Published in final edited form as:

Calcif Tissue Int. 2015 February ; 96(2): 129–137. doi:10.1007/s00223-014-9945-5.

Osterix-Cre transgene causes craniofacial bone development defect

Li Wang¹, Yuji Mishina¹, and Fei Liu^{1,*}

¹Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, MI 48109, USA

Abstract

The Cre/loxP system has been widely used to generate tissue-specific gene knockout mice. Inducible (Tet-off) *Osx-GFP::Cre* (*Osx-Cre*) mouse line that targets osteoblasts is widely used in the bone research field. In this study, we investigated the effect of *Osx-Cre* on craniofacial bone development. We found that newborn *Osx-Cre* mice showed severe hypomineralization in parietal, frontal, and nasal bones as well as the coronal sutural area when compared to control mice. As the mice matured the intramembranous bone hypomineralization phenotype became less severe. The major hypomineralization defect in parietal, frontal, and nasal bones had mostly disappeared by postnatal day 21, but the defect in sutural areas persisted. Importantly, Doxycycline treatment eliminated cranial bone defects at birth which indicates that Cre expression may be responsible for the phenotype. In addition, we showed that the primary calvarial osteoblasts isolated from neonatal *Osx-Cre* mice had comparable differentiation ability compared to their littermate controls. This study reinforces the idea that Cre positive litter mates are indispensable controls in studies using conditional gene deletion.

Keywords

Osx-Cre; Intramembranous bone; Craniofacial; Suture; Hypomineralization

INTRODUCTION

The Cre/loxP system has been widely used to generate tissue-specific gene knockout mice. Two strains of mice, one with a Cre insertion and the other with a loxP insertion, are bred to generate offspring with the tissue-specific gene knockout. The application of this technology has greatly advanced our understanding of the functions of numerous genes in a wide range of disciplines including bone research. Bone is a heterogeneous tissue dynamically regulated by osteoblasts and osteoclasts as well as other cell types. The availability of Cre mouse lines

*Corresponding author. feiliu@umich.edu, Phone: 734-936-0911, Fax: 734-647-2805.

DISCLOSURE

Dr. Fei Liu reports grant from NIH during the conduct of the study.

Dr. Yuji Mishina reports grants from NIH/NIDCR, personal fees from NIH/NICHD, other from NIH/NIEHS, personal fees from Shriners Hospital, grants from Department of Defense during the conduct of the study.

Dr. Li Wang has nothing to disclose.

targeting various bone cells has increased our ability to dissect cell type specific roles by targeting genes in both a cell and development stage specific manner.

In the past decade several Cre mouse lines have been generated which target osteoblasts [1–5]. *Osx-GFP::Cre* (*Osx-Cre*) mice are commonly used to target osteoblasts; they have a tTA and a tetracycline responsive element-controlled GFP/Cre fusion protein under the control of the Osterix (*Sp7*) promoter [2]. It has been shown that *Osx-Cre* is expressed during embryonic development from E14.5 and postnatally in both osteoblasts and sporadically in hypertrophic chondrocytes [2]. Breeding *Osx-Cre* mice with a floxed mouse line, where the target gene loci is flanked by loxP sites, generates a Cre/loxP knockout mouse with targeted gene deletion occurring in osteoblasts. Treatment with doxycycline can prevent the expression of the GFP-Cre fusion protein and recombination of the target gene. Therefore, this system allows cell type- and time-specific gene targeting [2, 6].

It is ideal to include both floxed and Cre transgenic littermate controls in Cre/loxP breeding experiments to ensure accurate interpretation of any observed phenotype. Mating schemes capable of generating genotypes including flox, Cre control, and conditional knockout (CKO) mice are inefficient at producing Cre control and CKO in one litter. For this practical reason many researchers did not include a Cre control group and used only a flox control group instead. This is indeed true for most of the gene targeting research with the *Osx-Cre* mouse line. When we used the *Osx-Cre* transgenic mouse line to generate conditional knockout mice for our gene of interest we found severely defective intramembranous bone development when compared to the flox control mice. Interestingly, a similar defective intramembranous bone development phenotype has been reported in several other CKO mouse models using *Osx-Cre* [7–9]. It has been documented that there is decreased body weight in young *Osx-Cre* transgenic mice resulting in delayed cortical bone expansion and accrual [10]. It is known that the Cre transgene itself may have non-specific effects [11]. This prompted us to question whether defective bone development in our CKO mouse models was due to the *Osx-Cre* itself. Therefore, we investigated the effect of *Osx-Cre* on early postnatal bone development. We showed that the *Osx-Cre* transgene itself had no effect on the trabecular bone parameters and minimal effect on the cortical parameters for one month old C57BL/6 background mice [12]. However, we noted that there is defective intramembranous bone development in *Osx-Cre* mice. This finding is of particular importance considering the increasing use of the *Osx-Cre* mouse line to generate osteoblast-specific knockouts. This study underscores the importance of including Cre positive littermate controls for comparison with CKOs.

MATERIALS AND METHODS

Animals and Genotyping

The *Osx-Cre* transgenic mouse line was described previously [2]. *Osx-Cre* mice were bred with Rosa 26 reporter mice [13] to generate Rosa26;*Osx-Cre* mice that bring reporter gene LacZ. *Osx-Cre* mice have been backcrossed to C57BL/6 background for at least 9 generations. Rosa 26 reporter mice have been backcrossed to C57BL/6 background for at least 5 generations. All mice used in this study were maintained and handled according to local, state, and federal regulation, and were approved by the Institutional Animal Care and

Use Committee at the University of Michigan. Mice were fed with 5001 or 5008 rodent diet (LabDiet). To suppress Cre activity, designated breeders were fed a diet containing 625 mg/kg doxycycline (Harlan, USA) to deliver a daily dose of 2–3 mg/mouse of doxycycline according to manufacturer's instruction. Mice were genotyped by PCR using tail DNA and primers (Cre and Rosa26) as previously described [14]. Primer sequences are available upon request.

Skeleton Preparation and Staining

Skeleton preparation and staining of mice at different ages was described in previous studies [15]. Briefly, after fixation with 95% ethanol for 48h, skeletons were stained with 0.015% Alcian blue in 80% ethanol and 20% acetic acid solution for overnight, and then digested by 2% KOH for 24h, followed by 0.015% Alizarin red in (1% KOH solution) staining for 24 h. Samples were finally maintained in 1:1 mixture of glycerol and 95% ethanol for analysis.

Primary Calvaria Culture and Histochemical Assay

Isolation and culture of calvarial cells from neonatal mice were described previously [12]. Cells were plated and cultured in 6-cm dish in alpha MEM containing 10% Heat-inactivated FBS till analysis. Alkaline phosphatase staining (ALP) was performed for osteoblast differentiation using a commercially kit (Sigma Diagnostics, USA), all procedures were according to the manufacturer's instructions. Modified Alizarin red staining (AR) was performed for mineralization analysis. In brief, cells were fixed for 1 hour with cold 70% ethanol and stained with 40 mM AR-S, pH 4.2, at room temperature for 10 minutes with rotation.

Histology and Fluorescence Microscopy

Histology was performed as described previously [12]. Primary calvarial osteoblasts isolated from *Osx*-Cre transgenic mice were observed with fluorescence microscope directly.

RNA extraction and qRT-PCR

RNA extraction and qRT-PCR were performed as previously described [12]. Total RNA of mouse frontal and nasal bones was extracted using the Trizol reagent (Invitrogen, USA), and 1 µg of total RNA (equal amount) per sample was reverse transcribed using first strand RT kit (Invitrogen, USA). Quantitative real time PCR (qRT-PCR) was performed using SYBR Green Supermix (Qiagen, USA) with primers that sequence information are available upon request. The level of target gene expression was normalized to the level of the reference gene 18S rRNA as described previously [12].

X-gal staining

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed for β-galactosidase activity assay. In brief, calvariae were dissected and rinsed with PBS, fixed in 4% PFA for 20 minutes at room temperature, rinsed with PBS again, and then stained 5 hours in X-gal solution containing 1 mg/mL X-gal (Invitrogen, USA), 5 mM potassium ferricyanide, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40 at 37°C.

Statistical analysis

Statistical differences were evaluated by Mann-Whitney test (Student's t-test). A value of $P < 0.05$ was considered to be statistical significance.

RESULTS

Intramembranous bone formation is defective in *Osx-Cre* mice

We previously characterized the effect of *Osx-Cre* transgene on the endochondrally formed femur and vertebra in one month old mice and found no adverse effect on the trabecular compartment of either bone [12]. In this study, we focused on the effect of the *Osx-Cre* transgene on intramembranous bone formation in the first three weeks postnatally. At birth there is no body weight difference between control mice and *Osx-Cre* mice (Fig 1). However, the body weight of *Osx-Cre* mice is significantly decreased after one week. As a first step to investigate the early bone development in these mice, we performed whole skeleton staining in newborn pups. Surprisingly, severe hypomineralization of nasal, frontal and parietal bone was observed in *Osx-Cre* positive skulls compared to controls (Fig 2A, 2B). This indicates defective intramembranous bone formation. The coronal sutural area had severe hypomineralization in *Osx-Cre* mice (Fig 2A). We performed skeleton staining on more than 20 *Osx-Cre* newborn pups and all pups showed a similar phenotype which indicates high penetrance. Histological examination in the nasal bone area showed significantly diminished mineralized tissue in the nasal bone of *Osx-Cre* mice (Fig 2B). Except for the craniofacial bone defects *Osx-Cre* mice had no obvious developmental defects in other major skeletal elements we examined including the humerus, radius and ulna, femur, tibia, fibula, ribs and vertebrae (Fig 2C) suggesting that endochondral bone formation was not affected at birth.

Hypomineralization of intramembranous bone in *Osx-Cre* mice gradually recovers during development

To further determine the extent of hypomineralization in *Osx-Cre* mice, we assessed skull development by performing skeleton staining at different postnatal time points. At day 7 hypomineralization in frontal, parietal and nasal bones of *Osx-Cre* mice persisted; however, it was much less severe compared to the defects present at birth (Fig 3A). The mineralization defect in coronal sutural area was the most prominent. At this stage, the staining pattern in other parts of the body was normal except that *Osx-Cre* mice had slightly shorter tibia and humerus (data not shown). At day 14 hypomineralization in frontal, parietal, and nasal bones was further diminished (Fig 3B). Minor, but still noticeable, defects existed in frontal and parietal bones. Hypomineralization persisted in coronal sutural area and started to become evident in the metopic sutural area. At day 21, the parietal bone in *Osx-Cre* mice appears normal. Very few minor porous spots were noticed in the frontal and nasal bones. The hypomineralization in coronal and metopic sutural areas continued to be evident (Fig 3C).

Cre and Osterix expression in *Osx*-Cre mice at different development stages

To determine the contribution Cre expression has in the observed hypomineralization phenotype, we measured Cre expression in the calvaria of *Osx*-Cre mice and found that there was higher Cre expression at birth relative to day 21 (Fig 4A). The association between Cre expression level and hypomineralization phenotype severity suggests higher Cre expression at birth may contribute to the phenotype. In addition, we measured the Osterix mRNA expression and found that Osterix mRNA levels did not differ between control and *Osx*-Cre mice (Fig 4B). We found decreased Osterix mRNA expression at day 21 compared to that at birth which is consistent with Cre expression. We measured osteoblast differentiation marker expression to determine to what extent defective osteoblast differentiation may contribute to the hypomineralization. As expected, the expression of Alkaline phosphatase, liver/bone/kidney (Alpl), Runt-related transcription factor 2 (Runx2), and Bone gamma carboxyglutamate protein (also known as osteocalcin, Ocn) was increased at later development stage (day 21) compared to at birth in both control and *Osx*-Cre groups (Fig 4C–4E). In contrast, Bone sialoprotein (Bsp) expression was decreased at later development stage (Fig 4F), indicating that calvaria at early development stage has more robust Bsp expression. However, we found no differential expression in any of those genes between control and *Osx*-Cre mice at either time point.

Doxycycline treatment rescues hypomineralization phenotype in *Osx*-Cre mice

To determine whether the hypomineralization phenotype in *Osx*-Cre mice was due to the expression of Cre protein, we took advantage of the inducible nature of Cre gene in *Osx*-Cre mice. The Cre expression in *Osx*-Cre mice is regulatable in a tet-off manner [2]. Therefore, treatment with doxycycline can prevent the expression of the GFP-Cre fusion protein and recombination of the target gene. We fed the *Osx*-Cre mice in mating units with doxycycline food in a continuous manner and analyzed the skeleton of the offspring of these mice. To determine whether indeed doxycycline treatment could prevent the expression of Cre recombinase, we crossed *Osx*-Cre mice with Rosa26 reporter mice and examined the effect of doxycycline treatment on LacZ expression. As expected, there was strong X-Gal staining in the frontal, parietal, nasal bone of Rosa26;*Osx*-Cre mice without doxycycline treatment. However, there was only minimal X-Gal positive staining in these areas in Rosa26;*Osx*-Cre mice with doxycycline treatment, indicating that doxycycline treatment effectively inhibited Cre expression (Fig 5A). Next, we performed skeleton staining for the control and *Osx*-Cre mice at birth with doxycycline treatment. We found that the hypomineralization phenotype in *Osx*-Cre mice was completely rescued after doxycycline treatment (Fig 5B), suggesting that the phenotype may be caused by the Cre expression.

Osx-Cre did not affect osteoblast *in vitro* differentiation

We previously showed that deletion of gene of interest with *Osx*-Cre greatly compromised *in vitro* osteoblast differentiation [12]. This prompted us to ask whether this was due to the effect of *Osx*-Cre itself. We digested primary osteoblasts from the parietal bone of neonatal control and *Osx*-Cre mice. In *Osx*-Cre mice, the Cre is fused with GFP under the control of Osterix promoter [2]. As expected, the primary calvarial osteoblasts isolated from *Osx*-Cre mice had positive nucleus GFP signal while it was absent in control cells (Fig 6A). We

cultured these calvarial osteoblasts in osteoblast differentiation medium and perform alkaline phosphatase staining at day 7 and Alizarin red staining at day 21. We observed similar staining pattern in Cre expressing osteoblast culture compared to that in control culture, suggesting that Cre expression in primary calvaria osteoblasts had no negative effect on both early and late osteoblast differentiation *in vitro*.

DISCUSSION

Davey et al. reported a cortical bone phenotype in skeletally immature *Osx*-Cre mice [10]. Razidlo et al. reported a minor craniofacial defect in the skulls of 5.5 week old *Osx*-Cre mice [7]. In this study, we reported a severe intramembranous bone formation defect in *Osx*-Cre mice during the early postnatal stage. Interestingly, the mineralization defect in these mice gradually recovered during development and largely disappeared by three weeks of age. Furthermore, doxycycline treatment can completely rescue these defects.

Cre recombinase is known to have possible toxic effects that can compromise normal cell cycle and survival. High levels of Cre recombinase expression in the nucleus of neuronal progenitors can compromise normal brain development [16]. High level myocardial expression of Cre recombinase can lead to dilated cardiomyopathy [17]. A similar mechanism may explain the hypomineralization phenotype in *Osx*-Cre mice. To support this notion, we found levels of Cre expression in the calvaria of *Osx*-Cre mice were more than two fold higher at birth than at three weeks of age. Increased calvarial Cre expression in newborn *Osx*-Cre mice is consistent with a higher Osterix expression level. Osterix mRNA levels did not differ between control and *Osx*-Cre mice at either age which suggests that introducing the GFP::Cre construct into exon 1 of the Osterix gene did not alter its endogenous expression. Importantly, we found that doxycycline treatment could completely rescue the defective mineralization phenotype with concomitant inhibition of Cre recombinase expression which further supports the idea that the hypomineralization phenotype in *Osx*-Cre mice was due to the Cre expression. Davey *et al.* also suggested that the Cre expression may directly contribute to the cortical bone phenotype in the skeletally immature *Osx*-Cre mice [10]. In *Osx*-Cre mice there is a Tet-controlled transactivator, tTA, cassette placed upstream of GFP::Cre. tTA is a fusion protein of an Escherichia coli Tet repressor (TetR) and the transcriptional transactivation domain of herpes simplex virus protein 16 (VP16AD) [18]. It has been reported that overexpression of tTA can result in disruption of transcriptional machinery and thus decreases the expression of other genes [19]. tTA expression in Osterix-expressing osteoblasts may lead to similar cellular damage; however, the rescue of the phenotype by doxycycline treatment argues against this possibility. tTA expression is under the control of the endogenous Osterix promoter and is not affected by doxycycline treatment which only prevents the binding of tTA to tetracycline-responsive element (TRE). This suggests that the expression of tTA is not responsible for functional defects in osteoblasts of *Osx*-Cre mice.

Although it is widely accepted that Osterix is expressed specifically by osteoblasts, some recent studies identified the Osterix expression in several non-osteoblast-lineage cells including olfactory glomerular cells, a subset of the gastric and intestinal epithelium, adipocytes, and perivascular cells in the bone marrow [20–22]. This raised the possibility

that the craniofacial bone development phenotype in *Osx*-Cre mice may be indirectly due to the non-osseous expression of GFP::Cre. Although it remains a possibility, normal body weight and general skeletal development observed in newborn *Osx*-Cre mice indicated defective localized intramembranous bone formation in craniofacial bones is not likely due to the systemic or indirect effect of Cre expression in other tissues.

Cre and GFP are fusion proteins under the control of the Osterix promoter, so higher levels of Cre expression detected shortly after birth could suggest increased GFP expression which may contribute to the hypomineralization phenotype. Although expression of GFP is considered to be innocuous for cells, cytotoxic effects have been demonstrated in both *in vitro* and *in vivo* studies [23, 24]. It remains to be determined the extent to which Cre or GFP contributed to the *in vivo* hypomineralization phenotype. Nevertheless, our current finding as well as others [10] underscored the importance of using appropriate controls for data analysis when the Cre/loxP system is used to generate tissue-specific gene knockout mice. Ideally, all CKO mice should have Cre positive littermate controls; however, the efficiency of producing both Cre control and CKO of the same gender in one litter from breeding *Flox/+;Cre* mice and *Flox/+* mice is extremely low. This practical limitation precluded the ideal approach for many study designs. If we thoroughly characterize the tissue specific effect due to Cre expression in a given tissue of interest, then we will have greater confidence when reaching conclusions from breeding experiments where *Flox/Flox* mice are used as controls. For example, we and others have shown that *Osx*-Cre itself has no effect on trabecular bone parameters [10, 12, 25]. Thus, any trabecular bone parameter changes observed in a given CKO mice with *Osx*-Cre is likely due to the deletion of target gene but not the expression of *Osx*-Cre itself. On the other hand, attributing any cortical bone parameter changes or intramembranous bone development at early stages to the gene of interest should be done cautiously. *Osx*-Cre positive littermate controls would be required to draw definitive conclusion in the latter case.

Primary calvaria osteoblast culture is a well-established cell culture system to study *in vitro* osteoblast differentiation. The observation that *Osx*-Cre caused significantly compromised osteoblast mineralization function *in vivo* raised the question about whether it would affect *in vitro* osteoblast differentiation and mineralization. It is important to address this because the conclusion will affect the interpretation for all *in vitro* primary calvarial osteoblast differentiation experiments which utilize *Osx*-Cre mice to delete any gene of interest. Our data demonstrated that both the early and terminal differentiation of primary calvarial osteoblasts isolated from neonatal *Osx*-Cre mice was comparable to control cells. It is unclear why *Osx*-Cre expression had adverse effect on mineralization in early postnatal calvaria *in vivo* but not in isolated primary calvarial osteoblasts *in vitro*. It remains to be determined whether the difference between *in vitro* culture and *in vivo* bone is due to the different Cre expression level. Nevertheless, our data validated the application of this *in vitro* osteoblast differentiation culture system when *Osx*-Cre is used to delete the gene of interest.

Acknowledgments

We thank Dr. Andrew McMahon for providing the *Osx*-Cre mice; Dr. Taylor Snider for critically reading and editing the manuscript. This study was funded by the National Institute Of Arthritis And Musculoskeletal And Skin Diseases of the National Institutes of Health under Award Number R01AR062030 (to F.L.) and R01DE020843 (to Y.M.).

References

1. Liu F, Woitge HW, Braut A, Kronenberg MS, Lichtler AC, Mina M, Kream BE. Expression and activity of osteoblast-targeted Cre recombinase transgenes in murine skeletal tissues. *Int J Dev Biol*. 2004; 48:645–653. [PubMed: 15470637]
2. Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development*. 2006; 133:3231–3244. [PubMed: 16854976]
3. Dacquin R, Starbuck M, Schinke T, Karsenty G. Mouse alpha1(I)-collagen promoter is the best known promoter to drive efficient Cre recombinase expression in osteoblast. *Dev Dyn*. 2002; 224:245–251. [PubMed: 12112477]
4. Zhang M, Xuan S, Buxsein ML, von Stechow D, Akeno N, Faugere MC, Malluche H, Zhao G, Rosen CJ, Efstratiadis A, Clemens TL. Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. *J Biol Chem*. 2002; 277:44005–44012. [PubMed: 12215457]
5. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schutz G, Tuckermann JP. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. *Cell Metab*. 2010; 11:517–531. [PubMed: 20519123]
6. Song L, Liu M, Ono N, Bringhurst FR, Kronenberg HM, Guo J. Loss of wnt/beta-catenin signaling causes cell fate shift of preosteoblasts from osteoblasts to adipocytes. *J Bone Miner Res*. 2012; 27:2344–2358. [PubMed: 22729939]
7. Razidlo DF, Whitney TJ, Casper ME, McGee-Lawrence ME, Stensgard BA, Li X, Secreto FJ, Knutson SK, Hiebert SW, Westendorf JJ. Histone deacetylase 3 depletion in osteo/chondroprogenitor cells decreases bone density and increases marrow fat. *PLoS One*. 2010; 5:e11492. [PubMed: 20628553]
8. Wu JY, Aarnisalo P, Bastepe M, Sinha P, Fulzele K, Selig MK, Chen M, Poulton IJ, Purton LE, Sims NA, Weinstein LS, Kronenberg HM. Gsalpha enhances commitment of mesenchymal progenitors to the osteoblast lineage but restrains osteoblast differentiation in mice. *J Clin Invest*. 2011; 121:3492–3504. [PubMed: 21804192]
9. Zhu W, Liang G, Huang Z, Doty SB, Boskey AL. Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development. *J Biol Chem*. 2011; 286:26794–26805. [PubMed: 21636574]
10. Davey RA, Clarke MV, Sastra S, Skinner JP, Chiang C, Anderson PH, Zajac JD. Decreased body weight in young Osterix-Cre transgenic mice results in delayed cortical bone expansion and accrual. *Transgenic Res*. 2012; 21:885–893. [PubMed: 22160436]
11. Schmidt-Supprian M, Rajewsky K. Vagaries of conditional gene targeting. *Nat Immunol*. 2007; 8:665–668. [PubMed: 17579640]
12. Liu F, Fang F, Yuan H, Yang D, Chen Y, Williams L, Goldstein SA, Krebsbach PH, Guan JL. Suppression of autophagy by FIP200 deletion leads to osteopenia in mice through the inhibition of osteoblast terminal differentiation. *J Bone Miner Res*. 2013; 28:2414–2430. [PubMed: 23633228]
13. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–71. [PubMed: 9916792]
14. Liu F, Lee JY, Wei H, Tanabe O, Engel JD, Morrison SJ, Guan JL. FIP200 is required for the cell-autonomous maintenance of fetal hematopoietic stem cells. *Blood*. 2010; 116:4806–4814. [PubMed: 20716775]

15. McLeod MJ. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology*. 1980; 22:299–301. [PubMed: 6165088]
16. Forni PE, Scuoppo C, Imayoshi I, Taulli R, Dastru W, Sala V, Betz UA, Muzzi P, Martinuzzi D, Vercelli AE, Kageyama R, Ponzetto C. High levels of Cre expression in neuronal progenitors cause defects in brain development leading to microencephaly and hydrocephaly. *J Neurosci*. 2006; 26:9593–9602. [PubMed: 16971543]
17. Buerger A, Rozhitskaya O, Sherwood MC, Dorfman AL, Bisping E, Abel ED, Pu WT, Izumo S, Jay PY. Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. *J Card Fail*. 2006; 12:392–398. [PubMed: 16762803]
18. Nishijima H, Yasunari T, Nakayama T, Adachi N, Shibahara K. Improved applications of the tetracycline-regulated gene depletion system. *Biosci Trends*. 2009; 3:161–167. [PubMed: 20103842]
19. Kuhnel F, Fritsch C, Krause S, Mundt B, Wirth T, Paul Y, Malek NP, Zender L, Manns MP, Kubicka S. Doxycycline regulation in a single retroviral vector by an autoregulatory loop facilitates controlled gene expression in liver cells. *Nucleic Acids Res*. 2004; 32:e30. [PubMed: 14966262]
20. Park JS, Baek WY, Kim YH, Kim JE. In vivo expression of Osterix in mature granule cells of adult mouse olfactory bulb. *Biochem Biophys Res Commun*. 2011; 407:842–847. [PubMed: 21463606]
21. Liu Y, Strecker S, Wang L, Kronenberg MS, Wang W, Rowe DW, Maye P. Osterix-cre labeled progenitor cells contribute to the formation and maintenance of the bone marrow stroma. *PLoS One*. 2013; 8:e71318. [PubMed: 23951132]
22. Chen J, Shi Y, Regan J, Karuppaiah K, Ornitz DM, Long F. *Osx-Cre* targets multiple cell types besides osteoblast lineage in postnatal mice. *PLoS One*. 2014; 9:e85161. [PubMed: 24454809]
23. Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells? *Biochem Biophys Res Commun*. 1999; 260:712–717. [PubMed: 10403831]
24. Huang WY, Aramburu J, Douglas PS, Izumo S. Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. *Nat Med*. 2000; 6:482–483. [PubMed: 10802676]
25. Liu Y, Berendsen AD, Jia S, Lotinun S, Baron R, Ferrara N, Olsen BR. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. *J Clin Invest*. 2012; 122:3101–3113. [PubMed: 22886301]

Figure 1. *Osx-Cre* mice have decreased body weight after postnatal day 7

(A) Representative images of control (Ctr) and *Osx-Cre* mice at birth (p0) and postnatal day 21 (p21). (B) Body weight at indicated time points. * $p < 0.05$, $n = 7$ per group. Data are mean $\pm$ SD.

Figure 2. Intramembranous bone formation is defective in *Osx-Cre* mice at birth

(A) There was severe hypomineralization in the frontal and parietal bone (arrows) and coronal sutural area (arrow heads) in *Osx-Cre* mice. (B) There was significant less mineralized area in the nasal bone of *Osx-Cre* mice (left panel). Arrow indicates the alizarin red negative staining area; H&E staining (right panel) showed much thinner nasal bone (arrowhead) in *Osx-Cre* mice. Inserts were the enlargement of indicated area. (C) *Osx-Cre* mice had normal whole skeleton, thoracic cage, arm, and leg development at birth.

Figure 3. Hypomineralization in *Osx-Cre* mice gradually recovers during development
Whole skull skeleton preparation of *Osx-Cre* and control mice at day7 (A), day 14 (B) and day 21 (C). P: Parietal bone; F: Frontal bone. Black arrows point to the sporadic porous holes in calvaria. Red arrows point to the unmineralized nasal bone. Black arrow heads point to the hypomineralized coronal sutural area. Red arrow heads point to the hypomineralized metopic sutural area.

Figure 4. Cre and osteoblast differentiation marker gene expression at different development stages

Quantitative PCR analysis of the mRNA expression of Cre (A), and osteoblast differentiation marker gene (B–F): Osterix (B), Alpl (C), Runx2 (D), Ocn (E), and Bsp (F) of parietal bone of 7-day-old control and *Osx-Cre* mice. * $p < 0.05$, N.S: Not statistically significant, $n = 5-7$ per group. Data are mean $\pm$ SD.

Figure 5. Doxycycline treatment can rescue hypo-mineralization phenotype of *Osx-Cre* mice
 The mating units were fed with or without doxycycline food as indicated. (A) X-gal staining and subsequent Alizarin Red staining of Rosa26 and Rosa26;*Osx-Cre* mice at birth. (B) Skull skeleton preparation of control and *Osx-Cre* mice fed with doxycycline food at birth.

Figure 6. *Osx-Cre* does not affect osteoblast *in vitro* differentiation

Primary calvarial osteoblasts were isolated from neonatal control (Ctr) and *Osx-Cre* mice. (A) Green fluorescence (Cre-GFP) and bright field (BF) images of cultured osteoblasts at day 21. (B) Alkaline phosphatase (ALP) staining at day 7. (C) Alizarin red S (AR) staining at day 21.