**INTRODUCTION**

One of the major processes of vertebrate skeletal development is endochondral bone formation, in which mesenchymal cells first condense and then differentiate into chondrocytes to form the cartilaginous shaft of the future bone. In the developing cartilage, proliferative chondrocytes differentiate to form first prehypertrophic and then hypertrophic chondrocytes (Cancedda et al., 1995; Linsenmayer et al., 1991; Vortkamp et al., 1996). This process is tightly regulated, which ensures proper longitudinal growth of long bones (Hunziker, 1994). As a result, specific spatial zones of chondrocytes with distinct patterns of proliferation, differentiation and cell morphology are laid down along the longitudinal axis of a developing long bone.

It has been shown that the proliferative and articular/resting chondrocyte region can be further divided into two zones (Zone I and Zone II) based on the distinct cell morphologies, developmental fates and functions (Abad et al., 2002; Iwamoto et al., 2000). Zone I corresponds to the epiphysis of developing long bones. Zone I cells are round and divide less frequently (Kobayashi et al., 2002; Long et al., 2001). They are articular chondrocytes that will become permanent articular cartilage and resting chondrocytes in the growth plate (Archer et al., 1994; Hunziker, 1994; Mitrovic et al., 1978; Pacifici, 1995). Articular cartilage maintains normal joint function throughout life, whereas resting chondrocytes contain cells similar to stem cells that are capable of generating new clones of proliferative zone chondrocytes (Abad et al., 2002). Zone II cells are highly proliferative chondrocytes in the growth plate that will exit the cell cycle and undergo hypertrophy (Howlett, 1979; Hunziker, 1994; Karp et al., 2000). Zone II cells are flattened and increase their cell size gradually the closer they are to the hypertrophic zone. These cells divide more frequently and line up with each other to form columns parallel to the long axis of the bone (Dodd, 1930; Long et al., 2001). Zone II depends on Zone I for a constant supply of proliferative chondrocytes. In the meantime, a population of undifferentiated chondrocytes remains in Zone I to maintain joint function at all times. Thus, the transition between Zone I and Zone II has to be carefully regulated. However, the underlying molecular mechanism is still poorly understood.

Signaling molecules have been shown to play important roles in regulating chondrocyte proliferation and differentiation (reviewed by de Crombrugghe et al., 2001; Karsenty, 2001). Disruption of the normal cell signaling during endochondral bone formation results in skeletal anomalies in both humans and mouse mutants (Gao et al., 2001; Li and Olsen, 1997). Indian hedgehog (Ihh)/parathyroid hormone-related peptide (PTHrP) negative feedback loop. We find that important cell cycle regulators such as cyclin D1 and p130, a member of the retinoblastoma family, exhibit complimentary expression patterns that correlate with the distinct proliferation and differentiation states of chondrocyte zones. Furthermore, we show that Wnt5a and Wnt5b appear to coordinate chondrocyte proliferation and differentiation by differentially regulating cyclin D1 and p130 expression, as well as chondrocyte-specific Col2a1 expression. Our data indicate that Wnt5a and Wnt5b control the pace of transitions between different chondrocyte zones.

**Key words:** Wnt5a, Wnt5b, Chondrocyte, Proliferation, Mouse
Recent studies have shown that PTHrP and Ihh signaling may also be involved in regulating the transition between Zone I and II, the two chondrocyte zones before prehypertrophy (Kobayashi et al., 2002). However, it was not clear which signaling molecules directly inhibit the transition between Zone I and II.

The mechanism by which signaling molecules control cell proliferation and differentiation involves key intracellular regulatory factors, which include cell cycle regulators and transcription factors. Cell cycle inhibitors such as p21, p57, p107 and p130, members of retinoblastoma (Rb) family, are involved in regulating chondrocyte proliferation and differentiation (Aikawa et al., 2001; Cobrinik et al., 1996; Su et al., 1997; Yan et al., 1997). These studies have shown that chondrocyte hypertrophy is delayed when cell cycle withdrawal is inhibited. Sox9 and Cbfa1 (Runx2 – Mouse Genome Informatics) are transcription factors that appear to have opposite activities in regulating chondrocyte differentiation (Bi et al., 2001; Takeda et al., 2001; Ueta et al., 2001). Sox9 inhibits chondrocyte hypertrophy whereas Cbfa1 enhances it. Although it is not clear how signaling molecules regulate Cbfa1 activity in the chondrocytes, it has been shown that Sox9 transcriptional activity can be significantly enhanced through phosphorylation by protein kinase A (PKA). Moreover, PTHrP signals through activating PKA and may inhibit chondrocyte maturation by increasing Sox9 activity (Huang et al., 2001). Sox9 is required for the expression of several cartilage-specific extracellular matrix components and it directly regulates Col2a1 (previously known as ColII) gene expression (Bell et al., 1997; Lefebvre et al., 1996; Mukhopadhyay et al., 1995; Zhou et al., 1995).

More recently, Wnt family members have been found to be involved in regulating skeletal development. Wnt proteins are important signaling molecules that have been shown to regulate cell proliferation and differentiation during embryonic development and tumor formation (for reviews, see Huelsken and Birchmeier, 2001; Peifer and Polakis, 2000). Studies carried out in chick have shown that ectopic Wnt5a expression delays chondrocyte differentiation, whereas ectopic Wnt4 expression promotes chondrocyte differentiation (Hartmann and Tabin, 2000). In addition, overexpression of Wnt14 in the chick limb leads to ectopic synovial joint formation (Hartmann and Tabin, 2001). However, direct evidence to show that endogenous Wnt gene function is required for chondrocyte proliferation or differentiation is lacking.

We have found that both Wnt5a and Wnt5b are expressed in the chondrocyte of developing long bones in mice. To understand the mechanism by which Wnt5a and Wnt5b regulate endochondral skeletal development in vivo, we have analyzed the skeletal development defects in mutant mice either that lack Wnt5a function (Yamaguchi et al., 1999) or overexpress Wnt5a or Wnt5b in chondrocytes. Our results indicate that Wnt5a is required for regulating chondrocyte proliferation and differentiation in both Zone I and Zone II, and Wnt5a signaling directly inhibits the transition from Zone I to Zone II. In addition, Wnt5b promotes cell proliferation and Zone II formation. Thus, Wnt5a and Wnt5b appear to coordinate longitudinal long bone growth by exerting opposite activities in regulating chondrocyte proliferation and chondrocyte-specific Col2a1 expression.

MATERIALS AND METHODS

Generation of transgenic mice
Col2a1-Wnt5a, Col2a1-Wnt5b transgenes were generated by subcloning mouse cDNAs of Wnt5a and Wnt5b (Gavin et al., 1999), respectively, into a Col2a1 transgenic vector (Long et al., 2001). Transgenic mice were generated by pronuclear injection according to standard procedures (Hogan et al., 1994). The resulting G0 embryos were harvested and analyzed. Embryos were genotyped by PCR using the following primers: GCAAGTGTCTGTGTTGTGGTG and CAGGGTTATTTCATACCTTAGAGACC for the Col2a1-Wnt5a mice; and GCAAGTGTCTGTGTTGTGGTG and CTATCTGACATAACTCT-GCCAAG for the Col2a1-Wnt5b mice.

Skeletal analysis
Embryos at E17.5 or E18.5 were dissected in phosphate-buffered saline (PBS). The embryos were then skinned, eviscerated and fixed in 95% ethanol. Skeletal preparation was performed as described previously (McLeod, 1980).

Histological analysis, in situ hybridization and immunohistochemistry
Embryos were dissected in PBS and fixed in 4% formaldehyde at 4°C overnight, dehydrated with increasing ethanol concentration, and embedded in paraffin wax. Serial sections of 6 μm were stained according to the Weigert-Safranin staining procedure (Prophet et al., 1994). BrdU labeling and radioactive 35 S RNA in situ hybridization were performed on serial sections as described (Si-Jacques et al., 1999). Whole-mount in situ hybridization were performed as described (Goodrich et al., 1996). RNA probes were has been described previously: Ihh (Bitgood and McMahon, 1995); Wnt5a and Wnt5b (Gavin et al., 1999); Pth (Goodrich et al., 1996); Osf2/Cbfa1 (Runx2 – Mouse Genome Informatics) (Ducy et al., 1997); osteocalcin (Desbois et al., 1994); and Pthlh, Pth/Pthrpr, Col2a1 and Col110a (ColX) (Lee et al., 1995).

For immunohistochemistry, sections were digested with 0.1% hyaluronidase for 15 minutes at 37°C, heated in citric buffer (pH 6.0, 10 mM) at 95°C for 25 minutes to enhance antigen accessibility. Slides were blocked with 4% horse serum (Sigma) at room temperature for 2 hours, incubated overnight at 4°C with primary antibodies: anti-cyclin D1 (Santa Cruz sc 8396) at 1:40; anti-p130 (BD Transduction Laboratories 610261) at 1:40; and anti-Sox9 at 1:30 (Huang et al., 2001). Signals were detected using the ABC kits (Vector laboratories) and DAB (Sigma), or Alexa Fluor 594-conjugated secondary antibodies purchased from Molecular Probes.

Primary chondrocyte isolation, culture, transfection and Luciferase assay
Primary mouse chondrocytes were prepared from the ventral rib cages of 0- to 3-day-old mice, according to the previously published protocol (Lefebvre et al., 1994). Cells were transfected using LipofectAMINE-PLUS (Invitrogen) according to the manufacturer’s instructions. The amount of DNA in each transfection was kept constant by addition of an appropriate amount of empty expression vector. The Col2a1 reporter construct pK159Axt6uc and the mutant Col2a1 reporter PKN159MTluc were gifts from Yoshikico Yamada (Krebshbach et al., 1996; Tanaka et al., 2000). To express Wnt5a or Wnt5b, the coding region of the genes were inserted into pIRESHrpGFP-1a expression vector (Stratagene). To determine Col2a1 reporter activity, 1.5×105 cells were co-transfected with a reporter construct (0.33 μg), pRLSV40 (Promega) as an internal control (0.016 μg) and the indicated plasmids in six-well plates. The transfected cells were harvested 48 hours later and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). All measurements were made using a Lumat LB9507 luminometer (EG&G Berthold).
RESULTS

Wnt5a regulates skeletal morphogenesis

A prominent feature of the Wnt5a mutant mouse is abnormal development of the skeletal system (Yamaguchi et al., 1999). Abnormalities are found in skeletal elements formed by endochondral ossification. As has been shown before, skeletal elements in the limb are significantly shortened and distal digits are missing in the Wnt5a–/– embryos (Yamaguchi et al., 1999). The Wnt5a–/– embryo also has a cleft palate, significantly shortened endocranial regions, irregular ossification centers in the vertebral bodies and fused or bifurcated ribs (data not shown). As endochondral skeletal development in long bones has been extensively studied and Wnt5a–/– embryos have very consistent phenotypes in the long bones, we focused our studies on this aspect of Wnt5a function. Histological analysis revealed that chondrocyte hypertrophy and skeletal ossification were significantly delayed in Wnt5a mutants (Fig. 1A,B). In the wild-type embryo, there was extensive chondrocyte hypertrophy and bone formation at 16.5 days post-coitum (dpc) in the radius and ulna. However, in the mutant, chondrocyte hypertrophy did not occur until 18.5 dpc in the same region (Fig. 1A). At 18.5 dpc, trabecular bone formation and blood vessel invasion in the humerus region were not complete and the periosteal bone matrix was reduced in the Wnt5a–/– embryos (Fig. 1B).

To understand the molecular mechanism by which Wnt5a regulates endochondral skeletal morphogenesis, Wnt5a expression was examined in the sections of developing long bones by in situ hybridization. Wnt5a was expressed at the boundary of proliferative and prehypertrophic chondrocytes and perichondrium/periosteum (Fig. 1C). Its expression domain overlapped with that of Ihh in the prehypertrophic region and flanked the hypertrophic chondrocytes that express Col10a1. In both the perichondrium and prehypertrophic chondrocytes, the expression of Wnt5a overlapped with that of Cbfa1, a transcription factor that determines osteoblast cell fate and regulates chondrocyte differentiation (Ducy et al., 1997; Kim et al., 1999; Komori et al., 1997; Otto et al., 1997; Takeda et al., 2001; Ueta et al., 2001). The expression pattern of Wnt5a, together with the histological analysis of the Wnt5a–/– embryos indicates that Wnt5a is a signaling molecule required for both chondrocyte and osteoblast differentiation during mouse endochondral skeletal morphogenesis.

Delayed Ihh expression and chondrocyte hypertrophy in Wnt5a mutant limbs

The defect in chondrogenesis in the Wnt5a mutant limb was detected by in situ hybridization from 11.5 dpc, when chondrogenesis has just started (Fig. 2A). We found that the expression of Sox9, the earliest marker for mesenchymal condensation, was not detected in the distalmost limb mesenchyme in the Wnt5a mutant. At 12.5 dpc, the distal zone that failed to express Sox9 expanded, indicating that Wnt5a activity is required for mesenchymal condensation in the distal limb bud.

Part of Wnt5a transcript was still produced in the Wnt5a mutant. It is worth noting that Wnt5a expression was still detected in the distal limb but missing in the Wnt5a mutant at 12.5 dpc, suggesting that Wnt5a may be required for its own expression in chondrocytes (Fig. 2B).

As Wnt5a expression overlapped with that of Ihh in the prehypertrophic region, and Ihh plays a key role in coordinating chondrocyte proliferation and differentiation, we examined whether Ihh expression was affected in the Wnt5a–/– limbs (Fig. 2A,B). At 11.5 dpc, Ihh expression was detected in the future humerus, radius and ulna in the wild-type embryo. In the Wnt5a mutant, Ihh expression was only weakly detected in the humerus and absent in the radius and ulna (Fig. 2A),...
although mesenchymal condensation occurred normally in the proximal limb. At 12.5 dpc, shortly after cartilage formation in the forelimb, Ihh was expressed strongly in the developing cartilage in the wild-type embryos, but no expression was detected in the Wnt5a mutant, and only weak expression was observed at 13.5 dpc (Fig. 2B and data not shown). At 15.5 dpc, strong expression of Ihh was observed in the humerus of the Wnt5a–/– embryo, but only patchy expression was detected in the ulna (Fig. 2C). Ihh expression correlated with the upregulation of patched (Ptch), a transcriptional target of hedgehog signaling (Goodrich et al., 1996) (Fig. 2C).

As limb skeletal morphogenesis progresses from the proximal to distal part of the limb, Ihh expression was first detected in the proximal limb. It is likely that the weaker expression of Ihh earlier in development was an indirect result of delayed chondrocyte differentiation and the proximodistal sequence of skeletal development was preserved in the Wnt5a mutant. This was confirmed by our observation that the expression of Pthrpr and Cbfa1 in the prehypertrophic chondrocytes was reduced and delayed and the expression of Col10a1, a hypertrophic chondrocyte marker, was absent in the Wnt5a–/– limb from 12.5 dpc to 15.5 dpc (Fig. 2B,C). Together, these results indicate that Wnt5a is required for the transition from proliferative chondrocytes to prehypertrophic chondrocytes. Moreover, as the phenotype of chondrocyte differentiation in the Wnt5a–/– limb is different from that in the Ihh–/– limb (St-Jacques et al., 1999), it is unlikely that Wnt5a acts through Ihh in regulating chondrocyte differentiation.

As PTHrP signaling suppresses chondrocyte differentiation at multiple steps (Kobayashi et al., 2002; Lanske et al., 1996), we examined Pthlh expression in Wnt5a mutants. Pthlh expression in the periarticular region of the Wnt5a–/– mutant was comparable with that in the wild-type embryo at 13.5 dpc (Fig. 2B). As Ihh signaling is required for the expression of Pthlh, this result suggests that normal Pthlh expression is maintained by very low levels of Ihh signaling in the Wnt5a mutant.

Since the skeletal elements in the Wnt5a mutant limb were significantly shortened, BrdU labeling experiments were performed in 14.5 dpc mouse embryos to assess chondrocyte proliferation in the distal humerus (Fig. 3A,B).
Undifferentiated chondrocytes are divided into two zones according to the cell shape, developmental fate and the known Ihh signalling domain (Abad et al., 2002; Iwamoto et al., 2000; Long et al., 2001) (Fig. 3A). In the wild-type embryo, Zone II is more highly proliferative than Zone I (Fig. 3B), as has been shown before (Long et al., 2001). In the Wnt5a mutant, the proliferation in Zone I was slightly increased. By contrast, the proliferation in Zone II was reduced by about 30% to a level similar to Zone I. These results indicate that Wnt5a is required for chondrocyte proliferation in Zone II where it is expressed. It is possible that Wnt5a acts indirectly by affecting Ihh expression as Ihh is a major positive factor for chondrocyte proliferation and Ihh expression was much weaker in Wnt5a–/– long bones (Fig. 2A,B). In addition to a proliferation deficiency, the cartilage morphology was severely distorted and the diameter of Zone I was reduced; the diameter of Zone II was increased in Wnt5a mutants (Fig. 3A).

Delayed osteoblast differentiation in Wnt5a mutants
To investigate bone development in Wnt5a–/– limbs, we examined expression of Cbfal, a transcription factor essential for osteoblast differentiation. Cbfal expression was significantly reduced in both chondrocytes and perichondrium in the Wnt5a mutants (Fig. 2B,C). In addition, the expression of osteocalcin, a marker for mature osteoblasts (Desbois et al., 1994), was not detected in Wnt5a mutants at 15.5 dpc, whereas its expression was readily detected in wild-type long bones (Fig. 2C). These results demonstrate that osteoblast differentiation is delayed in Wnt5a mutants, possibly as a result of delayed chondrocyte differentiation and Ihh expression.

As Wnt5a expression in chondrocytes and the perichondrium overlaps with that of Cbfal, it is also possible that Wnt5a regulates osteoblast development directly. However, endochondral ossification is dependent on Ihh. Thus, reduced Ihh signaling activity may secondarily result in delayed osteoblast differentiation. In the later case, restoring Ihh activity in the absence of Wnt5a function may be expected to rescue the osteoblast deficiency. To this end, Ihh was ectopically expressed in chondrocytes under the control of Col2a1 promoter/enhancer using the UAS-Gal4 system (Long et al., 2001). When these mice were mated to Wnt5a mutants (Fig. 4), we found that although the limb in the Wnt5a–/–; Col2a1-Gal4; UAS-Ihh mouse was slightly longer than the Wnt5a–/– limb, it was still much shorter than the limb of Col2a1-Gal4; UAS-Ihh mice. Osteoblast differentiation in the Wnt5a–/– limb was rescued by Ihh overexpression. Thus, Wnt5a may regulate osteoblast differentiation through indirectly affecting Ihh expression in chondrocytes.

In the cartilage, the gross phenotype of the Wnt5a–/–; Col2a1-Gal4; UAS-Ihh mouse was the combination of Wnt5a–/– and Col2a1-Gal4; UAS-Ihh mice. As ectopic Ihh expression is able to rescue Ihh–/– mice (A. McMahon, personal communication) to a level similar to Col2a1-Gal4; UAS-Ihh mice, our results indicate that Wnt5a and Ihh act in separate pathways in regulating chondrocyte proliferation and differentiation in the developing long bones.

Wnt5a delays chondrocyte differentiation in a dose-dependent manner
To understand better how Wnt5a regulates the differentiation of chondrocytes, Col2a1-Wnt5a transgenic mice were generated (Fig. 5A). In these transgenic mice, Wnt5a was expressed in non-hypertrophic chondrocytes under the control of rat Col2a1 promoter and enhancer (Nakata et al., 1993). When compared with the wild-type littermates, the Col2a1-Wnt5a transgenic mice showed severe skeletal defects. Skeletal elements in the limb were short and ossification was delayed. The cartilage was thicker and chondrocyte hypertrophy was significantly delayed in the developing long bones of the transgenic mouse (Fig. 5A).

At 14.5 dpc, Col10a1 expression domain was either reduced (Col2a1-Wnt5a number 17) or absent (Col2a1-Wnt5a number 25). The loss of Col10a1 expression correlated with higher levels of Wnt5a expression in individual transgenic embryos and a resulted increase in the severity of the phenotype (Fig. 5B). In addition, the prehypertrophic chondrocyte zone where Ihh and Pthrpr are normally expressed was either not detected (Col2a1-Wnt5a number 25) or remained at the midline of the skeletal element (Col2a1-Wnt5a number 17) (Fig. 5B). Thus, Wnt5a overexpression caused a delay in chondrocyte differentiation before hypertrophic development, and ectopic expression of Wnt5a alone was not sufficient to activate ectopic Ihh expression. Consequently, the region of undifferentiated

Fig. 3. Reduced chondrocyte proliferation in the Wnt5a mutant. (A) BrdU labeled cells on sections of the distal humerus of 14.5 dpc embryos are shown. The chondrocyte region is divided into two zones before hypertrophy. Zone I is adjacent to the joint and composed of round cells. Zone II is composed of mostly flattened and columnar chondrocytes adjacent to the prehypertrophic chondrocytes. In the mutant (right), because there are no regular columnar chondrocytes, Zone II is half the region between the Zone I on each end of the cartilage. Zone II was shorter and contained fewer BrdU-labeling cells in the mutant, especially in the central region. The diameter of the cartilage was reduced in Zone I but expanded in Zone II when compared with the wild type. (B) Quantification of BrdU labeling experiments shown in A. Cell proliferation rate was higher in Zone II than in Zone I in the wild-type embryo. In the mutant, proliferation in Zone I was slightly increased, but proliferation in Zone II was reduced by about 30% in the mutant to a level similar to that in Zone I.
chondrocytes was lengthened in Col2a1-Wnt5a transgenic embryos, even though the entire skeletal element was shorter (Fig. 5B). However, cell proliferation was decreased in both Zone I and II in the Col2a1-Wnt5a transgenic mice, as indicated by BrdU labeling (Fig. 5C). Thus, surprisingly, both loss and gain of Wnt5a activity resulted in a similar perturbation of chondrocyte proliferation and differentiation.

Wnt5b was also expressed in the cartilage, and ectopic expression of Wnt5b delayed chondrocyte differentiation. Wnt5b is the closest Wnt relative to Wnt5a (amino acid identity 79%). Interestingly, Wnt5b was also expressed in chondrocytes in a region between prehypertrophic and hypertrophic chondrocytes (Fig. 6A). To test whether Wnt5b plays a similar role as Wnt5a in regulating chondrocyte proliferation and differentiation, Col2a1-Wnt5b transgenic mice were generated. Examination of these transgenic mice at 17.5 dpc indicates that chondrocyte hypertrophy was delayed and bone ossification was also reduced (Fig. 6B). In the most severe examples, the skull was open, a phenotype that correlated with Col2a1 expression in the brain (Cheah et al., 1991) and was not observed in any of the Col2a1-Wnt5a transgenic mice. When the long bone phenotype of Col2a1-Wnt5b was further analyzed, we found that expression of both Ihh and ColX was undetectable at 15.5 dpc (Fig. 6C) indicating chondrocyte differentiation was delayed prior to hypertrophic development as observed in Col2a1-Wnt5a mice. However, in contrast to Col2a1-Wnt5a mice, chondrocyte proliferation was increased in both Zone I and II in the Col2a1-Wnt5b transgenic mice (Fig. 6D).

Wnt5a and Wnt5b act differently in regulating chondrocyte proliferation and Col2a1 expression

Chondrocyte differentiation in Wnt5a−/−, Col2a1-Wnt5a and Col2a1-Wnt5b mice was similarly delayed before chondrocyte hypertrophy. However, chondrocyte proliferation appeared to have been altered differentially by Wnt5a and Wnt5b overexpression. As chondrocytes have two zones with different properties of cell proliferation before hypertrophy, we examined whether chondrocyte differentiation had been arrested in different zones in Wnt5a−/−, Col2a1-Wnt5a and Col2a1-Wnt5b mice. To achieve this, we first analyzed the expression of several cell cycle regulators that have been shown to control chondrocyte proliferation and differentiation. p130, one of the Rb proteins, is involved in cartilage development (Cobrinik et al., 1996). Rb proteins impede G1/S transition by inhibiting the S-phase-promoting transcription factor E2F (Dyson, 1998; Weinberg, 1995). Cyclin D1 acts as an intracellular sensor for mitogens (Sherr and Roberts, 1999) and has been shown to be a transcriptional target of Wnt signaling (Megason and McMahon, 2002). Cyclin D1-CDK4 complex promotes G1/S transition partly through phosphorylating and inactivating Rb (Weinberg, 1995). We found that in the wild-type cartilage, cyclin D1 and p130 protein expression was complementary to each other in Zone I and Zone II (Fig. 7A). Zone I expressed a higher level of P130 but a lower level of cyclin D1. Conversely, Zone II expressed a higher level of cyclin D1 but a lower level of p130. p130 was also expressed at a higher level in differentiating chondrocytes when cells exit Zone II. These expression patterns correlate well with the different cell cycling rates in these two zones. In the Wnt5a mutant, cyclin D1 expression...
Wnt5a coordinates cartilage development

was decreased. This was at least partly due to reduced and delayed Ihh expression, as Ihh overexpression was able to increase cyclin D1 expression in the Wnt5a mutant (Fig. 7A). Zone I, which expressed higher levels of p130, was reduced in size in the Wnt5a mutant. In addition, p130 expression was decreased in chondrocytes undergoing hypertrophy in the Wnt5a mutant (Fig. 7A). These data suggest that Wnt5a may exert two effects by upregulating p130 expression: holding cells in Zone I and promoting the exit from Zone II to the hypertrophic zone. Indeed, when Wnt5a was overexpressed in chondrocytes, cyclin D1 expression was suppressed, but p130 expression remained high (Fig. 7B,C), indicating that most chondrocytes in Col2a1-Wnt5a mice were held in Zone I. As Ihh, Pthpr and Col10a1 are not detected in Col2a1-Wnt5a number 17, the expression of Ihh, Pthpr and Col10a1 is not detected. (C) BrdU-labeled cells on sections of the distal radius of 14.5 dpc embryos are shown. Fewer chondrocytes were labeled by BrdU in the Col2a1-Wnt5a embryos, as Wnt5a was expressed at a higher level. The columnar chondrocyte structure was disrupted in the Col2a1-Wnt5a embryo.

showed that more chondrocytes in Col2a1-Wnt5b mice were in Zone II, which is highly proliferative. Thus, Wnt5a overexpression appears to prevent chondrocytes from entering Zone II, whereas Wnt5b overexpression promotes entrance to Zone II and prevents cell cycle withdrawal of Zone II chondrocytes.

As Wnt5a and Wnt5b exhibited opposite activities in regulating chondrocyte proliferation, we then examined whether they also differentially regulated chondrocyte-specific gene expression. The transcription factor Sox9 has been shown to play an important role in chondrocyte hypertrophy (Bi et al., 2001; Huang et al., 2001) and to control the expression of several chondrocyte-specific genes (Bi et al., 1999; Lefebvre and de Crombrugghe, 1998). To test whether Wnt5a or Wnt5b regulates Sox9 pathway, we first examined Sox9 protein expression in chondrocytes using immunohistochemistry. Sox9 protein levels were decreased in both Wnt5a−/− and Col2a1-Wnt5a mice, and no alteration was observed in Col2a1-Wnt5b mice when compared with the wild-type littermate (Fig. 8A).

As Col2a1 is a direct target of Sox9, we probed the transcription activity of Sox9 by examining Col2a1 expression
in Wnt5a+/-, Col2a1-Wnt5a and Col2a1-Wnt5b mice. Col2a1 expression was increased in the cartilage of the Wnt5a+/- and Col2a1-Wnt5b mice, but decreased in the Col2a1-Wnt5a mice (Fig. 8B). To obtain a more quantitative assessment, luciferase assay of the Col2a1 reporter was performed in mouse primary chondrocytes (Fig. 8C). Col2a1-luciferase activity was suppressed by Wnt5a but increased by Wnt5b in a dose-dependent manner. As many Wnts, including Wnt1, signal through activating LEF/TCF-mediated transcription (van Noort and Clevers, 2002), we examined the effect of TCF1 and Wnt1 on the Col2a1 reporter. We found that surprisingly, Wnt1 inhibited Col2a1 reporter activity. TCF1 had no effect on the Col2a1 reporter or a mutant Col2a1 reporter, which has mutated Sox9-binding site (Tanaka et al., 2000) (Fig. 8D). As TCF1 activated LEF/TCF reporter (driven by LEF/TCF binding sites) in the primary chondrocytes (data not shown), these results suggest the effect of Wnt5a or Wnt5b was independent of LEF/TCF factors. However, these data indicate that Wnt5a and Wnt5b have opposite activities in regulating Col2a1 expression, which suggests that increased Sox9 activity may also contribute to the delay in chondrocyte differentiation in Wnt5a-/- and Col2a1-Wnt5b mice.

**DISCUSSION**

By manipulating the expression of Wnt5a and Wnt5b genetically in mice, we show that both Wnt5a and Wnt5b play important roles in regulating longitudinal growth of developing long bones and yet they exhibit opposite activities (summarized in Fig. 9). We provide the first evidence that chondrocyte zones with distinct cell proliferation and differentiation properties express different key cell cycle regulators such as cyclin D1 and p130. Our studies uncover the transition from articular/resting chondrocytes (Zone I) to highly proliferative chondrocytes (Zone II) as a new Wnt5a-regulated step required for proper morphogenesis of developing long bones. Wnt5a is also required for the transition from proliferative to hypertrophic chondrocytes. Wnt5b appears to counteract the activity of Wnt5a by promoting Zone II formation and inhibiting chondrocyte hypertrophy. These effects may result from opposite activities of Wnt5a and Wnt5b in modulating the cell cycle regulators cyclin D1 and p130, and Sox9-controlled Col2a1 expression.

The cartilage of a developing long bone in the limb has a characteristic morphology, with distinct spatial organization of proliferative and differentiated chondrocyte zones (Fig. 9). This morphology is established and maintained during normal development through molecular interactions that control transitions between different chondrocyte zones. Mutations
that block or delay such transitions will result in abnormal accumulation of chondrocytes in one of the two zones before chondrocyte hypertrophy, which often leads to short and thick cartilages. This phenomenon has been observed in many mouse mutants, including those with abnormal FGF (Iwata et al., 2000; Naski et al., 1998), PTHrP (Schipani et al., 1997) and Wnt signaling described here. We show that one function of Wnt5a signaling is to keep the chondrocytes in a state that is fated to become articular/resting chondrocytes. Thus, Wnt5a may play a pivotal role in the development and maintenance of articular/resting chondrocytes in the joint and cartilage growth plates.

**Wnt5a signaling is required for chondrocyte differentiation and may downregulate Sox9 transcription activity**

The sequential proliferation and differentiation of chondrocytes are tightly regulated by multiple signaling molecules and transcription factors. Chondrocyte differentiation can be regulated at two stages according to previous studies: the transition from proliferative to prehypertrophic chondrocytes; and the transition from prehypertrophic to hypertrophic chondrocytes (Crowe et al., 1999; Vortkamp et al., 1996). Ihh expression serves as a marker for prehypertrophic chondrocytes and Col10a1 is a marker for hypertrophic chondrocytes. Wnt5a is expressed in proliferative and prehypertrophic chondrocytes and is required for the first transitional event, as chondrocyte differentiation was significantly delayed and both Ihh and Col10a1 expression were greatly reduced in the Wnt5a mutant. Consistent with this, it has been shown in chick that overexpression of a dominant-negative Frizzled leads to delayed chondrocyte differentiation, suggesting that in chick, some endogenous Wnt signals through Frizzled to promote chondrocyte differentiation (Hartmann and Tabin, 2000).

Ihh expression was significantly reduced in the Wnt5a mutant mice described here because of delayed chondrocyte differentiation. However, the phenotypes in the Wnt5a, Col2a1-Wnt5a and Col2a1-Wnt5b mice are opposite to those in Ihh and Pthrpr mice in which chondrocyte differentiation is accelerated. Moreover, overexpression of Ihh in chondrocytes did not rescue the...
phenotype of skeletal shortening in Wnt5a mutants to an extent comparable with the wild-type mice. Thus, it is unlikely that Wnt5a or Wnt5b signal is directly mediated by Ihh function. It is likely that Wnt5a, Wnt5b and Ihh-PTHrP signals act in parallel pathways to regulate a common downstream factor that plays a key role in chondrocyte proliferation and differentiation. One such common downstream factor might be Sox9, an HMG box transcription factor that is found to regulate chondrocyte differentiation as well as being required for chondrocyte cell fate determination (Bi et al., 2001). Prior studies suggest that PTHrP signaling may inhibit chondrocyte differentiation through upregulating Sox9 activity (Huang et al., 2001). We have shown that the expression of Col2a1, a direct transcriptional target of Sox9 is increased in the Wnt5a mutant but decreased in Col2a1-Wnt5a transgenic mice. Thus, we conclude that Wnt5a may promote chondrocyte hypertrophy in part through decreasing the transcriptional activity of Sox9. Interestingly, we find Wnt5b, which is expressed at the boundary of prehypertrophic and hypertrophic chondrocytes, exhibits opposite activities in regulating Col2a1 expression, suggesting that Wnt5b may enhance Sox9 transcription activity in a dose dependent manner (Fig. 8). It is possible that Wnt5b plays a similar role to Pthlh in inhibiting chondrocyte hypertrophy through enhancing Sox9 transcription activity, which may influence chondrocyte differentiation by controlling the expression of chondrocyte specific genes (Bi et al., 1999; Lefebvre and de Crombrugghe, 1998). It will be interesting to investigate how Sox9 transcriptional activity is altered by Wnt5a and Wnt5b signaling and how Wnt and PTHrP signaling pathways interact with each other in chondrocytes.

**Proliferative chondrocytes can be divided into two zones with distinct proliferation properties and cell morphology**

Chondrocyte proliferation and differentiation are tightly associated with cell cycle progression and withdrawal. Zone I and Zone II chondrocytes have distinct morphology, developmental fate and cell cycling rate (Abad et al., 2002; Iwamoto et al., 2000; Long et al., 2001), which has prompted us to search for the underlying molecular mechanism. We find that a negative cell cycle regulator p130 and a positive regulator...
Wnt5a coordinates cartilage development

Figure 9. Proposed model for the action of Wnt5a and Wnt5b in regulating chondrocyte proliferation and differentiation. The morphology of a developing long bone is schematically shown on the left. Molecular interactions in regulating chondrocyte proliferation and differentiation are shown on the right. The major findings of this study are that Zone I and Zone II chondrocytes express p130 and cyclin D1 differentially, and that Wnt5a and Wnt5b regulate multiple transitional steps between different chondrocyte zones. Wnt5a expression is in purple. Wnt5b expression is in light blue. We show that Wnt5a promotes the expression of p130 but inhibits the expression of cyclin D1. Wnt5a may also downregulate Sox9 transcription activity. We propose these are the underlying mechanisms whereby Wnt5a inhibits the transition from Zone I to Zone II, but promotes the transition from Zone II to the prehypertrophic region. Conversely, Wnt5b enhances Sox9 transcription activity and cyclin D1 expression. This promotes Zone II formation but inhibits cell cycle withdrawal and chondrocyte hypertrophy. Thus, Wnt5a and Wnt5b act in parallel pathways to the Ihh and PTHrP negative feedback loop to coordinate chondrocyte proliferation and differentiation. Sox9 and cell cycle regulators may be the common targets.

Wnt5a and Wnt5b regulate chondrocyte proliferation through distinct pathways

It is likely that cell cycle regulators are also common targets of signaling molecules in regulating chondrocyte proliferation. Chondrocyte differentiation is delayed in both Col2a1-Wnt5a and Col2a1-Wnt5b transgenic mice. However, the differentiation delay in these two transgenic mice is caused by different regulatory activities of Wnt5a and Wnt5b in chondrocytes (summarized in Fig. 9). p130 and cyclin D1 expression is regulated by Wnt5a and Wnt5b in distinct manners. Wnt5a increases p130 expression but suppresses cyclin D1 expression. Thus, Zone I is expanded at the expense of Zone II and chondrocyte proliferation is decreased in Col2a1-Wnt5a mice. In addition, decreased p130 expression may also lead to delayed transition from proliferative to hypertrophic chondrocytes in the Wnt5a−/− embryo. Wnt5b appears to have some redundant activities with both Ihh and PTHrP signaling in promoting cyclin D1 expression and Sox9 transcription activity. As a result, entrance to Zone II is enhanced, whereas Zone II to hypertrophic chondrocyte transition was delayed. We conclude that Wnt5a overexpression delays chondrocyte hypertrophy indirectly as a result of prolonged residence of chondrocytes in Zone I, whereas Wnt5b overexpression directly delays chondrocyte hypertrophy by inhibiting cell cycle withdrawal and activating Sox9 activity.

In the Wnt5a−/− limb, chondrocytes enter Zone II prematurely but terminal differentiation is delayed as a combined result of decreased p130 expression and enhanced Sox9 activity in differentiating chondrocytes. This may cause reduced diameter of Zone I and enlarged diameter of Zone II (Fig. 3A). It is likely that the parallel columnar structure of chondrocytes in the proliferative chondrocyte zone ensures the normal longitudinal long bone growth and cartilage diameter. This process may be regulated by both Wnt5a and Wnt5b as suggested by the phenotypes of disrupted columnar chondrocyte organization, and short and thickened cartilages in Wnt5a−/−, Col2a1-Wnt5a and Col2a1-Wnt5b mice (Fig. 3A, Fig. 5C, Fig. 6D).

Taken together, Wnt5a and Wnt5b play a pivotal role in regulating the proper longitudinal bone growth by setting a proper pace during chondrocyte transition between different zones and controlling cell shape and polarity. We propose that when Zone I cells enter zone II, they express Wnt5a which signals back to maintain a stronger expression of p130 and a lower expression of cyclin D1 to prevent more cells from leaving Zone I. In addition, Wnt5a activates p130 expression and may suppress Sox9 activity to allow chondrocytes to leave Zone I and undergo hypertrophy. The newly formed hypertrophic chondrocytes express Wnt5b, which signals back to Zone II to prevent more cells from leaving Zone II (Fig. 9). It is likely that proper regulation of the transition between Zone I and II by Wnt5a is important in cartilage and bone development, and is essential in maintaining the articular cartilage throughout life.
The functional difference of Wnt5a and Wnt5b may rely on which of the Wnt receptors, namely Frizzleds, are expressed in chondrocytes. Vertebrate Wnt family comprises at least 18 different members and there are at least 10 different Frizzleds in the human genome (http://www.stanford.edu/~rnusse/wntgenes/mousewt.html). So far, three distinct Wnt signaling pathways have been identified (Niehrs, 2001; Winklbauer et al., 2001). It has been shown that the canonical Wnt pathway, which is mediated by β-catenin-LEF/TCF transcription complex, positively regulates cyclin D1 expression (Shutman et al., 1999; Tetsu and McCormick, 1999). Our data indicate that Wnt5b may signal through the canonical pathway, whereas Wnt5a antagonizes it. This is supported by recent studies that show a Wnt/Ca2+ pathway mediated by the Xenopus Wnt5a decreases β-catenin protein level (Saneyoshi et al., 2002). It is interesting to note that Wnt1, a canonical Wnt in other systems, acts similarly to Wnt5a in regulating Col2a1 reporter in the primary chondrocytes. It is likely that a single Wnt, for example Wnt5a or Wnt1, can elicit different responses depending on which Frizzled is expressed (He et al., 1997; Slusarski et al., 1997a; Slusarski et al., 1997b). We find that when human Frizzled 5 is expressed together with either Wnt5a or Wnt5b in the early Xenopus embryos, a secondary axis is induced (data not shown), indicating Wnt5a and Wnt5b both act as Wnt1 when Frizzled 5 is available. As Frizzled 5 shows little specificity with different Wnts (Deardorff et al., 2001), it is unlikely that Frizzled 5 is a major Frizzled involved in transducing Wnt5a/Wnt5b signals in chondrocytes. Since several different Frizzled genes are expressed in the developing cartilage (Xu et al., 2001), Wnt5a and Wnt5b signaling in chondrocytes may be very complex, and involve both canonical and non-canonical pathways at the same time.

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