Decreased body weight in young Osterix-Cre transgenic mice results in delayed cortical bone expansion and accrual.

Rachel A Davey¹, Michele Clarke¹, Stephen Sastra¹, Jarrod Skinner¹, Cherie Chiang¹, Paul H Anderson², Jeffrey D Zajac¹.

¹Department of Medicine, Austin Health, University of Melbourne, Heidelberg, Victoria, Australia, ²Musculoskeletal Biology Research, School of Pharmacy and Medical Sciences, Division of Health Sciences, University of South Australia, South Australia, Australia.

Address for Correspondence and Reprint Requests:

Dr Rachel Davey,
Department of Medicine, Austin Health,
University of Melbourne,
Studley Road,
Heidelberg, Victoria, 3084, Australia,
Ph: + 61 3 9496 5507,
Fax: + 61 3 9457 5485,
Email: r.davey@unimelb.edu.au

Key Words: osterix; Cre/loxP system; *Osx1-GFP::Cre* mice; cortical bone, growth, body weight

Short Communication

Abstract

Conditional gene inactivation using the Cre/loxP system has lead to significant advances of our understanding of the function of genes in a wide range of disciplines. It is becoming increasingly apparent in the literature, that Cre transgenic mice may themselves have a phenotype. In the following study we describe the bone phenotype of a commonly used Cre transgenic mouse line to study osteoblasts, the *Osx-GFP::Cre* (*Osx-Cre*) mice. Cortical and trabecular bone parameters were determined in the femurs of *Osx-Cre* mice at 6 and 12 weeks of age by microtomography (μ CT). At 6 weeks of age, *Osx-Cre* mice had reduced body weight by 22% ($P<0.0001$) and delayed cortical bone expansion and accrual, characterized by decreases in periosteal circumference by 7% ($P<0.05$) and cortical thickness by 11% ($P<0.01$), compared to wild type controls. Importantly, the cortical bone phenotype of the skeletally immature *Osx-Cre* mice at 6 weeks of age could be accounted for by their low body weight. The delayed weight gain and cortical growth of *Osx-Cre* mice was overcome by 12 weeks of age, with no differences observed between *Osx-Cre* and wild type controls. In conclusion, *Osx-Cre* expressing mice display a delayed growth phenotype in the absence of doxycycline treatment, evidenced by decreased cortical bone expansion and accrual at 6 weeks of age, as an indirect result of decreased body weight. While this delay in growth is overcome by adulthood at 12 weeks of age, caution together with appropriate data analysis must be considered when assessing the experimental data from skeletally immature Cre/loxP knockout mice generated using the *Osx-Cre* mouse line to avoid misinterpretation.

Introduction

The Cre/loxP system, which enables the deletion of a gene of interest in a tissue- and/or time-specific manner, has led to significant advances in our understanding of the function of target genes in a wide range of disciplines. The application of the Cre/loxP system is particularly advantageous in tissues that are heterogeneous in nature, such as bone. Our initial aim was to apply Cre/loxP technology in order to study the hormonal control of osteoblasts, the cells responsible for bone formation. Osteoblast development includes passage through three separate stages; 1) proliferation, 2) matrix development and maturation stage when collagen and other structural proteins are synthesized which is mineralized during the 3) mineralization stage (Owen et al. 1990). Our specific aim was to investigate the role of specific hormone receptors expressed by osteoblasts known to be important in bone development, growth and maintenance, using the Cre/loxP system. For this purpose we employed the *Osx-GFP::Cre* (*Osx-Cre*) mouse line which contains a tTA and a tetracycline responsive element-controlled GFP/Cre fusion protein under the control of the osterix (*Sp7*) promoter (Rodda and McMahon 2006). In the absence of doxycycline, a tetracycline analogue, it has been shown that the expression of the EGFP-Cre fusion protein is restricted to the osteoblast lineage throughout embryonic and early postnatal development (Rodda and McMahon 2006). Mating of the *Osx-Cre* mouse line with a floxed mouse line, in which the region of the target gene to be deleted is flanked by loxP sites, generates a Cre/loxP knockout mouse line, which when treated with doxycycline, prevents expression of the EGFP-Cre fusion protein and recombination of the target gene. This therefore also allows the deletion of the target gene to be controlled in a time specific manner by the withdrawal of doxycycline.

When using the Cre/loxP system, it is important to include both floxed and Cre transgenic littermate controls in all analyses to ensure accurate interpretation of the observed phenotype

as the insertion of loxP sites into the target gene in the floxed mouse, and random integration in the genome or knock-in of the Cre transgene in endogenous loci, together with high levels of Cre expression have been documented to have non-specific effects (Davey and MacLean 2006; Schmidt-Supprian and Rajewsky 2007). Accordingly, when we generated conditional knockouts of specific hormone receptors in osteoblasts by breeding floxed hormone receptor mouse lines with the *Osx*-Cre mice, we included both *Osx*-Cre and wild type littermate controls in our analyses. Of significant interest, we noted that the skeletally immature *Osx*-Cre mice had a cortical bone phenotype as an indirect result of the low body weight observed in these mice, which was overcome by adulthood. These findings are of particular importance given the increasing, widespread use of the *Osx*-Cre mouse line to generate osteoblast-specific knockouts. Whilst our data does not entirely invalidate the use of the *Osx*-Cre mouse line in bone research, caution however must be taken in interpreting and analyzing the data obtained from skeletally immature Cre/loxP knock out mice generated using this mouse line. Furthermore, this study reinforces that it is imperative that Cre transgenic littermate controls are included for direct side-by-side comparison with wild type and Cre/loxP knockouts.

Materials and Methods

Materials

Generation of the *Osx*-Cre mice has been described previously (Rodda and McMahon 2006). Briefly, the tTA regulated GFP::Cre construct was introduced into the first exon of the *Osx* gene using standard BAC recombination methods. The targeted BAC was then used to generate *Osx*-Cre mice by pronuclear injection. As bone mineral density is profoundly influenced by genetic background (Akhter et al. 1998; Beamer et al. 1996), the *Osx*-Cre mice were transferred to a congenic C57BL/6 background for at least 4 generations prior to use in experiments. Wildtype littermates were used as controls in all analyses. Mice were supplied with water and standard chow (Ridley Agriproducts) ad libitum, and were housed at 22 °C in a 12-h light/dark cycle in standard cages. The *Osx*-Cre mice were not treated with doxycycline in order to allow transgene expression and therefore recombinase activity, to be present from early development when osterix is expressed, in order to investigate the role of hormone receptors in bone growth and development. All procedures involving animals were approved by the Austin Health Animal Ethics Committee.

Bone Analyses

Femur length was measured using a digital calliper (Eta-lon Switzerland). The microarchitecture of the femora of 6 and 12 week old *Osx*-Cre mice were evaluated using a microtomographic imaging system (Skyscan 1174, Brussels, Belgium). Transverse CT slices of the femur were acquired using a 6.5 µm slice increment. Cortical parameters were determined in a 0.65 mm region of cortical bone, equivalent to 100 CT slices, centered on a CT slice located one third of the total femoral length from the distal end. Cortical thickness, periosteal circumference and medullary volume were determined using CTan software (v1.7,

Skyscan, Belgium). Trabecular bone parameters were determined in the metaphyseal region of the distal femur and were a function of femur length as previously described (Chiang et al. 2009; Davey et al. 2008). Trabecular bone parameters were delineated manually and evaluated in the volume of interest commencing from just below the growth plate extending 1.6 mm and 1.76 mm in *Osx*-Cre mice aged 6 and 12 weeks respectively. Images were reconstructed and thresholded using a specimen-specific threshold. Trabecular morphometric parameters (volume, thickness, number and separation) were computed using a direct 3D approach using CTan software (v1.7, Skyscan, Belgium).

Quantitative Real Time PCR (Q-PCR)

Cre mRNA expression was determined in the long bones of *Osx*-Cre mice at 6 and 12 weeks of age and in the brains of *Osx*-Cre mice at 12 weeks of age by Q-PCR as we have described (Turner et al. 2011). Osterix mRNA expression was determined in the long bones of wild type control and *Osx*-Cre mice at 6 and 12 weeks of age. We used Applied Biosystems TaqMan gene expression assays (Osterix: Mm00504574_m1, Cre: custom designed assay previously described (Chen et al. 2007)). Relative expression was calculated using the $\Delta\Delta C_T$ method, normalized to mouse β -actin (Mm00607939_s1) as a measure of cell number, to control for variability in cDNA concentration and expressed relative to a reference control, with $n \geq 8$ independent samples/group.

*Tissue Specificity of Cre-mediated Recombination in *Osx*-Cre Mice*

To confirm that the level of Cre expression detected in the *Osx*-Cre mice was sufficient to result in recombination of a target gene *in vivo*, *Osx*-Cre mice were bred with our floxed androgen receptor mouse line (Notini et al. 2005). mRNA was extracted from bone, brain and heart of *Osx*-ARKO mice at 6 weeks of age, cDNA synthesized and target gene deletion

determined by PCR as previously described (Notini et al. 2005).

Statistical Analyses

To ensure bone analyses were of sufficient power to detect significant differences, power calculations were performed. These calculations were based on the most variable bone histomorphometric parameter for both cortical (periosteal circumference) and trabecular (trabecular bone volume) bone for mice at 6 weeks of age, when bone turnover rates are high and more variable than those observed in adult mice. A sample size of 7 mice/group is required to detect a difference of 0.75mm in periosteal circumference (SD=0.5) and a sample size of 5 mice/group is required to detect a difference of 5% in trabecular bone volume (SD=2.8) between the groups at a significance level of 0.05 and a power of 80%. All experimental groups were of equal or greater sample size than indicated by these power calculations.

To compare means of two groups, unpaired Student's t-test was used, with Levene's test for homogeneity of variance. As changes in body weight can indirectly affect bone structure through mechanical loading, analysis of covariance (ANCOVA) was used to determine the influence of genotype on cortical and trabecular bone parameters after controlling for body weight. All data were analysed with SPSS for MacOSX. A value of $P < 0.05$ was considered significant.

Results and Discussion

Whilst it has been previously documented that the skeletons of *Osx*-Cre embryos do not differ significantly from wild type littermate controls (Rodda and McMahon 2006), we observed that the previously reported delay in growth in *Osx*-Cre mice (Berman et al. 2008; Walkley et al. 2008), was associated with delayed cortical bone expansion and accrual, and reduced body weight. The cortices of the long bones of *Osx*-Cre mice were smaller and thinner, characterized by decreased periosteal circumference and cortical thickness by 7% ($P<0.05$) and 11% ($P<0.01$) respectively, compared to wild type littermates at 6 weeks of age (Fig. 1a, 1b, 1c), while medullary volume was unaffected (Fig. 1d). The decreased cortical bone expansion and accrual observed in the *Osx*-Cre mice was overcome by 12 weeks of age, such that cortical bone parameters in the *Osx*-Cre mice did not differ from wild type controls (Fig. 1 b-d). The body weight of the *Osx*-Cre mice at 6 weeks of age was decreased by 22% compared to wild type ($P<0.0001$), and while the mean value for femur length was 5% shorter in *Osx*-Cre mice than in wild type mice, this did not reach statistical significance at the 0.05 level ($P=0.07$) (Fig. 3b). The reduced body weight in the *Osx*-Cre mice was also overcome by maturity at 12 weeks of age (Fig. 3a), which is consistent with previous reports documenting that the growth delay in *Osx*-Cre mice resolved with age, with *Osx*-Cre mice being a similar size to controls by 2 to 3 months of age (Berman et al. 2008). The delayed cortical bone expansion and accrual observed in the skeletally immature *Osx*-Cre mice at 6 weeks of age was not associated with any changes in trabecular bone parameters (trabecular bone volume, number, thickness and separation) (Fig. 2 a-d).

To our knowledge, this is the first report of a cortical bone phenotype in the skeletally immature *Osx*-Cre mouse. Previous studies utilizing the *Osx*-Cre mouse line to generate osteoblast-specific knockouts have not included littermate *Osx*-Cre transgenic controls in the

analysis of cortical and trabecular bone by micro CT and bone histomorphometry (Berman et al. 2008; Chang et al. 2008; Greenblatt et al. 2010; Walkley et al. 2008; Zhu et al. 2011). The importance of including *Osx*-Cre transgenic controls in these studies was perhaps overlooked given that the *Osx*-Cre appeared to overcome any delays in weight gain and growth by 2 months of age (Berman et al. 2008). However, it is interesting to note that Razidlo *et al*, did report as supplementary data, minor craniofacial defects in the skulls of 5.5 week old *Osx*-Cre, characterized by an extended snout (Razidlo et al. 2010).

It is well established that bone mass is highly correlated with body weight, and that mechanical loading is essential during growth for the formation of strong, weight-bearing bones (Bergmann et al. 2010; Turner et al. 2009). It is highly likely therefore, that the decrease in cortical bone expansion and accrual in the skeletally immature *Osx*-Cre mice at 6 weeks of age can be attributed to the low body weight observed in these mice. In support of this hypothesis, once corrected for changes in body weight using analysis of covariance, there were no significant differences in periosteal circumference or cortical thickness between the *Osx*-Cre and wild type control mice at 6 weeks of age. Whilst it is not common practice in the literature to present histomorphometric bone parameters per gram of body weight in mice, we have presented the cortical and trabecular bone parameters of the *Osx*-Cre mice in this manner in Figures 4a-g to illustrate the influence of body weight on the cortical and trabecular bone phenotype in the *Osx*-Cre mice. This indirect effect of low body weight to decrease cortical bone expansion and accrual in the skeletally immature *Osx*-Cre mice, whilst trabecular bone appears to be unaffected, may possibly be attributed to the rapid expansion of the cortical compartment that occurs during growth. It is interesting to note that following correction for body weight, that the trabecular parameters, trabecular number ($P<0.005$) and trabecular separation ($P<0.05$) were significantly increased in the *Osx*-Cre mice compared to

wild type controls at 6 weeks of age (Fig 4e and 4f). Whilst these changes in trabecular structure are modest and do not significantly effect trabecular bone volume, these data do suggest however, that in addition to the profound indirect effect of low body weight on cortical bone in *Osx-Cre* mice, the expression of the tTA regulated GFP::Cre transgene in osteoblasts may also be affecting osteoblast function. The effects of alterations in osteoblast function may be more likely to be detected in trabecular bone than cortical bone due to its higher surface area and increased metabolic activity.

The marked reduction in body weight of *Osx-Cre* mice at 6 weeks of age may be due to non-osseous expression of the tTA regulated GFP::Cre transgene. Whilst it is widely accepted that osterix is expressed specifically by osteoblasts, a recent study by Park *et al*, identified significant levels of osterix expression in the brain of mice (Park et al. 2011). Consistent with this finding, we found Cre to be expressed in the brains of *Osx-Cre* mice at similar levels to those expressed in bone at 6 weeks of age (Fig 5b). Furthermore, by mating a floxed mouse line with the *Osx-Cre* mice, we showed that the level of Cre expression in the bones and brain of *Osx-Cre* mice was sufficient to result in recombination of a floxed target gene *in vivo* (Fig 5c). The expression of the tTA regulated Cre::GFP transgene in the brain of *Osx-Cre* mice may therefore result in Cre toxicity or indirect gene alterations within the brain, such as recombination between cryptic or pseudo loxP sites (Schmidt-Supprian and Rajewsky 2007), which in turn may effect the central regulation of body weight resulting in the indirect effects observed on cortical bone.

Whilst the lower body weight in the *Osx-Cre* mice can account for their smaller bones at 6 weeks of age, this does not rule out the possibility that expression of the tTA regulated GFP::Cre transgene in bone and brain may at least in part, be contributing directly to the bone

phenotype, particularly as modest changes in the trabecular parameters number and separation were significant following correction for body weight. High levels of Cre expression in transgenic mice have been associated with genotoxic effects with observations of reduced neuronal proliferation and cell death when expressed at high levels in the central nervous system (Forni et al. 2006) and cardiomyopathy and heart failure when expressed at high levels in the heart (Buerger et al. 2006). The levels of Cre expression in the bones of *Osx*-Cre mice are 4.4 fold higher at 6 than 12 weeks of age (Fig. 5a), consistent with the decreased cortical bone growth phenotype, which resolved with age. The higher level of Cre expression in the bones of *Osx*-Cre mice at 6 weeks of age is not surprising given that the expression of osterix is also elevated in this age group, decreasing to a lower level of expression by 12 weeks of age ($P=0.07$) (Fig 5d), consistent with the age related decline in bone turnover ((Ferguson et al. 2003)). Osterix mRNA levels did not differ between control and *Osx*-Cre mice at either age (Fig 5d), suggesting that the knock-in of the tTA regulated GFP::Cre construct into exon 1 of the *osterix* gene did not alter its endogenous expression. Direct measures of bone formation using fluorochrome labeling however, are required to confirm that the high levels of Cre expression observed in the bones of the *Osx*-Cre mice at 6 weeks of age are directly affecting osteoblast function. The apparent resolution of the delayed weight gain and growth in the *Osx*-Cre may not be that surprising as it has been postulated that whilst it is likely many Cre-expressing mouse strains do not have a completely normal phenotype, they may overcome Cre toxicity through developmental selection and adaptation processes (Schmidt-Supprian and Rajewsky 2007).

Another potential mediator of the observed growth delay observed in the *Osx*-Cre mice is tTA expression. Consistent with many other studies using these mice (Chang et al. 2008; Greenblatt et al. 2010; Kolpakova-Hart et al. 2008; Razidlo et al. 2010; Rodda and McMahon

2006), in the present study we did not treat the *Osx*-Cre mice with doxycycline to suppress Cre expression, as we required Cre-mediated recombination of the target gene to occur from early in development in order to investigate the hormonal control of bone formation during development and growth. Overexpression of the same VP16 transactivation domain of the herpes simplex virus fused to the tTA protein used to generate the *Osx*-Cre mice (Kistner et al. 1996) has been shown to result in squelching of transcriptional machinery, thereby decreasing the expression of other cellular genes and attenuation of growth rate (Kuhnel et al. 2004; Strathdee et al. 1999; Zhou et al. 2009).

In conclusion, *Osx*-Cre mice expressing Cre from early embryonic development in the absence of doxycycline treatment, have delayed growth characterized by lower body weight and smaller bones of decreased periosteal circumference and cortical thickness compared to wild type controls at 6 weeks of age, whilst trabecular bone is unaffected. This delay in cortical bone expansion and accrual, and reduced body weight in *Osx*-Cre mice is overcome by 12 weeks of age when the mice are skeletally mature. Importantly, the cortical phenotype of the *Osx*-Cre mice at 6 weeks of age can be attributed to the indirect effect of low body weight as following statistical analysis correcting for body weight, these cortical parameters are not significantly different from wild type controls. The mechanism responsible for the low body weight in the *Osx*-Cre mice is not known, however, may at least in part be due to indirect gene alterations occurring in the brain as a result of non-osseous Cre transgene expression. When used properly with the appropriate controls and data analysis, the *Osx*-Cre mice can still be used to generate significant insight into osteoblast biology in adult, skeletally mature mice. Caution however, must be taken when interpreting the bone phenotype of young, skeletally immature Cre/loxP mice generated using the *Osx*-Cre mice as the bone effects observed are most likely due to the lower body weight and not as a result of target

gene deletion. This study further emphasizes the necessity for Cre transgenic controls to be included in all analyses, the exclusion of which, can lead to serious misinterpretation of the data.

Acknowledgements

The authors thank Rebecca Sawyer of Musculoskeletal Biology Research, School of Pharmacy and Medical Sciences, Division of Health Sciences, University of South Australia for excellent technical assistance. This study was funded by the National Health and Medical Research Council Australia (Project Grant 566503). CC was supported by a National Health and Medical Research Council of Australia, post-graduate scholarship.

References

- Akhter MP, DM Cullen, EA Pedersen, DB Kimmel,RR Recker (1998) Bone response to in vivo mechanical loading in two breeds of mice *Calcif Tissue Int* 63:442-9.
- Beamer WG, LR Donahue, CJ Rosen,DJ Baylink (1996) Genetic variability in adult bone density among inbred strains of mice *Bone* 18:397-403.
- Bergmann P, JJ Body, S Boonen, Y Boutsen, JP Devogelaer, S Goemaere, J Kaufman, JY Reginster,S Rozenberg (2010) Loading and skeletal development and maintenance *Journal of osteoporosis* 2011:786752. 2011/01/07
- Berman SD, E Calo, AS Landman, PS Danielian, ES Miller, JC West, BD Fonhoue, A Caron, R Bronson, ML Bouxsein, S Mukherjee,JA Lees (2008) Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage *Proceedings of the National Academy of Sciences of the United States of America* 105:11851-6. 2008/08/14
- Buerger A, O Rozhitskaya, MC Sherwood, AL Dorfman, E Bisping, ED Abel, WT Pu, S Izumo,PY Jay (2006) Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase *Journal of Cardiac Failure* 12:392-8. 2006/06/10
- Chang W, C Tu, TH Chen, D Bikle,D Shoback (2008) The extracellular calcium-sensing receptor (CaSR) is a critical modulator of skeletal development *Science signaling* 1:ra1. 2008/09/04
- Chen L, SM Kim, M Oppermann, R Faulhaber-Walter, Y Huang, D Mizel, M Chen, ML Lopez, LS Weinstein, RA Gomez, JP Briggs,J Schnermann (2007) Regulation of renin in mice with Cre recombinase-mediated deletion of G protein Gsalpha in juxtaglomerular cells *American journal of physiology. Renal physiology* 292:F27-37. 2006/07/11

- Chiang C, M Chiu, AJ Moore, PH Anderson, A Ghasem-Zadeh, JF McManus, C Ma, E Seeman, TL Clemens, HA Morris, JD Zajac, RA Davey (2009) Mineralization and bone resorption are regulated by the androgen receptor in male mice *J Bone Miner Res* 24:621-31.
- Davey RA, HE MacLean (2006) Current and future approaches using genetically modified mice in endocrine research *Am J Physiol Endocrinol Metab* 291:E429-38.
- Davey RA, AG Turner, JF McManus, WS Chiu, F Tjahjono, AJ Moore, GJ Atkins, PH Anderson, C Ma, V Glatt, HE MacLean, C Vincent, M Bouxsein, HA Morris, DM Findlay, JD Zajac (2008) The calcitonin receptor plays a physiological role to protect against hypercalcemia in mice *J Bone Miner Res* 23:1182-93.
- Ferguson VL, RA Ayers, TA Bateman, SJ Simske (2003) Bone development and age-related bone loss in male C57BL/6J mice *Bone* 33:387-98.
- Forni PE, C Scuoppo, I Imayoshi, R Taulli, W Dastru, V Sala, UA Betz, P Muzzi, D Martinuzzi, AE Vercelli, R Kageyama, C Ponzetto (2006) High levels of Cre expression in neuronal progenitors cause defects in brain development leading to microencephaly and hydrocephaly *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26:9593-602. 2006/09/15
- Greenblatt MB, JH Shim, W Zou, D Sitara, M Schweitzer, D Hu, S Lotinun, Y Sano, R Baron, JM Park, S Arthur, M Xie, MD Schneider, B Zhai, S Gygi, R Davis, LH Glimcher (2010) The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice *The Journal of clinical investigation* 120:2457-73. 2010/06/17
- Kistner A, M Gossen, F Zimmermann, J Jerecic, C Ullmer, H Lubbert, H Bujard (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice *Proceedings of the National Academy of Sciences of the United States of America* 93:10933-8. 1996/10/01

- Kolpakova-Hart E, B McBratney-Owen, B Hou, N Fukai, C Nicolae, J Zhou, BR Olsen (2008) Growth of cranial synchondroses and sutures requires polycystin-1 *Developmental Biology* 321:407-19. 2008/07/26
- Kuhnel F, C Fritsch, S Krause, B Mundt, T Wirth, Y Paul, NP Malek, L Zender, MP Manns, S Kubicka (2004) Doxycycline regulation in a single retroviral vector by an autoregulatory loop facilitates controlled gene expression in liver cells *Nucleic Acids Research* 32:e30. 2004/02/18
- Notini AJ, RA Davey, JF McManus, KL Bate, JD Zajac (2005) Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model *J Mol Endocrinol* 35:547-55.
- Owen TA, M Aronow, V Shalhoub, LM Barone, L Wilming, MS Tassinari, MB Kennedy, S Pockwinse, JB Lian, GS Stein (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix *J Cell Physiol* 143:420-30.
- Park JS, WY Baek, YH Kim, JE Kim (2011) In vivo expression of Osterix in mature granule cells of adult mouse olfactory bulb *Biochemical and Biophysical Research Communications* 407:842-7. 2011/04/06
- Razidlo DF, TJ Whitney, ME Casper, ME McGee-Lawrence, BA Stensgard, X Li, FJ Secreto, SK Knutson, SW Hiebert, JJ Westendorf (2010) Histone deacetylase 3 depletion in osteo/chondroprogenitor cells decreases bone density and increases marrow fat *PloS one* 5:e11492. 2010/07/16
- Rodda SJ, AP McMahon (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors *Development* 133:3231-44.

- Schmidt-Supprian M,K Rajewsky (2007) Vagaries of conditional gene targeting *Nature immunology* 8:665-8. 2007/06/21
- Strathdee CA, MR McLeod,JR Hall (1999) Efficient control of tetracycline-responsive gene expression from an autoregulated bi-directional expression vector *Gene* 229:21-9. 1999/03/30
- Turner AG, F Tjahjono, WS Chiu, J Skinner, R Sawyer, AJ Moore, HA Morris, DM Findlay, JD Zajac,RA Davey (2011) The role of the calcitonin receptor in protecting against induced hypercalcemia is mediated via its actions in osteoclasts to inhibit bone resorption *Bone* 48:354-61. 2010/09/21
- Turner CH, SJ Warden, T Bellido, LI Plotkin, N Kumar, I Jasiuk, J Danzig,AG Robling (2009) Mechanobiology of the skeleton *Science signaling* 2:pt3. 2009/04/30
- Walkley CR, R Qudsi, VG Sankaran, JA Perry, M Gostissa, SI Roth, SJ Rodda, E Snay, P Dunning, FH Fahey, FW Alt, AP McMahon,SH Orkin (2008) Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease *Genes and Development* 22:1662-76. 2008/06/19
- Zhou H, C Huang, M Yang, CP Landel, PY Xia, YJ Liu,XG Xia (2009) Developing tTA transgenic rats for inducible and reversible gene expression *International journal of biological sciences* 5:171-81. 2009/02/14
- Zhu W, G Liang, Z Huang, SB Doty,AL Boskey (2011) Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development *The Journal of biological chemistry* 286:26794-805. 2011/06/04

Figure Legends

Fig. 1: μ CT analysis of the femoral shaft in 6 and 12 week old control (white bars) and *Osx-Cre* (solid bars) mice. (a) Representative μ CT images of the femoral shaft. (b) Periosteal circumference (Peri Circum), (c) Cortical thickness and (d) Medullary volume. Values are mean $\pm$ SE, $n \geq 11$ for 6 week group and $n=7$ for 12 week group. * $P < 0.05$, ** $P < 0.01$ versus wild type at the same age, unpaired Student's t-test.

Fig. 2: μ CT analysis of the metaphysis in the femur in 6 and 12 week old control (white bars) and *Osx-Cre* (solid bars) mice. (a) Trabecular bone volume/tissue volume (BV/TV), (b) Trabecular number (TbN), (c) Trabecular thickness (TbTh) and (d) Trabecular separation (TbSep). Values are mean $\pm$ SE, $n \geq 10$ for 6 week group and $n=6$ for 12 week group.

Fig. 3: (a) Body weight and (b) femur length 6 and 12 week old control (white bars) and *Osx-Cre* (solid bars) mice. Values are mean $\pm$ SE, $n \geq 18$ for 6 week group and $n \geq 12$ for 12 week group. *** $P < 0.0001$ versus wild type at the same age, unpaired Student's t-test.

Fig. 4: Cortical and Trabecular parameters determined by μ CT analysis in 6 and 12 week old control (white bars) and *Osx-Cre* (solid bars) mice represented as per gram of body weight (bw). (a) Periosteal circumference (Peri Circum), (b) Cortical thickness, (c) Medullary volume, (d) Trabecular bone volume/tissue volume (BV/TV), (e) Trabecular number, (f) Trabecular thickness and (g) Trabecular separation. Values are mean $\pm$ SE, for cortical parameters (a-c): $n \geq 10$ for 6 week group and $n=6$ for 12 week group; for trabecular parameters (d-g): $n \geq 11$ for 6 week group and $n=6$ for 12 week group. * $P < 0.05$, ** $P < 0.01$ versus wild type at the same age, ANCOVA.

Fig. 5: (a) Cre mRNA expression in bone relative to β -actin in *Osx*-Cre mice at 6 and 12 weeks of age; (b) Cre mRNA expression in brain relative to β -actin in *Osx*-Cre mice at 12 weeks of age; (c) Detection of target gene deletion in Cre/loxP mice generated by breeding *Osx*-Cre mice with androgen receptor floxed mice. M: Marker, Lane 1: tibia, Lane 2: brain, Lane 3: heart (negative control tissue), Lane 4: RT-PCR negative control. Upper band – endogenous target gene (androgen receptor) expression, Lower band – recombined target gene (androgen receptor); (d) Osterix mRNA expression in bone relative to β -actin in wild type controls (white bars) and *Osx*-Cre (solid bars) mice at 6 and 12 weeks of age. * $P < 0.05$ versus 6 weeks within genotype, $n \geq 8$ /group, unpaired Student's t-test.

Fig.1

Fig. 2

Fig. 3

Fig.4

Fig. 5