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The Effect of Conditional Inactivation of Beta 1 Integrins using Twist 2 Cre, Osterix Cre and Osteocalcin Cre Lines on Skeletal Phenotype

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Abstract

Skeletal development and growth are complex processes regulated by multiple microenvironmental cues, including integrin-ECM interactions. The $\beta 1$ 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 $\beta 1$ integrin knockout results in embryonic lethality, studies of $\beta 1$ integrin function *in vivo* rely on tissue-specific gene deletions. While multiple *in vitro* studies indicate that $\beta 1$ integrins are crucial regulators of osteogenesis and mineralization, *in vivo* osteoblast-specific perturbations of $\beta 1$ integrins have resulted in mild and sometimes contradictory skeletal phenotypes. To further investigate the role of $\beta 1$ integrins on skeletal phenotype, we used the Twist2-Cre, Osterix-Cre and Osteocalcin-Cre lines to generate conditional $\beta 1$ 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 $\beta 1$ integrin disruption were smaller, had impaired skeletal development, especially

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in the craniofacial and vertebral tissues at E19.5, and did not survive beyond birth. Osterix-specific $\beta 1$ 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 $\beta 1$ 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 $\beta 1$ integrin expression in early mesenchymal condensations play an important role in skeletal ossification, while $\beta 1$ integrin-ECM interactions in pre-osteoblast, odontoblast- and hypertrophic chondrocyte- 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 $\beta 1$ integrin deletion had only minor effects on skeletal phenotype.

Keywords

Beta 1 integrins; skeletal phenotype; transgenic mice; Twist2-Cre; Osterix-Cre; Osteocalcin-Cre

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 $\alpha\beta$ heterodimeric receptors (2); $\beta 1$ integrins are the largest sub-family of integrins, as $\beta 1$ 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 indispensable for chondrogenesis and osteogenesis respectively (5, 6), and undergo osteoblastic differentiation under the regulation of Wnt/ β -catenin (7–9) and 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 anlagen 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).

$\beta 1$ integrins are believed to play a crucial role in bone formation as $\beta 1$ integrins are highly expressed in osteoblasts, osteoprogenitors and bone marrow stromal cells and are required for osteogenesis *in vitro*. Blocking of multiple $\beta 1$ integrins such as $\alpha 2\beta 1$ (18–22), $\alpha 5\beta 1$ (23–25), $\alpha 1\beta 1$ (20, 22) and $\alpha 3\beta 1$ (24) in these cells severely impairs *in vitro* osteogenic differentiation and mineralization. $\beta 1$ integrins also regulate cell survival, as disruption of fibronectin- $\alpha 5\beta 1$ interactions induces apoptosis in differentiated osteoblasts (26). Because $\beta 1$ integrins are essential for embryonic development (27), $\beta 1$ 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 $\beta 1$ integrins play a critical role in bone formation, *in vivo* deletion or functional perturbation of $\beta 1$ integrins in mature osteoblasts have yielded sometimes contradictory results and relatively mild skeletal phenotypes. For example, mice with dominant negative $\beta 1$ integrin ($\beta 1$ -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 14–365 days old with the same Osteocalcin-specific $\beta 1$ -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 $\beta 1$ integrin perturbations were studied. A third study of female mutants with osteoblast-specific $\beta 1$ -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 $\beta 1$ integrin ablation in $\beta 1$ 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 $\beta 1$ integrin perturbation studies have primarily focused on the phenotypes of cartilaginous tissues and endochondrally-derived long bones such as the femur or tibia and have not demonstrated severe bone phenotypes. Raducanu *et al.* demonstrated that conditional Prx1 Cre-mediated $\beta 1$ 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 $\beta 1$ 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 $\beta 1$ integrins in skeletal development and growth, especially with respect to $\beta 1$ 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 $\beta 1$ integrins on skeletal phenotype, in this study we generated mice with conditional $\beta 1$ 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 $\beta 1$ 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 $\beta 1$ integrin floxed ($itg\beta 1^{fl/fl}$) 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). $Itg\beta 1^{fl/fl}$ mice were mated with TW2-Cre, OSX-Cre or OC-Cre mice. Mice with $\beta 1$ integrin deletions under the respective promoters: $itg\beta 1^{fl/fl}$;TW2-Cre, $itg\beta 1^{fl/fl}$;OSX-Cre and $itg\beta 1^{fl/fl}$;OC-Cre were generated. In all studies, these conditional knockout mice were compared with littermates with a control genotype $itg\beta 1^{fl/fl}$ or $itg\beta 1^{fl/+}$ or with heterozygous conditional knockout genotype, $itg\beta 1^{fl/+}$;TW2-Cre, $itg\beta 1^{fl/+}$;OSX-Cre and $itg\beta 1^{fl/+}$;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 μ CT analysis and histological analysis.

μ CT analysis on E19.5 embryos

E19.5 embryos were imaged using a μ CT40 (46) using an X-ray intensity of 145 μ A, energy of 55 kVp, integration time of 200 ms, and resolution of 12 μ m. 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 $itg\beta 1^{fl/fl}$ E19.5 mouse embryo skeletons.

μ CT analysis on adult mouse calvariae and teeth

Male and female mice were anesthetized in an induction chamber filled with 4% isoflurane, maintained at 1.5% isoflurane and non-invasively imaged using a vivaCT40 (Scanco Medical) to determine calvarial and tooth structure. Scans were performed using an X-ray intensity of 145 μ A, energy of 55 kVp, integration time of 200 ms, and resolution of 30 μ m. The 2D greyscale tomograms were stacked and binarized by applying a Gaussian filter (sigma=1, support =1) using a threshold value of 450 mg HA/ccm to generate 3D reconstructions.

μ CT analysis and biomechanical testing on intact femurs

To test the effect of $\beta 1$ integrin deletion on bone development, female mouse femurs were harvested and stored at -80°C until ready for testing. The frozen femur specimens were thawed and imaged in PBS using a μ CT40 (Scanco Medical) to determine femur structure. The mid diaphysis and distal femur were imaged with an X-ray intensity of 145 μ A, energy of 55 kVp, integration time of 200 ms, and resolution of 12 μ m. The 2D tomograms for the mid diaphysis were stacked and binarized by applying a Gaussian filter (sigma = 0.8, support = 1) and a threshold value of 450 mg HA/ccm. An auto-contour algorithm (47) was applied to the μ CT scans of the distal femur, and the epiphysis and metaphysis were separately evaluated using threshold values of 450 and 540 mg HA/ccm, respectively. The 3D μ CT data were used to determine the moment of inertia of the femur mid diaphysis, which was combined with biomechanical testing data to calculate bone material property values. Mechanical analysis of the femurs was carried out by 3 point bending testing as previously described (48). Briefly, the femurs were loaded with the condyles facing up onto bending fixtures with lower span lengths of 6.2 mm. The upper fixture was aligned with the mid diaphysis which was imaged and analyzed by μ CT. The femora were loaded to failure at a rate of 0.5 mm/s at the mid diaphysis using the 858 Mini Bionix II testing system (MTS).

Femur fracture healing model

A unilateral femur fracture model was used to study fracture healing in 10–13 week old $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ littermate female mice. Mice were anesthetized under isoflurane, all hair was removed from the left hindlimb, and the skin was swabbed with alcohol and cycloheximide. A medial incision was then made along the femur and the underlying muscle was blunt dissected to expose the femur. The patella was dislocated to expose the condyles. To stabilize the fracture, a hole was made in the exposed condyles using a 25G needle, and a pre-cut 25G needle shaft was inserted into the intramedullary canal. A custom-made 3-point bending device was used to create a transverse fracture in the femur mid diaphysis. The patella was then repositioned over the condyles, the muscle was sutured and the skin incision was closed with wound clips (48).

Radiography, μ CT analysis and biomechanical testing of fracture calluses

At 2 and 5 weeks post-bone fracture, female mice were radiographically imaged using the MX-20 Radiography System (Faxitron Imaging) using an X-ray beam energy setting of 23kV and scan time of 15 seconds. Fractured femurs were harvested post-euthanasia from mice at 2 and 5 week. After harvest, the intramedullary pins were carefully removed from the fractured femurs and the fracture calluses were wrapped in PBS-soaked gauze and stored at -20°C . The fracture calluses were thawed under running water and μ CT imaged using a μ CT40 (Scanco Medical) with an X-ray intensity of 145 μA , energy of 55 kVp, integration time of 200 ms, and resolution of 12 μm . The 3D reconstructions were generated from 2D greyscale tomograms by applying a Gaussian filter ($\sigma = 0.8$, support = 1) and a global threshold value equivalent to 50% of the mineral density threshold for native intact cortical bone. Three-point bending testing on fracture calluses was performed using the same methods as described above for intact femurs. The calluses were loaded to failure at a rate of 0.5 mm/s at the mid diaphysis using the 858 Mini Bionix II testing system (MTS).

Histology

Female mouse bone samples were fixed in 10% neutral buffered formalin overnight, decalcified in 10% EDTA solution and transferred to 70% ethanol for storage at 4°C . Fracture callus samples were processed for embedding in Immunobed resin and sectioned to 2 μm thickness. Other bone samples were embedded in paraffin and sectioned at 5 μm thickness. Sections were deplasticized and stained with Safranin O/Fast Green to visualize cartilage or with Picrosirius Red to visualize collagen fibers.

Statistics

Statistical analyses were performed in Graphpad Prism. Comparisons between two groups were made using the t-test. Comparisons between 3 groups were made using one-way ANOVA with post-hoc Tukey. A p-value of less than 0.05 was considered significant.

Results

Deletion of $\beta 1$ integrins in osteochondro-progenitor cells results in embryonic lethality and severe growth and skeletal mineralization defects in $itg\beta 1^{fl/fl};TW2\text{-Cre}$ embryos

Heterozygous knockout ($itg\beta 1^{fl/+};TW2\text{-Cre}$) mice were mated with $\beta 1$ integrin floxed $itg\beta 1^{fl/fl}$ mice and eighty six mice produced from these matings were genotyped and analyzed. The intercrosses generated live $itg\beta 1^{fl/+}$, $itg\beta 1^{fl/fl}$ and heterozygous $itg\beta 1^{fl/+};TW2\text{-Cre}$ offspring in numbers close to the Mendelian ratio of 1:1:1 but no (0%) live knockout $itg\beta 1^{fl/fl};TW2\text{-Cre}$ mice (p-value < 0.0001 by Chi-square test (Table S1)), indicating that the $itg\beta 1^{fl/fl};TW2\text{-Cre}$ phenotype is lethal. Among the viable offspring, $itg\beta 1^{fl/fl}$ and heterozygous $itg\beta 1^{fl/+};TW2\text{-Cre}$ mice were indistinguishable from each other in terms of gross appearance, size or skeletal structure (Figure 1A). E11.5 and E13.5 $itg\beta 1^{fl/fl};TW2\text{-Cre}$ embryos harvested from timed matings between $itg\beta 1^{fl/+};TW2\text{-Cre}$ and $itg\beta 1^{fl/fl}$ mice were also indistinguishable from $itg\beta 1^{fl/fl}$ and heterozygous littermates (Figure S1). However, at E19.5, $itg\beta 1^{fl/fl};TW2\text{-Cre}$ embryos were much smaller ($< 70\%$ length) than $itg\beta 1^{fl/fl}$ littermates (Figure 1B), and displayed gross morphological abnormalities including large

abdominal growths (Figure 1B, $itg\beta 1^{fl/fl};TW2-Cre$ 1 and $itg\beta 1^{fl/fl};TW2-Cre$ 2) as well as cranial hemorrhaging (Figure 1B, $itg\beta 1^{fl/fl};TW2-Cre$ 3). μ CT analysis of E19.5 embryos revealed considerable skeletal mineralization in $itg\beta 1^{fl/fl}$ and heterozygous $itg\beta 1^{fl/+};TW2-Cre$ embryos, but severely reduced mineralization and impaired development in the skulls and vertebrae (Figure 1B, $itg\beta 1^{fl/fl};TW2-Cre$ 1 and $itg\beta 1^{fl/fl};TW2-Cre$ 3), or a complete lack of skeletal mineralization ($itg\beta 1^{fl/fl};TW2-Cre$ 2) in $itg\beta 1^{fl/fl};TW2-Cre$ embryos. Even in $itg\beta 1^{fl/fl};TW2-Cre$ embryos with mineralized long bones such as the femur, these limb bones were much shorter in comparison to $itg\beta 1^{fl/fl}$ bones (Figure 1B, $itg\beta 1^{fl/fl};TW2-Cre$ 1 and $itg\beta 1^{fl/fl};TW2-Cre$ 3). Although the observed growth and mineralization phenotypes in $itg\beta 1^{fl/fl};TW2-Cre$ mice were severe where they occurred, these phenotypes were not observed in all E19.5 $itg\beta 1^{fl/fl};TW2-Cre$ embryos (Table S2). It is noteworthy that all the $itg\beta 1^{fl/fl};TW2-Cre$ embryos appeared to have umbilical cord or vascular defects (Figure S2A), which may be the cause of embryonic lethality, although further analyses are required to fully establish if that is the case. Histology revealed a lack of Safranin O staining and hypertrophic chondrocytes in some vertebral skeletal elements of $itg\beta 1^{fl/fl};TW2-Cre$ embryos, indicating that impaired chondrocyte development may contribute to the defect in ossification (Figure S2B). Unfortunately, the lethality of the $itg\beta 1^{fl/fl};TW2-Cre$ phenotype precluded further analysis of postnatal skeletal development.

$itg\beta 1^{fl/fl};OSX-Cre$ mice are viable and fertile but display early and transient growth impairment as well as defects of the calvaria, femur and incisors

Heterozygous $itg\beta 1^{fl/+};OSX-Cre$ mice were mated with $\beta 1$ integrin floxed ($itg\beta 1^{fl/fl}$) mice to generate $itg\beta 1^{fl/fl};OSX-Cre$ conditional knockout offspring. This intercross gave rise to offspring in numbers which were approximately equal to the expected Mendelian ratios (Table S3). Although newborn pups appeared indistinguishable from each other, by the 21 day old weaning age, some animals in each litter were noticeably smaller than their littermates. After genotyping, we found that the growth rate of both male and female $itg\beta 1^{fl/fl};OSX-Cre$ mice was decreased at 2 weeks but began to increase and approach the growth rate of $itg\beta 1^{fl/fl}$ littermates at approximately 3 weeks (Figure 2A and B). As a result, $itg\beta 1^{fl/fl};OSX-Cre$ mice were smaller and weighed approximately 25–35% less than their littermates starting from 3 weeks old (Figure 2A and B). While it has been reported that young $OSX-Cre$ mice exhibit growth delays that were resolved at 12 weeks of age (50), we note that heterozygous $itg\beta 1^{fl/+};OSX-Cre$ littermates of $itg\beta 1^{fl/fl};OSX-Cre$ mice had normal weights (Figure 2A), demonstrating that the growth defect observed is a result of $\beta 1$ deficiency and not an effect of the $OSX-Cre$ transgene expression. The weight reduction in $itg\beta 1^{fl/fl};OSX-Cre$ mice reached its peak between 4–5 weeks and gradually declined, although $itg\beta 1^{fl/fl};OSX-Cre$ mice still weighed less than $itg\beta 1^{fl/fl}$ littermates at 9 weeks (Figure 2A). Radiographic analysis of $itg\beta 1^{fl/fl};OSX-Cre$ mice did not reveal any gross skeletal deformities, and confirmed that $itg\beta 1^{fl/fl};OSX-Cre$ mice were proportionally smaller than their littermates (Figure 2B).

Besides impaired growth, $itg\beta 1^{fl/fl};OSX-Cre$ mice also displayed defects in craniofacial mineralization at 3 weeks (Figures 2 and 3) which also became less pronounced with age (Figures 2 and 3). Although the calvarial mineralization and incisor length was normal in 11 day old $itg\beta 1^{fl/fl};OSX-Cre$ mice (Figure S3), maxillary and mandibular incisors in

itg β 1^{fl/fl};OSX-Cre mice were reduced in length at 3 weeks and displayed abnormal curvature and shape (Figure 2C). The severity of this incisor phenotype lessened with age but was still present at 8 weeks. Histological analysis 12 weeks old revealed uneven staining which may be indicative of odontoblast dysfunction in itg β 1^{fl/fl};OSX-Cre incisors (Figure 3B(i)).

To determine if the reduced weight in itg β 1^{fl/fl};OSX-Cre mice (Figure 2A) was due to a nutritional deficiency caused by incisor defects and difficulty for the mice to chew hard food pellets, we fed some litters of mice soft food. Even within litters that were provided with a soft diet, itg β 1^{fl/fl};OSX-Cre mice exhibited decreased weight compared to their littermates (data not shown), suggesting that non-nutritional factors contribute to the growth defect.

Itg β 1^{fl/fl};OSX-Cre mice displayed retarded mineralization in at 3 and 5 weeks, especially in the frontal and parietal bones, and the severity of this defect gradually decreased with age (Figure 3A). Itg β 1^{fl/fl};OSX-Cre calvariae appeared to be almost fully mineralized by 25 weeks although the skull size still remained smaller than itg β 1^{fl/fl} littermates. As expected, OSX-Cre control mice displayed normal calvarial mineralization and size (Figure 3A), indicating that the calvarial ossification and growth impairments were due to β 1 integrin deletion. Mineralization levels in heterozygous itg β 1^{fl/+};OSX-Cre mice appeared to be intermediate between itg β 1^{fl/fl} and itg β 1^{fl/fl};OSX-Cre mice (Figure 3A). Besides reduced mineralization in the bony plates of calvarial bones, itg β 1^{fl/fl};OSX-Cre mice also displayed delayed suture closure (Figure 3B(ii)) at 12 weeks old.

itg β 1^{fl/fl};OSX-Cre femurs display decreased cortical thickness and trabecular bone and have impaired bone density, structure and biomechanical properties

itg β 1^{fl/fl};OSX-Cre femurs analyzed by μ CT and histology displayed a reduction in cortical thickness at 8 weeks, although the cortical thickness defect appeared to improve at 12 weeks (Figure 4A, B and C). As Osterix is expressed in pre-osteoblast as well as pre-hypertrophic and hypertrophic chondrocyte lineage cells, we examined the femur growth plate. TRAP stained femur sections did not reveal any gross abnormalities in growth plate cartilage organization or in osteoclast number (Figure 4A), suggesting that the reduction in cortical thickness is osteo-lineage mediated. μ CT analyses further revealed significantly decreased cortical mineral density, bone volume and moment of inertia (Figure 4B and C). Three-point bending testing indicated inferior biomechanical properties in itg β 1^{fl/fl};OSX-Cre femurs as the maximum load, stiffness and work to failure were reduced by 36%, 43% and 64% respectively, although femur ultimate stress remained unchanged (Figure 4D).

β 1 integrin deletion in mature osteoblasts/osteocytes induces alterations to femur structure but does not affect bone density, biomechanics, fracture healing or *in vitro* mineralization of mouse BMSCs

β 1 integrin floxed (itg β 1^{fl/fl}) mice were mated with itg β 1^{fl/+};OC-Cre mice to generate offspring with Osteocalcin-driven β 1 integrin deletion (itg β 1^{fl/fl};OC-Cre). Offspring generated from these matings were viable, did not display gross abnormalities, and their genotype distributions conformed to the expected Mendelian ratios. OC-Cre lineage-tracing using the Rosa26 reporter confirmed that lineage-labeled cells were restricted to bone tissue, specifically in osteocytes and the periosteum (Figure S4). To assess the effect of β 1 integrin

deletion in osteoblasts and osteocytes on bone development and healing, we analyzed the structure of intact femurs in male 10-week old $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ littermates by μCT analysis. $itg\beta 1^{fl/fl};OC-Cre$ femurs had thicker cortices (Figure S5) with higher bone volumes but no change in bone density (Figure 5A and B). In addition, $itg\beta 1^{fl/fl};OC-Cre$ epiphyses displayed increased connectivity density, trabecular thickness (Figure 5A and B) and trabecular number. In contrast, there was reduced connectivity density and higher trabecular thickness in the metaphyses of animals with osteoblast-specific $\beta 1$ integrin deletion (Figure 5A and B). Despite this observed difference in bone structure, there were no changes in the biomechanical properties of $itg\beta 1^{fl/fl};OC-Cre$ femurs as measured by 3-point bending (Figure 5C). Ultimate stress, maximum load and work to failure values were not significantly different between $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ femurs. In order to examine the effect of $\beta 1$ integrin deletion on fracture healing potential, we created mid-diaphyseal femur fractures in $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ mice and evaluated their healing over 5 weeks. At 2 weeks post-fracture, both $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ mice displayed callus formation (Figure 5D). Five weeks after fracture, the calluses for both genotypes remodeled, and no gross structural differences could be observed in 3D μCT reconstructions (Figure 5D). μCT measures such as total callus bone volume and mineral density at 5 weeks post fracture were not different between $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ mice (Figure 5E). Three-point bending biomechanical testing on the fracture calluses at 5 weeks post-surgery also showed no differences in maximum load or stiffness (Figure 5F). Bone marrow stromal cells harvested from $itg\beta 1^{fl/fl}$ and $itg\beta 1^{fl/fl};OC-Cre$ mice exhibited no differences in alkaline phosphatase (ALP) activity at 14 days after culture in osteogenic media or in mineralization at 21 days after osteogenic induction (Figure 5G). These results indicate slight alterations to femur structure but no impairment of bone development, fracture healing, or osteoblastic mineralization in $itg\beta 1^{fl/fl};OC-Cre$ mice.

Discussion

The role played by $\beta 1$ integrins in skeletal progenitor and osteolineage cells on skeletal development is poorly understood. In this study, we focused on the skeletal phenotypes of transgenic mice with conditional $\beta 1$ integrin loss using Twist2 Cre, Osterix Cre and Osteocalcin Cre lines to generate conditional $\beta 1$ integrin deletions, where cre is expressed primarily but not exclusively in mesenchymal condensations, pre-osteoblasts, and mature osteoblasts respectively. Analysis of the skeletal phenotype in these mutants revealed reduced growth and dysfunctional skeletal development in E19.5 $itg\beta 1^{fl/fl};TW2-Cre$ embryos, an impairment in the calvarial and femoral mineralization, growth and incisor eruption of young $itg\beta 1^{fl/fl};OSX-Cre$ mice which lessened in severity as they aged, and minor structural changes in $itg\beta 1^{fl/fl};OC-Cre$ femurs with normal ossification, biomechanics and fracture healing.

We observed a lack of Safranin O staining in the under-ossified vertebrae of an $itg\beta 1^{fl/fl};TW2-Cre$ embryo, which suggests that the impaired $itg\beta 1^{fl/fl};TW2-Cre$ skeletal mineralization may be due to inhibited chondrocyte differentiation or function. The embryonic lethality of $itg\beta 1^{fl/fl};TW2-Cre$ mice is probably unrelated to bone development but it unfortunately prevented further investigation of $itg\beta 1^{fl/fl};TW2-Cre$ skeletons after birth. A previous study of $\beta 1$ integrin deletion in mesenchymal condensations of the limb

bud in $itg\beta 1^{fl/fl};Prx1$ -Cre mutants (51) yielded viable mice with disorganized articular cartilage, delayed primary and secondary ossification in the femur and short limbed dwarfism by 16 months of age, but no reported defects in the calvarial or vertebral skeletal elements (35). A major difference between the $Prx1$ -Cre (51) and TW2-Cre lines is that $Prx1$ is restricted to the limb bud mesenchyme but not the early E14.5 cranium or facial bone tissue (52), although $Prx1$ -Cre expression in a different $Prx1$ CreER-GFP line (53) has been reported at the calvarial sutures. In contrast, TW2-Cre is expressed in the pre-osteogenic cranial mesenchyme and also in the dermis (7, 38), and these differences in Cre expression pattern may account for the phenotypic differences observed. However, since the prenatal skeletal phenotype of $itg\beta 1^{fl/fl};Prx1$ -Cre mice has not been described, analysis of $itg\beta 1^{fl/fl};Prx1$ -Cre embryos or $itg\beta 1^{fl/fl};Sox9$ -Cre mutants which have not been generated, where $Sox9$ -Cre is expressed in neural crest cells (54) and mesenchymal condensations in regions of future endochondral bone formation, may further elucidate the role of $\beta 1$ integrin on skeletal formation during development in the limbs or in all skeletal elements, respectively.

Unlike the embryonically lethal $itg\beta 1^{fl/fl};TW2$ -Cre phenotype, $itg\beta 1^{fl/fl};OSX$ -Cre mice were viable and indistinguishable from $itg\beta 1^{fl/fl}$ littermates at birth but demonstrated early defects in incisor length, growth, calvarial and femoral mineralization, and femur biomechanics, which improved with age. $Itg\beta 1^{fl/fl};OSX$ -Cre incisors displayed uneven toluidine blue staining, a possible indicator of odontoblast dysfunction. Because Osterix expression occurs in odontoblasts (45) we speculate that the impairment of incisor eruption was odontoblast-mediated. Reduced growth in young $itg\beta 1^{fl/fl};OSX$ -Cre mice may be partially due to nutritional defects which occurred as a result of the short incisors. However, $itg\beta 1^{fl/fl};OSX$ -Cre mice were smaller even when fed soft food, suggesting that non-nutritional factors contribute to this phenotype as well.

The expression of Osterix in pre-hypertrophic chondrocytes, hypertrophic chondrocytes and preosteoblast lineage cells raises the possibility that the bone defects are due to dysfunction in chondrocytes, osteoblast precursors or osteoblasts. We speculate that the bone defects are due to osteolineage cell function for several reasons. Firstly, calvarial bones are formed by intramembranous ossification without the intermediate formation of a cartilage anlage; therefore the calvarial phenotype is unlikely to be chondrocyte-mediated. Secondly, mice expressing a dominant-negative $\beta 1$ integrin under the osteolineage-specific Osteocalcin promoter also display transient calvarial hypomineralization (29), which is consistent with the idea that the hypomineralized $itg\beta 1^{fl/fl};OSX$ -Cre calvarial phenotype is osteoblast lineage-mediated. Thirdly, we saw no disruption of the columnar organization of chondrocytes in the femoral growth plate which has been reported in $itg\beta 1^{fl/fl};Col2a1$ -Cre mutants due primarily to proliferating chondrocyte dysfunction (37). Fourthly, there were no gross abnormalities in TRAP staining in $itg\beta 1^{fl/fl};OSX$ -Cre femurs, suggesting that the femur phenotype may not be osteoclast-mediated.

Interestingly, disruption of ICAP-1, a protein which binds to (55) and inhibits $\beta 1$ integrin function (56) also results in impaired calvarial development due to delayed suture closure (57), suggesting that a correct balance of $\beta 1$ integrin activity may be required for proper calvarial development and growth. Because $\beta 1$ integrins constitute a large sub-family of integrins, the contributions of specific $\beta 1$ integrins for the phenotypes reported in this study

remain to be elucidated. However, some aspects of the $itg\beta 1^{fl/fl};OSX-Cre$ phenotype described here have also been observed mutants with integrin alpha subunit deficiencies. For example, $\alpha 11$, $\alpha 2$, and $\alpha 2 + \alpha 11$ integrin knockout mice exhibit proportional dwarfism attributed to altered IGF-1/GH signaling due to diminished GHRH production in the hypothalamus (58) and $\alpha 11$ knockout mice have short incisors (59). Although the mechanism by which $\alpha 11$ and $\alpha 2$ integrins regulate the IGF-1/GH axis is unclear, in light of the emerging understanding of bone as an organ with diverse endocrine functions (60, 61) it is conceivable that osteolineage disruption of the $\alpha 11$ and $\alpha 2$ integrins may contribute to the reduced growth in $itg\beta 1^{fl/fl};OSX-Cre$ mice.

In contrast to $itg\beta 1^{fl/fl};OSX-Cre$ mice, $itg\beta 1^{fl/fl};OC-Cre$ mice displayed only mild phenotypes which included alterations to the femur structure but no change in femur biomechanics, bone mineral density or fracture healing. One possible explanation for these findings is that $\beta 1$ integrins may not be required for mature osteoblast function and other integrins may compensate for the loss of $\beta 1$ integrin signaling. Indeed, mild skeletal phenotypes were observed in some other mutants with osteoblast-specific gene perturbations such as $\beta 1$ -DN expression under an osteocalcin promoter or $Col1a1$ -specific $\beta 1$ integrin deletion (30, 33, 34). However, it should be noted that approximately 10% of osteoblasts and osteocytes do not undergo OC-Cre mediated excision of floxed genes (39). Therefore, an alternative possibility is that although $\beta 1$ integrins may have an important role in osteoblast function, this small population of $\beta 1$ integrin positive osteoblasts and osteocytes may effectively compensate for the remaining $\beta 1$ integrin null osteoblasts and osteocytes. We speculate that a subpopulation of $\beta 1$ integrin positive cells may persist in $itg\beta 1^{fl/fl};OC-Cre$ mice because mature osteoblasts are short-lived and non-proliferative cells *in vivo* (62) which may be regularly replaced from the osteoprogenitor cell pool and further because $\beta 1$ integrins can be efficiently recycled through the endosomes and reused (63). In contrast, $itg\beta 1^{fl/fl};OSX-Cre$ mice would likely have completely knocked down $\beta 1$ integrin expression in osteoblasts as osterix expression occurs before osteocalcin expression. This study highlights the need for further investigation of the cell-ECM interactions of $itg\beta 1^{fl/fl};OSX-Cre$ osteo- and chondro- lineage cells to determine the tissue-specific role of $\beta 1$ integrins on cell adhesion, migration and integrin signaling to understand the mechanisms by which skeletal phenotype is altered in $itg\beta 1^{fl/fl};OSX-Cre$ mice.

In summary, we have shown that $\beta 1$ integrins expressed in mesenchymal condensations play an important role in embryonic skeletal development, and $\beta 1$ integrins in pre-osteoblast, pre-hypertrophic chondrocyte and odontoblast lineage cells contribute to the calvarial mineralization and femur development in young mice, but osteocalcin-specific $\beta 1$ integrin deletion had little effect on skeletal phenotype. In vitro characterization of skeletal cells with Osterix-specific $\beta 1$ integrin deletions will further our understanding of the role played by $\beta 1$ integrins in skeletal development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We deleted $\beta 1$ integrin (Itg $\beta 1$) in mice using Twist2, Osterix or Osteocalcin Cre lines
- Itg $\beta 1$ floxed-Twist2 Cre mice showed impaired embryonic skeletal development
- Itg $\beta 1$ floxed-Osterix Cre mice had defects in mineralization and mechanical properties
- Itg $\beta 1$ floxed-Osteocalcin mice displayed only minor femoral structural changes

Figure 1. $Itg\beta 1^{fl/fl};TW2-Cre$ mice have hypomineralized skeletons. (A) Radiographs indicate no gross skeletal differences between 10 week old female $Itg\beta 1^{fl/fl}$ and heterozygous conditional knockout $Itg\beta 1^{fl/+};TW2-Cre$ littermates. (B) (i) Stereoscopic images of E19.5 embryos. Labels for $Itg\beta 1^{fl/fl};TW2-Cre$ 1, 2 and 3 indicate 3 different embryos. Double white arrows indicate an abnormal large tissue mass in the abdominal region of conditional knockout $Itg\beta 1^{fl/+};TW2-Cre$ embryos 1 and 2, single white arrow indicates cranial hemorrhaging in $Itg\beta 1^{fl/+};TW2-Cre$ embryo 3. $Itg\beta 1^{fl/+};TW2-Cre$ embryos are also smaller than littermates.

(ii) and (iii) μ CT images of E19.5 embryos indicate reduced mineralization of $itg\beta 1^{fl/fl};TW2-Cre$ embryo skeleton. Scale bars indicate (i) 0.5 cm, (ii) 0.4 cm and (iii) 0.4 cm.

Figure 2. $Itg\beta 1^{fl/fl};OSX-Cre$ mice display impaired growth as well as craniofacial mineralization and incisor defects. (A) Weights of (i) male and (ii) female $Itg\beta 1^{fl/fl};OSX-Cre$ mice diverge significantly from their littermates between 24–27 days and reduced weight in $Itg\beta 1^{fl/fl};OSX-Cre$ mice continue into adulthood (n=4–5). * $p < 0.05$ compared to $Itg\beta 1^{fl/fl}$ and heterozygous conditional knockout $Itg\beta 1^{fl/+};OSX-Cre$ mice, # $p < 0.05$ compared to $Itg\beta 1^{fl/fl}$ by ANOVA with post-hoc Tukey test. (B) Radiographs of 5 week female littermates indicate that $Itg\beta 1^{fl/fl};OSX-Cre$ mice are proportionally smaller than their littermates and do

not display any gross skeletal deformities. Inset, dorsal view of $itg\beta 1^{fl/fl};OSX-Cre$ and $itg\beta 1^{fl/fl}$ female littermates. Scale bar 1 cm. (C) Frontal view of $itg\beta 1^{fl/fl};OSX-Cre$ and $itg\beta 1^{fl/fl}$ female littermate incisors at 3 and 5 weeks old. (D) Representative 3D μCT reconstruction displaying lateral and frontal views of female littermate skulls and incisors indicate that $itg\beta 1^{fl/fl};OSX-Cre$ mice have short and misaligned maxillary (yellow arrows) and mandibular (white arrows) incisors, scale bar 2 mm (n=5–6). The abnormal spacing between $itg\beta 1^{fl/fl};OSX-Cre$ mandibular incisors is indicated with a red asterix.

Figure 3.

(A) 3D μ CT reconstructions of dorsal view of calvaria and underlying skull base indicate significantly reduced calvarial mineralization in conditional knockout female *Itgβ1^{fl/fl};OSX-Cre* mice and an intermediate phenotype in heterozygous conditional knockout *Itgβ1^{fl/+};OSX-Cre* mice in comparison with *Itgβ1^{fl/fl}* littermates ($n=5-6$). Scale bar 2 mm. F- frontal bone, P – parietal bone, IP – interparietal bone, IF – interfrontal suture, C- coronal suture, S – sagittal suture, L – lambdoid suture. (B) Histology of cranial tissue in female mice reveals (i) uneven toluidine blue staining (asterisk) in *Itgβ1^{fl/fl};OSX-Cre* incisors and

(ii) delayed closure (black arrow) of the interfrontal suture in $itg\beta 1^{fl/fl};OSX-Cre$ mice (n=1).
A – ameloblasts, ES – enamel space, OB – odontoblasts, D – dentin. Scale bar 200 μ m.

Figure 4.

$\beta 1$ integrin deficiency in female $itg\beta 1^{fl/fl};OSX-Cre$ mice results in altered femur structure, reduced bone density and strength. (A) TRAP stained femur sections do not indicate gross abnormalities in osteoclast number or growth plate organization in conditional knockout $itg\beta 1^{fl/fl};OSX-Cre$ mice. Bottom panels are higher magnification images of the regions indicated with a box in the top panels. Scale bar 500 μm (top), 100 μm (bottom). (B) 3D μCT reconstructions femur cortex metaphysis and epiphysis at 12 weeks, scale bar 0.5mm. (C) μCT measures of cortical structure ($n=7-9$) in 12 week old femurs and (D)

Biomechanical testing measures by 3-point bending (n=7–9) of 12 week old femurs. *p < 0.05 and **p < 0.01 compared to $itg\beta 1^{fl/fl}$ by t-test.

Figure 5. $itg\beta1^{fl/fl};OC-Cre$ female mice have altered femur structure, but unchanged bone density, mineralization, biomechanical properties and fracture healing. (A) 3D μ CT reconstructions, (B) μ CT measures ($n=12-16$) and (C) biomechanical testing measures ($n=12-16$) for the femur cortex epiphysis, metaphysis and epiphysis at 12 weeks of conditional knockout $itg\beta1^{fl/fl};OC-Cre$ mice compared to $itg\beta1^{fl/fl}$ mice, * $p<0.05$, ** $p<0.01$ by t-test. Scale bar: (C) 500 μ m. (D) (i) 3D μ CT reconstructions, (i) Safranin O/fast green stained sections, (E) μ CT measures ($n=8-10$) and (F) biomechanical testing measures ($n=8-10$) of fractured

femur calluses indicate no differences in fracture healing. (G) *In vitro* ALP expression and mineralization (Alizarin red staining, ALZ) of BMSCs after 14 and 21 days of osteogenic induction respectively.