The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Dietary Calcium Restriction Affects Mesenchymal Stem Cell Activity and Bone Development in Neonatal Pigs[1–3]

Avanika Mahajan,[4] Lindsey S. Alexander,[4] Brynn S. Seabolt,[4] Daniel E. Catrambone,[5] James P. McClung,[5] Jack Odle,[4] T. Wayne Pfeiler,[6] Elizabeth G. Loboa,[6] and Chad H. Stahl[4]*

[4]Laboratory of Developmental Nutrition, Department of Animal Science, North Carolina State University, Raleigh, NC 27695; [5]Military Performance and Nutrition Divisions, U.S. Army Research Institute of Environmental Medicine, Natick, MA 01760; and [6]Joint Department of Biomedical Engineering at University of North Carolina-Chapel Hill and North Carolina State University, Raleigh, NC 27695

Abstract

The effects of dietary calcium (Ca) deficiency on skeletal integrity are well characterized in growing and mature mammals; however, less is known about Ca nutrition during the neonatal period. In this study, we examined the effects of neonatal Ca nutrition on bone integrity, endocrine hormones, and mesenchymal stem cell (MSC) activity. Neonatal pigs (24 ± 6 h of age) received either a Ca-adequate (1.2 g/100 g) or an ~40% Ca-deficient diet for 18 d. Ca deficiency reduced ($P < 0.05$) bone flexural strength and bone mineral density without major differences in plasma indicators of Ca status. There were no meaningful differences in plasma Ca, phosphate (PO_4), parathyroid hormone, or 1,25-dihydroxycholecalciferol due to Ca nutrition throughout the study. Calcium deficiency also reduced ($P < 0.05$) the in vivo proliferation of MSC by ~50%. In vitro studies utilizing homologous sera demonstrated that MSC activity was affected ($P < 0.05$) by both the Ca status of the pig and the sera as well as by their interaction. The results indicate that neonatal Ca nutrition is crucial for bone integrity and suggest that early-life Ca restriction may have long-term effects on bone integrity via programming of MSC. J. Nutr. 141: 373–379, 2011.

Introduction

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

Bone growth and development is a tightly coordinated process of bone formation and resorption. Bone formation is regulated by both the proliferation and differentiation rate of osteoprogenitor cells as well as the activity of mature osteoblasts; however, the rate of bone formation is more dependent on the number of osteoblasts present than on the activity of the individual osteoblasts (3). Mesenchymal stem cells (MSC)[7] in the bone marrow provide these osteoprogenitor cells but can also adopt an adipocytic lineage. The balance between adipocytic and osteogenic differentiation of MSC is currently thought to be of critical importance to bone health (4). The proliferation and differentiation of bone marrow-derived MSC are altered by the calcitropic hormones 1,25-dihydroxycholecalciferol and parathyroid hormone (PTH) (5–7). The impact of Ca nutrition on MSC activity, either directly or via its homeostatic hormones, during the neonatal period may be of great importance because of the greatest abundance of proliferating MSC during this period (8,9). Although several studies have explored the relationship between dietary Ca and bone development in older children and adults (10), limited studies have looked at early neonatal life (11,12). In particular, the effect of dietary Ca on the differentiation potential of MSC has not been determined.

[1] Supported in part by the North Carolina Agricultural Research Service. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations.
[2] Author disclosures: A. Mahajan, L. S. Alexander, B. S. Seabolt, D. E. Catrambone, J. P. McClung, J. Odle, T. W. Pfeiler, E. G. Loboa, and C. H. Stahl, no conflicts of interest.
[3] Supplemental Table 1 is available with the online posting of this paper at jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: chad_stahl@ncsu.edu.

[7] Abbreviations used: BMD, bone mineral density; BrdU, bromodeoxyuridine; Ca^+S, calcium-adequate sera; Ca^-S, calcium-deficient sera; MSC, mesenchymal stem cell; LPL, lipoprotein lipase; PTH, parathyroid hormone; pQCT, peripheral quantitative computed tomograph; SSIp, polar strength strain index.

Manuscript received August 11, 2010. Initial review completed September 23, 2010. Revision accepted December 31, 2010.
First published online January 19, 2011; doi:10.3945/jn.110.131193.

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

Materials and Methods

Piglets. All piglet protocols were approved by North Carolina State University's Institutional Animal Care and Use Committee. Fourteen 1-d-old cross-bred piglets (24 ± 6 h old, both male and female) were weighed and allotted on the basis of sex and body weight to 1 of 2 groups that received either a Ca-adequate (1.2% total Ca) or a Ca-deficient (0.7% total Ca) milk replacer. Nutrient requirements for this age of pigs were determined based on the composition of sow milk (17) and an extrapolation from NRC requirements for older pigs (18). The basal diet was designed to be similar to sow milk and contained no plant proteins (**Table 1**). The Ca-deficient group received the basal diet; Ca carbonate was added to the basal diet for the Ca-adequate group and was provided in slight excess of requirements to account for potentially reduced bioavailability and to ensure adequacy. All pigs were individually housed in raised cages and fed through a gravity-flow milk delivery system (19). All piglets were fed equal quantities 3 times daily and had their total daily intake restricted to match the growth rate of sow-reared pigs. Body weight and feed intake were recorded daily throughout the trial. Blood samples were collected initially and every 6 d by venipuncture using heparinized tubes, and plasma was obtained by centrifugation at 3500 × *g* at 4°C. Plasma samples were stored at –20°C until analysis. Serum was obtained from blood drawn at d 6, 12, and 18 by venipuncture and pooled within dietary treatment [Ca-adequate sera (Ca+S) and Ca-deficient sera (Ca−S)], filter sterilized, and heat inactivated prior to storage at −20°C. Prior to use in the cell culture experiments, equal quantities of sera from each day, within dietary treatment, were pooled. At the completion of the study, all pigs were orally given 20 mg bromodeoxyuridine (BrdU)/kg body weight in a small quantity of their milk replacer 12 h before tissue collection. Immediately prior to tissue collection, pigs were killed by penetrating captive bolt followed by exsanguination. Individual bone marrow, liver, kidney, and small intestine tissue samples were collected for subsequent gene expression analysis. Rear legs were collected and stored at 4°C for peripheral quantitative computed tomography (pQCT) and flexural strength analysis. Front legs were collected for isolation of bone marrow-derived MSC.

TABLE 1 Experimental diet composition on an as-fed basis[1]

	Diet	
	Calcium adequate[2]	Calcium deficient[3]
Ingredients, %		
Lactose	37.9	37.9
12/28 milk replacer fat[4]	18.2	18.2
13/60 milk replacer fat[4]	14.6	14.6
Delactosed whey	14.3	14.3
Sodium caseinate	11.3	11.3
Calcium carbonate	0.6	0
Potassium phosphate	0.2	0.2
Vitamin premix[5]	0.1	0.1
Mineral premix[6]	0.5	0.5
DL-Methionine	0.5	0.5
Potassium sorbate	0.5	0.5
Analyzed[7]		
Crude protein, %	27.7	27.7
Crude fat, %	18	18
Gross energy, *kJ/kg*	19,750	19,750
Lactose, %	36.4	36.4
Delactosed whey, %	20	20
Ca, %	1.2	0.6
PO_4, %	1	1

[1] Composition of the powdered milk replacer that was reconstituted at a rate of 175g/kg final liquid formula.
[2] Diet met pigs' requirements based on sow milk composition and an extrapolation from the NRC recommendations for older pigs (18).
[3] Diet was deficient in only in calcium.
[4] MSC, Dundee, IL.
[5] Vitamin premix provided per kg: 9.9 g retinyl acetate, 165 mg cholecalciferol, 36.7 mg DL-α-tocopherol, 5.1 g dimethylpyrimidinol bisulfate, 2.04 g thiamin, 8.38 g riboflavin, 4 g pyridoxine, 44 mg vitamin B-12, 30 g pantothenic acid, 33.1 g niacin, 2.76 g folic acid, 117 g ascorbic acid, and 66 mg biotin.
[6] Mineral premix provided 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.
[7] Diets were analyzed on a dry matter basis by Milk Specialties Co.

Plasma analysis. Plasma Ca concentrations were determined by flame absorption spectroscopy following dilution in 0.5% lanthium chloride. Inorganic phosphate (PO_4) concentrations were determined by the method of Gomori (20). The concentrations of 1,25-dihydroxycholecalciferol and PTH were determined utilizing commercially available kits (1,25-dihydroxy Vitamin D EIA, IDS and Porcine Intact PTH ELISA kit, Immutopics, respectively).

pQCT. Ex vivo pQCT scans were used to measure bone characteristics of the tibia. Scans of the tibia were taken at a translation speed of 10 mm/s at 10 and 50% of the approximated segment length proximal to the distal endplate of the tibia. Image processing and calculation of the various bone indices were performed using the manufacturer's software package (version 6.0 B, Stratee Medizintechnil). Total volumetric bone mineral density (BMD) and total bone area were calculated. Trabecular and cortical bone properties were assessed at the 10% proximal site. The following traits were assessed: trabecular area (mm^2), trabecular content (mg/mm^2), trabecular volumetric density (mg/cm^3), cortical area (mm^2), cortical content (mg/mm^2), and cortical volumetric density (mg/cm^3). Cortical bone properties were also assessed at the 50% proximal site; the following traits were assessed: cortical area (mm^2), cortical content (mg/mm^2), and cortical volumetric density (mg/cm^3). The ability of a bone to resist torsion was assessed using the polar strength strain index (SSIp); total and cortical bone SSIp measures were assessed at the 10 and 50% proximal sites, respectively.

Mechanical testing. Mechanical loading was conducted with a 3-point bending fixture adjusted to have a 30-mm span between lower supports and load applied from above at the center of the span, as previously described (21). The upper loading post was brought into contact with each specimen and ramp loading was applied at 50 mm/min until failure (22). Displacement and the resulting force were recorded at 10 Hz. Following mechanical testing, width, height, and cortical shell thickness at 4 locations were recorded for each specimen cross section at the midshaft diaphysis.

Maximum force and yield force were determined for each specimen from the force displacement data recorded during testing. Moments of inertia were calculated as elliptical shells (23). Flexural moduli were calculated using geometry data and linear portions of force displacement curves. The following 3-point bending equation was used to determine the modulus of elasticity: $E = \frac{FL^3}{48\delta I}$, where E is flexural elastic modulus, F is force, L is span between lower supports, δ is displacement, and I is cross section moment of inertia (23).

Gene expression. Total RNA was isolated from bone marrow, kidney, liver, and duodenum and relative quantities of transcripts of interest were

determined by semiquantitative real-time PCR as previously described (24). Primer oligonucleotides for *PPARG*, Runt related transcription factor (*Runx2*), lipoprotein lipase (*LPL*), osteocalcin (*OC*), osteoprotegerin (*OPG*), RANK ligand (*RANKL*), vitamin D receptor (*VDR*), calcitonin receptor (*CTR*), calcium-sensing receptor (*CaR*), PTH receptor (*PTHR*), 1α-hydroxylase (*CYP27B1*), 24 α-hydroxylase (*CYP24A1*), and sodium-phosphate cotransporter 2 (*NPT2*) were designed using PrimerQuest software (Integrated DNA Technologies; **Supplemental Table 1**). Primer sets and the control gene, 60S ribosomal RNA, were validated according to the specifications set forth by Livak and Schmittgen (25). Reactions were performed as previously described (24). At completion, all samples were subjected to a melt curve analysis to validate the absence of nonspecific products. The $2^{-\Delta CT}$ method was employed to normalize gene expression values prior to statistical analysis (25).

MSC isolation and immunocytochemistry. MSC were isolated based on adherence to plastic (6) and verified to be <5% positive for CD45 (AbD Serotec) and >95% positive for CD105 (Thermo Scientific). MSC were plated in basal media at 10^4 cells/cm^2 in 24-well plates and incubated for 48 h prior to fixation for immunocytochemical staining for BrdU. The MSC not utilized for immunocytochemistry were frozen in DMEM + 10% FBS + 5% DMSO and stored in LN_2 prior to use.

Cells were fixed and stained for the presence of BrdU as previously described (24). The in vivo rate of cell proliferation was determined by calculating the ratio of stained:unstained cells. A total of 5 fields of view (>200 cells total) from each of 2 replicate wells for each pig were counted and the mean taken per pig.

Cell culture in homologous sera. The MSC isolated from 6 individual pigs (3 Ca adequate and 3 Ca deficient) were thawed, resuspended in basal media, individually plated at 10^4 cells/cm^2 in 6-well plates, and given 48 h to attach prior to the initiation of treatments. The MSC from each individual pig were subjected to culture in 4 different media formulations for 6 d with a complete media change on d 3. The media formulations were as follows: *1*) Ca-adequate control = DMEM + Ca-adequate sera (Ca^+S); *2*) Ca-adequate adipogenic = DMEM + Ca^+S + adipogenic mix (500 nmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methyl-xanthine, 60 μmol/L indomethacin, 50 μmol/L β-mercaptoethanol, 1 mmol/L sodium pyruvate); *3*) Ca-deficient control = DMEM + Ca-deficient sera (Ca^-S); and *4*) Ca-deficient adipogenic = DMEM + Ca^-S + adipogenic mix. After 6 d of culture, cells were utilized for determination of cell proliferation, gene expression, and cytochemical staining.

In vitro cell proliferation. Cell proliferation for the in vitro culture study was determined using the CyQuant NF cell proliferation kit according to the manufacturer's instructions (Invitrogen Life Technologies). Proliferation was measured in duplicate wells for each pig.

Cytochemical staining. Cells were fixed and stained in duplicate for alkaline phosphatase activity (ALP) and the presence of neutral lipids on d 6. Staining for ALP was accomplished with a commercially available kit (ALP staining kit, Takara Bio) according to the manufacturer's instructions. Accumulation of neutral lipids was visualized by fixing the cells in 10% formalin and then staining with 0.2% Oil Red O for 1 h, followed by thorough rinsing with water. For cells to be considered positive for Oil Red O staining, at least 20% of the cell volume needed to be stained. Cells were counted using 6 fields of views for a total of at least 200 cells counted per well in duplicate wells for each pig.

Gene expression. Total RNA was isolated from MSC using the Ambion RNAqueous kit according to the instructions of the manufacturer and genomic DNA was removed by incubation with deoxyribonuclease I (DNA-free kit, Ambion). The generation of cDNA and analysis of the gene expression of *PPARG*, *Runx2*, *OC*, and *LPL* were conducted in the same manner as described above.

Statistical analysis. Data were analyzed using the GLM procedure of SAS (Version 9.1, SAS Institute). For the whole pig measures, dietary treatment was considered a fixed effect and initial body weight was used as a covariate for growth performance data. For the cell culture studies, media, sera Ca status, cell Ca status, and their interactions were considered fixed effects. For all individual pairwise comparisons of least square means, *P*-values were obtained with the pdiff option of SAS. Differences were considered significant at $P < 0.05$. Values reported in the text are least square means ± SE.

Results

Growth performance

Dietary Ca concentrations did not affect growth performance. Daily weight gain (0.22 ± 0.01 kg/d), daily feed intake (0.19 ± 0.01 kg/d), and feed conversion efficiency (1.15 ± 0.04) did not differ between the treatment groups.

Plasma macrominerals and hormones

There were no significant differences in the plasma concentrations of Ca and P throughout the 18-d feeding trial (**Fig. 1**). Plasma concentrations of 1,25-dihydroxycholecalciferol were greater ($P < 0.05$) in Ca-adequate compared with the Ca-deficient pigs on d 6. Plasma concentrations of PTH and 1,25-dihydroxycholecalciferol tended to be greater ($P = 0.12$) in Ca-deficient compared with the Ca-adequate pigs on d 18.

Bone integrity

The BMD and mechanical strength of tibial bones were greater (**Table 2**) among the Ca-adequate pigs ($P < 0.05$). The BMD was greater in both the trabecular and cortical fractions of the distal tibia and tended to be greater in the cortical bone at the mid-diaphysis in Ca-adequate pigs ($P = 0.1$). The cortical content in

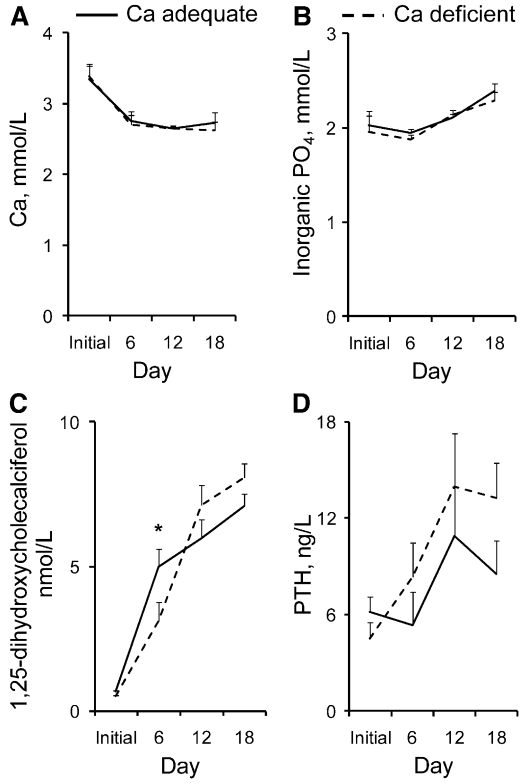

FIGURE 1 Effects of neonatal dietary Ca deficiency on plasma Ca (*A*), PO_4 (*B*), 1,25-dihydroxycholecalciferol (*C*), and PTH (*D*) concentrations in piglets. Values are least square means and SE, $n = 7$. *Different from Ca adequate, $P < 0.05$.

TABLE 2 Effect of neonatal Ca deficiency on bone integrity in piglets[1]

	Calcium adequate	Calcium deficient
Distal tibia		
Trabecular bone		
Bone area, mm^2	150.1 ± 3.61	159.7 ± 9.98
BMC, mg/mm^2	32.4 ± 0.57	31.7 ± 0.36
BMD, mg/mm^3	216.0 ± 3.22	199.5 ± 5.28*
Cortical bone		
Bone area, mm^2	27.46 ± 0.50	27.49 ± 1.06
BMC, mg/mm^2	12.6 ± 0.41	11.1 ± 0.49*
BMD, mg/mm^3	459.2 ± 10.37	403.6 ± 16.29*
Midshaft of tibia		
Trabecular bone		
Bone area, mm^2	12.75 ± 1.14	14.20 ± 0.85
BMC, mg/mm^2	1.16 ± 0.11	1.26 ± 0.06
BMD, mg/mm^3	90.51 ± 0.84	88.77 ± 1.06
Cortical bone		
Bone area, mm^2	53.57 ± 2.00	54.53 ± 0.43
BMC, mg/mm^2	32.2 ± 1.14	29.8 ± 1.54
BMD, mg/mm^3	601.1 ± 11.60	558.3 ± 21.40
SSIP	46.40 ± 2.25	36.87 ± 1.20*
Load at yield, *n*	423.1 ± 31.5	345.2 ± 14.4*
Maximum load, *n*	474.8 ± 31.8	404.2 ± 21.5*

[1] Values are least square means ± SE, $n = 7$. *Different from calcium adequate, $P < 0.05$.

the distal tibia was also greater in the Ca-adequate pigs ($P < 0.01$). The bones' ability to resist torsion, as assessed using the SSIp, and flexural strength were also greater among the Ca adequate pigs (Table 2).

In vivo cell proliferation

The percentage of BrdU labeled MSC isolated from humeral bone marrow was greater among the Ca-adequate pigs ($P < 0.05$). Piglets fed the Ca-adequate milk replacer had almost double the number of actively proliferating MSC as did the pigs fed the Ca-deficient diet (69 and 35% BrdU positive, respectively).

Tissue gene expression

In the bone marrow, the expressions of *CaR* and 1α-hydroxylase were 5-fold and 4-fold greater, respectively, in the Ca-deficient pigs compared with Ca-adequate pigs ($P < 0.05$) (**Fig. 2**). Expression of both *CTR* and *OPG* tended to be greater ($P < 0.1$) in the Ca-deficient pigs (Fig. 2). The levels of *VDR*, *PTHR*, *RANKL*, *Col1A1*, *PPARG*, *LPL*, and *OC* mRNA in bone marrow were not affected by dietary Ca. There were also no significant differences in the expression of *PTHR* and *VDR* in the liver and *PTHR*, *VDR*, *CTR*, *CaR*, *NPT2*, 1-α-hydroxylase, and 24-α-hydroxylase in the kidney between the 2 treatment groups. *CaR* levels tended to be greater in the duodenum of Ca-deficient pigs than in Ca-adequate pigs ($P = 0.1$). The expressions of *VDR*, *PTHR*, *Ca ATPase*, *TRPV6*, and calbindin in the duodenum were not affected by dietary treatment.

Cell culture

In vitro cell proliferation. MSC proliferation was significantly affected by treatment (control or adipogenic media), sera utilized (Ca^+S and Ca^-S), and tended to be affected by the interaction between treatment and sera ($P = 0.09$). As expected, adipogenic media reduced MSC proliferation by ~40% compared with control media ($P < 0.01$). A similar reduction in proliferation was seen with Ca^-S compared with Ca^+S ($P < 0.02$). There tended to be a greater reduction in proliferation in response to Ca^-S when the MSC were cultured in control rather than adipogenic media ($P = 0.09$). There was also a trend for increased proliferation in the Ca-deficient cells (~31%) compared with Ca-adequate cells ($P = 0.12$).

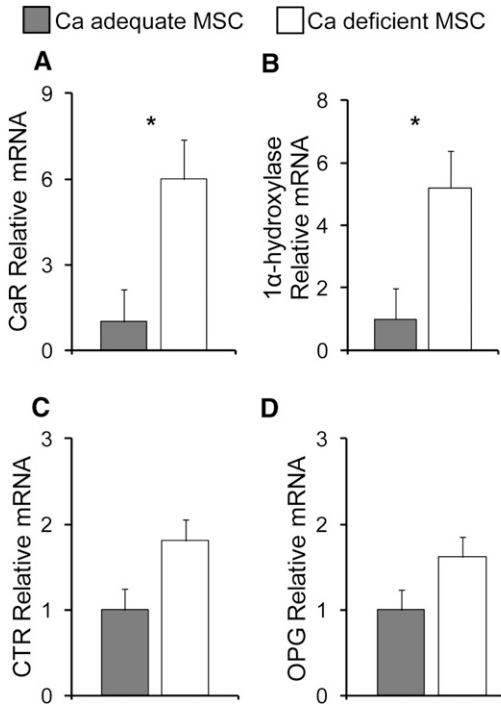

FIGURE 2 Effects of dietary Ca on gene expression of *CaR (A)*, 1α hydroxylase (*B*), *CTR (C)*, and *OPG (D)* in the bone marrow of piglets. Values are least square means and SE, $n = 7$, normalized to cDNA concentrations and the expression of 60S ribosomal RNA. *Different from Ca adequate, $P < 0.05$.

Cytochemical staining. Cells cultured in adipogenic media compared with control media had greater ALP staining ($P < 0.05$; 40.75 vs 18.5%). Surprisingly, there was greater ALP staining among MSC cultured in adipogenic media compared with those cultured in control media ($P < 0.05$). There was no effect of the Ca status of the sera or of the MSC on percentage of ALP^+ cells. A significant effect of both MSC and sera Ca status

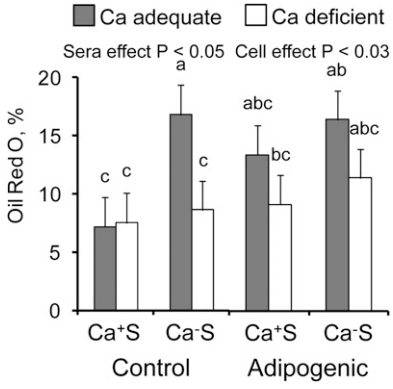

FIGURE 3 Effect of homologous sera and cell Ca status on Oil Red O staining of porcine MSC. Values are least square means and SE, $n = 4$. Means without a common letter differ, $P < 0.05$.

was seen with Oil Red O staining ($P < 0.05$) (**Fig. 3**). Surprisingly, treatment in adipogenic media did not affect Oil Red O staining ($P = 0.17$). Greater Oil Red O staining was seen among MSC from Ca-adequate pigs and among MSC cultured in $Ca^{-}S$ ($P < 0.05$) (Fig. 3). Although the interaction between MSC Ca status and sera Ca status was not significant ($P = 0.2$), individual pairwise comparisons demonstrated that Ca-adequate MSC accumulated more lipid when cultured in Ca-deficient sera compared with Ca-adequate sera ($P < 0.05$).

Gene expression. The expression of *PPARG* increased 1.8-fold in adipogenic media ($P < 0.05$). There also tended to be interactions between treatment and the Ca status of the sera ($P = 0.11$) as well as between treatment and the Ca status of the MSC ($P = 0.09$) (**Fig. 4**). Among cells cultured in adipogenic media, those in media utilizing $Ca^{-}S$ tended to express more *PPARG* than their counterparts cultured with $Ca^{+}S$ ($P = 0.07$). Additionally, MSC from Ca-deficient pigs also tended to express more *PPARG* under adipogenic conditions than their Ca-adequate counterparts ($P = 0.14$). Although adipogenic media caused a 100% increase in *LPL* expression, there were no significant interactions with either the Ca status of the sera or the MSC. The expression of *Runx2* was affected ($P < 0.05$) by treatment, the Ca status of the sera, and their interaction (Fig. 4). Surprisingly, treatment with adipogenic media increased the expression of *RunX2* as did culturing with $Ca^{+}S$. Among cells cultured in adipogenic media, those that received $Ca^{+}S$ had 1.4-fold greater expression of *RunX2*. There also tended to be ~70% greater *RunX2* expression among MSC isolated from Ca-adequate pigs compared with their Ca-deficient counterparts when cultured in control media ($P = 0.11$). Neither the Ca status of the MSC or sera nor treatment affected the expression of *OC*.

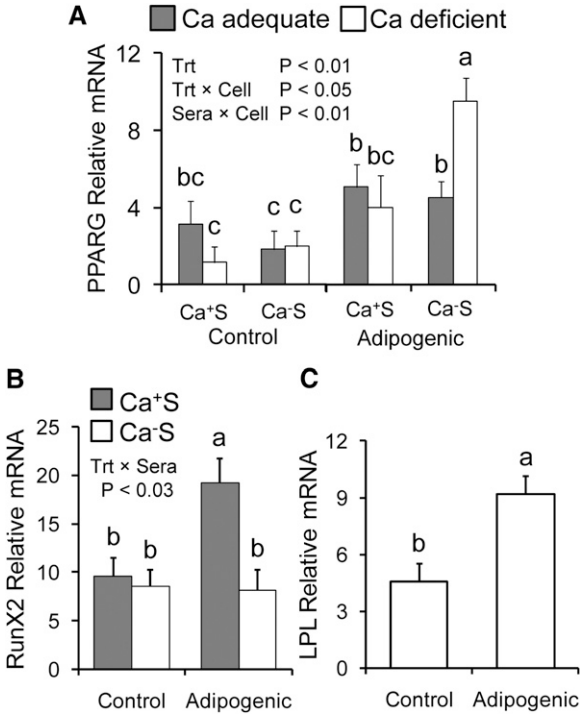

FIGURE 4 Effects of homologous sera and Ca status of MSC on the gene expression of PPARG (*A*), Runx2 (*B*), and LPL (*C*) in piglets. Values are least square means and SE, $n = 4$, normalized to cDNA concentrations and the expression of 60S ribosomal RNA. Means without a common letter differ, $P < 0.05$.

Discussion

In the present study, we examined the effects of dietary Ca deficiency during the early neonatal period by utilizing a physiologically appropriate animal model. Limited research has been conducted to examine the impact of dietary Ca deficiency in neonates (11,12) compared with studies employing older animals (26–30). Studies in older animals have clearly demonstrated that inadequate dietary Ca increases serum PTH and 1,25-dihydroxycholecalciferol levels, which result in bone loss (2,31–33). However, the role of PTH/1,25-dihydroxycholecalciferol -mediated pathways in mineral homeostasis is not fully elucidated in neonates. The anticipated increase in circulating levels of PTH associated with Ca deficiency was not seen in this study and, in additional contrast with Ca deficiency in older animals, there was no significant increase in circulating levels of 1,25-dihydroxycholecalciferol in our Ca-deficient neonates. Although these endocrine profiles do not match what would be expected in older animals, previous work in rats has suggested that during the early suckling period, the machinery to utilize 1,25-dihydroxycholecalciferol as an endocrine regulator of Ca homeostasis may be limited if not absent (34,35). The work of Hsu and Levine (36) also suggests blunted production of PTH in newborns. Therefore, the eucalcemia of our Ca-deficient neonatal pigs was likely not a result of the classical PTH/1,25-dihydroxycholecalciferol regulatory axis and certainly warrants future studies to elucidate the cause of its maintenance. Despite not seeing the changes in the circulating levels of classical regulators of Ca homeostasis in our Ca-deficient pigs, there was a significant reduction in BMD and bone integrity without a reduction in whole body growth or bone longitudinal growth indicative of a classical Ca deficiency.

To our knowledge, there have been no reported studies that have examined the effect of dietary Ca on bone-marrow derived MSC. We have demonstrated in this study that dietary Ca deficiency during the early neonatal period can result in dramatically reduced MSC proliferation in vivo. Because MSC provide the life-long supply of osteoprogenitor cells and MSC proliferation rates are greatest during early infancy (8), dietary restrictions that affect these cells could have both immediate as well as long-term consequences for bone health. Early-life nutrient restrictions affecting the activity of tissue-specific stem cells have previously been shown with satellite cells (muscle stem cells), resulting in permanent muscle growth deficits (37,38). The almost 50% reduction in proliferating MSC in our Ca-deficient pigs could have significantly affected the attainable peak BMD for these pigs. To evaluate if this change in MSC activity was due only to their endocrine environment or if they had been fundamentally altered (i.e. underwent a change in lineage allocation), we conducted a cell culture study utilizing homologous sera. Alterations in cell proliferation, lipid accumulation, and gene expression based on the Ca status of the MSC as well as the Ca status of the homologous sera strongly suggest that although the endocrine environment is in part responsible for changes in MSC activity, the MSC were somehow altered based on neonatal Ca deficiency. Further research is needed to determine whether this alteration of the MSC is a true developmental programming event or a carryover effect from the endocrine environment of the neonatal pigs from which they were isolated.

The Ca-deficient sera caused reduced ($P < 0.05$) proliferation, reduced ($P < 0.05$) *RunX2* expression, increased ($P < 0.05$) *PPARG* expression, and increased ($P < 0.05$) lipid accumulation in MSC. Based on the increased expression of

PPARG and the decreased expression of *RunX2* in the presence of Ca-deficient sera, it is tempting to hypothesize that Ca-deficient sera causes a shift of the MSC pool away from osteogenesis and toward an adipocytic phenotype. An increase in adipocytic and a concomitant decrease in osteogenic differentiation of MSC is suggested to be responsible in part for the bone loss seen during aging (39). The inverse relationship between *Runx2* and *PPARG* expression in MSC cultured in sera from Ca-deficient pigs may be indicative of shift away from osteogenesis, which could be responsible for the reduced BMD and bone strength in Ca-deficient pigs in this study. The changes in MSC activity caused by the Ca-deficient sera are particularly interesting, because the pooled sera utilized in the cell culture studies did not differ in Ca, phosphate, PTH, or 1,25-dihydroxycholecalciferol concentrations between Ca-adequate and Ca-deficient treatments. Further research is needed to identify other potential endocrine compounds that are altered in response to Ca deficiency and characterize their effect on MSC activity. This study demonstrates that neonatal Ca deficiency may have lifelong consequences for bone health via its impact on the activity of MSC. Our in vivo and in vitro experiments demonstrate that neonatal Ca deficiency has an inherent effect on MSC as well as one modulated through the endocrine environment.

As expected in our in vitro studies, the relative expression of *PPARG* was significantly greater in the media supplemented with adipogenic factors and there was a trend for greater lipid accumulation, consistent with a shift toward adipogenic differentiation. However, there was limited adipocytic differentiation among these cells. There are 2 potential explanations for the lack of clear adipocytic differentiation among these cells. First, cells were not allowed to reach confluence prior to adipocytic induction. Based on the number of MSC we were able to obtain for each pig as well as the limited amount of homologous sera we had available for media preparation, we were unable to allow the cells to reach confluence prior to adipocytic induction. A second potential explanation is that our adipogenic media was optimized for use with FBS. The substantial differences between neonatal porcine sera and FBS may require adjustment of the adipogenic agents to achieve a high percentage of adipocytic differentiation. Our adipogenic media did encourage differentiation, as evidenced by reduced proliferation, a trend for increase Oil Red O staining, and increased expression of both *PPARG* and *RunX2*, markers of adipocytic and osteogenic differentiation, respectively; however, this differentiation was not as specific as what is seen when using FBS-containing media.

We also assessed other physiological implications of dietary Ca deficiency by examining the expression of relevant genes in tissues related to Ca homeostasis. Increased expression of *CaR*, 1α-hydroxylase, *CTR*, and *OPG* was seen in the bone marrow of Ca-deficient pigs. Increased expression of *CaR* has been shown to affect bone formation by Ca-mediated increases in osteoblast and stromal cell proliferation (40) and inhibition of osteoclast formation (41). The increased *CaR* expression in the bone marrow might be a mechanism to attempt to preserve bone integrity during the short-term nutritional insult. Because calcitonin has been shown to inhibit bone resorption (42) and increase bone formation (43), elevated *CTR* in the Ca-deficient pigs may again be an attempt to limit bone resorption during this short-term dietary Ca deficiency. Elevated *OPG* in the bone marrow of Ca-deficient pigs may also have a similar goal as OPG inhibits osteoclast formation (44). Although the presence of 1α-hydroxylase in the kidney is necessary for synthesis of 1,25-dihydroxycholecalciferol to mediate its effects as an endocrine regulator of Ca and P homeostasis (45), it has also been suggested that 1,25-dihydroxycholecalciferol has autocrine and/or paracrine functions in bone marrow independent of circulating 1,25-dihydroxycholecalciferol (46). Although the circulating levels of 1,25-dihydroxycholecalciferol were unchanged based on dietary Ca level, the spike in the gene expression of 1α-hydroxylase in bone marrow could indicate increased local synthesis of 1,25-dihydroxycholecalciferol. Based on our previous study (6), 1,25-dihydroxycholecalciferol could directly alter MSC activity in the bone marrow.

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

Acknowledgments

The anti-BrdU antibody developed by S.J. Kaufmann was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. A.M., J.P.M., and C.H.S. designed research; all authors conducted research; A.M. and C.H.S. analyzed data; A.M. and C.H.S. wrote the paper; and C.H.S. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited

1. Ryan S. Nutritional aspects of metabolic bone disease in the newborn. Arch Dis Child Fetal Neonatal Ed. 1996;74:F145–8.
2. Stauffer M, Baylink D, Wergedal J, Rich C. Decreased bone formation, mineralization, and enhanced resorption in calcium-deficient rats. Am J Physiol. 1973;225:269–76.
3. Parfitt AM. Interpretation of bone densitometry measurements: disadvantages of a percentage scale and a discussion of some alternatives. J Bone Miner Res. 1990;5:537–40.
4. Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol. 2006;2:35–43.
5. Bellows CG, Wang YH, Heersche JN, Aubin JE. 1,25-dihydroxyvitamin D3 stimulates adipocyte differentiation in cultures of fetal rat calvaria cells: comparison with the effects of dexamethasone. Endocrinology. 1994;134:2221–9.
6. Mahajan A, Stahl CH. Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. J Nutr Biochem. 2009;20:512–20.
7. Rickard DJ, Wang FL, Rodriguez-Rojas AM, Wu Z, Trice WJ, Hoffman SJ, Votta B, Stroup GB, Kumar S, et al. Intermittent treatment with parathyroid hormone (PTH) as well as a non-peptide small molecule agonist of the PTH1 receptor inhibits adipocyte differentiation in human bone marrow stromal cells. Bone. 2006;39:1361–72.

8. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003;33:919–26.
9. Rickert CH, Strater R, Kaatsch P, Wassmann H, Jurgens H, Dockhorn-Dworniczak B, Paulus W. Pediatric high-grade astrocytomas show chromosomal imbalances distinct from adult cases. Am J Pathol. 2001;158:1525–32.
10. Heaney RP. Calcium, dairy products and osteoporosis. J Am Coll Nutr. 2000;19:S83–99.
11. Schanler RJ, Abrams SA, Sheng HP. Calcium and phosphorus deficiencies affect mineral distribution in neonatal miniature piglets. Am J Clin Nutr. 1991;54:420–4.
12. Lapillonne A, Salle BL, Glorieux FH, Claris O. Bone mineralization and growth are enhanced in preterm infants fed an isocaloric, nutrient-enriched preterm formula through term. Am J Clin Nutr. 2004;80: 1595–603.
13. Bustad LK, McClellan RO. Swine in biomedical research. Science. 1966;152:1526–30.
14. Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr. 1987;7:361–82.
15. Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T, et al. Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res. 2002;307:321–7.
16. Bosch P, Pratt SL, Stice SL. Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. Biol Reprod. 2006;74:46–57.
17. Heidebrecht AA, Mac VR, Ross OB, Whitehair CK. Composition of swine milk. I. Major constituents and carotene, vitamin A and vitamin C. J Nutr. 1951;44:43–50.
18. NRC. Nutrient requirements of swine. 10th ed. Washington, DC: National Academies Press; 1998.
19. Mathews SA, Oliver WT, Phillips OT, Odle J, Diersen-Schade DA, Harrell RJ. Comparison of triglycerides and phospholipids as supplemental sources of dietary long-chain polyunsaturated fatty acids in piglets. J Nutr. 2002;132:3081–9.
20. Gomori G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J Lab Clin Med. 1942;27:955–60.
21. Pfeiler TW, Lalush DS, Loboa EG. Semiautomated finite element mesh generation methods for a long bone. Comput Methods Programs Biomed. 2007;85:196–202.
22. McClung JP, Stahl CH, Marchitelli LJ, Morales-Martinez N, Mackin KM, Young AJ, Scrimgeour AG. Effects of dietary phytase on body weight gain, body composition and bone strength in growing rats fed a low-zinc diet. J Nutr Biochem. 2006;17:190–6.
23. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993;14:595–608.
24. Alexander LS, Mahajan A, Odle J, Flann KL, Rhoads RP, Stahl CH. Dietary phosphate restriction decreases stem cell proliferation and subsequent growth potential in neonatal pigs. J Nutr. 2010;140:477–82.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402–8.
26. Eklou-Kalonji E, Zerath E, Colin C, Lacroix C, Holy X, Denis I, Pointillart A. Calcium-regulating hormones, bone mineral content, breaking load and trabecular remodeling are altered in growing pigs fed calcium-deficient diets. J Nutr. 1999;129:188–93.
27. Ribovich ML, DeLuca HF. Intestinal calcium transport: parathyroid hormone and adaptation to dietary calcium. Arch Biochem Biophys. 1976;175:256–61.
28. Seto H, Aoki K, Kasugai S, Ohya K. Trabecular bone turnover, bone marrow cell development, and gene expression of bone matrix proteins after low calcium feeding in rats. Bone. 1999;25:687–95.
29. Medeiros DM, Plattner A, Jennings D, Stoecker B. Bone morphology, strength, and density are compromised in iron-deficient rats and exacerbated by calcium restriction. J Nutr. 2002;132:3135–41.
30. Talbott SM, Rothkopf MM, Shapses SA. Dietary restriction of energy and calcium alters bone turnover and density in younger and older female rats. J Nutr. 1998;128:640–5.
31. Marie PJ, Travers R. Continuous infusion of 1,25-dihydroxyvitamin D3 stimulates bone turnover in the normal young mouse. Calcif Tissue Int. 1983;35:418–25.
32. Raisz LG, Trummel CL, Holick MF, DeLuca HF. 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. Science. 1972;175:768–9.
33. Staal A, Geertsma-Kleinekoort WM, Van Den Bemd GJ, Buurman CJ, Birkenhager JC, Pols HA, Van Leeuwen JP. Regulation of osteocalcin production and bone resorption by 1,25-dihydroxyvitamin D3 in mouse long bones: interaction with the bone-derived growth factors TGF-beta and IGF-I. J Bone Miner Res. 1998;13:36–43.
34. Halloran BP, DeLuca HF. Appearance of the intestinal cytosolic receptor for 1,25-dihydroxyvitamin D3 during neonatal development in the rat. J Biol Chem. 1981;256:7338–42.
35. Mathews CH, Brommage R, DeLuca HF. Role of vitamin D in neonatal skeletal development in rats. Am J Physiol. 1986;250:E725–30.
36. Hsu SC, Levine MA. Perinatal calcium metabolism: physiology and pathophysiology. Semin Neonatol. 2004;9:23–36.
37. Jeanplong F, Bass JJ, Smith HK, Kirk SP, Kambadur R, Sharma M, Oldham JM. Prolonged underfeeding of sheep increases myostatin and myogenic regulatory factor Myf-5 in skeletal muscle while IGF-I and myogenin are repressed. J Endocrinol. 2003;176:425–37.
38. Moore DT, Ferket PR, Mozdziak PE. Early post-hatch fasting induces satellite cell self-renewal. Comp Biochem Physiol A Mol Integr Physiol. 2005;142:331–9.
39. Moerman EJ, Teng K, Lipschitz DA, Lecka-Czernik B. Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: the role of PPAR-gamma2 transcription factor and TGF-beta/BMP signaling pathways. Aging Cell. 2004;3:379–89.
40. Yamaguchi T, Chattopadhyay N, Kifor O, Brown EM. Extracellular calcium (Ca2+(o))-sensing receptor in a murine bone marrow-derived stromal cell line (ST2): potential mediator of the actions of Ca2+(o) on the function of ST2 cells. Endocrinology. 1998;139:3561–8.
41. Kanatani M, Sugimoto T, Kanzawa M, Yano S, Chihara K. High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. Biochem Biophys Res Commun. 1999;261:144–8.
42. Chambers TJ, Magnus CJ. Calcitonin alters behaviour of isolated osteoclasts. J Pathol. 1982;136:27–39.
43. Wallach S, Rousseau G, Martin L, Azria M. Effects of calcitonin on animal and in vitro models of skeletal metabolism. Bone. 1999;25: 509–16.
44. Hofbauer LC, Heufelder AE. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. J Mol Med. 2001;79:243–53.
45. Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M, Stewart PM. Expression of 25-hydroxyvitamin D3–1alpha-hydroxylase in the human kidney. J Am Soc Nephrol. 1999;10:2465–73.
46. Van Driel M, Koedam M, Buurman CJ, Hewison M, Chiba H, Uitterlinden AG, Pols HA, van Leeuwen JP. Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells. FASEB J. 2006;20:2417–9.