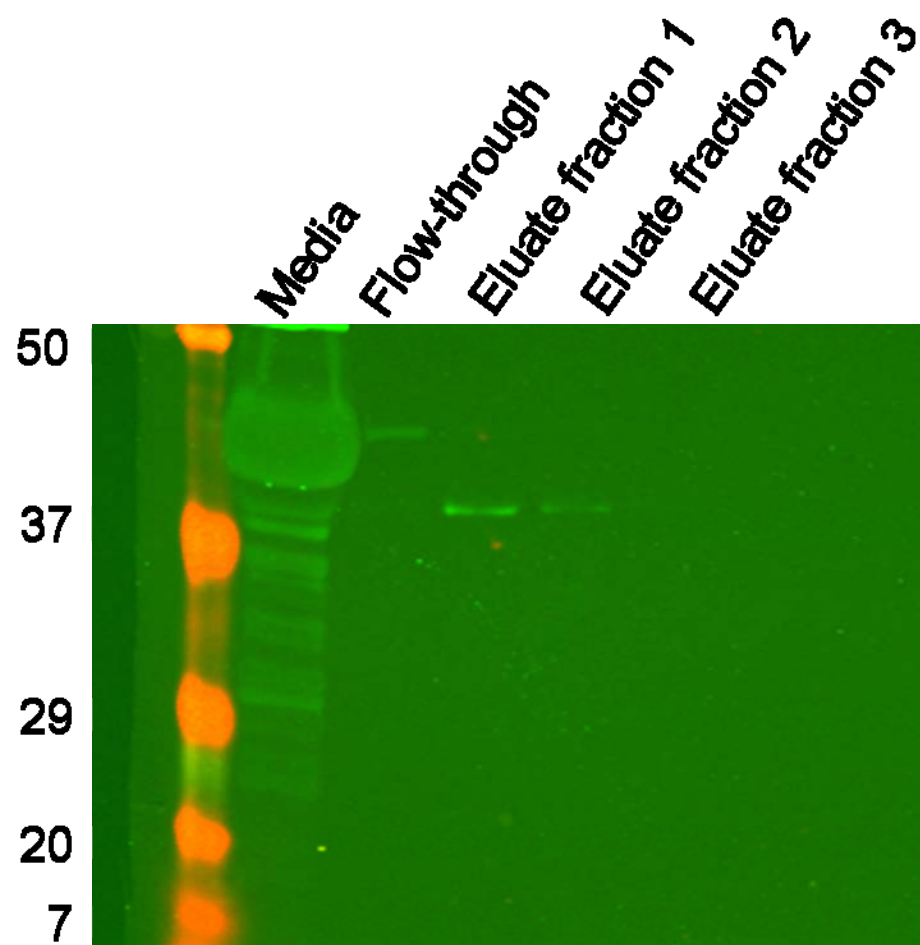
**Figure 15. Localization of Spp24 in the Kidney**

Panels A, B and C show H&E staining of rat kidneys. Panels C, D and E show confocal imaging of sections stained with an anti-Spp24 antibody and an alexaFluor-488 secondary antibody taken at 10x zoom. Spp24 is present in the tubules of the kidney cortex though clearly absent from glomeruli (F). Spp24 was not seen in the medulla (lower unstained area of D)



**Figure 16. Hydroxyapatite Binding of Spp24**

Media from Spp24 transduced cells was placed onto a hydroxyapatite column. Aliquots of media, flow-through and eluted fractions were tested by immunoblot. The eluted fractions showed the presence of Spp24 as detected by the anti-Spp24 antibody. Molecular weight standards and corresponding weights are shown on the left.



## **CHAPTER 3**

### **Secreted Phosphoprotein-24 (Spp24) Promotes Osteoblastogenesis but Requires BMP Ligand to Activate Runx2 and Osterix Transcription**

#### **Abstract**

Spp24 is a secreted phosphoprotein shown to accumulate in bone that displays homology to both cystatin C and TGF- $\beta$  receptor type-II. Spp24, based on several physical properties and on in vivo implantation studies, has been suggested to be the original bone morphogenetic protein which Urist described but never fully isolated. We undertook a study to examine the role Spp24 plays in the differentiation and function of osteoblasts by transducing primary human mesenchymal stem cells with adenovirus constructs expressing Spp24 and LacZ. The results showed 1835 of the 20,000 genes tested were regulated greater than two-fold. The expression of Spp24, though not resulting in expression of key osteoblast genes such as Runx2 or OSX did favor an osteoblast phenotype as did notable morphological changes and the presence of nodule formation. Spp24 appears to alter mesenchymal character by preventing numerous differentiation paths while it alters signaling pathways in a manner that primes the cells to react to stimuli that drive

differentiation. We examined how Spp24 affects BMP signaling by running parallel experiments that were supplemented by treatment with BMPs over the course of the 15 days. This allowed examination of how Spp24 effects are altered by osteoinductive signals but also how BMP changes cells over longer periods than typically examined in the literature.

## **Introduction**

Microarray has proven to be an invaluable tool in the study of osteoblast differentiation and the accompanying cellular changes of gene expression. Typically most studies utilize a relatively undifferentiated population of cells and provide the activating stimulus to initiate an osteoblast differentiation program with the recovered data illuminating the mechanism by which the stimulus acts (Huang, Yang et al. 2007). The studies have not only provided insightful information about how various stimuli act but also the behavior of mesenchymal stem cells (MSCs) (Marie and Fromigue 2006). MSCs have generated a lot of interest as they are easily isolated through bone marrow aspirates and have great potential with studies showing differentiation of MSCs into osteoblasts (Haynesworth, Goshima et al. 1992; Bruder, Jaiswal et al. 1997), adipocytes (Pittenger and Martin 2004), myocytes (Xu, Zhang et al. 2004), neurons and astrocytes (Sanchez-Ramos, Song et al. 2000; Hofstetter, Schwarz et al. 2002). Most of these studies in focus on early changes in MSC character as these are the initial steps in the determination of an osteoblastic fate.

Studies of osteoblast differentiation have implicated a large number of genes involved in the transition of cells to osteoblasts. Many studies have examined the changes that occur during bone morphogenetic protein (BMPs) induced differentiation (Korchynskyi, Decherling et al. 2003). Studies examining osteoblast differentiation in undifferentiated cells have shown multiple signaling pathways are involved in this complex process, indicating a complex signaling network with signaling crosstalk between Notch, Wnt and BMP pathways likely playing an important role (Canalis, Deregowski et al. 2005). Similarly, a large number of transcription factors have been shown to play important roles in altering the transcriptional activity of differentiating cells. Of principal importance are the transcription factors RUNX2 and OSX that are known to be essential to osteoblastogenesis (Komori, Yagi et al. 1997; Nakashima, Zhou et al. 2002).

Many signaling pathways have been implicated as regulators of osteoblast differentiation including Wnt signaling, hepatocyte growth factor (HGF), fibroblast growth factors (FGFs), and Notch. Wnt's are critical to bone development as illustrated by the absence of bone in wnt conditional knockout mice; instead the animals developed only cartilaginous skeletons with osteoblasts that were not fully differentiated (Hu, Hilton et al. 2005). Canonical Wnt signaling is activated by the binding of Wnts to a heterodimeric receptor complex composed of a G-protein coupled receptor, Frizzled, and low-density lipoprotein receptor related protein-5 or -6 (LRP5 or LRP6). Conflicting reports on the role of HGF have recently appeared in the literature. HGF has been shown to enhance osteoblastogenesis when adsorbed to hydroxyapatite surfaces (Zamboni, Camerino et al. 2000; Hossain, Irwin et al. 2005). Recently, HGF has also been shown to negatively affect



BMP signaling in osteoblast differentiation possibly through the inhibition of receptor-activated SMAD nuclear translocation (Standal, Abildgaard et al. 2007). The discrepancy between these reports is not fully understood however some of the difference is suggested to relate to the enhanced self renewal of MSC under transient treatment in some studies while longer studies with prolonged exposure are more affected by the inhibition of BMP signaling. Fibroblast growth factor 18 (FGF18) is an important factor in bone development as it enhances osteoblast differentiation and animals lacking FGF18 experience delayed bone development (Liu, Xu et al. 2002; Ohbayashi, Shibayama et al. 2002; Liu, Lavine et al. 2007). Notch signaling is another important pathway involved in osteoblast development that plays

Secreted phosphoprotein-2 (Spp24) is a secreted protein produced primarily in the liver and kidney that accumulates in the bone matrix (Sen, Walker et al. 1987; Hu, Coulson et al. 1995). The sequence of Spp24 reveals a cystatin homology domain in the N-terminal portion of the molecule that overlaps with a TGF- $\beta$  receptor II homology (T $\beta$ RII) domain (Hu, Coulson et al. 1995; Bennett, Khorram Khorshid et al. 2004). The C-terminal half contains a Spp24 signature domain conserved in over 20 species of vertebrates and the cartilaginous dogfish. Within the Spp24 domain is a SSEE sequence that is a conserved casein kinase recognition motif (Veis, Sfeir et al. 1997) and is also seen in SIBLING family proteins that play important roles in promoting matrix mineralization in the calcification of osseous tissues like bone and teeth (Wu and Veis 1990; Qin, Baba et al. 2004; Huq, Cross et al. 2005). Previous work in this lab has shown up-regulation of Spp24 in a study of the regulation of genes in the kidney under hypocalcemic conditions (Bajwa,

Horst et al. 2005). Recently, Benham, *et al.* showed that the T $\beta$ RII domain of Spp24 (called BMP binding peptide or BBP) when recombinantly produced in bacteria and placed in animal muscle pouches induced ectopic calcification and bone formation alone and co-implantation of BMP-2 with BBP enhanced the native osteoinductive of BMP-2 (Behnam, Phillips et al. 2005). They contend (Behnam, Phillips et al. 2005; Dagleish, Francis et al. 2006) that Spp24 is Marshall Urist's original bone morphogenetic protein/non-collagenous protein (BMP/NCP) (Urist 1965). The T $\beta$ RII domain of Spp24 has been implicated in the regulation of TGF $\beta$  family cytokines such as BMPs while the effect of the Spp24 is unclear but homology suggests it may be important in matrix calcification *in vivo*.

This study employed adenoviral over-expression of Spp24 as a means to investigate the long term effects of Spp24 on MSC differentiation to osteoblasts and on BMP-2/7 heterodimer driven differentiation. After transduction we used microarray analysis at 15 days to examine how the Spp24 over-expression changed the expression profile of these cells. Results showed changes in expression patterns that created a pre-osteoblastic condition in cells that while not fully committed to osteoblast differentiation had increased levels of genes associated with osteoblast differentiation and genes that prevented alternate pathways of mesenchymal cell differentiation.

## **Materials and Methods**

### *MSC Isolation and Culture*

Bone marrow aspirates were obtained from the femur of primary hip revision patients at the Virginia Commonwealth University/Medical College of Virginia Medical

Center via an IRB approved protocol. Bone marrow was transported to the VCU Orthopaedic Research Laboratory and combined with 2x volume of sterile saline and mixed by pipette. The bone marrow was layered over a Histopaque 1077 (Sigma) and centrifuged at 513g for 30 min at 4°C in a swinging bucket rotor with no brake. The layer in the gradient containing the mononuclear cells was collected by pipette and plated in plastic flasks for 48 hrs in DMEM Hi-glucose media containing 10% FBS and 1% penicillin/streptomycin. After 48 hrs the media was changed to remove non-adherent cells and fresh media with the addition of basic FGF at 2ng/ml (expansion media) was added. Cells were passaged three times before use in the following experiments.

For the generation of osteoblasts MSCs were cultured either in expansion media (as described above) or media containing 10 mM  $\beta$ -glycerol phosphate, 0.2 mM Ascorbic acid, and  $10^{-8}$  M Dexamethasone (osteogenic media) for 14 days. Additional experiments were performed where Dexamethasone was replaced with 10ng/ml of BMP-2/7 heterodimer.

#### *Adenoviral Transduction*

Mouse Spp24 cDNA and LacZ cDNA were cloned into CMV promoter controlled adenoviral constructs. The adenoviral constructs were used to transduce MSCs to a multiplicity of infection (MOI) of 100 viral particles per cell. Experimental groups examined Spp24 versus LacZ transduction as well as groups where both Spp24 and LacZ transduction was supplemented with 10ng/ml of BMP 2/7 heterodimer added with each change of media every 3 days.

#### *RNA Isolation*

RNA from virally transduced MSCs was collected using the TriZOL (invitrogen). After isolation contaminating DNA was removed from RNA samples using a 30 minute treatment with DNase I at 37°C. RNA was stored in RNase free DEPC-treated water.

#### *Microarray*

RNA was hybridized to Affymetrix human U133A GeneChips for microarray analysis at the VCU Nucleic Acid Core Facility. Gene profiles were further analyzed using the Affymetrix GCOS v1.4 software to identify biological processes, molecular function and cellular component effects of treatments.

### **Results**

#### *Spp24 in Osteoblasts*

MSCs were cultured 14 days supplemented with  $\beta$ -glycerol phosphate, ascorbic acid, and either BMP-2/7 or Dexamethasone to induce osteoblast differentiation (as seen by alizarin red staining in figure 17 A). These cells showed the presence of a smaller ~18 kDa size band, as detected by Western blot (figure 17 B) with an anti-Spp24 antibody, in the osteogenic media cultured cells while control cells treated with expansion media for the 14 days did not show a 18 kDa size band corresponding to Spp24 .

#### *Morphology and Histology*

Alizarin Red staining was used to examine the presence of calcium deposits as a measure of matrix mineralization (figure 18 A). The Spp24-transduced group showed weak Alizarin red staining that was more than that seen with the LacZ control transduced group. Both of the virally transduced groups that were treated with BMP 2/7 heterodimer

displayed strong alizarin red staining after 15 days however there was no distinguishable differences between the two. Alkaline phosphatase staining (figure 18 A), a marker of bone cell activity, was more intense when cells were transduced with an adenovirus containing Spp24 instead of LacZ, both between cells treated and untreated with BMP. In both alizarin red staining and alkaline phosphatase staining it appeared that Spp2 transduction without BMP treatment and LacZ transduction with BMP treatment showed comparable staining and Spp24 adenovirus combined with BMP treatment showed a greater intensity of staining. Cells transduced with control LacZ virus displayed no distinct morphological changes over the course of the experiments. They remained in a long tubular shape and grew to confluence in evenly distributed and parallel aligned mats of cells. Spp24 transduced cells exhibited distinguishable changes in cell morphology. Cells were observed to undergo significant rounding over the approximately two week time course (figure 18 B).

Cells coalesced and nodule formation was observed within the population of cells (figure 18 B&C). In cells treated with viral transduction and supplemented with BMP 2/7 heterodimer the cells, both Spp24 and LacZ transduced, showed the presence of osteoblast characteristics with nodule formation and osteoblast morphology. Nodule formation was comparable between BMP and Spp24 however when there BMP-2/7 was added to Spp24 there were more nodules seen than any other treatment.

### *Microarray*

In the experiment examining gene profile expression between Spp24 and LacZ transduced MSCs 57.2% of the 22,000 genes on the chip were present at detectable levels.

Of the detected genes 884 were up-regulated by at least 2-fold while 995 were 2-fold or greater down-regulated. Table 2 and 3 detail the most up- and down-regulated genes in these experiments. In addition to these there were also patterns of regulation as suggesting ectopic expression of Spp24 alters multiple aspects of MSC biology. Gene cluster analysis shows that among the genes classified in molecular functions, Spp24 affected MAP kinase activity, metal ion binding, cysteine-type endopeptidases, and protein serine/threonine kinase activity. Interestingly, among the genes clustered as biological properties there were numerous categories linked to osteoblast activity: osteoblast differentiation (up-regulation of TAZ; down-regulation of Msx, Runx2, and Osx), positive regulation of BMP signaling (up-regulation of BMPRII, Tribbles, Id2, and Id3), negative regulation of TGF $\beta$  signaling (down-regulation of Smad2, Smad3, TIEG, and Cerberus), and negative regulation of Wnt signaling pathway (downregulation of FGF18, Wnt5a, Axin, and APC). Interestingly there was also some negative regulation of BMP signaling with follistatin and chordin down-regulated while HGF antagonist was up-regulated. The Notch signaling pathway also displayed positive regulation as Notch, Jagged, and Hey2 were up-regulated. Groups of genes associated with alternate differentiation pathways of MSCs were regulated; genes for chondrocyte (Sox9, COMP), adipocyte (PPAR $\delta$ , C/EBP $\beta$ , adipsin), and myocyte (myosin, desmin) differentiation were all down-regulated.

Tables 4 and 5 detail the most highly regulated genes from microarrays that looked at Spp24 transduction compared to LacZ transduction when both were supplemented with BMP-2/7 heterodimer. Collagen expression patterns changed as was seen by two highly regulated genes, COL5a3 and COL4a3, being up- and down-regulated, respectively.

HOXA11 is another up-regulated gene important in skeletal patterning and mesenchymal cell differentiation.

## **Discussion**

Spp24 was shown to enhance BMP signaling and had an intrinsic osteogenic activity in *in vivo* implantation experiments conducted by Behnam, et al. (Behnam, Phillips et al. 2005). They found an 18.5 kDa fragment of Spp24 in their isolation of bone proteins similar to the ~18 kDa size fragment of Spp24 that was seen as the MSCs were exposed to conditions leading to osteoblast generation. To understand this activity we employed viral transduction as a method of over-expressing Spp24 in undifferentiated MSCs. Examination of morphological changes showed that the expression of Spp24 led to the limited formation of nodules characteristic of *in vitro* osteoblast cultures (Nefussi, Boy-Lefevre et al. 1985; Bhargava, Bar-Lev et al. 1988). Similarly cells transduced with Spp24 showed weak Alizarin red staining compared to the control transduced cells. These preliminary indications suggest that the over-expression of Spp24 may be pushing the cells to a more osteogenic phenotype; however it is worth noting that the typical experiments driving undifferentiated cells towards osteoblasts using BMPs, dexamethasone, or other known osteogenic molecules result in these changes in a shorter amount of time and this may indicate Spp24 is not as potent an osteogenic factor as some of the classical inducers of osteoblastogenesis.

To analyze exactly how Spp24 affects these changes in the MSCs we utilized microarrays. While the hallmarks of osteoblast differentiation, up-regulation of the

transcription factor Runx2 and its accompanying downstream transcription factor Osx were not seen there were significant changes that activate or potentiate pathways that will do affect osteoblast activity. The down-regulation of Runx2 and Osx shows the cells were not differentiated to osteoblasts, the down-regulation of significant effectors of alternate differentiation pathways, notably myocyte, adipocyte and chondrocyte, suggest that Spp24 may prevent these differentiation outcomes. An increase in TAZ demonstrates that Spp24 regulates a key step in initial osteoblast differentiation. TAZ is considered a critical rheostat that inhibits adipogenesis in MSCs while preserving the self-renewal aspect of the early osteoblast.

Numerous signaling pathways that have been implicated in osteoblast differentiation were seen to be regulated by Spp24 over-expression. Noteworthy among the regulated genes by Spp24 was the increase in Tribbles, a protein that affects cell cycle and migration control through its ability to target protein for proteasomal degradation. A Tribbles family member protein, Tribbles-3, was recently shown to interact with BMP receptor type II's cytoplasmic tail domain, and upon ligand binding Trb3 is released and targets Smurfs for proteasomal degradation. The degradation of Smurfs enhances the stability of Smads and their BMP signaling activity. The up-regulation of Tribbles may provide a mechanism for the osteoblast-like character of MSCs transduced with Spp24. The overall picture of Spp24's effects on MSC suggests a molecule that primes cells for an osteoblast phenotype despite not leading to fully committed differentiation while at the same time blocking alternate differentiation schemes.

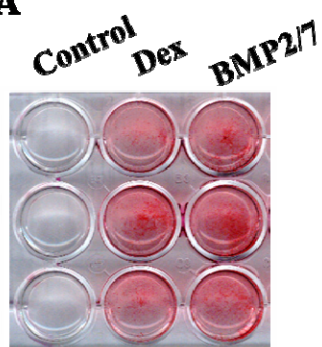
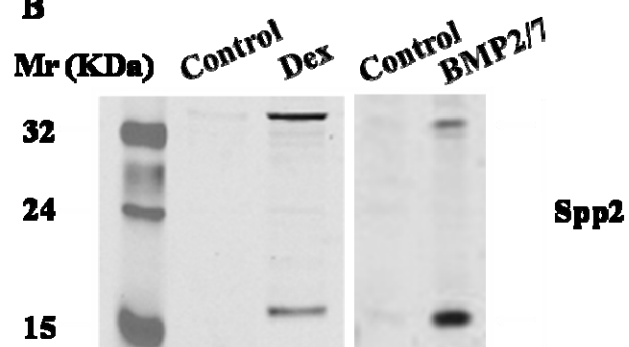


Overexpression of Spp24 with BMPs showed a number of similar genes, however there was a significant change that seemed to result in a more fully committed osteoblast cell. As seen in the table of highly regulated genes collagen expression patterns changed with COL1a1 also being up-regulated 2-fold. Additionally several MMPs were up-regulated. TGF $\beta$  expression decreased 2.4 fold when BMP treatment was combined with Spp24 transduction.

Spp24 is a secreted molecule that accumulates in the bone matrix and its function in bone is just beginning to be understood. It is predicted that Spp24 functions both independently of BMP ligands and in combination with BMPs once converted to a shorter active peptide. This study presents evidence for an important role of Spp24 in steering mesenchymal cell differentiation towards an osteoblast lineage. Additionally the interaction of Spp24 with TGF $\beta$  family ligands is an important function of Spp24, particularly relevant to bone mineralization is the interaction with BMPs. In conclusion, Spp24 appears to regulate early osteoblast differentiation by up-regulating the Notch/Jagged signaling pathway and by stimulating the rheostat gene TAZ. The combined effect of treatment of Spp24 with BMP accelerates BMP-mediated osteoblastogenesis and matrix mineralization.

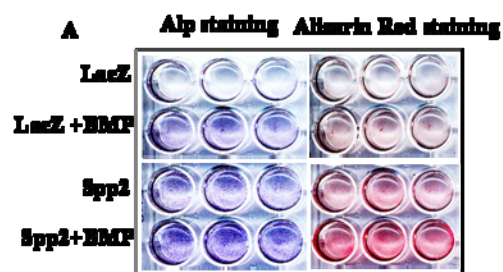
**Figure 17. Osteoblast Differentiation is Accompanied by a Conversion of Spp24 to Spp18**

Osteoinductive properties of Spp24 induced by Dex and BMP. MSCs were cultured in DMEM with 10% FBS, beta glycerophosphate, ascorbic acid and dex ( $10^{-8}$  M) and BMP2/7 (10ng/ml) for 15 days. Treatments were renewed every 3 days. Total cell extracts were used to analyze Spp24 expression. A) Osteoblast phenotype was examined by alizarin Red staining that showed the mineralization of extracellular matrix B) Western Blot results revealed that a smaller form of Spp24 was upregulated by both dex and BMP2/7 treatments. Cells also showed morphological changes during these time period.

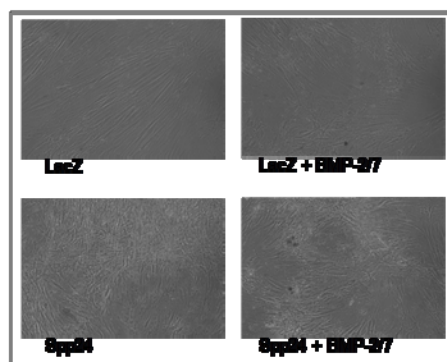
**A****B**

**Figure 18. Effect of Spp24 Overexpression on Mineralization and Nodule Formation**

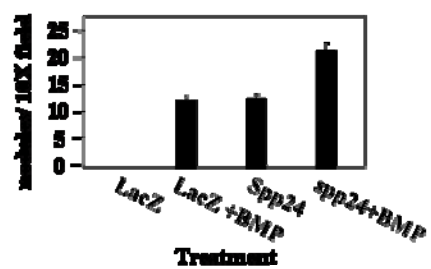
Effect of over expression of full length mouse Spp24 in MSC following 15 days adenoviral transduction. A) MSC cells after third passage were transduced with lacZ and spp24 adenoviral contrruct. Adenoviral LacZ used as a control transduction to adenoviral spp24. After transduction MSCs were cultured in DMEM with 10% FBS, beta glycerophosphate, ascorbic acid and with or without BMP2/7(10ng/ml) for 15 days. Renewal of media was done every 3 days interval. Results revealed that at day 14 mspp24 expression induced significant osteoblastogenesis assessed by alkaline phosphatase and alizarin Red staining. B-C) Morphological changes during osteogenesis and average counts of bone forming nodules per 10X power field. Adenoviral transduction was done either with LacZ control or Spp24 and both were treated vehicle and 10ng/ml BMP2/7 for 14 days. Medium was changed every 3 days interval. Seven independent fields were selected to get an average.



**B**



**C**



**Table 2. Genes Increased by Spp24 Transduction**

Genes highly up-regulated by Spp24 transduction compared to LacZ transduction of MSCs after 15 days.

Accession No.	Gene	Symbol	Fold Change
NM_007345	Zinc finger protein 236	ZNF236	15.8
NM_005768	Putative protein similar to Drosophila nessy	C3F	10.4
NM_002021	Flavin containing monooxygenase 1	FMO1	10.2
NM_080657	Radical S-adenosyl methionine domain containing 2	RSAD2	9
NM_006887	zinc finger protein 36, C3H type-like 2	ZFP36L2	9
NM_002317	Lysyl oxidase	LOX	8.8
NM_003242	Transforming growth factor, beta receptor II	TGFBR2	8.8
NM_001007097	Neurotrophic tyrosine kinase, receptor, type 2	NTRK2	8.4
NM_001850	Collagen, type VIII, alpha 1	COL8A1	8.2
NM_001033026	Chromosome 19 open reading frame 6	C19orf6	8
D38081	Thromboxane A2 receptor	-	8
NM_007368	RAS p21 protein activator 3	RASA3	8
NM_002872	Ras-related C3 botulinum toxin substrate 2	RAC2	7.8
NM_004587	Ribosome binding protein 1	RRBP1	7.6
AF091627	p73-like tumor protein	CUSP	7.6
NM_016593	Oxysterol 7alpha-hydroxylase	CYP39A1	7.6
U77949	Cdc6-related protein	HsCDC6	7.4
AF074480	CMP-N-acetylneuraminic acid hydroxylase	-	7.4
AF028333	Growth/differentiation factor-11	GDF11	7.2
NM_002983	Small inducible cytokine A3	SCYA3	7
NM_003999	Oncostatin M receptor	OSMR	6.8
NM_002028	Farnesyltransferase, CAAX box, beta	FNTB	6.8
NM_000201	Intercellular adhesion molecule 1	ICAM1	6.8
D84140	Immunoglobulin light chain V region	maB56	6.8
NM_001508	G protein-coupled receptor 39	GPR39	6.6
NM_182487	Olfactomedin-like 2A	OLFML2A	6.4
NM_003728	unc5 C	UNC5C	6.4
AF044286	Histone macroH2A1.1	-	6.4
NM_005623	Chemokine (C-C motif) ligand 8	CCL8	6.2
NM_024609	Nestin	NES	6.2
NM_002318	Lysyl oxidase-like 2	LOXL2	6.2

**Table 3. Genes Decreased by Spp24 Transduction**

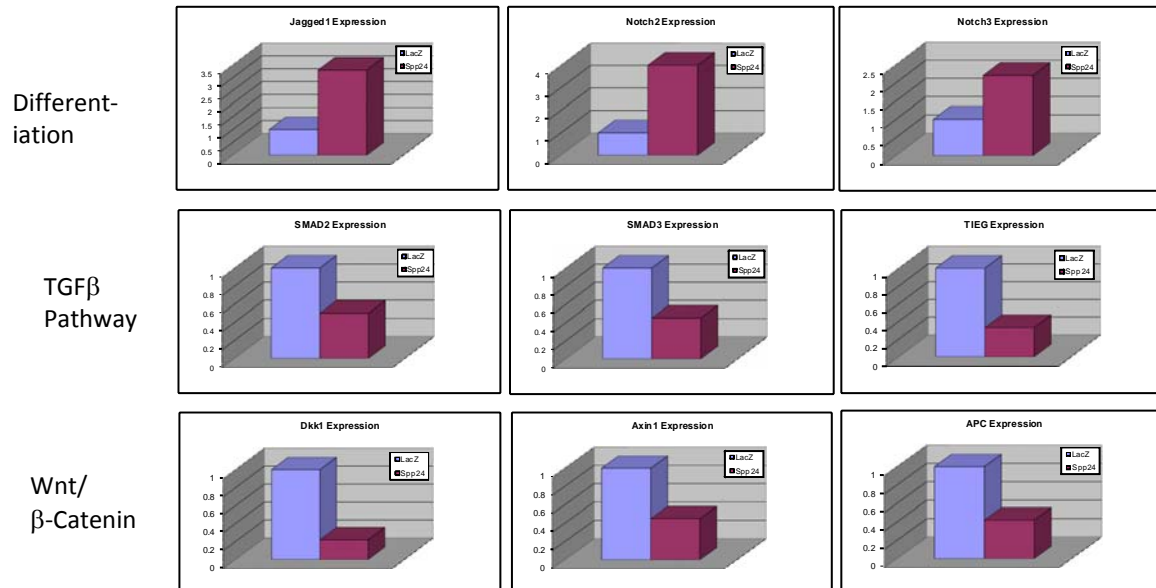
Genes highly down-regulated by Spp24 transduction compared to LacZ transduction of MSCs after 15 days.

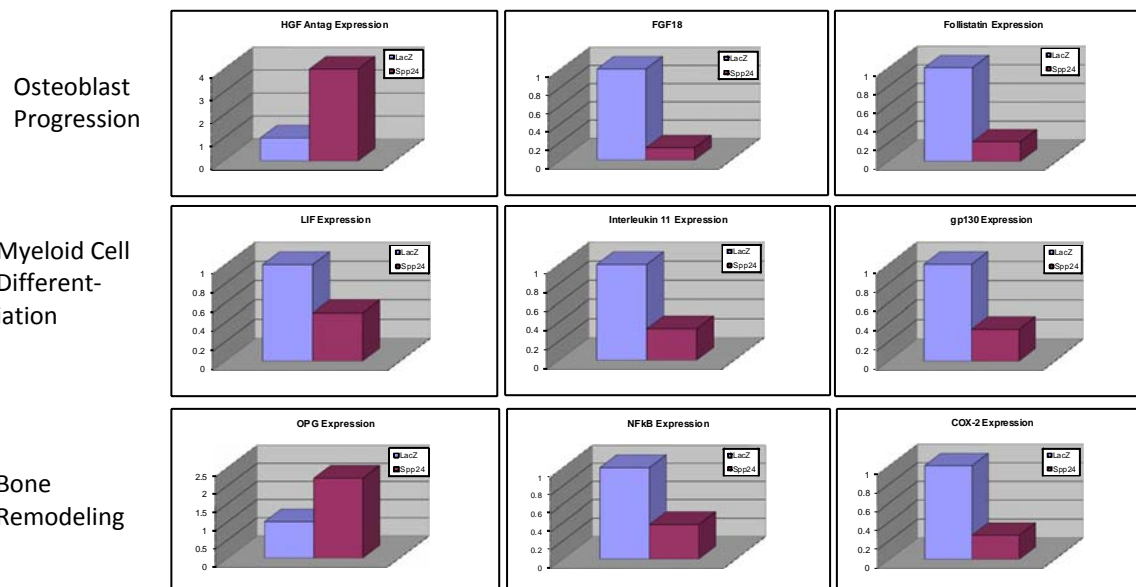


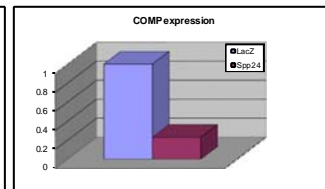
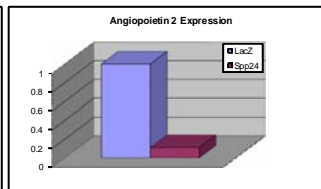
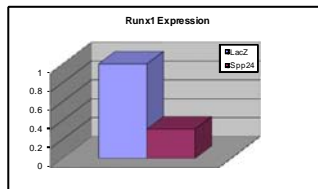
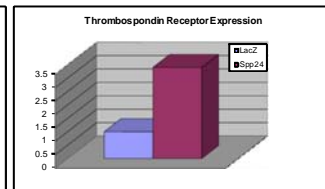
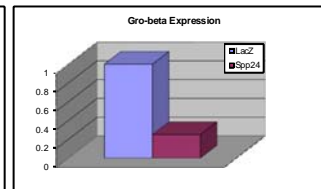
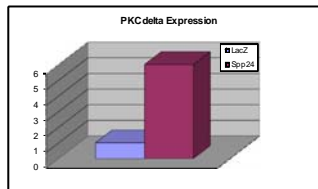
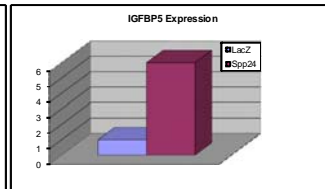
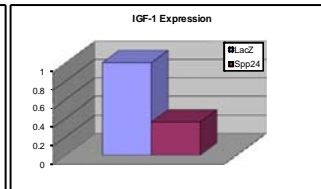
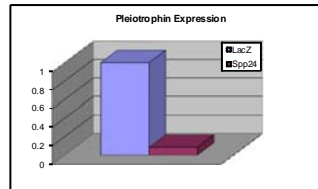
Accession No.	Gene	Symbol	Fold Change
NM_002825	Pleiotrophin	PTN	-11.8
NM_018518	Minichromosome maintenance complex component 10	MCM10	-10.8
NM_014246	Cadherin, EGF LAG seven-pass G-type receptor 1	CELSR1	-10.2
NM_006515	SET domain and mariner transposase fusion gene	SETMAR	-9.6
NM_001063	Transferrin	TF	-9.2
NM_004049	BCL2-related protein A1	BCL2A1	-9
NM_002888	Retinoic acid receptor responder 1 (tazarotene induced)	RARRES1	-8.8
AK021983	cDNA FLJ11921 fis, clone HEMBB1000318	-	-8.8
NM_001147	Angiopoietin 2	ANGPT2	-8.8
NM_003862	Fibroblast growth factor 18	FGF18	-7.4
BC000527	Ewing sarcoma breakpoint region 1	-	-7.4
NM_018700	Zinc-binding protein Rbcc728	Rbcc728	-7.2
NM_004543	Nebulin	NEB	-7.2
NM_001861	Cytochrome c oxidase subunit IV	COX4I1	-7
NM_022780	Required for meiotic nuclear division 5 homolog A	RMND5A	-7
NM_004876	Zinc finger protein 254	ZNF254	-6.6
NM_024115	Hypothetical protein MGC4309	-	-6.6
NM_021647	Microfibrillar-associated protein 3-like	MFAP3L	-6.6
X58987	D-1 dopamine receptor.	-	-6.6
NM_016619	Placenta-specific 8	PLAC8	-6.2
NM_025054	Valosin-containing protein (p97)/p47complex-interacting protein p135	VCIP135	-6
NM_015094	Hypermethylated in cancer 2	HIC2	-6
M27830	28S ribosomal RNA gene	-	-5.8
NM_004364	CCAATenhancer binding protein alpha	CEBPA	-5.8
NM_025154	unc-84 homolog A	UNC84A	-5.8
NM_014950	Zinc finger and BTB domain containing 1	ZBTB1	-5.8
xu43g07.x1	cDNA FLJ12327 fis, clone MAMMA1002140	-	-5.4
X80821	Ribosomal protein L18a homologue.	-	-5.4
NM_014645	Centrosomal protein 135kDa	CEP135	-5.2
U26662	Neuronal pentraxin II	NPTX2	-5.2
NM_002090	GRO3 oncogene	GRO3	-5.2

**Figure 19. Regulation of Genes by Spp24 Transduction**

Shown is the regulation of specific genes as found in our microarray study. Genes are grouped by similarity of function. TGF- $\beta$  signaling pathways were decreased; Notch and Jagged genes regulating differentiation were decreased; Wnt signaling was decreased; HGF signaling was increased; FGF-18 signaling was decreased; Osteoblast progression was halted; markers of myeloid fate were decreased; bone remodeling was increased; Early osteoblast development was seen with *Gro $\beta$*  increasing to delay mineralization; and finally matrix development was increased. These gene regulation patterns show how Spp24 alters MSC differentiation holding cells in a pre-osteoblast state

**A**

**B**

**C**Lineage  
Establish-  
mentEarly  
Osteoblast  
Different-  
iationMatrix  
Development

**Table 4. Genes Increased by Spp24 + BMP Treatment**

Highly up-regulated genes in cells transduced with Spp24 and treated with BMP-2/7 compared to cells transduced with LacZ and treated with BMP-2/7.

Accession No.	Gene	Symbol	Fold Change
NM_002155	Heat shock 70kD protein 6	HSPA6	19.8
NM_007345	Zinc finger protein 236	ZFP236	12.8
NM_0022144	Myodulin protein	LOC64102	11
M27830	28S ribosomal RNA gene	-	10.6
U88968	Alpha enolase like 1	ENO1L1	10.6
NM_005733	RAB6 interacting, kinesin-like	RAB6KIFL	10.6
NM_005523	Homeo box A11	HOXA11	10.2
NM_018492	PDZ-binding kinase	PBK	10
NM_001034	Ribonucleotide reductase M2 polypeptide	RRM2	10
NM_014750	discs, large homolog 7	DLG7	9.8
X51757	Heat-shock protein HSP70B	HSP70B'	9.8
NM_002421	Matrix metalloproteinase 1	MMP1	9.6
NM_018136	Abnormal spindle homolog microcephaly associated	ASPM	9
NM_000361	Thrombomodulin	THBD	9
NM_004887	chemokine (C-X-C motif) ligand 14	CXCL14	8.6
BC003186	GIN5 complex subunit 2	-	8.6
NM_001927	Desmin	DES	8.6
NM_003318	TTK protein kinase	TTK	8.2
NM_015719	Collagen, type V, alpha 3	COL5A3	8.2
NM_005397	Podocalyxin-like	PODXL	8.2
AF274954	PNAS-29	-	8.2
D88357	CDC2 delta T	-	7.8
NM_004417	Dual specificity phosphatase 1	DUSP1	7.8
NM_006240	Protein phosphatase, EF hand calcium-binding domain 1	PPEF1	7.8
AL117508	Epidermal Langerhans cell protein LCP1	-	7.6
NM_030945	Complement-c1q tumor necrosis factor-related protein	CTRP3	7.4
M10098	18S rRNA gene	-	7.2
U36189	p311 protein	hP311	7.2

**Table 5. Genes Decreased by Spp24 + BMP Treatment**

Genes down-regulated in cells transduced with Spp24 and treated with BMP-2/7 compared to cells transduced with LacZ and treated with BMP-2/7.

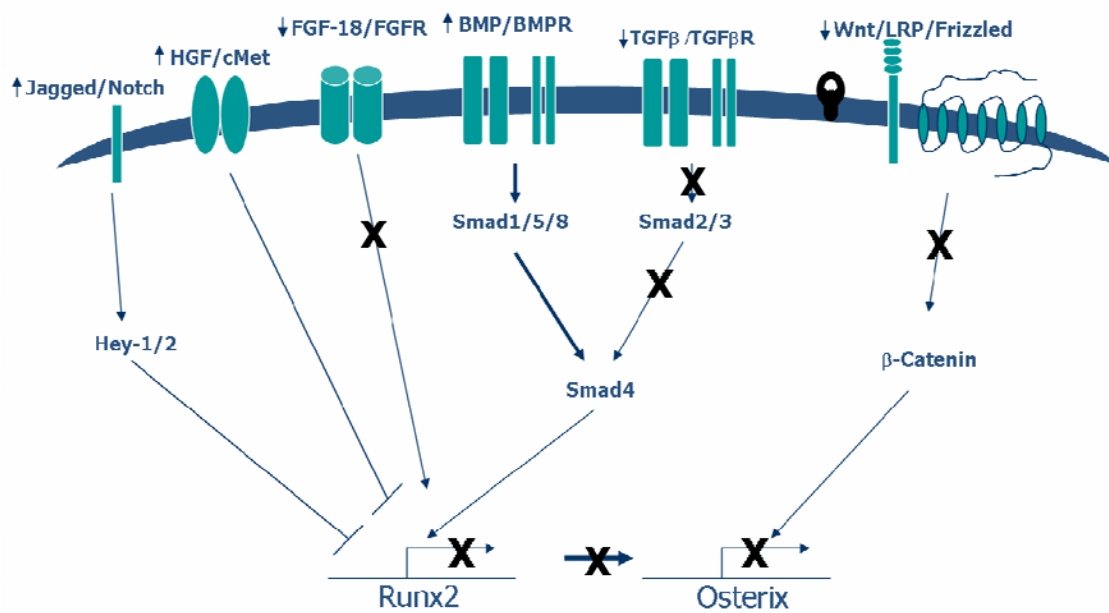


Accession No.	Gene	Symbol	Fold Change
NM_002825	Peiotrophin	PTN	-13
NM_138618	Rhesus blood group, CcEe antigens	RHCE	-12
NM_005000	NADH dehydrogenase 1 alpha subcomplex, 5	NDUFA5	-10.4
NM_031366	Collagen, type IV, alpha 3	COL4A3	-10
AK022215	cDNA FLJ12153 fis, clone MAMMA1000458	-	-9.6
NM_001508	G-Protein Coupled Receptor 39	GPR39	-9.4
NM_0022049	G-Protein Coupled Receptor 88	GPR88	-8.6
NM_003236	Transforming growth factor, alpha	TGFA	-8.6
NT_005612	Chromosome 3 genomic contig, reference assembly	-	-8
M60333	Human MHC class II HLA-DRA	-	-8
NM_080740	Suppressor of hairy wing homolog 1	SUHW1	-8
NM_002276	Keratin 19	KRT19	-7.8
U47054	Mono-ADP-ribosyltransferase	htMART	-7.6
NM_003810	Tumor necrosis factor superfamily, member 10	TNFSF10	-7.6
NM_004967	Integrin-binding sialoprotein	IBSP	-7.4
NM_007360	killer cell lectin-like receptor subfamily K, member 1	KLRK1	-7.4
NM_004139	Lipopolysaccharide-binding protein	LBP	-7.4
U32500	Type 2 neuropeptide Y receptor	-	-7.2
NM_021778	A disintegrin and metalloproteinase domain 28	ADAM28	-7.2
NM_013314	B cell linker protein	SLP65	-7
NW_922162	Chromosome 4 genomic contig	-	-7
NM_020904	Pleckstrin homology domain-containing, family A member 4	PLEKHA4	-7
NM_003566	Early endosome antigen 1	EEA1	-6.8
NM_003619	Protease, serine, 12 (neutrypsin, motopsin)	PRSS12	-6.8
U57059	Apo-2 ligand	-	-6.8
AL133386	Bone morphogenetic protein 5	BMP5	-6.6
NM_002125	Major histocompatibility complex, class II, DR beta 5	HLA-DRB5	-6.6
NM_000882	Interleukin 12A	IL12A	-6.6
NM_016619	Placenta-specific 8	PLAC8	-6.6

**Figure 20. Spp24 Effects on MSCs**

Spp24 transduction of MSCs elicited responses that blocked activation of critical osteoblast transcription factors RUNX2/CBFA1 and OSX. The effect of Spp24 on several important pathways in MSC differentiation are shown

### Effect of Spp2 on MSC Differentiation



## CHAPTER 4

### Conclusion and Future Directions

Studies in the literature have shown that Spp24 is both a protein that accumulates in bone and has a major effect on bone formation. We have examined the novel discovery of Spp24 in the kidney and described its expression in numerous tissues and cell lines. Additionally, we have investigated the effect of Spp24 on mesenchymal stem cells (MSCs) and how Spp24 alters BMP signaling on MSCs. These studies have provided great insight into the role or roles of Spp24 *in vivo*.

Investigations into the role of low calcium regulating genes in the kidney cortex led to the discovery that Spp24 is highly up-regulated under low serum calcium conditions. The previously unreported expression in the kidney cortex was strengthened by evidence of Spp24 seen in Western blots of kidney epithelial cells as well as in confocal microscopy showing expression in the kidney tubules. An interesting finding in Western blots of different cell models of both kidney and bone cells was the presence of higher size forms of Spp24. Sequence analysis showed there is a likelihood of multiple post-translational modifications including glycosylation, phosphorylation, and myristylation. There was also significant amount of protein seen in numerous tissues when a blot of multiple human

tissues was probed with the anti-Spp24 antibody. The presence in multiple tissues is not entirely surprising considering Spp24 is a secreted molecule that is part of a serum protein complex, but it does raise the question of what purpose that complex and Spp24 might serve in some of the distant tissues.

Sequencing of the rat Spp24 from liver RNA showed an additional base not present in the sequence published in GenBank. Clearly we will need to examine this further and report the results. The published sequence is for *Rattus Norvegicus* while we used Sprague-Dawley rats which could be a reason for the difference and it would be useful too compare sequences of RNA isolated from both animals. Also it would be necessary to sequence RNA from more animals to confirm our results. As a final step we need to express our cDNA in cells in culture before a sequence can be reported.

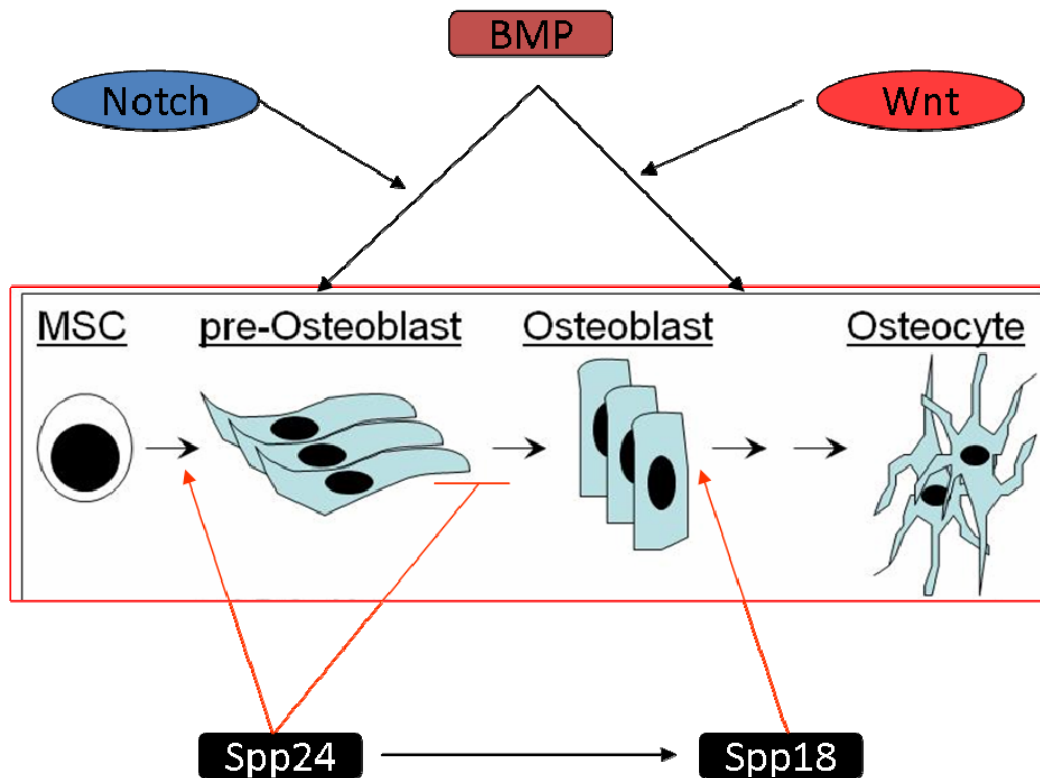
Spp24 has been found by both Senn, *et al.* and Behnam, *et al.* to be osteogenic and we investigated how Spp24 causes bone formation using microarray. Increased mineralization of cells cultured in vitro and distinct morphological changes accompanied the over-expression of Spp24 in MSCs. The unusual finding was the lacking of commitment of MSCs treated with Spp24 to an osteoblast lineage (no up-regulation of Runx2 or Osx) but instead there was a general trend to prevent full commitment to any lineage while instead activating other osteoblast related genes. The findings suggest Spp24 may be a molecule that causes cells to be primed to receive osteogenic signals. Our current understanding of Spp24's role in osteoblast differentiation is depicted in Figure 22. This activity of Spp24 is particularly interesting in a matrix protein that has also been shown to interact with BMPs. The method(s) of how Spp24 causes these cellular changes is still a

critical step that must be elucidated. Analysis of the sequence of Spp24 suggests many possible domains may play a role in this activity. Additional work in our lab has shown a dual effect of Spp24 on BMP activity; early activity represses BMP signaling while late potentiates BMP signaling. Accompanying the enhancing activity is the transition from larger molecular weight forms to a smaller ~18 kDa form.

**Figure 21. The Role of Spp24 in Osteoblast Development**

We believe Spp24 may act to in subtly different ways to promote the transformation of undifferentiated MSCs to osteoblast precursors where Spp24 holds these non-terminally differentiated cells. The combination of additional growth factors and the proteolytically digested 18 kDa form of Spp24 (Spp18) could then promote complete commitment to terminal osteoblastogenesis.

# Osteoblast Development





Investigating how Spp24 acts seems an area full of potentially exciting future directions for study. Clearly important are how Spp24 acts differently in its larger or smaller forms. Similarly, how the conversion to a smaller form occurs is important as well and may be related to the proteolytic cleavage necessary for the activation of SIBLING proteins (Qin, Baba et al. 2004). Based on the formation of an ~18.5 kDa form of Spp24 during osteoblast differentiation it seems likely that a protease produced during the commitment program of MSCs to osteoblasts may be involved in this mechanism. Numerous MMPs and other proteases are produced that may occupy this role.

We have generated numerous site-directed mutants based on an analysis of a sequence comparison of Spp24 from numerous species of vertebrates. These mutants will provide a mechanism to investigate a number of aspects of Spp24 biology. Mutants that block hypothesized cleavage sites that might be involved in the generation of a smaller, more osteogenic 18 kDa form could be vital to understanding how the conversion of forms alters Spp24's effect on differentiation and signaling. Additionally, mutants were generated to address how possible phosphorylation sites may alter how Spp24 affects mineralization in culture. We looked at both sites shown to be possible phosphorylation sites and the conserved SSEE domain related to SIBLING proteins' functions. Also, we will look at how mutating the TGF $\beta$  receptor domain and disrupting the structure will affect Spp24 activity.

One additional avenue for future directions is to employ transgenic animals to investigate the role of Spp24 *in vivo*. We are beginning to look at the generation of

transgenic and knockout animals. The generation of full length and ~18 kDa form transgenic animals with the genes expressed in bone via a collagen promoter could demonstrate the differences between the forms and how they affect bone. Knockout animals will likely exhibit decreased bone formation or mineralization. There may also be distant effects such as soft tissue calcification based on the function of Spp24 in the fetuin mineral complex.

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## **APPENDIX A**

### **Bacterial Protein Expression**

A cDNA sequence of human Spp24 was placed in the pQE-60 vector (Qiagen) for bacterial expression. To incorporate the Spp24 cDNA into the vector an Nco I restriction site was engineered at the 5' end of the mature Spp24 (without the native start codon and the signal peptide) and a BamH I site was engineered just at the 3' end of the sequence (except for the stop codon so that the bacterial protein product would contain the 6x His tag from the vector sequence). The engineered sites allowed incorporation of the cDNA into the vectors' existing restriction sites as well as eliminating the signal peptide and termination codon from the sequence so that the protein would not contain the signal peptide just like the mature protein following secretion from eukaryotic cells. The removal of the termination codon allowed the incorporation of a 6x His tag.

To express the protein the M-15[pREP4] strain of *E. coli* were transformed with the pQE-60/hSpp24 plasmid. M-15[pREP4] cells contain the pREP4 plasmid which confers kanamycin resistance as well as containing the Lac repressor (alternatively, another *E. coli* strain, SG13009[pREP4], also contains the pREP4 plasmid and can be used as a suitable expression host). IPTG is used to turn off the lac repressor and allow expression. A single colony was chosen and grown overnight in LB media containing ampicillin (50 µg/ml).

The following day LB media was inoculated with portions of the previous night's cultures. The cultures were then grown at 37° C with shaking until they reached an  $OD_{600} = 0.6$  absorbance units. Once they reached  $OD_{600} = 0.6$ , IPTG was added to turn off the repressive function of the lac repressor and allow expression of the protein. The bacteria were then allowed to express the protein for 3 hrs.

Cultures were then pelleted by centrifugation. The pellets were resuspended in 20mM phosphate buffer. Bacteria were lysed in a cell breaker. The lysate was centrifuged to pellet cell debris and insoluble material. The soluble fraction was saved to be run on a gel. The pelleted insoluble material was resuspended in 6M urea and the insoluble material was again pelleted by centrifugation. The proteins remaining in the 6M urea were placed on a Ni-sepharose column to be separated by affinity chromatography based on the interaction of the 6x His tag with the Nickel. After the protein had entered the column by gravity flow, 6M urea was used to wash the column. Washing was performed with sufficient volume to remove unbound additional protein as seen by a drop in the absorbance ( $A_{280}$ ) of column effluent to approximately the same absorbance as for 6M urea. At this point the protein was removed from the column by putting 6M urea containing EDTA. Fractions of the eluate were collected and also ran on a gel. The fractions containing the large bands of protein as evidenced on SDS-PAGE gels were pooled and dialyzed.

During expression of human recombinant Spp24 we found that the protein was largely insoluble and this fact often made isolation and utilization of Spp24 difficult. To demonstrate the protein was expressed and present in the insoluble material bacteria were

transformed, grown and induced. At the indicated time points a portion was taken, spun down, the supernatant decanted and the pellet was frozen. When samples had been collected over the whole time course the pellets were thawed and a sufficient volume of BugBuster (Novagen) reagent was added to solubilize most proteins. Following addition of BugBuster the insoluble material was pelleted by centrifugation. No Spp24 was found in the soluble portion (not shown). To look at the insoluble material Laemmli buffer was added to the pelleted material and samples were boiled for 10 min prior to electrophoresis. The samples were run, along with a sample from uninduced bacteria as a negative control. The gel was run and transferred to a nitrocellulose membrane. The membrane was incubated with an antibody raised against bovine Spp24 protein. An infra-red fluorescent secondary antibody was used to detect the protein. The blot was scanned using the Li-Cor Odyssey Imaging System. We found that following induction with IPTG expression of the protein was only seen in the insoluble portion.

We isolated the protein in large quantities and set about finding a suitable method to isolate a protein that could be soluble and properly folded. Initially we tried several buffers but none were able to resolubilize the protein. Based on the acidic isolation of Spp24 from tissues, specifically bone, we tried a citrate buffer at a pH of 2.5 but were still unable to solubilize Spp24. The proteins were solubilized in the buffer at a pH of 2.5 but the pH was raised to greater than 6 so that the necessary interactions between the 6xHis tag and the column could occur and allow binding of the protein to the column. Citrate did not work as a resolubilization buffer. As urea appeared the only suitable way to achieve binding of our protein to the column we sought ways to dialyze the eluted protein into

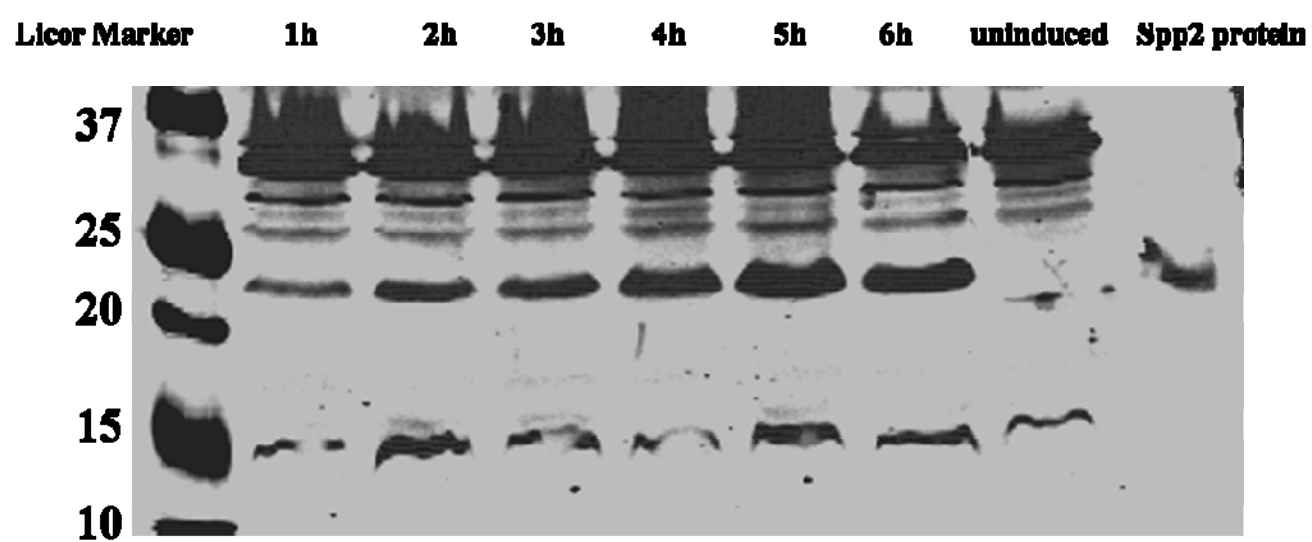
other solutions and maintain solubility after isolation. Dialysis against phosphate buffer and water both resulted in protein precipitation. We tried to slowly change the surrounding solution from 6M urea to a buffered solution by adding dropwise a phosphate buffer solution (we alternatively added the citrate buffer as well) in sufficient volume to remove the urea and leave the protein in dialysis in the buffer solution. This did not achieve our goal either resulting in protein precipitation after the buffer was slowly changed overnight at 4°C.

While we were able to achieve expression of the human Spp24 protein in bacteria we were not able to successfully solubilize the protein in any solution other than 6M urea. As other aspects of this project, namely adenoviral expression began to gain traction we shifted our focus from recombinant expression in bacteria due to the lack of a suitable solution to resuspend Spp24. This avenue may be an important method to consider at a later juncture as colleagues have reported implanting the insoluble material in animals and achieving ectopic bone formation, however with the systems now in place we are currently looking to investigate how this molecule acts before continuing with bacterial expression.



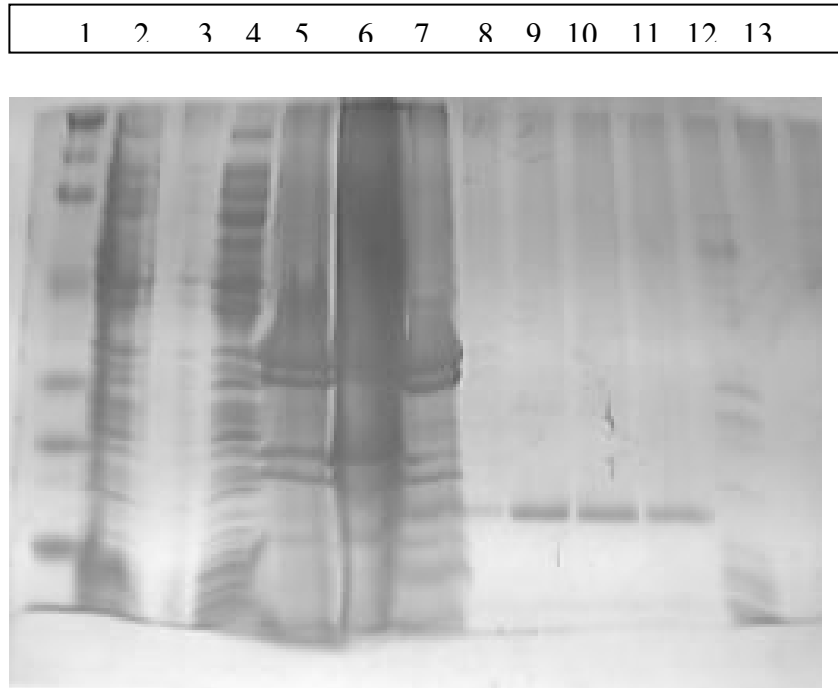
**Figure 22. Expression of Spp24 as an Insoluble Protein**

Western blot of insoluble material from bacterial expression cultures of Spp24 after isolation using BugBuster reagent. Notice the lack of a 24 kDa band in the uninduced lane as well as the recombinant Spp24 provided from a collaborator.



**Figure 23. Expression and Isolation of Recombinant Human Spp24**

Spp24 was not soluble in phosphate buffer (lane 4). Spp24, after solubilization in 6M urea and being loaded onto the column, was seen in eluate fractions #2 -5. Attempts to solubilize the protein in citric acid were unsuccessful.



1. Ladder
2. 10 ul of uninduced cultures
3. 1 ul of uninduced cultures
4. soluble protein from isolation
5. column input
6. pellet not solubilized in 6M urea
7. column flow through
8. fraction #2
9. fraction #3
10. fraction #4
11. fraction #5
12. solubilized protein (citric acid)
13. flow through from column (citric acid)

Spp24 was not soluble in phosphate buffer (lane 4). Spp24, after solubilization in 6M urea and being loaded onto the column, was seen in eluate fractions #2 -5. Attempts to solubilize the protein in citric acid were unsuccessful.

## **APPENDIX B**

### **Site Directed Mutagenesis**

A pCDNA3 construct containing mouse Spp24 with a 3x HA C-terminal tag was used as a template for site-directed mutagenesis reactions. Site-directed mutagenesis was performed using the Stratagene Quik-change II Site-Directed Mutagenesis kit (Stratagene) and protocol with minor modifications. Briefly, PCR reactions were set up with 125ng each of forward and reverse mutagenesis primers. 50ng of the pCDNA3 construct was used as template in a reaction with 2.5 U of *PfuUltra* HF DNA polymerase. The reaction conditions were as follows: an initial step of 30 secs at 95° C, followed by repeated cycles of 95° C for 30 sec, 55° C for 1 min, and 68° for 12 min. The cycles were performed 20 times for single amino acid mutations and 22 for multiple amino acid mutations. A final step at 68° C was performed for 10 min then *Dpn I* restriction enzyme (10U) was added to the reactions and incubated at 37° C for 1 hr to digest the methylated template DNA. The reactions were transformed into competent bacteria and plated overnight on LB/ampicillin plates at 37° C. Colonies were selected and plasmid preps made for sequencing using primers to the CMV promoter and the BGH poly-A sequences of the pCDNA3 construct. Primers were generated with a  $T_m \geq 78^\circ\text{C}$  using the Stratagene QuikChange® Primer  $T_m$  Calculator available online at [www.stratagene.com/QPCR/](http://www.stratagene.com/QPCR/)

tmCalc.aspx. Primers are listed in the accompanying table.

Mutations were chosen based on the material provided by a colleague showing the presence of highly conserved residues throughout Spp24 in over 20 species. Particularly we focused on residues known or likely to be phosphorylated including sites around the casein kinase II phosphorylation recognition motif and residues that may affect Spp24 structure including the TGF $\beta$  receptor type II homology domain. The mutation of N46 to W based on the discovery of an active, ~18 kDa form. An 18kDa form would likely be cleaved, from the N-terminus at somewhere around N46 and because N46 is conserved in all 22 species we chose this residue. We mutated it to a tryptophan in an effort to prevent any proteolysis at this side by hindering possible proteases' interaction through the incorporation of such a bulky residue. We also mutated L54 to alanine in an effort to completely change the character of this highly conserved amino acid. In examining possible phosphorylation we mutated S137 and T84. as a control for mutagenesis experiments we took a serine, S80, not shown to be phosphorylated and mutated it as well. We mutated two cysteines shown to be involved in two distinct disulfide bond interactions, C96 and C127. C127 is of particular interest based on Behnam *et al.*'s results that linearized BBP did not induce bone formation when implanted *in vivo*. We last examined the function of the SSEE domain by mutation S137, S137 and S138 together, S138 and E139 together, and finally E140.

**Figure 24. Site Directed Mutagenesis**

Part A illustrates the sites mutated (denoted with asterisks) on the mouse Spp24. Part B shows the primers used to generate the site-directed mutated pcDNA clones are listed in the table. The codons corresponding to the mutated amino acid(s) are highlighted in red.

A





B

Mutation	Fwd primer	Reverse Primer
N46W	gcc tca atg gca aag atg <b>TGG</b> tca cag tcc ctg agt cc	gg act cag gaa ctg cga <b>CCA</b> cac ctt tgc cac tga ggc
I54P	cc ctg agt cct tac <b>GCG</b> ttt cgg ccg acc cgg agc	gct ccg ggt cgc ccg aaa <b>CGC</b> gta agg act cag gg
S80A	gtc atg aac tta dag ttc <b>GCT</b> gtt cag gaa acc aca tgc ctg	cag gca tat ggt ttc ctg aac <b>AGC</b> gaa ctc taa gtt cat gac
T84V	g aac tta gaa ttc agt gtt cag gaa <b>GTC</b> aca tgc ctg ada g	c tct cag gca tgt <b>GAC</b> ttc ctg aac act gaa ctc taa gtt c
C96A	ggt gat ccg tcc acc <b>GCT</b> gcc ttc caa agg ggc	gcc cgt ttg gaa ggc <b>AGC</b> ggt gaa ggg atc acc
C127A	gat atg tgg gct cac <b>GCC</b> cgc tgg gcg tcc tc	ga gaa cgc cca ccg <b>GGC</b> ttg aac cca cac atc
S137A	ca tcc tca tct gag tcc aac <b>GCC</b> atg gag dag atg atg	cat cat ctc ctc act <b>GGC</b> gtt gaa ctc aaa taa gaa cg
SS137/82A	gg ccg tcc tca tct gag tcc aac <b>GCC GCT</b> gag dag atg atg ttt ggg gac	gtc ccc aaa cat cat ctc ctc <b>AGC GGC</b> gtt gaa ctc aaa taa gaa cgc cc
SE138/92Q	ccg tcc tca tct gag tcc aac acc <b>GCC CAG</b> gag atg atg ttt ggg gac	gtc ccc aaa cat cat ctc ctc <b>CTG GGC</b> gct gtt gaa ctc aaa taa gaa cgc
E140Q	g tcc aac aac agt gag <b>CAG</b> atg atg ttt ggg gac	gtc ccc aaa cat cat <b>CTG</b> ctc act gct gtt gaa c

## VITA

Samuel Cowan Ramage was born in Richmond, VA on March 20<sup>th</sup>, 1980. He graduated from the Collegiate School in Richmond, VA in 1998. He received a Bachelor of Science in Biology along with minors in Chemistry and Economics in 2002 from Duke University in Durham, NC. He entered graduate school at Virginia Commonwealth University in the Department of Biochemistry in 2004.

### **Honors and Awards**

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2007	Phi Kappa Phi member

### **Society Membership**

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 American Society of Bone and Mineral Research (ASBMR)  
 Orthopaedic Research Society (ORS)

### **Abstracts and Presentations**

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**SC Ramage** SC, PE Jones, WA Jiranek and MJ Beckman (2006) The Expression of RANKL by Fibroblasts within the Periprosthetic Membrane. Virginia Journal of Science.

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**SC Ramage**, AP Pacitti, O Korchynskyi, A Maiti, and MJ Beckman. Secreted Phosphoprotein 24 (Spp24) Promotes Osteoblastogenesis but Requires BMP Ligand to Activate Runx2 and Osterix Transcription (in preparation)

## **Chapters**

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