ABSTRACT


Both calcium and phosphorus tremendously impact bone health, and their deficiencies are associated with reduced bone parameters in growing mammals due to altered levels of calcitropic hormones (Parathyroid hormone (PTH) and 1,25(OH)\(_2\)D\(_3\)). However, effects of mineral deficiency on bone during the neonatal period are less characterized. Mesenchymal stem cell (MSC) differentiation into osteoblasts and adipocytes is a major determinant of bone quality and quantity. The aim of this research was to examine the effects of mineral nutrition and its endocrine regulators on MSC activity, bone parameters and gene expression during the neonatal period using pig as the model system. In the first study, 1,25(OH)\(_2\)D\(_3\) was found to stimulate adipocytic differentiation of MSC isolated from 2-week old piglet based on dose-dependent increase in adipogenic (PPARG, LPL and AP2) expression and lipid accumulation (Oil Red O staining) in cells treated in vitro for a period of 12 days. A trend for reduced Runx2 (osteogenic transcription factor) expression and a significant reduction in alkaline phosphatase (ALP) activity suggested inhibition of osteogenesis, however, the effects were unclear due to a dose-dependent increase in osteogenic marker osteocalcin expression with 1,25(OH)\(_2\)D\(_3\). Elevated levels of 1,25(OH)\(_2\)D\(_3\) during P deficiency seen in older animals could impact bone by altering MSC activity. In the second study, dietary P deficiency during the neonatal period (d0-d15) resulted in dramatically reduced in vivo MSC proliferation accompanied by increased adipogenic markers (PPARG and LPL) and reduced osteocalcin expression in the bone marrow. Although the reduction in growth performance,
bone growth and reduced PTH levels associated with classical P deficiency were observed, unlike older animals, circulating levels of 1,25(OH)2D3 did not change. The reduced PTH levels seen with P deficiency could impact MSC activity. In the third study, PTH was found to alter MSC activity depending on the mode of treatment as it caused increased adipogenic expression (PPARG, LPL) and lipid accumulation (Oil Red O) with continuous treatment and increased osteogenic expression (Runx2) and ALP activity by intermittent treatment. Also, PTH and 1,25(OH)2D3 acted antagonistically when added together as the ability of 1,25(OH)2D3 to stimulate adipogenesis was partly inhibited by PTH while the ability of PTH to cause osteogenic expression was partly inhibited by 1,25(OH)2D3. Since both calcitropic hormones affect MSC activity, we hypothesized that dietary calcium deficiency may alter developmental programming of bone during the neonatal period. In the fourth study, dietary Ca deficiency during the neonatal period (d0-d18) caused a dramatic reduction in the in vivo MSC proliferation. Although bone quality and quantity were significantly reduced, unlike older animals, the circulating levels of Ca, P and 1,25(OH)2D3 were unaltered and PTH levels were increased only by end of the study with calcium deficiency. In vitro studies utilizing homologous sera demonstrated that MSC activity was affected by both the calcium status of the animal and the sera, as well as by their interaction. Altered MSC activity maybe attributed to one or more of the 22 differentially expressed serum proteins identified by proteomic analysis of homologous sera. Overall, the data from these studies indicates that neonatal mineral nutrition is crucial for bone integrity and suggests that early life Ca and P restriction may have long-term effects on bone integrity via programming of MSC. Also, the
circulating levels of calcitropic hormones may affect in vivo bone development based on their ability to alter MSC differentiation potential in vitro as seen in these studies.
Role of Mineral Nutrition in Neonatal Bone Health: Effects on Porcine Mesenchymal Stem Cells

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Animal Science and Poultry Science

Raleigh, North Carolina

2009

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DEDICATION

This work is dedicated posthumously to my maternal grandparents, as well as my paternal grandfather. My maternal grandfather was one of the most influential, intelligent, charming and good human beings I have ever known. He showered a lot of his love and attention on me as I grew up. My maternal grandmother was a genuine, simple and affectionate person, who despite little education raised her children very well. I also admire my paternal grandfather, who was an intelligent man and a good human being, who loved me immensely and instilled in me good values and appreciation for little things in life. I know they would both be proud to see me achieve this goal.
BIOGRAPHY

Avanika Mahajan was born in Secunderabad and raised in New Delhi, the capital city of India. She is the eldest daughter of Drs. Usha and Arun Kumar and has one younger sister, Aranika. She received her B. Tech. degree in Biotechnology from Guru Gobind Singh Indraprashtha University, Delhi in 2004. Avanika began her Ph.D. studies with Dr. Chad Stahl at Iowa State University in 2005 and then moved to North Carolina State University along with Dr. Stahl in 2007. Following the completion of her degree, Avanika will be joining the Keck School of Medicine of University of Southern California, Los Angeles as a Postdoctoral Research Associate. Beyond the halls of academia, her interests include music and dancing.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my thanks to those who helped me with various aspects of conducting research and the writing of this thesis. First and foremost my Major Professor, Dr. Chad Stahl for his guidance, patience and support throughout this research. I would like to thank him for letting me pursue my degree under your guidance. He has been an outstanding mentor by always being willing to listen and help, making me a better scientist. I would also like to thank Dr. Jack Odle and Dr. Marcelo for their support, guidance and career advice throughout my session at NCSU. Thanks also to Dr. Elizabeth Loboa for letting me collaborate with her lab for pursuing an exciting research project as well as for her guidance. I would like to thank everyone in my lab including Ani Qu (Iowa State University), Lindsey Alexander and Brynn Seabolt for their support, assistance and a good time all these years. I would also like to thank the undergrads in the lab especially Nadia Hassounah and Mohammed Khan for their assistance with my research projects. I appreciate all the career advice from Dr. Sunny Liu that helped me broaden my perspective. Many people helped me in my research at NC State, and in this regard I would like to thank Weifang, Christopher Lassiter, Tiffany and Ryan Odle for their help with our animal studies. Most importantly, I thank Seth McCullen and Dr. Lin Xi for their invaluable time and help with my research projects. I would also like to thank graduate students of my department for their friendship and support. I would like to acknowledge the persistent technical help that I received from Marian Correll and Chris Brown.

My special thanks to my husband Anup Gokarn without whose support and
encouragement, I would not have been able to accomplish my degree. I thank him for his consistent belief in me as well as for motivating me to do better. Finally, I would like to thank my parents Drs. Usha and Arun Kumar and my sister Aranika for instilling the right values in me, exposing me to the best of educational environment and making me the person I am. They have been my pillar of strength and motivation all these years of pursuing my degree. I would not have been able to accomplish all of this without the love and support of my family.
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CHAPTER 1

Literature Review

Introduction

Mineral nutrition has a tremendous impact on bone health. Both Calcium (Ca) and Phosphorus (P) are required for optimum bone growth, and their deficiencies can lead to a plethora of bone diseases like rickets, osteomalacia and osteoporosis. The deficiency of Ca and P has been shown to cause reduced bone strength, bone growth, bone mineral density and bone mineral content (Schanler et al., 1991; Stauffer et al., 1973). This is primarily attributed to increased bone resorption caused by calcitropic hormones, parathyroid hormone (PTH) and 1,25(OH)_{2}D_{3} whose levels are altered during mineral deficiency. Also, both PTH and 1,25(OH)_{2}D_{3} have been shown to alter the differentiation potential of mesenchymal stem cells (MSC) in vitro (Atmani et al., 2003; Mahajan and Stahl, 2009; Rickard et al., 2006). Since MSC give rise to the pool of osteoprogenitor cells, they play an important role in bone development. Although the effects of mineral deficiency on bone are well characterized, there are only a few studies examining their impact on neonatal bone health (Hsu and Levine, 2004). It is valuable to evaluate their effects on bone development in neonates because of the rapid bone mass acquisition rates (Holick and Dawson-Hughes, 2004), highest number of proliferating MSC (Stenderup et al., 2003) as well as increasing evidence for link between bone quantity and quality achieved in infancy and osteoporosis (Fewtrell et al., 1999). Moreover, there are no studies examining the effect of mineral nutrition on MSC population in neonates. Piglets serve as an excellent model system for studying the effect of nutritional factors on neonatal bone development because of similar physiology, bone remodeling cycle
(Bustad and McClellan, 1966; Lapillonne et al., 2004) and lineage allocation of MSC (Bosch et al., 2006a; Ringe et al., 2002a). Therefore, this chapter summarizes the literature relevant to the effects of mineral nutrition in association with the calcitropic hormones on bone parameters as well as MSC phenotype in both neonatal as well as young growing pigs.

**Structure and Function of Bone**

Bone is a specialized connective tissue that provides mobility, shape and support for the body, protects various organs and helps in muscle locomotion. It is the storage site for essential minerals like Ca and P, and regulates their extracellular levels by responding to hormone signals. It also performs immune, cellular and regenerative functions by development and storage of multipotent stem cells in the bone marrow. Bone is composed of both an organic and inorganic phase. Approximately 95% of the organic phase consists of type I collagen and the remaining 5% is composed of other non-collagenous proteins like osteocalcin, osteonectin, osteopontin and glycosaminoglycans. The inorganic phase consists of crystalline mineral salts, primarily Ca and P present in the form of hydroxyapatite crystals. However, bone is also a living tissue and consists of three kinds of cells: 1) Osteoblasts (bone-forming cells), 2) Osteoclasts (bone-resorbing cells) and 3) Osteocytes (bone-maintenance cells). Osteoblasts are the fully differentiated cells that secrete the collagenous and the non-collagenous proteins to form the bone matrix. They also regulate mineralization of the bone. Osteoclasts are the large, multi-nucleated cells that cause bone resorption while osteocytes are the mature osteoblasts that get embedded in the bone matrix and help in its maintenance. Morphologically, there are two different kinds of bone in the body: 1)
Trabecular (cancellous): found in outer parts of most bones; and 2) Cortical (compact): found in the inner parts of most bones. Cortical bone is comprised of dense calcified tissue and primarily plays a structural role, while trabecular bone is loosely organized with a porous matrix having a spongy appearance. It primarily plays a metabolic role as it is vascularised and comprises the bone marrow that harbors mesenchymal stem cells and hematopoietic stem cells.

**Bone Development**

*Bone Modeling*

Formation of the bone is called ossification. It occurs by either a direct (intramembranous) or an indirect (endochondral) process leading to the formation of membranous and cartilaginous bone. Intramembranous ossification occurs during embryonic development. It involves direct transformation of mesenchymal cells into osteoblasts and is restricted to cranial and facial bones, and parts of the mandible and clavicle. Weight-bearing and joint-forming bones are formed by endochondral ossification. Bone formation is initiated from embryonic mesenchyme that transforms into cartilage moulds. This is followed by increased proliferation and elaborate matrix formation by chondrocytes near the ends of these cartilage moulds to increase longitudinal growth. Chondrocytes then undergo hypertrophy and eventually disappear remaining only as a scaffold for further bone growth. Thereafter, osteoblasts differentiate from mesenchyme and mineralize the bone matrix laid down by chondrocytes on the surface of cartilage moulds to form trabeculae. Osteoclast precursors derived from hematopoietic stem cells get attracted to the trabeculae through the vasculature.
They then fuse with themselves to give rise to mature multi-nucleated osteoclasts (Kondo et al., 2004). These mature osteoclasts then resorb most of the trabeculae, followed by laying down of new bone on the remaining trabeculae by the osteoblasts.

**Bone Remodeling**

The process of initial bone development where formation precedes and exceeds resorption is called bone modeling and is dominant during the first two decades of our lives. During adult skeletal development in the next three decades, bone formation is coupled with bone resorption. The failure of formative phase of bone recycling to keep up with the resorptive phase in the later decades of life can lead to reduced skeletal strength and increased risk of fracture. Remodeling occurs by a concerted efforts by group of cells consisting of osteoblasts and osteoclasts called the basic multicellular units (BMU) that maintain a balance between resorption by osteoclasts and formation by osteoblasts by a complex and less understood mechanism. The life span of a single BMU is 6-7 months and approximately 10% of the adult skeleton is remodeled every year (Litwack, 2002). The BMU respond to multiple autocrine, paracrine and endocrine signals as well as mechanical stimulation to tightly couple bone resorption and bone formation. Bone remodeling is critical to maintain optimum health and accelerated resorption or defective bone matrix formation can cause various bone defects like osteoporosis, osteopenia, osteopetrosis and rheumatoid arthritis.
Cellular Aspects

RANKL, a member of the tumor necrosis factor family, serves as the signal for osteoclast formation. It is originally expressed in the osteoblasts and binds to the RANK receptor on the cell surface of the osteoclast precursors to cause their transition into mature osteoclasts (Anderson et al., 1997). Osteoclast maturation can be inhibited by the decoy receptor osteoprotegerin that is produced by the osteoblasts and binds to RANKL to block its activity (Yasuda et al., 1998). Therefore, osteoclast formation is dependent on the RANK/RANKL/OPG signaling axis (Figure 1.1). Some of the markers expressed by the immature osteoclast are TRAP (Tartrate-resistant alkaline phosphatase), calcitonin receptor (CTR) and beta-3 integrin. Osteoblasts follow a different course as they differentiate from MSC, and require the presence of primary osteogenic transcription factor called Runx2, osterix and several components of the Wnt signaling pathways (Ducy et al., 1997; Hu et al., 2005; Nakashima et al., 2002). Commonly used markers to characterize mature osteoblasts are matrix protein Col1A1 (Type I collagen), non-collagenous protein osteocalcin and a key enzyme involved in bone mineralization called the alkaline phosphatase. Some osteoblasts get embedded in the bone matrix to give rise to the bone maintenance cells called the osteocytes which then make up the majority of bone cells (Nijweide et al., 1996).
Neonatal Bone Health

Bone undergoes different stages throughout the life span of a human being. During the first two decades of life, bone grows in both length and width. Rate of bone acquisition as a percentage of body mass is highest during infancy continuing up to puberty with reduced growth during other periods of childhood and adulthood (Holick and Dawson-Hughes, 2004). During infancy, average whole body bone mineral content and total body mineral density increase by 389% and 157% respectively (Koo et al., 1998). Bone mass accumulates linearly from infancy through puberty followed by some additional bone mass accumulation leading to peak bone mass in young adulthood. This has been primarily attributed to increased bone
formation over resorption. Several studies suggest that peak bone mass achieved in early adulthood is correlated to the bone mass accumulated in early post-natal period. For instance, a study demonstrated that at 8-12 yrs of age, whole body BMC for prematurely born infants was significantly lower than children of same age born at term (Fewtrell et al., 1999). Therefore, this is especially important for premature or low-birth weight infants that have inadequate bone health to begin with. A study (Cooper et al., 2001) has directly linked the growth rate in childhood with the risk of hip fracture in later life. They found that the risk of hip fracture was elevated in babies born short as well as those with slow rate of childhood growth. Growth is more sensitive to nutrition and endocrine profile in early periods compared to later life, and under nutrition is a more important determinant of body size during early periods compared to later life (Mccance and Widdowson, 1962). This is because of the rapid rate of cell division and tissue growth during the neonatal period. One of the potential predictors of bone health are mesenchymal stem cells (MSC) since they give rise to osteoblasts. It has been shown that during infancy, the number of proliferating MSC are at its peak, and that with aging they undergo an accelerated senescence and reduced life span (Stenderup et al., 2003). Therefore, it is important to maximize the potential of these cells for bone growth during infancy. Based on increasing evidence linking peak bone mass to bone strength and risk of fracture in later life, osteoporosis is being viewed as a pediatric disease. Therefore, it is critical to maximize skeletal mineral acquisition relatively early in life.

**Mineral Nutrition and Bone Health**

Adequate bone health is vital for normal functioning of the body and is dependent on
optimum bone integrity, which in turn depends on the quantity and the quality of the bone. Quantity of bone is determined by the bone mineral content and bone mineral density, while quality of bone depends on the organization of the matrix proteins comprising the microarchitecture of the bone. Apart from genetics and environment, adequate bone health is majorly dependent on optimum nutrition intake. Ca and P are the two primary minerals essential for bone development and maintenance, and comprise approximately 80% of the bone mineral content. The deficiency of Ca and P has been shown to cause reduced bone strength, bone growth, bone mineral density and bone mineral content (Cromwell et al., 1995; Stauffer et al., 1973). This is primarily attributed to increased bone resorption caused by calcitropic hormones PTH and 1,25(OH)₂D₃ whose levels are altered during mineral deficiency. Circulating levels of PTH are elevated during Ca deficiency and reduced during P deficiency, while those of 1,25(OH)₂D₃ are elevated during both Ca and P deficiencies (Hernandez et al., 1996; Stauffer et al., 1973; Tanaka and Deluca, 1973). Nutritional inadequacies can lead to the onset of several bone diseases. Softening of bones due to defective or inadequate mineralization caused by nutritional deficiency of vitamin D causes rickets in children and osteomalacia in adults. However, one of the most widespread bone diseases that affects over 10 million people in US alone, is the skeletal disease osteoporosis. This disease is characterized by increased susceptibility to fractures due to reduced bone mass and structural deterioration (Consensus Development Conference, 1991), and not only impairs an individual’s quality of life but also diminishes life expectancy. Osteoporotic fractures occur commonly at the hip, spine and the wrist. These fractures can be associated with significant morbidity and excess mortality. For instance, up to 20% of patients
diagnosed with hip fracture die within 6 months (Sexson and Lehner, 1987). A third of the surviving patients become permanently disabled (Bonar et al., 1990). Spine fractures also result in significant pain and disability, thus greatly diminishing the quality of life lead by such individuals. The economic burden on the society due to this disease is equally high as total costs estimated for all osteoporotic fractures is approximately $20 billion in the USA and $30 billion in the European Union (Cummings and Melton, 2002). The lifetime risk for osteoporosis for a 50-year-old man and woman is 20% and 50% respectively. The nutritional factors are critical for prevention and treatment of osteoporosis, of which the most important ones are Ca, P and vitamin D. Other dietary components that affect bone development are magnesium, zinc, copper, iron, fluoride, vitamins A, C, and K. Vitamin D and Ca are often administered as supplements for therapeutic treatment of osteoporosis, and have been shown to reduce the risk of hip fracture and other non-vertebral fracture in the elderly (Chapuy et al., 1994). Also, intermittent doses of PTH given subcutaneously have been shown to exert anabolic effects on bone (Dobniq and Turner, 1995), and has been shown to reduce fracture risks in post-menopausal women (Neer et al., 2001). Also, both PTH and 1,25(OH)2D3 have been shown to alter the differentiation potential of mesenchymal stem cells (MSC) in vitro (Mahajan and Stahl, 2009; Rickard et al., 2006). Because MSC give rise to osteoblasts, alteration of lineage allocation based on PTH or 1,25(OH)2D3 treatment can have serious implications for bone health. Therefore, the role of mineral nutrition in bone health is of utmost importance.
**Mesenchymal Stem Cells: Impact on Bone**

Mesenchymal stem cells (MSC) are pluripotent stem cells that reside primarily in the bone marrow and have the capacity to self renew and differentiate into distinct mesenchymal lineages. They have been shown to form bone, cartilage and adipose tissue in vivo (Owen, 1988), and, differentiation of MSC into the osteogenic, adipogenic and chondrogenic lineage *in vitro* has also been well established (Jiang et al., 2002; Pittenger et al., 1999). Studies also have demonstrated that MSC can give rise to myoblasts and even early progenitors of neural cells (Wakitani et al., 1995; Woodbury et al., 2000). MSC are easily isolated from the bone marrow based on their ability to attach to plastic and a concomitant lack of adherence of hematopoietic stem cells (Friedenstein et al., 1970). Similarities between the mode of differentiation into various mesenchymal lineages *in vitro* between porcine and human derived MSC (Ringe et al., 2002a) suggests that porcine MSC can serve as a valuable model system to study the impact of nutrition on bone health. MSC differentiation potential *in vitro* has also been shown to be altered by the calcitropic hormones PTH (Nishida et al., 1994) and 1,25(OH)₂D₃ (Atmani et al., 2003; Beresford et al., 1992). Increasing evidence suggests that osteoporosis and age-related bone loss may be attributed to increased adipogenic differentiation at the expense of osteogenic differentiation causing reduced bone volume and increased bone marrow adiposity (Beresford et al., 1992). This may result from increased differentiation of MSC into adipocytes (fat cells) at the expense of osteoblasts (bone cells) *in vivo* (Figure 1.2). The two major transcription factors that determine the fate of MSC are Runt related transcription factor (Runx2) and peroxisome proliferator activated receptor gamma (PPARγ). Osteogenesis is switched on by Runx2 (Komori, 2003) while PPARG is
the master regulator of adipocytic differentiation (Tontonoz et al., 1994). Since functional alteration of MSC can cause age-related bone loss or degenerative bone diseases, MSC can be an important target for nutritional interventions like Ca and vitamin D, hormone therapy including estrogens and selective estrogen receptor modulators (SERMs), and drugs like biphosphonates employed for increased bone strength and treatment of osteoporosis. Identification of nutrients that favor osteogenic differentiation of MSC in vivo can lead to novel therapies for increased bone formation, treatment of bone disorders as well as development of MSC as a tool for tissue engineering. MSC is not only important for normal bone development, but can also serve as a valuable tool for stem cell therapy for the treatment of various diseases. For example, MSC have been utilized for spine fusion (Muschler et al., 2003), repair of segmental bone defects (Quarto et al., 2001) and craniotomy defects (Krebsbach et al., 1998) by local delivery of marrow stem cells to the site of injury. Apart from orthopedic medicine, MSC have also been utilized for therapeutic treatment of broad spectrum of other diseases like muscular dystrophy (Gussoni et al., 1999), spinal cord injury (Teng et al., 2002) and coronary artery disease (Orlic et al., 2001).
Figure 1.2 Mesenchymal stem cell differentiation

**Pig as a Model System**

Pigs are being increasingly used as a model system by nutritionists to evaluate the effects of nutritional factors on the skeletal system. The growing pig is an excellent model system to investigate changes in bone remodeling, physiology and metabolism in response to diets varying in mineral nutrition (Eklou-Kalonji et al., 1999; Pointillart et al., 1995). The guidelines for use of an animal model system to study osteoporosis also recommend using a non-rodent species for research (Thompson et al., 1995). This is because the nutritional behavior and bone remodeling cycle in pigs are more similar to humans compared to mice or rats. Several features of the pig skeleton are similar to humans. Both undergo bone remodeling by utilizing local groups of osteoblasts and osteoclasts called the BMU (bone multicellular units) (Spurrell, 1965). The rate of bone removal and deposition in both cortical
and trabecular fractions is also comparable to that of humans (Mosekilde et al., 1987). Also, they reach a peak bone mass at the age of 2.5-3 years (Bouchard et al., 1997), and are one of the few species to undergo spontaneous fractures similar to humans (Spencer, 1979). Like humans, pigs also have the haversian canal system consisting of narrow tubes containing blood vessels and nerves that are surrounded by rings of bone (lamellae) located in the compact bone. This is of significance since it suggests similar endocrine influences on bone parameters amongst pigs and humans. The ovariectomized calcium-restricted Sinclair S1 minipig has been shown to undergo a significant reduction in bone mass, increased bone resorption and a disrupted bone structure (Mosekilde et al., 1993a, b), suggesting its potential to serve as a model for studying bone remodeling changes as well as post-menopausal bone loss that affects millions of women every year worldwide. Other desirable traits in pigs are similar diet (omnivorous), gastrointestinal tract and reproductive cycle compared to humans (Bustad and McClellan, 1966). Pigs also undergo a rapid increase in body mass during the neonatal period (3-4 fold increase in a span of 2-weeks) thus facilitating the study of physiological processes rapidly. However, there are a few disadvantages of using pigs rather than rodents for research such as larger housing area, increased labor expenses for animal care, and difficult animal handling due to size and aggressive nature of pigs. Regardless, biologically, the pig is an excellent model system to examine the effects of nutrition on bone health.
**Calcium Homeostasis**

Calcium is required for the normal growth and development of the body. Intracellular calcium is vital for various cellular functions like mediating morphological, gene expression and electrochemical responses through signal transduction pathways, while extracellular Ca helps in the normal functioning of muscle and nervous tissue, and normal development and maintenance of bones and teeth. Majority of Ca in the body is found in the bone (99%) and the remaining 1% is found in the extracellular fluids, blood, various organs and intracellular space. Therefore, a set of highly specialized proteins mediate Ca transport across plasma membrane and other cell organelle membrane. The net extracellular Ca balance depends majorly on the balance between the absorption in the small intestine, release from the bone due to resorption and the reabsorption in the kidney. Ca transport through the small intestine depends on 2 modes of transport (Figure 1.3). The paracellular transport is the passive mode of transport and is dependent on increased concentrations of Ca on the luminal side. The transcellular transport is the active mode of transport, which consists of 3 steps: 1) transport of Ca into the intestinal cells across the brush border membrane by the use of specialized Ca channel proteins like TRPV6 and TRPV5 (Transient receptor proteins), 2) intracellular Ca transport by vitamin D regulated protein called Calbindin-9k (Choi and Jeung, 2008) and 3) Expulsion of Ca from the cell into the blood stream by plasma membrane Ca ATPase (Wasserman, 1997). Both TRPV5 and TRPV6 belong to the class of transient receptor potential channels that perform diverse physiological functions. Their structure consists of six transmembrane spanning region and a hydrophobic pore region. Although there is 80% similarity in the sequence of TRPV5 and TRPV6, they have different expression pattern and
distinct functions in Ca transport. Calbindin is often co-expressed with TRPV5 in various tissues. Two major calbindins, calbindin-D9k and calbindin-D28K influence Ca transport in the mammalian intestine and kidney. In the intestine, calbindin enhances Ca absorption through a vitamin D mediated mechanism, and so helps in adapting to a low Ca diet. The extrusion of Ca from the basolateral membrane of the enterocyte and into the extracellular fluid occurs against a steep electrochemical gradient via PMCA (plasma membrane calcium ATPase) and NCX (Na+/Ca2+ exchanger). NCX activity is not affected by vitamin D, however, PMCA expression in the intestine has been shown to be regulated by vitamin D previously (Cai et al., 1993). Extrusion of Ca from enterocytes may also be mediated by calbindin mediated mechanism since calbindin-D28K is actively co-expressed with tubulin in the presence of 1,25(OH)2D3 (Nemere et al., 1991). The kidney also plays an important role in reabsorption of Ca to restore approximately 98% of the filtered Ca. Like the duodenum, kidney also employs two pathways for Ca transport: 1) paracellular (passive) transport across the tight junctions primarily mediated by electrical or chemical gradients, and 2) transcellular transport consisting of passive influx across luminal membrane followed by binding to calbindin-D28K and cytosolic transport leading to extrusion at the peritubular membrane by active transport via calcium binding protein such as calbindin-D28K. Approximately 55% and 10% of the Ca reabsorption occurs in the proximal convoluted tubule and proximal straight tubule respectively. Another 10% is reabsorbed in the distal convoluted tubule, while the thick ascending loop accounts for 20% Ca reabsorption. Paracellular transport occurs primarily in the proximal tubule and the thick ascending loop and is coupled with Na+ transport (Suki and Rouse, 1981), while transcellular (active) transport is the primary mode
in the distal tubule and is not dependent on Na transport (van Os, 1987). The rate of reabsorption is regulated by calcitropic hormones PTH and 1,25(OH)₂D₃ specifically in the parts of nephrons utilizing active mode of transport (Cotanzo and Windhager, 1992).

![Diagram of Ca transport](image)

Figure 1.3 Transport of Ca across an enterocyte.

The plasma levels of ionized Ca are tightly regulated by the concerted action of PTH and 1,25(OH)₂D₃ (Figure 1.4). During reduced Ca levels in the plasma, PTH is released by the parathyroid gland in response to activated calcium-sensing receptors (CaR). PTH then acts on bone to release Ca and kidney to increase re-absorption of urinary Ca and the production
of 1,25(OH)_2D_3. Elevated 1,25(OH)_2D_3 levels increase intestinal absorption of Ca and also release further Ca into circulation by its actions on bone. (Flynn, 2003; Prentice et al., 2003).

![Figure 1.4 Physiological effects of calcitropic hormones](image)

**Calcium and Bone**

Adequate dietary intake of Ca is important throughout life to maximize bone mineral content and reduce the risk of fractures (reviewed by (Heaney, 2000)). Dietary Ca is an important determinant of peak bone mass by its influence on skeletal retention of Ca (Matkovic et al., 1990; Matkovic et al., 1977). Also, increased dietary Ca intake results in reduced bone resorption rate and increased bone mineralization rate (Hasling et al., 1990). Positive effects
of dietary Ca on bone also have been demonstrated by several studies in post-menopausal women who are more susceptible to bone loss and fracture due to their altered endocrine profile. For instance, a significant reduction in bone loss from femoral neck and improved Ca balance was seen with adequate dietary Ca intake (Aloia et al., 1994). Ca has been shown to reduce the risk of osteoporotic fractures as shown by several studies (Chan et al., 1987; Chapuy et al., 1994; Dawson-Hughes et al., 1990). Although several studies have explored the relationship between dietary Ca and bone development in older children and adults, limited studies have looked at early neonatal life (Lapillonne et al., 2004; Schanler et al., 1991). Because the proliferation and differentiation of bone marrow derived MSC are dramatically altered by the calcitropic hormones (Bellows et al., 1994; Mahajan and Stahl, 2009; Rickard et al., 2006), the impact of Ca nutrition on MSC activity, either directly or via its homeostatic hormones during neonatal bone development is of great importance.

**Phosphate Homeostasis**

Phosphorus (P) is a macromineral that is vital for normal growth and development of the body. It exerts most of its biological functions in the body in the form of phosphate. Approximately 85% of P in the body is found in bones and teeth, followed by 14% in soft tissues and only 1% in the extracellular space. The extracellular P is present in both and organic and inorganic forms, however, inorganic form of P is considered clinically relevant. P serves various functions in the body at both cellular and tissue level. Within the cell, it forms an important constituent of biomolecules like ATP, nucleic acids and phospholipids in the cell membrane. However, the extracellular P is important primarily for proper mineralization of bone. P homeostasis in the body is maintained by the interplay between intestinal absorption, renal excretion, and
exchanges with bone and the intracellular space. The plasma levels of P are loosely regulated by hormonal factors vitamin D, PTH and the phos photonins. In the small intestine, P is absorbed by both passive paracellular diffusion and active transport using the sodium-phosphate co-transporter NPT1 (Broer et al., 1998). More than 70% of the unretained P is reabsorbed in the proximal tubule of the kidney. This reabsorption is facilitated by renal transport of P by NPT2 which is in turn regulated by hormones PTH and 1,25(OH)2D3. The 1,25(OH)2D3 increases while PTH and phophatonins decrease renal reabsorption (Takeda et al., 2000). Phosphatons like FGF23 and PHEX are novel factors that have been found to greatly influence P homeostasis. FGF23 regulates P reabsorption in the kidney by regulating the expression of sodium phosphate transporters (NPT-2a and NPT-2c) and synthesis of 1,25-dihydroxyvitaminD3 in the duodenum (Berndt et al., 2005). PHEX indirectly regulates renal P transport and vitamin D metabolism and is an important determinant of osteoblast differentiation (Shih et al., 2002; Tenenhouse, 1999).

**Phosphate and Bone**

Dietary P deficiency can have tremendous impact on bone health and growth performance. We have previously demonstrated reduced body weight, lower ash percentage and weaker bones in pigs due to 20% deficiency in dietary P based on NRC requirements (Alexander et al., 2008). Several other studies have also shown reduced skeletal growth due to dietary P deficiency (Harrison et al., 1980; Jendza et al., 2005; Veum et al., 2001). However, little research examined the effects of this mineral during the neonatal period of growth. Specifically, there has been no investigation into the impact of dietary P on the potential for nutritional programming of MSC. A study demonstrated reduced bone mineralization and
higher incidence of broken bones due to P deficiency during the early growth period in chickens and showed that bone integrity is more dependent on the P status of the animals in early life (Driver et al., 2006). Schanler et al (Schanler et al., 1991) also demonstrated inadequate mineral distribution in the tibia and vertebra due to P deficiency in neonatal miniature piglets. Because early life P restriction impacts bone growth potential, one of the mechanisms of reduced bone growth could be alteration of MSC differentiation potential. Hormones known to regulate P homeostasis like PTH and 1,25(OH)₂D₃ also have been shown to alter MSC differentiation (Bellows et al., 1994; Mahajan and Stahl, 2009; Rickard et al., 2006). Therefore, further studies to examine the effects of P on MSC activity are warranted.

1,25-DihydroxyvitaminD₃

Vitamin D can act as both nutrient and hormone to perform various roles in the normal growth and development of the body. It is a major regulator of Ca and P homeostasis in the body. It is vital for bone development as it affects mineralization, osteoblast and osteoclast formation as well as directly regulates transcription of several genes important for bone development. Its deficiency can cause impaired mineralization and bone softening culminating into bone diseases like rickets in children, and osteomalacia and osteoporosis in adults. Vitamin D has also been associated with the prevention or treatment of other disease conditions in the body like cardiovascular diseases, diabetes mellitus type I and cancer. It also mediates enhanced immunity, paracrine regulation of cell proliferation and differentiation and insulin secretion from beta-pancreatic cells. Vitamin D is either obtained
from the diet or synthesized in the skin with the aid of UV light. The level of sunshine that may lead to optimum vitamin D synthesis is questionable due to the increased risk of skin cancer caused by UV radiation as well as the dependence of rate of synthesis on multiple factors like age of individual, skin pigmentation, extent of clothing and pollution.

**Physiological Synthesis of 1,25(OH)\(_2\)D\(_3\)**

Vitamin D is a fat-soluble steroid hormone that mediates most of its effects on the body through its active form called 1,25-dihydroxyvitaminD\(_3\) (1,25(OH)\(_2\)D\(_3\)). Vitamin D\(_3\) or cholecalciferol is synthesized in the skin by the action of sunlight. It is then taken to liver by the blood stream where it is hydroxylated to 25-dihydroxyvitaminD\(_3\) by 25-hydroxylase (CYP27A). It is further hydroxylated in the kidney to 1,25(OH)\(_2\)D\(_3\) by 1-alpha hydroxylase (CYP27B1), which is then released into circulation to act on several target organs. The 1,25(OH)\(_2\)D\(_3\) can also undergo degradation in the kidney. The 1,25(OH)\(_2\)D\(_3\) gets converted to 24,25(OH)\(_2\)D\(_3\) by 24-hydroxylase (CYP24A1) which is further catabolized to cause complete degradation.

**Physiological Effects of 1,25(OH)\(_2\)D\(_3\)**

The 1,25(OH)\(_2\)D\(_3\) exerts its effects on the classical target organs bone, kidney and small intestine to maintain Ca homeostasis. Circulating levels of 1,25(OH)\(_2\)D\(_3\) are elevated due to Ca deficiency induced secondary hyperparathyroidism. The elevated 1,25(OH)\(_2\)D\(_3\) levels enhance calcium absorption in the duodenum by activating calcium binding protein calbindin-D\(_{9k}\). Calbindin-D\(_{9k}\) mRNA levels are induced in the rat digestive tract within 1
hour of 1,25(OH)_2D_3 administration. (Perret et al., 1985). The 1,25(OH)_2D_3 also stimulates rapid increase in Ca transport within seconds through non-genomic pathway also termed as transcaltachia. This is primarily mediated by activation of voltage-gated Ca channels or activation of protein kinase A or protein kinase C (Nemere et al., 2004). In the kidney, 1,25(OH)_2D_3 increases the re-absorption of Ca back into circulation. This is through 1,25(OH)_2D_3 mediated increase in transcellular Ca\(^{2+}\) transport. Increased Ca transport by the administration of 1,25(OH)_2D_3 is accompanied by substantial increase in calbindin-D\(_{28k}\) cytosolic concentrations (Bindels et al., 1991) suggesting direct induction of calbindin-D\(_{28k}\) by 1,25(OH)_2D_3. A 1,25(OH)_2D_3 response element has also been proposed for the calbindin-D\(_{28k}\) suggesting direct transcriptional control (Minghetti et al., 1989). The effects of 1,25(OH)_2D_3 on bone are at both mineralization and cellular levels. The 1,25(OH)_2D_3 triggers osteoclast activity to increase bone resorption and Ca release. However, it is also directly involved in osteoblast formation and increased longitudinal bone growth. The 1,25(OH)_2D_3 generates biological responses in wide range of organs including bone, kidney and small intestine through genomic and non-genomic pathways (Figure 1.5). Genomic pathways include action through the nuclear vitamin D receptor (VDR\(_{nuc}\)) while non-genomic pathways are mediated via the putative membrane receptor (VDR\(_{mem}\)). The VDR\(_{nuc}\) is a member of the nuclear receptor superfamily and is a ligand-activated transcription factor. The 1,25(OH)_2D_3 bound VDR\(_{nuc}\) associates with other nuclear hormone receptors, most prominent being the retinoid-X receptor (RXR). The VDR/RXR heterodimer binds to the vitamin D response elements (VDREs) present on the promoter region of several genes and facilitates recruitment and activity of co-activators and chromatin remodeling complexes to
initiate transcription (Belandia et al., 2002; Bettoun et al., 2004; Carlberg, 2003). Some of the genes upregulated by genomic pathway are osteocalcin and osteopontin that increase osteoblast formation, calbindin that facilitates intestinal absorption and 24-hydroxylase that causes 1,25(OH)_{2}D₃ degradation (MacDonald et al., 1993; Nagpal et al., 2005). They also decrease PTH and PTH related protein synthesis via VDRE mediated mechanism (Nagpal et al., 2005). The activation of VDR_{mem} by 1,25(OH)_{2}D₃ triggers secondary messengers such as cAMP and Ca that trigger signal transduction pathways to cause several physiological changes such as initiation of Ca and P uptake in the intestinal cells (Nemere et al., 2004), opening of voltage-gated Ca and chloride channels in osteoblasts and skeletal muscle (De Boland and Boland, 1994; Zanello and Norman, 2004a, 2003, 2004b, 1997) and opening of Ca-dependent K channels in kidney (Edelman et al., 1986). The responses by non-genomic pathways can be generated in seconds as compared to genomic pathways that can take hours.
Figure 1.5 Cellular pathways of nuclear and membrane bound vitamin D receptors

Effects of 1,25(OH)2D3 on MSC

Dihydroxy-cholecalciferol (1,25(OH)2D3) is a key hormone involved in bone development and mineral homeostasis; however, the role of 1,25(OH)2D3 in differentiation of MSC has been controversial. Treatment of MSC cultures with 1,25(OH)2D3 has been shown to both induce (Beresford et al., 1992) and inhibit (Atmani et al., 2002; Atmani et al., 2003; Zhang et al., 2006) osteogenic differentiation, as well as induce (Atmani et al., 2003) and inhibit (Kelly and Gimble, 1998; Kong and Li, 2006) adipocytic differentiation in different model systems. Reduced bone mineral density during aging occurs in part due to a shift in the differentiation potential of MSC to favor adipocytic differentiation as opposed to osteogenic differentiation (Cao et al., 2005; Moerman et al., 2004). Altering the MSC phenotype in
favor of osteogenic differentiation could help maximize bone integrity in young growing animals as well as help maintain bone integrity in the aged. Although there have been limited studies with porcine MSC (Bosch et al., 2006b; Ringe et al., 2002b; Vacanti et al., 2005; Zou et al., 2004), the role of 1,25(OH)\textsubscript{2}D\textsubscript{3} in porcine MSC differentiation into adipogenic or osteogenic lineages has not been evaluated.

**PTH**

PTH is a polypeptide consisting of 84 amino acids that is released from the parathyroid gland and acts as an endocrine regulator of Ca and P homeostasis in the body. In the bone, PTH increases resorption by causing Ca release and regulates the activity of both osteoblasts and osteoclasts. Increased PTH levels during Ca deficiency (secondary hyperparathyroidism) leads to excessive bone resorption that contributes to the manifestation of diseases like rickets, osteomalacia and osteoporosis.

**Physiological Effects of PTH**

Primary target organs of PTH are bone, small intestine and kidney. Deficiency of Ca in the body causes secondary hyperparathyroidism resulting in increased PTH secretion into the blood stream. Secretion of PTH is chiefly controlled by serum Ca through a negative feedback mechanism. Low serum Ca levels are sensed by CaR located on parathyroid cells which triggers the phospholipase C pathway through a G-protein coupled mechanism to increase intracellular concentration of calcium, which triggers vesicle fusion and exocytosis of parathyroid hormone. PTH acts on the bone to cause resorption and demineralization. As
bone is degraded, both Ca and P are released into circulation, however slightly more Ca than P is released. PTH also enhances the absorption of calcium in the intestine by increasing the production of active form of vitamin D (1,25(OH)₂D₃) in the kidney. This is mediated through up-regulation of 1-alpha-hydroxylase that converts vitamin D to its active form (1,25(OH)₂D₃). This activated form of vitamin D increases the absorption of calcium by the intestine via calbindin. Apart from vitamin D mediated increased absorption, PTH also directly impacts Ca and P transport across the duodenum. It was found to directly enhance Ca uptake in rat and chick enterocytes as well as Ca transport in the perfused chick duodenal loop (Massheimer et al., 2000; Nemere and Norman, 1986; Picotto et al., 1997). PTH was also shown to enhance the uptake and transport of phosphate in a perfused duodenal loop system (Nemere, 1996). Such effects of PTH maybe mediated by G-protein coupled receptor mediated increase in secondary messengers like cAMP, IP₃ and DAG that open Ca channels, trigger intracellular Ca influxes or alter gene expression (Gentili et al., 2001; Massheimer et al., 2000). The presence of PTH receptor (PTHR) has also been detected in enterocytes further supporting the direct actions of PTH in the small intestine. (Watson et al., 2000). In the kidney, PTH enhances reabsorption of calcium from distal tubules and the thick ascending limb and reduces re-absorption of P from the proximal tubule hence increasing its excretion from the body. PTH has been shown to produce both stimulatory and inhibitory effects on osteoblasts depending on the mode of administration. In other words, PTH affects both bone formation and resorption thus closely regulating the coordinated mechanism of bone remodeling. Intermittent delivery of PTH has been shown to increase bone mass, strength, mineral density, micro-architecture and fracture healing (Compston, 2007; Hock
and Gera, 1992; Neer et al., 2001; Rubin et al., 2002), whereas continuous administration of PTH results on bone resorption and hypercalcemia (Hock and Gera, 1992; Marx, 2000). This is of utmost significance from the perspective of mineral nutrition since elevated levels of PTH during Ca deficiency may reflect continuous exposure as oppose to P deficiency during which the levels are greatly suppressed. The mechanism of differential response of bone to PTH depending on the mode of administration is not fully understood. Continuous administration of PTH induces bone resorption by activating osteoclasts through the RANKL/OPG/RANK regulatory axis. PTH stimulates the expression of RANKL (receptor activator of nuclear factor-κB) and inhibits expression of OPG (osteoprotegerin) in osteoblasts to activate osteoclast formation and activity through RANK receptor (Lee and Lorenzo, 1999; Teitelbaum, 2000). Intermittent PTH treatment, depending on the species utilized, has been shown to increase osteoblast activity (Dobign and Turner, 1995; Meng et al., 1996), decrease osteoblast apoptosis (Jilka et al., 1999); and stimulate proliferation and differentiation of osteoprogenitor cells residing in the bone marrow (Kostenuik et al., 1999; Nishida et al., 1994). Interestingly, increased expression of PTH/PTHrP receptor was found to be associated with enhanced osteogenic and reduced adipogenic differentiation both in vivo and ex vivo (Menuki et al., 2008). Also, daily treatment with PTH increased osteoblast numbers as well as reduced the number of adipocytes in the bone marrow of ovariectomized monkeys (Sato et al., 2004). PTH mediates its effects at cellular level by activating intracellular signal transduction pathways primarily via two secondary messengers: cAMP and phospholipase (PLC) (Abou-Samra et al., 1992). cAMP and phospholipase activate transcription factors CREB and AP-1 respectively to regulate transcription of PTH target
genes. PTH regulates the expression of diverse proteins including growth factors like TGFβ, IGF-1 and BMPs (Canalis et al., 1989; Raisz, 1988); transcription factors like Runx2 (Krishnan et al., 2003); and regulatory molecules like alkaline phosphatase (ALP) (Majeska and Rodan, 1982), osteocalcin (Noda, 1989; Termine et al., 1981), OPG (Lee and Lorenzo, 1999) and Col1A1 (Partridge et al., 1989).

**Effects of PTH on MSC**

Because osteoblasts are derived from MSC, one of the ways PTH could modulate bone development is by affecting the activity of the MSC pool in the bone marrow. Both in vitro (Beresford et al., 1992; Jaiswal et al., 2000; Spinella-Jaegle et al., 2001) and in vivo (Akune et al., 2004; Sabatakos et al., 2000; Wronski et al., 1981) studies suggest that there is an inverse relationship between osteogenic and adipogenic differentiation of MSC and that the bone loss seen during osteoporosis and aging is attributed in part to increased adipogenesis at the expense of reduced osteogenesis (Burkhardt et al., 1987; Meunier et al., 1971; Moerman et al., 2004). Therefore, it is tempting to hypothesize that PTH can alter bone development by altering MSC activity. Little work has been done to demonstrate the effects of PTH on MSC populations. Although inhibition of adipogenesis in MSC with intermittent PTH treatment has been demonstrated (Rickard et al., 2006), effects were examined in the presence of adipogenic induction media only at extremely high concentrations of PTH compared to the physiologically levels. Specifically, no work has looked into the effects PTH on porcine MSC activity. Because pig is an excellent model system for studying the impact of nutrition
on bone health, further research to examine the effects of PTH on porcine MSC populations is warranted.

In conclusion, Ca and P deficiencies tremendously impact the endocrine profile (PTH and 1,25(OH)2D3) and these endocrine hormones have been shown to have a profound impact on MSC activity. Because the activity of MSC is of critical importance to bone growth and development, and the controls over MSC lineage allocation and activity are not well understood, it is important to understand the impact of mineral nutrition in MSC activity in relation to bone growth.

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CHAPTER 2

Title: Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells.*

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*This work was supported in part by the Biotechnology Research and Development Corporation (Peoria, IL) and the North Carolina Agricultural Research Service.

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Key words: Mesenchymal stem cells, dihydroxy-cholecalciferol, pig, adipogenesis, osteogenesis
ABSTRACT

Dihydroxy-cholecalciferol (1,25(OH)₂D₃) has been shown to have pleiotropic effects on the differentiation of mesenchymal stem cells (MSC) based on species and culture conditions. We have examined the effects of 1,25(OH)₂D₃ on the differentiation of porcine MSC under culture conditions designed to promote proliferation in order to attempt to mimic the conditions in young, rapidly growing animals. The MSC were isolated from bone marrow of a young pig and grown in basal media (BM) containing DMEM + 10% fetal bovine serum and antibiotics. Cells received either BM, BM + 10⁻⁸ M 1,25(OH)₂D₃, or BM + 10⁻⁷ M 1,25(OH)₂D₃ with complete media changes every 3d for a total of 12d of culture. On days 3, 6, 9, and 12 viable cell numbers were determined, and samples were collected for gene expression analysis and cytochemical staining. There was a treatment-based reduction in cell numbers on 6d, 9d and 12d (P < 0.05). The concentrations of mRNAs encoding peroxisome proliferator-activated receptor gamma (PPARγ), lipoprotein lipase (LPL), and adipocyte-binding protein 2 (AP2) were increased (P < 0.05) in a manner indicative of adipocytic differentiation by treatment with 1,25(OH)₂D₃ in a dose-dependent manner. However, the mRNA levels of osteocalcin (OC), a late stage marker of osteoblastic differentiation, was also increased (P < 0.05) by treatment with 1,25(OH)₂D₃. An increased percentage of lipid filling, based on Oil Red O staining, and decreased alkaline phosphatase activity (ALP), was also seen with 1,25(OH)₂D₃ treatment. These data suggest that 1,25-(OH)₂D₃ stimulates the differentiation of porcine MSC towards an adipocytic phenotype.

Key words: Mesenchymal stem cells, cholecalciferol, pig, adipogenesis, osteogenesis
Introduction

Mesenchymal stem cells (MSC) are known to be pluripotent cells that have the capacity to self renew and differentiate into distinct mesenchymal lineages. Differentiation of MSCs into osteoblasts, adipocytes and chondrocytes has been well established [1-9]. Studies have also demonstrated that MSC can give rise to myoblasts and even early progenitors of neural cells [10,11]. Similarities between the mode of differentiation into various mesenchymal lineages in vitro between porcine and human derived MSC [4,5,12,13], as well as the physiological similarities between pigs and humans [14-16] suggest that pigs can serve as an important animal model system to study the impact of nutrition on bone health as well as for MSC-based tissue engineering studies.

Dihydroxy-cholecalciferol (1,25(OH)2D3) is a key hormone involved in bone development and mineral homeostasis; however, the role of 1,25(OH)2D3 in differentiation of MSC has been controversial. Treatment of MSC cultures with 1,25(OH)2D3 has been shown to both induce [17] and inhibit [18-20] osteogenic differentiation, as well as induce [19] and inhibit [21,22] adipocytic differentiation in different model systems. Reduced bone mineral density during aging occurs in part due to a shift in the differentiation potential of MSC to favor adipocytic differentiation as opposed to osteogenic differentiation [23,24]. Altering the MSC phenotype in favor of osteogenic differentiation could help maximize bone integrity in young growing animals as well as help maintain bone integrity in the aged. Although there have been limited studies with porcine MSC (Bosch et al., 2006b; Ringe et al., 2002b; Vacanti et al., 2005; Zou et al., 2004), the role of 1,25(OH)2D3 in porcine MSC
differentiation into adipogenic and osteogenic lineages is unclear. In the present study, we examined the effect of 1,25(OH)\textsubscript{2}D\textsubscript{3} on in vitro porcine MSC differentiation at concentrations similar to the circulating levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} in the plasma of young pigs and in young pigs fed a mineral deficient diet [25]. We utilized both gene expression analysis and cytochemical staining to make distinctions between osteoblastic and adipocytic lineages. Our data is supportive of 1,25(OH)\textsubscript{2}D\textsubscript{3} stimulating adipocytic differentiation in porcine mesenchymal stem cells.

Materials and Methods

Mesenchymal Stem Cell Isolation and Culture

All animal protocols were approved by Iowa State University’s Institutional Animal Care and Use Committee. Bone marrow from the humerus and radial bones of an 18d old cross-bred pig was harvested under aseptic conditions and dispersed by passage through a 20-gauge needle with DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with antibiotics (100 U penicillin G, 100 μg streptomycin, and 0.25 μg amphotericin B/mL) (Sigma-Aldrich, St. Louis, MO). The dispersed marrow was then washed by repeated centrifugation at 900 × g for 5 min followed by discarding of the supernatant and re-suspension in DMEM. This step was repeated three times. After washing, cells were re-suspended in basal media (BM) consisting of DMEM + 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) + antibiotics. Cell viability and number were determined by trypan blue staining. Erythrocytes were lysed prior to cell counting by the addition of 4% acetic acid. Cells were then plated at a density of 5 × 10\textsuperscript{4} nucleated cells/cm\textsuperscript{2} in T-75 culture
flasks (Corning, Corning, NY) in BM. The flasks were incubated at 37°C in a humidified environment containing 5% CO₂. Hematopoietic cells and all other non-adherent cells were removed with complete media changes with vigorous washing every 3d. When cells reached approximately 80% confluency, they were harvested using 0.25% Trypsin in 1mM EDTA, counted and plated in BM at 10³ cells/cm² for study. The cells were allowed to attach to the plates for 2d prior to receiving the treatment media containing 1,25(OH)₂D₃ (Sigma-Aldrich, St. Louis, MO). Cells received either BM, BM + 10⁻⁸ M 1,25(OH)₂D₃, or BM + 10⁻⁷ M 1,25(OH)₂D₃. The 1,25(OH)₂D₃ was provided in 95% ethanol, and equal amounts of 95% ethanol (0.1% volume) were added to all treatments. Media was completely changed every 3d for a total of 12d of culture.

**Cell Proliferation**

Cell proliferation was measured using the MTS based CellTiter 96® AQ™ueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions. This assay employs a colorimetric method for determining the number of viable cells in culture. Cells cultivated in 24-well plates were utilized for this assay and cell numbers were assessed by adding 100 µl MTS reagent to 500 µl media in each well, followed by incubation at 37°C for 2 h. Absorbance was then read at 490 nm to quantitate the colored formazan product produced by active cells, and absolute cell numbers were determined using a standard curve.
Cytochemical Staining

Cells plated in 6-well plates were fixed and stained in duplicate for alkaline phosphatase activity (ALP) and the presence of neutral lipids using Oil Red O on day 3, 6, 9 and 12 of culture. Staining for ALP was accomplished with a commercially available kit (ALP staining kit, Takara Bio Inc., Otsu, Shiga, Japan) according to manufacturer’s instructions. Briefly, cells were fixed by incubating with fixation solution (Citrate buffer pH 5.4 containing 60% acetone and 10% methanol) for 10 min followed by washes in PBS and staining in premixed Bromo-Chloro-Indolyl phosphate/ Nitro Blue Tetrazolium chloride substrate for 40 min at 37°C. Plates were then thoroughly washed with water and observed under the microscope. Accumulation of neutral lipids was visualized by fixing the cells in 10% formalin and then staining with 0.2% Oil Red O for 1 hr followed by thorough rinsing with water.

Gene expression analysis

Total RNA was extracted from samples on days 3, 6, 9 and 12 of culture using the Ambion RNAqueous kit (Ambion, Austin, TX) followed by treatment with deoxyribonuclease I (Ambion DNA-free kit) to eliminate genomic DNA. Purified RNA was reverse transcribed with Superscript Reverse transcriptase III (Invitrogen Life Technologies) using both oligodT (Integrated DNA Technologies, Coralville, IA) and random hexamer primers (Invitrogen Life Technologies) in a 1:1 ratio. The resulting cDNA was then incubated for 20 min at 37°C with RNase H (Invitrogen Life Technologies) to remove RNA. Both starting RNA and final cDNA concentrations were quantified using a fluorescence-
based quantification kit (Invitrogen Life Technologies) and only cDNA samples generated by reactions with greater than 90% efficiency were utilized.

Semi-quantitative real-time PCR was performed using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer oligonucleotides were designed using PrimerQuest software available from Integrated DNA Technologies. The forward and reverse primers for a portion of the 18S ribosomal subunit were: TTAGAGTGTTCAAAAGGCGGCGA and TCTTGGCAATGCTTTCGCTCTGG; for peroxisome proliferator-activated receptor gamma (PPARγ): AATTAGATGACAGCGACCTGGCGA and TGTCTTGAAATGTCCTCGATGGCTG; for lipoprotein lipase (LPL): ACCGGTGCAACAACTTGCTATG and ACTTTGTAAGGCGAATCCTGACGA; for adipocyte-binding protein 2 (AP2): GGCACACACAGCAACCTGA and GGGCGGTCCTCCTCTCAAG; for Runx related transcription factor 2 (Runx2): CAAGTGCGGTGAAGACTTTCTCCA and AGGCTGTTCATGATGGCCATAGTCCCT; for osteocalcin (OC): TACCCAGATCCTGGAGCCC and TATGCCATAGAAGCCCG; and for retinoid X receptor alpha (RXRα): TTCACCAAGCACATCTGTGCCATC and TGTCGATCGAGCTCTTGTTGT respectively. Optimal primer concentrations for each primer set were determined prior to quantification by real-time PCR, and the linearity of amplification for each gene of interest over a 2 log range of cDNA concentrations was verified to be similar to that of the control gene, 18S ribosomal RNA. The control gene was verified to be unaffected by 1,25(OH)D3 treatment. Reactions were performed using 12.5 µl of 2x SYBR Green Supermix (Bio-Rad), 200–900 nM of each primer and 25 ng of cDNA in
a final volume of 25μl. All samples were subjected to 40 amplification cycles (1 cycle consisted of 30 s of melting at 95°C followed by 30 s of annealing and extension at 65°C). At the completion of the amplification cycles, all samples were subjected to a melt curve analysis to validate the absence of nonspecific products. The 2-ΔCT method of Livak and Schmittgen (2001) was employed to normalize gene expression values prior to statistical analysis [26].

Statistical analysis

All treatments had 6 independent replicates (except for the treatment groups on day 12 where n = 5) for gene expression and 3 independent replicates for cell proliferation experiments. A randomized complete block design was employed and data was analyzed using the GLM procedure of SAS® (Version 9.1, SAS Institute Inc., Cary, NC) with 1,25(OH)2D3 treatment, length of culture and the interaction of the two considered as fixed effects. Differences with P < 0.05 were considered to be statistically significant.

Results

Cell Proliferation:

Cell numbers increased linearly up to 6d regardless of treatment followed by a significant reduction in cell proliferation between day 6 and day 9 (Table 1). A statistically significant reduction in cell numbers (15% less than BM control) was seen with 10^{-7} M 1,25(OH)2D3 by 6d of culture (P < 0.05). On day 12, both 10^{-7} M 1,25(OH)2D3 and 10^{-8} M
1,25(OH)\textsubscript{2}D\textsubscript{3} treatment groups had significantly lower (8 and 10%, respectively) cell numbers (P < 0.03 and P < 0.01, respectively) compared to the BM control.

**Cytochemical Staining:**

The appearance of rounded, lipid-filled adipocyte-like cells amongst the spindle shaped MSC was seen by day 3 in all treatment groups. However, the proportion of lipid-filled cells was considerably greater in the 1,25(OH)\textsubscript{2}D\textsubscript{3} treated cultures as compared to the control (Fig. 1). This higher proportion of Oil Red O stained cells with 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment was also seen at day 6, 9 and 12. In the 1,25(OH)\textsubscript{2}D\textsubscript{3} treated cultures, a pronounced change in the morphology of these lipid-filled cells was seen over the course of the study. These cells increased in size and had lipid droplets, which occupied a larger proportion of the cytoplasm. The morphological and cytochemical differences seen with 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment are suggestive of an increase in adipocytic differentiation of these cells.

Alkaline phosphatase (ALP) staining of the cultured cells was carried out to identify osteoblast-like cells (Fig 1). The proportion of cells staining positive for ALP activity decreased in 1,25(OH)\textsubscript{2}D\textsubscript{3} treated cultures on all days. There appeared to be a greater reduction in the proportion of cells with ALP activity with the higher 1,25(OH)\textsubscript{2}D\textsubscript{3} dosage.

**Gene Expression**

Treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} caused significantly higher (P < 0.001) levels of PPAR\textsubscript{g} message in a dose-dependent fashion (Fig. 2). The 10\textsuperscript{-7} M 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment
group had approximately 4-fold higher (P < 0.001) concentrations of PPARγ mRNA than the control group at day 3 which increased to a 5-fold increase (P < 0.001) by day 12. There was no significant increase in the concentration of PPARγ mRNA in the control cells over the course of the study. Increased expression levels of LPL, another early marker of adipocytic differentiation, was also seen with 1,25(OH)2D3 treatment in a dose-dependent manner (P < 0.001). The levels of LPL mRNA increased over the course of the study (P < 0.001) and there was approximately 10, 14, and 16-fold more LPL message (P < 0.001) in the cells treated with 10−7 M 1,25(OH)2D3 compared to controls on days 6, 9 and 12, respectively (Fig 2). The levels of LPL message in the 10−7 M 1,25(OH)2D3 treatment group on day 12 of culture were roughly 15-fold higher (P < 0.001) than the levels seen in this treatment group after just 3 days of culture. The expression of mRNA coding for AP2, a late marker for adipocytic differentiation, followed a pattern very similar to LPL. There was a 2, 4 and 6-fold increase (P < 0.05) in AP2 gene expression levels for 10−7 M 1,25(OH)2D3 treated cells compared to controls at day 6, 9 and 12 respectively (Fig 2). The expression pattern of PPARγ, LPL and AP2 in response to treatment with 1,25(OH)2D3 is suggestive of an induction of adipocytic differentiation.

The mRNA levels of Runt related transcription factor 2 (Runx2), an osteoblast-specific transcription factor, significantly rose (P < 0.001), regardless of treatment, from day 3 to day 6 (approximately 3-fold), and then remained constant (Fig 3). Overall, there was a significant treatment based reduction (P < 0.005) in Runx2 expression. Expression of Runx2 significantly decreased (P < 0.05) with 10−8 M 1,25(OH)2D3 when compared with the control group on day 12, however there were no significant treatment-based differences on day 3, 6
and 9. We also measured the levels of osteocalcin (OC), a late differentiation marker for mature osteoblasts. Surprisingly, OC gene expression increased over time and in a dose-dependent manner (P < 0.001) with 1,25(OH)_{2}D_{3} treatment (Fig 3). The OC gene expression levels in control cultures did not change significantly over the course of the study, but in cultures treated with 1,25(OH)_{2}D_{3}, it increased significantly over time and with respect to the control cultures. The response peaked on day 12 where there was a greater than 10-fold increase (P < 0.001) in expression, compared with the controls in 10^{-8} M 1,25(OH)_{2}D_{3} treated cultures and a 20-fold increase (P < 0.001) in 10^{-7} M 1,25(OH)_{2}D_{3} treated cultures.

Expression of Retinoid X Receptor (RXR) significantly changed over time (P < 0.001) with an increase in expression from day 3 to day 6 followed by a reduction in expression from day 6 to day 12. Overall, there was a 1,25(OH)_{2}D_{3} treatment-based increase (P < 0.02) in the expression of Retinoid X Receptor (RXR), however there were no significant differences on individual days (Fig 3).

**Discussion**

Dihydroxy-cholecalciferol (1,25(OH)_{2}D_{3}) is an established regulator of mesenchymal stem cell differentiation; however, the role of 1,25(OH)_{2}D_{3} in adipogenic differentiation, one of the possible fates of MSC, has been controversial. Although the process is being studied in murine cultures, little work has focused on the role of the 1,25(OH)_{2}D_{3} in porcine MSC differentiation [4,12,13]. In pre-adipocytic 3T3-L1 murine cells, 1,25(OH)_{2}D_{3} was shown to both stimulate [27] and inhibit [22] adipocytic differentiation. In fetal rat calvaria cells, adipocytic differentiation was shown to be stimulated by 1,25(OH)_{2}D_{3} (Bellows and
Heersche, 2001; Bellows et al., 1994b), while it was inhibited in murine bone marrow stromal cells [22] and rat bone marrow stromal cells [17]. The cytochemical staining and pattern of gene expression seen in this study supports that treatment with 1,25(OH)₂D₃ stimulates adipocytic differentiation in MSC isolated from porcine bone marrow.

Treatment with 1,25(OH)₂D₃ caused a dose-dependent decrease in cell proliferation indicating a shift toward cell differentiation. In this study, 1,25(OH)₂D₃ treatment increased both the number of cells which stained positive for lipid droplets as well as the amount of staining per cell in a dose-dependent fashion. Although cells treated with 1,25(OH)₂D₃ contained lipid filled droplets after 3d of treatment, these cells retained the characteristic spindle-shape of MSC. While the control cultures also had some cells containing Oil Red O stained lipid droplets after 3d of treatment, there were considerably fewer as compared to the treated cultures. Morphological differences in cells treated with 1,25(OH)₂D₃ were prominent after 6d of culture, when the smaller lipid filled droplets seen after 3d appeared to coalesce into larger droplets which occupied a majority of the cytoplasm. The cells in the control cultures retained their spindle-shape over the length of the study and a lower percentage of these cells contained stained lipid droplets on all days. Lipid filling and related morphological changes have been seen previously with 1,25(OH)₂D₃ treatment of rodent cells [19], however, in our porcine-derived MSC these changes occurred in a more rapid fashion. Among the 1,25(OH)₂D₃ cells, there was also reduced alkaline phosphatase activity detected by staining. This is indicative of a reduction in the population of MSC that would be capable of undergoing osteoblastic differentiation, which is supportive of our conclusion that 1,25(OH)₂D₃ stimulated adipocytic differentiation in this study.
In addition to our cytochemical staining results, gene expression analysis further supports the role of 1,25(OH)_{2}D_{3} in stimulating adipocytic differentiation of porcine MSC. The commitment of MSC to the adipocytic lineage is known to be determined by the expression of a PPARγ, a nuclear hormone receptor that mediates a cascade of signal transduction events that stimulates the expression of early and late adipogenic markers [30-32]. In this study, a dose-dependent increase in the mRNA levels of PPARγ was seen in response to 1,25(OH)_{2}D_{3} by 3d of treatment. While this is supportive of the initiation of adipocytic differentiation, it potentially conflicts with that of Hida et al. [33] which showed an inhibitory effect of 1,25(OH)_{2}D_{3} on PPARγ protein levels in 3T3-L1 cells. This differential response may be due to cell type or species specific differences, but regardless of its cause it highlights the importance of using appropriate animal models if extrapolations to humans are to be made. In addition to the increase in the PPARγ message, the expression of both LPL and AP2 mRNA also increased with 1,25(OH)_{2}D_{3} treatment. An increase in the expression of LPL, an early marker of adipocytic differentiation, in 3T3-L1 cells with 1,25(OH)_{2}D_{3} treatment has been previously shown [27]. The mRNA levels of AP2, a lipid binding protein expressed in the late stages of adipocytic differentiation [34], was also increased in our porcine MSC with 1,25(OH)_{2}D_{3} treatment and we saw a significant increase in the expression of PPARγ by 1,25(OH)_{2}D_{3} treated MSC prior to an increase in AP2 message, we examined the expression of RXRα in these cells. We found that the expression of RXRα was not affected by 1,25(OH)_{2}D_{3} in this study, suggesting that the expression of RXRα is not a critical control point for the stimulation of AP2 gene expression.
Because adipocytic differentiation is thought to be inversely related to osteogenic differentiation in MSC [17], we also examined the expression of an osteogenic transcription factor, Runx2, and an osteoblast marker, osteocalcin. We saw an overall decrease in expression of Runx2 with 1,25(OH)_{2}D_{3} treatment, which would be supportive of 1,25(OH)_{2}D_{3} inhibiting osteoblastic differentiation. However, unlike the genes examined for adipogenesis, dose-dependent response on individual days was lacking, thus making the data inconclusive. Also, the expression of OC mRNA significantly increased with 1,25(OH)_{2}D_{3} in a dose-dependent manner. The OC gene is known to be differentially regulated by 1,25(OH)_{2}D_{3} in different species. In rats and humans, OC mRNA levels are highly stimulated by 1,25(OH)_{2}D_{3} [35-37] (Beresford et al., 1984; Owen et al., 1991; Yoon et al., 1988). However, the regulation of the OC gene by 1,25(OH)_{2}D_{3} is different in mouse where 1,25(OH)_{2}D_{3} appears to down regulate the expression of OC [38,39]. Our data shows that in porcine MSC, OC gene expression is upregulated by 1,25(OH)_{2}D_{3} suggesting similar gene regulation as seen in rats and humans. We also measured the expression of RXRa since it is known to play a defined role in the transcription of both osteogenic and adipogenic genes. RXRa is known to dimerize with Vitamin D receptor to cause increased expression of OC [40]. Also, increased expression of the AP2 gene has been shown to require the binding of a heterodimeric complex formed by PPARγ and RXRa [41]. Although there was an overall treatment based increase in the expression of RXRa, it is difficult to derive any conclusions based on gene expression data due to lack of consistent dose-dependent response with 1,25(OH)_{2}D_{3} treatment on individual days. The role of 1,25(OH)_{2}D_{3} in osteoblastic differentiation of porcine MSC remains unclear. While the ALP staining results in the
current study strongly suggest a decrease in the percentage of cells that could take on an osteoblastic phenotype with 1,25(OH)₂D₃ treatment, it may be possible that with treatment, osteoblastic differentiation is triggered in a small osteoblastic progenitor population of the MSC which are responsible for the increase in OC gene expression seen in this study. Another possibility is that the hormone may be causing increased OC gene expression without any increase in protein expression thus preventing them from terminally differentiating into osteoblasts. This, however, is not in agreement with literature that suggests that OC is expressed only in mature osteoblasts [42,43]. The active metabolite 1,25(OH)₂D₃ can exert its function through the nuclear VDR to either upregulate or downregulate gene expression. On the other side, 1,25(OH)₂D₃ has also been shown to provoke biological effects in cells through non-genomic signaling by increasing intracellular Ca²⁺[44,45,46]. Calcium has been shown to exert a bi-phasic regulatory role in human adipocytic differentiation [47] and also been shown to regulate adipogenesis in murine cells [48]. Although we suggest the signaling of 1,25(OH)₂D₃ through the nuclear VDR to cause an increase in adipogenesis by altered gene expression, 1,25(OH)₂D₃ may also act through the calcium signaling pathway to regulate adipogenesis. Further studies to examine this effect of 1,25(OH)₂D₃ in porcine MSC is warranted.

The antagonistic relationship between adipocytic and osteogenic differentiation from MSC suggests that increasing adipocytic differentiation would have negative consequences for bone health [17,49-54]. The bone loss seen during aging and the resulting disease, osteoporosis, has been attributed in part to an increase in adipocytic and a concomitant decrease in osteoblastic differentiation of the MSC in bone marrow [55]. Elucidating the role
of altered MSC differentiation on bone integrity is difficult since bone integrity is effected by genetics, nutrition, and their interaction [56-59]. Interestingly, 1,25(OH)₂D₃ levels and its signaling are also effected by genetics, nutrition and their interaction [60-61].

Our study has demonstrated an increase in adipocytic differentiation for porcine MSC with treatment with 1,25(OH)₂D₃. However, the effect of 1,25(OH)₂D₃ on osteogenic differentiation was not clear. Elucidation of the role of 1,25(OH)₂D₃ in the differentiation of MSC could lead to novel interventions to maximize bone integrity and for the treatment of bone wasting diseases.

**Literature Cited**


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Table 1. Effect of 1,25(OH)₂D₃ on cell proliferation.¹

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 3 (×10⁴)</th>
<th>Day 6 (×10⁴)</th>
<th>Day 9 (×10⁴)</th>
<th>Day 12 (×10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.9 ± 1.4</td>
<td>26.5 ± 2.8ᵃ</td>
<td>36.6 ± 3.2ᵃ</td>
<td>45.4 ± 2.1ᵃ</td>
</tr>
<tr>
<td>10⁻⁸M</td>
<td>8.5 ± 0.9</td>
<td>25.2 ± 8.8ᵃ</td>
<td>36.0 ± 4.1ᵃ</td>
<td>41.7 ± 1.6ᵇ</td>
</tr>
<tr>
<td>10⁻⁷M</td>
<td>9.0 ± 0.7</td>
<td>22.6 ± 1.8ᵇ</td>
<td>32.7 ± 1.7ᵇ</td>
<td>40.8 ± 2.1ᵇ</td>
</tr>
</tbody>
</table>

¹ All cells were plated at an initial density of 1×10³ cells/cm² in 24 well plates (2,000 cells/well) and allowed 2d of growth in BM prior to receiving treatments. Cell number was determined using a standard curve of freshly counted MSC utilizing a MTS-based assay and are presented as means ± standard deviation. ² The treatment groups are control (ethanol as carrier), 10⁻⁸M 1,25(OH)₂D₃ and 10⁻⁷M 1,25(OH)₂D₃ in basal media.ᵃᵇ Values within a Day not sharing a common superscript are significantly different (P < 0.05).
Figure 1. Cytochemical staining of porcine mesenchymal stem cells treated with 1,25(OH)₂D₃. Alkaline phosphatase and Oil red O staining of Control, 10⁻⁸M 1,25(OH)₂D₃ (1X Vit D) and 10⁻⁷M 1,25(OH)₂D₃ (10X Vit D) treated cells. Oil red O staining pictures are from day 3, 6, 9 and 12. Pictures for relative ALP staining differences between control and treatment groups are representative for all days.
Figure 2. Effect of 1,25(OH)₂D₃ on adipocyte-related gene expression during MSC differentiation. MSC were cultured in the presence of (i) control; (ii) 10⁻⁸M and (iii) 10⁻⁷M 1,25(OH)₂D₃ for 12 days. Expression of PPARγ, LPL and AP2 mRNA as determined by real-time PCR. P < 0.0001 for treatment, days of culture and treatment × days of culture. Within a day, columns not sharing a common letter are significantly different (P < 0.05).
Fig 3. Effect of 1,25(OH)\(_2\)D\(_3\) on osteoblast-related gene expression during MSC differentiation. 1,25(OH)\(_2\)D\(_3\) Expression of Runx2, (p < 0.0001 for treatment; p < 0.006 for days of culture), OC (p < 0.0001 for treatment, days of culture and treatment × days of culture) and RXRα (p < 0.0001 for treatment; p < 0.02 for days of culture and p < 0.002 for treatment × days of culture) mRNA as determined by real-time PCR. Within a day, columns not sharing a common letter are significantly different (P < 0.05).
CHAPTER 3

Dietary phosphorus impacts mesenchymal stem cell proliferation and gene expression in the bone marrow.
ABSTRACT

Although dietary P deficiency reduces skeletal growth and MSC are essential for bone development, the effect of P on early cell programming has not been examined. Neonatal pigs (24 ± 6h post-partum) received either a P adequate diet or a diet with 25% less $P_{\text{available}}$ over a 15 d trial. Classical P deficiency profile was seen in the form of reduced body growth ($P < 0.01$), feed efficiency ($P < 0.05$) and bone mineral content ($P < 0.05$). Also, as expected, plasma concentrations of both P ($P < 0.01$) and PTH ($P < 0.05$) were significantly reduced in P deficient animals when compared to their P adequate counterparts with no significant differences in 1,25(OH)$_2$D$_3$ plasma levels. In addition, P deficiency also induced a greater than 50% reduction in MSC proliferation ($P < 0.01$) in vivo. The mRNA expression levels of PPARγ and LPL were significantly elevated in the bone marrow of P deficient animals while osteocalcin mRNA levels were 3-fold greater ($P < 0.01$) in the P adequate fed animals. The expression of 1-alpha-hydroxylase was also elevated in the bone marrow of P deficient animals compared to P adequate animals. The results indicate that early life restriction in dietary P impacts in vivo proliferation of MSC and suggests the possible role of altered MSC activity in inducing the bone integrity changes seen with P deficiency in this study.
INTRODUCTION

Dietary P deficiency has been shown to tremendously impact bone health and growth performance. Deficiency of P in the diet causes reduced growth performance, bone mineral content and bone growth in older pigs (Alexander et al., 2008; Jendza et al., 2005; Veum et al., 2001). However, little research has been done to examine the effects of this mineral during the neonatal period of growth. Reduced bone mineralization and higher incidence of broken bones due to P deficiency have been seen during the early growth period, and bone integrity at maturity has been shown to be more dependent on the P status of the animals in early life (Driver et al., 2006). P deficiency also has been shown to cause inadequate mineral distribution in the tibia and vertebra in neonatal miniature piglets (Schanler et al., 1991). Since early life P restriction impacts bone growth potential, one of the mechanisms of reduced bone growth could be alteration of MSC differentiation potential. There have been no studies evaluating the impact of dietary P on the potential for nutritional programming of these tissue specific stem cells. Hormones known to regulate P homeostasis like PTH and 1,25(OH)_2D_3 also have been shown to alter MSC differentiation potential (Atmani et al., 2003; Kelly and Gimble, 1998; Rickard et al., 2006). Therefore, further studies to examine the effects of P on MSC activity are warranted.
MATERIALS AND METHODS

*Animals*

All animal protocols were approved by North Carolina State University’s Institutional Animal Care and Use Committee. Twenty, 1 day old piglets (28± 4 h post-partum, both male and female) were weighed and allotted based on body weight (BW) and sex into 1 of 2 dietary treatment groups: 1) Nutritionally adequate diet, and 2) 25% P deficient diet. Pigs were pair-fed a soy-based liquid milk replacer over a 15 d trial period (Table 3.1). Diets were formulated based on sow milk composition (Klobasa et al., 1987) and a soy-based milk replacer was utilized to achieve desired P deficiency due to the low bioavailability of P from soy protein sources. Pigs were housed individually in raised cages and provided milk replacer 3 times per day using a gravity-flow delivery system (Mathews et al., 2002). Body weight and feed intake were recorded daily throughout the trial. Blood samples were collected initially and every 5 days by venipuncture using heparinized tubes (Vacutainer Plus, BD Vacutainer, Franklin Lakes, NJ), and plasma was obtained by centrifugation at 3,500 x g and 4°C. Plasma samples were stored at –20°C until analysis. At the completion of the study, all pigs were given 25 mg of BrdU/kg BW by IP injection 4 h prior to euthanasia to allow for adequate incorporation of BrdU in proliferating MSC nuclei. Bone marrow was harvested from each animal and snap-frozen in liquid nitrogen for subsequent gene expression analysis. Front legs were collected for isolation of bone marrow derived MSC. Radial bones with attached ulnae were collected and stored at 4°C for determination of mineral content.
Mineral content of the radii was determined by drying at 60°C for 24 h followed by ashing at 600°C for 24 h.

**Plasma Analysis**

Plasma Ca concentrations were determined by flame absorption spectroscopy (Shimadzu AA6701F, Atomic Absorptiometry Flame Emission Spectrophotometer) following dilution in 0.5% lanthium chloride. Inorganic P concentrations were determined with a microplate spectrophotometer (Biotek Synergy HT, KC4 Software) based on modification of a previous method (Gomori, 1942). Briefly, plasma was deproteinated with 12.5% trichloroacetic acid and assayed using Elon solution (p-methylaminophenol sulfate). The concentrations of 1,25(OH)₂D₃ and PTH were determined by utilizing commercially available kits (Porcine Intact PTH ELISA kit Immutopics, San Clemente, CA; and 1,25(OH)₂D₃ EIA kit, IDS, Fountain Hills, AZ).

**MSC Isolation**

MSC were isolated as described previously (Mahajan and Stahl, 2009). Briefly, bone marrow was harvested from the humerus of each pig under aseptic conditions and dispersed by successive passage through 20 and 18-gauge needles and suspended in DMEM (Invitrogen Life Technologies) supplemented with antibiotics (100 U penicillin G, 100 μg streptomycin, and 0.25 μg amphotericin B per mL) (Sigma-Aldrich, St. Louis, MO). The dispersed marrow was then washed three times by repeated centrifugation at 900 × g for 5 min followed by re-suspension in DMEM. After washing, cells were re-suspended in basal media (BM) consisting of DMEM + 10% heat inactivated fetal bovine serum (Invitrogen Life
Technologies) + antibiotics. Cell viability and number were determined by trypan blue staining after lysing erythrocytes by the addition of 4% acetic acid. Cells were plated at a density of $5 \times 10^4$ nucleated cells/cm$^2$ in T-75 culture flasks (Corning, Corning, NY) in BM. The flasks were incubated at 37°C in a humidified environment containing 5% CO$_2$. Hematopoietic cells and all other non-adherent cells were removed with complete media changes with vigorous washing every 24 hours. After three days, MSC were harvested using 0.25% Trypsin in 1mM EDTA, counted and plated in BM at $10^3$ cells/cm$^2$ in 24 well plates and incubated for 48 hours prior to fixation for immunocytochemical staining for BrdU.

**Determination of in vivo cell proliferation**

Cells for immunocytochemical staining were fixed in 0.6% hydrogen peroxide (H$_2$O$_2$) in methanol at 4°C for 10 min. After fixation, cells were washed 3 times with PBS at room temperature. DNA was then denatured by incubation in pre-warmed (37°C) HCl for 1 h. The acid was neutralized by 2 washes in 1X TBE (pH 8.5) followed by 3 washes in 1X PBS. The cells were incubated with anti-BrdU (1:100 in 0.1% BSA in 1X PBS; G$_3$G$_4$, Developmental Studies Hybridoma Bank, University of Iowa) primary antibody in a humidified chamber at 4°C overnight. The cells were then washed with PBS 3 times followed by incubation with goat anti-mouse HRP conjugated secondary antibody (1:500 in 1% BSA in PBS; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. After 3 washes with PBS, wells were covered with 0.5 mg/ml of diaminobenzidine (DAB) and 0.02% H$_2$O$_2$ in PBS for approximately 2 minutes for achieving the desired color intensity. The in vivo rate of cell proliferation was determined by calculating the ratio of stained to
unstained cells. A total of 5 fields of view (> 200 cells total) from each of 2 replicate wells per animal were counted and averaged per animal.

**Analysis of Gene Expression**

Total RNA was isolated from bone marrow (RNeasy Midi Kits, Qiagen, Valencia, CA) and genomic DNA was removed by treatment with deoxyribonuclease according to manufacturer’s instructions (DNA-free kit, Ambion, Austin, TX). Total RNA was reverse-transcribed with Superscript Reverse transcriptase III (Invitrogen Life Technologies, Carlsbad, CA) using both oligo dT (Integrated DNA Technologies, Coralville, IA, USA) and random hexamer primers (Invitrogen Life Technologies) in a 1:1 ratio. The resulting cDNA was treated with RNase H (Invitrogen Life Technologies) to remove residual RNA. Both starting RNA and final cDNA concentrations were quantified using fluorescence-based quantification kits (Invitrogen Life Technologies). Relative quantities of transcripts of interest were determined by semi-quantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System, BioRad). Primer oligonucleotides for PPARγ (peroxisome proliferators activated receptor gamma 2), Runx2 (Runt related transcription factor), LPL (lipoprotein lipase), osteocalcin (OC), osteoprotegerin (OPG), RANKL, vitamin D receptor (VDR), calcitonin receptor (CTR), PTH receptor (PTHR), 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) were designed using PrimerQuest software (Integrated DNA Technologies; Table 3.2). Optimal primer concentrations for each primer set were determined prior to quantification by real-time PCR. Reactions were performed using 12.5 µl of 2× SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 200–900 nM of
each primer and 100 ng of cDNA in a final volume of 25 µl. All samples were subjected to 40 amplification cycles (one cycle consisted of 30 s of melting at 95°C, followed by 30 s of annealing and extension at 65°C). At completion, all samples were subjected to a melt curve analysis to validate the absence of nonspecific products. A modification of the $2^{-\Delta\Delta Ct}$ method was employed to normalize gene expression values prior to statistical analysis (Livak and Schmittgen, 2001) (Table 3.2).

Statistics

Data were analyzed using the GLM procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC) with dietary treatment considered as a fixed effect.

RESULTS

Dietary P restriction resulted in changes in plasma hormone concentrations and growth performance after day 5 of treatment. On day 5, piglets fed the P adequate diet had plasma P concentrations (6.1 mg/dL) that were at least 1.5 fold greater ($P < 0.01$) than those of their deficient counterparts (3.6 mg/dL), and this difference increased to almost a 2-fold thereafter and remained until the end of the study on day 15 (Figure 3.1). There was a significant increase ($P < 0.05$) in plasma Ca levels with dietary P deficiency on day 5 (113.4 mg/L vs. 102.1 mg/L), with no significant differences on any other day (Figure 3.1). Plasma PTH concentrations were significantly reduced ($P < 0.02$) in the P deficient animals on day 5, and this effect only increased over time and remained until the end of the trial (Figure 3.1).
There were no differences in plasma levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} throughout the 15 d trial period (Figure 3.1).

P deficiency resulted in a significant reduction (P < 0.01) in growth and feed efficiency as both the average daily gain and feed efficiency of adequate animals was almost double compared to deficient counterparts (Figure 3.2). Reduction in growth performance with P deficiency was accompanied by a significant reduction in bone growth. P deficiency resulted in a significant reduction (P < 0.01) in the fresh weight (19.8 g vs 15g) as well as the total mineral content of the radial bone compared to adequate counterparts (Figure 3.3). The fat residue of bone based on dry weight minus ash content was significantly higher. There was a significant difference (P < 0.01) in the number of proliferating MSC in vivo based on dietary treatment. P deficiency resulted in a greater than 2 fold reduction in the total number of proliferating cells compared to P adequate diet (Figure 3.4).

Transcript levels of 1-alpha-hydroxylase were elevated (P < 0.1) in the bone marrow of the P deficient animals. There was also a trend (P = 0.1) for increased gene expression of PPAR\textgamma\textsubscript{2} and LPL mRNA in the bone marrow of animals fed the P deficient diet. Osteocalcin gene expression was increased 3 fold (P < 0.01) in the bone marrow of the P adequate animals compared to their deficient counterparts (Figure 3.5). There were no changes in the mRNA expression levels of CALCR, VDR, PTHR, 24-hydroxylase, OPG and RANKL in the bone marrow based on dietary treatment.
DISCUSSION

In this study, we have examined the effects of dietary P restriction on bone growth and MSC activity by utilizing the neonatal pig as a model system. A classical dietary P deficiency profile was achieved based on dramatic reduction in plasma P levels, growth performance and bone mineral content (Alexander et al., 2008; Harper et al., 1997). We have also previously demonstrated similar effects induced by 20% P deficiency in relatively older pigs (Alexander et al., 2008). Circulating levels of PTH and 1,25(OH)2D3 were measured as indicators of P status. Similar to previous P restriction studies, dietary P deficiency resulted in a significant reduction in plasma PTH levels. Dietary P deficiency can cause reduction in PTH levels either directly through post-transcriptional modification by altering RNA-protein binding (Kilav et al., 1995), or via increase in the renal synthesis of 1,25(OH)2D3 levels by upregulation of 1-alpha-hydroxylase (Zhang et al., 2002). Further, dietary P can also affect PTH levels by directly affecting the size and proliferation of parathyroid cells (Wang et al., 1996).

However, in this study, circulating levels of 1,25(OH)2D3 did not change with dietary treatment throughout the trial period. Elevation in 1,25(OH)2D3 plasma concentrations with dietary P deficiency has been shown previously in older animals (Alexander et al., 2008). However, the role of 1,25(OH)2D3 in regulating mineral homeostasis in not fully elucidated in neonates. Previous studies have demonstrated the inability of neonatal endocrine system to utilize 1,25(OH)2D3 as a regulator of calcium homeostasis partly due to reduced expression of vitamin D receptor in the intestine (Halloran and DeLuca, 1981; Mathews et al., 1986).
Although MSC are critical for postnatal bone growth, there have been no studies looking into the effects of dietary P on MSC programming. There was a dramatic reduction in the *in vivo* MSC proliferation with P deficiency in our study. Such a large reduction can have a tremendous impact on bone formation, and maybe directly associated with reduced bone mineral content and bone growth seen in animals fed P deficient diet. Effects of nutrient restrictions on tissue-specific stem cells have been demonstrated previously with satellite cells whose in vivo activity is altered in response to severe dietary restrictions causing permanent muscle growth deficits (Jeanplong et al., 2003; Moore et al., 2005). We have previously demonstrated adipocytic differentiation of MSC based on 1,25(OH)₂D₃ treatment (Mahajan and Stahl, 2009). Although we did not see any differences in 1,25(OH)₂D₃ levels in plasma based on P deficiency in this study, increased 1-alpha-hydroxylase expression in the bone marrow of P deficient animals is suggestive of local synthesis of 1,25(OH)₂D₃ that can alter bone development by its autocrine or paracrine signaling. Increased bone loss is often attributed to increased adipogenesis at the expense of osteogenesis (Beresford et al., 1992; Moerman et al., 2004). Therefore, reduced bone parameters seen with P deficiency may in part be attributed to increased bone adiposity at the expense of bone formation. These results suggest that increased local synthesis of 1,25(OH)₂D₃ in neonates and elevated circulating levels of 1,25(OH)₂D₃ in older animals maybe one of the modes of dietary P deficiency induced changes in MSC activity. We examined the expression of osteogenic and adipocytic markers in the bone marrow in order to evaluate relative MSC differentiation. Reduced mRNA levels of osteocalcin message coupled with the elevated levels of transcripts for PPARγ2 and LPL is suggestive of a shift of
the MSC population in the bone marrow towards adipocytic differentiation at the expense of osteogenic differentiation. Such changes may directly be associated with reduced bone parameters seen with P deficiency. Based on our previous study (Mahajan and Stahl, 2009), such changes may have been induced by locally produced 1,25(OH)2D3 in the bone marrow. Also, lack of change in OPG and RANKL expression indicates that osteoclast activity may not be affected by dietary P. In such case, P deficiency may alter bone development by altering bone formation through changes in MSC activity without affecting bone resorption.

In conclusion, dietary P status during early post-natal development can have a profound impact on bone growth. This may in part be attributed to the reduced MSC proliferation and increased adipogenic differentiation resulting in reduced bone formation. Such dietary P induced changes in bone maybe attributed to local synthesis of 1,25(OH)2D3 in the bone marrow, however, further studies are warranted to elucidate the mechanism.

ACKNOWLEDGEMENT

This work has been submitted as a part of the manuscript:

Dietary P restriction decreases stem cell proliferation and subsequent growth potential in the neonatal pig. Lindsey S. Alexander, Avanika Mahajan, Jack Odle, Kyle L. Flann, Robert P. Rhoads, and Chad H. Stahl. (J Nutr)

LITERATURE CITED


Rickard, D. J. et al. 2006. Intermittent treatment with parathyroid hormone (pth) as well as a non-peptide small molecule agonist of the pth1 receptor inhibits adipocyte differentiation in human bone marrow stromal cells. Bone 39: 1361-1372.


Wang, Q., E. Paloyan, and A. M. Parfitt. 1996. Phosphate administration increases both size and number of parathyroid cells in adult rats. Calcif Tissue Int 58: 40-44.

Table 3.1 Experimental diet composition

<table>
<thead>
<tr>
<th>Ingredients, g/kg</th>
<th>Phosphorus adequate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Phosphorus deficient&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td>Isolated soy protein</td>
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<td>350</td>
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<tr>
<td>7/60 milk replacer high fat&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>250</td>
</tr>
<tr>
<td>Potassium phosphate</td>
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<tr>
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<tr>
<td>Sucrose</td>
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<td>11</td>
</tr>
<tr>
<td>Vitamins&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Minerals&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Methionine</td>
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<td>2</td>
</tr>
<tr>
<td>Salt</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Antibiotics</td>
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<td>10</td>
</tr>
<tr>
<td>Xanthum gum</td>
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<tr>
<td>Analyzed&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>Crude protein, g/kg</td>
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<td>304</td>
</tr>
<tr>
<td>Calcium, g/kg</td>
<td>16.2</td>
<td>16.5</td>
</tr>
<tr>
<td>Phosphorus, g/kg</td>
<td>9.3</td>
<td>7.1</td>
</tr>
</tbody>
</table>
Table 3.1 Continued

1Diet met NRC requirements
2Diet 25% deficient in Phosphorus
3MSC, Dundee, IL
4Provided all vitamins (Vitamin A (33 075 kIU/kg), Vitamin D₃ (6615 kIU/kg), Vitamin E (55125 kIU/kg), Vitamin K (5.1 g/kg), thiamin (2 g/kg), riboflavin (8.4 g/kg), pyridoxine (4 g/kg), Vitamin B₁₂ (0.04 g/kg), pantothenic acid (30 g/kg), niacin (33 g/kg), folic acid (2.8 g/kg), ascorbic acid (117 g/kg), biotin (0.07 g/kg)) and minerals (Na, Cl, K, Mg, S, Cu, Zn, Se, Co, I, Fe, Mn), with the exception of calcium and phosphate at levels to meet or exceed the animals requirements
5Diets were analyzed by DairyOne Forage Laboratory, Ithaca, NY
Table 3.2 Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</tr>
<tr>
<td></td>
<td>R: 5' TGCTTTGAATGTCTCGCATGGCT 3'</td>
</tr>
<tr>
<td>Runx2/CBFA1</td>
<td>F: 5' CAAGTGCGGCTGCAAACCTTCTCCA 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' AGGCTGTTTGATGCCATAGTCCCT 3'</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>F: 5' TACCCAGATCCTCTGGAGGCC 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' TATGCCATAAGAGCGGCCGATA 3'</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>F: 5' ACCGTTGCAACAACCTTGGCTATG 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' ACTTTTGAGGGCATCTGAGCAGA 3'</td>
</tr>
<tr>
<td>Parathyroid hormone receptor</td>
<td>F: 5' TACTGTTTTCTGCAACGGGGAGGTA 3'</td>
</tr>
<tr>
<td></td>
<td>R&quot; 5' GCGCTTAAAGTCCAGTGCAATGT 3'</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>F: 5' TGTCGAGAGAGGCTGCATTGAA 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' TCATCTTCCCAGAAGCAGTCCATCA 3'</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>F: 5' AGGAGTGGAAGTATGCACTTGGCC 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' GGAGCGGCCAAGAATAGCAAA 3'</td>
</tr>
<tr>
<td>Vitamin D receptor</td>
<td>F: 5' TTGCACACACCTCAAGCACAAGG 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' TGC TCT ACG CCA AGA TGA TCC AGA 3'</td>
</tr>
<tr>
<td>Calcitonin receptor</td>
<td>F: 5' ATGACAGCGCGTGATGTATGTAA 3'</td>
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<tr>
<td></td>
<td>R: 5' TGCTCATGCCATTACAGGGAGT 3'</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>F: 5' AACGGCAACACAGCTCAACAAGA 3'</td>
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<tr>
<td></td>
<td>R: 5' TGCTCAAGGTAGTTAGCATGT 3'</td>
</tr>
<tr>
<td>RANKL</td>
<td>F: 5' TGGATCACAGCACATCAGAGCAGA 3'</td>
</tr>
<tr>
<td></td>
<td>R: 5' TGGTACAAAGAGGACAGACTCAGT 3'</td>
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Figure 3.1 Effect of dietary P on (A) plasma inorganic phosphate levels, (B) plasma Ca levels, (C) plasma PTH concentrations, and (D) dietary P on plasma 1,25(OH)₂D₃ concentrations. Values presented are least square means and standard error. * Indicates a significant treatment effect (P < 0.05). P adequate, n = 9; P deficient, n = 9.
Figure 3.2 Effect of dietary P on growth performance. Values presented are least square means and standard error. P adequate, n = 9; P deficient, n = 9.
Figure 3.3 Effect of dietary P on bone measures. Values presented are least square means and standard error. a,b Values not sharing a common superscript are significantly different (P < 0.05). P adequate, n = 9; P deficient, n = 9.
Figure 3.4 Effect of dietary P on MSC proliferation. Values presented are least square means and standard error. a,b Values not sharing a common superscript are significantly different (P < 0.05). P adequate, n = 9; P deficient, n = 9
Figure 3.5 Effect of dietary P on gene expression in bone marrow. Values presented are least square means and standard error. Values not sharing a common superscript are different (P < 0.1). Values without superscripts have P = 0.1. P adequate, n = 9; P deficient, n = 9.
CHAPTER 4

Title: Parathyroid hormone and its interaction with 1,25 Dihydroxycholecalciferol affects porcine mesenchymal stem cell activity.

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Key words: Mesenchymal stem cells, calcitropic hormones, pig
Abstract

Parathyroid hormone (PTH) is a key hormone for the homeostatic regulation of Ca and P and has significant effects on bone integrity. In this study, we have examined the effects of either continuous or intermittent PTH treatment as well as the interaction between PTH and 1,25(OH)\textsubscript{2}D\textsubscript{3} on porcine MSC (pMSC) activity. MSC were isolated individually from the bone marrow of 3 pigs (2 weeks of age). In the first study, MSC were treated with PTH either continuously or intermittently (1h/day). In our second study we examined the effect of a range of PTH doses in the presence of 1,25(OH)\textsubscript{2}D\textsubscript{3} concentrations sufficient to promote adipogenesis. After 6d, cell proliferation, gene expression and cytochemical differences were determined. The expression of PPAR\textgreek{g} and LPL was increased with continuous PTH treatment while that of Runx2 was increased by both continuous and intermittent PTH exposure (P < 0.05). Intermittent PTH treatment also significantly increased (P < 0.05) alkaline phosphatase (ALP) activity and decreased cell proliferation, while continuous PTH treatment significantly (P < 0.05) increased Oil Red O\textsuperscript{+} staining and decreased MSC proliferation compared to the control. We have previously showed that 1,25(OH)\textsubscript{2}D\textsubscript{3} stimulates adipocytic differentiation of pMSC. Because during Ca deficiency there would also be an increase in PTH we wanted to examine the combined effect of these hormones on pMSC. The ability of 1,25(OH)\textsubscript{2}D\textsubscript{3} to stimulate adipogenesis was partly inhibited by co-treatment with PTH as seen by reduced (P < 0.05) gene expression of PPAR\textgreek{g} and LPL. The increased expression of osteocalcin by 1,25(OH)\textsubscript{2}D\textsubscript{3} alone was also inhibited by PTH, while the increased gene expression of Runx2 and the percentage of ALP\textsuperscript{+} cells by PTH was
inhibited by 1,25(OH)₂D₃ (P < 0.05). These data suggest that PTH alters pMSC activity differentially based on mode of administration and that PTH and 1,25(OH)₂D₃ may act antagonistically on pMSC.

Introduction

Parathyroid hormone (PTH) is a key hormone for maintaining calcium and phosphate homeostasis in the body. Circulating levels of PTH have been shown to increase during calcium deficiency (Naveh-Many et al., 1995; Persson et al., 1993; Rader et al., 1979) and decrease during phosphate deficiency (Alexander et al., 2008; Riond et al., 2001). Because deficiencies in either of these nutrients negatively impacts bone growth and development, it is necessary to examine the effect of PTH on mesenchymal stem cells (MSC) which provide the life-time supply of bone forming cells. PTH has been employed as a therapeutic agent for increasing bone formation, however, its effects on bone are affected by the mode of administration. Intermittent dosing with PTH increases bone mass, bone strength, bone mineral density and even improves micro-architecture and regenerative capacity (Compston, 2007; Hock and Gera, 1992; Neer et al., 2001; Rubin et al., 2002) whereas, continuous treatment increases bone resorption and leads to bone loss (Hock and Gera, 1992; Marx, 2000). The mechanism behind this differential response of bone to PTH is not fully understood. At the cellular level, intermittent PTH treatment, has been shown to increase osteoblast activity (Dobnim and Turner, 1995; Meng et al., 1996), decrease osteoblast apoptosis (Jilka et al., 1999); and stimulate proliferation and differentiation of osteoprogenitor cells residing in the bone marrow (Kostenuik et al., 1999; Nishida et al.,
1994). Interestingly, increased expression of PTH/PTHrP receptor was found to be associated with enhanced osteogenic and reduced adipogenic differentiation both in vivo and ex vivo (Menuki et al., 2008). Also, daily treatment with PTH increased osteoblast numbers as well as reduced the number of adipocytes in the bone marrow of ovariectomized monkeys (Sato et al., 2004). Antagonistic effects are observed with continuous PTH treatment, as it stimulates bone resorption due to increased osteoclast activity. PTH mediated increase in RANKL:OPG ratio by direct stimulation of osteoblasts leads to increased osteoclast activity (Ma et al., 2001; Neer et al., 2001). Because osteoblasts are derived from MSC, a potential mechanism by which PTH could modulate bone development is by affecting the activity of the MSC pool. Both in vitro (Beresford et al., 1992; Jaiswal et al., 2000; Spinella-Jaegle et al., 2001) and in vivo (Akune et al., 2004; Sabatakos et al., 2000; Wronski et al., 1981) studies suggest that there is an inverse relationship between osteogenic and adipogenic differentiation by MSC, and that the bone loss seen during osteoporosis and aging is attributed, at least in part, to increased adipogenesis (Burkhardt et al., 1987; Meunier et al., 1971; Moerman et al., 2004).

Another hormone that is affected by PTH and that has been shown to impact bone is 1,25 dihydroxycholecalciferol (1,25(OH)₂D₃). Both PTH and 1,25(OH)₂D₃ are primary regulators of calcium homeostasis, and synergistically increase bone resorption during hypocalcemia. The effects of 1,25(OH)₂D₃ on MSC are pleiotropic, as it has been found to both induce (Beresford et al., 1992) and inhibit (Atmani et al., 2002; Atmani et al., 2003; Zhang et al., 2006) osteogenic as well as induce (Atmani et al., 2003) and inhibit (Kelly and Gimble, 1998; Kong and Li, 2006) adipogenic differentiation of these cells. Previously, we
have demonstrated that physiological levels of 1,25(OH)₂D₃ stimulate adipocytic
differentiation of porcine MSC (Mahajan and Stahl, 2009). Since both PTH and 1,25(OH)₂D₃
are central in mineral homeostasis and bone development, it is important to examine the
effects of interaction of these two hormones on MSC activity. Based on various studies
discussed, effects of PTH and 1,25(OH)₂D₃ on osteoblasts and MSC proliferation and
differentiation have been found to vary depending on species, stage of differentiation and
culture conditions. Although many studies have been conducted using rats or mice as the
model system, none have examined the effect of PTH alone or in association with
1,25(OH)₂D₃ on porcine MSC in vitro. It is necessary to employ a non-rodent large animal
model system having similar bone remodeling and nutritional behavior as humans, for the
study of bone diseases like osteoporosis and better understanding of the impact of nutrition
on bone development. Pig serves as an excellent model system in this regard (Bustad and
McClellan, 1966; Miller and Ullrey, 1987). The objective of this study, was to determine the
effects of PTH mode of treatment and the interaction of PTH with 1,25(OH)₂D₃ on porcine
MSC activity.

Materials and Methods

Mesenchymal Stem Cell Isolation and Culture

All animal protocols were approved by North Carolina State University’s Institutional
Animal Care and Use Committee. MSC were isolated from three 14d old cross-bred pigs as
previously described (Mahajan and Stahl, 2009). Cells were plated in DMEM + 10% heat-
inactivated FBS (BM) (Invitrogen) in 6-well plates at 10³ cells/cm². For the study examining
intermittent vs continuous PTH treatment, complete media changes were made every 2 days until cells reached confluency (9 days post-plating). For the intermittent PTH treatment, cells were incubated for 1 h/day in bovine PTH (1-34) (500 pg/ml) (Sigma-Aldrich, St. Louis, MO) in BM followed by complete substitution with BM alone. For continuous treatment, bovine PTH (1-34) was added at the same concentration every 2d at the time of media change. Cells were harvested after 6 days of treatment. Our second study examined the interaction of PTH and 1,25(OH)2D3 since both of these hormones would be elevated during dietary Ca deficiency. For this study, cells were given 2 days to attach prior to the initiation of the following treatments: 1) Control: BM + vehicle, 2) PTH: BM + 500 pg/ml bovine PTH (1-34), 3) VD: BM + 100 nM 1,25(OH)2D3 (Sigma-Aldrich, St. Louis, MO) 4) 100X PTH-VD: BM + 500 pg/ml bovine PTH (1-34) + 100 nM 1,25(OH)2D3, 5) 10X PTH-VD: BM + 50 pg/ml bovine PTH (1-34) + 100 nM 1,25(OH)2D3, 6) 1X PTH-VD: BM + 5 pg/ml bovine PTH (1-34) + 100 nM 1,25(OH)2D3. The media was completely changed every 3 days, and cells were harvested after 6 days of treatment.

Cell Proliferation

Cells were pulsed with 10 μM BrdU for 4 hours, followed by immunocytochemistry to assess the percentage of BrdU-positive cells. Briefly, cells were fixed in methanol + 1% H2O2, followed by denaturation of DNA by incubation in pre-warmed (37°C) HCl for 1 h. The acid was then neutralized with 2 washes of TBE (pH 8.5) followed by 3 additional washes in PBS. Cells were incubated in primary antibody (G3G4; Developmental Studies Hybridoma Bank, University of Iowa) (1:100, 0.1 % BSA in 1X PBS) overnight followed by
incubation in a goat anti-mouse HRP-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) (1:500, 1% BSA in 1X PBS) for 2 h. Stained cells were visualized by incubation with 0.5 mg/ml of diaminobenzidine and 0.02% H₂O₂ in PBS for 5 minutes (37). Approximately 400 cells were counted in at least 5 fields of view per animal and the percentage proliferation was determined by calculating the ratio of stained to unstained cells.

Cytochemical Staining

Cells plated in 6-well plates were fixed and stained in duplicate for alkaline phosphatase activity (ALP) and the presence of neutral lipids using Oil Red O. Staining for ALP was accomplished using a commercially available kit (ALP staining kit, Takara Bio Inc., Otsu, Shiga, Japan) according to manufacturer’s instructions. Accumulation of neutral lipids was visualized by fixing the cells in 10% formalin and then staining with 0.2% Oil Red O for 1 hr followed by thorough rinsing with water.

Gene expression analysis

Total RNA was extracted from cells using the Ambion RNeAqueous kit (Ambion, Austin, TX) and genomic DNA was removed by treatment with deoxyribonuclease I (Ambion DNA-free kit). Purified RNA was reverse transcribed with Superscript Reverse transcriptase III (Invitrogen Life Technologies) using both oligodT (Integrated DNA Technologies, Coralville, IA) and random hexamer primers (Invitrogen Life Technologies) in a 1:1 ratio. The resulting cDNA was then incubated with RNase H (Invitrogen Life
Technologies) to remove RNA. Both starting RNA and final cDNA concentrations were quantified using a fluorescence-based quantification kit (Invitrogen Life Technologies). Semi-quantitative real-time PCR was performed using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer oligonucleotides were designed using PrimerQuest software available from Integrated DNA Technologies (Table 1). Optimal primer concentrations for each primer set were determined prior to quantification by real-time PCR, and the linearity of amplification for each gene of interest over a 2 log range of cDNA concentrations was verified to be similar to that of the control gene, 18S ribosomal RNA. The control gene was verified to be unaffected by treatment. Reactions were performed using 12.5 μl of 2x SYBR Green Supermix (Bio-Rad), 200–900 nM of each primer and 25 ng of cDNA in a final volume of 25μl. All samples were subjected to 40 amplification cycles (1 cycle consisted of 30 s of melting at 95°C followed by 30 s of annealing and extension at 65°C). At the completion of the amplification cycles, all samples were subjected to a melt curve analysis to validate the absence of nonspecific products. Gene expression was normalized to cDNA concentration and the control gene (18S) using a modification of the 2^{-ΔΔCT} method of Livak and Schmittgen (2001) [26].

Statistical analysis

All treatments had 3 biological replicates as well as technical duplicates for analysis. Data were analyzed with multivariate analysis of variance (ANOVA) using the GLM procedure of SAS® (Version 9.1, SAS Institute Inc., Cary, NC). Duncan’s multiple range test
was used to compare treatment means. Data are expressed as means ± SEM. Differences with P < 0.05 were considered to be statistically significant.

**Results**

Continuous vs Intermittent PTH treatment

*Cell proliferation and cytochemical staining*

Regardless of mode of treatment, PTH significantly (P < 0.05) reduced the proliferation of MSC. There was a 3 fold and 2 fold reduction in proliferating cells (P < 0.05) compared to the controls with intermittent and continuous PTH treatments, respectively (Figure 1). Continuous PTH treatment resulted in an almost 2 fold increase (P < 0.05) in the number of cells stained positive for Oil Red O when compared to control and intermittent PTH treatment groups (Figure 1). The intermittently treated group had significantly (P < 0.03) greater percentage of ALP positive cells compared to the control cultures (~ 4-fold) and tended to have greater ALP activity when compared to continuously treated cultures (P < 0.1).

*Gene expression*

The mRNA expression levels of adipogenic and osteogenic markers were measured at day 6. Regardless of mode of administration, PTH increased (P < 0.05) the mRNA expression of both Runx2 and LPL (Figure 2). Continuous PTH treatment also caused a significant increase (P < 0.05) in the mRNA abundance of PPARγ compared with both the control cultures (greater than 2 fold) and the intermittently treated cultured (greater than 1.5
fold). The levels of LPL message were approximately 1.5-fold (P < 0.05) greater with continuous PTH treatment compared with the control group and there was a trend (P = 0.1) for increased expression compared to the intermittent group as well. Neither the mRNA expression of osteocalcin nor PTHR changed based on PTH treatment, when compared with the control.

Interaction of PTH and 1,25(OH)₂D₃

*Cell proliferation and cytochemical staining*

The proliferation rate of the PTH alone group, while not significantly different from the control group, was greater (P < 0.05) than all other treatments (Figure 3). Also, the number of cells were significantly less (P < 0.05) in the 100X PV treated cells with a trend for reduction (P < 0.1) in 10X PV and 1X PV treated cells when compared to control cells. The ALP activity was the highest in control and PTH treated cells, with little activity in vitamin D and 1X PV treated cells and no activity in 10X PV and 100X PV treated cells.

*Gene Expression*

The expression of relevant adipogenic and osteogenic markers was measured in cultures after 6 days of treatment. Consistent with our previous study, the mRNA expression of PPARG was significantly elevated (P < 0.05) in the 1,25(OH)₂D₃ treated cultures compared to the control (9-fold) (Figure 4). The addition of any dose of PTH to the 1,25(OH)₂D₃ dramatically reduced this increase in *PPARG* (P < 0.05), however both 1X and 100X dose still tended to have greater levels of PPARG message than did the control (P < 0.05). The 1X
dose also had a trend for increased PPARG message relative to the PTH alone treatment (p < .1). The LPL message levels in the 1,25(OH)₂D₃ treated cultures was dramatically increased compared to the PTH and control cultures (P < 0.05). There was a dose-dependent reduction in this increase in LPL message abundance with PTH treatment. Both 10X (P < .1) and 100X (P < .05) dose of PTH reduced LPL while 1X dose caused no change in expression compared to cultures treated with 1,25(OH)₂D₃ alone. The expression caused by the 100X PTH in the presence of 1,25(OH)₂D₃ was significantly more than both the control and PTH treatment alone. A similar effect was seen with the abundance of OC mRNA, with the 1,25(OH)₂D₃ group having significantly greater levels of this message, but PTH treatment reducing this effect regardless of dose levels (P < .05) (Figure 4). The addition of any dose of PTH to 1,25(OH)₂D₃ caused OC mRNA expression greater than control cultures while 1X dose caused expression greater than PTH treatment alone (P < .05). PTH alone caused a significant increase in the concentrations of Runx2 and PTHR mRNAs compared to control and 1,25(OH)₂D₃ treatment (P < .05). The addition of 1,25(OH)₂D₃ completely eliminated this increase (P < .05).

Discussion

The objectives of this study, were to characterize the effects of intermittent or continuous PTH treatment on porcine MSC, and examine the interaction of PTH with 1,25(OH)₂D₃ on porcine MSC activity. We pursued these objectives to gain a better understanding of how dietary interventions may impact bone growth and development, because PTH/1,25(OH)₂D₃ axis is the classical bone mineral homeostatic pathway and
mesenchymal stem cells provide all of the osteoprogenitor cells. A secondary objective was
to provide further cellular characterization for a highly biologically appropriate animal model
for nutrition and bone physiology studies, the pig. While rodent model have predominated
the field, and are irreplaceable for certain genetic models, they less closely resemble humans
than do pigs in several critical areas. While both humans and pigs undergo bone remodeling
by utilizing local groups of osteoblasts and osteoclasts called the BMU (bone multicellular
units) (Spurrell, 1965), rats are limited in their ability to do so. The rate of bone removal and
deposition in pigs is also comparable to that of humans (Mosekilde et al., 1987) and are one
of the few species to undergo spontaneous fractures similar to humans (Spencer, 1979).
Conversely in rats, the relationship between increased bone fragility and susceptibility to
fractures is unclear and they do not seem to be an appropriate model for osteoporotic
fractures (Peng et al., 1997). Lack of haversian system in young rats unlike pigs is also
another limitation to assess the effects of ovariectomy or other hormonal influences on bone.

We examined the impact of mode of PTH treatment on MSC because of varying
levels of in vivo PTH exposure as a function of mineral nutrition. PTH exposure in vivo is
cyclic in nature during adequate mineral nutrition as oppose to Ca deficiency during which
PTH levels are continuously elevated. As would be expected based on human in vivo and in
vitro studies, porcine MSC responded differently to PTH treatment based on its mode of
administration. The decreased proliferation coupled with increased ALP activity compared
with untreated MSC is suggestive of intermittent PTH treatment stimulating osteogenesis,
whereas the decreased proliferation, increased lipid accumulation, and greater abundance of
PPARG and LPL message seen with continuous administration of PTH is suggestive of it
stimulating adipogenesis. A few studies have examined the effect of PTH treatment on MSC proliferation and differentiation potential (Rickard et al., 2006; Yang et al., 2009). Although Rickard et al, who demonstrated an inhibition of adipogenesis with intermittent treatment and no differences with continuous treatment (Rickard et al., 2006), they demonstrated these effects in the presence of adipogenic induction media unlike the proliferative media used in our study. Also, the concentrations of PTH employed were drastically higher compared to the physiologically relevant levels used in our study (Kumar et al., 2009; Riond et al., 2001). Other confounding factors could be the differences in the model system employed and the duration of the study. Since there is an inverse relationship between osteogenic and adipogenic differentiation of MSC (Beresford et al., 1992), increased expression of adipogenic markers with continuous PTH treatment could be associated with the inhibitory effects of PTH in vivo on bone. Increase in the ALP activity of cells when treated intermittently with PTH is consistent with several other studies conducted with bone marrow stromal cells (Nishida et al., 1994; Rickard et al., 2006; Yang et al., 2009). However, there was in increase in Runx2 expression with both treatment modes. This could be a result of highly elevated mRNA levels in the small proportion of committed osteoprogenitor cells amongst other progenitors pushed towards adipogenesis that may constitute a higher proportion in the total number of cells. Lack of change in osteocalcin levels is consistent with other studies (Rickard et al., 2006; Yang et al., 2009), and maybe due to the short duration of the culture in which we may not expect fully differentiated osteoblasts.

Regardless of treatment mode, cell proliferation was found to be significantly reduced with PTH compared to control cultures. A reduction in the number of proliferating
cells in rat bone with intermittent PTH has been previously shown (Onyia et al., 1997). This is also supportive of studies that have demonstrated that the stimulatory effects of intermittent PTH treatment on bone and osteoblast numbers may not be attributed to increased proliferation of osteoprogenitor cells (Dobnig and Turner, 1995; Jilka et al., 1999). Since there is an inverse relationship between proliferation and differentiation, drastic reduction in proliferation with PTH treatment may indicate a switch to differentiation. This is consistent with increased expression of osteogenic and adipogenic markers with PTH treatment in our study.

The results support previous in vivo and in vitro work demonstrating that intermittent PTH treatments stimulate bone formation while continuous elevated PTH results in bone loss. The anabolic effects of PTH when given intermittently, on bone formation and bone strength have been demonstrated previously (Compston, 2007; Hock and Gera, 1992; Neer et al., 2001; Rubin et al., 2002). Increased bone resorption, hypercalcemia and bone loss due to continuous PTH treatment have also been observed in vivo (Hock and Gera, 1992; Marx, 2000). Therefore, we hypothesize that apart from the calcium release, increased osteoclast activity and reduced osteoblast activity in the bone caused by hyperparathyroidism, increased bone marrow adiposity at the expense of reduced osteogenesis could be another mechanism of bone loss.

Apart from PTH, 1,25(OH)₂D₃ is another hormone that is important for bone development. Both PTH and 1,25(OH)₂D₃ are important regulators of osteoblast and osteoclast activity that in turn determines the balance between bone resorption and bone formation. They have both been also demonstrated to alter MSC differentiation potential in
various model systems (Atmani et al., 2003; Mahajan and Stahl, 2009; Rickard et al., 2006; Yang et al., 2009). At molecular level, both have been shown to affect cell differentiation and gene expression through rapid influxes of calcium signaling cascades (Edelman et al., 1986; Yamaguchi et al., 1987). Therefore, it is important to examine the interactive effects of these two hormones on MSC differentiation potential. In this study, 1,25(OH)₂D₃ was found to increase the expression of adipogenic marker genes like PPARγ and LPL compared to the control cultures. This is consistent with our previous work showing adipocytic differentiation of porcine MSC with 1,25(OH)₂D₃ treatment (Mahajan and Stahl, 2009). However, reduced expression of both adipogenic markers by 1,25(OH)₂D₃ in the presence of PTH suggests an inhibitory effect of PTH on 1,25(OH)₂D₃ induced adipogenic expression. Addition of PTH to 1,25(OH)₂D₃, depending on the PTH concentration, caused expression of PPARγ and LPL significantly higher than both the control and PTH alone treated cultures suggesting that PTH may not completely inhibit the effects of 1,25(OH)₂D₃. Lack of a significant increase in adipogenic expression with PTH alone in this study unlike the continuous treatment with PTH every 48 hours suggests that frequency of media change and the number of cells at the time of treatment induction play a critical role in cell differentiation. Increased osteocalcin expression due to 1,25(OH)₂D₃ was again consistent with our previous study, and like PPARγ and LPL, its expression was partly inhibited by PTH. Although Sammons et al demonstrated an increased osteocalcin expression by combination of PTH-1,25(OH)₂D₃, they did not examine the effects of treatment with PTH and 1,25(OH)₂D₃ alone (Sammons et al., 2004). Therefore the greater stimulatory effects of 1,25(OH)₂D₃ alone, on osteocalcin expression may have still been consistent. However, the expression of osteogenic
transcription factor Runx2 was elevated with PTH treatment, and the presence of 1,25(OH)₂D₃ partially inhibited the PTH mediated increase. Also, 1,25(OH)₂D₃ alone did not reduce Runx2 expression, which is consistent with our previous study. Therefore, 1,25(OH)₂D₃ inhibited Runx2 expression only in the presence of PTH. Thus, these results indicate the importance of PTH-1,25(OH)₂D₃ interaction in altering MSC phenotype. Co-effects of PTH and 1,25(OH)₂D₃ on osteogenesis have been examined by a few studies and are found to be diverse. Sammon et al demonstrated increased osteogenesis in human MSC (Sammons et al., 2004), while Costa et al observed no differences in alveolar bone cell cultures (Costa and Fernander, 2000) by the PTH-1,25(OH)₂D₃ combination. Also, Li et al observed a co-stimulatory release of calcium from serum-free calvaria cultures causing increased bone resorption (Li and Farach-Carson, 2001). Although the interactive effects of PTH and 1,25(OH)₂D₃ on osteogenesis have been examined, there have been no studies looking at their effect on adipogenic potential of MSC. This is the first study to demonstrate differential regulation of 2 primary adipogenic markers based on co-treatment with PTH and 1,25(OH)₂D₃.

Inhibitory effects of 1,25(OH)₂D₃ on PTH actions maybe through the inhibition of one or more enzymes like adenylate cyclase or phospholipase C whose activation through the G-protein coupled mechanism results in PTH-PTHr mediated effects on cells (Abou-Samra et al., 1992). Although 1,25(OH)₂D₃ mediates its physiological effects through both genomic and non-genomic pathways (Lips, 2006), antagonistic effects of PTH on 1,25(OH)₂D₃ might be through the VDRₙuc mediated effects on gene expression since genes examined in this study like osteocalcin are known to have a VDRE in their promoter
region (Yoon et al., 1988). Also PPARG increases cellular adipocytic expression by binding with RXR alpha that also dimerizes with VDR to alter gene expression by the genomic pathway (Tontonoz et al., 1994). Nevertheless, inhibition of one or more components of the non-genomic pathway may also be a mode of PTH mediated inhibition. Further studies to elucidate the molecular mechanism of this antagonistic activity of PTH and 1,25(OH)2D3 are warranted.

In conclusion, we have demonstrated the differential effects of PTH on MSC differentiation potential depending on the mode of treatment and interaction with 1,25(OH)2D3. Since bone loss is partly attributed to increased adipogenesis at the expense of osteogenesis, understanding the co-impact of PTH and 1,25(OH)2D3 on MSC differentiation potential can lead to therapeutic strategies for bone development.

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Table 1. Primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARG</td>
<td>F: 5' AATTAGATGACAGCGACCTGGCGA 3' R: 5' TGTCTTGAATGTCTCGATGGGCT 3'</td>
</tr>
<tr>
<td>Runx2</td>
<td>F: 5' CAAGTGCGGTGCAAACCTTTTCTCCA 3' R: 5' AGGCTGTTTGATGCCATAGTCCCT 3'</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>F: 5' TACCCAGATCCTCTGGAGCCC 3' R: 5' TATGCCATAGAAGCGCCGATA 3'</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>F: 5' ACCGTTGCAACAACCTTGGGCTATG 3' R: 5' ACTTTTGAGGGCATTGAGCACGA 3'</td>
</tr>
<tr>
<td>Parathyroid hormone receptor</td>
<td>F: 5' TACTGTTTCTGCAACGGGAGGTA 3' R&quot; 5' GCGTTAAAGTCCAGTGCCAATGT 3'</td>
</tr>
<tr>
<td>18S</td>
<td>F: 5' TTAGAGTGTTCAAAGCAGGCCCGA 3' R: 5' TCTTGGCAAATGCCTTCGG 3'</td>
</tr>
</tbody>
</table>
Figure 1. Effect of PTH on cell proliferation and cytochemical staining. Percentage of cells stained positive for A) BrdU, B) Oil Red O, and C) ALP activity. MSC were cultured in the presence of 500 pg/ml PTH either continuously (every 48 hr) or intermittently (1h/day) for 6 days post-confluency. Data is based on 6 fields of views consisting of at least 200 cells/animal/treatment group. N = 3 per treatment group. Values presented are least square means and standard error. Columns not sharing a common letter are significantly different (P < 0.05).
Figure 2. Effect of PTH on MSC gene expression. Expression of (a) PPARγ, (b) Runx2, (c) LPL, (d) osteocalcin and (e) PTHR mRNA as determined by real-time PCR. MSC were cultured in the presence of 500 pg/ml PTH either continuously (every 48 hr) or intermittently (1h/day) for 6 days post-confluency. N= 3 per treatment group. Values presented are least square means and standard error. Columns not sharing a common letter are significantly different (P < 0.05).
Figure 3. Effect of PTH and 1,25(OH)\_2D\_3 on cell proliferation and ALP activity staining. MSC were cultured in the presence of 500 pg/ml PTH alone (PTH), 1,25(OH)\_2D\_3 alone (vitamin D) or PTH (5 (1X PV), 50 (10X PV) or 500 (100X PV) pg/ml) + 1,25(OH)\_2D\_3 for 6 days. Data is based on 6 fields of views consisting of at least 200 cells/animal/treatment group. Number of cells stained positive for ALP activity was either zero (undetected) (-), few (+) or numerous (++) N = 3 per treatment group. Values presented are least square means and standard error.
Figure 4. Effect of PTH and 1,25(OH)₂D₃ on MSC gene expression. Expression of (a) PPARγ, (b) Runx2, (c) LPL, (d) osteocalcin and (e) PTHR mRNA as determined by real-time PCR. MSC were cultured in the presence of 500 pg/ml PTH alone (PTH), 1,25(OH)₂D₃ alone (vitamin D) or PTH (5 (1X PV), 50 (10X PV) or 500 (100X PV) pg/ml) + 1,25(OH)₂D₃ for 6 days. N = 3 per treatment group. Values presented are least square means and standard error.
CHAPTER 5

Title: Dietary calcium affects developmental programming of bone.

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Abstract

Effects of dietary calcium (Ca) deficiency on skeletal integrity are well characterized in growing and mature mammals; however, little work has examined Ca nutrition during the neonatal period. In this study, we examined the effects of neonatal Ca nutrition on bone integrity, endocrine parameters, and mesenchymal stem cell (MSC) differentiation potential. Neonatal pigs (24 ± 6h post-partum) received either a Ca adequate or a 30% Ca deficient diet for 18 days. Ca deficiency reduced (P < 0.05) bone flexural strength and BMD, and elevated (P < 0.05) plasma PTH levels by the end of the study. There were no significant differences in plasma Ca, P, or 1,25(OH)₂ vitamin D based on Ca nutrition throughout the study. Calcium deficiency also reduced (P < 0.05) the in vivo proliferation of MSC by approximately 50%. In vitro studies utilizing homologous sera demonstrated that MSC activity was affected (P < 0.05) by both the Ca status of the animal and the sera, as well as by their interaction. Proteomic analysis of the sera utilized in the cell culture experiments identified 22 differentially expressed (>1.3-fold) proteins including albumin, alpha-fetoprotein, fetuinA and Apo-AI. The results indicate that neonatal Ca nutrition is crucial for bone integrity and suggest that early life Ca restriction may have long-term effects on bone integrity via programming of MSC.

Keywords: Calcium, Mesenchymal stem cells, differentiation potential, bone, neonate, pig
Introduction

Maximizing bone integrity during growth and development is critical for the prevention of osteoporosis, and as a result much research has examined the impact of dietary calcium (Ca) on bone mineral accretion during growth and development. However, few studies have examined the impact of dietary Ca during the neonatal period, which is surprising due to the frequency of metabolic bone disease seen among premature and low birth weight humans (1). In older humans and other animals, the detrimental effects of calcium deficiency on bone are primarily attributed to increased bone resorption mediated by secondary hyperparathyroidism (2).

Bone growth and development is a coordinated process of bone deposition by osteoblasts and bone resorption by osteoclasts. Bone formation is regulated by both the proliferation and differentiation rate of osteoprogenitor cells as well as the activity of mature osteoblasts; however the rate of bone formation is more dependent on the number of osteoblasts present than on the activity of the individual osteoblasts (3). Mesenchymal stem cells (MSC) in the bone marrow provide these osteoprogenitor cells, but can also adopt an adipocytic lineage. The balance between adipocytic versus osteogenic differentiation of MSC is of critical importance to bone health (4). The proliferation and differentiation of bone-marrow derived MSC are dramatically altered by the calcitropic hormones dihydroxycholecalciferol (1,25(OH)2D3) and parathyroid hormone (PTH) (5-7). The impact of Ca nutrition on MSC activity, either directly or via its homeostatic hormones, during the neonatal period is of great importance because during this developmental period there are the highest number of proliferating MSC (8, 9). Although several studies have explored the
relationship between dietary Ca and bone development in older children and adults (10),
limited studies have looked at early neonatal life (11, 12). In particular, there have been no
studies examining the effect of dietary Ca on the differentiation potential of MSC.

Piglets serve as an excellent model system for studying human neonatal nutrition,
particularly for nutritional studies targeting bone development, because of similar size and
function of their gastrointestinal tract, similar physiology, and bone remodeling cycle (12-
14), as well as similarity in the lineage allocation of their MSC (15, 16). To identify the
potential impact of dietary Ca on the developmental potential of bone, we have examined the
impact of dietary Ca deficiency on bone integrity, endocrine parameters, in vivo MSC
proliferation, and in vitro MSC differentiation potential utilizing neonatal pigs.

Materials and Methods

Animals

All animal protocols were approved by North Carolina State University’s Institutional
Animal Care and Use Committee. Fourteen 1 day old cross-bred piglets (26 ± 2 h post-
partum, both male and female) were weighed and allotted on the basis of gender and BW to 1
of 2 dietary treatment groups differing in Ca intake: Ca adequate (1.2 %) and Ca deficient
(0.59 %). Nutrient requirements for pigs of this age were determined based on the
composition of sow’s milk (17) and an extrapolation from NRC (1998) requirements for
older pigs. The diets were designed to be as similar as practically possible to the sow’s milk
and contained no plant proteins (Table 1). Calcium for the deficient group was derived from
the basal diet only; additional Ca was added to the Ca adequate diet in the form of Ca
carbonate. Phosphorus was added equally to both diets in the form of potassium phosphate. All animals were individually housed in raised cages and fed through a gravity-flow milk delivery system (18). All piglets were fed equal quantities 3 times daily and had their total daily intake restricted in order to match the growth rate of sow-reared pigs. Body weight and feed intake were recorded daily throughout the trial. Blood samples were collected initially and every 6 days by venipuncture using heparinized tubes (Vacutainer Plus, BD Vacutainer, Franklin Lakes, NJ), and plasma was obtained by centrifugation at 3,500 x g at 4°C. Plasma samples were stored at –20°C until analysis. At the completion of the study, all pigs were given 20mg BrdU/kg body weight orally in a small quantity of their milk replacer 12 hrs before euthanization to allow for adequate incorporation of BrdU in proliferating MSC nuclei. Bone marrow, liver, kidney, and small intestine were harvested from each animal and snap-frozen in liquid nitrogen for subsequent gene expression analysis. Rear-legs were collected and stored at 4°C for peripheral quantitative computed tomography (PQCT) and flexural strength analysis. Front legs were collected for isolation of bone marrow derived MSC.

**Plasma Analysis**

Plasma Ca concentrations were determined by flame absorption spectroscopy (Shimadzu AA6701F, Atomic Absorptiometry Flame Emission Spectrophotometer) following dilution in 0.5% lanthium chloride. Inorganic P concentrations were determined by the method of Gomori (1942) modified for use with a microplate spectrophotometer (Biotek Synergy HT, KC4 Software) (19). Briefly, plasma was deproteinated with 12.5%
trichloroacetic acid and assayed using Elon solution (p-methylaminophenol sulfate). The concentrations of 1,25(OH)₂D₃ and PTH were determined utilizing commercially available kits (IDS, Fountain Hills, AZ and Immutopics, San Clemente, CA, respectively).

**Peripheral quantitative computed tomography**

Ex vivo PQCT (Stratec Medizintechnik, Pforzheim, Germany XCT SA Plus) scans were used to measure bone characteristics of the tibia. The left hind limb was placed into the scanning gantry on a small animal positioning platform. Initial scout scans were utilized at a scan speed of 30 mm/sec to identify the total bone length and distal end plate of the tibia. Scans of the tibia (single axial slices of 0.5 mm thickness, voxel size 0.1 mm, measure diameter 90 mm) were then taken at a translation speed of 10 mm/s at 10% and 50% of the approximated segment length proximal to the distal endplate of the tibia. Image processing and calculation of the various bone indices were performed using the manufacturer’s software package (version 6.0 B). For all scans, a threshold algorithm was used to separate the tibia from the soft tissue background using a <169mg/cm³ threshold. Once separated from the soft tissue background, total volumetric bone mineral density and total bone area were calculated. Trabecular bone in the tibia was identified at the 10% site by a threshold of 650 mg/cm³ and after concentrically peeling off 10% of the total bone voxels. Trabecular and cortical bone properties were assessed at the 10% proximal site using a filter with thresholds between 200 and -100 mg/cm³ and 500 mg/cm³, respectively. The following parameters were assessed at the proximal 10% site: trabecular area (mm²), trabecular content (mg/mm³), trabecular volumetric density (mg/cm³), cortical area (mm³), cortical content (mg/mm³) and cortical
volumetric density (mg/cm$^3$). Cortical bone properties were assessed at the 50% proximal site using a filter with a threshold of 500 mg/cm$^3$. The following parameters were assessed at the proximal 50% site: cortical area (mm$^3$), cortical content (mg/mm$^3$) and cortical volumetric density (mg/cm$^3$). The ability of a bone to resist torsion was also assessed using the polar strength strain index (SSIP). The total and cortical bone SSIP parameters were assessed at the 10% and 50% proximal sites using a threshold algorithm (contour mode 31) with values of < 200 mg/cm$^3$ and <500mg/cm$^3$, respectively.

**Mechanical Testing**

Mechanical load testing was conducted with a three-point bending fixture (Instron Corp., Norwood, MA). The fixture was adjusted to have a 30 mm span between lower supports and load was applied from above at the center of the span. Force was applied by an 858 Mini Bionix II load frame, using a 1.5 kN load cell (MTS Systems Corp., Eden Prairie, MN) in a similar method as previously described (20). The upper loading post was brought into contact with each specimen and ramp loading was applied at 50 mm/minute until failure (21). Displacement and the resulting force were recorded at 10 Hz. Following mechanical testing, width, height, and cortical shell thickness at four locations were recorded for each specimen cross section at the midshaft diaphysis. Maximum force and yield force were determined for each specimen by the force-displacement data recorded during testing. Moments of inertia were calculated as elliptical shells (22). Flexural moduli were calculated using geometry data and linear portions of force-displacement curves. The following three-
point bending equation was used to determine the modulus of elasticity: \( E = \frac{FL^3}{48\delta I} \), where \( E \) is flexural elastic modulus, \( F \) is force, \( L \) is span between lower supports, \( \delta \) is displacement, and \( I \) is cross section moment of inertia (22).

**Gene Expression**

Total RNA was isolated from bone marrow, kidney, liver and the duodenum of the small intestine (RNeasy Midi Kits, Qiagen, Valencia, CA) and genomic DNA was removed by treatment with deoxyribonuclease I according to manufacturer’s instructions (DNA-free kit, Ambion, Austin, TX). Total RNA was reverse-transcribed with Superscript Reverse transcriptase III (Invitrogen Life Technologies, Carlsbad, CA) using both oligo dT (Integrated DNA Technologies, Coralville, IA, USA) and random hexamer primers (Invitrogen Life Technologies) in a 1:1 ratio. The resulting cDNA was then incubated for 20 min at 37°C with RNase H (Invitrogen Life Technologies) to remove residual RNA. Both starting RNA and final cDNA concentrations were quantified using fluorescence-based quantification kits (Invitrogen Life Technologies). Relative quantities of transcripts of interest were determined by semiquantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System, BioRad). Primer oligonucleotides for peroxisome proliferators activated receptor gamma 2 (PPARG), Runt related transcription factor (Runx2), lipoprotein lipase (LPL), Osteocalcin (OC), osteoprotegerin (OPG), RANKL, vitamin D receptor (VDR), calcitonin receptor (CTR), calcium-sensing receptor (CaR), PTH receptor (PTHR), 1α-hydroxylase (CYP27B1), 24α-hydroxylase (CYP24A1) and sodium-phosphate cotransporter
2 (NPT2) were designed using PrimerQuest software (Integrated DNA Technologies; Table 2). Optimal primer concentrations for each primer set were determined prior to quantification by real-time PCR, and the linearity of amplification for each gene of interest over a 2-log range of cDNA concentrations was verified to be similar to that of the control gene, 60S ribosomal RNA. The control gene was verified to be unaffected by dietary treatment.

Reactions were performed using 12.5 µl of 2× SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 200–900 nM of each primer and 100 ng of cDNA in a final volume of 25 µl. All samples were subjected to 40 amplification cycles (one cycle consisted of 30 s of melting at 95°C, followed by 30 s of annealing and extension at 65°C). At completion, all samples were subjected to a melt curve analysis to validate the absence of nonspecific products. A modification of the 2^−ΔCT method was employed to normalize gene expression values prior to statistical analysis (23).

MSC isolation and immunocytochemistry

MSC were isolated as explained previously (6). MSC were plated in basal media (BM) at 10^3 cells/cm^2 in 24 well plates and incubated for 48h prior to fixation for immunocytochemical staining for BrdU. The MSC not utilized for immunocytochemistry were frozen in DMEM + 10% FBS + 5% DMSO and stored in LN2 prior to use.

Cells for immunocytochemical staining were fixed in 0.6% hydrogen peroxide (H_2O_2) in methanol at 4°C for 10 min. After fixation, cells were washed 3 times with PBS at room temperature. DNA was then denatured by incubation in pre-warmed (37°C) HCl for 1 h. The acid was neutralized by 2 washes in 1X TBE (pH 8.5) followed by 3 washes in 1X PBS.
The cells were then incubated with anti-BrdU (1:100 in 0.1% BSA in 1X PBS; G3G4, Developmental Studies Hybridoma Bank, University of Iowa) primary antibody in a humidified chamber at 4°C overnight. The cells were washed three times with PBS followed by incubation with a goat anti-mouse HRP conjugated secondary antibody (1:500 in 1% BSA in PBS; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. After 3 washes with PBS, wells were covered with 0.5 mg/ml of diaminobenzidine (DAB) and 0.02% H2O2 in PBS for approximately 2 min for achieving the desired color intensity. The in vivo rate of cell proliferation was determined by calculating the ratio of stained to unstained cells. A total of 5 fields of view (> 200 cells total) from each of 2 replicate wells per animal were counted and averaged per animal.

**Cell culture in homologous sera**

The MSC isolated from 6 individual pigs (3 Ca adequate and 3 Ca deficient) were thawed, resuspended in BM and individually plated at 10^3 cells/cm^2 in 6 well plates, and given 48 h to attach prior to the initiation of treatments. The MSC from each individual animal were subjected to culture in 4 different media formulations for 6 days. The treatment media was completely changed after 3 days of culture. The sera utilized was obtained by pooling sera collected on day 6, 12, and 18 within dietary treatment (calcium adequate sera (Ca+S) and calcium deficient sera (Ca-S)), filter sterilizing and heat inactivating it (56°C, 30 min) prior to storage at -20°C. The media formulations were as follows: 1) Ca adequate control (CaC) = DMEM + Ca+S, 2) Ca adequate adipogenic (CaA) = DMEM + Ca+S + adipogenic mix (500nM dexamethasone, 0.5mM 3-isobutyl-1-methyl-xanthine, 60μM indomethacin, 50 μM
beta-mercaptoethanol, 1mM sodium pyruvate), 3) Ca deficient control (CdC) = DMEM + Ca-S, 4) Ca deficient adipogenic (CdA) = DMEM + Ca-S + adipogenic mix. After 6 days of culture, cells were utilized for determination of cell proliferation, gene expression and cytochemical staining.

In vitro cell proliferation

Cell proliferation for the in vitro culture study was determined using the CyQuant NF cell proliferation kit according to the manufacturer’s instructions (Invitrogen Life Technologies). Proliferation was measured in duplicate wells/animal/treatment group.

Cytochemical staining

Cells were fixed and stained in duplicate for alkaline phosphatase activity (ALP) and the presence of neutral lipids on day 6. Staining for ALP was accomplished with a commercially available kit (ALP staining kit, Takara Bio, Otsu, Shiga, Japan) according to manufacturer's instructions. Accumulation of neutral lipids was visualized by fixing the cells in 10% formalin and then staining with 0.2% Oil Red O for 1 h, followed by thorough rinsing with water. Cells were counted using 6 fields of views for a total of at least 200 cells counted per well in duplicate wells/animal/treatment group.

Gene Expression

Total RNA was isolated from MSC using the Ambion RNAqueous kit (Ambion, Austin, TX) according to the instructions of the manufacturer and genomic DNA was removed by
incubation with deoxyribonuclease I (DNA-free kit, Ambion, Austin, TX). The generation of
cDNA and analysis of the gene expression of PPAR\(\gamma\), Runx2, OC and LPL were conducted
in the same manner as described for the tissue gene expression work.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (Version 9.1, SAS Institute
Inc., Cary, NC) with media, sera and cell type considered as a fixed effect. Differences were
considered significant at \(P < 0.05\).

**Proteomic analysis**

Proteomic analysis of the pooled serum from Ca adequate and Ca deficient animals
that was utilized in the cell culture studies was carried out by Applied Biomics (Hayward,
CA). Total protein was extracted from the serum samples using 7 M urea, 2 M thiourea, 4%
CHAPS, and 30 mM Tris-\(\text{HCl}\), pH 8.5. After quantification of the extracted proteins, equal
amounts from each sera were labeled with either Cy3 or Cy5 dyes, combined and subjected
to isoelectric focusing (pH 3–10) followed by SDS-PAGE. Images of the gel were obtained
using Typhoon TRIO (GE Healthcare, Piscataway, NJ) and analyzed by Image Quant
software (version 5.0, GE Healthcare, Piscataway, NJ). The fold-changes were calculated
using the DeCyder software (version 6.0, GE Healthcare, Piscataway, NJ). Protein spots of
interest that were differentially expressed between the Ca-adequate and Ca-deficient serum
by greater than 1.3-fold were picked by Ettan Spot Picker (GE Healthcare, Piscataway, NJ)
and subjected to in-gel trypsin digestion, peptide extraction and desalting prior to MALDI-
TOF/TOF analysis (ABI 4700, Applied Biosystems, CA). The NCBI database was searched for protein identification and candidates with protein and ion confidence interval scores greater than 95% were considered significant.

Statistics

Data were analyzed using the GLM procedure of SAS (Version 9.1, SAS Institute Inc., Cary, NC) with dietary treatment considered as a fixed effect. Initial BW was used as a covariate for growth performance data. Differences were considered significant at P < 0.05.

Results

Growth Performance

Dietary Ca concentrations did not affect growth performance. There were no differences seen in the average body weight (Ca adequate 5.75 ± 0.16 kg, Ca deficient 5.65 ± 0.15 kg), average daily weight gain (0.22 ± 0.01 kg/d), average daily feed intake (0.19 ± 0.01 kg/d) and the feed efficiency (1.15 ± 0.04) of the two dietary treatment groups at the end of the study.

Plasma macrominerals and hormones

There were no significant differences in the plasma concentrations of Ca and P throughout the 18d feeding trial (Figure 1). Plasma PTH levels were significantly increased in the Ca deficient animals (P < 0.05) after 18 days of feeding with no significant differences at earlier
time-points (Figure 1). Although not statistically significant, there was a trend for increased 1,25(OH)₂D₃ in the Ca deficient pigs on d18 (P < 0.12).

**Bone integrity**

The tibial bones of pigs receiving the Ca adequate milk replacer had greater BMD and mechanical strength (P < 0.05, Figure 2). There was significantly (P < 0.05) greater BMD in both the trabecular and cortical fractions of the distal tibia as well as a trend (P < 0.1) for increased BMD in the cortical bone at the mid-diaphysis. The cortical content in the distal tibia was also significantly (P < 0.05) increased in the Ca adequate animals. The bones ability to resist torsion, as assessed using the polar strength strain index (SSIp), and flexural strength were also significantly greater (P < 0.01) in the Ca adequate pigs.

**In vivo cell proliferation**

There was a significant difference (P < 0.05) in the percentage of BrdU labeled MSC isolated from humeral bone marrow based on dietary treatment. The piglets fed the Ca adequate milk replacer had almost double the number of actively proliferating MSC as did the pigs fed the Ca deficient diet (Figure 3).

**Tissue gene expression**

In the bone marrow, the mRNA expression of CaR and alpha-1-hydroxylase was increased 6-fold and 5-fold respectively (P < 0.03), in the Ca deficient animals when compared with Ca adequate animals (Figure 4). Both CTR (P < 0.06) and OPG (P < 0.1) showed a trend for
increased levels of transcripts in the Ca deficient animals (Figure 4). The levels of VDR, PTHR, RANKL, Col1A1, PPARG, LPL and OC mRNA in bone marrow were not affected by dietary Ca. There were also no significant differences in the message levels of PTHR and VDR in the liver; and PTHR, VDR, CTR, CaR, NPT2, 1-α-hydroxylase and 24-α-hydroxylase in the kidney for the two dietary groups. In the duodenum, there was a trend (P < 0.1) for elevated CaR mRNA levels in the Ca deficient animals with no significant differences in the expression of VDR, PTHR, Ca ATPase, TRPV6 or calbindin.

**Cell culture**

In vitro cell proliferation

MSC proliferation was significantly affected by treatment (control or adipogenic media) and sera (Ca+S and Ca-S) (Figure 5). Within Ca+S, adipogenic media caused significantly lower proliferating cells compared to control cultures (P < 0.004). Also, Ca+S had greater number of proliferating cells than the Ca-S in control media (P < .005, ~2-fold), but not in adipogenic media. Overall, there was a trend for increased proliferation in the deficient cells (~ 1.5-fold) compared to adequate cells (P = 0.12).

Cytochemical staining

For ALP activity, there was a treatment effect as cells cultured in adipogenic media had more ALP staining than the control (Figure 5, P < 0.001). There was no effect of the sera and the animal type on percentage of ALP+ cells. Based on oil red O staining, regardless of treatment, Ca adequate cells accumulated more lipid when cultured in Ca deficient sera
compared to Ca adequate sera (P < 0.05). Overall, adequate cells had significantly more stained lipid droplets than deficient cells (P < 0.03) and cells grown in the presence of Ca-S had more staining than Ca+S (P < 0.05). Also, there was a trend for adipogenic treatment group having more staining than the control group (P < 0.11).

Gene expression

There was a significant treatment × sera × animal effect for the expression of PPARG as the deficient cells had 2-fold greater expression than the adequate cells when cultured in adipogenic media in the presence of Ca-S (P < .005) (Figure 6). Regardless of media type, deficient cells expressed at least 2-fold higher expression in the Ca-S when compared with Ca+S (P < .02). Deficient cells also had higher levels of PPARG compared to adequate cells in the presence of Ca-S (P < .02). As expected, adipogenic media caused significantly higher expression than the control (P < .0001, 1.5-fold). Also, when cultured in adipogenic media, cells tended to express significantly more PPARG in the presence of Ca-S compared to Ca+S (P < .07). Levels of LPL were not affected by sera type in the deficient cells; however, in adequate cells, Ca+S tended to cause more expression compared to Ca-S (P = .12) (Figure 6). Also, LPL expression tended to be higher in the adequate cells when compared with deficient cells in the control media (P < .12), but not the adipogenic media. Like PPARG, there was an overall treatment effect for LPL as the expression with adipogenic media was 2-fold higher than the control (P < .03). However, this effect varied based on cell type as it was significant in cells isolated from the deficient animals (P < .02), but not the adequate animals. Expression of Runx2 was significantly affected by treatment in the Ca+S, but not Ca-S
(Figure 6). In the presence of Ca+S, there was more expression with the adipogenic media compared to control (P < .02). Also, within adipogenic media, cells cultured in Ca+S expressed atleast 2-fold more Runx2 when compared to Ca-S (P < .01). There was a treatment × animal effect as in the control media, the adequate cells tended to express almost 2-fold greater Runx2 than deficient cells (P = 0.1). For osteocalcin, there was greater expression in the adequate cells when compared to deficient cells in the control (P < .03) media (Figure 6). Also, significantly more osteocalcin was produced by adequate cells in the control media compared to adipogenic media (P < 0.05).

**Proteomic analysis**

The total protein content of the sera from Ca deficient animals was substantially greater than that of the Ca adequate pigs (59 vs. 40 mg/mL). Based on DIGE analysis, 24 proteins were found to be differentially expressed by greater than 1.3 fold (Figure 7). Of these 24 proteins, 22 were identified by MALDI analysis. The proteins identified with greater concentration in the Ca deficient serum were albumin, pre-albumin (transthyretin), alpha-fetoprotein and fetuinA, with transthyretin having the greatest fold increase (2.6 fold). Apolipoprotein A-I, apolipoprotein A-IV and haptoglobin were found at greater concentrations in the serum from Ca adequate animals, with haptoglobin having the largest difference (1.9-fold).

**Discussion**

In the present study, we have examined the effects of dietary Ca deficiency during the early neonatal period by utilizing a physiologically appropriate animal model. Limited research
has been conducted to examine the impact of dietary Ca deficiency in neonates (11, 12) compared with studies employing older animals (24-26). Studies in older animals have clearly demonstrated that inadequate dietary Ca increases serum PTH and 1,25(OH)₂D₃ levels which result in bone loss (2, 27-29). However, the role of PTH/1,25(OH)₂D₃ mediated pathways in mineral homeostasis in not fully elucidated in neonates. The anticipated increase in circulating levels of PTH associated with Ca deficiency was not observed until the end of this study, and in additional contrast with Ca deficiency in older animals, there was no significant increase in circulating levels of 1,25(OH)₂D₃ in our Ca deficient neonates (25). Although these endocrine profiles do not match what would be expected in older animals, previous work in rats has suggested that during the early suckling period, the machinery to utilize 1,25(OH)₂D₃ as an endocrine regulator of Ca homeostasis may be limited if not absent (30, 31). Also, the work of Hsu and Levine (32) suggests blunted production of PTH in newborns. Therefore, the eucalcemia of our Ca deficient neonatal pigs was likely not a result of the classical PTH/1,25(OH)₂D₃ regulatory axis, and certainly warrants future studies to elucidate the mechanism by which Ca is conserved. Despite not observing changes in the circulating levels of classical regulators of Ca homeostasis in Ca deficient animals, the provision of Ca deficient diets affected bone health, as there was a significant reduction in BMD and bone integrity without a reduction in whole body growth or bone longitudinal growth.

Regardless of developmental period and the model utilized, there have been no previously reported studies that have examined the effect of dietary Ca on bone-marrow derived MSC. We have demonstrated in this study that dietary Ca deficiency during the
early neonatal period can result in dramatically reduced MSC proliferation in vivo. Because MSC provide the life-long supply of osteoprogenitor cells and MSC proliferation rates are greatest during early infancy (8), dietary restrictions that impact these cells could have both immediate as well as long-term consequences for bone health. Effects of nutrient restrictions on tissue-specific stem cells have been demonstrated previously with satellite cells whose in vivo activity is altered in response to severe dietary restrictions causing permanent muscle growth deficits (33, 34). The almost 50% reduction in proliferating MSC observed in our Ca deficient animals could have significantly impacted the attainable peak BMD for these animals. In order to evaluate whether this change in MSC activity was due only to the endocrine environment or if the cells had been fundamentally altered (i.e. underwent a change in lineage allocation) we conducted a cell culture study utilizing homologous sera. As expected, the relative abundance of mRNA for both the adipogenic transcription factor PPARG and adipogenic marker LPL were significantly greater in the adipogenic media when compared to the control media. A trend for increased lipid accumulation based on oil red O staining in adipogenic medium was also consistent with this pattern indicating a shift toward adipogenic differentiation. In adipogenic media, deficient cells expressed more PPARG than adequate cells in the presence of Ca-S. This 3-way interaction suggests altered MSC potential based on the interaction of differentiation agents, serum markers and inherent cell potential. The greatest differences in expression were found within the adipogenic media. Evidenced by increased expression of PPARG and an almost 2.5-fold reduced expression of osteogenic transcription factor Runx2, we hypothesize a shift of MSC pool away from osteogenesis and toward an adipocytic phenotype in the presence of Ca-S in the adipogenic media. Since the
bone loss seen during aging has been attributed in part to an increase in adipocytic and a concomitant decrease in osteogenic differentiation of MSC (35), the inverse relationship between Runx2 and PPARG may be associated with the reduced bone strength in Ca deficient animals observed in this study. In support of above hypothesis, increased lipid accumulation in the presence of Ca-S when compared with Ca+S, regardless of media type, also suggests Ca-S induced adipogenic differentiation. Significant reduction in the number of proliferating MSC when cultured in Ca-S is also supportive of increased MSC differentiation and matches well with the reduced in vivo MSC proliferation in the Ca deficient animals. This highlights the importance of the endocrine environment in determining the fate of bone-marrow derived MSC that can alter neonatal bone development. Interestingly, the osteogenic transcription factor Runx2 was elevated in the adipogenic media when compared with control media. Runx2 expression has been shown to precede osteoblast differentiation in vivo (36) and the correlation between Runx2 mRNA/protein levels and expression of its target genes specific to osteoblast differentiation have been poorly correlated (37, 38). Therefore, increased expression in adipogenic media may have been restricted to mRNA or unmodified protein levels. It is also possible that the adipogenic induction reagents may not have been sufficient to drive all differentiation toward adipogenesis in the presence of pig serum factors. The interaction of these chemicals utilized for adipocytic differentiation with one or more differentially expressed factors in the serum based on dietary differences may have pushed a small MSC population toward an osteogenic phenotype. This is supported by increased ALP activity of cultured cells in the presence of adipogenic media. However, there was no change in the expression of late osteoblast differentiation marker osteocalcin with
adipogenic treatment suggesting the absence of mature osteoblasts. This is expected due to the short duration of culture in our study. Osteocalcin expression was elevated 5-fold in the adequate cells when compared with the deficient cells in control media. This in agreement with the in vivo data indicating stronger bones in Ca adequate animals. Higher Runx2 expression in adequate cells cultured in control media is again consistent with our hypothesis and is suggestive of increased osteogenic differentiation in adequate animals.

While the effects observed in our cell culture experiments that were based on the Ca status of the host animals support developmental programming of bone by dietary Ca, differences caused solely by the Ca status of the sera were of particular interest. Because the Ca adequate and Ca deficient sera utilized in the cell culture study were a compilation of samples obtained at days 6, 12, and 18 of this study, there were no differences in Ca, P, PTH, or 1,25(OH)₂D₃ concentrations that could explain the observed sera effects. In order to better characterize the factors that may have been responsible for such effects, we conducted a proteomic analysis of the sera. Twenty-two proteins were found to vary by greater than 1.3 fold and the sera themselves differed in overall protein content by almost 1.5 fold. While there was a variety of the types of proteins that varied in their expression, albumin, its precursor transthyretin (prealbumin), and its fetal homologue α-fetoprotein had increased expression in the sera obtained from the Ca deficient animals. Although albumin is abundantly found in the serum and has many functions, it has been shown to impact bone. Albumin induces bone resorption in vitro in rats (39), and exerts anabolic effects on bone (40). Expression of MSC differentiation markers PPARG (41) and Runx2 (42) examined in this study have also been shown to be reduced in vitro in the presence of albumin. While its
effects on bone are unclear, albumin could affect MSC differentiation. Interestingly, profetal, a drug based on α-fetoprotein has been shown to decrease stromal precursor cell colony formation in the bone marrow (43). It is possible to hypothesize that the decrease in vivo MSC proliferation observed with dietary Ca deficiency as well as in vitro reduction in the number of proliferating cells cultured in the presence of Ca deficient sera, may be caused by the increased α-fetoprotein levels. The increased circulating levels of IgG4, clusterin and mannan-binding lectin A in the Ca deficient animals could be suggestive of an inflammatory response to lower dietary Ca levels. Many of the proteins affected by dietary Ca that are discussed, are acute phase proteins whose levels are sensitive to stress, inflammation or disease. Modified levels of such proteins may be a direct outcome and an indicator of altered levels of cytokines or growth factors that may directly affect bone or MSC differentiation. In contrast, these proteins may also directly alter the cytokine/growth factor levels that affect bone physiology. Further studies are warranted to examine the role of these proteins in altering MSC mediated bone formation. Higher levels of fetuin A during dietary Ca deficiency maybe a feedback mechanism to increase bone formation since this protein is among the most abundant non-collagenous proteins of the bone (44, 45). Transferrin, whose levels were elevated in the serum of Ca adequate animals, has been shown to increase the formation and activity of both osteoblasts and osteoclasts by increasing mitochondrial biogenesis (46). Lipoproteins with greater expression in the serum of Ca adequate animals were ApolipoproteinA-I (ApoAI) and ApolipoproteinA-IV (ApoAIV). They are important constituents of HDL, which has been associated with increased bone formation and inversely correlated with risk of osteoporosis (47-49). This suggests a positive role of Apo-AI and
Apo-A-IV in bone formation that may contribute to differences in bone integrity and MSC differentiation observed in this study. The total protein differences (Ca deficient > Ca adequate) observed in the serum of animals from the 2 dietary groups may have been due to the increase in expression of major serum proteins like albumin and its precursor in the serum derived from Ca deficient animals. Regardless, the differential proteomic profile of the 2 sera highlights the impact of dietary Ca levels in regulating levels of proteins in the blood that maybe critical to bone strength and BMD either independently or in association with MSC differentiation potential.

We also assessed the physiological implications of dietary Ca modulation by studying the expression of relevant genes in bone marrow, small intestine, liver and kidney. Increased expression of CaR was observed in both the bone marrow and small intestine of the Ca deficient animals. Increased CaR levels in osteoblasts, osteoclasts or stromal cells have been previously shown to affect bone formation by Ca-mediated increased osteoblast and stromal proliferation (50) and inhibition of osteoclast formation (51). Therefore, elevated CaR levels in the bone marrow of Ca deficient animals may be a feedback mechanism to increase bone formation by the action of Ca. Also, CaR is expressed in the intestinal villus and crypt cells that participate in Ca absorption in the gut (52). Since we did not observe any significant differences in the circulating levels of 1,25(OH)2D3 throughout the study and PTH did not change until the end of the study, increased CaR levels in the duodenum of the deficient animals may be responsible for similar plasma Ca levels for adequate and deficient animals by causing increased Ca uptake. Since calcitonin has been shown to inhibit bone resorption (53) and increase bone formation (54), elevated CTR in the Ca deficient animals
may again be a feedback mechanism for calcitonin induced bone formation. Elevated OPG levels in the bone marrow of Ca deficient animals may also be a feedback response to minimize bone resorption by known functions of OPG that include inhibition of osteoclast formation (55). While the presence of 1α-hydroxylase in the kidney is necessary for synthesis of 1,25(OH)₂D₃ to mediate its effects as an endocrine regulator of Ca and P homeostasis (56), expression of 1α-hydroxylase has also been detected in skeletal tissue that in turn has led to the suggestion that 1,25(OH)₂D₃ has autocrine and/or paracrine function in bone marrow independent of circulating 1,25(OH)₂D₃ (57). Again, since we did not see a rise in circulating levels of 1,25(OH)₂D₃, the huge spike in the expression of 1α-hydroxylase could be a potential mechanism of increased local synthesis of 1,25(OH)₂D₃. Such locally produced 1,25(OH)₂D₃ could directly alter MSC activity in the bone marrow.

In summary, dietary Ca deficiency during the early neonatal period has significant effects on bone that do not appear to be mediated by the classical Ca homeostatic regulation through the PTH/1,25(OH)₂D₃ axis. We have demonstrated that this early life dietary Ca deficiency dramatically altered the in vivo activity of bone marrow derived MSC. Our in vitro studies also suggest that the MSC isolated from the Ca deficient animals may have been fundamentally altered, providing strong evidence for developmental programming of bone by early life Ca nutrition. With increasing support for viewing osteoporosis as a pediatric disease with elderly onset, as well as increasing evidence of altered MSC lineage allocation causing the reduced bone formation and concomitant increase in bone adipose content seen with aging and osteoporosis, understanding the importance of early life nutrition on the
programming of MSC is critical. This study highlights the importance of Ca nutrition during neonatal bone development in this regard and these results warrant the further examination of dietary Ca recommendation of human neonates to assure maximum bone growth, particularly those born prematurely or at a low-birth weight.

Literature Cited


Table 1. Experimental diet composition

<table>
<thead>
<tr>
<th>Ingredients, %</th>
<th>Basal Diet(^1)</th>
</tr>
</thead>
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<tr>
<td>Sodium caseinate</td>
<td>11.25</td>
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<tr>
<td>Delactosed whey</td>
<td>14.32</td>
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<tr>
<td>Potassium phosphate</td>
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<tr>
<td>Calcium carbonate</td>
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<tr>
<td>12/28 Milk Replacer fat</td>
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<tr>
<td>13/60 Milk Replacer fat</td>
<td>14.62</td>
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<tr>
<td>Vitamin premix(^2)</td>
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<tr>
<td>Prestarter mineral(^2)</td>
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</tr>
<tr>
<td>Methionine</td>
<td>0.49</td>
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<tr>
<td>Potassium sorbate</td>
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</tr>
</tbody>
</table>

Analized\(^3\)

| Crude protein, %            | 27.69            |
| Crude fat, %                | 18               |
| Gross energy, Kcal/Kg       | 4717.61          |
| Lactose, %                  | 36.37            |
| Delactosed whey, %          | 20               |
| Ca, %                       | 0.59             |
| P, %                        | 0.77             |

\(^1\)Diet met NRC requirements
\(^2\)Milk Specialties, Dundee, IL
\(^3\)Provided all vitamins (Vitamin A, Vitamin D\(_3\), Vitamin E, Vitamin K, thiamin, riboflavin, pyridoxine, Vitamin B\(_12\), pantothenic acid, niacin, folic acid, ascorbic acid, biotin) and minerals (Na, Cl, K, Mg, S, Cu, Zn, Se, Co, I, Fe, Mn), with the exception of calcium and phosphate at levels to meet or exceed the NRC requirements.

\(^3\)Milk Specialties, Dundee, IL
Table 2 Primer sequences for real-time PCR

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<th>Primer</th>
<th>Sequence</th>
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<td>PPARG</td>
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<tr>
<td></td>
<td>R: 5' TGTCTTTGAATGTCCTCGATGGGCT 3'</td>
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<tr>
<td>Runx2/CBFA1</td>
<td>F: 5' CAAGTGCGGTGCAAATTTTCTCCA 3'</td>
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<td></td>
<td>R: 5' AGGCTGTGGTGATGCCATAGTCCCT 3'</td>
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<tr>
<td>Osteocalcin</td>
<td>F: 5' TACCAGATCCTTGAGGCC 3'</td>
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<td></td>
<td>R: 5' TATGCCATAGAAGCGCCGATA 3'</td>
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<td></td>
<td>R: 5' ACTTTGTAGGCCATCTGAGCACGA 3'</td>
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Table 2 Continued
Figure 1. Effect of dietary Ca on (A) plasma inorganic P concentrations and (B) plasma Ca concentrations, (C) plasma PTH concentrations, and (D) plasma 1,25(OH)₂D₃ concentrations. Values presented are least square means and standard error. Ca adequate, n = 7; Ca deficient, n = 7. *Indicates significant treatment effect (P < 0.05).
**Figure 2.** Effect of dietary Ca on tibial bone integrity. Ex vivo peripheral quantitative computed tomography scans were used to measure A) Bone area, B) BMC (bone mineral content) and C) BMD (bone mineral density) at the 10% site of the bone and, D) Bone Area, E) BMC (bone mineral content) and F) BMD (bone mineral density) at the 50% site of the bone. Three-point bending test was used to calculate G) Polar strength strain index, and H) flexural strength. Values presented are least square means and SE. Ca adequate, n = 7; Ca deficient, n = 7. *Indicates significant treatment effect (P < .05).
Figure 3. **Effect of dietary Ca on the proliferation of MSC in vivo.** Mesenchymal stem cells isolated from humeral bone marrow were fixed and immunocytochemically stained for BrdU. Percentage of positively stained cells is based on 5 fields of view per replicate well per animal. Values presented are least square means and SE. Ca adequate, n = 7; Ca deficient, n = 7. *Indicates significant treatment effect (P < .05).
**Figure 4. Effect of dietary Ca on gene expression in bone marrow.** Gene expression was measured using semi-quantitative real-time PCR. Expression of A) CaR (p<0.05), B) CYP27B1 (p<0.05), C) CTR (p<0.05) and, D) OPG (p<0.1) in the bone marrow was increased in the Ca adequate animals. Values presented are least square means and standard error. Ca adequate, n=6; Ca deficient, n=6. *Indicates significant treatment effect (p<0.05)
Figure 5. Effect of homologous sera and cell type on expression of adipogenic and osteogenic markers in MSC. Cells from Ca adequate or Ca deficient animals were cultured in either control or adipogenic differentiation media utilizing serum from either calcium adequate or the calcium deficient animals. Expression of PPARG (trt p<.0001, trt*cell p<.05, sera*cell p<.01, trt*sera*cell p<.11), Runx2 (trt*sera p<.03), LPL (trt p<.0001, sera p<.08) and OC (cell p<.03) was measured using semi-quantitative real-time PCR. Values presented are least square means and standard error. Ca adequate, n=4; Ca deficient, n=4.
Figure 6. Effect of homologous sera and cell type on proliferation and cytochemical staining of MSC. MSC proliferation (trt P < .0001, sera P < .001, cell P < .011), ALP activity (trt P < .001) and Oil Red O staining (trt P < .01, sera P < .01, cell P < .002) were measured. Proliferation is based on fluorescence measured in duplicate wells per animal per treatment group. Oil Red O was extracted from duplicate wells per animal per treatment group and values were normalized to proliferation data. Values presented are least square means and standard error. Ca adequate, n=4; Ca deficient, n=4.
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**Figure 7.** Proteomic analysis of sera from Ca adequate and Ca deficient neonatal pigs. Differentially expressed proteins in homologous sera were examined by 2-D DIGE analysis. Protein spots with greater than 1.3 fold differential expression are labeled in the merged image. Proteins identified by MALDI-TOF/TOF analysis are listed in table. M.W=molecular weight, P.I = isoelectric point. C.I.= confidence interval. Negative fold change indicates more expression in Ca deficient sera. Protein score C.I. > 95% are considered significant.
CHAPTER 6
CONCLUSIONS

Collectively, the data from these studies indicate that mineral (calcium and phosphate) nutrition and calcitropic hormones can have a tremendous impact on neonatal bone health in part due to the altered MSC activity. Because PTH and 1,25(OH)₂D₃ levels in vivo are altered in response to mineral deficiency and are known to directly affect bone growth, altered MSC activity seen in response to treatment with calcitropic hormones in vitro can be of physiological significance. Based on the increased adipogenic differentiation of MSC due to physiologically relevant 1,25(OH)₂D₃ levels in vitro, similar effects in vivo could cause reduced bone formation. This is because bone loss seen with aging and osteoporosis is often attributed to increased marrow adiposity that in turn is caused by increased adipogenic differentiation of MSC at the expense of osteogenic differentiation (Beresford et al., 1992). The 1,25(OH)₂D₃ has been shown to have pleiotropic effects on bone as its increased levels in response to mineral deficiency has been shown to cause bone resorption while on the other hand it has been supplemented into diet for treatment of osteoporosis. Therefore, altered MSC activity can be one of the modes of 1,25(OH)₂D₃ mediated changes in bone characteristics and understanding such effects can help formulate 1,25(OH)₂D₃ levels in diet suitable for optimum bone growth. The variable effects of PTH on MSC in vitro depending on the mode of treatment again can be physiologically relevant because PTH has been shown to exhibit antagonistic effects on bone in vivo depending on the mode of treatment. Increased bone mass and strength when administered intermittently
and enhanced bone resorption and hypercalcemia due to continuous infusion may in part be
due to the PTH mediated effects on MSC activity. Therefore, in addition to bone resorption,
increased adipogenic expression caused by continuous administration of cells with PTH in
our study may result in increased marrow adiposity that can serve as an additional
mechanism for reduced bone formation. Although the synergistic effects of PTH and
1,25(OH)₂D₃ on bone resorption during Ca deficiency are well characterized, their interactive
effects on MSC activity has not been examined previously. It is important to understand the
physiological interaction of these hormones as determinants of bone formation. The
antagonistic effects of PTH and 1,25(OH)₂D₃ on MSC activity seen in vitro in our study
suggests that therapeutic treatments for improving bone formation including a combination
of PTH and 1,25(OH)₂D₃ need to be assessed for their in vivo effects on MSC activity in
order to formulate an optimum dose. Further studies looking into the in vivo effects of PTH,
1,25(OH)₂D₃ individually and in combination on MSC activity are warranted. Dietary
reduction in P levels caused a dramatic reduction in MSC proliferation in vivo that can have
tremendous impact on bone health and may have been associated with reduced bone growth
that resulted from dietary P deficiency. The reduced mRNA levels of osteocalcin coupled
with increased adipogenic expression is suggestive of a shift towards increased adipocytic
differentiation at the expense of osteogenic differentiation from MSC. Such a shift in the
lineage allocation of MSC maybe directly associated with reduced bone growth and bone
mineral content seen in piglets fed P deficient diet. Also, P homeostasis may be differentially
regulated in the neonate based on unaltered 1,25(OH)₂D₃ levels in circulation and the
possibility of local synthesis of 1,25(OH)₂D₃ in the bone marrow, which in turn may favor
adipocytic differentiation of MSC based on our in vitro results, thus reducing bone formation. Further studies to elucidate the mechanism of P deficiency mediated alteration in MSC lineage allocation as well as validation of local synthesis of 1,25(OH)2D3 are warranted. Effects of Ca deficiency on bone development during the neonatal period are less characterized. Delayed increase in PTH levels accompanied with unaltered Ca, P and 1,25(OH)2D3 levels in vivo in neonatal piglets fed Ca deficient diet is suggestive of altered Ca homeostasis mechanisms during early postnatal growth. Therefore, reduced bone strength and bone mineral density seen with dietary Ca deficiency in this study may not be attributed to bone resorption mediated by calcitropic hormones. Significant reduction of proliferating MSC in vivo seen in Ca deficient animals as well as altered MSC differentiation potential in vitro suggest that altered MSC activity may have in part contributed to the differences in bone parameters seen in this study based on dietary Ca levels. Also, the in vitro altered MSC activity based on differences in the cell type and sera utilized suggest that MSC may have been fundamentally altered and/or its activity may have been affected by the endocrine environment in vivo depending on dietary Ca levels of the animals. Such changes in MSC proliferation and lineage allocation may have resulted due to the effects of one or more of the differentially expressed proteins identified by the proteomic analysis of the homologous sera. Further studies to examine the individual effects of the differentially expressed proteins on MSC activity are warranted. Therefore, this study highlights the importance of Ca nutrition during neonatal bone development and warrants further examination of dietary Ca recommendation of human neonates to assure maximum bone growth, particularly those born prematurely or at a low-birth weight.
The essential role of MSC activity for skeletal tissue growth and development suggests the importance of determining the impact of mineral nutrition on progression of these cells. Because both Ca and P have been shown to result in marked reductions in skeletal tissue growth and bone mineralization, it is important to investigate the possible impact of these essential dietary components on MSC activity in early life when these bone-forming stem cells are most abundant and actively proliferating. Because PTH and 1,25(OH)₂D₃ are primary regulators of mineral homeostasis, their impact on MSC activity is of significance. Our studies suggest that dietary P and Ca status as well as circulating levels of calcitropic hormones during early post-natal development can have tremendous impacts on subsequent growth potential that can significantly impact both human health. It is valuable to evaluate their effects on bone development in neonates because of the rapid bone mass acquisition rates (Holick and Dawson-Hughes, 2004), highest number of proliferating MSC (Stenderup et al., 2003) as well as increasing evidence for link between bone quantity and quality achieved in infancy and osteoporosis. Appropriate dietary interventions for optimizing early skeletal growth can reduce the risk of fracture in later life and help prevent osteoporosis. It can be especially important for premature infants or those born at low-birth weight for maximizing BMD and bone integrity since they have reduced BMC compared to those born at term (Fewtrell et al., 1999). Swine production can also be improved as dietary interventions for low birth weight piglets can improve growth performance. Therefore, such studies can lead to strategies for maximizing bone integrity, prevention of bone-diseases like osteoporosis as well as development of MSC as therapeutic tool for cell and gene therapy.
Further work is needed to elucidate the mechanisms for mineral nutrition mediated effects on bone development, as well as the effects of these minerals and calcitropic hormones in altering MSC activity.

Literature Cited


