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Skeletal Defects in Osterix-*Cre* Transgenic Mice

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Abstract

Cre/*loxP* recombination is a powerful strategy widely used for *in vivo* conditional gene targeting. This technique has made possible many important discoveries of gene function in normal and disease biology. However, due to the transgenic nature of most *Cre* mouse strains undesired phenotypes occasionally occur in *Cre* mice. Here we report skeletal defects in Osterix-*Cre* (*Osx-Cre*) transgenic mice including delayed calvarial ossification and fracture calluses at multiple skeletal sites. These data suggest that *Osx-Cre* containing controls should be used for both *in vivo* and *in vitro* skeletal analyses of conditional knockout mice generated with this *Osx-Cre* mouse strain.

Keywords

Osterix-*Cre*; Skeletal defects; Mouse; Transgenic

Introduction

Application of the Cre/*loxP* site-specific recombination system has been widely used for chromosome modification and gene targeting studies in mice for almost two decades. When recombination is induced *in vivo*, not only can chromosomal deletions, duplications, inversions or translocations be generated, but conditional gene modification in a cell type, tissue or developmental stage specific manner can also be achieved (Yu and Bradley 2001, Kuhn and Torres 2002). Therefore, the Cre/*loxP* system has been a powerful tool in studies aimed at understanding basic biological processes as well as human diseases associated with genetic abnormalities.

Currently, one frequent application of the Cre/*loxP* strategy is to generate conditional knockout (CKO) mice that require generation both of a mouse strain harboring a *loxP*-flanked region of a target gene (floxed) and of a second mouse strain expressing the Cre recombinase constitutively. The latter is often achieved by making a *Cre* transgenic mouse line, where most of the time the insertion of a *Cre*-containing construct in the mouse genome is random and unknown. As a result, it can occasionally disrupt or enhance the function of an endogenous gene, causing undesired phenotypes in transgenic *Cre* mice

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themselves (Palmiter and Brinster 1986). An example of such a mouse line is the Osterix-*Cre* (*Osx-Cre*) transgenic mouse (Rodda and McMahon 2006).

The *Osx-Cre* transgenic mice were originally generated by the McMahon group (Rodda and McMahon 2006) and are now available at the Jackson Laboratory. Osterix (*Osx*) is a zinc finger-containing transcription factor required for osteoblast differentiation and bone formation (Nakashima et al. 2002). It is expressed in both endochondral and membranous osteoblasts and functions downstream of *Runx2*, another master transcription factor essential for osteogenesis (Komori et al. 1997). Although both *Runx2* null and *Osx* null mutant mice exhibit similar bone defects characterized by a lack of bone formation and an absence of differentiated osteoblasts, *Osx* appears to strictly control osteoblast differentiation while *Runx2* has a much earlier and broader effect on invasion of mesenchymal cells, osteoclasts and blood vessels into hypertrophic cartilage matrix as well (Komori et al. 1997, Nakashima et al. 2002). Clearly, expression of these transcription factors represents different stages in mesenchymal cell differentiation resulting in different cell behavior and fates. Therefore, mice expressing the *Cre* transgene under the control of the *Runx2* or *Osx* promoter have become powerful genetic tools for studying skeletogenesis as well as specific stages in bone development (Rauch et al. 2010, Rodda and McMahon 2006). Here we report that *Osx-Cre* transgenic mice exhibit unexpected skeletal phenotypes that could confound bone studies using these mice. We suggest that proper controls must be used for *in vivo* and *in vitro* analyses involving this *Osx-Cre* mouse line.

Materials and Methods

Animals

Osx-Cre transgenic mice were kindly provided by Dr. Andy McMahon at Harvard University (Rodda and McMahon 2006) and also obtained from the Jackson Laboratory. Mice harboring floxed alleles of *Pkd1* were described previously (Starremans et al. 2008). C57BL/6 mice were purchased from Charles River.

Skeletal staining

After euthanasia, mice were skinned and eviscerated. Skeletal specimens were then incubated in a staining solution containing 0.006% Alizarin red S, 0.005% Alcian blue, 0.13% glacial acetic acid and 62% ethanol. Stained skeletal samples were further cleared in 1.8% and 0.3% potassium hydroxide solutions and stored in 100% glycerol. All animal work was conducted according to protocols approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Micro-CT Scanning

After euthanasia, skulls of P6 pups were skinned, separated from the trunk and subjected to high-resolution micro-CT scanning (Xradia MicroXCT-200 system, Pleasanton CA, USA). Images were reconstructed and generated by the Xradia software.

Results

Osx-Cre transgenic mice exhibit multiple bone defects

The *Osx-Cre* mice provided by Dr. Andy McMahon were crossed to C57BL/6 mice in our laboratory for three generations. Although these mice carry the tetracycline-controlled Tet-Off regulation of *Osx-Cre*, we did not administer any doxycycline to the mice throughout our examination in order to observe the effect of actively expressed *Osx-Cre*. To examine whether the *Osx-Cre* transgene alone affects normal bone development, we analyzed skeletons from newborn to one-week-old pups. Newborn *Osx-Cre* pups were indistinguishable from their wild type (WT) littermates. However, body weight comparison at P6 showed that *Osx-Cre* pups were significantly lighter than their WT littermates, weighing 3.60g and 4.13g on average, respectively (n=8 for WT, n=9 for *Osx-Cre*, $P<0.01$). Alcian blue and Alizarin red staining of skeletons revealed that *Osx-Cre* pups exhibited multiple skeletal defects including delayed calvarial and clavicular mineralization and frequent fracture callus spots on the scapula, ribs and fibula (Figure 1a-f, mu). To confirm that the delayed calvarial mineralization in *Osx-Cre* pups is not an artifact from the skeletal staining method, which can occasionally occur, micro-CT analysis was carried out on the calvaria of P6 mice. As shown in Figure 1v-x, *Osx-Cre* mice exhibited delayed and reduced calvarial mineralization at P6. To determine whether the observed skeletal defects in *Osx-Cre* mice were associated with the *Osx-Cre* transgene expression or due to normal variation among newborn littermates, we examined the frequency of skeletal defects in both *Osx-Cre* pups and their WT littermates. Over 83% (10 out of 12) P6 *Osx-Cre* pups had a scapula callus defect with 42% having bilateral calluses. Delayed calvarial mineralization was seen in 67% of *Osx-Cre* pups (8/12) (Figure 1y). In contrast, none of the 13 WT pups exhibited scapula calluses or obviously delayed calvarial ossification (Figure 1y). These results suggest that the observed bone defects are highly associated with the expression of the *Osx-Cre* transgene, although the phenotype is not fully penetrant.

Mouse genetic backgrounds can often affect the manifestation of a skeletal defect. To determine whether the bone phenotype in our *Osx-Cre* mice is affected by the mixed mouse strain background, we purchased the same line of *Osx-Cre* mice from the Jackson Laboratory where they had been backcrossed to C57BL/6 mice for at least 10 generations. At age P6, these *Osx-Cre* mice showed similar skeletal defects as described above (Figure 1g-l), including reduced body weight (WT: 3.43g n=21, *Osx-Cre*: 2.60g n=12, $P<0.01$), indicating that the skeletal defects observed in the *Osx-Cre* mice are not related to their mixed strain background.

In studies using the Cre/*loxP* CKO model, mice with homozygously floxed target gene (fl/fl) are often chosen as controls for the CKO mice that would in addition express a *Cre* transgene (Ogata et al. 2011, Kamiya et al. 2008). To assess to what extent the bone defects in *Osx-Cre* mice could affect analysis of bone phenotypes in other CKO mice generated with this deleter strain, we examined the skeleton of polycystin 1 (*Pkd1*) CKO mice using *Osx-Cre*. Not surprisingly, homozygous *Pkd1* CKO mice (*Osx-Cre*:*Pkd1*$^{fl/fl}$) also had fracture calluses often on the rib, clavicle and scapula (Figure 2a-c) as well as delayed calvarial mineralization (data not shown) at early ages. As this phenotype was not seen in

Pkd1$^{fl/fl}$ mice (Figure 2d-f), *Pkd1*$^{fl/fl}$ mice would not be the correct controls to use in these experiments. Instead, *Osx-Cre* or *Osx-Cre:Pkd1*$^{fl/+}$ mice need to be used.

Discussion

Here we report that the *Osx-Cre* transgenic mice available from the Jackson Laboratory exhibit skeletal defects including delayed calvarial ossification and fracture healing calluses at multiple skeletal sites. Decreased body weight and delayed cortical bone development have been reported in young adults of these *Osx-Cre* mice (Davey et al. 2012). Modest increase in trabecular number and separation has also been shown after body weight correction in these mice (Davey et al. 2012). Taken together, the skeletal abnormalities in *Osx-Cre* mice appear to occur both in membranous and long bones.

Although the phenotype is mild, it raises the issue of proper controls when this *Cre* line is used for skeletal analyses. Several skeletal studies have been published using this *Osx-Cre* transgenic mouse strain (Greenblatt et al. 2010, Razidlo et al. 2010, Zhu et al. 2011). However, some of the studies describing skeletal abnormalities or defective cellular functions did not include *Osx-Cre* in their controls. Our data suggest that heterozygous knockouts (expressing both *Osx-Cre* and heterozygous floxed target gene) or *Osx-Cre* mice are necessary controls for *Osx-Cre* mediated CKO mice. Only when such controls are used, can the possibility that some of the observed skeletal abnormalities are due to expression of the *Cre* transgene rather than deletion of the target gene be excluded. Moreover, primary osteoblast isolation and culture are often carried out from calvariae in P0-P3 pups for *in vitro* analyses. As *Osx-Cre* pups clearly had delayed calvarial ossification *in vivo*, it is highly likely that primary osteoblasts isolated from *Osx-Cre* mice have impaired differentiation, proliferation or osteogenic function. Therefore, *in vitro* primary osteoblast analyses should also include cells from *Osx-Cre* mice to control for the effect of the *Osx-Cre*.

While it is clear that transgenic *Osx-Cre* mice display unexpected skeletal defects, the reason why they occur remains unknown. Recently, several studies have shown that in addition to osteoblasts, osteocytes and hypertrophic chondrocytes, *Osx-Cre* also targets many other cell types including stromal cells, adipocytes and perivascular cells in the bone marrow (Chen et al. 2014), brain cells (Park et al. 2011), and lung cells (our unpublished data). Whether any of these cell types, particularly bone marrow stromal cells and brain cells, contribute to the observed skeletal defects in *Osx-Cre* mice awaits further investigation. In addition to the non-skeletal tissue expression of *Osx-Cre*, it is also possible that these undesired phenotypes are caused by impaired osteoblast function due to insertional effects of the transgene. It has been estimated that 5% of established transgenic lines result in insertional mutation effects (Meisler 1992). Several possibilities may explain such effects. First, the site at which the *Cre* transgene inserts into the mouse genome could possibly disrupt the normal function of an endogenous gene. Second, deletion or duplication of large chromosomal DNA regions could occur during transgene integration (Palmiter and Brinster 1986). Third, prokaryotic vector sequences have been shown to be inhibitory for some mammalian genes and could have a disruptive effect (Townes et al. 1985). Fourth, the *Osx* promoter, GFP and *Cre* containing bacterial artificial chromosome used to generate this

Osx-Cre line (Rodda and McMahon 2006) is a relatively large piece of foreign DNA that potentially could exert an interruptive effect.

In our study, *Osx-Cre* mice were backcrossed to C57BL/6 mice for either three (our laboratory) or more than ten generations (Jackson Laboratory). Although mouse genetic backgrounds play important roles in the formation of certain skeletal defects (Lee et al. 2010), it does not appear to affect the manifestation of skeletal defects in these *Osx-Cre* mice. Due to the various genetic backgrounds in which mice carrying floxed alleles of interest are often developed, CKO mice using *Cre* are also frequently generated with mixed backgrounds. Therefore, it is of great importance to be aware of undesired defects in a *Cre* deleter mouse line and properly control for such defects in the associated analyses.

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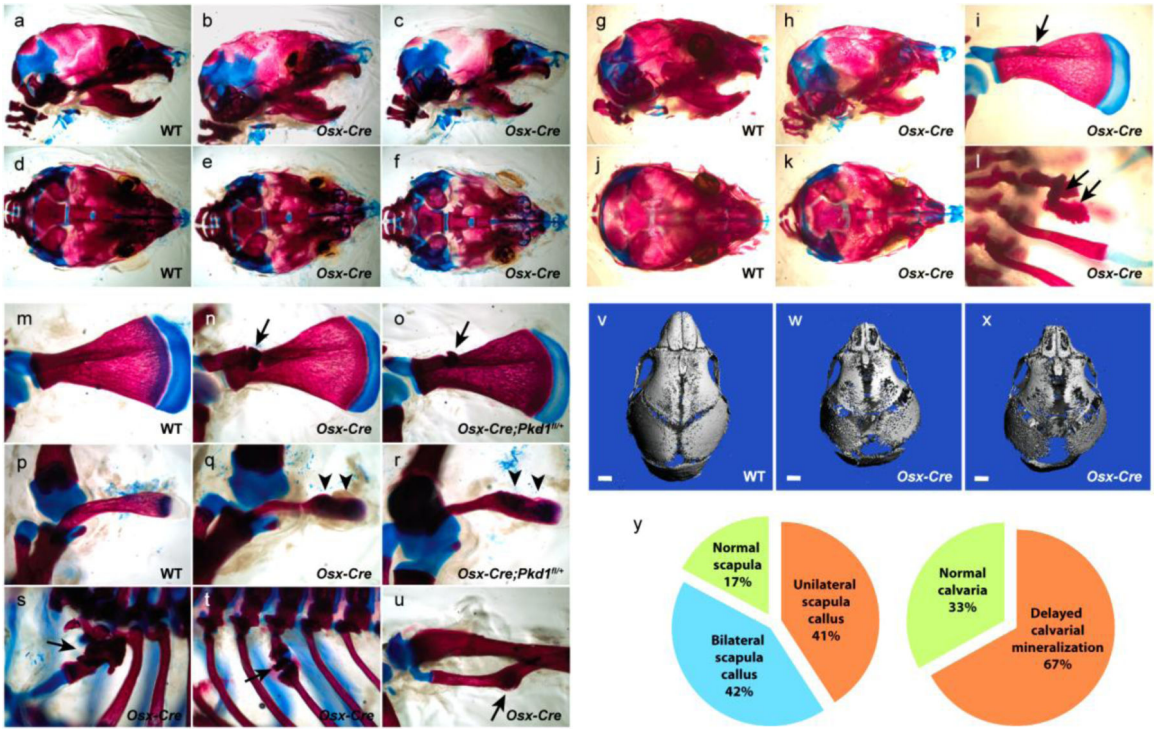

Figure 1. Skeletal defects in *Osx-Cre* transgenic mice

Alcian blue and Alizarin red skeletal staining shows delayed calvarial (b, c, e, f, h, k) and clavicular (q, r) mineralization in *Osx-Cre* pups compared to WT littermate controls (a, d, g, j, p). Fracture callus spots are frequently observed on the scapula (i, n, o), ribs (l, s, t) and fibula (u) in *Osx-Cre* but not in control pups (m, p)Micro-CT analysis of the skull further confirms delayed calvarial mineralization in *Osx-Cre* pups (v-x). The occurrence of scapula calluses and delayed calvarial mineralization in *Osx-Cre* transgenic mice has a high frequency of 83% and 67%, respectively (y). These skeletal defects are observed in *Osx-Cre* pups both on a mixed (a-f, m-u) and C57BL/6 background (g-l). Arrow: fracture callus; arrowhead: delayed clavicular mineralization.

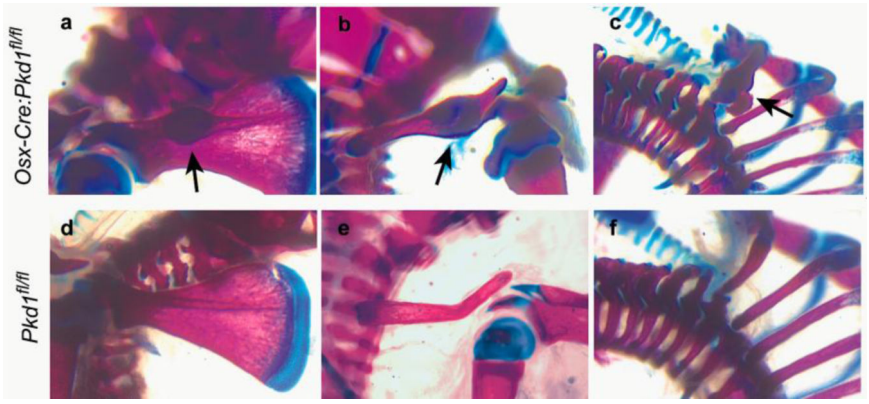

Figure 2. Skeletal defects in *Osx-Cre*:*Pkd1*$^{fl/fl}$ conditional knockout mice

Alcian blue and Alizarin red skeletal staining shows frequent fracture calluses on the scapula (a), clavicle (b) and ribs (c) in P6 *Osx-Cre*:*Pkd1*$^{fl/fl}$ conditional knockout mice, but none in *Pkd1*$^{fl/fl}$ pups (d-f).