Bone 68 (2014) 131–141

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

The effect of conditional inactivation of beta 1 integrins using twist 2 Cre, Osterix Cre and osteocalcin Cre lines on skeletal phenotype

Asha Shekaran [a,b,1], James T. Shoemaker [a], Taylor E. Kavanaugh [a], Angela S. Lin [b,c], Michelle C. LaPlaca [a], Yuhong Fan [b,d], Robert E. Guldberg [b,c], Andrés J. García [b,c,*]

[a] Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, 313 Ferst Drive, Atlanta, GA 30332, USA
[b] Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, 315 Ferst Drive, Atlanta, GA 30332, USA
[c] School of Mechanical Engineering, Georgia Institute of Technology, 801 Ferst Drive, Atlanta, GA 30332, USA
[d] School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, GA 30332, USA

ARTICLE INFO

Article history:
Received 10 May 2014
Revised 13 August 2014
Accepted 16 August 2014
Available online 27 August 2014

Edited by: Ms. J. Aubin

Keywords:
Beta 1 integrins
Skeletal phenotype
Transgenic mice
Twist2-Cre
Osterix-Cre
Osteocalcin-Cre

ABSTRACT

Skeletal development and growth are complex processes regulated by multiple microenvironmental cues, including integrin–ECM interactions. The β1 sub-family of integrins is the largest integrin sub-family and constitutes the main integrin binding partners of collagen I, the major ECM component of bone. As complete β1 integrin knockout results in embryonic lethality, studies of β1 integrin function *in vivo* rely on tissue-specific gene deletions. While multiple *in vitro* studies indicate that β1 integrins are crucial regulators of osteogenesis and mineralization, *in vivo* osteoblast-specific perturbations of β1 integrins have resulted in mild and sometimes contradictory skeletal phenotypes. To further investigate the role of β1 integrins on skeletal phenotype, we used the Twist2-Cre, Osterix-Cre and osteocalcin-Cre lines to generate conditional β1 integrin deletions, where Cre is expressed primarily in mesenchymal condensation, pre-osteoblast, and mature osteoblast lineage cells respectively within these lines. Mice with Twist2-specific β1 integrin disruption were smaller, had impaired skeletal development, especially in the craniofacial and vertebral tissues at E19.5, and did not survive beyond birth. Osterix-specific β1 integrin deficiency resulted in viable mice which were normal at birth but displayed early defects in calvarial ossification, incisor eruption and growth as well as femoral bone mineral density, structure, and mechanical properties. Although these defects persisted into adulthood, they became milder with age. Finally, a lack of β1 integrins in mature osteoblasts and osteocytes resulted in minor alterations to femur structure but had no effect on mineral density, biomechanics or fracture healing. Taken together, our data indicate that β1 integrin expression in early mesenchymal condensations play an important role in skeletal ossification, while β1 integrin-ECM interactions in pre-osteoblast, odontoblast- and hypertrophic chondryocyte-lineage cells regulate incisor eruption and perinatal bone formation in both intramembranously and endochondrally formed bones in young, rapidly growing mice. In contrast, the osteocalcin-specific β1 integrin deletion had only minor effects on skeletal phenotype.

Introduction

Skeletal development, growth, function and repair are complex processes mediated by multiple cell types, such as osteoblasts, chondrocytes and osteoclasts, which are regulated by diverse microenvironmental cues including cell-extracellular matrix (ECM) interactions. Adhesion to ECM is primarily mediated by the integrin receptor family, which also regulates crucial cell functions such as survival, migration and differentiation [1]. Integrins comprise 24 different αβ heterodimeric receptors [2]; β1 integrins are the largest sub-family of integrins, as β1 integrins associate with 12 different α subunits [2].

During embryonic skeletal development, osteoblasts and chondrocytes differentiate from common precursor cells, found in condensed mesenchymal tissue. Bones are developed from these mesenchymal cells by two distinct mechanisms: endochondral bone or intramembranous ossification [3,4].

The condensed mesenchymal precursor cells are bipotential and co-express Sox-9 and Runx2 which are indispensible for chondrogenesis and osteogenesis respectively [5,6], and undergo osteoblastic differentiation under the regulation of Wnt/β-catenin [7–9] and

* Corresponding author at: Rae S. and Frank H. Neely Chair and Regents' Professor Woodruff School of Mechanical Engineering Petit Institute for Bioengineering and Bioscience Georgia Institute of Technology 2310 IBB Building 315 Ferst Drive Atlanta, GA 30332-0363, USA.
E-mail address: andres.garcia@me.gatech.edu (A.J. García).
URL: http://www.garcialab.gatech.edu/ (A.J. García).
[1] Current address Bioprocessing Technology Institute, 20 Biopolis Way, #06-01 Centros, Singapore 138668.

http://dx.doi.org/10.1016/j.bone.2014.08.008
8756-3282/

Osterix [10]. Pre-osteoblasts cells start to express Osterix, which is essential for osteoblast differentiation and mineralization during development [10] and postnatal growth [11]. Osterix is also expressed in prehypertrophic and hypertrophic chondrocytes and odontoblasts and regulates hypertrophic chondrocyte maturation [12]. Osterix+ pre-osteoblasts undergo further differentiation, giving rise to mature osteoblasts which express osteocalcin, an osteoblast-specific marker [13].

Although the cartilage anlages of long bones are formed in mice prenatally, primary ossification and secondary ossification occur after birth [14]. Once formed, diaphyseal lengthening of long bones occurs at the cartilaginous growth plate [15], while diametric expansion occurs through intramembranous ossification at the periosteum. Calvarial bone formation begins with mesenchymal cell aggregation [16] followed by osteoblastic differentiation of these cell masses near the cartilaginous skull base in the neural crest-derived frontal and mesoderm-derived parietal bones which extend superiorly [17].

β1 integrins are believed to play a crucial role in bone formation as β1 integrins are highly expressed in osteoblasts, osteoprogenitors and bone marrow stromal cells and are required for osteogenesis *in vitro*. Blocking of multiple β1 integrins such as α2β1 [18–22], α5β1 [23–25], α1β1 [20,22] and α3β1 [24] in these cells severely impairs *in vitro* osteogenic differentiation and mineralization. β1 integrins also regulate cell survival, as disruption of fibronectin-α5β1 interactions induces apoptosis in differentiated osteoblasts [26]. Because β1 integrins are essential for embryonic development [27], β1 integrin functions *in vivo* are typically studied using tissue-specific gene deletions or expression of a dominant negative mutant.

Although *in vitro* data strongly suggests that β1 integrins play a critical role in bone formation, *in vivo* deletion or functional perturbation of β1 integrins in mature osteoblasts have yielded sometimes contradictory results and relatively mild skeletal phenotypes. For example, mice with dominant negative β1 integrin (β1-DN) expression in mature osteoblasts under the rat osteocalcin promoter [28] displayed thinner parietal bones at 35 days old which reached normal thickness by 90 days old in male mice [29]. However, in a second study of male and female mice from 14 to 365 days old with the same osteocalcin-specific β1-DN expression, the mutants displayed increased body weight and a largely normal appendicular skeletal phenotype with only mild and transient differences bone mass, mechanics and histomorphometry [30]. However, some of these contradictions in the literature may be due to differences in the background strains of mice in which the β1 integrin perturbations were studied. A third study of female mutants with osteoblast-specific β1-DN expression at 63 days old reported reductions in the cancellous bone mass in the femoral metaphysis and lumbar vertebral body, decreased femoral strength and tibial curvature as well as an increased osteoclast surface in response to hindlimb unloading [31]. Two studies of osteoblast-specific β1 integrin ablation in β1 integrin-floxed;Col1a1Cre [32] mice showed no change in skeletal development or growth but displayed altered responses to hindlimb unloading [33,34].

Other *in vivo* conditional β1 integrin perturbation studies have primarily focused on the phenotypes of cartilaginous tissues and endochondrally-derived long bones such as the femur or the tibia and have not demonstrated severe bone phenotypes. Raducanu et al. demonstrated that conditional Prx1 Cre-mediated β1 integrin inactivation in the limb bud mesenchyme resulted in knee joint articular cartilage abnormalities, delayed secondary ossification, and shortened long bones and blood vessel calcification in the hind limb at 16 months [35], but the cranial phenotype has not been described in these mutants. Similarly, although chondrocyte-specific β1 integrin perturbations using the Col2a1 Cre line [36] were shown to induce chondrodysplasia, delayed femoral primary and secondary ossification, perinatal lethality, and short-limbed dwarfism in survivors [37]. However, much remains unknown about the role of β1 integrins in skeletal development and growth, especially with respect to β1 integrin function in early mesenchymal condensations and osteoprogenitors, and their contributions to the development of intramembranously formed bones such as calvarial bones.

In order to further investigate the effect of β1 integrins on skeletal phenotype, in this study we generated mice with conditional β1 integrin deletions restricted primarily to the mesenchymal condensation, pre-osteoblast, or mature osteoblast lineages respectively by crossing the Twist2-Cre [38], Osterix-Cre [8], or osteocalcin-Cre [39] lines with a β1 integrin-floxed mouse line [40]. Twist 2 is not bone specific and is detected as early as E9.5 in the pharyngeal arches and somites [41], and Twist2-Cre is expressed in E11.5 in limb mesenchymal condensations [42], as well as in the mesenchymal cells which give rise to the craniofacial skeleton by E12.5 [43]. Osterix (Sp7) expression is not bone specific and is primarily restricted to pre-osteoblasts, but is also expressed in prehypertrophic and hypertrophic chondrocytes as well as odontoblasts [10,11,44,45]. Because osteocalcin is a mature osteoblast marker expressed by osteoblasts and osteocytes, osteocalcin-Cre is expressed later than Osterix-Cre, from E17.5 onwards in mouse embryos [39].

Materials and methods

Mouse crosses and genotyping

Homozygous β1 integrin floxed (itgβ1$^{fl/fl}$) mice, B6;129-*Itgb1^{tm1Efu}*/J, as well as TW2-Cre mice, B6.129X1-*Twist2$^{tm1.1(cre)Dor}$*/J, and OSX-Cre mice, B6.Cg-Tg(Sp7-tTA,tetO-EGFP/cre)1Amc/J, were purchased from Jackson Laboratories, and OC-Cre mice were kindly provided by Thomas Clemens (Johns Hopkins University). Itgβ1$^{fl/fl}$ mice were mated with TW2-Cre, OSX-Cre or OC-Cre mice. Mice with β1 integrin deletions under the respective promoters: itgβ1$^{fl/fl}$;TW2-Cre, itgβ1$^{fl/fl}$;OSX-Cre and itgβ1$^{fl/fl}$;OC-Cre were generated. In all studies, these conditional knockout mice were compared with littermates with a control genotype itgβ1$^{fl/fl}$ or itgβ1$^{fl/+}$ or with heterozygous conditional knockout genotype, itgβ1$^{fl/+}$;TW2-Cre, itgβ1$^{fl/+}$;OSX-Cre and itgβ1$^{fl/+}$;OC-Cre. All offspring of OSX-Cre mice were given doxycycline in their drinking water once they were weaned to prevent continuous tetO-controlled transgene expression, which has been reported to cause side effects such as malocclusion. Mice were tail clipped after weaning and genotyped by PCR analysis of genomic DNA extracted using the Qiagen DNeasy Kit. All protocols were approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology in adherence to federal guidelines for animal care.

Timed mating and embryo harvest

Timed matings were performed by placing breeders together the evening prior to the onset of the 12-hour dark cycle. Females were checked the following morning for plugs. Plugged females were single housed and euthanized at E11.5, E13.5 or E19.5 to harvest embryos. Embryos were tailed clipped for genotyping and fixed in 10% neutral buffered formalin for μCT analysis and histological analysis.

μCT analysis on E19.5 embryos

E19.5 embryos were imaged using a μCT40 [46] using an X-ray intensity of 145 μA, energy of 55 kVp, integration time of 200 ms, and resolution of 12 μm. The 2D greyscale tomograms were stacked and binarized by applying a Gaussian filter (sigma = 1, support = 1) and global threshold value of 150 mg HA/ccm to generate 3D reconstructions. The threshold was chosen based on the mineral densities of the control itgβ1$^{fl/fl}$ E19.5 mouse embryo skeletons.