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Effects of Sex and Notch Signaling on the Osteocyte Cell Pool

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Abstract

Osteocytes play a fundamental role in mechanotransduction and skeletal remodeling. Sex is a determinant of skeletal structure, and female C57BL/6J mice have increased osteoblast number in cancellous bone when compared to male mice. Activation of Notch in the skeleton causes profound cell-context dependent changes in skeletal physiology. To determine the impact of sex and of Notch signaling on the osteocyte cell pool, we analyzed cancellous and cortical bone of 1 to 6 month old C57BL/6J or 129SvJ/C57BL/6J mice and determined the osteocyte number/area. There was an age-dependent decline in osteocyte number in cancellous bone of male but not female mice, so that 6 month old female mice had a greater number of osteocytes than male littermates. Although differences between male and female mice were modest, female mice had ~10-15% greater number of osteocytes/area. RNA sequence analysis of osteocyte-rich preparations did not reveal differences between sexes in the expression of genes known to influence bone homeostasis. Neither the activation of Notch1 nor the concomitant inactivation of *Notch1* and *Notch2* in *Osterix (Sp7)* or *Dentin matrix protein 1 (Dmp1)* expressing cells had a pronounced and consistent effect on cancellous or cortical bone osteocyte number in either sex. Moreover, inactivation of *Notch1* and *Notch2* in *Dmp1* expressing cells did not influence the bone loss in a muscle immobilization model of skeletal unloading. In conclusion, cancellous bone osteocytes decline with age in male mice, cortical osteocytes are influenced by sex in younger mice, but osteocyte cell density is not affected substantially by Notch signaling.

Keywords

Notch; bone remodeling; sex; osteocytes; mechanotransduction

INTRODUCTION

Osteocytes are cells with a dendritic morphology that communicate through a canalicular network with each other and with osteoblasts and lining cells (Dallas et al., 2013).

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Osteocytes play a fundamental role in mechanotransduction, and osteocyte ablated mice exhibit bone loss and microstructural deterioration, verifying the critical function played by these cells in bone remodeling (Tatsumi et al., 2007). Osteocytes have distinct biology from osteoblasts, their cells of origin prior to becoming embedded in the trabecular and cortical bone matrix. Moreover, osteocytes are a primary source of signals that regulate bone remodeling. For example, osteocytes are a rich source of receptor activator of nuclear factor kappa B ligand (Rankl) and can signal osteoclast precursors to differentiate into bone resorbing cells (Nakashima et al., 2011; Xiong et al., 2011; Xiong et al., 2015). Osteocytes are the most prevalent source of the Wnt antagonist sclerostin and Wnt/ β -catenin signaling in osteocytes plays a critical role in bone homeostasis. Mice harboring osteocyte-specific inactivation of β -catenin are profoundly osteopenic (Kramer et al., 2010). Moreover, activation of Wnt/ β -catenin signaling in osteocytes induces a bone anabolic response and triggers Notch signaling (Tu et al., 2015). Female C57BL/6J mice have a higher number of osteoblasts/trabecular perimeter than male mice, and since osteoblasts are the cellular source of osteocytes, the osteocyte cell density may also be influenced by sex (Zanotti et al., 2014).

Notch are single-pass transmembrane receptors that play a critical role in skeletal development and homeostasis, and in osteoblast and osteoclast differentiation (Engin et al., 2008; Hilton et al., 2008; Zanotti and Canalis, 2010; Zanotti et al., 2008). Notch interactions with its ligands, which are Jagged1 and 2, and Delta Like1, 3 and 4, result in a series of proteolytic cleavages and the release of the Notch intracellular domain (NICD) (Borggreffe and Liefke, 2012; Fortini, 2009; Zanotti and Canalis, 2010). The NICD translocates to the nucleus and interacts with recombination signal binding protein for immunoglobulin kappa J region (Rbpjk) and mastermind-like proteins to regulate transcription (Kovall, 2008; Nam et al., 2006; Schroeter et al., 1998; Wilson and Kovall, 2006). Recently, we demonstrated that Notch1 plays a unique role in osteocyte function, and its activation in these cells leads to an inhibition of bone resorption in cancellous bone and an enhancement of bone formation in cortical bone (Canalis et al., 2013a). However, it is not known whether Notch has an impact on the osteocyte cell pool, or whether this is influenced by sex.

The intent of the present study was to define the influence of sex and Notch signaling on the osteocyte cell density using models of conditional activation or inactivation of Notch previously reported by our laboratory (Canalis et al., 2013a; Canalis et al., 2013b; Zanotti and Canalis, 2014). To activate Notch, we used the *Rosa^{Notch}* mouse model, where a STOP cassette, placed between the *Rosa26* promoter and the Notch1 NICD coding sequence, is flanked by *loxP* sites (Murtaugh et al., 2003; Stanger et al., 2005). To inactivate Notch, we used dual *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice, where Cre recombination results in the downregulation of *Notch1* and *Notch2*. *Rosa^{Notch}* and *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice were crossed with mice expressing the Cre recombinase under the control of the *Osterix* (*Sp7*) or the *Dentin matrix protein1* (*Dmp1*) promoter (Lu et al., 2007; Rodda and McMahon, 2006). *Osterix* expressing cells are undifferentiated cells of the osteoblastic lineage that eventually differentiate into osteocytes, cells known to express *Dmp1* (Chen et al., 2014; Lu et al., 2007; Maes et al., 2010; Nakashima et al., 2002). The osteocyte cell density was established by histomorphometry of cancellous and cortical bone. In addition, we compared gene profiles in osteocyte-enriched preparations from C57BL/6J male and

female mice, and tested the consequences of the *Notch1* and *Notch2* inactivation in osteocytes on the skeletal response to unloading.

MATERIALS AND METHODS

Wild Type Mice

Experiments in wild type mice were conducted in male and female littermate-matched C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME).

Rosa^{Notch} Mice

Rosa^{Notch} mice were obtained from Jackson Laboratory in a 129SvJ/C57BL/6J genetic background (Murtaugh et al., 2003; Stanger et al., 2005). In these mice, the *Rosa26* locus is targeted with a DNA construct encoding Notch1 NICD, preceded by a STOP cassette flanked by *loxP* sites, cloned downstream of the *Rosa26* promoter. Expression of NICD from the targeted *Rosa26* locus occurs following the excision of the STOP cassette by Cre recombination of *loxP* sequences (Buchholz et al., 1996; Sauer and Henderson, 1988). To study the consequences of the Notch induction in undifferentiated cells of the osteoblastic lineage on the osteocyte cell pool, homozygous *Rosa*^{Notch} mice were bred with hemizygous mice expressing the Cre recombinase under the control of the *Osterix* (*Sp7*) promoter (*Osx-Cre*^{+/-}) in a C57BL/6J genetic background (Jackson Laboratory) (Rodda and McMahon, 2006). Because the expression of Cre is under the control of the tet-off cassette, moms were treated with 645 mg/Kg of doxycycline from the time of conception to delivery. To activate *Notch1* in osteocytes, transgenic mice expressing the Cre recombinase under the control of a ~10 kb fragment of the *Dmp1* promoter (*Dmp1-Cre*) in a C57BL/6J genetic background were obtained from J. Feng (Dallas, TX) (Lu et al., 2007). Homozygous *Rosa*^{Notch} mice were mated with hemizygous *Dmp1-Cre*^{+/-} mice. All mating schemes created 50% *Cre*^{+/-};*Rosa*^{Notch} experimental and 50% *Rosa*^{Notch} littermate controls. Male and female experimental and control littermate-matched mice were compared at 3 months of age. Genotyping was carried out by polymerase chain reaction (PCR) in tail DNA extracts (Table 1). Deletion of the *loxP*-flanked STOP cassette by the Cre recombinase was documented by PCR in DNA from tibiae at 1 month of age using specific primers, as previously published (Table 1) (Canalis et al., 2013b). The induction of NICD and Notch target gene expression was documented by quantitative reverse transcription (qRT)-PCR in femurs and calvariae from 1 month old mice, as published (Canalis et al., 2013a; Canalis et al., 2013b).

Notch1 and Notch2 Conditional Mice

Conditional *Notch1* and *Notch2* mice were provided by F. Radtke (Lausanne, Switzerland) and T. Gridley (Scarborough, ME), respectively (McCright et al., 2006; Radtke et al., 1999). For the creation of the *Notch1* conditional allele, a region of the *Notch1* locus containing the 3.5 kb upstream of the putative transcriptional start site and the first exon was flanked by *loxP* sequences, so that Cre recombination results in the removal of the Notch1 signal peptide (Radtke et al., 1999). For the creation of the *Notch2* conditional allele, exon 3 was flanked by *loxP* sequences so that Cre recombination leads to a frameshift and consequent truncation of the Notch2 protein (McCright et al., 2006). *Notch1* and *Notch2* conditional mice were mated to create dual *Notch1*^{loxP/loxP} and *Notch2*^{loxP/loxP} mice in a 129SvJ/

C57BL/6J background. *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice were mated with either *Osx-Cre* or *Dmp1-Cre* mice to create *Osx-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* or *Dmp1-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* to cross with *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice so that 50% of the progeny would be inactive for both *Notch* genes (*Notch1^{-/-};Notch2^{-/-}*), and 50% would serve as controls (*Notch1^{loxP/loxP};Notch2^{loxP/loxP}*). Moms carrying *Osx-Cre* alleles were treated with doxycycline throughout their pregnancy as described for crosses with *Rosa^{Notch}* mice. Male and female experimental and control littermate-matched mice were compared at 3 months of age. Genotyping was carried out in tail DNA by PCR using specific primers (Table 1). Deletion of *loxP*-flanked sequences by the Cre recombinase directed under the control of the *Osterix* (*Sp7*) promoter was documented by PCR in DNA extracted from tibiae of 1 month old and calvariae of 3 month old mice using specific primers (Table 1) as previously reported (Zanotti and Canalis, 2014). Deletion of *loxP*-flanked sequences by the Cre recombinase directed under the control of the *Dmp1* promoter was documented in femurs from 1 month old mice, as previously reported (Canalis et al., 2013a).

Muscle Immobilization Protocol

The effect of muscle immobilization on the skeleton was tested in 9 and 12 week old *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* and littermate control male mice. For this purpose, control and experimental mice were injected with botulinum toxin A (BtxA) (Allergan Inc., Irvine, CA) at a dose of 2 units/100 g of body weight administered in equal amounts in the quadriceps and the triceps surae muscle groups or injected with saline under anesthesia (Grimston et al., 2011; Manske et al., 2010; Poliachik et al., 2010; Warner et al., 2006). The contralateral limb was used as an internal control. Experimental and control mice were sacrificed 3 weeks post-BtxA injection, when bone loss following muscle paralysis reaches its nadir (Grimston et al., 2011). The degree of muscle paralysis was assessed 3 days post-injection using the digital abduction scoring test, where the degree of abduction of the digits in the paralyzed limb is assessed and scored using a scale of 0 – 4, with 0 reflecting normal muscle function and 4 indicating total paralysis (Aoki, 2001).

All animal experiments were approved by the Animal Care and Use Committees of Saint Francis Hospital and Medical Center or UConn Health.

Microcomputed Tomography

Bone microarchitecture of femurs from experimental and control mice was determined using a microcomputed tomography instrument (μ CT 40; Scanco Medical AG, Bassersdorf, Switzerland), which was calibrated weekly using a phantom provided by the manufacturer (Bouxsein et al., 2010; Glatt et al., 2007). Femurs were scanned in 70% ethanol at high resolution, energy level of 55 kVp, intensity of 145 μ A and integration time of 200 ms. Trabecular bone volume fraction and microarchitecture were evaluated starting approximately 1.0 mm proximal from the femoral condyles. A total of 160 consecutive slices were acquired at an isotropic voxel dimension of 216 μ m³ and a slice thickness of 6 μ m and chosen for analysis. Contours were manually drawn every 10 slices a few voxels away from the endocortical boundary to define the region of interest for analysis. The remaining slice contours were iterated automatically. Trabecular regions were assessed for total

volume, bone volume, bone volume fraction (bone volume/total volume, BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and separation, connectivity density (Conn.D) and structure model index (SMI), using a Gaussian filter ($\sigma = 0.8$) and user defined thresholds (Bouxsein et al., 2010; Glatt et al., 2007). For analysis of femoral cortical bone, contours were iterated across 100 slices along the cortical shell of the femoral midshaft, excluding the marrow cavity. Analysis of bone volume/total volume (BV/TV), porosity, cortical thickness, cross-sectional tissue (TA) and bone (BA) areas, periosteal (P.peri) and endocortical (P.endo) perimeters, and material density were performed using a Gaussian filter ($\sigma = 0.8$) and user defined thresholds. The terminology and units used are in accordance with guidelines published by the Journal of Bone and Mineral Research (Bouxsein et al., 2010).

Bone Histomorphometric Analysis

For cancellous bone histomorphometry, femurs were embedded in methyl methacrylate, and longitudinal sections, 5 μm thick, were cut on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with 0.1% toluidine blue. Bone volume/tissue volume, bone area and osteocyte number were measured in a defined area between 360 μm and 2160 μm from the growth plate, using OsteoMeasureXP software (OsteoMetrics, Atlanta, GA). Complete cancellous bone histomorphometric parameters on the consequences of the Notch activation and inactivation in *Osterix* and *Dmp1* expressing cells in the mice being reported have been published previously by this laboratory (Canalis et al., 2013a; Canalis et al., 2013b; Zanotti and Canalis, 2014). For cortical histomorphometry, femurs embedded in methyl methacrylate were cut through the mid-diaphysis with an EXAKT Precision Saw at Alizee Pathology (Baltimore, MD). Slides were ground using an EXAKT 400 CS micro grinding system to a thickness of $\sim 15 \mu\text{m}$, surface polished and stained with hematoxylin/eosin and analyzed at a magnification of x400 using OsteoMeasureXP software. Stained sections were used to draw the cortical bone to determine cortical thickness and area, and to count osteocytes within the cortex.

Osteocyte number in cancellous and cortical bone was expressed as cells/bone area measured. The terminology and units used for histomorphometry are those recommended by the Histomorphometry Nomenclature Committee of the American Society of Bone and Mineral Research (Dempster et al., 2013; Parfitt et al., 1987).

Osteocyte-enriched Femurs

Osteocyte-enriched cells were obtained following a modification of a previously described method (Halleux et al., 2012). Femurs were removed aseptically from 1 month old experimental and control mice; the surrounding tissues were dissected, the proximal epiphyseal end was excised, and the bone marrow was removed by centrifugation. The distal epiphysis was excised, and femurs were digested for 20 min at 37°C with type II bacterial collagenase pretreated with *N*- α -tosyl-L-lysyl-chloromethyl ketone hydrochloride and subsequently exposed to 5mM EDTA for 20 min at 37°C. The resulting osteocyte-enriched cortical femurs were processed for RNA extraction prior to RNA Sequence (RNASeq) analysis.

RNA Sequence Analysis

Differences in gene expression in the osteocyte cell population from male and female C57BL/6J mice were determined by RNASeq analysis (Marguerat and Bahler, 2010; Wang et al., 2009). Osteocyte enriched preparations from male and female littermate mice were obtained and high quality nucleic acids were extracted. The Agilent Tape Station 2200 High Sensitivity RNA Assay was used to verify total RNA integrity (Agilent Technologies, Santa Clara, CA). RNA libraries were made using the Illumina TruSeq Stranded mRNA sample preparation kit following manufacturer protocol (Illumina, San Diego, CA) at the Center for Genome Innovation, Institute for Systems Genomics (University of Connecticut, Storrs, CT). Eight samples were individually barcoded, quantified using qPCR (KAPA Biosystems, Wilmington, MA), pooled in equimolar ratios and sequenced on High Output Illumina NextSeq 500 sequencing run (paired end 150bp reads; version 2 chemistry). The Computational Biology Core within the Institute for Systems Genomics at the University of Connecticut (Storrs, CT) performed quality control and data analyses on these samples.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical differences were determined by analysis of variance with post-hoc analysis by Holm-Sidak.

RESULTS

Influence of Sex on Osteocyte Cell Density

In accordance with previous results establishing the skeletal microarchitectural and histomorphometric sex-dependent changes in C57BL/6J maturing mice, trabecular bone volume increased or remained stable between 1 and 6 months of age in male mice and declined in female mice (Table 2) (Zanotti et al., 2014). As a consequence, female C57BL/6J mice had lower cancellous bone volume than male littermates at 3 and 6 months of age. Cortical bone thickness and area increased in both sexes between 1 and 3 months of age although the increase was greater in male mice. Cortical thickness remained stable in male mice but continued to increase in female C57BL/6J mice so that it was not different between sexes at 6 months of age. The findings confirm that cancellous bone volume declines more rapidly in female than in male C57BL/6J mice, whereas cortical bone gains occur in both sexes through maturity (Glatt et al., 2007; Zanotti et al., 2014). The decline in cancellous bone in female mice is possibly due to greater resorptive activity than in male mice. There was an age-dependent decline in osteocyte cell density in cancellous bone in male, but not in female mice ($p < 0.05$). Therefore, at 6 months of age, female C57BL/6J mice had a greater number of osteocytes/bone area (Table 2). There was a modest but significant decrease in osteocyte cell number in cortical bone as the mice matured ($p < 0.05$), but this decline was not influenced by sex. A modest, although significant, difference in osteocyte number between sexes was noted in cortical bone where female mice had ~10-15% greater number of osteocytes/bone area than male mice in the first 3 months of life (Table 2).

To establish possible mechanisms or consequences of the differences in osteocyte cell density between male and female mice, RNASeq analysis of osteocyte-rich preparations was

performed but revealed modest differences in gene expression (Table 3). Expression of genes known to affect bone remodeling was not different between sexes.

Influence of Notch on Osteocyte Cell Density

To induce the conditional activation of Notch in cells of the osteoblastic lineage with the potential to differentiate into osteocytes, as well as in mature osteocytes, homozygous *Rosa^{Notch}* mice were mated with hemizygous *Osx-Cre* or *Dmp1-Cre* mice. Recombination of *loxP* sites flanking the STOP cassette and induction of Notch target gene expression in bone extracts was previously documented (Canalis et al., 2013a; Canalis et al., 2013b). In accordance with previous publications, activation of Notch in either *Osterix* or *Dmp1* expressing cells caused increased cancellous bone volume and bone area in 3 month old mice of both sexes (Canalis et al., 2013b). Cortical bone was not affected in *Osx-Cre^{+/-};Rosa^{Notch}* mice, and *Dmp1-Cre^{+/-};Rosa^{Notch}* mice had a cortical structure that could not be separated from cancellous bone (Table 4). Activation of Notch in *Osterix* expressing cells increased osteocyte number in cancellous bone of male but not female mice and had no effect in osteocyte number in cortical bone in either sex. Activation of Notch in *Dmp1* expressing cells did not have an effect on osteocyte cell density in cancellous bone in either sex.

To inactivate *Notch1* and *Notch2* in osteoblast precursors or mature osteocytes, female *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* were bred with either male *Osx-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* or male *Dmp1-Cre^{+/-};Notch1^{loxP/loxP};Notch2^{loxP/loxP}* mice to generate *Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* experimental and *Notch1^{loxP/loxP};Notch2^{loxP/loxP}* littermate controls. Recombination of the *Notch1* and *Notch2* conditional alleles was documented in bone extracts as previously reported (Canalis et al., 2013a; Zanotti and Canalis, 2014). Inactivation of *Notch1* and *Notch2* in *Osterix* expressing cells caused modest changes in mature mice, whereas inactivation of *Notch1* and *Notch2* in *Dmp1* expressing cells increased cancellous bone in 3 month old male and female mice. Inactivation of *Notch1* and *Notch2* in *Osterix* expressing cells did not alter osteocyte cell density in male or female mice in either cancellous or cortical bone (Table 5). Inactivation of *Notch1* and *Notch2* in *Dmp1* expressing cells increased osteocyte number in cancellous bone of male but not female mice, and did not alter osteocyte number in cortical bone.

Confirming the results obtained in C57BL/6J mice, 3 month old 129SvJ/C57BL/6J female mice had greater number of osteocytes/cancellous bone area under basal and under conditions of Notch activation and inactivation (Tables 4 and 5). However, the magnitude of the difference in cancellous bone osteocyte cell density between sexes was variable and not uniformly statistically significant.

Effect of Immobilization on the Skeleton of Osteocyte Notch1/2 Inactivated Mice

In previous work, we reported a protective effect of the Notch activation in osteocytes on the skeleton during immobilization (Canalis et al., 2013a). To test whether the preferential *Notch1* and *Notch2* inactivation in osteocytes affected the bone loss that occurs during immobilization, the quadriceps and triceps surae muscle groups of 9 week or 12 week old

Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-} male mice and sex-matched littermate controls were injected with BtxA or saline. Mice were sacrificed 3 weeks later, the known nadir of the effect of BtxA-induced immobilization on bone mass (Grimston et al., 2011). Muscle paralysis was confirmed by the digital abduction test, and saline-injected mice scored 0, whereas mice injected with BtxA scored (means $\pm$ SEM; $n=14$) 3.6 ± 0.2 , respectively, confirming adequate paralysis (Aoki, 2001). μ CT revealed an ~60% decrease in femoral cancellous bone volume in control and *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* mice following BtxA administration (Table 6). Connectivity density was markedly decreased, structure model index revealed a tendency toward rod-like trabeculae, and cortical bone revealed a decrease in thickness and bone area following immobilization. There were no obvious differences between BtxA treated controls and *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* mice at cancellous sites, although a greater decrease in cortical thickness was observed in one of two experiments in *Notch1* and *Notch2* null mice. These observations confirm that BtxA-induced immobilization causes bone loss and indicate that the preferential inactivation of *Notch1* and *Notch2* in osteocytes does not modify the loss of cancellous bone and has a modest impact at cortical sites.

DISCUSSION

Our study verifies previous work demonstrating structural differences in cancellous and cortical bone between male and female C57BL/6J mice. Our findings also demonstrate a sex-dependent decline in cancellous bone osteocyte cell density as mice mature, so that osteocyte number/bone area is greater in fully mature female than in male C57BL/6J mice. In younger mice (1 to 3 months of age), the osteocyte number/cortical bone area also was higher in female than in male mice. These observations reveal compartment-specific influences of age and sex on osteocyte cell density in the skeleton. Although these observations may not apply to mice of other genetic compositions, the results of these studies suggest that osteocyte cell density is modestly influenced by age and sex. Moreover, the same tendency of a higher number of osteocytes/cancellous bone area was noted in 3 month old female 126SvJ/C57BL/6J mice under basal and under conditions of Notch misexpression. There are no immediate explanation for these findings, and RNASeq analysis failed to demonstrate meaningful differences in the gene expression profile in osteocyte-rich preparations from either sex. The results reported are in line with the sex-dependent differences in osteoblast number in C57BL/6J mice (Zanotti et al., 2014). A terminal fate of the osteoblast is its differentiation into osteocytes and since female C57BL/6J mice have a greater number of osteoblasts, one may have expected a greater number of osteocytes in female than in male mice. However, the results do not exclude alternate destinies for osteoblasts or a differential rate of osteoblast differentiation into osteocytes between male and female C57BL/6J mice.

Recent work has confirmed a rapid turnover of osteoblasts *in vivo*, and a selected number of these cells differentiate into osteocytes (Mizoguchi et al., 2014). Work from this and other laboratories has demonstrated that Notch activation arrests osteoblast cell differentiation (Engin et al., 2008; Hilton et al., 2008; Zanotti et al., 2008). Because of this reason, we expected a Notch-dependent effect on the osteocyte cell pool. The present work confirms that Notch activation in cells of the osteoblastic lineage with the capacity to differentiate into

osteocytes or its activation in mature osteocytes causes an increase in cancellous bone. However, it demonstrates that Notch1 activation does not have a consistent or substantial effect on osteocyte cell density in either sex, although this does not exclude direct effects of Notch on osteocyte function. Notch activation in mature *Dmp1* expressing cells did not cause obvious morphological changes in these cells. The conditional activation of Notch in “osteocyte precursors” and in mature osteocytes was achieved by expressing the Cre recombinase under the control of the *Osterix* or *Dmp1* promoter, for the removal of a STOP cassette and release of the NICD. *Osterix* was used to direct Cre because *Osterix* expressing cells eventually differentiate into mature osteoblasts and osteocytes (Chen et al., 2014; Maes et al., 2010). Inactivation of *Notch1* and *2* in *Osterix* expressing cells did not alter cancellous or cortical bone osteocytes of male or female mice while the inactivation of *Notch1* and *Notch2* in *Dmp1* expressing cells caused a modest increase in osteocyte cell density in cancellous bone of male mice. The results suggest that Notch activity in osteocyte precursors or in mature osteocytes does not play a major role in the control of the osteocyte cell pool.

A central function of osteocytes is to sense mechanical signals and transduce these into biological signals that regulate skeletal cell function (Dallas et al., 2013). Recently, we demonstrated that mechanical stimulation of the MLOY4 osteocytic cell line using fluid flow shear stress induces Notch signaling (E. Canalis, unpublished observations), leading to the hypothesis that Notch regulates mechanotransduction in osteocytes. This function is not necessarily dependent on osteocyte cell density and may prove relevant to the mechanisms of Notch action in bone.

Because activation of Notch in osteocytes protects from the bone loss observed following the unloading of the skeleton, we postulated that the *Notch* inactivation in *Dmp1* expressing osteocytes would lead to a greater bone loss following immobilization (Canalis et al., 2013a). However, microarchitectural analyses revealed a similar loss of cancellous and cortical bone in BtxA paralyzed limbs of control mice and of *Notch1/Notch2* inactivated mice. The protective effect of the Notch activation in osteocytes from the consequences of skeletal unloading is probably related to a substantial suppression of bone resorption and an osteopetrotic phenotype. The fact that the *Notch1* and *Notch2* inactivation did not influence the effects of skeletal unloading may suggest that Notch plays only a limited function in the unloaded skeleton.

CONCLUSIONS

In conclusion, there is an age-dependent decline in cancellous bone osteocytes in male but not in female mice, and cortical osteocyte density is higher in younger female mice but osteocytes are only modestly affected by Notch signaling in either skeletal compartment.

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ABBREVIATIONS

The abbreviations used are:

BA	bone area
BV/TV	bone volume/total volume
BtxA	botulinum toxin A
Conn.D	connectivity density
Dmp1	Dentin matrix protein 1
NICD	Notch intracellular domain
Osx	Osterix
P.peri	periosteal perimeter
P.endo	periosteal endocortical perimeter
qRT	quantitative reverse transcription
PCR	polymerase chain reaction
Rankl	receptor activator of Nuclear factor kappa B ligand
Rbpjκ	recombination signal binding protein for immunoglobulin kappa J region
RNASeq	RNA sequence
SMI	structure model index
TA	tissue area
Tb.N	trabecular number
Tb.Th	trabecular thickness
μCT	microcomputed tomography

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Table 1

Primers used for allele identification by PCR.

Genotyping				
Allele	Strand	Sequence 5'- to -3'	Amplicon Size (base pairs)	
<i>Dmp1-Cre</i>	Forward	CCCGCAGAACCTGAAGATG	534	
	Reverse	GACCCGGCAAAACAGGTAG		
<i>Notch1^{loxP}</i>	Forward	CTGACTTAGTAGGGGGGAAAC	<i>Notch1^{wt}</i> = 300 <i>Notch1^{loxP}</i> = 350	
	Reverse	AGTGGTCCAGGGTGTGAGTGT		
<i>Notch2^{loxP}</i>	Forward	GCTCAGCTAGAGTGTGTTCTTGTG	<i>Notch2^{wt}</i> = 400 <i>Notch2^{loxP}</i> = 500	
	Reverse	TTTGTGGCCGTAACTTTCTCATG		
<i>Osx-Cre</i>	Forward	GCGGTCTGGCAGTAAAACTATC	100 <i>Notch2^{loxP}</i> = 500	
	Reverse	GTGAAACAGCATTGCTGTCACTT		
<i>Rosa^{Notch}</i> allele	Forward	GGAGCGGAGAAATGGATATG	WT = 600	
	Wild type reverse	AAAGTCGCTCTGAGTTGTTATTG		
	<i>Rosa^{Notch}</i> reverse	GCGAAGAGTTTGTCTCTCAACC		
<i>LoxP</i> Recombination				
<i>Notch1</i>	Forward	CTGACTTAGTAGGGGGGAAAC	370	
	Reverse	TAAAAAGAGACAGCTGCCGAG		
<i>Notch2</i>	Forward	GCTCAGCTAGAGTGTGTTCTTGTG	450	
	Reverse	ATAACGCTAAACGTGCACCTGGAG		
<i>Rosa^{Notch} STOP</i>	Forward	TTCCGGGTCTTTCCAGTGG	492 296	
	Reverse absent <i>loxP</i> recombination	AGCCTCTGAGCCCAAGAAAGC		
	Reverse present <i>loxP</i> recombination	GCCGACTGAGTCTCTCGCC		

Table 2

Osteocyte number in cancellous and cortical bone of 1, 3 and 6 month old male and female C57BL/6J wild type mice.

	1 Month		3 Month		6 Month	
	Male	Female	Male	Female	Male	Female
<i>Cancellous Bone</i>						
Bone Volume/Tissue Volume (%)	8.9 ± 1.3	5.9 ± 0.7	17.4 ± 1.6	6.1 ± 1.2 [*]	12.5 ± 1.6	2.6 ± 0.7 [*]
Bone Area (mm ²)	0.21 ± 0.03	0.14 ± 0.02	0.40 ± 0.04	0.14 ± 0.03 [*]	0.23 ± 0.03	0.05 ± 0.01 [*]
Osteocytes/Bone Area (mm ²)	790 ± 53	850 ± 33	598 ± 32	749 ± 68	495 ± 42	859 ± 8.0 [*]
<i>Cortical Bone</i>						
Thickness (µm)	190 ± 11	173 ± 4	355 ± 14	308 ± 9 [*]	360 ± 11	342 ± 5
Bone Area (mm ²)	0.43 ± 0.03	0.37 ± 0.02	1.01 ± 0.08	0.76 ± 0.04 [*]	1.06 ± 0.80	0.85 ± 0.03 [*]
Osteocytes/Bone Area (mm ²)	1191 ± 38	1298 ± 24 [*]	1007 ± 30	1153 ± 38 [*]	1044 ± 33	1014 ± 20

Bone histomorphometry was performed on femurs from wild type C57BL/6J male and female mice. Values are means ± SEM; n = 4 to 6.

* Significantly different between sexes, $p < 0.05$.

Table 3

RNASeq analysis of male and female osteocytes.

Gene Name	Ensemble Gene IDs	Fold Change in Females
Mx1	ENSMUSG00000000386	0.564194103
Ms4a4c	ENSMUSG000000024675	0.600576567
Ifi44	ENSMUSG000000028037	0.630366194
Il1rn	ENSMUSG000000026981	0.631570758
Ptx3	ENSMUSG000000027832	0.633671403
Ebf3	ENSMUSG000000010476	0.752762452
Kdm5c	ENSMUSG000000025332	1.326466707
Kdm6a	ENSMUSG000000037369	1.517834561
Eif2s3x	ENSMUSG000000035150	1.549843905
Vldlr	ENSMUSG000000024924	1.551111996
Olfm2	ENSMUSG000000032172	1.671919491
Pi15	ENSMUSG000000067780	1.673890087
Lppr5	ENSMUSG000000033342	1.832092082
Lgi2	ENSMUSG000000039252	1.85071977
Zfp804a	ENSMUSG000000070866	2.49317683
Shank2	ENSMUSG000000037541	2.859312896
Greb1	ENSMUSG000000036523	3.76399671
Aldh1a2	ENSMUSG000000013584	4.263146685
A2m	ENSMUSG000000030111	5.861513757

RNA was extracted from osteocyte-rich preparations of male and female mice (n = 4) and processed for RNASeq analysis as described in Materials and Methods.

Table 4

Osteocyte number in cancellous and cortical bone of 3 month old male and female conditional *Osx-Cre^{+/-};Rosa^{Notch}* and *Dmp1-Cre^{+/-};Rosa^{Notch}* mice and controls.

<i>Osx-Cre^{+/-};Rosa^{Notch}</i>				
	Males		Females	
	Control	Notch	Control	Notch
<i>Cancellous Bone</i>				
Bone Volume/Tissue Volume (%)	10.5 ± 1.5	25.3 ± 2.0 *	3.8 ± 0.5 [#]	25.7 ± 1.3 *
Bone Area (mm ²)	0.27 ± 0.04	0.66 ± 0.05 *	0.10 ± 0.01 [#]	0.67 ± 0.03 *
Osteocytes/Bone Area (mm ²)	552 ± 23	697 ± 46 *	783 ± 63 [#]	821 ± 26 [#]
<i>Cortical Bone</i>				
Cortical Thickness (μm)	330 ± 9	325 ± 28	288 ± 10	302 ± 12
Bone Area (mm ²)	0.85 ± 0.04	0.91 ± 0.10	0.72 ± 0.03	0.77 ± 0.06
Osteocytes/Bone Area (mm ²)	1167 ± 30	1169 ± 58	1179 ± 10	1212 ± 20
<i>Dmp1-Cre^{+/-};Rosa^{Notch}</i>				
<i>Cancellous Bone</i>				
Bone Volume/Tissue Volume (%)	6.3 ± 0.3	16.0 ± 3.0 *	4.2 ± 0.4	17.1 ± 4.6 *
Bone Area (mm ²)	0.17 ± 0.01	0.42 ± 0.08 *	0.11 ± 0.01	0.44 ± 0.12 *
Osteocytes/Bone Area (mm ²)	554 ± 26	625 ± 32	589 ± 40	684 ± 55
<i>Cortical Bone</i>	Could not discern cortical from trabecular bone.			

Bone histomorphometry was performed on femurs from 3 month old male and female *Osx-Cre^{+/-};Rosa^{Notch}* and *Dmp1-Cre^{+/-};Rosa^{Notch}* mice and control littermates. Values are means ± SEM; n = 5 to 6 for *Osx-Cre^{+/-};Rosa^{Notch}* and 4 to 7 for *Dmp1-Cre^{+/-};Rosa^{Notch}*.

* Significantly different from controls, $p < 0.05$.

[#] Significantly different between sexes.

Table 5

Osteocyte number in cancellous and cortical bone of 3 month old male and female conditional *Osx-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* and *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* null mice and controls.

<i>Osx-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}</i>				
	Males		Females	
	Control	<i>Notch1,2^{-/-}</i>	Control	<i>Notch1,2^{-/-}</i>
<i>Cancellous Bone</i>				
Bone Volume/Tissue Volume (%)	8.9 ± 1.8	11.4 ± 1.8	4.3 ± 0.8 [#]	6.3 ± 1.4
Bone Area (mm ²)	0.23 ± 0.06	0.24 ± 0.03	0.10 ± 0.02 [#]	0.14 ± 0.03
Osteocytes/Bone Area (mm ²)	647 ± 59	785 ± 20	894 ± 80 [#]	1075 ± 70 [#]
<i>Cortical Bone</i>				
Cortical Thickness (μm)	307 ± 16	279 ± 13	307 ± 13	284 ± 7
Bone Area (mm ²)	0.83 ± 0.06	0.76 ± 0.03	0.81 ± 0.08	0.68 ± 0.03
Osteocytes/Bone Area (mm ²)	1471 ± 46	1525 ± 34	1384 ± 78	1535 ± 91
<i>Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}</i>				
<i>Cancellous Bone</i>				
Bone Volume/Tissue Volume (%)	7.8 ± 0.8	11.9 ± 0.8 [*]	2.7 ± 0.2 [#]	8.2 ± 0.8 ^{*#}
Bone Area (mm ²)	0.20 ± 0.02	0.31 ± 0.02 [*]	0.06 ± 0.0 [#]	0.17 ± 0.02 ^{*#}
Osteocytes/Bone Area (mm ²)	578 ± 25	746 ± 27 [*]	789 ± 90 [#]	678 ± 51
<i>Cortical Bone</i>				
Cortical Thickness (μm)	326 ± 6	385 ± 12 [*]	324 ± 21	293 ± 10 [#]
Bone Area (mm ²)	0.93 ± 0.04	1.12 ± 0.09	0.87 ± 0.12	0.71 ± 0.04 [#]
Osteocytes/Bone Area (mm ²)	1073 ± 70	981 ± 46	1146 ± 67	1162 ± 34 [#]

Bone histomorphometry was performed on femurs from 3 month old male and female *Osx-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* and *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* null mice and control littermates. Values are means ± SEM; n = 4 to 6 for *Osx-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* and n = 5 to 8 for *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}*.

* Significantly different from controls, $p < 0.05$.

Significantly different between sexes.

Table 6

Effect of botulinum toxin A-induced immobilization on femoral microarchitecture assessed by μ CT in 12 and 15 week old male conditional *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* mice and controls.

Experiment A	Control		<i>Dmp1-Cre^{+/-};Notch1,2^{-/-}</i>	
	Saline <i>n</i> = 4	BtxA <i>n</i> = 3	Saline <i>n</i> = 4	BtxA <i>n</i> = 5
<i>Distal Femur Trabecular Bone</i>				
Bone Volume/Tissue Volume (%)	9.6 ± 2.1	4.0 ± 0.5 *	7.9 ± 1.0	4.1 ± 0.3 *
Trabecular Separation (μm)	248 ± 9	249 ± 14	232 ± 8	238 ± 5
Trabecular Number (1/mm)	4.0 ± 0.1	4.1 ± 0.3	4.3 ± 0.1	4.2 ± 0.1
Trabecular Thickness (μm)	407 ± 3	263 ± 17 *	372 ± 20	238 ± 4 *
Connectivity Density (1/mm ³)	154 ± 31	69 ± 15 *	177 ± 24	88 ± 16 *
Structure Model Index	2.2 ± 0.3	2.6 ± 0.1	2.4 ± 0.1	2.5 ± 0.1
Density of Material (mg HA/cm ³)	963 ± 10	916 ± 6 *	963 ± 6	909 ± 6 *
<i>Femoral Midshaft Cortical Bone</i>				
Bone Volume/Tissue Volume (%)	88.8 ± 0.8	85.9 ± 0.6	88.0 ± 0.8	83.4 ± 1.7 *
Porosity (%)	11.2 ± 0.8	14.1 ± 0.6	12.0 ± 0.8	16.6 ± 1.7 *
Cortical Thickness (μm)	166 ± 3	129 ± 10 *	156 ± 7	123 ± 8 *
Total Area (mm ²)	2.1 ± 0.2	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Bone Area (mm ²)	0.94 ± 0.08	0.79 ± 0.02 *	0.99 ± 0.02	0.85 ± 0.02 *
Periosteal Perimeter (μm)	5.1 ± 0.3	5.1 ± 0.1	5.0 ± 0.1	5.0 ± 0.1
Endocortical Perimeter (mm)	3.7 ± 0.2	4.0 ± 0.2	3.5 ± 0.1	3.8 ± 0.1
Density of Material (mg HA/cm ³)	1171 ± 8	1149 ± 7	1168 ± 13	1164 ± 3
Experiment B				
	Saline <i>n</i> = 3	BtxA <i>n</i> = 2	Saline <i>n</i> = 4	BtxA <i>n</i> = 3
<i>Distal Femur Trabecular Bone</i>				
Bone Volume/Tissue Volume (%)	14.7 ± 3.5	6.0 ± 0.5 *	14.8 ± 4.8	5.7 ± 0.4 *
Trabecular Separation (μm)	199 ± 7	225 ± 5	193 ± 9	213 ± 13
Trabecular Number (1/mm)	5.0 ± 0.2	4.4 ± 0.1	5.2 ± 0.2	4.7 ± 0.2
Trabecular Thickness (μm)	43 ± 6	33 ± 1	45 ± 3	31 ± 3 *
Connectivity Density (1/mm ³)	374 ± 47	173 ± 30 *	397 ± 36	214 ± 25 *
Structure Model Index	1.6 ± 0.4	2.8 ± 0.1 *	1.8 ± 0.1	2.8 ± 0.1 *
Density of Material (mg HA/cm ³)	996 ± 8	957 ± 9 *	999 ± 5	967 ± 4 *
<i>Femoral Midshaft Cortical Bone</i>				
Bone Volume/Tissue Volume (%)	91.8 ± 0.4	90.4 ± 0.3	90.6 ± 0.2	85.9 ± 0.1 **

Experiment B	Control		<i>Dmp1-Cre^{+/-};Notch1,2^{-/-}</i>	
	Saline <i>n</i> = 3	BtxA <i>n</i> = 2	Saline <i>n</i> = 4	BtxA <i>n</i> = 3
Porosity (%)	8.2 ± 0.4	9.6 ± 0.3	9.4 ± 0.2	14.2 ± 1.0 ^{*#}
Cortical Thickness (μm)	179 ± 9	151 ± 5 [*]	160 ± 4	116 ± 6 ^{*#}
Total Area (mm ²)	1.8 ± 0.2	1.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.2
Bone Area (mm ²)	0.88 ± 0.05	0.74 ± 0.06 [*]	0.92 ± 0.04	0.75 ± 0.05
Periosteal Perimeter (μm)	4.8 ± 0.2	4.6 ± 0.1	4.9 ± 0.1	4.9 ± 0.2
Endocortical Perimeter (mm)	3.5 ± 0.2	3.4 ± 0.1	3.6 ± 0.1	3.8 ± 0.2
Density of Material (mg HA/cm ³)	1158 ± 1	1135 ± 12	1170 ± 12	1137 ± 16

μCT was performed on femurs from *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}* mice and control littermates. In experiment A and B, 9 week and 12 week old mice, respectively, were injected with saline or BtxA and sacrificed 3 weeks later at 12 and 15 weeks of age. Values are means ± SEM

^{*}Significantly different between saline and BtxA injection, $p < 0.05$ by unpaired *t* test.

[#]Significantly different between control and *Dmp1-Cre^{+/-};Notch1^{-/-};Notch2^{-/-}*.