Developmental Biology 362 (2012) 76–82

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Indian hedgehog requires additional effectors besides Runx2 to induce osteoblast differentiation

Xiaolin Tu [a,1,2], Kyu Sang Joeng [a,b,1,3], Fanxin Long [a,b,c,*]

[a] Department of Medicine, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA
[b] Division of Biological and Biomedical Sciences, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA
[c] Department of Developmental Biology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA

ARTICLE INFO

Article history:
Received for publication 28 October 2011
Revised 17 November 2011
Accepted 21 November 2011
Available online 2 December 2011

Keywords:
Indian hedgehog
Ihh
Runx2
Osteoblast
Bone

ABSTRACT

Indian hedgehog (Ihh) is indispensable for osteoblast differentiation during embryonic development of the endochondral skeleton. In the absence of Ihh, cells of the osteoblast lineage fail to activate the expression of Runx2, a transcription factor integral to osteoblast differentiation. However, it is hitherto unclear whether the lack of Runx2 expression is solely responsible for the failure of osteoblast formation in Ihh-null embryos. Here, by creating a mouse allele that expresses Runx2 in a Cre-dependent manner, we show that force-expression of Runx2 in the skeletogenic cells restores bone formation in the Runx2-null, but not in the Ihh-null embryo. Thus, the mechanism through which Ihh induces osteoblast differentiation requires other effectors in addition to Runx2.

Introduction

Most elements of the mammalian skeleton originate from a cartilage intermediate through endochondrial ossification (Kronenberg, 2003). In this process, the skeletogenic mesenchymal cells condense to form a cartilage anlage composed of chondrocytes and several layers of surrounding fibroblastic cells that constitute the perichondrium. Following the initial phase of proliferation, chondrocytes located at the center of the anlage exit the cell cycle and undergo hypertrophy (increase in cell size). It is at this time that the bone-forming osteoblasts differentiate from the perichondrium adjacent to the hypertrophic chondrocytes. Thus, osteoblast differentiation during endochondral ossification is tightly coupled with chondrocyte development.

Indian hedgehog (Ihh) is a key signal emanating from the chondrocytes to induce osteoblast differentiation. Among the three mammalian Hedgehog proteins, Ihh is uniquely expressed by chondrocytes transitioning to the fully hypertrophic state, commonly known as the prehypertrophic and early hypertrophic chondrocytes (Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). Genetic deletion of Ihh in the mouse resulted in a complete lack of osteoblasts in the endochondral skeleton (St-Jacques et al., 1999). Similarly, ectopic induction of osteoblast differentiation by precocious hypertrophic chondrocytes in a chimeric mouse model also required Ihh (Chung et al., 2001). Moreover, studies of Smoothened (Smo), which encodes a 7-pass transmembrane protein indispensable for Hh signaling in the receiving cell, demonstrated a cell-autonomous requirement for Smo in the perichondrium for osteoblast differentiation (Long et al., 2004). These studies support a direct role for Ihh signaling in osteoblastogenesis.

The mechanism through which Ihh induces osteoblast differentiation is not well understood. Analyses of the $Ihh^{-/-}$ embryo have revealed that the perichondrium is severely hypoplastic, and that none of the known markers for the osteoblast lineage is detectable, indicating that the differentiation process is arrested at a very early stage (Hu et al., 2005; St-Jacques et al., 1999). Although our previous work has shown that Ihh exerts its osteogenic effect through both Gli3 suppression and Gli2 activation (Hilton et al., 2005; Joeng and Long, 2009), the relevant target genes for either Gli2 or Gli3 are not known.

Runx2, a runt-domain transcription factor, is an attractive candidate as an important mediator for the osteogenic activity of Ihh. Molecular and genetic studies have established the essential role of Runx2 in osteoblast differentiation (Ducy et al., 1997; Lee et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Importantly, similar to Ihh removal, deletion of Runx2 in the mouse leads to no osteoblasts, and hypoplasia of the perichondrium (Komori et al., 1997; Otto et al., 1997). Moreover, Runx2 expression in the perichondrium was abolished in the $Ihh^{-/-}$ embryo. These findings raise the possibility

* Corresponding author at: Box 8127, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110, USA.
E-mail address: flong@wustl.edu (F. Long).
[1] These authors contributed equally to the paper.
[2] Current address: Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA.
[3] Current address: Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.11.013

that force-expression of Runx2 in the perichondrium may be sufficient to restore osteoblast differentiation in Ihh$^{-/-}$ embryos. Here we test this possibility by genetic means.

Results

Generation of a mouse strain expressing Runx2 in a Cre-dependent manner

To create a versatile tool to express Runx2 in a tissue-specific manner, we modified the Rosa26 genomic locus through homologous recombination so that Runx2 expression can be achieved following Cre-mediated recombination (Fig. 1A). The modified allele was termed R26^{Runx2}. As expected, mice carrying either one or two copies of the allele (genotypes designated R26$^{Runx2/+}$ or R26$^{Runx2/Runx2}$, respectively) were completely normal. When these mice were crossed with a Col2-Cre transgenic line that targets both chondrocyte and osteoblast lineages in the endochondral skeleton, they produced progenies with the genotype of Col2-Cre; R26$^{Runx2/+}$ (or C2Cre; R26$^{Runx2/+}$) that were viable and possessed a relatively normal skeleton at E18.5 (Fig. 1B1–B2).

Forced expression of Runx2 rescues bone formation in Runx2-null mice

To determine the efficacy of Runx2 expressed from the R26^{Runx2} allele, we tested whether activation of the allele in the skeletogenic cells could functionally replace the endogenous Runx2 alleles in the embryonic skeleton. Specifically, we generated Runx2-null embryos (Runx2$^{-/-}$), and those that also carried the Col2-Cre transgene, and one or two R26^{Runx2} alleles (Runx2$^{-/-}$; C2Cre; R26$^{Runx2/+}$, or Runx2$^{-/-}$; C2Cre; R26$^{Runx2/Runx2}$, respectively). When analyzed at E18.5 by whole-mount skeletal staining, the Runx2$^{-/-}$ embryos, exhibited no alizarin red staining throughout the body except for the zeugopod (Fig. 1B3). This staining pattern was consistent with the previous reports that the Runx2$^{-/-}$ embryos possessed no bone and only a small amount of mineralized cartilage in the zeugopod. Importantly, the Runx2$^{-/-}$ embryos expressing one or two R26^{Runx2} alleles exhibited progressively more alizarin red staining characteristic of bone (distinguishable from mineralized cartilage by a more intense red color) (Fig. 1B4–B5). The restoration of bone formation in the endochondral skeleton but not the skull was consistent with the specific targeting of Col2-Cre to the former. Thus, activation of the R26^{Runx2} allele in the endochondral skeleton is sufficient to restore bone formation in the Runx2-null embryo.

To corroborate the findings above, we conducted further analyses of the tibia. H&E staining of longitudinal sections from E18.5 embryos confirmed that activation of the R26^{Runx2} allele in the wild-type background (C2Cre; R26$^{Runx2/+}$) did not overtly alter the morphology of the cartilage, the bone collar or the marrow (Fig. 2A–B). On the other hand, the Runx2$^{-/-}$ tibia lacked a bone collar or a marrow cavity, but possessed an elongated region of hypertrophic chondrocytes (Fig. 2C). In contrast, the Runx2$^{-/-}$ embryos expressing one or two R26^{Runx2} alleles formed a bone collar (Fig. 2D–E). Surprisingly, a marrow cavity was only observed in the embryos expressing two R26^{Runx2} alleles, revealing a dependence of marrow formation on Runx2 dosage (see Discussion). Moreover, no trabecular bone was observed within the marrow cavity of the Runx2$^{-/-}$ embryos expressing two R26^{Runx2} alleles. Nonetheless, these results confirm that activation of a single R26^{Runx2} allele is sufficient to induce osteoblast differentiation within the perichondrium in the absence of endogenous Runx2.

The efficacy of the R26^{Runx2} allele was further demonstrated by molecular analyses of the tibia in E18.5 embryos. In situ hybridization confirmed that Runx2 was normally expressed in the perichondrium and the primary spongiosa, and at a lower level in the prehypertrophic and early hypertrophic cartilage (Fig. 3A1). Notably, prominent Runx2 signals were also detected in the perichondrium and the cartilage of the Runx2$^{-/-}$ embryo (which did not possess a primary spongiosa) (Fig. 3A2), indicating that the Runx2-null allele produced a mutant mRNA sufficiently stable to be detected by the in situ probe (see Methods). Moreover, activation of either one or two R26^{Runx2} alleles did not noticeably increase the overall Runx2 signal,

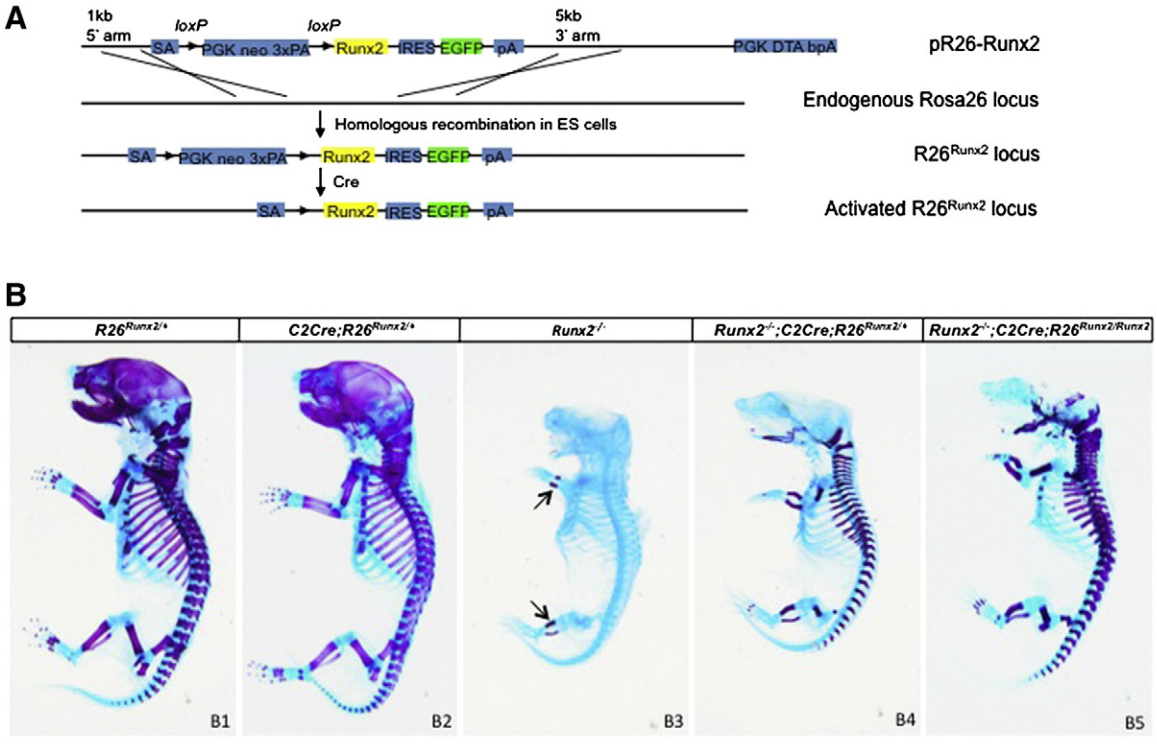

Fig. 1. (A) Diagram for generation of the R26^{Runx2} allele. (B) Whole-mount skeletal staining of E18.5 mouse embryos. Arrows denote mineralized cartilage.