

ABSTRACT

ALEXANDER, LINDSEY. Dietary Phosphate Influences Satellite Cell Activity and Subsequent Growth Potential in the Neonatal Pig. (Under the direction of Dr. C. H. Stahl.)

Satellite cells and mesenchymal stem cells (MSC) are critical to the growth and development of muscular and skeletal tissues. Satellite cells provide the myonuclei that allow for muscle fiber hypertrophy and MSC supply the osteoprogenitor cells responsible for skeletal growth. Despite the impact of dietary phosphate (PO_4) on muscular and skeletal tissue growth, relatively little research has examined the impact of PO_4 nutrition during the neonatal period when there is the highest number of satellite cells in muscle and MSC in bone marrow. The objective of the following experiments was to examine the possibility of nutritional programming of tissue specific stem cell activity by dietary PO_4 .

In the first experiment 20 piglets obtained at 24 h of age, were allotted into 1 of 2 treatment groups and were pair-fed, a soy-based liquid milk replacer that either met PO_4 requirements or was approximately 25% deficient in available PO_4 over 15 d. Lower sera PO_4 and PTH concentrations were observed ($P < 0.05$) in PO_4 restriction. Phosphate deficiency reduced growth ($P < 0.05$), feed efficiency ($P < 0.05$), bone mineral content ($P < 0.05$) and stem cell proliferation ($P < 0.05$). Dietary PO_4 impacted expression of genes associated with adipogenesis and osteogenesis in bone marrow and those associated with vitamin D metabolism in the kidney. We demonstrated that dietary PO_4 deficiency reduces in vivo proliferation of stem cells, suggesting that dietary PO_4 during early post-natal development can impact future growth potential by influencing stem cell activity.

The PO_4 requirement of the neonatal pig is unknown, therefore a second experiment was performed to determine how differing inclusion levels of PO_4 into the neonatal diet

impacted stem cell kinetics. Twenty-one male and female piglets were obtained at 24 h of age and allotted into 1 of 3 dietary treatment groups. Pigs were pair-fed a milk-replacer diet that was either PO₄ adequate, 25% in excess of the PO₄ requirement, or 25% deficient in PO₄ content for 12 d. Excess dietary PO₄ increased sera PO₄ and PTH ($P < 0.05$) concentrations and improved ($P < 0.05$) feed conversion efficiency. Dietary PO₄ deficiency resulted in wider bones ($P < 0.05$) with lesser dry matter percentages ($P < 0.05$). Excess dietary PO₄ increased proliferation of MSC ($P < 0.05$) but not satellite cells. Expression of those genes associated with osteogenesis in bone marrow and Ca and P regulation in the thyroid were affected by dietary PO₄. It was determined that access to provision of PO₄ that is more available and in excess of what is supplied by the sow may have potential beneficial effects on stem cell activity and subsequent growth.

In the final experiment, satellite cells isolated from pigs in Experiment 2 were cultured in proliferating and differentiating media over 7 d to determine the impact of dietary PO₄ on satellite cell progression through the myogenic lineage. Increased proliferation was noted in cells from PO₄ excess pigs. Immunofluorescent staining for Pax7, MyoD, and myogenin demonstrated increases in positive staining for all 3 proteins between 3 d and 5 d, but decreased by 7 d. Cells isolated from PO₄ excess pigs had greater ($P < 0.05$) DNA:protein content at 5 d, though DNA:protein content was higher in cells from PO₄ deficient pigs by 7 d. . Increased MyoD and myogenin gene expression ($P < 0.05$) were noted at 7 d in cells from PO₄ deficient pigs. These data are suggestive of possible premature differentiation of satellite cells during PO₄ restriction.

From these experiments it can be concluded that early life dietary PO_4 influences tissue specific stem cell activity by triggering a nutritional programming event that permanently alters the behavior of these cells. Gaining an understanding of how early life nutrition impacts muscle and bone growth will allow us to clarify the possible long-term effects on growth potential in the pig.

Dietary Phosphate Influences Satellite Cell Activity and Subsequent Growth Potential in the Neonatal Pig

by
Lindsey Sharone Alexander

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APPROVED BY:

Dr. Chad H. Stahl
Committee Chair

Dr. Jack Odle

Dr. Paul E. Mozdziak

Dr. Jerry Spears

BIOGRAPHY

Lindsey Alexander was born on June 29, 1983 in Chicago, IL to Bryant and Sharon Alexander. She received a B.S. in Animal and Poultry Sciences in 2005 from Tuskegee University in Tuskegee, AL. She went on to Iowa State University in Ames, IA where she earned an M.S. in Animal Science under the direction of Dr. Chad H. Stahl. In 2007, she pursued her Ph.D. in Animal Science at North Carolina State University in Raleigh, NC.

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CHAPTER I: General Introduction

Lower production costs and the need of the consumer for high quality pork with minimal fat have driven the swine industry to produce pigs with improved lean growth efficiency. Efficient lean growth is characterized as rapid muscle weight gain with minimal fatty tissue accretion. A major determinant of muscle mass is muscle fiber number (1, 2), which is established in utero. Since muscle fiber number is determined prenatally restricted maternal nutrition and increased intrauterine crowding are believed to negatively impact muscle development (3-5), consequently lowering individual birth weights within a litter. Multiple studies have demonstrated the positive correlation between fiber number and growth rate (6, 7) noting that nutritional interventions in later life cannot overcome the damaging effects of lower birth weights on growth performance (8). However, many attempts have been made to maximize lean growth in the neonatal phase (9), when the majority of mass acquired is skeletal muscle. Postnatal muscle growth occurs through hypertrophy of existing fibers which is achieved through the myonuclear donation of satellite cells. *In vivo* reductions in satellite cell number, whether through nutrient restriction or mechanical stress, have been shown to adversely affect muscle growth by inhibiting increases in myonuclear number and fiber volume. These data demonstrate the crucial role of satellite cells in postnatal muscle growth.

Although dietary phosphate (PO_4) influences muscular growth and early life PO_4 nutrition has been shown to have a greater impact on growth than later in life (10), relatively little research has examined the impact of PO_4 nutrition on satellite cell activity during the neonatal period when there is the highest proportion of satellite cells in muscle and any

effects of altered nutrition would be more pronounced. Understanding the role dietary PO_4 may play in the developmental programming of muscle tissue is of great concern to animal agriculture. The increased cost of dietary PO_4 supplements and the need to reduce PO_4 excretion from animal production has driven research toward developing strategies to minimize the environmental impact of swine production while maintaining the production efficiency. By gaining a better understanding of how early life PO_4 nutrition impacts muscle growth we can clarify the possible long-term effects on growth potential as well as develop strategies to reduce PO_4 excretion while maximizing production efficiency.

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CHAPTER II: Literature Review

Muscle Growth and Development

Prenatal myogenesis and postnatal growth performance

Within the United States, pork ranks third in annual meat consumption behind poultry and beef (1) and remains one of the preferred meats in the world, accounting for approximately 50% of daily meat protein intake worldwide (1, 2). Lower production costs and the need of the American consumer for high quality pork with minimal fat have driven the swine industry to produce leaner pigs with greater feed conversion efficiencies. The application of various nutritional strategies and selective breeding techniques has allowed for the production of pigs with high lean efficiency resulting in improved growth rates and carcass composition traits.

Efficient lean growth is characterized as rapid muscle weight gain with minimal fatty tissue accretion. A major determinant of muscle mass is muscle fiber number (3-5), which is determined in utero, where the development of skeletal muscle begins. As thoroughly reviewed by Du, within the embryo, mesenchymal stem cells receive signals to commit to the myogenic lineage (6, 7) through the Wnt and Sonic hedgehog signaling pathways. These pathways mediate expression of Pax3, Pax7, and Gli, which further direct the expression of the muscle regulatory factors (MRFs), proteins whose expression are indicative of myoblast proliferation which will lead to eventual myotube formation and fusion (7).

Embryonic myogenesis is a biphasic process (8, 9) wherein multinucleated primary muscle fibers are formed during its initial stages and a second longer stage results in the formation of secondary muscle fibers that proliferate and fuse on the surface of the primary

fibers. Secondary fiber hyperplasia begins approximately 50 days following conception (9-11, Figure 1) and any reduction in total fiber number is believed to be the result of a reduced secondary fiber population. While primary fibers tend to be more resistant to nutritional alterations, studies in various species have shown that secondary fibers are more sensitive to changes in nutrition (12-14).

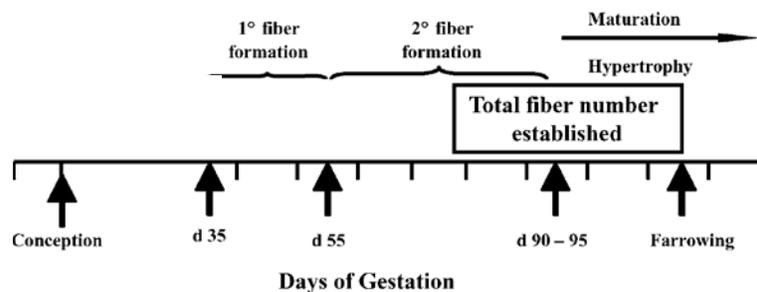


Figure 1. Gestational muscle fiber formation in the pig (Foxcroft, GR et al. 2006. J. Anim. Sci.)

Because muscle fiber number is determined prenatally and multiple studies within various species have demonstrated the positive correlation between fiber number and growth rate (10, 15-17), maternal nutrition is thought to influence fetal muscle development and subsequently affect postnatal growth performance. In guinea pigs, Ward and Stickland (18) demonstrated the differential effects of maternal nutrition on different muscle types based on the proportion of secondary fibers present. Maternal nutrient restriction resulted in fetuses with decreased fiber number in those muscles with a greater quantity of secondary fibers while undernutrition had no impact on primary fiber number. Dwyer et al. (10) found that increasing maternal feed intake in the sow for 30 days post conception improved the mean number of secondary fibers formed within a litter. In the previous year Dwyer et al (17) demonstrated the impact of muscle fiber number on postnatal growth performance. High muscle fiber number was associated with increased growth rate and improved feed

conversion efficiency in later life when it is thought that growth is more impacted by genotype (19). These results are in agreement with work that shows that pigs selected for fast growth tend to be more efficient at feed conversion and contain less fat, even at equivalent live weights of those pigs with lesser fiber numbers (10, 20-22). However, it should be noted that additional studies have displayed differences in fetal muscle development in response to maternal nutrition without corresponding disparities in later life growth performance. Despite differences in total fiber number, Cerisuelo et al. (23) demonstrated that offspring of sows that received additional feed mid-gestation only exhibited differences in growth and feed efficiency in the nursery phase. These differences were not apparent by the grower-finisher phase, a point when the aforementioned studies have described the greatest correlation between growth performance and muscle fiber number (17, 19). Similarly, Nissen et al. (24) found that increased feed intake by sows during early to mid-gestation did not significantly increase fiber number or fiber area in either low or high birth weight offspring. Moreover, additional maternal feed intake resulted in lower muscle deposition rates, an effect which was more pronounced in lighter birth weight pigs.

Although the role of maternal nutrition in intrauterine programming of fetal myogenesis is disputed, uterine crowding is also thought to influence fetal muscle fiber type and number. Foxcroft et al. (25) reviewed the impact of intrauterine growth retardation or “runting” on pre- and postnatal growth, suggesting that uterine capacity can affect developmental potential. Town et al. (26) performed unilateral oviduct ligation to reduce viable embryos in utero in sows and compared fetal muscle development to fetuses of unmodified sows. Muscle weight, muscle cross-sectional areas and secondary fiber numbers

were reduced in unmodified sows, substantiating the negative impact of intrauterine crowding on fetal myogenesis. These data imply that crowding within the uterus contributes to intra litter variations in birth weights and the runt pigs within a litter. Nissen et al. (27) demonstrated that intra litter differences in daily gain, muscle mass, and muscle deposition were positively correlated to starting birth weights. Similar results were illustrated by Tristan et al. (28) between litters.

The extent to which maternal nutrition and intrauterine crowding contribute to programming of fetal myogenesis is unclear. Still, these data suggest that the impact of each is far more pronounced in smaller fetuses, resulting in lower birth weights and poorer growth performance relative to their larger littermates. Nutritional interventions in the grower-finisher phase, where muscle fiber number is thought to have the greatest association with rate of growth, have attempted to make gains in lean muscle accretion to overcome the negative impact of lower birth weights. Bee (22) found that growth rates of low birth weight barrows with ad libitum access to feed did not differ from their high birth weight counterparts; however, low birth weight animals were less efficient at feed conversion due to increased intake. Additionally, more fat deposition was noted in the lower birth weight animals, indicative of the restricted lean muscle accretion that occurs in low birth weight pigs relative to high birth weight pigs at similar body weights. These results indicate that nutritional interventions implemented later in life cannot overcome the detrimental effects of low birth weight on muscle development.

The neonatal phase is one in which the majority of mass acquired is skeletal muscle. Rapid skeletal muscle growth in the neonate is a result of an increased rate of protein

synthesis; however, this phenomenon decreases with age. Multiple studies conducted by the Davis lab (29-31) have validated the increased response of protein synthesis in skeletal muscle during the neonatal period which permitted more efficient utilization of amino acids for growth. Attempts to improve growth during the neonatal phase have led to the use of manufactured liquid diets. Many studies have (32, 33) demonstrated the efficacy of feeding a manufactured liquid diet to improve growth performance in neonatal pigs, with corresponding increases in protein deposition, illustrating the importance of neonatal nutrition in maximizing muscle growth.

Satellite cells and myogenic progression

Muscle fiber hyperplasia terminates in utero, therefore postnatal growth is dependent upon the hypertrophy of existing fibers. With increases in fiber size are corresponding increases in DNA content through myonuclear addition (34-36). Because myonuclei are post mitotic, nuclear addition to fibers is accomplished through the activity of satellite cells. Initially identified in 1961, satellite cells mediate skeletal muscle regeneration and hypertrophy. Located between the sarcolemma and basement membrane of terminally differentiated muscle fibers, once activated, satellite cells will proliferate, differentiate and fuse to existing fibers and donate nuclei to the growing myofiber. Because each myonucleus within a fiber regulates gene transcription and protein synthesis within a finite volume of cytoplasm, the addition of myonuclei is necessary for an increase in fiber size (Figure 2).

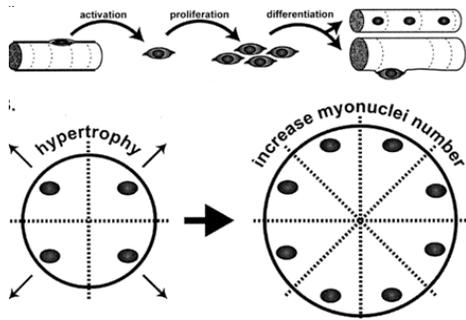


Figure 2. Muscle fiber hypertrophy (Hawke, T.J. 2005. Exerc. Sport. Sci. Rev.)

Commitment of satellite cells down the myogenic lineage is regulated in both embryonic myogenesis and in adult skeletal muscle by a group of transcription factors that function to signal activation and proliferation of satellite cells, followed by differentiation. Expression of paired box transcription factors (Pax3, Pax7) identifies satellite cells as such and functions as a marker of quiescence or activation. Expression of either paralogue varies based on muscle type (Figure 3). Pax3 and Pax7 also serve as upstream regulators of members of the muscle regulatory factor (MRF) family (37, 38). Muscle regulatory factors, Myf5 and MyoD act as myogenic determinants during embryonic development, while myogenin and MRF4 function in differentiation of myoblasts in later life. Muscle progenitor cells of mouse embryos lacking Pax3 and Pax7 demonstrated major skeletal muscle deficits and a lack of activation of Myf5 and MyoD, preventing the cells from entering the myogenic program, resulting in embryo death (39). Overexpression of Pax3 also prevents Myf5 and MyoD expression, seemingly arresting satellite cell progression by keeping cells in a quiescent state (40-42).

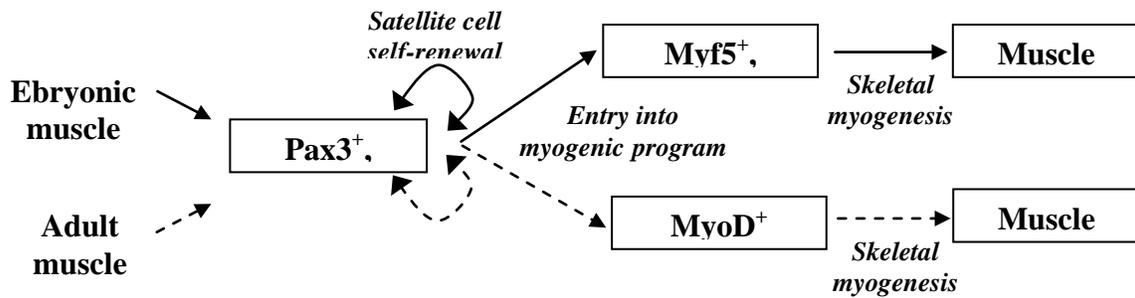


Figure 3. Pax3 and Pax7 ensure satellite cell survival

Sabourin and Rudnicki (42) wrote an extensive review examining the hierarchical relationship of MRF family members and clarifying their function in embryonic muscle development. Through the use of MRF knock-out mice it was determined that inactivation of MyoD results in a normal muscle development likely due to an upregulation in Myf5 expression (43), while the absence of Myf5 results in perinatal death (44). Yablonka-Reuveni et al (45) demonstrated delayed differentiation in cells from MyoD-null mice. Both MyoD and Myf5 are necessary for myoblast formation and normal progression down the myogenic lineage. The absence of both results in death due to lack of muscle formation (46). Embryos lacking myogenin do not form myofibers (47), while the absence MRF4 triggers compensatory increases in myogenin expression resulting in normal muscle formation (48). As summarized in the review (42), the MRFs clearly function in a hierarchical manner, with Myf5 and MyoD functioning as primary myogenic determinants whose expression is required to set embryonic myogenesis in motion, followed by expression of the secondary MRFs, myogenin and MRF4, whose expression signifies differentiation through myotube formation and fusion. The aforementioned studies demonstrate the crucial role of satellite

cells through expression of these markers in both embryonic and postnatal muscle growth (Figure 4).

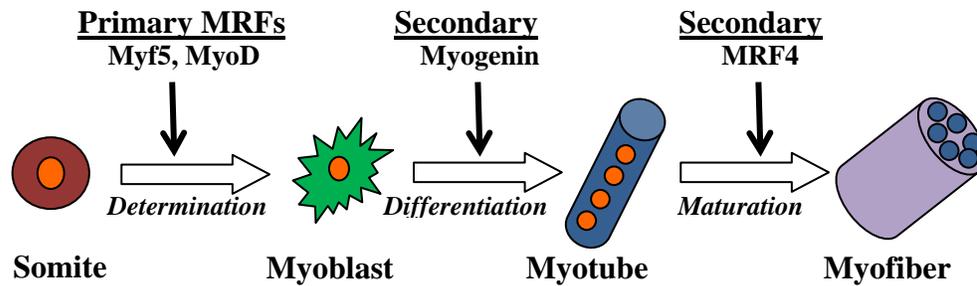


Figure 4. Main events involved in myogenesis and differentiation

Satellite cells and muscle hypertrophy

Reductions in satellite cell number, whether through nutrient restriction or mechanical stress, have been shown to adversely affect muscle growth by inhibiting increases in myonuclear number and fiber volume. Induction of compensatory hypertrophy in specific muscles through ablation of its synergistic partner has been verified (49, 50). Rosenblatt et al. has extensively covered the harmful effect of satellite loss on postnatal muscle growth through the use of synergistic muscles in growing young mice and rats. Removal of the tibialis anterior (TA) in young growing rats and mice resulted in an increase in muscle mass (Figure 5) and myonuclei number of the extensor digitorum longus (EDL) due to overloading (52).

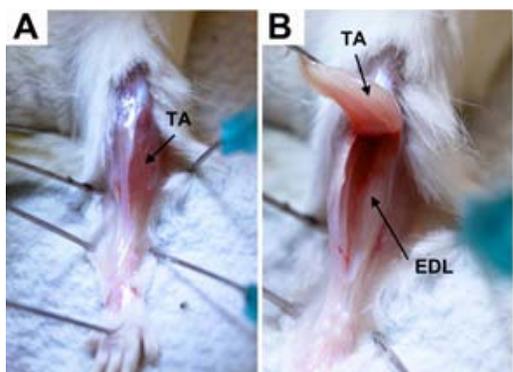


Figure 5. (A) Tibialis anterior and (B) extensor digitorum longus of the mouse (Oishi, PE et al. 2011. JoVE)

Gamma irradiation causes reproductive death of satellite cells and when the EDL of rats were exposed to irradiation prior to the removal of the TA, hypertrophy of the EDL was diminished, though not entirely eliminated (52). A similar experiment was performed in adult mice and normal hypertrophy of the EDL was completely eradicated (51), suggestive of the inverse relationship between age and satellite cell number. The importance of satellite cell activity on normal muscle growth was also seen in turkeys, with dramatically reduced muscle growth and satellite cell activity following irradiation (53). The aforementioned studies suggest that any reduction in satellite cell activity or myonuclear accretion results in impaired postnatal muscle development. The studies performed in the young animal are also indicative of the sensitivity of immature muscle to alterations in normal muscle growth.

Severe nutrient restriction has also been shown to alter satellite cell kinetics *in vivo* (54-57) leading to permanent muscle growth deficits. Halevy et al (54, 55) demonstrated marked decreases in satellite cell number and activity following total nutrient deprivation for 48 h posthatch in chicks and turkey poults. Likewise, underfed sheep had reduced muscle size and decreased expression of MRFs involved in the progression of satellite cells through their myogenic lineage relative to their adequately fed counterparts (56). All nutrient

restricted animals were able to increase satellite cell activity and muscle size upon restoration of their feeding programs; however, these compensatory responses were not sufficient in making equivalent gains to animals that initially received adequate diets. These data show that the alteration of satellite cell activity by nutrient restriction causes permanent growth deficits. Severe nutrient deprivation adversely affected the pool of satellite cells, thereby reducing proliferative capacity and subsequent growth potential.

Dietary phosphate

Phosphate regulation

Phosphate (PO_4) is integral to cellular biology because it functions in various biological processes including energy metabolism, cell signaling, muscle function and bone mineralization (58). Because of the importance of PO_4 as a major structural component of bone in addition to its critical role in various cellular processes, PO_4 restriction results in impaired bone mineralization and reduced muscle growth. Approximately 85% of body P is found in bone. The remaining 15% is distributed throughout the soft tissue and intracellular fluid, the majority of which is associated with proteins, lipids, nucleic acids and other organic compounds. Maintenance of PO_4 homeostasis is achieved through the coordinated actions of intestinal absorption, bone storage pools and renal reabsorption (59, 60). Intestinal absorption of PO_4 occurs through dual mechanisms, a paracellular pathway that largely functions when intestinal PO_4 concentrations are high and a transcellular pathway that is mediated by sodium dependent carrier (NPT2) molecules when intestinal PO_4 concentrations are low (61).

Phosphate contributions from bone storage pools are determined by the rate bone remodeling (58) while the kidneys serve as primary regulators of PO_4 homeostasis due to their ability to

increase or decrease PO_4 reabsorptive capacity utilizing carrier molecules similar to those present in the intestine (62). “Phosphate sensors” have been identified in unicellular organisms and respond to changes in extracellular PO_4 by altering intracellular protein metabolism, subsequently regulating transcription in response to modifications in PO_4 concentrations (58, 63). The proteins produced allow for increased PO_4 retention in the cell. A similar mechanism of action is believed to take place in cells located in the intestines, kidneys, and parathyroid glands of multicellular animals, although not much is known about these sensors. Berndt et al. (64) put forth the theory of a signaling axis in which intestinal PO_4 sensors regulate reabsorption or excretion of PO_4 from the kidney depending on intestinal PO_4 concentrations. These actions occur in direct response to dietary PO_4 and happen independent of parathyroid hormone (PTH) and 1,25 $(\text{OH})_2$ vitamin D, which were previously considered to be the main regulators of Ca and PO_4 balance.

The critical interplay of PTH and 1,25 $(\text{OH})_2$ vitamin D in maintaining PO_4 homeostasis has been established (65, 66). Katai et al. (66) found that PO_4 transport activity in rats is decreased during 1, 25 $(\text{OH})_2$ vitamin D deficiency, but uptake was increased following administration of 1, 25 $(\text{OH})_2$ vitamin D to deficient rats. Similarly, Marks et al. (67) demonstrated increased PO_4 absorption and NPT2 protein abundance in the jejunum of mice and rats with increased 1, 25 $(\text{OH})_2$ vitamin D. Although many studies show that responses to dietary PO_4 by 1, 25 $(\text{OH})_2$ vitamin D are due to transcriptional control of 25-Hydroxyvitamin D_3 1 α -hydroxylase activity by PO_4 concentrations (68), Segawa et al. (69) and Capuano (70) demonstrated adaptations to a low PO_4 diet independent of 1, 25 $(\text{OH})_2$ vitamin D in 1, 25 $(\text{OH})_2$ vitamin D receptor (VDR) null mice. Both investigations noted 1,

1,25 (OH)₂ vitamin D independent increases in NPT2b protein abundance and mRNA transcripts in the intestines. While it can be concluded that dietary PO₄ transport across the intestinal epithelium is due to NPT2 protein, the mechanism by which this occurs does not seem entirely dependent on 1,25 (OH)₂ vitamin D.

Moallem et al. (71) established the role of dietary PO₄ in altering mRNA expression in the parathyroid glands, affecting PTH secretion. Lotscher et al (72) suggested that PTH facilitates the intracellular movement of sodium phosphate cotransporters from the apical membrane of the proximal tubules in the kidneys to assist in PO₄ excretion or reabsorption, depending on PO₄ needs. Administration of PTH to PO₄ restricted wild type mice causes further reductions in PO₄ transport across the brush border membrane and decreases in NPT2 protein abundance in the kidneys. Conversely, NPT2 null mice were unresponsive to the inhibitory actions of PTH on PO₄ reabsorption suggesting that NPT2 is the main regulatory target of PTH in renal PO₄ handling (62). Since PO₄ homeostasis is tightly regulated by 1,25 (OH)₂ Vitamin D via the intestines and PTH via the kidney, changes in circulating levels of these hormones are contingent upon the PO₄ needs of the animal (73).

In recent years, a group of phosphaturic peptides identified as “phosphatonins” have been implicated in the regulation of PO₄ status. First discovered in patients with disorders associated with renal PO₄ wasting (tumor-induced osteomalacia, autosomal-linked hypophosphatemic rickets, X-linked hypophosphatemic rickets) (74), overexpression of these peptide factors have been shown to modify PO₄ status by inducing phosphaturia by altering 1,25 (OH)₂ vitamin D metabolism (75) and/or reducing sodium-phosphate cotransporter abundance in the proximal tubules of the kidneys (76). Phosphatonins have been shown to

adversely affect bone mineralization *in vitro* (77). Of the many phosphonins discovered, fibroblast growth factor 23 (FGF23), is the most extensively studied. It functions in a well defined feedback loop with that involves PO_4 , PTH, $1,25(\text{OH})_2$ Vitamin D. It is secreted in response to high dietary PO_4 , increasing PO_4 excretion by acting on the parathyroid to decrease PTH release and on the kidney to decrease activity of 25-Hydroxyvitamin D_3 1- α -hydroxylase. All can be regulated by dietary PO_4 intake and mediate the changes in PO_4 reabsorption by the kidney in response to changes in dietary PO_4 .

Dietary phosphate and animal agriculture

The majority of PO_4 in animal feedstuffs is in the poorly available form of phytate, therefore the inclusion of additional PO_4 sources to swine diets has been necessary to meet PO_4 needs. Because inadequate dietary PO_4 is detrimental to growth, it is common practice for producers to supplement animal diets with inorganic PO_4 sources. Increased public and governmental concern over the environmental impact of excess PO_4 in the excreta of pigs has led the Environmental Protection Agency to introduce regulations limiting the amount of PO_4 in swine effluent (78). This has driven research to minimize the environmental impact of swine production (78, 80) by focusing on more accurately defining the PO_4 requirements of pigs, as well as developing and evaluating technologies that could minimize PO_4 excretion from pig production (81-84). Additionally, the dramatic increase in the cost of dietary inorganic PO_4 sources has further motivated producers to reduce the levels of inorganic PO_4 added to swine diets. Although there is a great deal of interest in reducing PO_4 excretion by production animals, such efforts could result in sub-clinical PO_4 deficiencies that adversely affect growth potential. The prevention of dietary PO_4 deficiency is critical to maintaining

the profitability of animal agriculture as well as animal well being. While the effects of dietary PO₄ restriction in growing pigs, such as reduced growth, poor bone integrity, and reduced feed efficiency have been established (81, 82, 84) and a great deal of PO₄ nutrition research in swine has focused on identifying requirements for optimizing production efficiency while minimizing PO₄ excretion (85-87), few research efforts have examined the mechanisms behind PO₄ utilization and the impact on lean muscle accretion.

Early life PO₄ restriction has been shown to have a greater impact on growth potential than PO₄ deficiencies experienced later in life. Driver et al (88) found that broilers initially receiving PO₄ deficient diets were unable to make compensatory gains and had poorer bone integrity even when receiving an adequate diet later in life compared to those animals that received the adequate diet throughout life. Additionally, it was concluded that carcass quality in the broiler is dependent on the interaction between Ca and PO₄ inclusion levels and the age of the bird. This study illustrated reduced weight gain and bone integrity in chickens fed a P deficient diet earlier in life as opposed to those that received adequate dietary PO₄ early on, indicative of the importance of early life nutrition on growth performance and bone integrity to yield improved meat quality.

Although muscle tissue contains the second largest PO₄ pool in the animal, little research has examined the impact of PO₄ utilization on the response of muscle growth and carcass characteristics during early life dietary PO₄ restriction. Severe nutrient restriction reduces satellite cell number and activity and decreases muscle size and the expression of muscle regulatory factors. Nutritionally induced reductions in satellite cell activity in early

life result in permanent growth deficits. This suggests that early life nutritional restrictions can have lifelong consequences, ostensibly through its impact on satellite cell activity.

Despite the importance of satellite cells to muscular development relatively little research has examined the impact of PO_4 nutrition during the neonatal period when satellite cells numbers are highest. Meisires and Doumit (89) noted higher rates of proliferation and differentiation among satellite cells isolated from 1 week old pigs compared to those at 7 weeks. It is also estimated that 80% of satellite cells are present in 2 day old mice, whereas only 10% are left by day 10 (90). Early life PO_4 restriction has been shown to have a greater impact on growth potential than PO_4 deficiencies experienced later in life and also to be of greater importance for muscular growth compared to Ca (91) during dual deficiency.

Adequate PO_4 nutrition, especially in early life, is essential for proper muscle growth and satellite cells are critical to postnatal muscle development and are highest in the young animal. Taken together, these factors would suggest that there is a mechanism by which dietary PO_4 influences activity of satellite cells, although the mechanism has yet to be elucidated.

The industrialization of animal agriculture to maximize production efficiency and meet the growing demands of the consumer has led to increased output from these facilities. Environmental concerns over the amount of PO_4 in the excreta have led to research examining nutritional strategies to reduce PO_4 levels without sacrificing animal performance. Preventing dietary PO_4 deficiency is critical to maintaining the profitability of animal agriculture as well as animal well being. Because the greatest rate of growth occurs in early

life, clarifying how PO_4 nutrition impacts immature muscle would be beneficial in maximizing growth performance and increasing animal sustainability.

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CHAPTER III:
Dietary phosphate restriction decreases stem cell proliferation and subsequent growth potential in neonatal pigs

Published in Journal of Nutrition: Lindsey S. Alexander¹, Avanika Mahajan¹, Jack Odle¹, Kyle L. Flann², Robert P. Rhoads², and Chad H. Stahl¹
¹Laboratory of Developmental Nutrition, Department of Animal Science, North Carolina State University, Raleigh, NC 27695; ²Department of Animal Science, University of Arizona, Tucson, AZ, 85719

ABSTRACT: While mesenchymal stem cells (MSC) and satellite cells are essential for postnatal muscle and bone development and phosphate (PO_4) restriction reduces both muscle and skeletal tissue growth, no research has investigated the possible mechanism by which this mineral may impact early cell programming. Twenty piglets obtained at 1 d of age (1.8 ± 0.3 kg) received either a PO_4 adequate diet or a 25% less $\text{PO}_{4\text{available}}$ diet over a 15 d trial. Feed intake and body weight (BW) were recorded daily and blood samples collected every 5 d. After 15 d, pigs were given an intraperitoneal (IP) injection of bromodeoxyuridine (BrdU) 4 h prior to tissue collection. As expected, PO_4 deficiency resulted in reduced growth ($P < 0.05$), feed conversion efficiency ($P < 0.05$) and bone mineral content ($P < 0.05$), as well as lower plasma concentrations of both PO_4 ($P < 0.01$) and parathyroid hormone (PTH) ($P < 0.05$). In addition to these classical indicators of P deficiency, there was also reduced proliferation of both MSC ($P < 0.01$), and satellite cells ($P < 0.05$) *in vivo*. The expression of osteocalcin messenger RNA (mRNA) in bone marrow was also 2 fold greater ($P < 0.01$) within the PO_4 adequate treatment group. These data indicate that in addition to reductions in muscle and bone growth, dietary PO_4 impacts proliferation of tissue specific stem cells *in vivo*. Nutritional programming of tissue specific stem cells by dietary PO_4 may have profound implications for life-long growth potential.

INTRODUCTION

Dietary phosphate (PO_4) restriction reduces both muscular and skeletal tissue growth (1, 2). Satellite cells are responsible for post-natal muscle growth by providing the myonuclei that allow for muscle fiber hypertrophy (3, 4) and mesenchymal stem cells (MSC) provide the life-time supply of osteoprogenitor cells responsible for skeletal growth (5, 6). Surprisingly, despite the importance of satellite cells and MSC for muscular and skeletal development relatively little research has examined the impact of PO_4 nutrition during the neonatal period when there is the highest enrichment of satellite cells in muscle and MSC in bone marrow. Early life PO_4 restriction has been shown to have a greater impact on growth potential than PO_4 deficiencies experienced later in life (7) and also to be of greater importance for muscular and skeletal growth compared to Ca (8) during dual deficiency. While severe nutrient restriction has been shown to alter satellite cell kinetics *in vivo* (9, 10) leading to permanent muscle growth deficits, and modifications of the *in vitro* nutrient environment has been shown to influence the progression of MSC down alternative lineages (11, 12), there has been little investigation into the potential for nutritional programming of these tissue specific stem cells by dietary PO_4 .

Understanding the role dietary PO_4 may play in the developmental programming of bone and muscle tissue is of particular importance for formulating dietary strategies for preterm and low birth weight human infants, but is also of great concern to animal agriculture. The Environmental Protection Agency has introduced regulations limiting the amount of PO_4 in swine effluent (13), which coupled with the dramatic increase in the cost of dietary PO_4 supplements has driven research to minimize the environmental impact of swine

production (14, 15) by more accurately defining the PO₄ requirements of pigs, as well as developing and evaluating technologies that could minimize P excretion from pig production (16, 17). Efforts to minimize PO₄ excretion in pig production could result in sub-clinical PO₄ deficiencies that adversely affect growth potential. By understanding the role of dietary PO₄ in the developmental programming of bone and muscle tissue, better strategies to reduce PO₄ excretion while maximizing production efficiency could be developed.

The objective of the present study was to determine the impact of early life PO₄ restriction on tissue-specific stem cell activity and the subsequent changes in growth and endocrine parameters. Our hypothesis is that early life dietary PO₄ restriction could negatively impact the stem cells responsible for post-natal bone and muscle growth and have life-long implications for musculoskeletal growth and development. The use of the neonatal pig as our model allows for this work to have dual benefit to both animal agriculture and human health.

MATERIALS AND METHODS

Animals

All animal protocols were approved by North Carolina State University's Institutional Animal Care and Use Committee. Thirteen female and 7 male piglets obtained at 24-32 h of age (1.8 ± 0.3 kg), after being allowed to consume colostrum, were weighed and allotted into 1 of 2 treatment groups based on body weight (BW) and gender. Over a 15 d trial period, and pigs were pair-fed based on mean intake, a soy-based liquid milk replacer that either met P requirements or was approximately 25% deficient in available PO₄ (**Table 1**). A soy-based

milk replacer was utilized due to the low bioavailability of PO₄ from soy protein sources compared to animal protein sources, which allowed us to meet dietary protein requirements while maintaining a PO₄ deficient diet. Diets were formulated based on sow milk composition (18, 19). Pigs were housed individually in raised cages and provided milk replacer 3 times/d at 0800, 1600, and 2300 h via a gravity-flow liquid feed delivery system. Feed intake and BW were recorded daily and blood samples collected every 5 d by venipuncture using heparinized tubes (Vacutainer Plus BD Vacutainer, Franklin Lakes, NJ). Plasma was obtained by centrifugation at 3,500 × g at 4°C and subsequently stored at -20°C until further analysis. After 14 d of receiving their dietary treatments, all pigs were given 25 mg of bromodeoxyuridine (BrdU)/kg BW by intraperitoneal (IP) injection 4 h prior to tissue collection. Bone marrow and muscle tissue was harvested under aseptic conditions for the isolation of MSC and satellite cells, respectively. These tissues, in addition to kidney, were also sampled for gene expression analysis. 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 concentrations of PO₄, calcium (Ca), parathyroid hormone (PTH), and 1,25-dihydroxycholecalciferol were determined for all samples. Phosphate concentrations were determined by the method of Gomori (20). Calcium concentrations were determined by flame absorption spectroscopy following dilution in 0.5% lanthium chloride. The concentrations of PTH and 1,25-dihydroxycholecalciferol were determined using

commercially available kits (Porcine Intact PTH ELISA kit Immutopics, San Clemente, CA; and 1,25-dihydroxycholecalciferol EIA kit, IDS, Fountain Hills, AZ).

Isolation of stem cells

All stem cells were isolated from humeral bone marrow and loin muscle from individual pigs. The procedure for isolation of satellite cells was modified from methods developed by Allen et al. and Rhoads et al. (21, 22). Briefly, minced muscle tissue was incubated at 37°C for 1 h in 1.25 g protease type XIV from *Streptomyces griseus*/L PBS (approximately 40 mL buffer/5 g tissue). Satellite cells were separated from muscle fibers by differential centrifugation and then filtered through a 100 µm cell strainer to rid the satellite cells of debris. They were then collected by centrifugation at 1500 × g for 5 min, resuspended in 20 mL of proliferative media (DMEM + 10% heat inactivated fetal bovine serum (FBS) + antibiotics) and placed in a sterile uncoated 15 cm plate at 37°C for 2 h. Non-adherent cells were plated in fresh media into 6-well tissue culture plates coated with poly-l-lysine (MP Biomedicals, Solon, OH) and fibronectin (Roche Applied Science, Indianapolis, IN) at a density of 1,000 cells/cm². All cultures were incubated at 37°C in a humidified environment containing 5% CO₂.

The MSC were isolated from bone marrow according to the method of Mahajan and Stahl (11). Briefly, cells from dispersed marrow were plated at a density of 5 × 10⁴ nucleated cells/cm² in T-75 culture flasks in proliferative media. Hematopoietic cells and all other non-adherent cells were removed with complete media changes with vigorous washing every 24 h.

Determination of in vivo cell proliferation

The percentage of satellite cells and MSC with BrdU incorporated into their DNA was identified by immunocytochemistry. Prior to incubation with the primary antibody (G₃G₄; Developmental Studies Hybridoma Bank, University of Iowa), DNA was denatured by incubation in hydrochloric acid for 1 h, followed by acid neutralization with 2 washes of TBE (pH 8.5) followed by 3 washes in PBS. A goat anti-mouse HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) was utilized, and stained cells were visualized by incubation with 0.5 g/L of diaminobenzidine and 0.02% H₂O₂ in PBS for 5 min (23). Approximately 400 cells were counted per animal and the percentage proliferation was determined by calculating the ratio of stained to unstained cells.

Analysis of Gene Expression

Total RNA was isolated from bone marrow, muscle, and kidney using RNeasy Midi Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (Ambion DNA free-kit, Austin, TX), and the RNA was then reverse transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen, Carlsbad, CA) to ensure the removal of residual RNA. Primer sets were designed using software (Integrated DNA Technologies, Coralville, IA) for the examination of 1- α -hydroxylase, peroxisome proliferator-activated receptor gamma 2 (PPAR γ 2), osteocalcin, and lipoprotein lipase (LPL) messenger RNAs (mRNA) in bone marrow; for Pax7, MyoD, and myogenin mRNAs in

muscle tissue; and for calcitonin receptor (CALCR), 1- α -hydroxylase, 24-hydroxylase, and vitamin D receptor (VDR) mRNAs in kidney tissue (Supplemental Table 1).

Primer sets were validated according to the specifications set forth by Livak and Schmittgen (24). Relative quantities of the transcripts of interest were determined by semi-quantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System and SybrGreen Supermix, Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions included 40 cycles of 20 s of melting at 95°C followed by 20 s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis. Gene expression was normalized to cDNA concentration, determined using a fluorescence-based quantification kit (Oligogreen, Invitrogen Life Technologies), using a modification of the $2^{-\Delta CT}$ method (24).

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

An effect of PO₄ restriction on plasma hormone concentrations and growth performance was apparent after 5 d on trial. Pigs fed the adequate diet had plasma PO₄ concentrations nearly 2 fold greater than those of their PO₄ deficient counterparts at 5 d, an effect that remained until trial completion (**Figure 1A**, $P < 0.01$). There was a significant effect of diet on plasma Ca at 5 d, but this difference was not apparent at any other day

(Figure 1B, $P < 0.05$). Parathyroid hormone concentrations were almost undetectable until 15 d in PO_4 deficient pigs (Figure 1C). Phosphate adequate pigs had approximately 1.7, 4.3, and 2.3 fold greater ($P < 0.05$) plasma PTH concentrations than the PO_4 deficient pigs at 5 d, 10 d, and 15 d, respectively. There were no differences based on dietary treatment in plasma 1,25-dihydroxycholecalciferol concentrations throughout the 15 d trial period (Figure 1D).

Phosphate deficiency resulted in reduced growth ($P < 0.05$) and feed efficiency ($P < 0.05$) (**Table 2**). This reduction in feed efficiency was due to reduced growth and not to a reduction in feed intake, as the piglets in this study were pair-fed. Over the 15 d trial period, animals receiving the adequate diet gained almost twice as much on a daily basis than the PO_4 restricted piglets (0.176 vs. 0.098 kg/d, $P < 0.05$); consequently there was 39.4% poorer feed efficiency among PO_4 deficient piglets ($P < 0.05$). Likewise, PO_4 deficiency resulted in less bone growth (Table 2). While the percentage of mineral content did not differ between dietary treatment groups (Table 2), the total mineral content of radial bone of PO_4 deficient pigs (1.50 g) was 22% less ($P < 0.05$) than that of the PO_4 adequate pigs (1.99 g). Along with the reductions in both bone and muscle growth observed during PO_4 deficiency, there were correspondingly less proliferating MSC (2.3% vs. 0.7%, $P < 0.01$) and satellite cells (4.8% vs. 2.2%, $P < 0.05$) *in vivo*. Phosphate adequate pigs had 2.3 fold and 1.2 fold greater proliferation of MSC and satellite cells, respectively, than did PO_4 deficient pigs (Table 2).

Within bone marrow, there was a trend for higher concentrations of 1- α -hydroxylase (Figure 2A; $P = 0.07$), PPAR γ 2 (Figure 2B; $P = 0.1$), and LPL (Figure 2C; $P = 0.11$) mRNAs in pigs fed the PO_4 deficient diet, while osteocalcin expression was 2 fold greater (Figure 2D; $P < 0.01$) in PO_4 adequate fed pigs. Pigs fed the PO_4 adequate diet had reduced expression of

CALCR (Figure 2E; $P < 0.05$), 24-hydroxylase (Figure 2F; $P < 0.05$), and VDR (Figure 2G: $P < 0.05$) in the kidney while increased expression of 1- α -hydroxylase (Figure 2H; $P < 0.05$) was observed. Expression of CALCR, 24-hydroxylase, and VDR was 1.3 fold, 2.3 fold, and 1.6 fold greater in PO_4 deficiency when compared to their adequate counterparts, respectively. Phosphate adequate pigs had 1.2 fold greater expression of 1- α -hydroxylase than PO_4 deficient pigs. No differences in the expression of Pax7, MyoD, and myogenin in muscle tissue were observed between the 2 dietary treatment groups (data not shown).

DISCUSSION

The objective of this study was to determine the impact of PO_4 restriction on growth and tissue specific stem cell activity in the neonatal pig. The neonatal pig was chosen due to the increased sensitivity of the young animal to nutrient deficiencies as well as the greater abundance of tissue specific stem cells present in a young, rapidly growing animal. Although MSC and satellite cells are critical to postnatal bone and muscle development and dietary PO_4 restriction reduces muscle and skeletal tissue growth, little research has investigated the potential role of dietary PO_4 on tissue-specific stem cell activity.

While the level of dietary PO_4 deficiency utilized in this study is relatively minor in older pigs (25), it was severe for the neonatal pigs. As is expected in a severe dietary PO_4 deficiency, we saw acutely lower plasma PO_4 concentrations (Figure 1A), dramatic reductions in growth (Table 2), poorer feed conversion efficiency (Table 2), as well as less bone mineralization (Table 2). Although the percentage of mineral present in the radial bones did not differ between dietary treatments, pigs receiving the PO_4 adequate milk replacer had

greater total bone mineral content, a reflection of their significantly larger size relative to their PO₄ deficient counterparts.

In addition to growth rate and bone mineral content, endocrine hormones associated with Ca and PO₄ homeostasis were also measured as indicators of PO₄ status. Similar to previous PO₄ restriction studies, reductions in dietary PO₄ decreased plasma PTH concentrations ($P < 0.05$; Figure 1C; 25, 26). Although the mechanism responsible for this reduction is not clearly understood, a direct effect of dietary PO₄ deficiency in altering PTH mRNA expression, as well as reducing parathyroid cell proliferation independent of serum Ca and 1,25-dihydroxycholecalciferol levels has been suggested (27-29). Our current study is supportive of this mechanism rather than a reduction in PTH due to increased serum 1,25-dihydroxycholecalciferol (30, 31), because plasma 1,25-dihydroxycholecalciferol concentrations did not differ between dietary treatment groups throughout the trial period (Figure 1D). While 1,25-dihydroxycholecalciferol concentrations have been previously shown to increase in response to dietary PO₄ deficiency (25), the absence of this increase was not entirely unexpected in our study. Mineral regulation via the PTH/1,25-dihydroxycholecalciferol axis has not been fully elucidated in neonates. Halloran and DeLuca (32) demonstrated that intestinal Ca uptake is unresponsive to 1,25-dihydroxycholecalciferol during the early suckling period in rats, and that that active transport of Ca as well as increased concentrations of intestinal 1,25-dihydroxycholecalciferol receptors were not detected until 2 to 3 weeks postpartum (32, 33) suggesting that the homeostatic regulation of Ca and PO₄ by 1,25-dihydroxycholecalciferol is absent in the neonate.

Our kidney gene expression data further supports that there may be altered or immature mineral homeostatic mechanisms in the neonatal pig. The increased concentrations of 24-hydroxylase ($P < 0.05$) mRNA in the kidneys of PO_4 deficient pigs (Figure 2F) conflict with the results of Wu et. al (34), who found that PO_4 deprivation reduces mRNA expression of 24-hydroxylase, the enzyme involved in 1,25-dihydroxycholecalciferol catabolism. The decrease in 1- α -hydroxylase mRNA with PO_4 deficiency that we report in this study is also contrary to what has been previously reported, as PO_4 restriction generally causes an increase in the message of 1- α -hydroxylase as well as an increase in circulating 1,25-dihydroxycholecalciferol (25, 30, 31). Of these studies, ours is the only one which examined PO_4 restriction in neonates.

While the aforementioned changes in growth performance, bone mineral content, and plasma indicators of mineral status are typical of dietary PO_4 deficiency (25, 35), the *in vivo* reduction of MSC ($P < 0.05$) and satellite cell ($P < 0.05$) proliferation resulting from dietary PO_4 restriction in the pig (Table 2) has not been previously reported. Although very few studies have examined the impact of nutrition on MSC proliferation or differentiation *in vivo* (36), several *in vitro* studies have demonstrated links between nutritional status and alterations in MSC differentiation (11, 12, 37). Nutrient restriction has been shown to reduce satellite cell number and activity (10, 38) and decrease muscle size and the expression of myogenic regulatory factors (9). Nutritionally induced reduction in satellite cell activity resulted in permanent growth deficits, which suggests that an early life nutritional event can have lifelong consequences via actions on tissue-specific stem cells.

The competitive nature of osteoblastic and adipocytic differentiation of MSC has been established (39-41), therefore within the bone marrow from which our MSC were isolated, we measured the relative quantity of transcripts for genes associated with osteogenesis (osteocalcin) and adipogenesis (PPAR γ 2 and LPL), as well as 1 α -hydroxylase, due to possible autocrine or paracrine function of 1,25-dihydroxycholecalciferol in bone marrow (42, 43). The decreased quantities of osteocalcin mRNA coupled with trends for increased PPAR γ 2 and LPL mRNAs is suggestive of a shift of the MSC population in the bone marrow away from osteogenic and towards adipocytic differentiation, which is supported by our bone mineral content data. Although the mechanism needs to be elucidated, based on the increased concentration of 1 α -hydroxylase expression in bone marrow coupled with our previous *in vitro* work (11) demonstrating that 1,25-dihydroxycholecalciferol stimulates adipocytic differentiation of MSC it is tempting to hypothesize that dietary PO₄ deficiency may increase bone marrow 1,25-dihydroxycholecalciferol concentrations which in turn alter the lineage allocation of MSC.

In the present study, we demonstrated that dietary PO₄ deficiency results in the reduced proliferation of both satellite cells and MSC *in vivo*; suggesting that dietary PO₄ status during early post-natal development can have profound impacts on future growth potential. These findings have significant implications for both human health and animal agriculture. One in every eight births in the U.S. is premature, and pre-term infants have lower BMC than term infants (44) and are at greater risk of PO₄ deficiency. A similar problem is encountered in swine production with low birth weight piglets. Without appropriate dietary intervention, these neonatal PO₄ deficiencies could have lifelong

consequences for both lean body mass accrual and achieving maximal BMD and bone integrity. In humans this could lead to increased risk of both obesity and osteoporosis while in animal agriculture this would cause reduced growth performance and therefore reduced sustainability. Further work is needed to elucidate the development of the mineral homeostatic mechanisms in the neonate, as well as to determine the mechanisms by which dietary PO₄ is altering tissue-specific stem cell activity.

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Table 1. Experimental diet composition on an as fed basis¹.

Ingredients,	PO ₄ adequate Diet ²	PO ₄ deficient Diet ³
	Composition, %	
Isolated soy protein	35.0	35.0
7/60 milk replacer high fat ⁴	25.0	25.0
Potassium phosphate	1.6	0
Lactose	28.1	29.4
Sucrose	4.2	4.5
Limestone	2.0	2.0
Dicalcium phosphate	1.1	1.1
Vitamin Pre-mix ^{4,5}	0.1	0.1
Mineral Pre-mix ^{4,6}	0.5	0.5
Methionine	0.2	0.2
Salt	0.3	0.3
Antibiotic Pre-mix ⁷	1.0	1.0
Xanthum gum	1.0	1.0
Analyzed ⁸		
Crude protein	30.9	28.4
Calcium	1.52	1.54
Phosphorus	0.87	0.66

¹Composition of the powdered milk replacer that was reconstituted at a rate of 175g/kg final liquid formula

²Diet met pigs requirements based on sow milk composition.

³Diet was 25% deficient in phosphate.

⁴Milk Specialties Corporation, Dundee, IL

⁵Liqui-Wean Vitamin Premix contains per kg: 9.9 g retinyl acetate, 165 mg cholecalciferol, 36.7 mg α -tocopherol, 117 g ascorbic acid, 29 g D-pantothenic acid, 33 g niacin, 8.4 g riboflavin, 5.1 g menadione, 66 mg biotin, 44 g vitamin B-12, 2.04 g thiamine, 4 g vitamin B-6, and 2.76 g folic acid.

⁶Liqui-Wean Mineral Premix contains per kg: 271 g calcium, 140 mg phosphate, 610 mg sodium, 18.34 g chloride, 129 mg potassium, 14.6 g magnesium, 26.54 g sulfur, 1.85 g copper, 20 g zinc, 68 mg selenium, 124 mg cobalt, 437 mg iodine, 20.8 g iron, 5.44 g manganese, and 60 g choline.

⁷Antibiotic Pre-mix provides per kg: 7.72 g oxytetracycline and 15.44 g neomycin base.

⁸Diets were analyzed on a dry matter basis by DairyOne Forage Laboratory, Ithaca, NY)

Table 2. Impact of PO₄ restriction on growth performance, bone mineral content, and stem cell activity in neonatal pigs¹

	PO₄ adequate	PO₄ deficient	SE
Weight gain, kg/d			
0-5 d	0.114*	0.064	0.012
5-10 d	0.131*	0.063	0.013
10-15 d	0.283*	0.167	0.018
Cumulative	0.176*	0.098	0.010
Feed conversion efficiency, kg gain/kg feed			
0-5 d	1.15*	0.682	0.119
5-10 d	0.669*	0.345	0.081
10-15 d	0.936*	0.634	0.073
Cumulative	0.881*	0.534	0.055
Bone mineral content			
Fresh weight, g	19.76*	14.98	0.679
Ash weight, g	1.99*	1.50	0.108
Ash percentage, %	31.77	30.68	0.888
BrdU positive stem cells			
Satellite cells, %	4.84*	2.24	0.789
MSC, %	2.32*	0.70	0.228

¹ Values presented are least square means

*Different from PO₄ deficient piglets (P < 0.05)

Table 3. Primers used for quantification of gene expression by real-time PCR

Gene name	Accession number	Primer sequence
VDR	EF405627	F: 5' TTGCCAAACACCTCAAGCACAAGG 3' R: 5' TGCTCTACGCCAAGATGATCCAGA 3'
CALCR	M74420	F: 5' TGCTCATGCCATTACTAGGGCAGT 3' R: 5' ATGACAGGGCCGTGGATGATGTAA 3'
PPAR γ 2	NM_015869.4	F: 5' AATTAGATGACAGCGACCTGGCGA 3' R: 5' TGTCTTGAATGTCCTCGATGGGCT 3'
1- α -hydroxylase	AB018743	F: 5' AGGAGTGAAGTATGCACTTGGCCT 3' R: 5' GGAGCGGCCCAAAGAAATAGCAAA 3'
24-hydroxylase	AF245504	F: 5' TGTGACGAGAGAGGCTGCATTGAA 3' R: 5' TCATCTTCCCGAACGTGCTCATCA 3'
Lipoprotein lipase	NM_214286.1	F: 5' ACCGTTGCAACAACCTGGGCTATG 3' R: 5' ACTTTGTAGGGCATCTGAGCACGA 3'
Osteocalcin	NM_199173	F: 5' TACCCAGATCCTCTGGAGCCC 3' R: 5' TATGCCATAGAAGCGCCGATA 3'
Pax7	AY653213	F: 5' CAACCACATCCGCCACAAGATAGT 3' R: 5' AGAGGATCTTGGAGACACAGCCAT 3'
MyoD	NM_001002824	F: 5' GCGTGCAAACGCAAGACCACTAA 3' R: 5' AGTCTCGAAGGCCTCGTTGACTTT 3'
Myogenin	NM_001012406	F: 5' TGACCCTACAGATGCCCACAATCT 3' R: 5' GTTGGGCATGGTTTCATCTGGGAA 3'

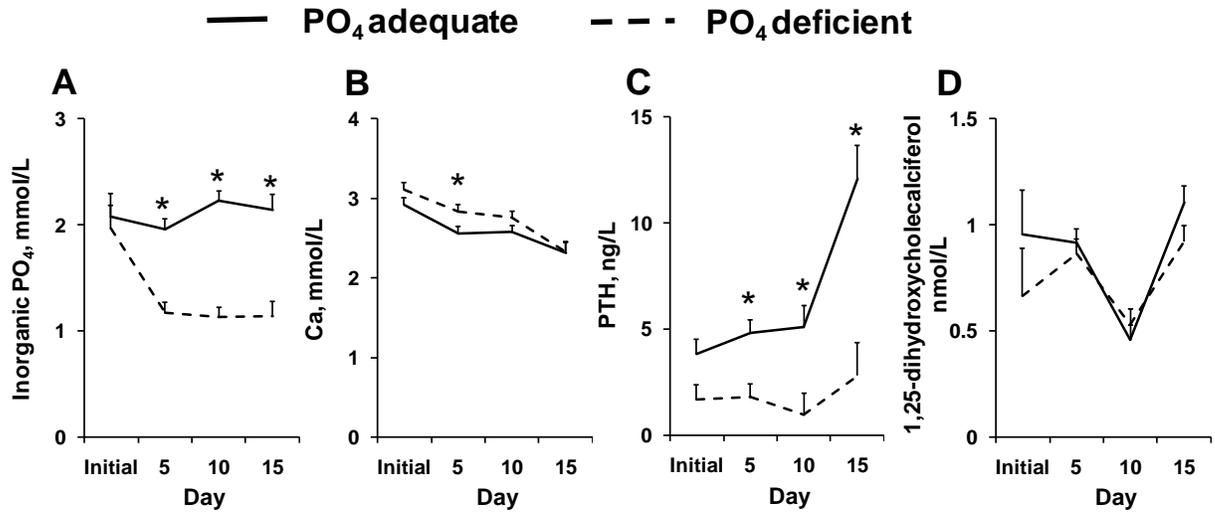


Figure 1. Effect of dietary PO₄ on plasma inorganic PO₄ concentrations (A), Ca concentrations (B), PTH concentrations (C), and 1,25-dihydroxycholecalciferol concentrations (D) in neonatal pigs. Plasma was collected by venipuncture every 5 d throughout the 15 d trial. Values presented are least square means and standard error. *Different from PO₄ deficient piglets (P < 0.05). PO₄ adequate, n = 9; PO₄ deficient, n = 9.

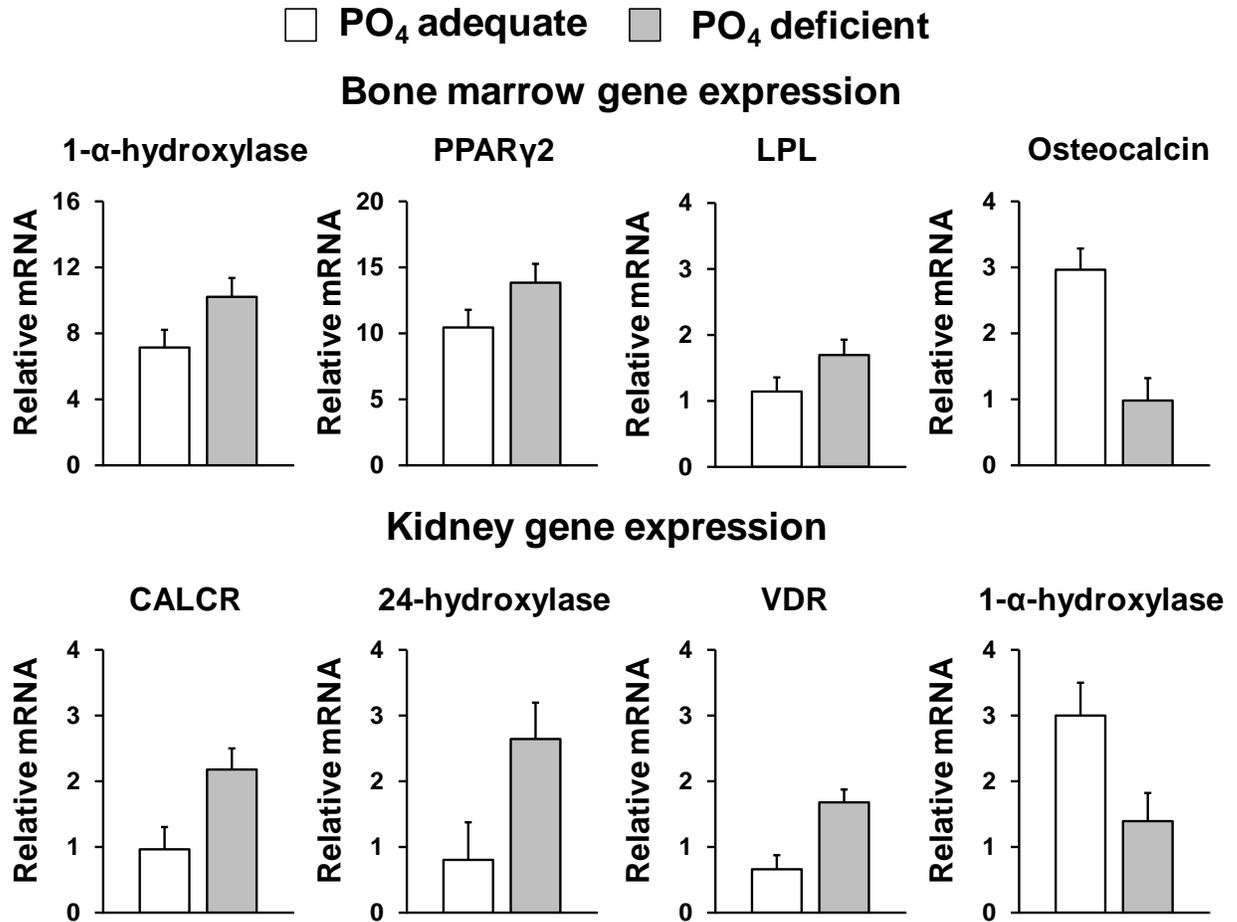


Figure 2. Effect of dietary PO₄ on the gene expression of 1- α -hydroxylase (A), PPAR γ 2 (B), LPL (C), and osteocalcin (D) in bone marrow and the gene expression of CALCR (E), 24-hydroxylase (F), VDR (G), and 1- α -hydroxylase (H) in kidneys of neonatal pigs. Values presented are least square means and standard error of values normalized to cDNA concentrations. * Different from PO₄ deficient piglets (P < 0.05). PO₄ adequate, n = 9; PO₄ deficient, n = 9.

CHAPTER 4:
Dietary phosphate status impacts stem cell activity and growth potential in neonatal pigs

To be submitted to the Journal of Nutrition: Lindsey S. Alexander¹, Brynn S. Seabolt¹, R.P. Rhoads² and Chad H. Stahl¹

¹Laboratory of Developmental Nutrition, Department of Animal Science, North Carolina State University, Raleigh, NC 27695

²Department of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

ABSTRACT: The critical nature of satellite cells to post natal muscle growth and mesenchymal stem cells (MSC) to bone development has been covered extensively. Our previous work has linked phosphate (PO_4) nutrition to activity of these stem cells. The present study sought to determine the impact of different inclusion levels of dietary PO_4 on stem cell behavior in the neonatal pig. Twenty-one piglets (1 d of age, 1.8 ± 0.2 kg BW) were pair-fed liquid diets that were either PO_4 adequate, 25% in excess of PO_4 or 25% deficient in PO_4 content for 12 d. Growth performance was monitored daily and blood samples collected every 6 d. At 12 d, individual pigs were orally dosed with BrdU for in vivo stem cell proliferation determination. Phosphate deficiency resulted in reduced ($P < 0.05$) sera PO_4 and PTH concentrations. Excess PO_4 improved ($P < 0.05$) feed conversion efficiency and radii of PO_4 deficient fed pigs were wider ($P < 0.05$) and had greater dry matter percentages ($P < 0.05$). Phosphate restriction reduced in vivo satellite cell and MSC proliferation ($P < 0.05$) and altered expression of genes in the thyroid and bone marrow. Further work is needed to better understand early nutritional programming of tissue specific stem cells and the potential benefits of emphasizing early PO_4 nutrition for future growth potential of the neonatal pig.

INTRODUCTION

Numerous studies have demonstrated the importance of dietary phosphate (PO_4) to lean muscle growth and bone integrity. Our previous work in the neonatal pig has linked early PO_4 nutrition to the activity of satellite cells and mesenchymal stem cells (MSC) (1), which are the progenitor cells responsible for muscle and bone development), respectively. During the neonatal phase, animals have the greatest abundance of these tissue specific stem cells, in addition to experiencing the greatest growth (2) compared to any other period in their lifetime. Because of this, the young, rapidly growing animal is also more susceptible to the detrimental effects of nutrient deficiencies. Multiple studies have documented the responsiveness of these tissue specific stem cells to different nutritional modifications (1, 3-5).

Although dietary phosphate (PO_4) restriction reduces both muscular and skeletal tissue growth (6-8), very little work has investigated the possible nutritional programming of tissue specific stem cell by dietary PO_4 . We have demonstrated that early dietary PO_4 deficiency reduced proliferation of satellite cells and mesenchymal stem cells (MSC) in vivo, resulting in poorer growth and reduced bone integrity (1).

Identifying the mechanism by which dietary PO_4 may influence developmental programming of muscle and skeletal tissue has implications in both human nutrition and animal production as it relates to nutritional strategies to treat preterm infants and the environmental impact of PO_4 resulting from animal agriculture. The objective of the present study was to determine the impact of different inclusion levels of dietary PO_4 on stem cell activity and expression of myogenic transcription factors and the subsequent changes in

growth, bone integrity, and endocrine parameters in the neonatal pig. Changes in satellite cell activity relative to dietary PO₄ could offer insight into approaches for maximizing future growth.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. Twenty-one male and female piglets were obtained at 24 h of age (1.8 ± 0.2 kg) and allotted into 1 of 3 dietary treatment groups by gender and body weight. Pigs were pair-fed a milk-replacer diet at 170 g/L that was either PO₄ adequate, 25% in excess of the PO₄ requirement, or 25% deficient in PO₄ content for 12 d. Diets were formulated based on sow milk composition and NRC requirements (9, **Table 1**). Pigs were individually housed and fed 5 times a day every 3 h. Feed intake and body weight were recorded daily and blood collected every 6 d by venipuncture using heparinized tubes (Vacutainer Plus BD Vacutainer, Franklin Lakes, NJ). Sera was obtained by centrifugation at 3500 x g and stored at -20°C until analysis. At trial completion, pigs were administered 25 mg of bromodeoxyuridine (BrdU)/kg body weight in their respective milk-replacer diets 12 h prior to tissue collection. Bone marrow and muscle tissue were harvested for the isolation of mesenchymal stem cells (MSC) and satellite cells, respectively. The thyroid and a sample of bone marrow were also collected for gene expression analysis. Radial bones with attached ulnae were measured for length and width. 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

Sera concentrations of PO₄, calcium (Ca), and parathyroid hormone (PTH) were determined for all samples. Phosphate concentrations were determined by the method of Gomori (10). Calcium concentrations were determined by flame absorption spectroscopy following dilution in 0.5% lanthium chloride. Sera concentrations of PTH were determined using commercially available kits (Porcine Intact PTH ELISA kit Immutopics, San Clemente, CA).

Isolation of stem cells

All stem cells were isolated from individual pigs. The procedure for isolation of satellite cells was modified from methods developed by Allen et al (11) and Rhoads et al. (12). Briefly, minced muscle tissue was incubated at 37°C in 1.25 g protease type XIV from *Streptomyces griseus* (Sigma, St. Louis, MO)/L PBS at approximately 40 mL buffer/5 g tissue. Satellite cells were separated from muscle fibers by differential centrifugation and were then collected by centrifugation at 1500 × g for 5 min. Cells were then resuspended in 20 mL of proliferative media (DMEM + 10% heat inactivated fetal bovine serum (FBS) + antibiotics) and plated in fresh media onto 145cm² tissue culture plates coated with poly-L-lysine (MP Biomedicals, Solon, OH) and fibronectin (Sigma, St. Louis, MO). Pax7 staining greater than 92% was used to validate satellite cell preps.

The MSC were isolated from bone marrow according to the method of Mahajan and Stahl (13). Cells from dispersed marrow were plated at a density of 5 × 10⁴ nucleated cells/cm² in T-75 culture flasks in proliferative media. Hematopoietic cells and all other non-adherent cells were removed with complete media changes with vigorous washing every 24

h. All cultures were incubated at 37°C in a humidified environment containing 5% CO₂. Mesenchymal stem cells were validated using CD105 and CD45 staining. Preps that stained greater than 95% for CD105 and less than 5% for CD45 were used.

In vivo cell proliferation

The percentage of satellite cells and MSC with BrdU incorporated into their DNA was identified by immunocytochemistry (1). Approximately 200 cells were counted per animal and the percentage proliferation was determined by calculating the ratio of stained cells to total cells.

Analysis of Gene Expression

Total RNA was isolated from bone marrow and the parathyroid using RNeasy Midi Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Genomic DNA contamination was removed by treatment with deoxyribonuclease (Ambion DNA free-kit, Austin, TX), and the RNA was then reverse transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen, Carlsbad, CA) to ensure the removal of residual RNA. Primer sets (**Table 2**) were designed using software (Integrated DNA Technologies, Coralville, IA) for the examination of 1- α -hydroxylase, peroxisome proliferator-activated receptor gamma 2 (PPAR γ 2), osteocalcin, lipoprotein lipase (LPL), adipocyte protein 2 (AP2), insulin-like growth factor 1 (IGF1), runt-related transcription factor 2 (Runx2), and receptor activator of nuclear factor kappa-B ligand (RANKL) messenger RNAs (mRNA) in bone marrow; and parathyroid hormone (PTH), calcitonin, and calcium sensing receptor (CasR) in the parathyroid.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (Version 9.2, 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

An effect of dietary PO_4 on circulating PO_4 concentrations was noted by 6 d on trial. Sera PO_4 concentrations of those animals receiving the PO_4 deficient diet remained lower ($P < 0.05$) relative to the other treatment groups at 6 d and 12 d (**Table 3**). Sera PO_4 concentrations of pigs receiving the excess diet were numerically higher than those of pigs receiving the adequate diet at on both sample days. Dietary PO_4 restriction resulted in higher ($P < 0.1$) sera Ca concentrations at 6 d, likely the result of lower sera PO_4 concentrations. At 12 d, sera Ca concentrations did not differ between dietary treatment groups (Table 2). An effect of dietary PO_4 on sera PTH was apparent at 6 d. Animals receiving the adequate or excess diets had sera PTH concentrations that were approximately 8 and 10 times higher than the deficient group, respectively (**Table 3**). By trial completion, dose dependent PTH concentrations were noted between all three groups (4.96 pg/ml vs. 37.55 pg/ml vs. 52.03 pg/ml), with PO_4 restriction resulting in lower ($P < 0.05$) circulating PTH concentrations relative to PO_4 excess, though the adequate group did not differ from either (**Table 3**).

There were no obvious differences in growth performance within the first 6 d on trial. In the last 6 d, excess PO_4 improved growth ($P < 0.05$, **Figure 1A**) relative to adequate fed animals and tended ($P = 0.12$) to increase gain when compared to the PO_4 deficient pigs.

Although intake did not differ between groups during this period, PO₄ excess fed pigs were more efficient ($P < 0.05$) at feed conversion (**Figure 1B**). Additional dietary PO₄ resulted in improved ($P < 0.05$) feed conversion efficiency relative to PO₄ restriction, though only a trend ($P = 0.17$) existed compared to PO₄ adequate pigs. Despite the differences observed in intake and feed efficiency throughout the trial, additional PO₄ only tended to increase gain relative to the adequate ($P = 0.17$) and deficient ($P = 0.11$) groups (**Figure 1**).

Dietary PO₄ deficiency resulted in wider bones ($P < 0.05$, Figure 2B) with lesser dry matter percentages ($P < 0.05$, **Figure 2C**) when compared to the other treatment groups. Radii of these pigs also tended to be shorter in length ($P = 0.1$, **Figure 2A**) and contain less mineral ($P = 0.1$, Figure 2D). Similar to our previous work, PO₄ deficiency resulted in reduced ($P < 0.05$) proliferation of both satellite cells and MSC (Figure 3). While satellite cell proliferation did not differ (**Figure 4A**) between pigs receiving the adequate or excess PO₄ diets, excess dietary PO₄ increased ($P < 0.05$) proliferation of MSC (Figure 4B) relative to the deficient and adequate fed pigs. Within bone marrow, osteocalcin expression tended to be higher ($P < 0.1$, Figure 3C) in PO₄ excess fed pigs compared to those receiving the deficient diet. Phosphate excess also resulted in decreased ($P < 0.05$) mRNA expression of RANKL (**Figure 3D**) compared to PO₄ adequacy. Expression of calcitonin in the thyroid was higher ($P < 0.05$, Figure 2A) in PO₄ adequacy and tended to be higher ($P < 0.1$) in PO₄ excess when both groups are compared to pigs receiving the deficient diet. Additionally, CasR mRNA expression tended ($P < 0.1$, **Figure 3B**) to be diminished in PO₄ deficiency.

DISCUSSION

We have previously demonstrated an in vivo reduction in satellite cell proliferation during PO₄ restriction. The increased sensitivity of the neonatal pig to nutrient deficiencies as well as the greater abundance of tissue specific stem cells present in a young, rapidly growing animal made it an ideal model to determine the impact of PO₄ status on the activity of satellite cells and MSC.

Phosphate excess had an effect on growth performance relative to the adequate group, most notably in the final 6 d on trial. As is typical in PO₄ deficiency, pigs tended to have reduced growth and poorer feed conversion efficiency. The differences in growth performance in the present study were far less dramatic than those previously observed (1). The bioavailability of PO₄ employed in the present study was much higher than the soy-based source formerly used, which allowed for a less severe deficiency resulting in growth comparable to sow reared piglets.

Concentrations of endocrine hormones associated with PO₄ status were reflective of dietary PO₄ restriction. At 6 d, we observed slightly lowered sera Ca during PO₄ adequacy and excess despite equal inclusion within each treatment diet. This was likely a consequence of elevated PO₄ levels. By trial completion, sera Ca concentrations in PO₄ adequacy and excess remained lowered relative to the deficient group, but not significantly so, making the increases in sera PTH at 12 d a response to dietary PO₄. We previously observed a direct effect of dietary PO₄ on sera PTH independent of sera Ca, a phenomenon that has been substantiated in previous works (18, 19). Calcium and PO₄ balance is tightly regulated by the interplay of PTH and 1, 25 (OH)₂ vitamin D in the adult animal, however, we and others

have shown that mineral regulation by $1, 25 \text{ (OH)}_2$ vitamin D is absent in the neonate (1, 14, 15), therefore PTH seems to be the major regulatory hormone of Ca and PO_4 balance in the neonatal animal.

The only observed differences in bone due to PO_4 restriction were in width and dry matter percentage, with only trends for longer bones ($P = 0.11$) in PO_4 excess and greater ash percentage ($P = 0.11$) in adequacy relative to PO_4 restricted pigs. Growth of long bones in the young animal occurs from the growth plates located at the ends of the bone which is consistent with our results since radii of pigs lacking adequate dietary PO_4 tended to be shorter than pigs receiving the adequate or excess diet. Lower dry matter percentage during PO_4 restriction is indicative of greater water content within the bone, a large portion being associated with protein. This corresponds with lesser mineral content in found PO_4 deficient pigs.

We also found that PO_4 excess had no impact on satellite cell proliferation. As was expected, restriction reduced proliferation; however, additional PO_4 did not improve proliferation relative to pigs receiving the adequate diet. This could account for the comparable growth performance of the adequate and excess groups. Although the impact of excess dietary PO_4 on growth in the neonatal pig has not been previously reported, nutrient deficiencies have been shown to reduce satellite cell number and activity (16, 17) and decrease muscle size and the expression of those genes associated with myogenic progression (18, 19). The permanent growth deficits resulting from reductions in satellite cell number are indicative of the critical nature of early life nutrition on growth and nutritional programming of tissue specific stem cells. It is possible that the inclusion level of the dietary PO_4 coupled

with the protein source used in the study was not enough to distinguish differences between the PO₄ adequate and excess groups. However, the trends observed in growth suggest that addition of PO₄ at levels higher than those used in the present study will be beneficial to muscle growth and development. Mesenchymal stem cell proliferation did increase with additional PO₄, but did not result in a marked difference between bone traits of adequate or excess fed pigs.

Because of the divergent fates of MSC into either osteoblastic or adipocytic lineages (20, 21), we once again looked at genes associated with these respective lineages. There were no differences observed in those genes associated with adipogenesis. Osteocalcin mRNA expression tended to be increased ($P < 0.1$) in PO₄ excess fed pigs compared to deficient pigs. This was not unexpected because increased PO₄ has previously promoted expression of those genes associated with osteogenesis (1). RANKL expression in adequacy was almost double ($P < 0.05$) that of excess fed pigs, who had the lowest expression levels among all 3 groups. This particular protein is responsible for maturation, activation, and differentiation of osteoclasts. Its expression on the surface of osteoblasts is increased by enhanced PTH levels (22), associated with heightened bone resorption. This is contrary to what was observed in the present study since sera PTH concentrations were highest in excess, though only significant when compared to the deficient group and mRNA transcripts of PTH within the thyroid did not differ (data not shown) between any dietary treatment groups. CaSR expression in the thyroid was highest in adequacy. Parathyroid cells and thyroid C-cells are sensitive to changes in circulating Ca and it is this response to sera Ca that regulates the production and release of PTH or calcitonin from the parathyroid and thyroid,

respectively (23). Expression of both calcitonin and CaSR was increased in PO₄ adequacy and excess although excess PO₄ was not significantly different from either the adequate or deficient fed pigs. This corresponds with the lowered sera Ca observed in both the PO₄ adequate and excess groups.

As with our previous work, we were able to depress growth performance, alter bone integrity traits and change sera indicators of PO₄ status through PO₄ restriction. In our earlier study, we found that PO₄ deficiency reduced stem cell proliferation; therefore there was the possibility that additional PO₄ could improve proliferation of these cells, thus enhancing the growth and development of bone and muscle. Because animals experience the greatest rate of growth during the neonatal period, a point where the majority of the mass acquired is skeletal muscle, nutritional inputs in early life are critical to maximizing future growth.

In the present study, we have demonstrated the potential benefits of providing additional dietary PO₄ to the neonatal pig. We observed trends for improved growth performance and bone integrity. We also established that PO₄ excess increases MSC proliferation and expression of genes associated with osteogenesis. Early PO₄ nutrition has implications for both human health and animal agriculture. Approximately 13% U.S. births are premature and pre-term infants have lower BMC than term infants (24) and are at greater risk of PO₄ deficiency, which is potentially detrimental to lifetime development. Similarly, prospective growth of low birth weight piglets is negatively impacted by the absence of adequate PO₄ nutrition. Because nutritional interventions in later life have been found to have far less impact on growth than those applied in early life (25, 26), it is critical that appropriate dietary interventions occur early on to maximize lean body mass accrual and

bone integrity. Lack of adequate PO_4 nutrition in humans this could lead to increased risk of both obesity and osteoporosis while in animal agriculture this would cause reduced growth performance and therefore reduced sustainability. Inclusion of PO_4 in excess of PO_4 needs could provide compensatory gains in the low birth weight neonate that matches or surpasses that of their average weigh counterparts.

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Table 1. Experimental diet composition on an as fed basis¹.

Base Diet ^{2,3}	
Ingredients,	Composition, %
Whey	21.6
Whey protein concentrate	5.0
Edible lard	24.3
Flow agent	0.22
Emulsifier	0.37
Soy protein isolate	30.0
Calcium carbonate	1.2
Dicalcium phosphate	1.0
Calcium chloride	0.33
Vitamin E premix	0.04
Mineral premix	0.5
Vitamin premix	0.08
Artificial flavor	0.03
Potassium sorbate	0.5
Dextrose	14.6
D, L methionine	0.18

¹Composition of the powdered milk replacer that was reconstituted at a rate of 175g/kg final liquid formula

²Diet was 25% deficient in phosphate.

³Milk Specialties Corporation, Dundee, IL

Table 2. Primers used for quantification of gene expression by real-time PCR

Gene name	Primer sequence
AP2	F: 5' GGCCAAACCCAACCTGA 3' R: 5' GGGCGCCTCCATCTAAG 3'
IGF-1	F: 5' TTCGCATCTCTTCTACTTGGCCCT 3' R: 5' CGTACCCTGTGGGCTTGTTGAAAT 3'
PPAR γ 2	F: 5' AATTAGATGACAGCGACCTGGCGA 3' R: 5' TGTCTTGAATGTCCTCGATGGGCT 3'
Runx2	F: 5' CAAGTGCGGTGCAAACCTTTCTCCA 3' R: 5' AGGCTGTTTGATGCCATAGTCCCT 3'
RANKL	F: 5' TGGATCACAGCACATCAGAGCAGA 3' R: 5' TGGTACCAAGAGGACAGACTCACT 3'
LPL	F: 5' ACCGTTGCAACAACCTTGGGCTATG 3' R: 5' ACTTTGTAGGGCATCTGAGCACGA 3'
Osteocalcin	F: 5' TACCCAGATCCTCTGGAGCCC 3' R: 5' TATGCCATAGAAGCGCCGATA 3'
PTH	F: 5' ATGCATAACCTGGGCAAACACCTG 3' R: 5' TAGAAGCTCCGAGGGCAACAAAGT 3'
Calcitonin	F: 5' TCCAGAGCTAAGCGGTFCAGTAAT 3' R: 5' TTTCTTGCCAGGTGTTTCAGGC 3'
CaSR	F: 5' CATCAAGTTCCGAAACACGCCCAT 3' R: 5' GCAGAGCACGAAGCTAATGCCAAA 3'

Table 3. Effect of dietary PO₄ on sera measures

	Sera Measures					
	PO ₄ (mg/dL)		Ca (mg/L)		PTH (pg/mL)	
	d6	d12	d6	d12	d6	d12
PO ₄ deficient	5.27 ^a	5.00 ^a	116.62 ^a	112.22	1.52 ^a	4.96 ^a
PO ₄ adequate	6.14 ^b	6.73 ^b	99.17 ^b	108.69	13.57 ^b	37.55 ^{ab}
PO ₄ excess	6.34 ^b	6.99 ^b	102.87 ^b	104.53	15.94 ^b	52.03 ^b
<i>P-value</i>	< 0.01	<0.01	<0.05	=0.6	<0.01	=0.1

^{a, b}Values not sharing a common superscript are different (P < 0.05)

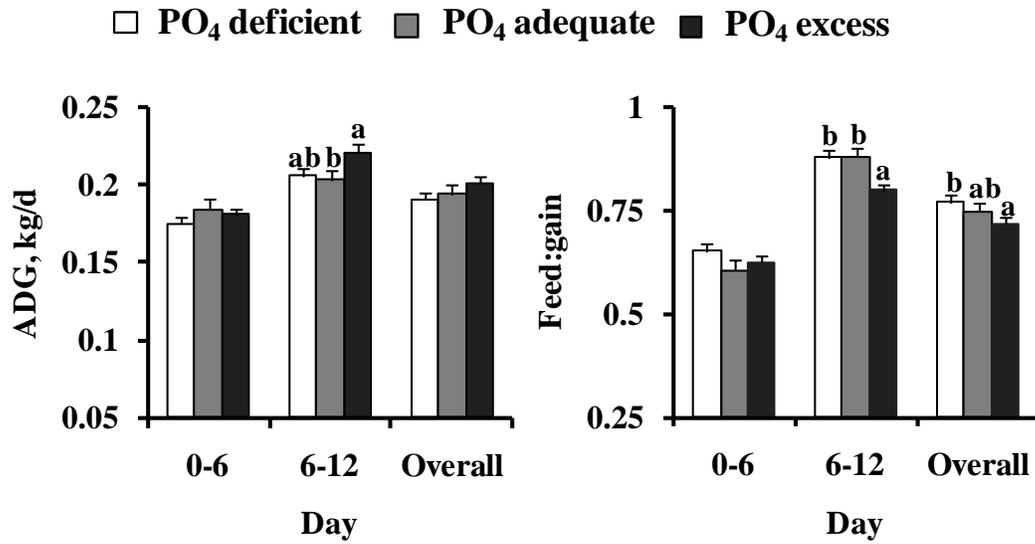


Figure 1. Effect of dietary PO₄ on growth performance (A) Average daily gain, kg/d (B), Feed efficiency (feed:gain). Body weight and intake were recorded everyday over the entire trial. Values presented are least square means and standard error. ^{a, b}Values not sharing a common superscript are different (P < 0.05). PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

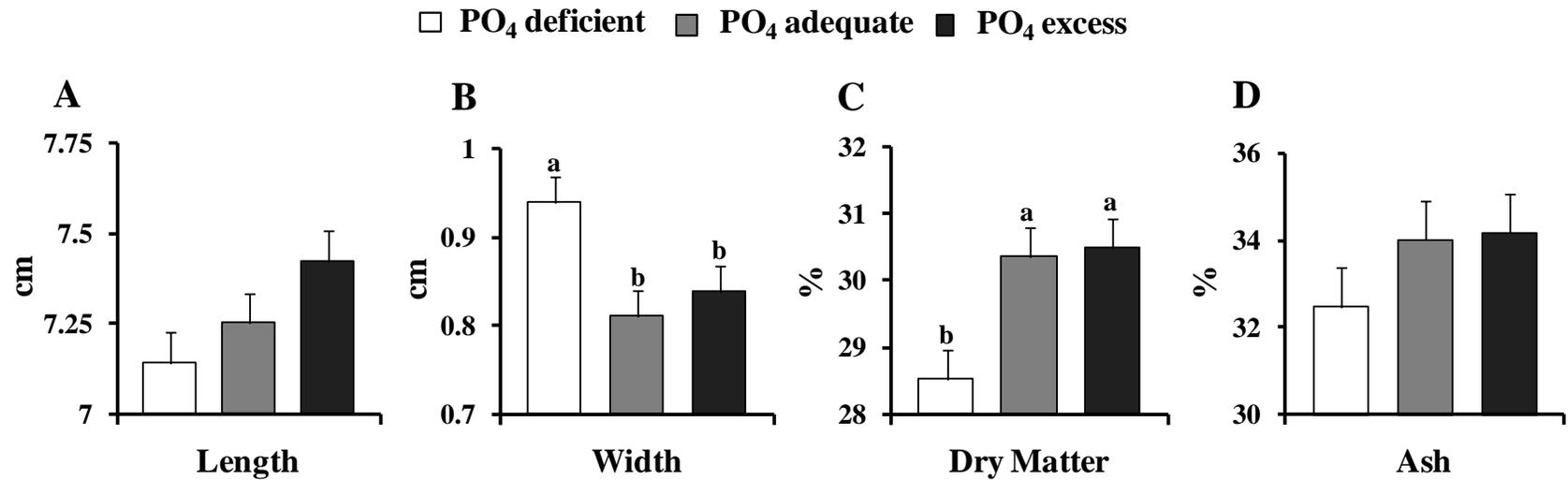


Figure 2. Effect of dietary PO₄ on bone parameters (A), Length (mm) (B), width (mm) (C), dry matter percentage (D) ash percentage of radii collected at trial completion neonatal pigs. Values presented are least square means and standard error of values normalized to cDNA concentrations. ^{a, b}Values not sharing a common superscript are different (P < 0.05). PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

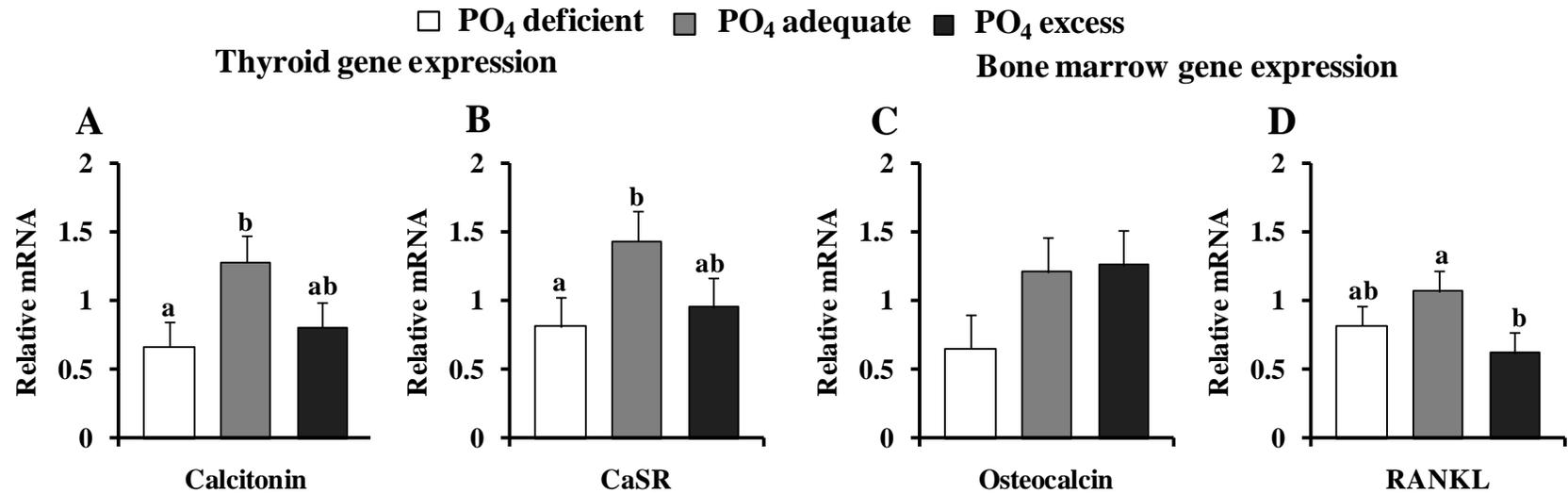


Figure 3. Effect of dietary PO₄ on the gene expression of (A), calcitonin and (B), CaSR) in bone marrow and the gene expression of (E) osteocalcin and (F) RANKL in the thyroid of neonatal pigs. Values presented are least square means and standard error of values normalized to cDNA concentrations. ^{a, b}Values not sharing a common superscript are different (P < 0.05). PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

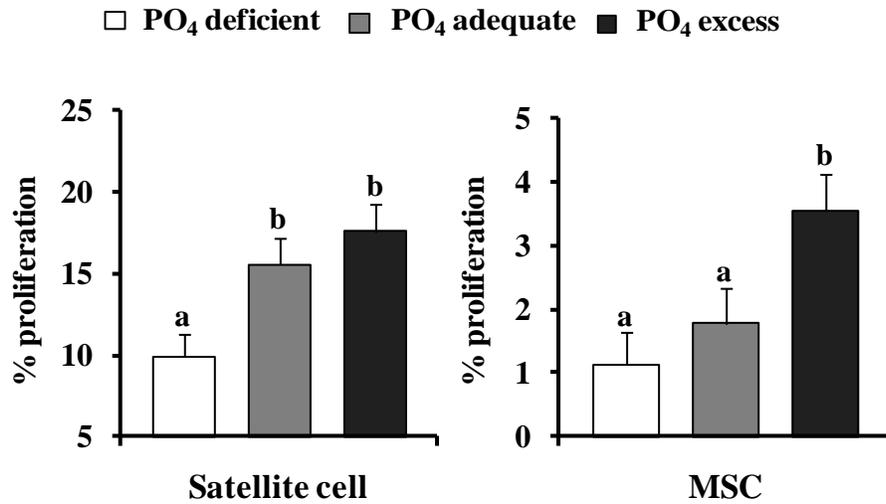


Figure 4. Effect of dietary PO₄ on in vivo stem cell proliferation. (A) Satellite cell proliferation and (B) MSC. ^{a, b}Values not sharing a common superscript are different (P < 0.05). PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

**CHAPTER V:
Dietary phosphate supplementation to neonatal pigs impacts satellite cell proliferation
and progression through their myogenic lineage**

To be submitted to the Journal of Nutritional Biochemistry: Lindsey S. Alexander and Chad H. Stahl¹

¹Laboratory of Developmental Nutrition, Department of Animal Science, North Carolina State University, Raleigh, NC 27695;

Abstract

Despite the importance of satellite cells to muscle fiber hypertrophy and dietary PO₄ to muscle development, little research has examined the impact of PO₄ nutrition on activity of satellite cells. Satellite cells isolated from 21 pigs that were fed a milk-replacer diet that was either PO₄ adequate (PA), 25% in excess of the PO₄ requirement (PE), or 25% deficient in PO₄ (PD) content for 12 d. The isolation procedure was performed according to methods developed by Allen et al. and Rhoads et al. Cells were cultured in proliferative media for 3 d, just prior to confluence, and were switched to differentiation media for an additional 4 d, totaling 7 d in culture. Cell proliferation was determined at 1 d and 2 d after plating. Immunofluorescent staining was performed at 3, 5, and 7 d in culture for Pax7, MyoD, and myogenin. DNA:protein content was determined at 5 d and 7 d. Greater proliferation ($P < 0.05$) was noted in cells isolated from PE cells 1 d after plating. Staining of cells did not significantly differ between treatment groups for either protein. DNA content was higher ($P < 0.05$) in PE cells relative to PD and PA cells at 5 d. Myogenin and MyoD expression spiked in PD cells at 7 d and were higher ($P < 0.05$) than PA and PE cells. The differences in cell behavior based on dietary PO₄ would suggest a PO₄ nutrition in programming of satellite cell activity. By gaining a better understanding of how early we can clarify the possible long-term effects on growth potential in the pig.

Introduction

Satellite cells provide the myonuclei that allow for muscle fiber hypertrophy (1, 2). Once activated, satellite cells will proliferate, differentiate and fuse to existing fibers and donate nuclei to the growing myofiber. Commitment of satellite cells down the myogenic lineage is regulated by Pax7, MyoD, and myogenin, a group of transcription factors that, when expressed sequentially, function to signal myogenic progression. Expression of these transcription factors have been shown to be altered by nutrient restriction (3) and likely initiate the changes in satellite cell activity due to nutrition. Because dietary PO₄ restriction reduces muscular growth (4-6), investigating possible nutritional programming of satellite cells by dietary PO₄ through modifications in satellite cell proliferation and expression of myogenic transcription factors is necessary to determine a probable mechanism by which this occurs.

The important role of satellite cells in muscle growth and the necessity of dietary PO₄ for proper muscle development have lead to the examination of the impact of PO₄ nutrition during the neonatal period when there is the greatest proportion of satellite cells in muscle. Multiple studies have illustrated the importance of early life nutrition on satellite cell kinetics and subsequent growth (7, 8). We have previously demonstrated in vivo reductions in satellite cell proliferation during PO₄ restriction, suggestive of a mechanism by which dietary PO₄ alters stem cell activity with corresponding decreases in growth (9).

This study examined in vitro behavior of satellite cells isolated from pigs that received 3 different levels of dietary PO₄. The objective was to determine the impact of different inclusion levels of dietary PO₄ on stem cell activity and expression of myogenic

transcription factors and t. Changes in satellite cell activity relative to dietary PO₄ could offer insight into approaches for maximizing future growth and determining if alterations in early nutrition permanently alter cell behavior.

Materials and Methods

Animals and satellite cell isolation

All animal protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. Twenty-one male and female piglets obtained at 24-32 h of age were allotted into 1 of 3 dietary treatment groups by gender and body weight. Pigs were pair-fed a milk-replacer diet that was either PO₄ adequate (PA), 25% in excess of the PO₄ requirement (PE), or 25% deficient in PO₄ (PD) content for 12 d. At trial completion, satellite cells were isolated from the loin muscle of individual pigs according to modified methods developed by Allen et al. (9) and Rhoads et al (10). Briefly, minced muscle tissue was incubated at 37°C for 1 h in 1.25 g protease type XIV from *Streptomyces griseus*/L PBS (approximately 40 mL buffer/5 g tissue). Satellite cells were separated from muscle fibers by differential centrifugation and then filtered through a 100 µm cell strainer to rid the satellite cells of debris. They were then collected by centrifugation at 1500 × g for 5 min, resuspended in 20 mL of proliferative media (DMEM + 10% heat inactivated fetal bovine serum (FBS) + antibiotics) and plated in 145 cm² tissue culture plates coated with poly-l-lysine (MP Biomedicals, Solon, OH) and fibronectin (Roche Applied Science, Indianapolis, IN). Satellite cells were identified in each culture by Pax7 staining greater than 92%. All cultures were incubated at 37°C in a humidified environment containing 5% CO₂.

When cells reached approximately 60% confluence, they were harvested using 0.25% trypsin in 1 mM EDTA (Invitrogen, Carlsbad, CA) and frozen down for later use.

Cell proliferation

Cryopreserved satellite cells from individual animals from each treatment group (n=15) were cultured in proliferation media (DMEM + 10% FBS + antibiotics) in 15 cm plate, coated with poly-l-lysine and fibronectin until 60% confluence. Cells were subsequently trypsinized and replated at 2500 cells/sq. cm² (5000 cells/well) in 24-well plates. Satellite cells were cultured in proliferative media for 3 d and switched to differentiation media (DMEM + 2 % horse serum (HS) + antibiotics) for an additional 4 d, totaling 7 d in culture. Cell proliferation was determined at 1 d and 2 d after initial plating using Click-iT® EdU Alexa Fluor® 488 HCS Assay (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Cells were pulsed with EdU (5-ethynyl-2'-deoxyuridine) for 2 h, fixed and permeabilized, followed by staining to detect EdU incorporated cells. Approximately 200 cells were counted per animal and proliferation was measured by calculating the ratio of EdU stained cells to the total number of cells.

Immunofluorescent staining

Immunofluorescent staining was performed at 3, 5, and 7 d in culture. Cells were fixed in 2% paraformaldehyde for 30 min on ice. Following fixation, a block consisting of 1% bovine serum albumin (BSA) and 0.1% triton X-100 in phosphate buffered saline (PBS) was applied for 1 h at room temperature. One well per animal was designated for co-staining of Pax7 and MyoD and another well for co-staining of myogenin and MyoD. The primary antibodies and their dilutions in PBS containing 1% BSA were as follows: (1) mouse

monoclonal anti-Pax7 (1:50; AbD Serotec, Raleigh, NC), (2) rabbit polyclonal anti-MyoD (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and (3) mouse monoclonal anti-myogenin (1:50; AbCam, Cambridge, MA). Primary antibodies were incubated overnight at 4°C followed by a wash in PBS and incubation in secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted in PBS containing 1% BSA as follows (1) DyLight 488 AffiniPure donkey anti-mouse IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) and (2) Texas Red AffiniPure goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained using a 1:1000 dilution of 4,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO) in PBS for 2 min.

DNA:protein content

DNA:protein content was determined at 5 d and 7 d. Three hundred microliters of lysis buffer (10mM Tris HCl, pH 8; 0.1 mM EDTA, pH 8; 0.5% (w/v) SDS) were added to 4 wells per animal with each well being treated a technical replicate. The lysate was split, using 150 µl for DNA isolation and 150 µl for protein concentration determination. To isolate DNA, proteinase K (Sigma, St. Louis, MO) and RNase A (Omega Bio-Tek, Norcross, GA) were added to lysed cells and incubated at 42°C for 3 h. Phenol chloroform isoamyl (Sigma, St. Louis, MO) was added to the lysate and centrifuged. The aqueous layer was removed followed by the addition of sodium acetate and 100% ethanol. After overnight precipitation at 4°C, samples were centrifuged at maximum speed for 10 min. The resulting pellets were washed 3 times in 70% ethanol, allowed to dry, and reconstituted in 10 µl of DNase-free water.

Protein concentrations were determined using the Lowry's assay (11). Lysed samples were diluted 1:5. An alkaline copper reagent was added to the lysate and samples were allowed to sit for 10 min at room temperature. Folin-Ciocalteu's Phenol reagent (Sigma, St. Louis, MO) was then added and incubated for 30 min at room temperature. Samples were transferred to a 96-well plate and read at 750 nm using a microplate spectrophotometer (PowerWave HT Microplate Scanning Spectrophotometer, Bio-Tek, Winooski, VT) against a BSA standard. Sample concentrations were determined using a spectrophotometer at A_{260} .

Gene expression analysis

Total RNA was extracted using the commercially available Ambion RNAqueous Micro Kit (Ambion, Austin, TX). Genomic DNA was eliminated by treatment with deoxyribonuclease (Ambion DNA free-kit, Austin, TX). Purified RNA was then reverse transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen, Carlsbad, CA) to ensure the removal of residual RNA. Primer sets were designed using software (Integrated DNA Technologies, Coralville, IA) for the examination of Pax7, MyoD, and myogenin mRNAs. The forward and reverse primers (in that order) for each gene were as follows: Pax7: CAACCACATCCGCCACAAGATAGT and AGAGGATCTTGGAGACACAGCCAT; MyoD: GCGTGCAAACGCAAGACCACTAA and AGTCTCGAAGGCCTCGTTGACTTT; Myogenin: TGACCCTACAGATGCCCAATCT and GTTGGGCATGGTTTCATCTGGGAA

Primer sets were validated according to the specifications set forth by Livak and Schmittgen (12). Relative quantities of the transcripts of interest were determined by semi-

quantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System and SybrGreen Supermix, Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions included 40 cycles of 20 s of melting at 95°C followed by 20 s of annealing and extension at 60°C. Following amplification, all samples were subjected to a melt curve analysis. Gene expression was normalized to the adequate group at 3 d for each gene, using a modification of the $2^{-\Delta CT}$ method.

Statistical analysis

Data were analyzed using the GLM procedure of SAS (Version 9.2, SAS Institute Inc., Cary, NC) with dietary treatment considered as a fixed effect. Replicate was also used as a fixed effect since satellite cell isolations did not occur on the same day. Differences were considered significant at $P < 0.05$.

Results

Greater proliferation ($P < 0.05$) was noted in cells isolated from PE cells 1 d after plating relative to the other treatment groups (Figure 1). After 48 h, groups did not differ in proliferation rate, but there was an increase in proliferation of PD cells between 1 d and 2 d, while proliferation remained the same in PA cells and decreased in cells isolated from PD fed pigs. Immunofluorescent staining was performed for those proteins associated with myogenic progression at 3, 5, and 7 d. At 3 d, Pax7⁺ staining percentages in PA ($P = 0.14$) and PE ($P = 0.1$) cells tended to be higher than PD cells (Figure 2). Almost all cells were Pax7⁺ within each group by 5 d, averaging around 96.2%. Pax7 staining percentages decreased in all groups (2.34% vs. 3.06% vs. 1.72) by 7 d. Similar to Pax7 staining at the same time point, PD cells tended to have a lower percentage of MyoD⁺ cells relative to PA

($P = 0.13$) and PD ($P = 0.1$) cells at 3 d (Figure 2). MyoD staining did not differ at any other time. Myogenin staining only differed at 5 d, with PA cells tending ($P=0.11$) to have a higher percentage of myogenin⁺ cells relative to PD cells.

DNA and protein content were determined at 5 d and 7 d. DNA content was higher ($P < 0.05$) in PE cells relative to PD and PA cells at 5 d (Figure 3). Although DNA content increased in all groups by 7 d, PD cells had higher ($P < 0.05$) DNA content than PA cells while PE cells did not differ from either group. At 5 d, PD cells had greater protein content than either PA or PE cells, though not significantly so (705.14 ng/ μ l vs. 647.23 ng/ μ l vs. 648.21 ng/ μ l). Protein content decreased in all groups at 7 d. Phosphate excess cells had greater ($P < 0.05$) DNA:protein content relative to the other groups at 5 d (Figure 3). At 7 d, DNA:protein content increased in all groups with PE cells being the highest ($P < 0.05$) relative to PA cells and PE cells not differing from either group.

Gene expression was analyzed at 3 d, 5 d, and 7 d. Pax7 expression increased over time but did not differ between groups at any time point. Expression was highest in PE cells at 5 d but by 7 d, expression levels of Pax7 in PD cells had surpassed PE cells. MyoD expression did not differ between groups at 3 d and 5 d, but there was a spike in expression at 7 d in PD cells. Expression of MyoD in PD cells at 7 d was a little over 2-fold greater ($P < 0.05$) than expression in PE cells. MyoD expression in PA cells did not differ from either group. A similar pattern was observed in myogenin expression. At 5 d, myogenin expression in PD cells was 3-fold higher ($P < 0.05$) than PA cells and tended to be higher than PE cells ($P = 0.1$). Myogenin expression also spiked in PD cells at 7 d, with levels being approximately 3-fold higher ($P < 0.05$) than PA and PE cells.

Discussion

We have previously demonstrated an in vivo reduction in satellite cell proliferation during PO₄ restriction. The increased sensitivity of the neonatal pig to nutrient deficiencies as well as the greater abundance of tissue specific stem cells present in a young, rapidly growing animal made it an ideal model to determine the impact of PO₄ status of satellite cells on progression of these cells through the myogenic lineage. The current study allows further exploration of the hypothesis that early life dietary PO₄ causes a nutritional programming event that permanently alters satellite cell kinetics, thus effecting future growth potential.

An effect ($P < 0.05$) of excess PO₄ on proliferation was observed within the first 24 h on trial, which suggests that excess nutriture may increase the proliferative capacity of satellite cells. Although proliferation did not differ between treatment groups at 2 d, it should be noted that PD cells experienced a sharp increase in proliferation relative to 1 d (Figure 1). The delayed increase in proliferation could be a response of PD cells to the sera rich proliferation media. Studies in whole animals that have caused reductions in satellite cell activity due to nutrient deprivation have reported compensatory responses to nutrient restriction, including rapid increases in satellite cell proliferation following the restoration of a normal diet (7, 8).

Postnatal muscle growth is dependent upon the hypertrophy myofibers. Along with increases in fiber size are corresponding increases in DNA content through myonuclear addition (13-15). Myonuclei are post mitotic so nuclear addition to fibers is accomplished through the activity of satellite cells. Because each myonucleus within a fiber regulates a finite volume of cytoplasm, the addition of myonuclei is necessary for an increase in fiber

size. We calculated DNA and protein content at 5 d and 7 d in culture. At 5 d, PO₄ excess cells had higher DNA content ($P < 0.05$) and lower protein content (Figure 3). This resulted in a higher DNA to protein ratio, which is suggestive of greater proliferation in dietary PO₄ excess. This corresponds with Pax7 gene expression data that demonstrated higher expression in PO₄ excess cells at 5 d, though not significantly (Figure 4). There was an unexpected decrease in total protein content at 7 d in all groups, as we would assume protein content would increase following increases in DNA content. While the decrease in total protein content corresponds to the reduction in protein staining, the higher DNA:protein content in PD cells does not correlate with the increased ($P < 0.05$) expression of MyoD and myogenin 7 d.

A possible explanation for the lack of consistency in protein content and staining relative to gene expression could be cell loss due to apoptosis or necrosis. Another plausible explanation could be the action of micro-RNAs, which decrease translation of protein-coding genes through mechanisms that have yet to be clarified. In recent years, multiple micro-RNAs designated “myogenic micro-RNAs” have been implicated as regulators of muscle development and differentiation (16, 17). Muscle specific micro-RNAs, mir-1, mir-133, and mir-206 have been shown to regulate different stages of myogenesis and directly target proteins associated with myogenic progression (18, 19). There is the possibility that the increases in MyoD and myogenin expression at 7 d did not translate to protein synthesis due to interference from micro-RNAs.

The sequential expression of Pax7, MyoD, and myogenin in the myogenic progression of satellite cells has been covered extensively (20, 21). Proper expression of

these proteins is critical to normal muscle development and any modifications in expression pattern can alter muscle growth (33, 34). After 3 d in proliferative media, Pax7⁺ and MyoD⁺ staining tended to be less in PO₄ deficient cells (P = 0.1, Figure 3). Myogenin staining did not significantly differ between treatments at any of the time points examined, though myogenin⁺ staining was less in PO₄ deficient cells at all points. The percentage of cells staining for myogenin did not exceed Pax7⁺ or MyoD⁺ cells until 7 d. Halevy et al. (22) examined protein expression patterns of Pax7, MyoD, and myogenin in myogenic cultures derived from 9-d old posthatch chickens. With increases in Pax7⁺/MyoD⁻ cells, the number of Pax⁺/MyoD⁺ cells decreased with a portion transitioning to myogenin⁺. The Pax⁺/myogenin⁺ phenotype was present in a very small number of cells and was suggested to represent a population of cells that maintain their ability to proliferate. Pax7 expression is known to be higher during proliferation and declines upon differentiation of satellite cells whereas MyoD acts as an intermediary between proliferation and terminal differentiation. Reductions in cells staining positive for either protein were not unexpected by 7 d in culture, though it was surprising to see percentages of myogenin⁺ cells remain relatively unchanged between 5 d and 7 d. Halevy et al. (22) noted transient increases in myogenin after 3 d in culture, however, protein levels declined after 6 d, an event that was attributed to a possible decline in the number of cells that can undergo differentiation. Free nuclei versus nuclei in tubes were counted to determine tube formation and we found very little tube formation in all groups. A more likely explanation for the absence of differences in myogenin staining observed between 5 d and 7 d could be the lack of tube formation.

The data in the present study validate a possible role of early life PO₄ nutrition in programming of satellite cell activity. The higher proliferation of PE cells at 1 d and the large increase in MyoD and myogenin expression between 5 d and 7 d in PD cells might indicate a greater proliferative capacity of excess PO₄ cells, whereas premature differentiation occurs in satellite cells isolated from PO₄ restricted pigs. By gaining a better understanding of how early life PO₄ nutrition impacts satellite cell activity, we can clarify the possible long-term effects on growth potential in the pig.

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□ PO₄ deficient ■ PO₄ adequate ■ PO₄ excess

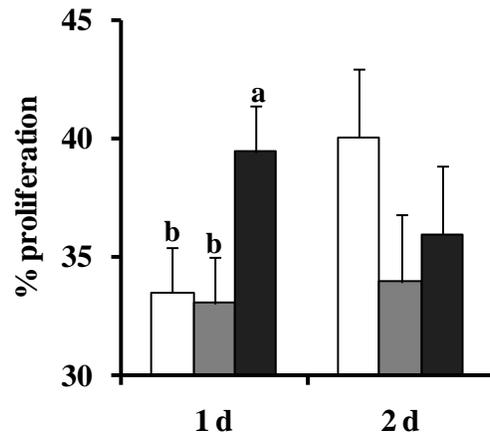


Figure 1. Effect of dietary PO₄ on in vitro satellite cell proliferation^{a, b} Values not sharing a common superscript are different ($P < 0.05$). PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

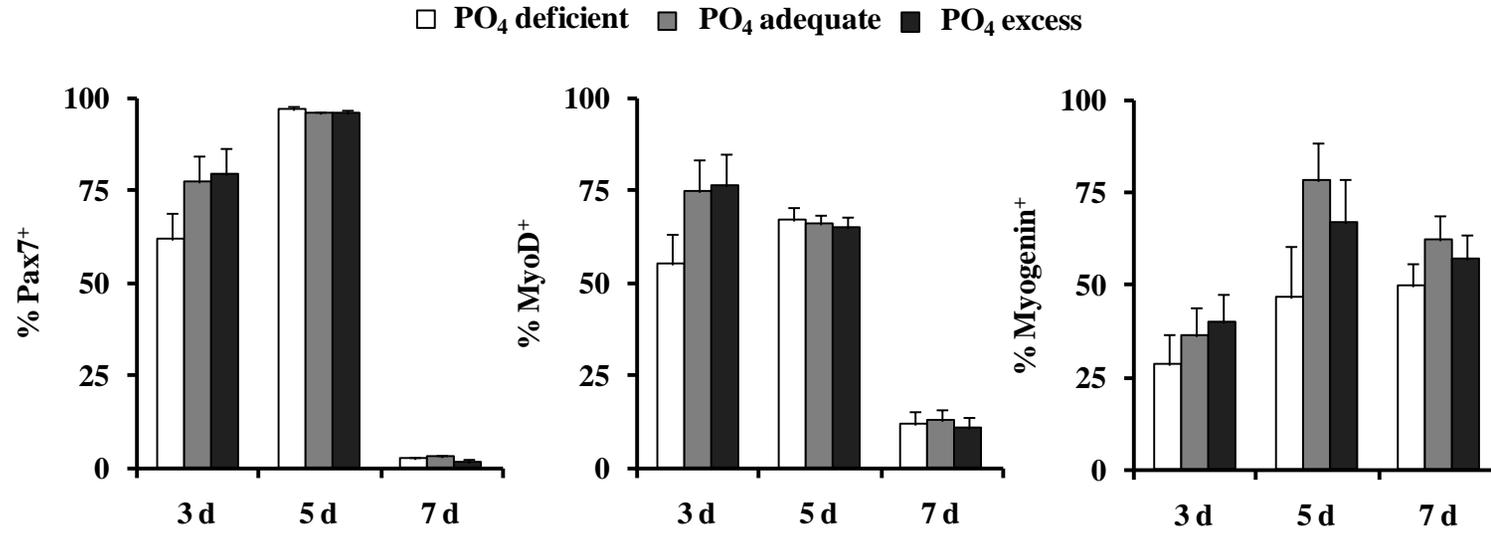


Figure 2. Effect of dietary PO₄ on protein staining of myogenic markers
^{a, b}Values not sharing a common superscript are different (P < 0.05).
 PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

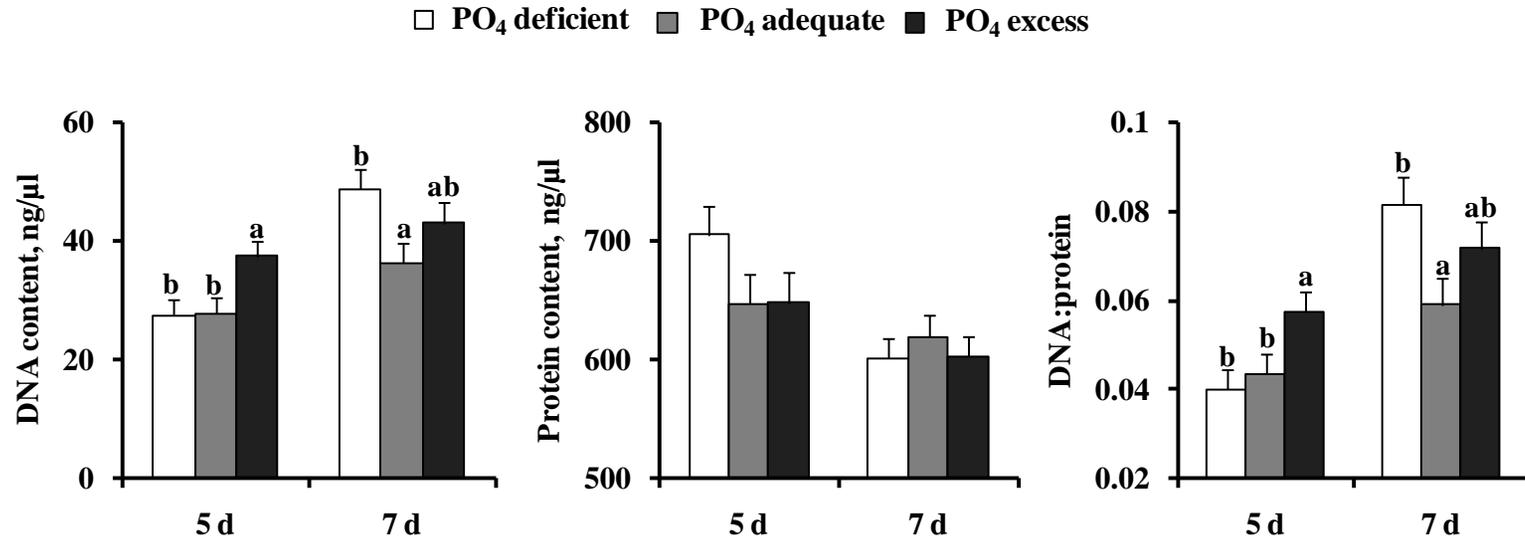


Figure 3. Effect of dietary PO₄ on DNA and protein content
^{a, b}Values not sharing a common superscript are different (P < 0.05).
 PO₄ excess, n=7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

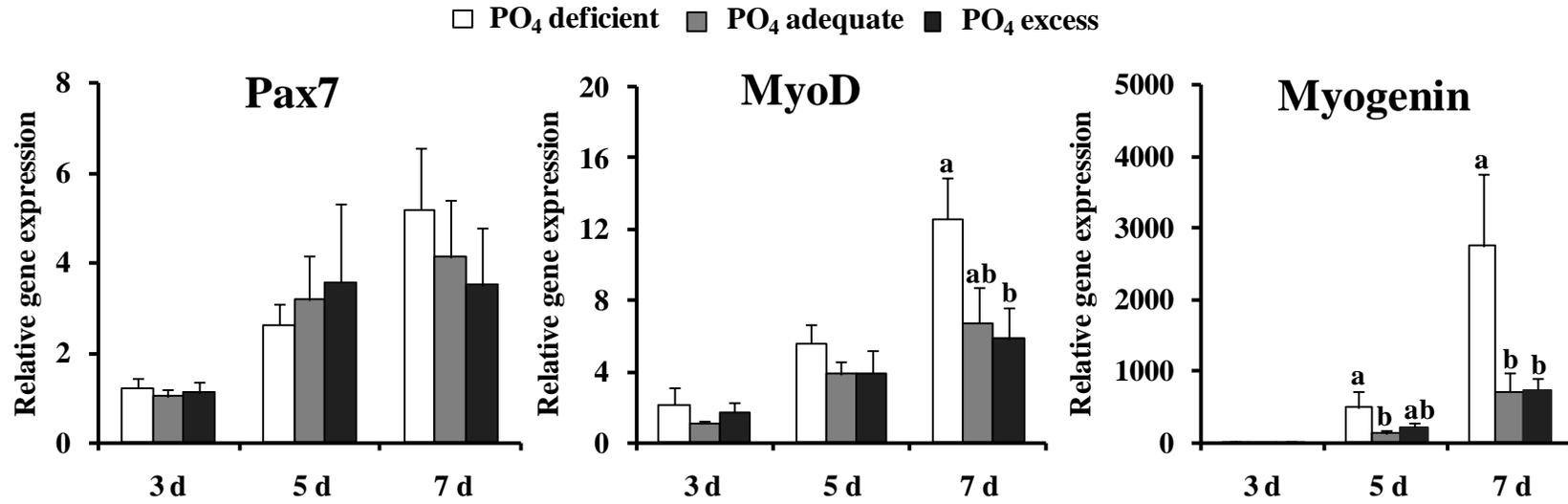


Figure 4. Effect of dietary PO₄ on satellite cell gene expression
^{a, b}Values not sharing a common superscript are different ($P < 0.05$).
 PO₄ excess, n = 7; PO₄ adequate, n = 7; PO₄ deficient, n = 7.

CHAPTER VI: General conclusions

The objectives of these studies were to evaluate the impact of dietary phosphate (PO_4) on satellite cell proliferation and subsequent growth potential in the neonatal pig. When examining the impact of PO_4 restriction on satellite cell proliferation, pigs demonstrated the typical responses to PO_4 deprivation. The diet was effective in reducing plasma PO_4 and PTH. Pigs also demonstrated reduced growth, feed conversion efficiency and bone mineral content. Most importantly, the PO_4 deficiency utilized in the study was effective in reducing satellite cell proliferation in vivo. The second study added an additional treatment to determine the impact of PO_4 in excess of the adequate diet in the first trial. The third PO_4 inclusion level could potentially increase proliferation of satellite cells in the hopes of maximizing growth and bone integrity. There were differences in our sera indicators of PO_4 status, which validated the effectiveness of the diets fed. Phosphate restriction negatively impacted growth performance and bone parameters. Satellite cell proliferation was once again reduced in PO_4 deficiency. Although there were no dramatic differences between PO_4 adequate and PO_4 excess pigs based on the indicators of PO_4 status measured, there were trends for increased satellite cell proliferation, growth performance and bone mineral with excess PO_4 . The second study demonstrated the potential benefits of providing additional dietary PO_4 neonatal pig. The objective of the final experiment was to further verify that early dietary PO_4 causes a nutritional programming event that permanently alters satellite cell kinetics. To achieve this, satellite cells isolated from pigs in experiment 2 were cultured in different media over 7 d to determine the impact of PO_4 status on lineage progression of

satellite cells. Cells isolated from PO₄ excess pigs had greater proliferation after 1 d and higher DNA:protein content at 5 d. While Pax7 gene expression continued to increase in all groups until the final day of the trial, cells isolated from PO₄ deficient pigs greatly increased expression of MyoD and myogenin at 7 d. This would suggest that cells from PO₄ excess pigs have a greater proliferative capacity and cells from PO₄ deficient pigs may differentiate prematurely. Taken together, these studies illustrate nutritional programming of satellite cells by dietary PO₄ and emphasize the importance of early life nutrition in maximizing future growth.

Phosphate nutrition is critical to muscle growth and satellite cells are responsible for muscle fiber hypertrophy, yet very little research has examined the impact of PO₄ utilization on the response of muscle growth during the neonatal phase when satellite cell numbers are greatest. Nutritionally induced reductions in satellite cell activity in early life result in permanent growth deficits, substantiating that early life nutritional restriction can have lifelong consequences. Preventing dietary PO₄ deficiency is critical to maintaining the profitability of animal agriculture as well as animal well being. Because the greatest rate of growth occurs in early life, clarifying how PO₄ nutrition impacts immature muscle would be beneficial in maximizing growth performance and increasing animal sustainability.