2009

Analysis of Secreted Phosphoprotein-24 and its Effects During Osteoblast Differentiation in a Mesenchymal Stem Cell Model

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ANALYSIS OF SECRETED PHOSPHOPROTEIN-24 AND ITS EFFECTS DURING OSTEOBLAST DIFFERENTIATION IN A MESENCHYMAL STEM CELL MODEL

A thesis submitted in partial fulfillment of the requirements for the degree of Master's of Biochemistry at Virginia Commonwealth University.

by

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August, 2009

ACKNOWLEDGEMENT

I would like to express my deepest and sincerest sense of gratitude to the following group of people who have made the realization of this thesis possible.

First and foremost, I would like to thank my advisor Dr. Matthew J. Beckman for accepting me into his laboratory. He has been a fountain of knowledge for me and because of it we have had many enriching conversations. I have taken his earnest advice with deep consideration. I am also very pleased of the mile markers he set out for me to accomplish. I would also like to thank him for taking the time to teach me and to continually challenge in every step of this process. His ability to stay calm in the face of adversity gave me the room to learn from mistakes. His ambition of seeking the truth and being a discoverer has given me motivation necessary to complete this thesis and become the scientist that I am.

I would also like to thank my lab mates Aparna Maiti and Benjamin Woolbright. Aparna has been the source of scientific knowledge and technical training for me. She has been thorough in teaching proper scientific technique and has not been accepting of anything less. She was very tough on the execution of experimentation and I know there were many times when I drove her crazy but I am a better person because of it. Ben has also been a strong critic of my work and because of it I am one step closer to perfection. I will always remember the good times and laughs we shared.

Lastly, without the support of my family and friends I would not be where I am today. I would like to thank them for the unconditional love they have given me through the years in times of joy and sorrow. They continue encourage me to set my goals high and be the best person that I can be. Their unrelenting belief that I am capable of doing anything I set my mind to do is an inspiration to me and the constant force the drives me to make them proud.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATION

AA	ascorbic acid
Ab	antibody
Bad	Bcl-2 antagonist of cell death
Bcl-2	B-cell lymphoma 2 (apoptosis regulator)
BMD	bone mineral density
BMP	bone morphogenetic protein
bFgF	basic fibroblast growth factor
βGP	β–glycerol phosphate
Ca^{2+}	calcium
cAMP	cyclic-adenosine monophosphate
CREB	cAMP response element-binding
Dex	dexamethasone
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GH	growth hormone
hMSC	human mesenchymal stem cell
IGF-1	insulin-like growth factor I
IHC	immunohistochemistry
kDa	kilo daltons
RNA	ribonucleic acid
MSC	mesenchymal stem cell
Osx	osterix
PCR	polymerase chain reaction
PBS	phosphate buffered solution
PKA	protein kinase A
PO_4^{3-}	phosphate ion
PTH	parathyroid hormone
Runx2	human homolog to *runt* transcription factor in drosophila
SPP2	secreted phosphoprotein gene
Spp18	secreted shosphoprotein-18
Spp24	secreted shosphoprotein-24

ABSTRACT

ANALYSIS OF SECRETED PHOSPHOPROTEIN-24 AND ITS EFFECTS DURING OSTEOBLAST DIFFERENTIATION IN A MESENCHYMAL STEM CELL MODEL

By Jochen Alexander Granja Vasquez, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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Musculoskeletal diseases, in particular osteoporosis, are increasingly becoming more prevalent in the U.S. due to the ageing population (Figure1). It is estimated that one-sixth of 300 million people in U.S. suffer from bone disorders or loss. About 10 million of those people above age 50 suffer from osteoporosis. Patients that suffer from osteoporosis have high morbidity and mortality rates. For instance, patients have decreased bone

mineral density (BMD), a measurement of bone density that reflects the strength of bone as represented by calcium content. A decrease in BMD typically leads to an increased risk of bone fractures. In particular, hip fractures have an associated 20% mortality rate 1 year after injury among senior citizens [1]. Patients that suffer from musculoskeletal diseases and from bone injuries, not associated with disease, account for 130 million hospital visit per year. Not to mention, 245 billion dollars of healthcare expenditure [2].

Over that last 30 years, there has been much improvement in the field of bone research and its application to medicine. It has changed the quality of life and prolonged the life expectancy of patients suffering from bone disease. However, many details remain unknown about the underlying mechanism that control bone metabolism, formation, and healing. Furthermore, current effective therapies to combat bone disorders have limitations including unwanted side effects and prohibitive costs. For example, treatment with glucocorticoids which is a known inducer of osteoblastogenesis in vitro has been shown to produce an osteoporotic phenotype in vivo. Recognizing the importance of bone health and its affordability to the public makes the advancement of therapeutic targets work worth doing. Work in this field will eventually lead to the prevention, treatment, and cure for bone disease.

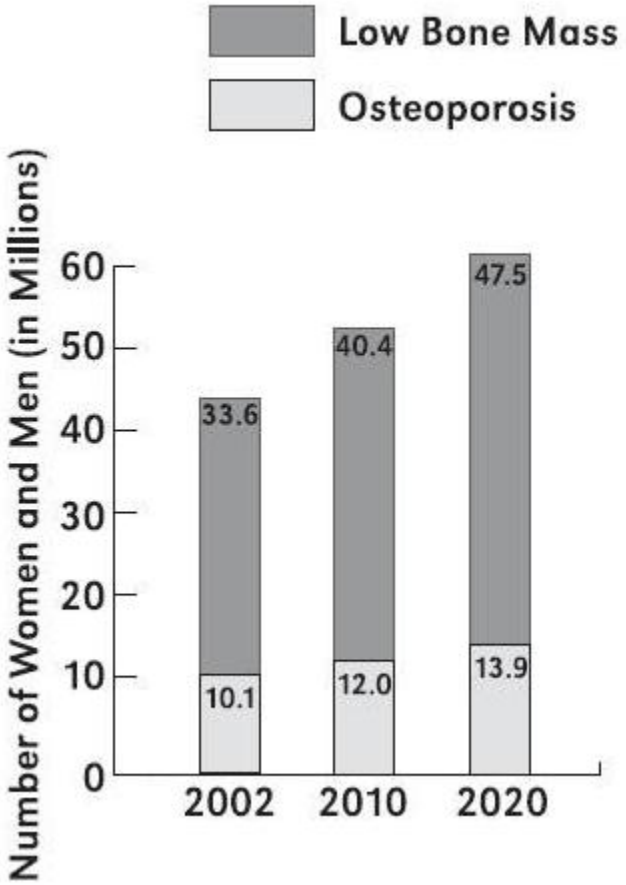
A potential therapeutic candidate that maybe involved directly or indirectly with bone formation is secreted phosphoprotein-24 (Spp24). The following research aims to establish an importance and role for Spp24 in bone differentiation. A novel antibody that detects Spp24 which we have developed and characterized, has allowed us to feasibly study the protein. Our results demonstrate localization of Spp24 in different tissue, the

processing of the protein during osteoblastogenesis, and have allowed us to conceptualize possible functions based on our data.

Figure 1. Prevalence of Osteoporosis and/or Low Bone Mass in Both Sexes 50 Years of Age or Older

Source: Bone Health and Osteoporosis: A Report of the Surgeon General

Data from the National Health and Nutrition Examination Survey (NHANES) is conducted by National Center for Health Statistics, a part of the Center for Disease Control and Prevention. This survey is conducted on a nationally representative sample of Americans. As part of NHANES, BMD of the hip was measured in 14,646 men and women over 20 years of age throughout the United States from 1988 until 1994. Based on the WHO definitions of normal to osteoporotic BMD, a percentage was derived for those individuals above the age of 50 who have osteoporosis and low bone mass. These percentages where then applied to the total population of patients over the age of 50. Projections for 2010 and 2020 are base on population forecasts for these years due to both expected growth and ageing of population.


Low Bone Mass
Osteoporosis
Number of Women and Men (in Millions)
60
50
40
30
20
10
0
33.6
10.1
40.4
12.0
47.5
13.9
2002
2010
2020

CHAPTER 1

GENERAL INTRODUCTION

Bone Biology and Physiology

The skeleton is a highly dynamic and metabolic organ responsible for a vast variety of functions including structural support of the body, locomotion, protection of vital organs, and maintenance of calcium/phosphorous homeostasis. It is composed of three cell types having different functions. The first cell type is the bone forming osteoblast which secretes an inorganic/organic-matrix. The inorganic matrix is known as hyroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The organic portion of the matrix, know as osteoid, is composed of various collagenous (particularly collagen type I) and non-collagenous proteins. The second cell type is an osteoclast. It is involved in breaking down bone tissue so that the metabolites can be taken into the extracellular fluid through a process known as resorption. The last cell is an osteocyte and is a former osteoblast. After osteoblasts have secreted the matrix the cells become embedded in their own bone matrix that has mineralized. Osteocytes primarily maintain the integrity of bone through their cytoplasmic extensions which form a network. This network serves to acquire nutrients, remove waste, and as a mechanosensory mechanism to sense different types of stimuli. Osteoblast and osteoclast cells engage in a coupled rate constant cycle of formation and resorption known as remodeling or turnover. This remodeling process is responsible for homeostasis, growth,

and repair of the skeleton. A peak BMD is reached during adolescence and early adulthood via the remodeling process. After which, around the age of 25-30 the process of formation begins to slow and resorption remains constant or increases. Eventually the constant or increased rate of resorption causes a negative balance between the coupled processes that result in phenotypic states known as osteopenia (bone thinning or bone loss) and a diseased state known as osteoporosis (severe bone loss).

There are two mechanism of bone formation during development. The first is known as intramembranous ossification which involves the recruitment of mesenchymal stem cells (MSC's) that differentiate into osteoblast. Intramembranous ossification is responsible for the development of flat bones like the skull and mandible. The second mechanism is endochondral ossification and requires cells to first differentiate into chondrocytes. After chondrocytes have finished building a template for bone architecture, they are later replaced by invading osteoblast to mineralize the bone and is the main mechanism for long bone development [3].

The metabolic and homeostatic functions of bone are tightly regulated by hormones, cytokines, and transcription factors via local (autocrine and paracrine) and endocrine signaling. These signaling molecules (i.e., growth hormone (GH), bone morphogenetic protein (BMP), and secreted phosphoprotein-24 (Spp24) Table 1.) effect bone development, proliferation, differentiation, turnover, and fracture healing. Further information of some specific molecules that regulate bone metabolism will be discussed in subsequent sections of the general introduction.

Although, many molecules have been implicated in being involved, directly or indirectly, associations of these molecules with well-defined bone phenotypes and functions is not well described. In particular, Spp24 is a relatively novel protein that is not well understood, and may be an important signaling mediator controlling bone formation including differentiation, development, and fracture healing.

Bone Pathologies

There is a large number and variety of bone diseases. All of which cause phenotypic abnormal bones. Risk factors of bone diseases include genetics, age, diet, exercise, and hormonal imbalance. This section will only briefly introduce three.

Osteoporosis is the most common of these diseases and could be the result of one or more of the aforementioned risk factor. Typically osteoporosis is associated with low BMD and characteristically has higher osteoclastic than osteoblastic activity. Osteoporosis greatly increases the risk of fractures particularly of the hip, spine, and wrist. Typically osteoporosis does not present any symptoms unless there is a bone fracture.

The second most common bone disease is Paget's disease. Paget's has been found to have strong familial predispositions. Characteristics of Paget's include overactive osteoclast activity which the body tries to compensate by increasing osteoblastic activity. This ultimately leads to disorganized formation and structure of bone. Although both Paget's and Osteoporosis share common characteristics including increased osteoclastic activity and fragile bones, they are completely different disease. Difference between Paget's and Osteoporosis include the areas they affect, bone structure, and symptoms.

Paget's normally affects one or two bones in the body whereas osteoporosis affects the whole skeleton. Since both diseases can be associated with negative bone loss it might be easy to assume that bone structure is similar. Although this can be the case, Paget's can also cause an enlargement of a bone, part of the compensation mechanism already discussed, whereas Osteporosis causes net negative bone loss weakening bone structure. Finally, symptoms of Paget's include hearing loss, vision impairment, and pain from pinched nerves or inflamed joints associated with the enlargement of particular bones. As mentioned, Osteoporosis is typically asymptomatic.

Lastly, Rickets (which affects children) and Osteomalacia (which affects adults) are related diseases that are not very common in the United States. These diseases are typically caused by vitamin D insufficiency due to lack of sun exposure or nutrition. Vitamin D is central to the mineralization of bone. Therefore, an insufficiency would lead do abnormal bone development [1].

Stem Cells and Bone Marrow Derived Mesenchymal Stem Cells

Stem cells are only cell type with the unique ability to both proliferate without limitation (i.e. self-renewal) and differentiate into a specific cell lineage (i.e. totipotent cells or unlimited cell plasticity). Besides being the cells where all organisms originate from, in the adult human being they are the body's natural healers capable of repairing, replacing, or regenerating damaged or diseased tissue. For this reason, stem cells are the target and future for tissue engineering and regenerative medicine. The ethical issues and government sanctions surrounding the use and isolation of embryonic stem cells has

resulted in research efforts to focus on tissue specific adult stem cells which do not have these limitations. Of the adult stems cells suitable for tissue engineering and regenerative medicine, adult human bone marrow derived (MSC's) are the primary focus for this study. They have been known to differentiate into various tissues like bone, cartilage, fat, and muscle depending upon the host of signaling cues it receives from cytokines and growth hormone in an endocrine, paracrine, and autocrine fashion [4]. Also, other studies show MSC's ability to trans-differentiate into other lineages (i.e. hepatocytes & neurocytes) [5]. This demonstrates the pluripotent potential MSC's have and successive impact in field of tissue engineering and regenerative medicine.

The term mesenchyme, as found in the Online Etymology Dictionary, is derived from greek words "meso" and "khymos" meaning "middle" and "juice," respectively. Found in the middle of the three germ layers of mammalian systems, MSC's originate from the mesoderm which gives rise to connective tissue. Although MSC's come from the mesoderm, proliferation continues in repositories of the adult body, namely bone marrow [4]. Mesenchymal stem cells have been found in a variety of adult tissues including periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle, dermis, brain, liver[5].

Gene Markers of MSC's through Mature Osteoblast

The hematopoietic stem cell lineage has been well characterized with specific cell surface markers to identify the stage of differentiation a population of cells is in. The same thing has not been done with the mesenchymal stem cell lineage but is slowly being

discovered. The isolation of MSC's usually leads to a diverse and heterogeneous population of cells. Mesenchymal stem cells usually express specific cell surface markers such as Stro1, C44, and CD105 [6]. These are the most common markers but other markers have been identified. If one or more of these markers are expressed then chances are there truly is a specific population of MSC's. If none of the markers are expressed, it does not mean there is not a specific MSC population because MSC's are multipotent. Some markers have a redundancy among cell lineages but this is not necessarily always the case. The reason for this is simply because the great heterogeneity and diversity MSC's have.

Once MSC's begin to differentiate they began to loose some of their cell surface markers and begin to gain osteoblast specific cell surfaces marker. The most common markers for the osteoblast lineage are collagen type 1, alkaline phosphatase (ALP), and osteocalcin [7]. Each marker represents different stages of osteoblastogenesis as early, mid, and late stage, respectively.

Osteoblast Differentiation

BMP mediated

In 1965, Marshall Urist was the first to realize during his studies of bone that a partially purified extract of non-collagenous protein from demineralized bone matrix contained a potent molecule capable of inducing bone formation. He termed this molecule bone morphogenetic protein (BMP). Urist never completely isolated and purified the molecule which he suspected to be a potent inducer of bone. Over the next forty years, scientist used similar starting materials to discover molecules that had osteogenic properties.

Consequently, 20 BMP's have been identified, characterized, and named after Urist's original BMP [8]. However, Urist argued that there remained a molecule within his partially purified extract which was not a BMP molecule. The molecule was later identified as a fragment of Spp24, which migrated with the known BMP's and enhanced its osteogenic potential [9].

Bone morphogenetic proteins are ubiquitous growth factors that are members of the transforming growth factor β (TGFβ) superfamily. They have been implicated to play important functions in embryonic development and cellular signaling in the adult animal [8]. Importantly, BMP's 2 and 7 are morphogens that induce osteoblast differentiation. In fact the use of recombinant BMP2 has been FDA approved for clinical purposes. Finally, an in vivo study of a rodent model has shown that recombinant heterodimer BMP2/7 is an even more powerful morphogen than its homodimer equivalents [10].

Signal transduction of BMP occurs when dimerized ligand bind to dimerized serine/threonine kinases type I and type II receptors forming a heterotetrameric activated receptor complex. Afterwhich, Smad proteins 1, 5, or 8 along with co-Smad 4 relay the signal to the nucleus [8]. There Smad proteins activate master genes, Runx2 and Osterix, which control osteoblastogenesis.

Dexamethasone mediated

As mentioned systemic hormones and local factors regulate bone remodeling. Of those hormones glucocorticoids are important regulators of remodeling. If administered in an excessive and continuous amount, the predominant effect in *in vivo* studies is to

increase resorption and decrease formation. For this reason, glucocorticoids are never used as a continuous treatment to heal fractures or disease for clinical applications. As evidence, there have been a number of studies demonstrating the long term effect of glucocorticoid treatments induces osteoporosis. The mechanism of how this occurs is not very clear but it has been shown to decrease genes important to osteoblastogenesis such as IGF-1 and type 1 collagen [11].

However, Bellows et al. and later Cheng et al. demonstrated that a very low physiological concentration (10 nM) of dexamethasone, a synthetic glucocorticoid, was capable of inducing differentiation which resulted in an osteoblast phenotype using an in vitro model [12, 13]. The mechanism of differentiation is unknown. There are studies that suggest that it may have a common pathway to BMP signaling yet, others that indicate an independent signaling mechanism [14]. Although there are competing theories, glucocorticoids such as dexamethasone provide an easy, practical, and cost effective way of differentiating MSC's in vitro for a wide array of studies in the laboratory.

Other mediators: Parathyroid hormone mediate (PTH) & Vitamin D (1,25-hydroxycholecalciderol)

Parathyroid hormone is synthesized and secreted from the parathyroid gland. It is the major hormone responsible for the regulation of serum calcium (Ca^{2+}) concentration. When the body senses low serum Ca^{2+} it signals to the parathyroid gland to start secreting PTH. PTH then acts on bone by increasing resorption and thereby increasing serum Ca^{2+}. It also increases Ca^{2+} renal reabsorption, further increasing serum Ca^{2+}. The last method by

which PTH increases serum Ca^{2+} is through an indirect effect. PTH stimulates production of vitamin D which increases Ca^{2+} absorption from the intestines to the extracellular fluid which effectively increases serum Ca^{2+}.

Although the effects of PTH catabolism are well known in the scientific and medical community, its anabolic effects are not. Furthermore, the mechanism regulating its anabolic effects is not well understood. Its most well characterized mode of action during molecular signaling is through the cAMP-CREB pathway which increases osteoblast numbers through an anti-apoptotic effect. PTH binds the PTH recptor, a G-protein coupled recptor, stimulates $G\alpha_s$-mediated activation of adenylyl cyclase which stimulates cyclic-AMP (cAMP) production. This subsequently phosphorylates and activates protein kinase A (PKA). Protein kinase A is responsible for the inactivation of the pro-apoptotic protein Bad as well as activation of the cAMP response element-binding (CREB). CREB then mediates expression of survival gene, Bcl-2 [15, 16].
Although this has been the most studied pathway other pathways may also be involved [15]. Parathyroid hormone has been found to directly stimulate proliferation of precursor cells or osteoprogenitors of osteoblasts. Additionally, the means of administration of PTH in in vivo studies greatly influences its catabolic versus anabolic effects. Intermittent administration has been reported to have anabolic affects while increasing collagen type I synthesis. The opposite holds true for the continuous administration of PTH. Continuous administration causes catabolic effects and inhibition of collagen type I synthesis.

Vitamin D synthesis begins with an analog of cholesterol, 7-dehydrocholesterol. In the skin when 7-dehydrocholesterol is exposed to UV radiation it becomes cholecalciferol.

The product then gets hydroxylated in the liver by 25-hydroxylase. After which it is further hydroxylated to its active form 1,25-hydroxycholecalciferol by 1α-hydroxylase in the kidney. Vitamin D has very similar physiological functions as PTH. Its biggest role is in bone mineralization by providing Ca^{2+} and phosphate through the remodeling process. It has also been shown to have direct effects on osteoblastic progenitors by inhibiting their proliferation [17] and mediating their differentiation [18-20]. Again, this molecule's mode of action is not well understood but it is very likely that signaling occurs through the vitamin D receptor (VDR).

Secreted Phosphoprotein-24 Protein Characterization

Secreted phosphoprotein-24(Spp24) is a non collagenous, secreted bone matrix protein, and has a molecular mass of 24kDa in its native full length form. It is transcribed from the SPP2 gene and mapped to human chromosome 2q37.1 [21]&[22]. The transcript is expressed in the liver [22], kidney [23], and to some degree bone [24] (i.e. periosteum). However, most of Spp24 is synthesized in the liver. How this expression is regulated is not really known. We believe Spp24 expression might be a downstream target of GH/IGF-1 signaling axis that induces osteoblastogenesis. Many studies have shown the impact GH/IGF-1 signaling axis has on the induction of osteoblastogenesis including BMP mediate osteoblastogenesis[25-29]. However, no one has ever showed the effect of GH/IGF-1 signaling axis in the context of Spp24 signaling. This hypothesis merits further research but is not the topic of this investigation.

The bovine protein sequence contains a signal peptide that directs the protein down the secretory pathway and a serine rich phosphorylation site. The serine rich phosphorylation motif is common to other secreted phosphoproteins that are phosphorylated by secretory pathway protein kinases [24]. Another hypothesized function for the phosphorylation modification is it may signal for Spp24 cleavage to occur but needs further investigation. Spp24 also has a cystatin domain that is homologous with members of the cystatin superfamily (cysteine/thiol protease inhibitors) such as cystatins, stefins, fetuins, cathelicidins, and kininogens [30] &[31]. Within its cystatin domain Spp24 contains a TGF-β receptor II homology 1 domain (TRH1). Finally, the protein contains a domain that slightly overlaps the cystatin domain known as the Spp24 domain. It is unique only to Spp24 and is highly conserved among vertebrate species. This domain is poorly characterized and much less understood.

Based on its cystatin sequence homology it has been predicted to have functions similar to members of the cysteine protease inhibitors. Cysteine protease inhibition is important in bone because osteoclast are thought to release cysteine proteases such as cathepsins that help mediate bone resorption by breaking down collagen matrix and other non-collagenous proteins under acidic conditions[24]. It may also play a role in the serum fetuin mineral complex that prevents soft tissue calcification, in particular vascular tissue [32-34]. The cystatin domain of the fetuin mineral complex is speculated to prevent soft tissue calcification by binding and inhibiting the growth of Ca^{2+}/ PO_4^{3-} crystals after osteoclasts have freed the inorganic ions through resorption [35, 36]. This function is not only important in the prevention of soft tissue calcification but may also act as mineral chaperons in the

vasculature that provide and deliver Ca^{2+} & PO_4^{3-} to boney tissue that may have a need for ions [37]. Paul A. Price characterized fetuin mineral complex and showed it contains three proteins that are consistently present upon purification: fetuin-A, matrix-Gla, and Spp24 [38]. Knock outs of fetuin-A and matrix-Gla have been shown to cause gross calcification of soft tissues in mice. Another protein that has similar function of inhibiting soft tissue calcification is osteopontin and knocking it out has the same effect. There currently is no knock out model for Spp24 but it is hypothesized to have a similar effect in the context of its association to the fetuin mineral complex. However, in a recent study by Van den Bos et al. neither Spp24 nor fetuin, both molecules comprising a cystatin domain, were found to inhibit the activity of cysteine proteases, specifically, cathepsin B and K. [39]. This suggests that fetuin and Spp24 have another mechanism whereby it inhibits soft tissue calcification other then its proposed cysteine protease inhibition function. Lastly, another predicted function is it may act as a precursor molecule like kininogens that produce biologically more potent molecule (e.g. bradykinin) after cleavage [24].

Clearly, more work is necessary to unravel the biological properties of Spp24 so that it may one day be used as an adjuvant for bone therapies.

Table 1. Hormones, Growth Factors, and Transcription Factors Affecting Bone and/or Osteoblast Development

Table 1 summarizes information about the name, type, and action of various regulators of bone development.

Name	Type	Action
Growth Hormone (GH)	Hormone	Important regulator of skeletal growth. Acts by stimulating expression of IGF-1.
Parathyroid Hormone (PTH)	Hormone	Maintains calcium homeostasis. It is known to stimulate resorption and can stimulate formation of bone but only when adminstered intermittently.
Estrogen	Hormone	Besides its hormonal effects in pubertal growth and osteoporotic phenotype in postmenopasual women deficient in estrogen there is evidence of it being an inhibitor of osteoclast resorption and stimulator of osteoblast proliferation.
Glucocorticoids	Hormone	Can cause an osteoporotic phenotype when administer continuosly in vivo. However, in vitro MSC's can terminally differentiate into osteoblast.
Vitamin D	Hormone	homeostasis and affects sketetal development as shown by knock out mice defient in either 25-hydroxyvitamin D -1 α-hydroxylase, vitamine D receptor, or both.
Bone Morphogenetic Protein (BMP)	Growth Factor	BMP family proteins play a crucial role in mediating growth, differentiation, and apoptosis in different cell types.
Insulin-like Growth Factor I (IGF)	Growth Factor	A ubiquitous growth prompoting polypeptide important in regulating cellular proliferation and differentiation via endocrine, paracrine, and autocrine signaling. Circulating IGF is expressed abundantly in liver and bone.
Secreted Phosphoprotein-24 (Spp24)	Growth Factor	A fragment of the protein has been shown to increase rate of calcification. We have shown it to differentiate MSC into osteoblasts.
Runx2	Transcription Factor	A necessary but not suffient transcripiton factor for osteoblastogenesis. May play a more significant role in early differentiation.
Osterix (Osx)	Transcription Factor	A zinc finger-containging transcription factor required for terminal differentiation of osteoblast. Null mice have impared differentiation and no bone is formed.

CHAPTER 2

DEVELOPMENT AND PRELIMINARY CHARACTERIZATION OF SECRETED PHOSPHOPROTEIN-24 ANTIBODY

ABSTRACT

Since the isolation of secreted phosphoprotein-24 (Spp24) in 1995 [24], not much is actually known about the key characteristics of the protein such as its structure, localization, and physiological function. What is known about its structure is the homology that exists with cystatins and cathelecidins. This reasoning is based on the primary sequence of bovine Spp24. Analysis of the primary sequence has led to speculation that Spp24 may have similar functions as its homologs. Over the last 14 years, the protein has been studied to vaguely address some of these characteristics using recombinant forms. Examples of the recombinant forms are a secreted isoform of mature bovine Spp24 sequence with an N-terminal Met-$(His)_6$ [40]or a full length mouse Spp24 sequence with a C-terminal 3x Hemagglutinin (HA) tagged isoform as done in our lab. These studies have also yielded a lot of data demonstrating processing and lability of the protein under different conditions as well as providing some data on Spp24's function [9, 40, 41]. We have also shown that the protein is secreted into the extracellular peri-membranous space of MG-63 osteoblast-like cells using confocal fluorescence but taken all together it has failed to completely address these unknown characteristics. In order to elucidate the

structure, localization, and physiological function of endogenous Spp24 there exist the need for an anti-body that recognizes the endogenous form. This prompted us to develop one.

INTRODUCTION

Secreted phosphoprotein-24, an extracellular bone matrix protein is highly conserved among vertebrate species. Spp24 proteins have 50% to 90% sequence homology in particular the secretory peptide, the cystatin domain, and the variable arginine rich C-terminal region of the Spp24 domain [42]. There is also a continuous stretch of residues, SerSerGluGlu, which is identically conserved among 21 vertebrate species. Bovine Spp24, when compared to its orthologs, has a highly rich polyserine region that is interrupted and variable to its counterparts. Here we describe a comparative in silico (ExPASy online) analysis of the primary sequence of bovine, mouse, and human spp24. This includes key structural features, physical, and chemical properties that allowed us to analyze and develop the most optimal antibody (Figure 2).

The protein has been shown to be involved in bone turnover and neogenesis and is increasingly gaining more interest in the field of bone research. New research is emerging suggesting that secreted phosphoprotein-24 can regulate cytokine signaling (i.e. Spp24 is pseudoreceptor capable of binding and modulating BMP signaling) for bone development, differentiation, and fracture healing [42]. Our lab has shown it to be a potent bone morphogen that has dual functions (i.e. inhibits and stimulates) with the BMP signaling interface as demonstrated by other researchers [41] & [9].

There is a need for a reliable and readily available anti-body that detects Spp24 to study structural functional relationships of the protein as well as studying the localization of the protein. To this end, we developed a novel antibody using mouse sequence that can detect residues 170-183 which correspond to the epitope SDESRSEQFRDRSL. Two rabbits, YZ27 and YZ28, where used for its development. The two antibodies, anti-Spp24 27A and 28B, have been characterized via ELISA and through comparison of western blots including an antibody developed by Brochmann-Murray et. al. which we call Murray's antibody.

MATERIALS AND METHODS

In Silico: Comparative Analysis of Primary Spp24 Sequence in Bovine, Mouse, and Human

UniprotKB/Swiss-Prot was used to find primary sequence of Spp24 from bovine, mouse, and human. ExPASy alignment tool was used to find, highlight homologous domains, and key structural features in mouse and human sequence based on bovine sequence characterized by Hu et al. Kyte & Doolittle hydrophobicity plots where done using ExPASy online and corroborated with the algorithms used by YenZym™. Other parameters analyzed by our lab include surface probability, protein flexibility, and antigenic index (algorithmic data was provided by YenZym™).

In Vivo Antibody Development

Based on the in silico analysis, the C-terminal peptide of mouse Spp24 (CSDESRSEQFRDRSL) was the decided to be the most optimal epitope and synthesized. The N-terminal cysteine of the peptide was assigned for single point, site directed conjugation to keyhole limpet hemocyanin (KLH). Finally, it was injected into rabbits YZ27 and YZ28 for antiserum production (YenZym™).

Characterization via ELISA

Microtiter wells are coated with 0.1 µl/ 100 µl/ well of peptide conjugated cys.-GIgG (goat immunoglobulin) to capture antibodies. After which, 100µl of serially diluted (1:1,000, 1:10,000, and 1:100,000) antiserum/antibody are added to each well and incubated for 30 mins at 37°C. Each dilution was done twice to give an n=2. This is followed by decanting antiserum/antibody and 2 washes with wash buffer containing PBS with 0.05% Tween-20. One hundred micro liters of horse radish peroxidase HRP-conjugated goat anti-rabbit (1:2000) was added to each well and incubated for 30 mins at 37°C. After a similar wash step as mentioned above, 100 µl of ABST (2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) substrate solution was added to each well and incubated at room temperature for 10 to 20 mins. The plate was then read at an absorbance of 405.

Western Blot Detection of Spp24 with Anti-Spp24 #27A, 28B, and Anti-Murray's

Human bone marrow derived MSCs were isolated by adherence to plastic then expanded and passaged three times before use in experiments. The use of these cells from human patients followed an approved IRB protocol. Mesenchymal stem cells with adeno-viral mediated over-expression were lysed on days 7,8, and 9 using mammalian protein extraction reagent (MPER) containing Pierce Halt protease inhibitor. Samples were then sonicated and centrifuged at 14,000 rpms for 15 mins. Supernatant containing solubilized protein was quantified via Bio-Rad Bradford protein assay and samples where diluted to equal concentrations of total protein. Samples where then boiled for 5 mins at 95°C in the

presence of β-mercaptoethanol/Laemmli buffer. Proteins were separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes where then blocked with Licor blocking solution and incubated with primary antibody Spp24 (1:1000 dilution). The bound antibodies were detected with 800 nm fluorescence's labeled goat anti-rabbit IgG on Licor Odyssey scanner.

RESULTS

Antibody Development

Murray's antibody was made to detect the epitope having A.A. residues 175-188 of bovine sequence, GEPLYEPSREMRR [40]. In contrast our Spp24 27A and 28B antibodies were made to mouse sequence CSDESRSEQFRDRSL which correspond to A.A. residues 170-183. This epitope choice was the result of selecting the most optimal epitope based on the hydrophobicity plot, surface probability, protein flexability, and antigenic index (Figure 2).

Analysis of the primary sequence revealed little homology and overlap between epitopes of bovine sequence versus both mouse and human sequences (Figure 3). The bovine epitope was found to have a single serine and tyrosine residue where as both mouse and human have four serines. Additionally, bovine epitope has two proline residues which are known to confer characteristic kinks or turns in the secondary structure of a protein. Moreover, there is a two A.A. difference between the epitopes of mouse and human sequence. If exposed to both mouse and human epitopes, 27A and 28B antibodies have the ability to detect and cross react with each epitope. This is not the case with the bovine epitope due to very little sequence homology. This will result in little to no detection of mouse and human epitope.

ELISA

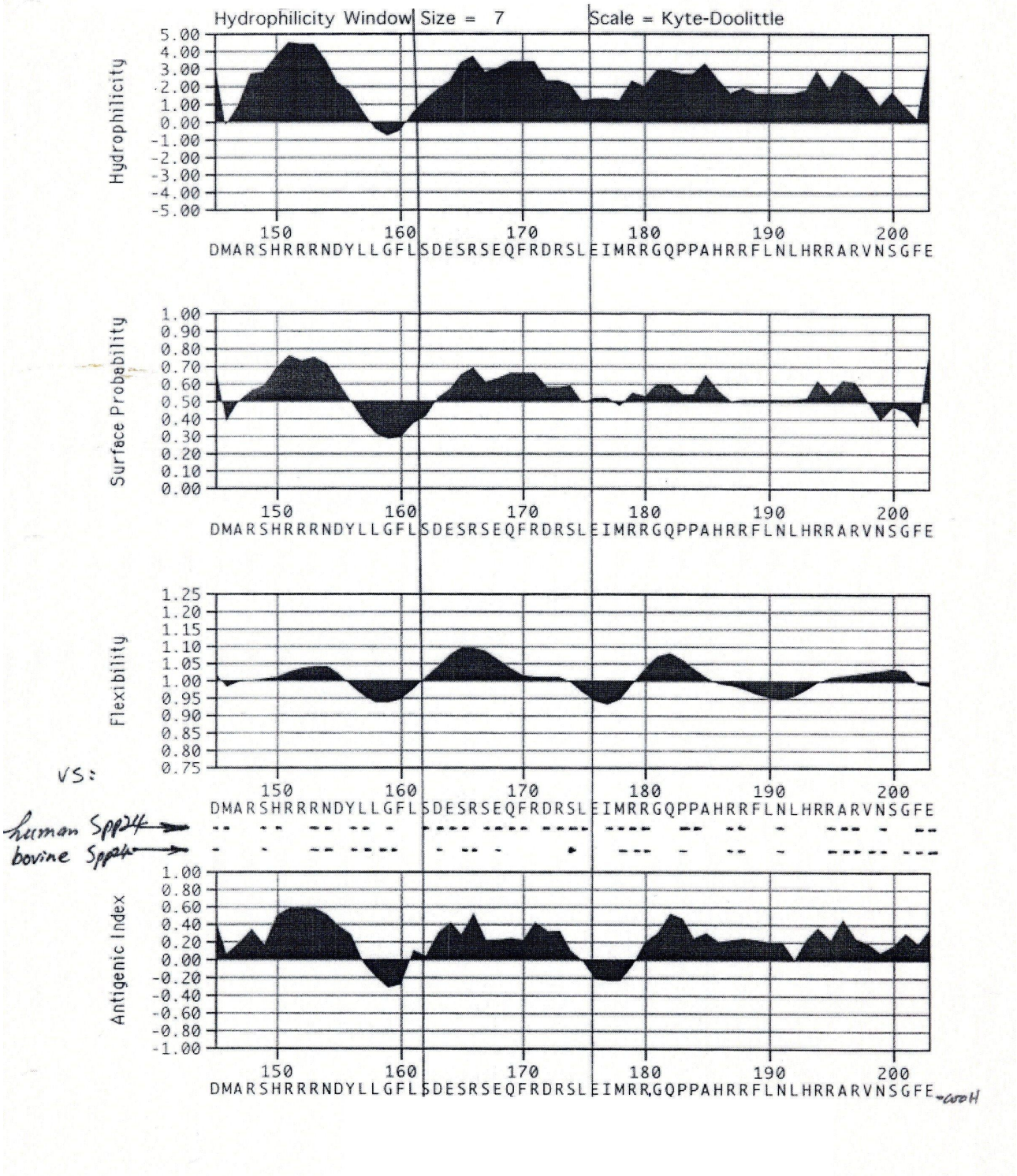
At 20 minutes of incubation, using all three dilutions of antiserum, both 27A and 28B antibody showed significant increase in response to the peptide cys-conjugated goat IgG when compared to pre-immune serum. The most concentrated (1:1,000) forms of both antibodies showed the greatest response as expected (Figure 4). From this point onward pre-immune serum was not used as ELISA confirmed it could not bind and detect the Spp24 antigen.

Western Blot Comparing Detection of Spp24 with Anti-Spp24 #27A, 28B, and Anti-Murrays

Western blots of protein collected on days 7, 8, and 9 after overexpressing Spp24 in MSC's showed 27A and 28B antibody having detection of the expected and correct size (24kDa) protein. Murray's antibody however had no marked detection of a 24kDa protein. Finally, there is some specificity that can be confirmed with Murray's antibody. Detection of a smaller fragment of Spp24, about 18kDa in size, is seen with all three antibodies (Figure 5).

Figure 2. In Silico: Comparative Analysis of Primary Spp24 Sequence in Bovine, Mouse, and Human
Source: YenZym™ Algorithms

Figure shows different plots of various properties to determine the best epitope for mouse Spp24 sequence including: hydrophobicity, surface probability, protein flexibility, and antigenic sequence. The sequence alignment comparison of conserved A.A.'s, when compared to mouse sequence, is denoted by a dash. The A.A.'s residue numbering is based on the mouse sequence of 203 A.A.'s.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Scale = Kyte-Doolittle
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
150
160
170
180
190
200
DMARSHRRRNDYLLGFLSDESRSEQFRDRSLEIMRRGQPPAHRRFLNLHRRARVNSGFE
Surface Probability
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Flexibility
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
VS:
human Spp24
bovine Spp24
Antigenic Index
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00

Figure 3. Sequence Alignment Stack of Primary Spp24 Sequence in Bovine, Mouse, and Human

ExPASy alignment tool was used to do sequence alignment. Figure contains a key with highlighted regions of interest.

Stack of three species:

Signal peptide (yellow)

Cystatin domain (+---------+)

Cysteine knots/Disulfide bridges (cyan & green)- ***NOTE: From C to C is the TRHI domain**

Serine rich sequence (underlined)

SSEE- only four residues in sequence that are identically conserved among 21 species

Rabbit anti-mouse Spp24 27A & 28B epitope- ***NOTE: Can cross react with human epitope**

V.S.

Murray's Rabbit anti-bovine Spp24 epitope

```
                          10        20        30        40        50        60
Ruler                     ---------+---------+---------+---------+---------+
SPP24_BOVIN(Q27967)       ----MEKM--AMKMLVIFVLGMNHWTCTGFPVYDYDPASLKEALSASVAKVNSQSLSPYL  54
SPP24_MOUSE(Q8K1I3)       ----MEQA--MLKTLALLVLGMHYWCATGFPVYDYDPSSLQEALSASVAKVNSQSLSPYL  54
SPP24_HUMAN(Q13103)       MISRMEKMTMMMKILIMFALGMNYWSCSGFPVYDYDPSSLRDALSASVVKVNSQSLSPYL  60
                              **:    :* * ::.***::* .:*********:**::******.**********

                          70        80        90        100       110       120
Ruler                     ---------+---------+---------+---------+---------+---------+
SPP24_BOVIN(Q27967)       FRAFRSSVKRVNALDEDSLTMDLEFRIQETTCRRESEADPATCDFQRGYHVPVAVCRSTV  114
SPP24_MOUSE(Q8K1I3)       FRATRSSLKRVNVLDEDTLVMNLEFSVQETTCLRDSG-DPSTCAFQRGYSVPTAACRSTV  113
SPP24_HUMAN(Q13103)       FRAFRSSLKRVEVLDENNLVMNLEFSIRETTCRKDSGEDPATCAFQRDYYVSTAVCRSTV  120
                          *** ***:***:.***:.*.*:*** ::**** ::*  **:** ***.* *..*.*****

                          130       140       150       160       170       180
Ruler                     ---------+---------+---------+---------+---------+---------+
SPP24_BOVIN(Q27967)       RMSAEQVQNVWVRCHWSSS-SGSSSSEEMFFGDILGSSTSRNSYLLGLTPDRSRGEPLYE  173
SPP24_MOUSE(Q8K1I3)       QMSKGQVKDVWAHCRWASS-SESNSSEEMMFGDMARSHRRRNDYLLGFLSDESRSEQFRD  172
SPP24_HUMAN(Q13103)       KVSAQQVQGVHARCSWSSSTSESYSSEEMIFGDMLGSHKWRNNYLFGLISDESISEQFYD  180
                          ::*  **:.* .:* *:** * * *****:***:  *   **.**:*: .*.* .* : :

                          190       200       210
Ruler                     ---------+---------+---------+-
SPP24_BOVIN(Q27967)       PSRE-MRRNFPLGNRRYSNPWPRARVNPGFE  203
SPP24_MOUSE(Q8K1I3)       RSLEIMRRGQPPAHRRFLNLHRRARVNSGFE  203
SPP24_HUMAN(Q13103)       RSLGIMRRVLPPGNRRYPNHRHRARINTDFE  211
                           *   ***  * .:**: *   ***:*..**
```

Figure 4. ELISA

This figure is a graphical representation of the immune response to the antigenic sequence elicited by 27A and 28B antibodies at 20 mins of incubation with different serially diluted serum solutions versus pre-immune serum in an n=2.

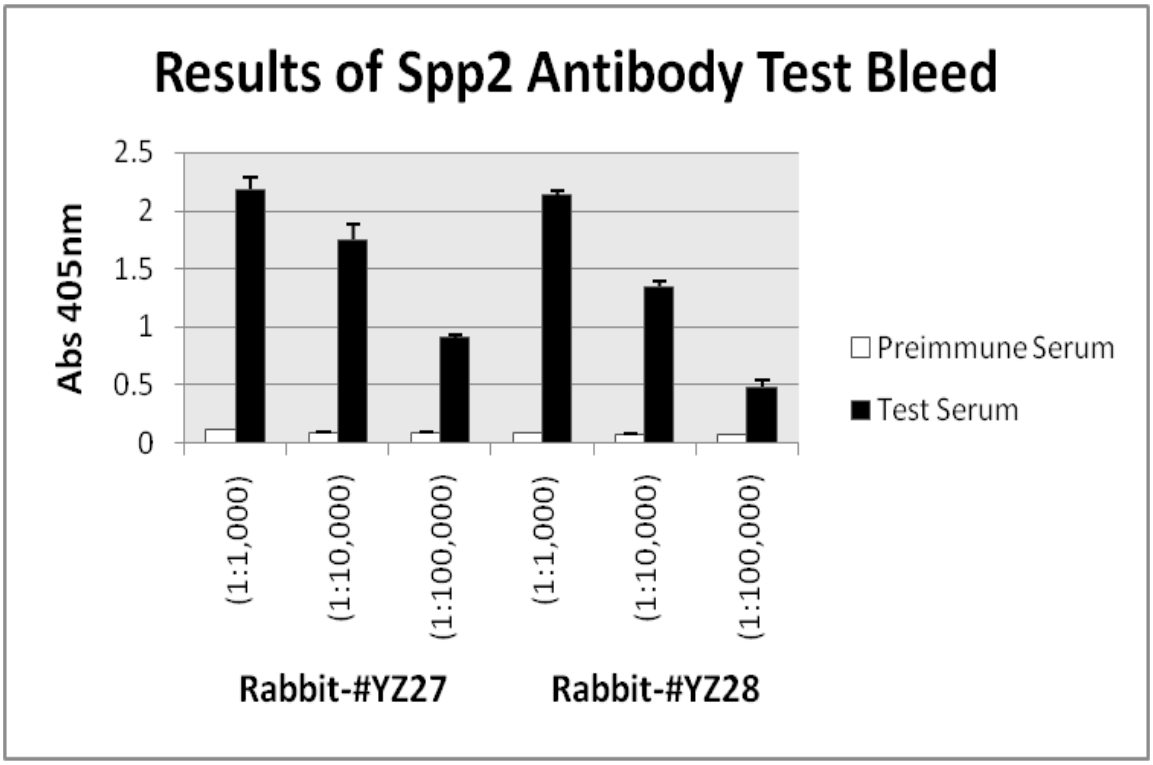
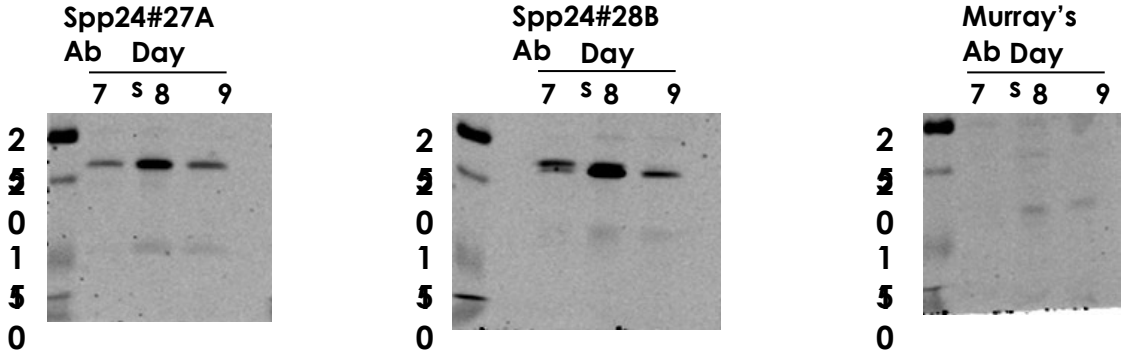

Results of Spp2 Antibody Test Bleed
Abs 405nm
2.5
2
1.5
1
0.5
0
(1:1,000)
(1:10,000)
(1:100,000)
(1:1,000)
(1:10,000)
(1:100,000)
Rabbit-#YZ27
Rabbit-#YZ28
Preimmune Serum
Test Serum

Figure 5. Comparison of Spp24 Detection with Antibodies 27A, 28B, and Murray's

Western blots showing detection of Spp24's 24 kDa protein with 27A and 28B but not Murray's. Also, all three antibodies detect similar 18kDa fragments of Spp24.


Spp24#27A
Ab Day
7 S 8 9
250
150
Spp24#28B
Ab Day
7 S 8 9
250
150
Murray's
Ab Day
7 S 8 9
250
150

DISSUCUSSION

The lack of an anti-body for the endogenous protein may have led Brochmann-Murray et al. to develop one. Brochmann-Murry et al. mentions the development of an anti-body that recognizes C-terminal end, residues 175-188, of bovine sequence [40]. However, they have never included any data in the literature where they use their own anti-body. All of their data for detection of a 24kDa protein relies on the recombinant bovine Spp24 sequence with N-terminal $Met(His)_6$ tagged protein created in E.coli. Wanting to know why they had not shown data with Murray's antibody we requested and tested Murray's antibody. We found detection of an 18 kDa fragment but not full length 24 kDa. The inability of Murray's to detect the 24kDa protein may be due differences in sequences between species and the location of where the epitope was made. There is a small over lap of sequence homology upon examination of the epitopes recognized by Murray's versus mouse specific 27A and 28B. In fact, neither mouse nor human sequences have the two prolines that bovine sequence has. Perhaps, the folding of bovine sequence when compared to mouse or human is different due to these prolines. This may preclude Murray's antibody to detect the epitopes of the native structure of mouse and human sequence. However, the last three residues of the bovine epitope are identically conserved among all three species and this may confer a little more specificity in recognizing the 18 kDa form of Spp24. Furthermore, it may be possible that the epitope of 18 kDa fragment in mouse and human sequence is more accessible to Murray's antibody because the protein is folded differently than the native 24 kDa structure. These inconstancies raise questions

about the ability of Murray's antibody to detect mature Spp24 in mouse and humans. For this reason we believe our 27A and 28B antibody are better to study Spp24.

After analysis of the primary sequence of Spp24 in bovine, mouse, and human as well as the epitopes each antibody recognizes, it was easy to predict that Murray's antibody would have difficulty in detecting endogenous level Spp24 in human MSC's or over-expressed mouse Spp24. The ELISA data showed positive results of an immune response by our 27A and 28B antibodies. Taken together along with the western blot data we are more confident that our antibodies are better suited than Murray's to study Spp24. We hope that this novel antibody can help us better understand and describe the localization in tissues, its properties, and a structural functional relationship of Spp24.

CHAPTER 3

LOCALIZATION, PROCESSING, AND PROPERTIES OF SECRETED PHOSPHOPROTEIN-24

ABSTRACT

In this study we are the first to describe the spatial localization of Spp24 in different tissues using immunohistochemistry (IHC) a non-collagenous bone matrix protein, in bone tissue and its expression in many different tissues using western blot analysis. Detection of the 24 kDa protein, Spp24, was found in most tissue types including liver, brain, lung, kidney, spleen, testis, heart, bladder, blood, and bone upon western blot analysis. However, it is important to note that bone was the only tissue with expression of an 18 kDa fragment of Spp24, henceforth referred to as Spp18. This suggests that bone is a tissue type where Spp24 is metabolically processed and this smaller fragment is thought to be a biologically more potent or active molecule important for bone induction. This idea of a biologically more active molecule is based on previous studies that demonstrate a smaller fragment of Spp24 is capable of enhancing osteogenic properities[9, 42].

Furthermore, IHC of liver and kidney sections showed a specific staining pattern using our Spp24 antibody (Figure 7). More importantly, both mouse bone and human bone marrow sections confirmed that only a subset of cells, found in bone marrow but not cortical bone, showed detection with Spp24 antibody (Figure 8). The finding that Spp24 is

not found in the osteocytes of cortical bone but rather in precursor cells of the bone marrow suggests that it is an important molecule for bone development and remodeling. Human mesenchymal stem cells (hMSC's) were chosen as a eukaryotic model system to assess expression and possible function of Spp24. Adenoviral Spp24 overexpression of hMSC's over a two week time course allowed a feasible method to study the protein due to low endogenous Spp24 protein expression. The protein profile demonstrated the processing of Spp24 and the appearance of Spp18 at day 7. In addition, shortly after the appearance of Spp18 we can see progression of hMSC's to mature osteoblast assessed by matrix calcification and visualized via alizarin red stain. The loss of detection of the molecule towards the end of the differentiation time course supports the hypothesis of mature osteoblast having no expression of Spp24 possibly because it no longer needs it and becomes degraded. We then tested Spp24's stability after being subjected to a number of freezing and thawing cycles at room temperature. After which, we checked for degradation. In support of the idea that proteolytic susceptibility of Spp24 may be calcium dependent, work done by Brochmann-Murray et al.[40], we show Spp24 overexpresion in MSC's lysates are degraded by 4mM calcium solution.

INTRODUCTION

Although Spp24's exact function in bone biology is not yet known, there is evidence supporting the notion that size of the protein correlates to its role in enhancing or inhibiting bone morphogenetic protein (BMP) signaling. Bone morphogenetic protein is the common morphogen for induction of bone differentiation. Benhanm et al. [9]demonstrated a synthetic19 amino acid (BMP binding peptide or BBP), cyclic peptide made from Spp24's TRH1 domain having modest BMP affinity (K_d~3 x 10^{-5}), was capable of enhancing ectopic bone formation when implanted into mouse skeletal muscle. In contrast, Sintuu et al. [41] provided data in which FL-Spp24 inhibits BMP-2 induced ectopic calcification. Therefore, it maybe necessary for Spp24 to undergo processing into a smaller peptide before it can become a more bio-active molecule.

Previously, tissue specific protein expression has not been shown with this molecule. To address this issue we developed an anti-body to mouse Spp24 that cross reacts with a similar epitope on human Spp24. We utilize the novel anti-Spp24 27a for immunohistochemistry and western blot analysis to further our understanding of Spp24 and help elucidate a role for Spp24 in bone.

MATERIALS AND METHODS

Multi Tissues Blot

Different organ tissues were collected from c57 black 6 mice and stored at -70°C. They were indivdually flash frozen with liquid nitrogen and grinded with mortar and pestal. Bone was decalcified using EDTA before flash freezing. An extraction buffer was prepared containing 20mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, and 1mM EGTA. The buffer was then added to each organ to extract protein. Samples were then sonicated and centrifuged at 14,000 rpms for 15 mins. Supernant containing solubilized protein was quantified via Bio-Rad Bradford protein assay and samples where diluted to equal concentrations of total protein. Samples where then boiled for 5 mins at 95°C in the presence of β-mercaptoethanol/Laemmli buffer. Proteins were separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes where then blocked with Licor blocking solution and incubated with primary antibody Spp24 (1:1000 dilution). The bound antibodies were detected with 800 nm fluorescence's labeled goat anti-rabbit IgG on Licor Odyssey scanner.

Immunohistochemistry

Paraffin sections from bone of c57 black mice were cut to 3 microns and mounted on slides. Human bone marrow tissue was provided by the Department of Anatomical Pathology and prepared similarly. Sections were then deparaffinized in three changes of

Xylene for 5 minutes each. Rehydratiation of sections was conducted in decreasing grades of alcohol for 5 changes of 3 minutes each: 100% two changes, 95%, 80% and then rinsed in distilled water.

Hematoxylin and Eosin Stain

After rehydration slides are stained with hematoxylin rinsed and dipped into an acidic alcohol solution to remove non-specific binding of the stain. The slides were then rinsed and a carbonate solution is added to bring out the blue hue commonly found with the stain. Eosin was used as the counter stain. Finally slides where dehydrated, cleared with xylene and covered with a cover slip.

Immunohistochemistry using 27A Antibody

After rehydration slides were treated with 3% Hydrogen Peroxide containing sodium azide for 3 to 5 minutes to block endogenous peroxidase activity. Paraffin slides required HIER (Heat Induced Epitope Retrieval) therefore placed in a steamer for heat targeted retrieval. A pH of 6 was used for 20 minutes to retrieve the epitope. Slides were allowed to cool for 20 minutes. Slides were loaded onto Dako Corporation auto-immunostainer. Nonspecific protein binding sites were blocked by incubation in the serum free protein block for 5 minutes. Slides were then rinsed for 2 minutes in Dako wash buffer. Spp24 mouse specific rabbit polyclonal antibody was added to each test slide using an optimal dilution of 1:50 and conjugated to a secondary anti-rabbit IgG. A negative control slide was also made that contained secondary alone. Slides were then rinsed with

Dako wash buffer as previously mentioned. Horseradish peroxidase (HRP) Envision+™ polymer was incubated with each slide and control for 20-50 minutes followed by a rinse step. After the application of the HRP polymer specific antibody and conjugated polymer enzyme complex slides were visualized utilizing a precipitated enzyme product/substrate 3,3'-diaminobenzidine chromogen (DAB+). Finally, slides were counterstained using Gill's III Hematoxylin for 30 seconds, dehydrated, cleared, and a coverslip was placed on each. The entire procedure was done at room temperature.

Cell Culture

Human bone marrow derived MSCs were isolated by adherence to plastic then expanded and passaged three times before use in experiments. The use of these cells from human patients followed an approved IRB protocol. Human MSC's were purified from primary hip patients in accordance with an approved IRB protocol. Cells were expanded in culture conditions of DMEM medium containing 10% FBS, 1% P/S antibiotic and 2 ng/ml of FGF-basic for 3 passages before switching to osteogenic conditions. Osteogenic medium conditions involved removing bFGF from the DMEM and supplementing with 2 mM ascorbic acid and 10 mM β-glycerol phosphate for a 15 day period. The cells were maintained in a incubator at 37°C with a humidified atmosphere and 5% CO2 composition in air.

Adenoviral Overexpression of Spp24 Time course

Human MSC's were transduced 24 hours before day 1 using an adenovirus construct containing Spp24 with HA tag at a multiplicity of infection (MOI)=100. Mesenchymal stem cells were allowed to differentiate for a 2 week time course under osteogenic conditions. Day 0 were the uninfected control cells. Cells were lysed each day using mammalian protein extraction reagent (MPER) containing Pierce Halt protease inhibitor. Lysates were prepared for western blot as previously mentioned and completed using the same conditions. Spp24 was detected using anti-Spp24.

Spp24 Stability and Lability Assays

Spp24 Stability After Freezing and Thawing

Protein extracts from MSC's where taken at day 9. Cells were differentiate using dexamethasone. Twenty micro-liter aliquots of protein extract where added to 5 Eppendorf tubes and frozen at -20°C for storage. Each tube was thawed at room temperature and refrozen a different number cycle ranging from 1-5 times. Protein extracts were then run through a western blot as described. To normalize α-tubulin was used as a loading control.

Spp24 Calcium Dependent Proteolysis Assay

Human MSC's where transduced with Adeno-Spp24HA. Cell lystates where collected at 0, 1, 2, 4, 6, and 24 hrs in presence or absence of 4mM calcium solution.

Lysates were prepared for western blot as previously mentioned and completed using the same conditions. Spp24 was detected using anti-Spp24HA (1:5000 dilution).

RESULTS

Western blot Analysis of Multiple Tissues, Spp24 Overexpression Time course, and Calcium Dependent Proteolysis of Spp24

Spp24 was found to have no detection in muscle and very faint detection in intestines. Most other tissues had detection including liver, brain, lung, kidney, spleen, testis, heart, bladder, blood, and bone. Bone however, was the only tissue with expression of Spp18 (Figure 6). To further explore the fragmentation of Spp24, hMSC's were chosen as a model system whereby overexpression of Spp24 demonstrated the fragmentation pattern of Spp24 as cells underwent differentiation. The western blot revealed a possible cleavage event beginning at day 7. Additionally, the presence of a doublet band and Spp18 was revealed. Shortly after the appearance of Spp18 there was noticeable increase in calcification to the cellular matrix occurring at day 9 and was visualized via alizarin red stain (calcification data shown in Chapter 4). The detection of 18 kDa fragment remained until day 12 after which complete expression were lost for both Spp24 and Spp18 (Figure 9). The stability and degradation of Spp24 was tested after 5 freeze and thaw cycles but was not affected by these conditions (Figure 10A). To corroborate Brochmann-Murray et. al. findings of a calcium dependent mechanism for proteolysis of Spp24, hMSC lysates that had been infected with Adeno-Spp24HA and co-incubated with 4mM calcium. Lysates completely degraded after 1 hr of calcium treatment. Also, other non-specific proteins where not degraded by the presences of calcium (Figure 10B).

H&E and IHC Analysis

Immunohistochemistry of liver and kidney sections showed specific staining patterns using our Spp24 27A antibody when compared to just the application of secondary anti-rabbit IgG. The liver had very high protein expression throughout most of the tissue except for the walls of the hepatic vein. Spp24 was also found to localize in the kidney, specifically the cortex but not in the glomeruli or medulla (Figure 7).

Hematoxylin and eosin stain of mouse bone depicted cancellous bone in purple and cortical bone in pink, respectively. The IHC of mouse bone showed specific Spp24 expression of a subset of cells in the marrow space of cancellous bone. Based on these results we then went on to look at human bone marrow. Human bone marrow also had a similar pattern of staining and only a specific number of cells expressed Spp24 (Figure 8).

Figure 6. Multi-Tissue Blot

Western blot showing detection of Spp24 with 27A Ab in different tissues. Only bone has expression of 18 kDa fragment. (n=1)

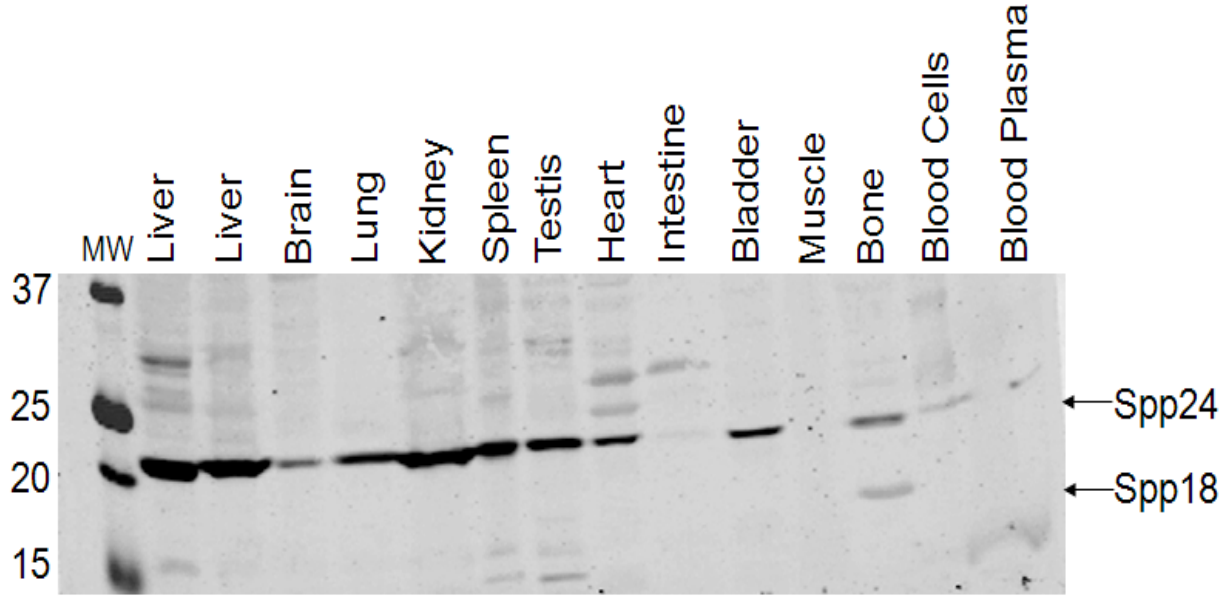
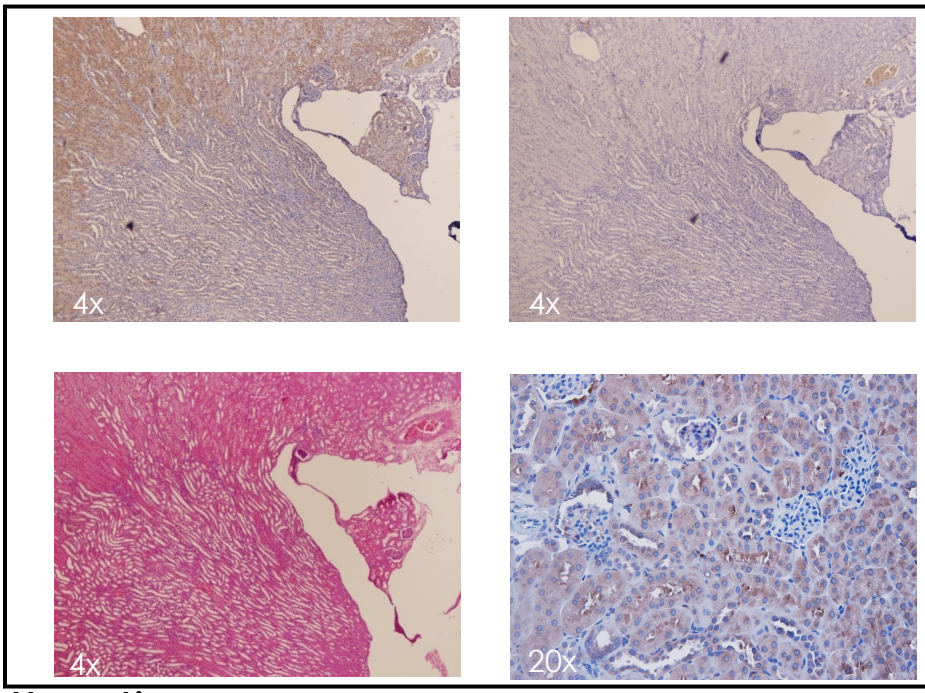

MW
Liver
Liver
Brain
Lung
Kidney
Spleen
Testis
Heart
Intestine
Bladder
Muscle
Bone
Blood Cells
Blood Plasma
37
25
20
15
Spp24
Spp18

Figure 7. IHC and H&E Staining of Kidney and Liver

For each box beginning from top left corner to right corner: IHC with primary Spp24 27A Ab. Then section with secondary alone. Bottom left corner: H&E stain followed by IHC with primary Spp24 27A Ab at 20x.

Mouse Kidney



Mouse Liver

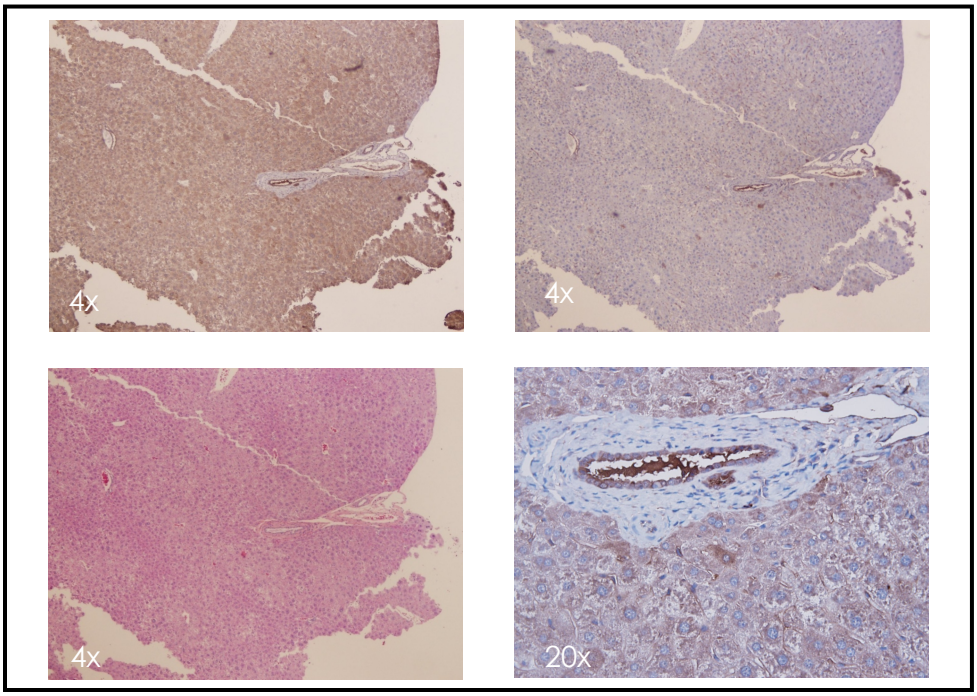
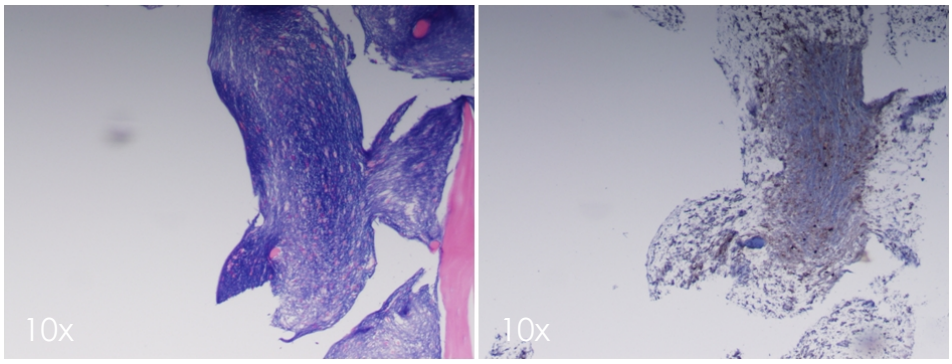


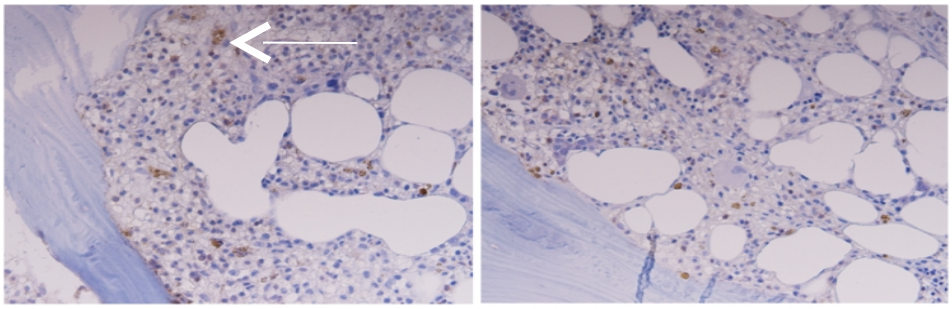
Figure 8. IHC and H&E Staining of Mouse Bone and Human Bone Marrow

Beginning with top left to right and ending with bottom left to right: H&E of bone where cortical bone is stained with eosin (pink) and trabecular bone is stained with hematoxylin (bluish purple).

Mouse Bone



Human Bone Marrow



Immunohistochemistry of Spp24 in Human Bone Marrow samples

Figure 9. Adenoviral Overexpression of Spp24 Time Course

Western blot showing 15 day time course of Spp24 processing. Spp18 appears at day 7. Both Spp24 and Spp18 detection is abolished after day 12. (n=1)

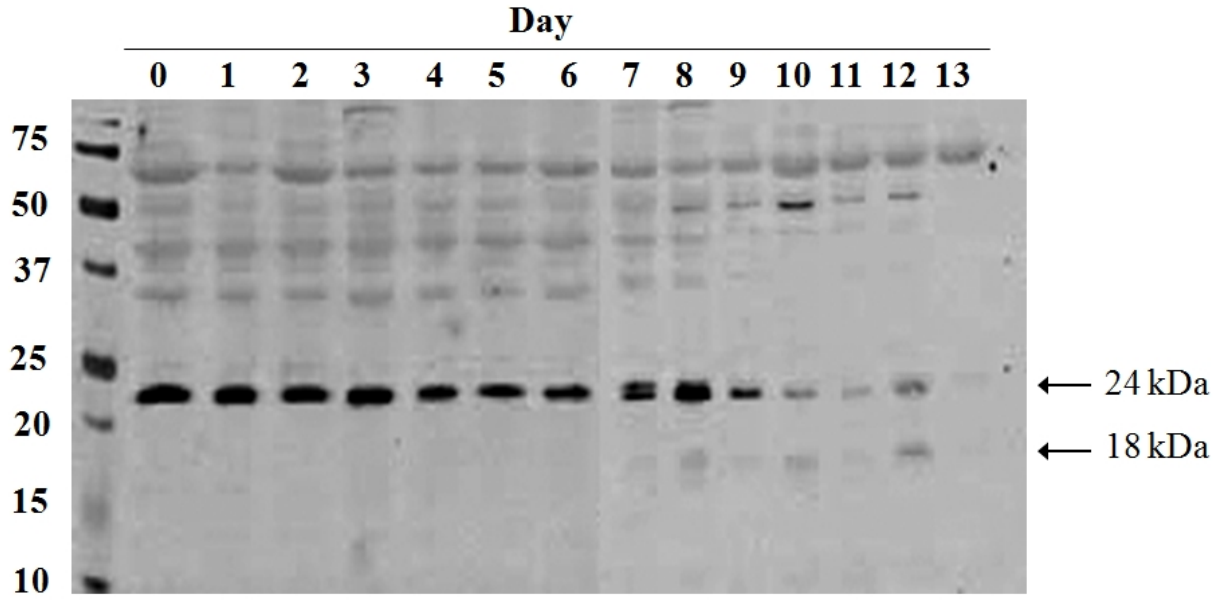
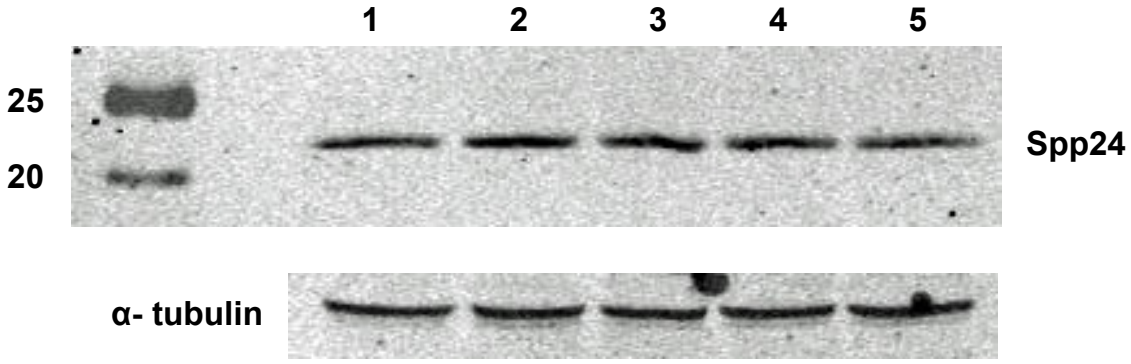

Day
0 1 2 3 4 5 6 7 8 9 10 11 12 13
75
50
37
25
20
15
10
24 kDa
18 kDa

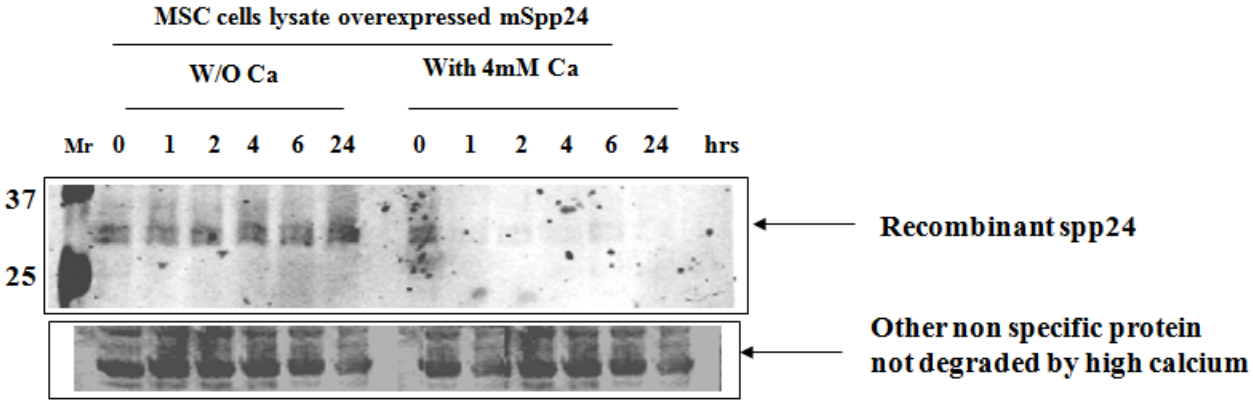
Figure 10. Stability and Lability Assays

A: Spp24 protein after 1 to 5 different freeze and thaw cycles. B: Spp24 degradation after exposure to 4mM Ca^{+} solution compared to no degradation of non-specific protein.

A.


1
2
3
4
5
25
20
Spp24
α- tubulin

B..


MSC cells lysate overexpressed mSpp24
W/O Ca
With 4mM Ca
Mr 0 1 2 4 6 24 0 1 2 4 6 24 hrs
37
25
Recombinant spp24
Other non specific protein
not degraded by high calcium

DISSCUSION

Transcription of Spp24 has been found in liver [22], kidney [23], and bone [24]; it was expected that subsequent protein expression be found in these and only these tissues, using both IHC and western blot. This study proved otherwise, protein detection of Spp24 was found in almost all tissues types and previously has not been shown. Why detection is found in tissues other than where the transcript is found is not known and much less known about what role Spp24 plays in these other tissues. However, a possible explanation for its detection in many tissues types is due to liver secretion of Spp24 into the blood stream and subsequently migrating to other tissues. Elucidating a role for Spp24 in bone has been a central focus in our lab. Hence, the multi-tissue blot finding of an 18 kDa cleavage product in bone and previous literature leads us to propose that bone is a key tissue where the molecule is processed and can become a metabolically active protein.

To specify the spatial localization of Spp24 expression in bone we preformed IHC on mouse bone and later human bone marrow. Interestingly, we found only a small population of cells positively stained Spp24 within the bone marrow of mouse but not cortical bone. Furthermore, human marrow also displayed a similar staining pattern. Based on the absence of detection in cortical bone where mature osteoblast that later become osteocytes reside, we reasoned that the populations of cells that expressed Spp24 were progenitor cells for osteoblast.

We then decided to study the molecule in an hMSC model. The use of an adenoviral-Spp24 construct in hMSC's made it possible to highly express Spp24, which is

naturally low in abundance. The western blot in which we overexpressed Spp24 during the differentiation time course revealed the smaller fragment, Spp18, at day 7. Day 7 also illustrated the appearance of a doublet band around 24 kDa's. This doublet may represent a possible modification that in turn signals for cleavage to occur but further research is needed. Shortly after the appearance of the Spp18, noticeable increase in calcification to the cellular matrix occurred at day 9 (data shown in Chapter 4). This result suggests that Spp24 can act as a bone morphogen under osteogenic condition. Also, detection of both Spp24 and Spp18 is abolished by day 12. This may bolster the contention that in mature osteoblast Spp24 expression is absent.

Secreted Phosphoprotein-24 may have its own mode of action if it is capable of driving differentiation under osteogenic conditions but it is not know how this works. However, to address its effects in combination with BMP signaling we think the time course data depicts the mature Spp24 as pre-bio-activated form of the molecule, much like kininogens. This is followed by proteolytic step to produce Spp18 and is what we call the bio-activation step. Mature Spp24 may play an inhibitory role with respects to BMP signaling as Sintuu et al. [41] demonstrated at the beginning of differentiation. However, this inhibition may be required for the proper context of signaling cues and cellular environment to be established before the molecule gets processed; in other words bioactivated. After which differentiation can proceed. Post-bioactivation we clearly see increased calcification occurring possibly due to the smaller fragments interaction with BMP as demonstrate by Benham et. al.[9]. To understand the dual function of Spp24 in the context BMP signaling differentiation further investigations are need.

Brochmann-Murray et. al.[40] demonstrated that *E. coli* extracts expressing a His-tagged form of Spp24 when combined and incubated with MC3T3-E1 cell extracts in the presence of calcium were susceptible to proteolysis. But, in the absence of calcium the opposite was true. In order to support the claim of a possible calcium dependent mechanism that is responsible for the proteolysis of Spp24 we tested our hMSC model sytem. Our model system differed than theirs in that we overxpressed adeno-Spp24HA in a eukaryotic system but calcium conditions were the same. Our results still confirmed the idea of a possible calcium dependent mechanism. Finally, although Spp24 seems to be very labile, its stability after several freezing and thawing cycles seems to be unaffected.

CHAPTER 4

SPP24 MEDIATED DIFFERENTIATION FROM MESENCHYMAL STEM CELL TO OSTEOBLAST OR Spp24 EFFECTS IN MSC MODEL

ABSTRACT

Data from our lab has shown that Spp24 can differentiate mesenchymal stem cells to an osteoblast. How Spp24 achieves this phenomenon is not yet known. Spp24 could play a role in the calcification of matrix and regulate genes important for this process, or Spp24 could be a powerful morphogen inducing MSC's to differentiate into osteoblast subsequently causing osteogenic effects. The most likely possibility is that it participates in both of these processes.

This study looks at the ability of Spp24 to regulate the processes that orchestrate proliferation of MSC's, differentiation of MSC's to osteoblasts, and matrix calcification. Using Draq5 which is a fluorescent dye that stains nuclear DNA we compare proliferation rates of MSCs induced by treatment with BMP, Dexamethasone and Spp24. The differentiation analysis specifically compares ALP stain/activity and matrix calcification assessed with Alizarin red stain. These methods are standard ways of assessing differentiation. Furthermore, similar morphological changes are noticeable after induction with BMP, Dex, or Spp24. Finally, we show data where Spp24 regulates master genes, Runx2 and Osx, important to osteoblastogenesis. Analyses were monitored in experiments throughout the days of the entire differentiation process or days 3 and 9. Days 3 and 9 are

important time points because they represent what we believe to be a pre and post bio-activation status of Spp24 protein, respectively. Our results show that Spp24 is capable of regulating proliferation, genes important to osteoblast differentiation, and matrix calcification.

INTRODUCTION

Recent studies on Spp24 have suggested that the TRH1 domain of Spp24 is capable of binding BMP ligand which is suggestive that it can modulate BMP signaling [42]. For this reason we wanted to investigate how Spp24 could effect BMP mediate osteoblastogenesis. Previously, our lab has shown that short interfering RNA targeting Spp24 (siSpp24) can completely abrogate BMP mediated osteoblastogenesis when compared to an off-target control (siCnt) during a 15 day differentiation time frame. Runx2 and Osterix are master regulatory transcription factors for the osteoblastogenesis process. Spp24 had a differential effect on Runx2 and Osterix. Treatment of mesenchymal stem cells with siSpp24 resulted in a reduced capacity of BMP to stimulate Runx2. This was interesting in light of the fact that BMP is known to increase Runx2 expression in the differentiating MSC. Osterix was effected differentially, as Osterix increases were unaffected by BMP. However, baseline levels were almost completely abrogated. Experts in the field who have developed knock out mice have reported that knock outs of one of these two genes leads to incomplete or no bone formation [43]. This shows the importance these two genes have in controlling osteoblastogenesis. If these genes require Spp24 for proper osteoblastogenesis signaling, then it likely that Spp24 itself is also required for the differentiation process.

If by silencing Spp24 we see abrogation of osteogenesis through the regulation of masters genes, what would happen if we over expressed Spp24 in an MSC model? We

hope to demonstrate that Spp24 over-expression will lead to differentiation involving the regulation of master genes and matrix calcification. These studies will give us an insight of how Spp24 might be causing its effects. This understanding is central to the therapeutic potential it may have in bone disease and fracture healing.

MATERIALS AND METHODS

Draq5 Proliferation Assay

Human bone marrow derived MSCs were isolated by adherence to plastic then expanded and passaged three times before use in experiments. The use of these cells from human patients followed an approved IRB protocol. Five micro molar concentration of Draq 5 is needed to stain nuclear DNA. Once solution is made it is added directly to the media of a 6 well plate containing differentiate MSC's. Incorporation of dye into the cell takes about 30 minutes at room temperature and 5-10 minutes at 37°C. Fluorescence intensity of wells is measured using Licor Odyssey scanner.

Phenotypic Marker Comparison of Differentiation Mediate by Dex, BMP, and Spp24

Alizarin Red Stain

Alizarin red solution prepared as a 1% aqueous solution: Dissolve 1 g of alizarin red S in 50 mL of distilled water, then dilute to 100 mL. The pH should be around 4.1-4.3 and is heated to 50°C. Wells containing differentiate MSC's are fixed with 4% paraformaldehyde. Stain is applied for 10 minutes. It is then washed a minimum of 3 times with PBS to rid any non-specific staining.

ALP stain and Activity

After induction of differentiation, cells were washed with phosphate-buffered saline twice (PBS), fixed with 4% paraformaldehyde, and stained for alkaline phosphatase for 10 minutes. Staining solution was removed and washed twice with PBS. Then destaining solution of 95% ethanol was used to remove stain. The plate reader was used to quantitate the absorbance of the stain in solution at 405 nm. ALP activity was normalized using intensity of Draq5 fluorescence which represents total DNA content.

RT-PCR

Human mesenchymal stem cells were infected with AdLacZ or AdSpp24. 24 hours after infection, cells were treated with and without 10ng/mL BMP2/7, which was replenished every three days. After treatment, cells were lysed with Trizol reagent and RNA was extracted at 24-48 hours for one experiment and days 3 & 9 for the second experiment. DNA protection was done via RQ1 RNAse free DNAse (Promega.) 1ug of RNA was reverse transcribed with Superscript II RT (Invitrogen) was performed with primers for hSpp24 for 35 cycles and subsequently PCR amplified with the indicated primer. Osterix was amplified for 32 cycles and Runx 2 was amplified for 29 cycles. GAPDH was used as a loading control and was amplified for 27 cycles in all experiments.

RESULTS

Comparison of Proliferation and Differentiation with Different Inducers

Dexamethasone treatment showed significant increase in proliferation over control beginning at day 12 and a concomitant increase in calcification on day 12 (Figure 11A). Similar results occurred when the cells were treated with BMP or AdSpp24. Proliferation of cells with BMP treatment are significantly increased on day 9 and calcification begins to occur around day 12. Significant increases in proliferation occurred with Spp24 over-expression as early as day 3 and calcification as early as day 9 (Figure 11B).

The comparison of proliferation on days 3 and 9 show Dex and Spp24 to have the most significant proliferative effects on day 9. Also, BMP seems to more directly regulate ALP activity than the other treatments but Spp24 does have significant effects on day 9 (Figure 11C). For these experiments, significance was set at $p<0.05$. Experiments were completed in an n of 3 so that statistical evaluation could be used.

Regulation of Runx2 and Osterix by BMP and Spp24 Over-expression

Taq-man based qPCR was used for a 48 hour analysis of early Runx2 expression after AdSpp24 treatment revealed repressed expression of Runx2 as early as 24 hours whereas BMP activated Runx2 by 48 hours. Interestingly, Spp24 repressed the BMP enhancement of Runx2 at 24 and 48 hours (Figure 12A). This data suggest Spp24 acts as a

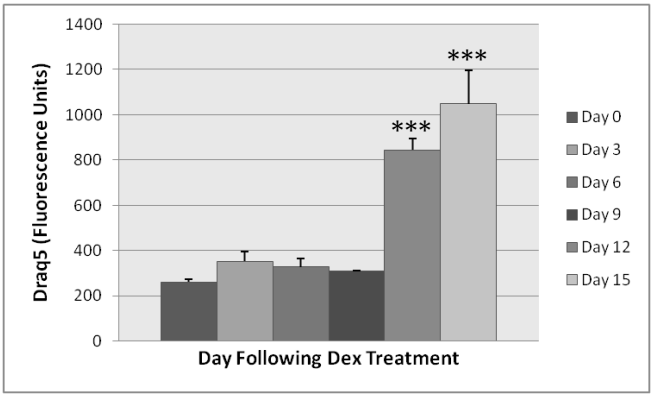
repressive agent towards BMP signaling during early osteoblastogenesis. The molecular mechanism behind this is still unknown, however Spp24 acting as a pseudoreceptor to BMP has been suggested [42].

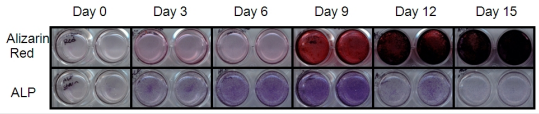
Analysis at days 3 and day 9 of Runx2 and Osx revealed interesting data. First, BMP increased Runx2 expression both at day 3 and 9, as expected. It also increased Osterix expression at day 3 versus LacZ control. As seen in the other experiment, Runx2 was repressed by Spp24 treatment alone. In stark contract, Spp24 seemed to increase Osterix expression over control at day 9. The most intriguing part of the data came from the co-treatment of BMP and Spp24. Co-treatment with both osteoblastogenic molecules increased expression of both Runx2 and Osterix over LacZ control at day 9 (Figure 12B). This could possibly be because of a different effect of Spp24 during day 9 or post bio-activation during osteoblastogenesis. If Spp24 is enhancing Runx2 and Osterix at day 9, it could explain the enhancement of BMP-mediated osteoblastogenesis.

Figure 11. Proliferation and Differentiation Assays

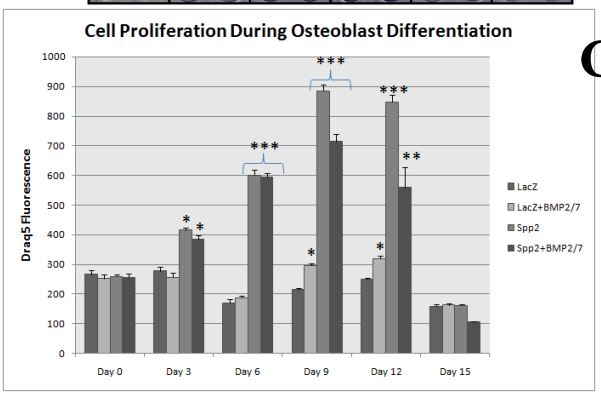
Figure A: Shows proliferation (Draq5) and differentiation (alizarin red & ALP stains) following Dex treatment.
Figure B: Shows proliferation (Draq5) and differentiation (alizarin red) following LacZ control, 10ng/mL BMP2/7, Ad-Spp24, and combo treatment.
Figure C: Shows proliferation (Draq5) and differentiation (ALP activity) at days 3 and 9 following Dex, 10ng/mL BMP2/7, and Ad-Spp24 treatment compared to control.
NOTE: All experiments are n=3

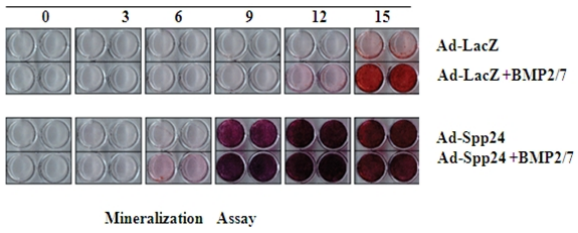
A.





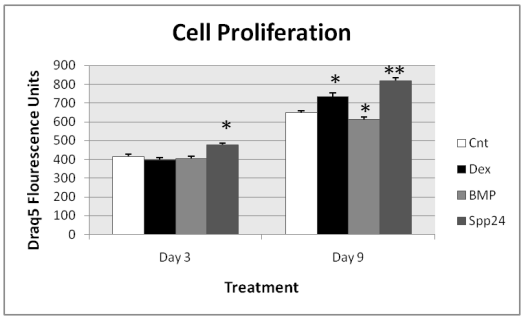
B.





Mineralization Assay

C.



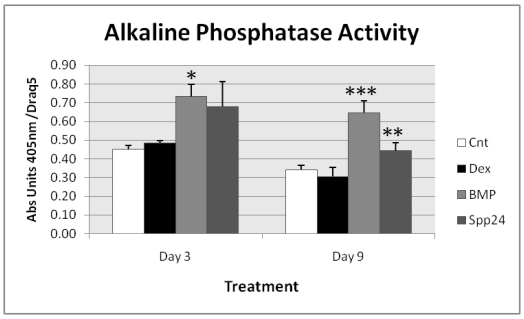
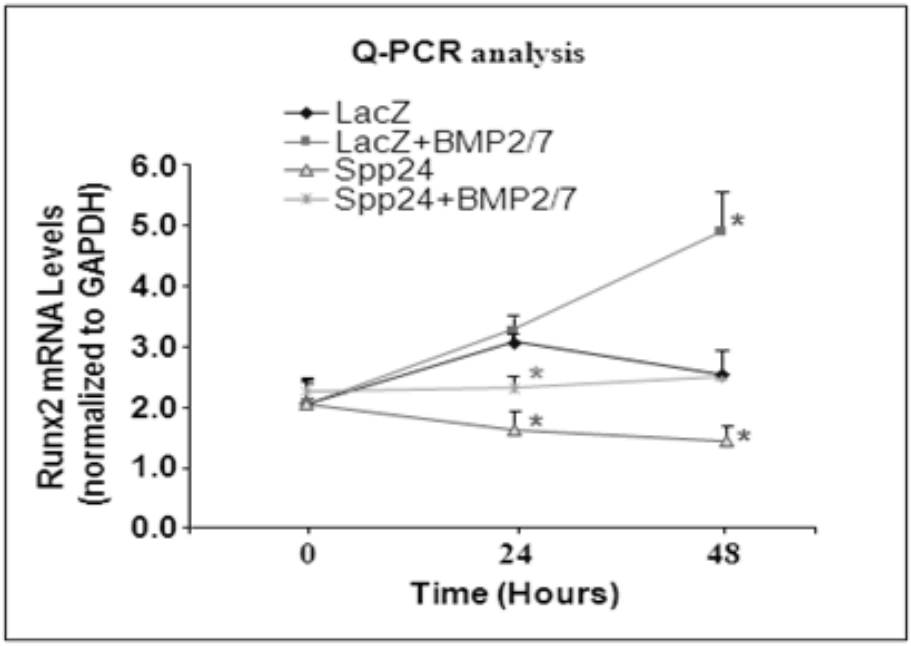


Figure 12. Regulation of Runx2 and Osx by BMP and Spp24 Over-Expression

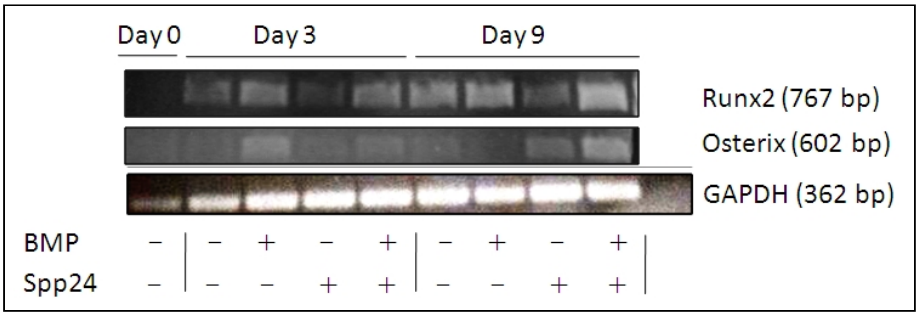
Figure A: MSCs were infected with AdLacZ or AdSpp24. 24 hours post infection, cells were treated with and without BMP2/7. 24 and 48 hours after BMP treatment, RNA was isolated for Taq-man based qPCR measurements of Runx2 expression. (n=3)

Figure B: MSCs were treated with either AdSpp24 or AdLacZ in the presence and absence of 10ng/mL BMP2/7. RNA was extracted at days 3 and 9. The samples were reverse transcribed using Superscript II (Invitrogen) PCR amplification was performed with primers for hSpp24 for 35 cycles. Osterix was amplified for 32 cycles, Runx 2 was amplified for 29 cycles, and Trb3 was amplified for 33 cycles. GAPDH was used as a loading control and was amplified for 27 cycles in all experiments. (n=1)

A.


Q-PCR analysis
LacZ
LacZ+BMP2/7
Spp24
Spp24+BMP2/7
Runx2 mRNA Levels (normalized to GAPDH)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
24
48
Time (Hours)
*
*
*
*

B.


Day 0
Day 3
Day 9
Runx2 (767 bp)
Osterix (602 bp)
GAPDH (362 bp)
BMP
Spp24

DISCUSSION

Draq5 fluorescence, a marker of proliferation, increased throughout different time points of differentiation with BMP, Dex, and AdSpp24. Some proliferation was noted as early as day 3 and as late as day12. All three treatments also showed alkaline phosphatase activity, an indicator of osteoblastogenesis, and both Dex and Spp24 showed significant increase at day 9 with Spp24 showing a significant increase as early as day 3. Finally, an increase in alizarin red stain, a marker of calcium deposition, along with the morphological changes of the cell strongly suggests that each treatment could differentiate cells from MSC to osteoblast. These studies clearly demonstrates that Spp24, like BMP and Dex, has osteogenic and morphogenic properties. All treatments were capable of inducing proliferative effects, differentiating MSC's, and producing a calcified matrix. Differences such as the onset and regulation of effects may be due to the fact that each may have slightly differences in their mechanism of action to achieve osteoblastogenesis and osteogenesis. While the mechanism of BMP has been well studied, further exploration into Spp24 will have to be done before a specific mechanism can be defined.

The most interesting thing about this study was Spp24's ability to regulate the master genes of osteoblastogenesis. Runx2 is repressed early on at day 3 and Osterix is enhanced at day 9, a point post bio-activation of Spp24. Since Osterix is downstream of Runx2, a potential mechanism could be Spp24 activating Osterix independently of Runx2.

This would entail Spp24 affecting Osterix and Runx2 differently, which our results support. This mechanism could explain the repression of Runx2 followed by the enhancement of Osterix.

CHAPTER 5

GENERAL CONCLUSIONS

The studies presented in this thesis demonstrate the development of an anti-body which helped determine localization of Spp24 in different tissues as well as understand properties and events taking place during differentiation of an osteoblast. These studies have also established Spp24 as a key player in the differentiation pathway of osteoblast. Spp24 regulates master genes of osteoblastogenesis Runx2 and Osterix, as well as increasing phenotypic markers of the mature osteoblast that play a role in calcification of the secreted matrix. Based on these results we were able to propose a mechanism. Our mechanism describes Spp24 repressing Runx2 expression. Eventually repression is lost and BMP signaling can enhance Runx2 expression at day 9 or post bio-activation. Concomitantly, Spp24 can increase Osterix expression which leads to morphogenic and osteogenic effects of Spp24. Further work is needed to completely elucidate Spp24 mechanism of action.

Future work may include a dual tag vector system that would allow the monitoring of the N-terminal and C-terminal side of the protein. This would give us better understanding of the protein processing taking place in the cell. Another possibility may be to use FRET analysis in order to elucidate protein-protein interactions taking place especially in its relationship with BMP signaling.

Spp24 presents an exciting chapter in the field of tissue engineering and regenerative medicine as it displays a lot of potential for osteoblastogenesis and hopefully,

osteogenesis. It may one day act as an adjuvant therapy along with current recombinant BMP techniques that aid in fracture healing.

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VITA

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