The Transcription Factor Hand1 Is Involved In Runx2-Ihh-Regulated Endochondral Ossification

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Abstract

The developing long bone is a model of endochondral ossification that displays the morphological layers of chondrocytes toward the ossification center of the diaphysis. Indian hedgehog (Ihh), a member of the hedgehog family of secreted molecules, regulates chondrocyte proliferation and differentiation, as well as osteoblast differentiation, through the process of endochondral ossification. Here, we report that the basic helix-loop-helix transcription factor Hand1, which is expressed in the cartilage primordia, is involved in proper osteogenesis of the bone collar via its control of Ihh production. Genetic overexpression of Hand1 in the osteochondral progenitors resulted in prenatal hypoplastic or aplastic ossification in the diaphyses, mimicking an Ihh loss-of-function phenotype. Ihh expression was downregulated in femur epiphyses of Hand1-overexpressing mice. We also confirmed that Hand1 downregulated Ihh gene expression in vitro by inhibiting Runx2 transactivation of the Ihh proximal promoter. These results demonstrate that Hand1 in chondrocytes regulates endochondral ossification, at least in part through the Runx2-Ihh axis.

Introduction

The vertebrate skeleton develops through two tightly controlled processes: intramembranous ossification and endochondral ossification. Intramembranous ossification is the direct differentiation of condensed mesenchymal cells into the osteoblasts of bone tissue. Endochondral ossification is characterized by condensation of mesenchymal cells to produce a cartilage primordium surrounded by the perichondrium, consisting of prechondroblasts, osteoblasts, and fibroblasts [1,2]. The long bones, developed by endochondral ossification, consist of two cartilaginous epiphyses connected by a bony diaphysis. The process of ossification begins in two locations; the primary ossification is located in the center of the future diaphysis and the secondary ossification is located in the center of the epiphysis.
Indian hedgehog (Ihh) is a member of the hedgehog family of secreted molecules, which controls chondrocyte proliferation and differentiation as well as osteoblast differentiation. Ihh is detected in the chondrocytes of the early cartilage primordium [3]. Ihh−/− mice display severely shortened long bones, fused digits, delayed calcification, and a failure of cortical bone and bone collar formation [4]. Ectopic expression of Ihh in chondrocytes induces expression of Runx2 (Runt-related transcription factor 2), a master molecule for osteoblast differentiation, throughout the perichondrium that induces bone collar formation [5]. Temporary attenuation of Ihh activity decreased Runx2 expression and produced mice with shortened limbs, trunk and skull bones [6]. Deletion of Runx2 disables the expression of Ihh; however, the addition of Runx2 restores Ihh expression [2]. Thus, Runx2 positively regulates Ihh expression in chondrocytes, and, in turn, Ihh also positively regulates Runx2 expression in the perichondrium; disruption of the latter process results in impaired chondrocyte differentiation and osteoblastogenesis.

Basic helix-loop-helix (bHLH) transcription factors played the crucial roles during embryonic development. Hand1 and Hand2, highly conserved bHLH proteins, are expressed in the developing limb bud [7,8,9]. Genomic regions enriched in Hand2 chromatin complexes were identified in early limb buds [10]. In Hand2 transgenic mice, bones of the zeugopod, in both forelimbs and hindlimbs, were shortened and malformed [8]. However, little is known about the role of Hand1 and Hand2 in the development of the endochondral bones. Here, we demonstrate that Hand1-overexpressing mice show aplastic or hypoplastic ossification in the long bones, partially mimicking the bone phenotype observed in Ihh−/− mice. Hand1 inhibits Ihh expression by suppressing Runx2 transactivation of the Ihh promoter. Our data indicate that Hand1 acts as a negative regulator of endochondral ossification.

**Materials and Methods**

**Hand1 conditionally-overexpressing mice**

The transgene vector CAG-lox-CAT-lox-Hand1 was constructed by inserting a Hand1 cDNA into the CAG-CAT-(cDNA insert)-poly(A) cassette to generate a transgenic line, CAG-CAT Hand1Tg/+ (Stock No. RBRC01369, RIKEN). For conditional activation of Hand1, Twist2-Cre knock-in males [11] were crossed with CAG-CAT Hand1Tg/+ females. Rosa26 Reporter (R26R) (Stock No. 6148, The Jackson Laboratory) mice have been described previously [12].

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Tokyo Medical and Dental University (Permit Number: 0160215A). All efforts were taken to minimize pain experienced by the mice. Animals were housed with no more than 4 per cage. All animals were maintained in an HEPA (high-efficiency particulate arrestance)-filtered rack with a 12-hour light/dark cycle. Animals were fed an autoclaved laboratory rodent diet. Animals were sacrificed by carbon dioxide inhalation.

**Bone staining**

Skeletal preparations were stained using alcian blue for cartilage and alizarin red for ossified bones, as described previously [13].

**Histology and immunohistochemistry**

Bone samples were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin, as previously described [14]. To unmask antigens, tissue sections were boiled in 10 mM citrate
buffer [for phospho-Histone H3, and Spp1 (Secreted phosphoprotein 1)], or incubated in 700 U/mL proteinase K solution (Nacalai Tesque) (for Sox9) at 37°C for 5 minutes, or treated with 1mg/mL hyaluronidase (Sigma-Aldrich) at 37°C (for Runx2). Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector) and Immpact DAB peroxidase substrate (Vector). Sections were counterstained with Hematoxylin QS (Vector) or Methyl Green Nuclear Counterstain (Vector). The following primary antibodies were used: anti-Spp1 antibody (RB9097-PO; Thermo Scientific), anti-Ihh antibody (sc-1196; Santa Cruz), anti-RUNX2/CBFA1 antibody (PA1224; Boster Biological), anti-HAND1 antibody (GTX11846; GeneTex), anti-SOX9 antibody (AB5535; Millipore), and anti-phospho-Histone H3 (Ser10) antibody (06–570; Millipore).

For alcian blue staining, sections were treated with 3% acetic acid, stained with 1.5 mg/mL alcian blue 8GX in 3% acetic acid solution, and then counterstained with Eosin Y (Sigma-Aldrich). For von Kossa staining, sections were incubated in 1% silver nitrate overnight under UV light, then incubated in 5% sodium thiosulfate, and counterstained with Eosin Y.

Real-time quantitative PCR (qPCR)

Total RNA was extracted from tissue or cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche). Real-time quantitative PCR (qPCR) was performed using the LightCycler FastStart DNA MasterPLUS SYBR Green 1 kit (Roche). Amplification of single products was confirmed by monitoring dissociation curves. All data were normalized to Ppia (peptidylprolyl isomerase A) expression.

Primer sequences used for amplification were as follows: Hand1 forward: 5′-CTTTAATCTCTTTCGCGCAG-3′, Hand1 reverse: 5′-CAAGGATGCACAAGCAGGT-3′; Ppia forward: 5′-CGCGTCTCCTTCGAGCTGTTTG-3′, Ppia reverse: 5′-TGTAAAGTCACCACCTGGCACAT-3′; Runx2 forward: 5′-GCTCACGTCGCTCATCTTG-3′, Runx2 reverse: 5′-TATGGCGTCAAACAGCCTCT-3′; and Ihh forward: 5′-TGACAGAGATGCCAGCTGAG-3′, Ihh reverse: 5′-AGAGCTCACCCCCAACTACA-3′.

Luciferase assay and stable cell lines

ATDC5 (RIKEN) and COS1 (RIKEN) cells were grown in Dulbecco’s Minimal Essential Medium (DMEM)/Ham’s F12 or DMEM high glucose media respectively, supplemented with 10% fetal bovine serum. Luciferase assays were performed as previously described [15], plhhluc [2], expression vectors for Hand1, Hand2, Tcf15 [16], Runx2 [17], Id1 [18] and Tcf21 [19] were described previously.

ATDC5 cells were transfected with myc-Hand1 or myc-pcDNA3.1 using FuGENE 6 (Roche). For stable transfections, cells were selected using 200 μg/ml neomycin (Sigma-Aldrich) and individual clones were amplified prior to analysis. Immunostaining and qPCR verified the presence of the transgene.

Statistical analysis

All experiments were performed independently with a minimum of three replicates. Data were analyzed using an unpaired Student’s t-test and expressed as the mean ± standard deviation (SD). P-values less than 0.05 were considered significant for all experiments; asterisks denote significance.
Results and Discussion

Overexpression of Hand1 causes developmental defects in the limbs

To investigate the role of Hand1 in the development of the endochondral bones, conditional Hand1 transgenic mice (Hand1^{Tg+}; Twist2-Cre), whose Hand1 overexpression is driven by the Twist2 promoter in the osteochondral progenitors, were generated. During endochondral ossification, Twist2 promoter-driven Cre expression is detected in the chondrocytes of the growth plate cartilage and the osteoblasts in the perichondrium, periosteum, and endosteum [11]. Hand1^{Tg+}; Twist2-Cre mutants were slightly dwarfed at postnatal day 1 (P1) (Fig 1A and S1 Fig). All Hand1 mutants displayed preaxial polydactyly in the autopod (Fig 1A and S1 Table, n = 54). By P21, Hand1 mutants were severely dwarfed (Fig 1B), and only 33% (n = 18/54) grew to adulthood. Bone staining showed hypoplastic ossification of the zeugopod; malformed, duplicated or malarticulated radii; and mirror-image duplication of digits in Hand1 mutant forelimbs (Fig 1C and S1 Table). In Hand1 mutant hindlimbs, aplastic ossification of tibiae, “C”-shaped fibulae, and distal phalangeal duplications were noted (Fig 1C and S1 Table). In addition, incomplete fusion of the xiphoid process and the hypoplastic supraoccipital bone were observed in the endochondral bones of Hand1 mutants (S1 Fig). A range of malformations in endochondral ossification was already present as early as E16.5 (Fig 1D and S1 Fig). These findings suggest that Hand1 overexpression may interfere with the commitment of limb mesenchyme cells to the cartilage fate and/or control the development of endochondral ossification.

To further investigate the role of the closely related bHLH protein Hand2 in limb development, we examined the skeletal phenotype of Hand2^{Tg+}; Twist2-Cre mice. Hand2 mutants (100%, n = 10) were perinatal lethal, accompanied by skeletal abnormalities similar to those seen in Hand1 mutants (S2 Fig). Patients with 4q trisomy: dup(4)(q35.2-q31.22) manifest preaxial polydactyly [20]. The trisomic region contains HAND2, and overdosage of Hand2 is a major cause of the limb phenotypes of 4q trisomy [21]. Since Hand1-overexpressing mice have limb and skeletal phenotypes, the HAND1 coding region (5q33.2) may be one of the candidate regions for preaxial defects and short stature in humans.

Hand1 mutants show delayed and hypoplastic ossification

Since malformation of long bones was observed in Hand1 and Hand2 mutants, we focused our attention on these bone elements. von Kossa staining revealed hypoplastic bone collars in the trabeculae of Hand1 mutant femurs at E16.5 (Fig 2A), whereas the growth plate showed no significant difference from wild-type (Wt) (S3 Fig). Expression of Sox9, an essential transcription factor in chondrocyte differentiation, and the number of dividing chondrocytes in the femoral epiphysis of Hand1 mutants was not significantly different from Wt at E16.5 (S3 Fig).

Delayed onset of secondary ossification was also observed in Hand1 mutants (Fig 2B). At P7, secondary ossification was observed in Wt femurs, whereas only the beginning of chondrocyte hypertrophy was visible in Hand1 mutant femurs (Fig 2B). In Wt, Spp1 (secreted phosphoprotein 1), also known as osteopontin, was expressed in mature osteoblasts in the region of the secondary ossification center, whereas Spp1 was not expressed in the cartilaginous epiphyses in Hand1 mutants (Fig 2B). At P21, femurs of Hand1 mutants showed the presence of secondary ossification (Fig 2B). These results suggest that Hand1 overexpression affects primary ossification at the prenatal stage of endochondral ossification and secondary ossification may also be controlled, at least in part, by Hand1.

Hand1, Runx2, and Ihh are expressed in the cartilage primordia

The above results suggested that the bone abnormalities in Hand1 mutants are induced by a primary defect in the regulation of endochondral ossification. To examine whether the Hand1
expression pattern is compatible with such a phenotype, immunostaining was performed in the cartilage primordium of the forelimb. At E12.5, Hand1 was strongly expressed in the distal and proximal parts of immature chondrocytes (Fig 3A). At E16.5, no detectable 
Hand1 signal in Wt femoral epiphyseal cartilage was demonstrated by qPCR, whereas 
Hand1 was overexpressed in mutant femurs (Fig 3B). These results suggest a potential role for Hand1 at the primordial stage of cartilage development.

The phenotypic abnormalities noted in long bone ossification in Hand1 mutants are partially reminiscent of those observed in Ihh−/− mice [4] and Runx2−/− mice [22], which exhibit delayed primary ossification and failure of bone collar formation. Given the expression of Hand1 in the cartilage primordia and the defective ossification seen in Hand1 mutants, we
examined the expression of Runx2 and Ihh in the cartilage primordium of the forelimb at E12.5. In contrast to Hand1 expression, Ihh was mainly expressed in the central part of the cartilage primordium (Fig 3A). Runx2 was expressed in the whole cartilage template, although more strongly in the centrally located chondrocytes (Fig 3A). Taken together, these results suggest that Runx2 is coexpressed with Ihh and Hand1 in the cartilage primordium, while the expression pattern of Ihh is opposite to that of Hand1.

Ihh expression is decreased in the epiphyseal cartilage of Hand1 mutants

To further analyze whether Hand1 genetically regulates the expression of Ihh contributing to endochondral ossification, we examined gene expression by qPCR of femoral epiphyseal cartilage. Expression of Ihh and Runx2 was significantly decreased in Hand1 mutant epiphyseal cartilage at E16.5 and P1 (Fig 3B and 3C). We also confirmed by immunohistochemistry that the Ihh-positive region was decreased in Hand1 mutant hypertrophic chondrocytes at E16.5 (Fig 3D). During endochondral ossification, osteoprogenitor cells in the perichondrium give rise to osteoblasts. Osteoblasts then enter the cavity via periosteal buds and deposit osteoid on the calcified matrix as a scaffold [1]. The immunohistochemistry analysis of Runx2 showed reduction in the number of Runx2-positive osteoblasts in the peristeum and the perichondrium of Hand1 mutant femurs (Fig 3D). Hand1 mutant femurs showed more fibrous periosteum and less cellular periosteum (Fig 3D). These results indicate that Hand1 affects Ihh and Runx2 expression in the early development of the cartilage. It is also possible that Hand1 is involved in the regulation of cell population expressing Runx2 in the periosteum.

Hand1 negatively regulates Ihh expression in vitro

The observation that the Ihh expression level was decreased in the epiphyseal cartilage of Hand1-overexpressing mice suggested that Hand1 could directly regulate Ihh expression. To
address this possibility, we examined the effect of Hand1 on Ihh expression in vitro using ATDC5, a chondroprogenitor cell line, stably transfected with a Hand1 expression vector. Hand1 overexpression was confirmed in Hand1-transfected ATDC5 cells (Fig 4A). Expression of Ihh and Runx2 in Hand1-overexpressing cells was significantly decreased (Fig 4A).

Runx2 directly binds and activates the Ihh promoter [2]. We have previously shown that Hand1 and Hand2 directly bind and inhibit Runx2 transactivation [15]. Because both Hand1 and Runx2 were expressed in the distal part of the immature chondrocytes of the cartilage primordium, and the expression pattern of Ihh was opposite to that of Hand1 (Fig 3A), it is possible that Hand1 negatively regulates Ihh expression through inhibited Runx2 activity. To address this possibility, we tested the effect of Hand1 on the transcriptional activity of the Ihh...
Fig 4. Hand1 inhibits the Ihh promoter through Runx2 transactivation. (A) qPCR analysis of Hand1 (a), Ihh (b), and Runx2 (c) transcripts in ATDC5 cells stably transfected with an empty vector (Control) or Hand1 expression vector. (B) Luciferase assays. COS1 cells were transiently cotransfected with pIhh-luc reporter and the indicated expression vectors. Luciferase data (b) is shown as a percentage of Runx2 activation (normalized to 1.0). The data represent the mean ± SD. (C) Model for transcriptional regulation of endochondral ossification by Hand1. Hand1 inhibits Runx2-dependent Ihh expression, which normally promotes Runx2 expression in the perichondrium and the periosteum, which, in turn, is required for osteoblast differentiation.

doi:10.1371/journal.pone.0150263.g004
Overexpression of Hand1 or Hand2 in COS1 cells does not affect the expression level of Runx2 [15]. Hand1 alone did not affect Ihh promoter activity; however, it inhibited Runx2-dependent activation of the Ihh promoter (Fig 4B). Transfection of a Hand2 showed similar results to Hand1 (Fig 4B). To ensure this mechanism was unique to Hand proteins, we tested other tissue-specific bHLH proteins Id1, Tcf15, and Tcf21 in this assay. None of these proteins inhibited Runx2 transactivation of the Ihh promoter (Fig 4B). Runx2 induces the differentiation of perichondrial cells via the Ihh-Gli pathway [6]. These results suggest that Hand1 inhibits Ihh expression in the cartilage and consequently decreases Runx2 expression in the perichondrium and the periosteum, where Runx2 is required for osteoblast differentiation (Fig 4C). Interestingly, misexpression of Hand2 induces shortened and malformed limb in the absence of direct DNA binding [8]. Indeed, Hand1 and Hand2 do not require direct DNA binding to inhibit Runx2 transactivation function [15].

In summary, our results indicate that Hand1 is involved in proper osteogenesis of the bone collar via its control of Ihh production. Genetic overexpression of Hand1 and Hand2 in the osteochondral progenitors resulted in prenatal hypoplastic or aplastic ossification in the diaphyses. Hand1 and Hand2 overexpressing mice could provide unique animal models for understanding the molecular basis of limb development.

Supporting Information
S1 Fig. Defective ossification in Hand1 mutants. (DOCX)
S2 Fig. Defective ossification in Hand2 mutants. (DOCX)
S3 Fig. Chondrocyte morphology in femoral epiphyseal cartilage. (DOCX)
S1 Table. Skeletal phenotypes observed in Hand1 mutant mice at P1. (DOCX)
S1 Text. Supplemental Experimental Procedures. (DOCX)

Acknowledgments
We are grateful to Toshihisa Komori, Eiji Hara, David Ornitz, Eric N. Olson, and Rhonda Bassel-Duby for reagents and mice. We thank Eriko Matsumoto and Mariko Mizuguchi for technical assistance.

Author Contributions
Conceived and designed the experiments: NF. Performed the experiments: LEL HK. Analyzed the data: YS MN. Contributed reagents/materials/analysis tools: YS MN NF. Wrote the paper: LEL NF.

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