

## ARTICLE

## Wnt7b can replace Ihh to induce hypertrophic cartilage vascularization but not osteoblast differentiation during endochondral bone development

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Indian hedgehog (Ihh) is an essential signal that regulates endochondral bone development. We have previously shown that Wnt7b promotes osteoblast differentiation during mouse embryogenesis, and that its expression in the perichondrium is dependent on Ihh signaling. To test the hypothesis that Wnt7b may mediate some aspects of Ihh function during endochondral bone development, we activated Wnt7b expression from the R26-Wnt7b allele with Col2-Cre in the *Ihh*<sup>-/-</sup> mouse. Artificial expression of Wnt7b rescued vascularization of the hypertrophic cartilage in the *Ihh*<sup>-/-</sup> mouse, but failed to restore orthotopic osteoblast differentiation in the perichondrium. Similarly, Wnt7b did not recover Ihh-dependent perichondral bone formation in the *Ihh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup> embryo. Interestingly, Wnt7b induced bone formation at the diaphyseal region of long bones in the absence of Ihh, possibly due to increased vascularization in the area. Thus, Ihh-dependent expression of Wnt7b in the perichondrium may contribute to vascularization of the hypertrophic cartilage during endochondral bone development.

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## INTRODUCTION

Indian hedgehog (Ihh) signaling is critical for endochondral bone development. Ihh is expressed by prehypertrophic and early hypertrophic chondrocytes, signaling to chondrocytes and perichondral cells.<sup>1-2</sup> The studies of *Ihh* knockout mice have demonstrated the importance of Ihh in chondrocyte proliferation and maturation, osteoblast differentiation and cartilage angiogenesis.<sup>2</sup> Other genetic studies with smoothed (*Smo*), an essential transducer of hedgehog signaling, showed that direct Ihh input in chondrocytes and osteoprogenitors is required for chondrocyte proliferation and osteoblast differentiation, respectively.<sup>3-4</sup> However, direct Ihh signaling in endothelial cells is not necessary for vascularization of the hypertrophic cartilage, indicating that Ihh controls cartilage angiogenesis likely through a secondary signal.<sup>4</sup>

Wnt proteins are a family of glycoproteins playing critical roles in many aspects of animal development. During embryonic skeletal development, Wnt/ $\beta$ -catenin

signaling is required for osteoblast differentiation, as demonstrated by genetic studies of both  $\beta$ -catenin and the Wnt coreceptors Lrp5/6.<sup>5-9</sup> During endochondral bone development, osteoblast differentiation first occurs within the perichondrium surrounding the prehypertrophic and hypertrophic cartilage in response to Ihh. We term this process Ihh-dependent orthotopic perichondral ossification. Although Wnt/ $\beta$ -catenin signaling has been shown to function downstream of Ihh during orthotopic perichondral ossification in the long bones,<sup>7-8</sup> it is not known whether Wnt signaling can replace Ihh in this process. We have previously shown that Wnt7b is not only expressed in the domain of orthotopic perichondral ossification, but also required to ensure the timely initiation of the process.<sup>7</sup> Interestingly, Wnt7b has also been shown by others to play important roles in proper vascularization of the central nervous system.<sup>10</sup> A potential role for Wnt signaling in skeletal vascularization has not been examined.

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Here we test the hypothesis that Wnt7b may mediate *Ihh* function in inducing orthotopic perichondral ossification and hypertrophic cartilage vascularization. To this end, we artificially expressed Wnt7b in the endochondral skeleton of *Ihh*<sup>-/-</sup> embryos. We provide evidence that Wnt7b is sufficient to induce vascularization of the hypertrophic cartilage in the absence of *Ihh*.

## MATERIALS AND METHODS

### Mouse strains

*Ihh*<sup>+/-</sup>, *Gli3*<sup>+/-</sup>, *Col2-Cre3* and *R26-Wnt7b* mouse strains were previously described.<sup>2-3,11-12</sup> All animal studies were approved by Washington University Animal Studies Committee.

### Analyses of mouse embryos

For whole mount skeletal staining, we modified the McLeod method.<sup>13</sup> Briefly, mouse embryos were isolated at E18.5. After removing skin, the embryos were fixed with 95% ethanol overnight and incubated in acetone overnight. Next, embryos were stained with staining solution (0.03% Alcian blue, 0.005% Alizarin Red S type, 10% glacial acetic acid, and 70% Ethanol) for 2–3 days at room temperature. Stained embryos were incubated with 95% ethanol 24 h, and then, cleared with 1% KOH. Finally, clear skeleton were stored in glycerol solution (8 part glycerol: 2 part 1% KOH). For histological and molecular analyses, embryonic limbs were harvested in cold PBS and fixed in 10% buffered formalin overnight at room temperature. Subsequently, the fixed limbs were decalcified with 14% EDTA/PBS (pH 7.4) for 3 days. Finally, the limbs were processed and embedded in paraffin for sectioning (6 μm thickness). Limb sections were stained with hematoxylin and eosine (H&E) for basic histological analysis. Alcian blue and picro-sirius red<sup>14</sup> staining were performed to distinguish cartilage and bone matrix on sections. Radioactive *in situ* hybridization using <sup>35</sup>S-labeled riboprobes was performed as previously described.<sup>3-4,7,15</sup>

## RESULTS

### Wnt7b expression in perichondrium requires *Ihh*

Previously, in E14.5 mouse embryos, we have shown that Wnt7b is expressed by perichondral cells flanking the prehypertrophic and early hypertrophic cartilage, and that the expression is critically dependent on *Ihh* signaling. To corroborate this finding, we performed *in situ* hybridization on long bone sections from mouse embryos of later stages. At E15.5, Wnt7b was normally expressed most robustly in the perichondrium flanking the prehypertrophic and early hypertrophic chondrocytes, but also at a lower level in the perichondrium/periosteum flanking the emerging marrow cavity (Figure 1a1 and 1a2). Interestingly, at E18.5, Wnt7b expression was more restricted to the

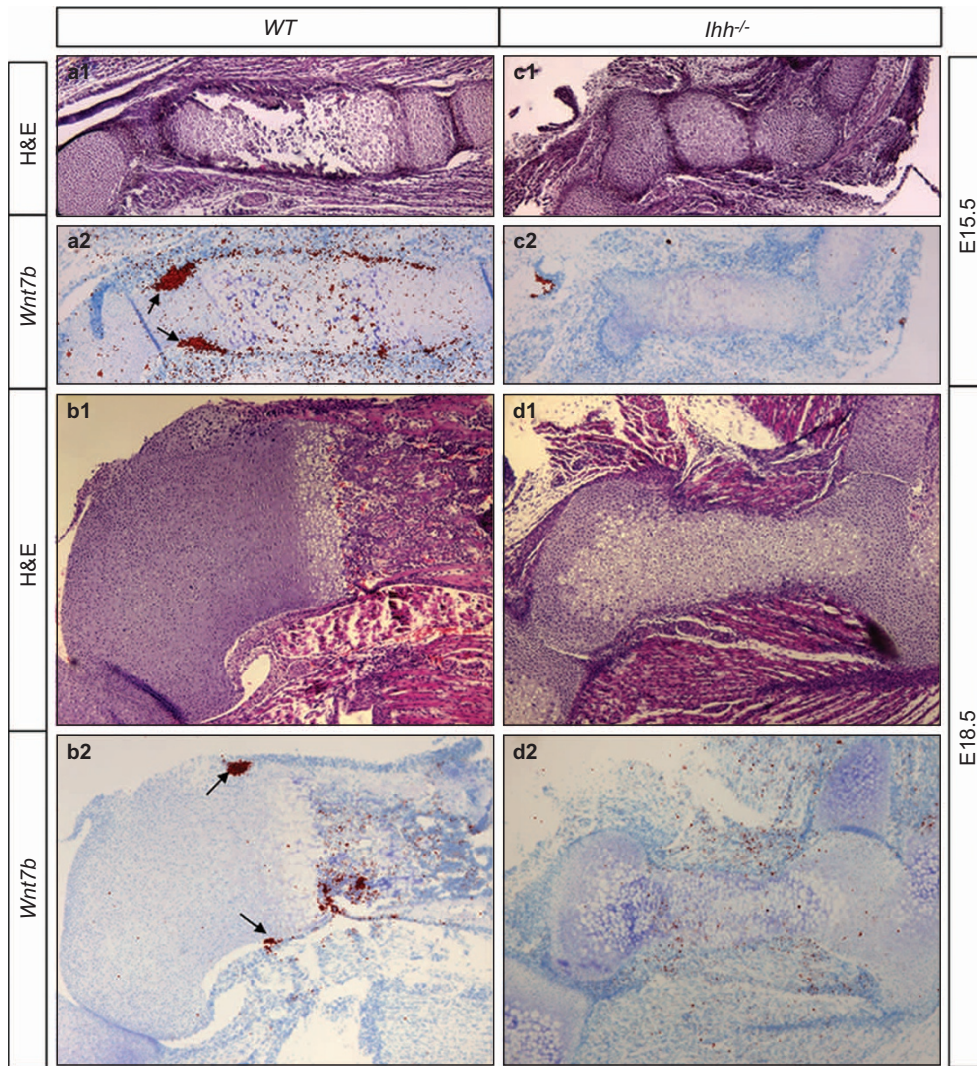
perichondrium surrounding the prehypertrophic and early hypertrophic chondrocytes, with no obvious expression in the diaphyseal periosteum (Figure 1b1 and 1b2). In contrast, Wnt7b was not detected in *Ihh*<sup>-/-</sup> embryos at either stage (Figure 1c1, 1c2, 1d1 and 1d2). Thus, Wnt7b is expressed in the osteogenic perichondrium in an *Ihh*-dependent manner.

### Overexpression of Wnt7b increases embryonic bone formation

To study the role of Wnt7b on bone formation, we took advantage of the *R26-Wnt7b* mouse strain that can overexpress Wnt7b in a Cre-dependent manner.<sup>12</sup> Specifically, we generated *Col2-Cre3;R26*<sup>Wnt7b/+</sup> mice (termed *C2Wnt7b* mice) by using the *Col2-Cre3* transgenic line that targets both osteoblasts and chondrocytes.<sup>3</sup> Whole-mount skeletal staining at E18.5 indicated that the long bones in *C2Wnt7b* embryos were slightly shorter but noticeably thicker than those in the normal littermate with the genotype of *R26*<sup>Wnt7b/+</sup> (Figure 2a and b). Histological analyses showed not only an increase in the thickness of the cortical bone, but also a presumptive marrow cavity filled with bone in the *C2Wnt7b* animal (Figure 2c, 2c1, 2d and 2d1). On the other hand, the growth plate cartilage appeared to be relatively normal. Consistent with histology showing extra bone mass within the presumptive marrow cavity of *C2Wnt7b* mice, *in situ* hybridization confirmed that the area contained an abnormally large number of osteoblasts expressing the well-known markers including Osterix (*Osx*), bone sialoprotein (*Bsp*), and osteocalcin (*OC*) (Figure 3). These results confirm that the *R26-Wnt7b* mouse strain is functional and that Wnt7b stimulates bone formation in the mouse embryo.

### Wnt7b induces hypertrophic cartilage vascularization in *Ihh*<sup>-/-</sup> embryos

To test whether Wnt7b mediates *Ihh* function during endochondral bone development, we produced *Col2-Cre3;Ihh*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> (termed *Wnt7b-rescue*) embryos by crossing the *Col2-Cre3;Ihh*<sup>+/-</sup> and *Ihh*<sup>+/-</sup>; *R26*<sup>Wnt7b/Wnt7b</sup> mice. The *Ihh*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> embryos, like the *Ihh*<sup>-/-</sup> embryos as previously reported, showed little vascularization of the hypertrophic cartilage at E18.5, even though the control littermate had developed a bone marrow cavity (Figure 4a1, 4a2, 4b1 and 4b2). The long bones of the *Wnt7b-rescue* embryos appeared to be similar in gross morphology to those of *Ihh*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> embryos (Figure 4b1 and 4c1, and data not shown). However, they displayed clear vascularization in their hypertrophic cartilage, as evident by the presence of red blood cells, even though a marrow cavity was not formed (Figure 4c1 and 4c2). To gain further insight about the vascularization phenotype, we performed *in situ* hybridization with



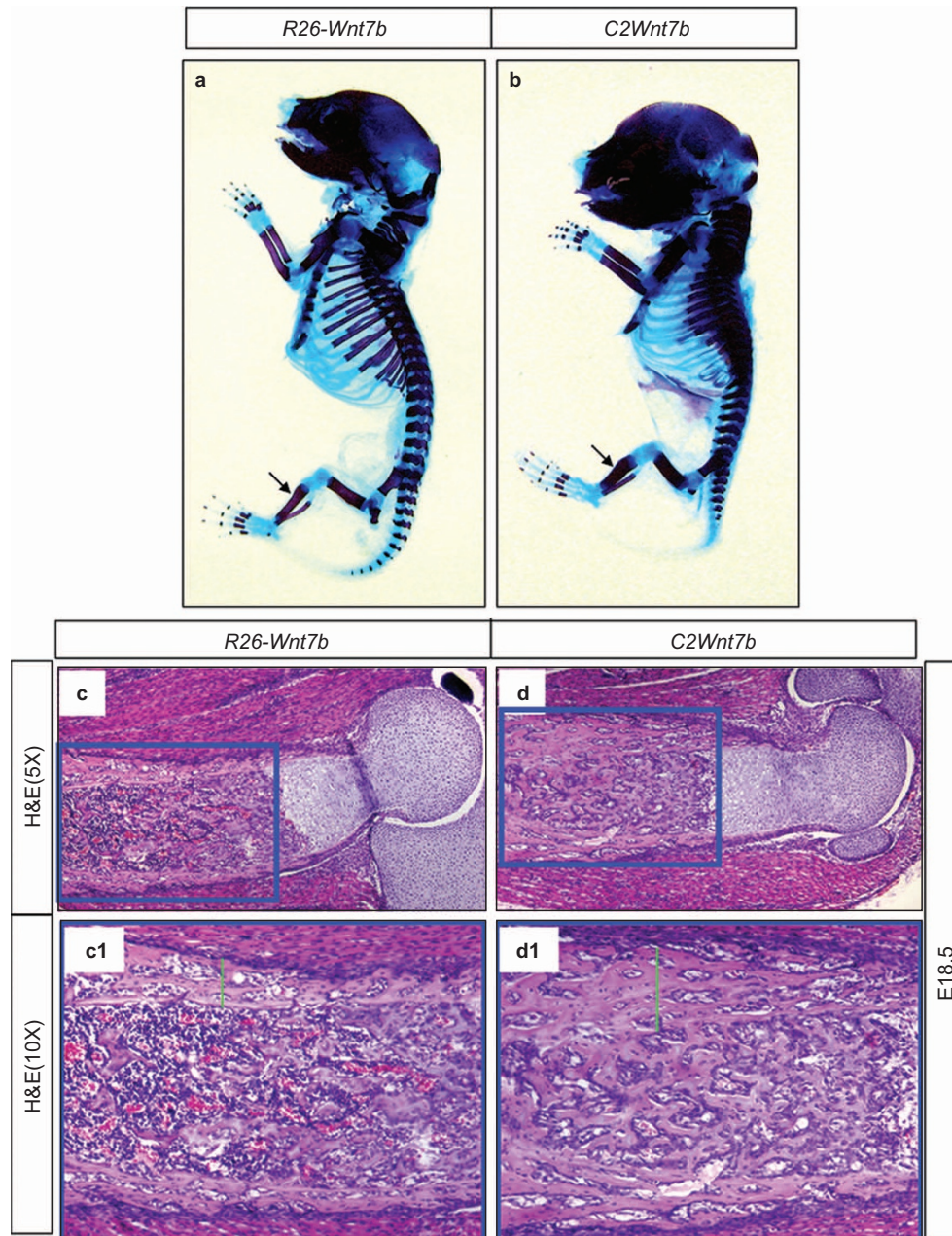
**Figure 1.** Expression of Wnt7b in developing long bones of mouse embryos. (a1, a2, b1, b2) H&E staining (a1, b1) and Wnt7b in situ hybridization (a2, b2) on longitudinal sections of the humerus in WT embryos at E15.5 (a1, a2) and E18.5 (b1, b2). (c1, c2, d1, d2) H&E staining (c1, d1) and Wnt7b in situ hybridization (c2, d2) on longitudinal sections of the humerus in *lhh*<sup>-/-</sup> embryos at E15.5 (c1, c2) and E18.5 (d1, d2). *In situ* hybridization signal in red. Arrows denote expression in perichondrium flanking prehypertrophic and early hypertrophic cartilage. WT, wild-type.

molecular markers for hypertrophy and vascularization. Normally, *Col10a1* is most robustly expressed by the early and intermediate hypertrophic chondrocytes, whereas *Mmp13* demarcates the terminal hypertrophic cells as well as osteoblast-lineage cells, and *Mmp9* marks the leading edge of vascular invasion in the bone marrow cavity (Figure 4d1–4d3). In the *lhh*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> embryo, the core of the cartilage element contained a hypertrophic domain expressing *Col10a1* peripherally and *Mmp13* centrally in a concentric manner, but no *Mmp9* expression was detected, confirming no vascularization of the hypertrophic cartilage (Figure 4e1–4e3). In contrast, expression of *Col10a1* and *Mmp13* in the hypertrophic cartilage of Wnt7b-rescue mice assumed a linear instead of concentric arrangement more similar to the normal pattern (Figure 4f1 and 4f2). More importantly, the Wnt7b-rescue

cartilage expressed *Mmp9* within the hypertrophic region, indicative of vascular invasion (Figure 4f3). Likely as a result of the vascularization, the *Mmp13*-positive domain was no longer contiguous in the central hypertrophic region in the Wnt7b-rescue embryo, in contrast to the *lhh*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> mutant (Figure 4e2 and 4f2). Overall, forced expression of Wnt7b was sufficient to induce hypertrophic cartilage angiogenesis in the absence of *lhh*.

Wnt7b expression does not rescue *lhh*-dependent osteoblast differentiation in *lhh*<sup>-/-</sup> mutants

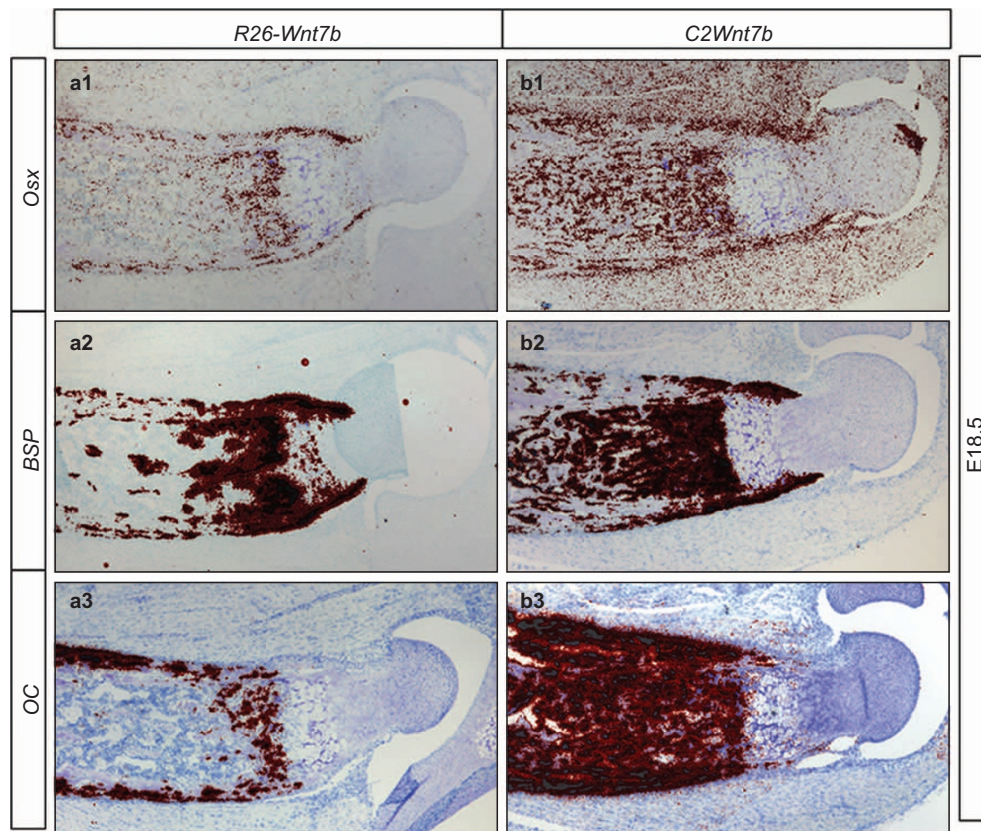
We next analyzed the potential rescue of osteoblast differentiation in Wnt7b-rescue embryos. Histology indicated no cortical bone (bone collar) or primary spongiosa in the Wnt7b-rescue embryo (Figure 4c1 and 4c2). To gain more insight, we performed molecular analyses for osteoblast



**Figure 2.** Wnt7b increases bone formation during endochondral bone development. (a, b) Whole-mount skeletal staining of E18.5 embryos from  $R26^{Wnt7b/+}$  (a) versus  $Col2-Cre3; R26^{Wnt7b/+}$  (b) embryos. Arrows point to tibia that show marked size difference. (c, d) H&E staining of longitudinal sections through the distal half of humerus. (c1, d1) Higher magnification of boxed regions shown in c and d. Green line in c1, d1 denotes cortical thickness.

differentiation. *In situ* hybridization revealed normal expression patterns for Runx2, AP and Osx in E18.5  $lhh^{+/-}$  embryos. These molecules were detected in the primary spongiosa, the diaphyseal periosteum (red arrow) as well as the perichondrium flanking the prehypertrophic and hypertrophic cartilage (blue arrow) (Figure 5a1–5a3). The last domain represents the area wherein *lhh*-dependent osteoblast differentiation (orthotopic perichondrial ossification) occurs during normal development. In contrast, no such expression was detected in the

perichondrium of  $lhh^{-/-}; R26^{Wnt7b/+}$  long bones, even though the hypertrophic cartilage expressed Runx2 (Figure 5b1–5b3). Compared to  $lhh^{-/-}; R26^{Wnt7b/+}$  embryos, the long bones of Wnt7b-rescue embryos also lacked the osteoblast markers in the perichondrium flanking the prehypertrophic and hypertrophic cartilage (blue arrow) (Figure 5c1–5c3). Interestingly, however, Runx2, AP and Osx were detected in the diaphyseal perichondrium in the Wnt7b-rescue embryo, typically on one side of the long bone (red arrow), and also within the presumptive



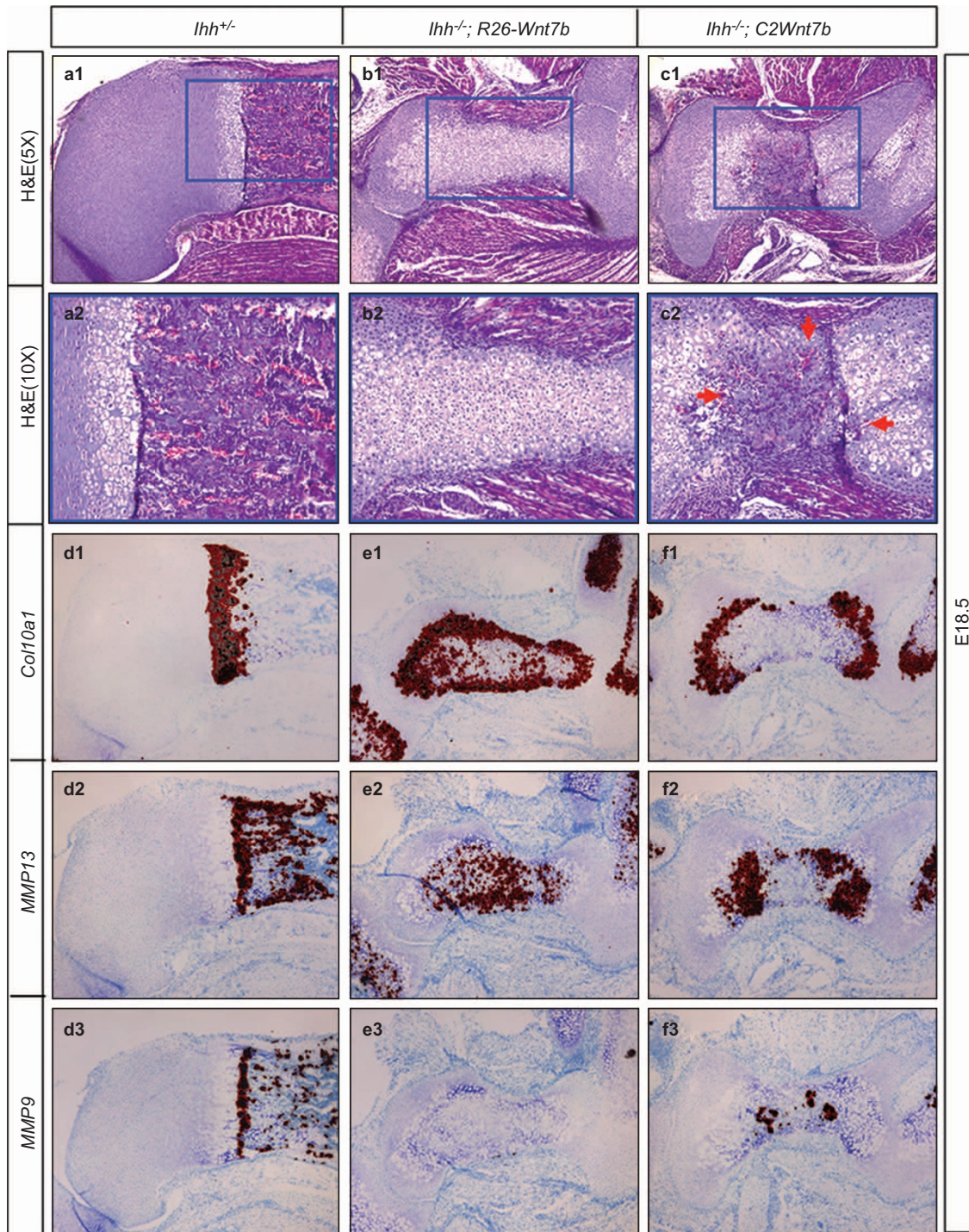
**Figure 3.** Molecular analyses of osteoblast differentiation at E18.5. *In situ* hybridization of osteoblast markers on sections of distal humeri from  $R26^{Wnt7b/+}$  (a1–a3) versus  $Col2-Cre3; R26^{Wnt7b/+}$  (b1–b3) littermate embryos at E18.5. *In situ* hybridization signals shown in red.

marrow region (asterisk) (Figure 5c1–5c3). Because expression of the osteoblast markers in these areas coincided with vascularization, the induction of osteoblast differentiation may be secondary to the increased vascularization in response to Wnt7b. A formal proof for this hypothesis awaits further studies. In summary, Wnt7b is not sufficient to restore orthotopic perichondral ossification in the  $Ihh^{-/-}$  embryo, but appears to induce osteoblast differentiation secondary to the enhanced vascularization.

Wnt7b expression does not rescue  $Ihh$ -dependent osteoblast differentiation in  $Ihh^{-/-}; Gli3^{-/-}$  embryos  
Wnt7b expression in  $Ihh^{-/-}$  mutant enhanced hypertrophic cartilage angiogenesis, but failed to rescue  $Ihh$ -dependent osteoblast differentiation. This phenotype was remarkably similar to that of the  $\Delta NGli2$ -rescue embryo.<sup>16</sup> Because removal of  $Gli3$  in the  $\Delta NGli2$ -rescue embryo rescued  $Ihh$ -dependent orthotopic perichondral ossification, we hypothesized that the process can be similarly restored by simultaneous removal of  $Gli3$  and expression of Wnt7b. To test this hypothesis, we generated  $Col2-Cre3; Ihh^{-/-}; Gli3^{-/-}; R26^{Wnt7b/+}$  embryos (termed double-rescue embryo) by crossing  $Col2-Cre3; Ihh^{+/-}; Gli3^{+/-}$  with  $Ihh^{+/-}; Gli3^{+/-}; R26^{Wnt7b/Wnt7b}$  mice.

We first performed histological analyses on the long bones at E18.5. The length and organization of the growth plate was largely restored in the double-rescue embryo (Figure 6a1, b1, a2 and b2). This was consistent with our previous finding that removal of  $Gli3$  restored growth plate morphology in the  $Ihh^{-/-}$  embryo.<sup>15</sup> Interestingly, the double-rescue embryo formed a bone collar detectable by either H&E or sirus red staining (Figure 6b1 and 6b2). Careful examination revealed that bone was formed at the diaphyseal region surrounding the presumptive bone marrow cavity, but not in the perichondrium surrounding the hypertrophic zone where ossification normally occurs (Figure 6b4). The presumptive marrow cavity also contained bone trabeculae in the double-rescue embryo (Figure 6b3). The diaphyseal bone formation in the double-rescue mouse was similar to that observed in  $Ihh^{-/-}; Gli3^{-/-}$  embryos, but appeared to be more robust<sup>15</sup> (data not shown). However, because our current mating strategy did not produce  $Ihh^{-/-}; Gli3^{-/-}$  littermate embryos for direct comparison, we cannot rule out the possibility that the apparent quantitative difference might be due to differences in genetic background.

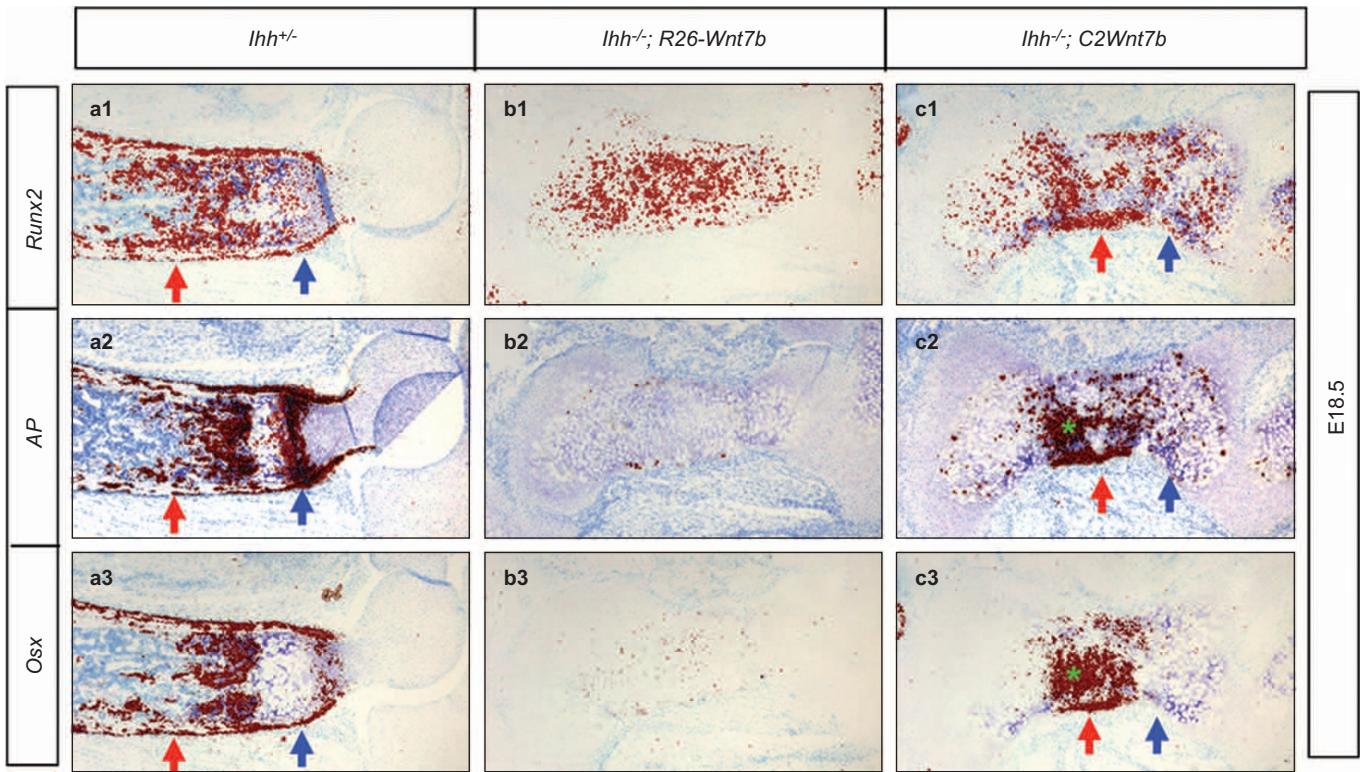
To confirm the bone phenotype at the molecular level, we examined the expression of osteoblast markers



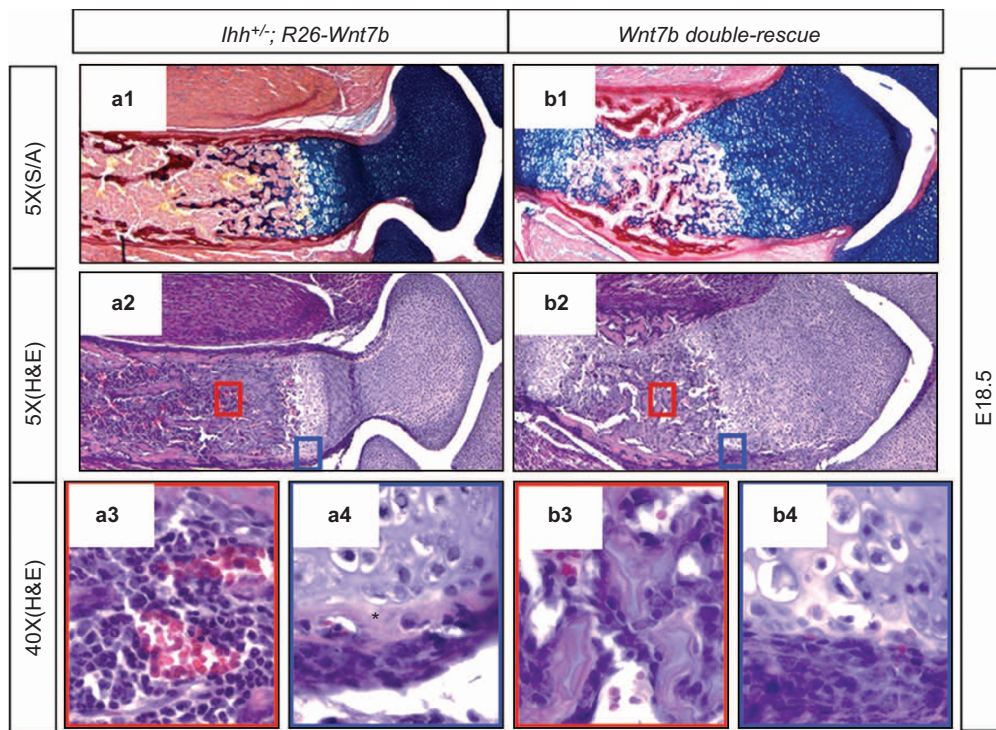
**Figure 4.** Analyses of hypertrophic cartilage vascularization in the humerus of E18.5 littermate embryos. (a1–c1) H&E staining of humerus sections from *lhh*<sup>+/-</sup> (a1), *lhh*<sup>-/-</sup>; *R26*<sup>*Wnt7b*/+</sup> (b1) and *lhh*<sup>-/-</sup>; *Col2-Cre3*; *R26*<sup>*Wnt7b*/+</sup> (c1) embryos. (a2–c2) High-magnification images of boxed areas in a1–c1. Red arrows point to red blood cells. (d1–d3, e1–e3, f1–f3) *In situ* hybridization on humerus sections. Hybridization signal shown in red.

including Runx2, Osx, Bsp and OC. These markers were normally expressed by all osteoblasts associated with the primary spongiosa, diaphyseal periosteum (red arrow) and the perichondrium flanking the prehypertrophic and hypertrophic cartilage (blue arrow) (Figure 7a1–7a4).

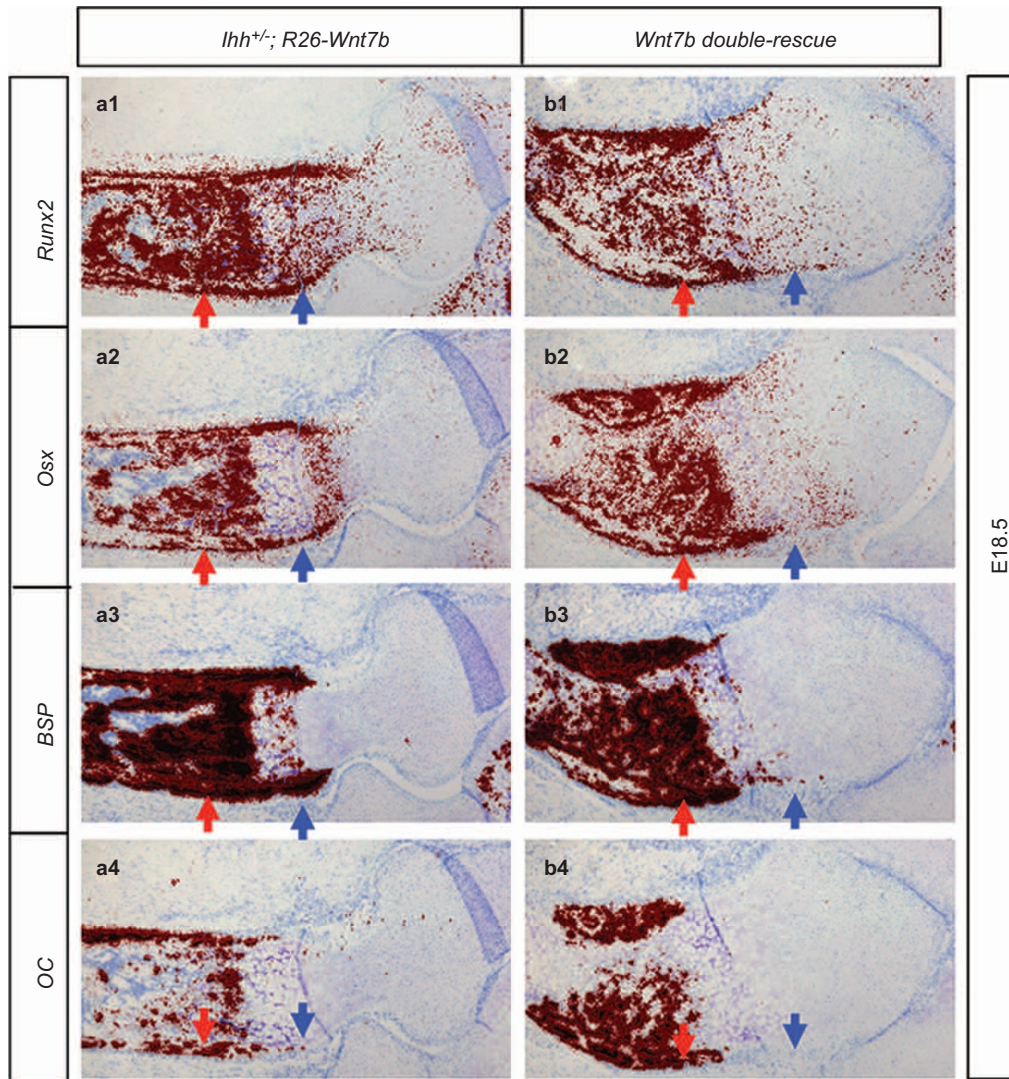
However, in the double-rescue embryo, although the markers were expressed in the diaphyseal region including the periosteum (red arrow), they were not present at any significant level in the presumptive region for orthotopic perichondral ossification (blue arrow) (Figure 7b1–7b4).



**Figure 5.** Molecular analyses for osteoblast differentiation in E18.5 littermate embryos. *In situ* hybridization of Runx2 (a1–a3), AP (b1–b3) and Osx (c1–c3) on humerus sections. Distal end to the right. Blue arrows: perichondrium flanking hypertrophic chondrocytes; red arrows: diaphyseal perichondrium. Asterisk denotes signal in presumptive marrow cavity.



**Figure 6.** Morphological analyses of *lhh*<sup>+/-</sup>; R26<sup>Wnt7b/+</sup> versus *Col2-Cre3*; *lhh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup>; R26<sup>Wnt7b/+</sup> (*Wnt7b* double rescue) littermate embryos at E18.5. (a1, b1) Sirius Red and Alcian Blue staining of longitudinal sections through the humerus. Distal end to the right. (a2, b2) H&E staining. (a3, a4, b3, b4) Higher magnification images of boxed areas in a2 and b2.



**Figure 7.** *In situ* hybridization for osteoblast markers in *Ihh*<sup>+/-</sup>; *R26*<sup>Wnt7b/+</sup> (a1–a4) versus *Col2-Cre3; Ihh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup>; *R26*<sup>Wnt7b/+</sup> (Wnt7b double rescue) (b1–b4) littermate embryos at E18.5. Shown are humerus sections with distal end to the right. Blue arrow: perichondrium flanking hypertrophic chondrocytes; red arrow: perichondrium flanking diaphysis.

Thus, Wnt7b expression coupled with *Gli3* deletion did not restore orthotopic perichondral ossification in the *Ihh*<sup>-/-</sup> embryo.

## DISCUSSION

We have investigated the potential role of Wnt7b in mediating the multiple functions of *Ihh* signaling during endochondral bone development. By examining mouse embryos at multiple embryonic stages, we confirmed the Wnt7b is expressed by perichondrial cells undergoing *Ihh*-dependent ossification and that the expression is dependent on *Ihh*. Through genetic experiments that force-expressed Wnt7b, we found that Wnt7b was sufficient to induce hypertrophic cartilage vascularization that is otherwise absent in *Ihh*<sup>-/-</sup> embryos. On the other hand, Wnt7b forced-expression failed to activate perichondrial

osteoblast differentiation at the orthotopic position when *Ihh* was absent, even when the transcription repressor *Gli3* was removed. These results provide additional insights about the mechanisms of *Ihh* function during endochondral skeletal development.

How *Ihh* regulates Wnt7b expression is not understood at present. We have noticed in the Wnt7b promoter potential *Gli* binding sites conforming to the consensus sequence TGG GTG GTC as previously defined by others,<sup>17–18</sup> so it will be of interest to determine whether these sites mediate direct regulation of Wnt7b expression by *Ihh*. Alternatively, the control of *Ihh* over Wnt7b expression may be indirect.

It is noteworthy that Wnt7b expression caused osteoblast differentiation within the diaphyseal perichondrium in the absence of *Ihh*. Although at the histological level,



bone tissue was not evident in the Wnt7b-rescue embryo, molecular analyses revealed upregulation of Runx2, AP and Osx within a small domain of the perichondrium at the diaphysis, as well as in the presumptive marrow cavity. Interestingly, expression of the osteoblast markers was invariably detected asymmetrically on one side of the long bone section, which exhibited more advanced vascularization (judged by histology) than the other side. A similar concurrence between diaphyseal ossification and vascularization was observed in *Ihh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup> embryos as we have previously reported.<sup>15</sup> Indeed, when Wnt7b was force-expressed in the background of *Ihh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup>, diaphyseal bone formation was most prominent, as indicated by both histology and molecular analyses. These results support the hypothesis that vascularization was sufficient to activate the osteogenic program within diaphyseal perichondrium independent of *Ihh*. Future studies are necessary to elucidate the osteogenic signals associated with vascular invasion.

In contrast to the diaphyseal perichondrium, the perichondrium flanking the prehypertrophic and hypertrophic cartilage requires *Ihh* signaling to undergo osteoblast differentiation. We have previously shown that simultaneous activation of *Gli2* and removal of *Gli3* can replace *Ihh* to activate osteogenesis within this domain.<sup>15–16</sup> Others reported that *Gli1* also plays a stimulatory role in this process.<sup>19</sup> Downstream of the *Gli* transcription factors, however, the critical effectors responsible for osteoblast differentiation are not known. We previously demonstrated that forced-expression of *Runx2* failed to restore orthotopic perichondral ossification in *Ihh*<sup>-/-</sup> embryos.<sup>20</sup> Similarly, here we show that Wnt7b cannot replace *Ihh* in this process. Thus, induction of perichondral ossification by *Ihh* likely involves multiple downstream effectors, and a full understanding of the process requires further investigation.

In contrast to osteoblast differentiation, vascularization of the hypertrophic cartilage was induced by forced-expression of Wnt7b in the absence of *Ihh*. The evidence for Wnt7b as an angiogenic signal previously came from studies of the central nervous system, but a similar role in other systems has not been demonstrated.<sup>10,21</sup> The present study indicates that Wnt7b normally produced by the perichondrium may function as a relay signal for the chondrocyte-derived *Ihh* to induce blood vessel invasion of the hypertrophic cartilage. It is not known at present whether a direct input of Wnt signaling in endothelial cells is required for cartilage angiogenesis. To answer that question would require manipulation of Wnt signal reception or transduction directly in the endothelium.

### Conflict of Interest

The authors declare no conflict of interest.

### Acknowledgements

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