Development 127, 3141-3159 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4388

Dual roles of Wnt signaling during chondrogenesis in the chicken limb

Christine Hartmann and Clifford J. Tabin*

Harvard Medical School, Department of Genetics, Boston, Massachusetts 02115, USA
*Author for correspondence (e-mail: Tabin@rascal.med.harvard.edu)

Accepted 5 May; published on WWW 22 June 2000

Summary

Long bones of the appendicular skeleton are formed from a cartilage template in a process known as endochondral bone development. Chondrocytes within this template undergo a progressive program of differentiation from proliferating to postmitotic prehypertrophic to hypertrophic chondrocytes, while mesenchymal cells immediately surrounding the early cartilage template form the perichondrium. Recently, members of the Wnt family of secreted signaling molecules have been implicated in regulating chondrocyte differentiation. We find that *Wnt-5a*, *Wnt-5b* and *Wnt-4* genes are expressed in chondrogenic regions of the chicken limb: *Wnt-5a* is expressed in the perichondrium, *Wnt-5b* is expressed in a subpopulation of prehypertrophic chondrocytes and in the outermost cell layer of the perichondrium, and *Wnt-4* is expressed in cells of the joint region. Misexpression experiments demonstrate that two of these Wnt molecules, *Wnt-5a* and *Wnt-4*, have opposing effects on the differentiation of chondrocytes and that these effects are mediated through divergent signaling pathways. Specifically, *Wnt-5a* misexpression delays the maturation of chondrocytes and the onset of bone collar formation, while *Wnt-4* misexpression accelerates these two processes. Misexpression of a stabilized form of β-catenin also results in accelerated chondrogenesis, suggesting that a β-catenin/TCF-LEF complex is involved in mediating the positive regulatory effect of Wnt-4. A number of the genes involved in Wnt signal tranduction, including two members of the *Frizzled* gene family, which are believed to encode Wnt-receptors, show very dynamic and distinct expression patterns in cartilaginous elements of developing chicken limbs. Misexpression of putative dominant-negative forms of the two Frizzled proteins results in severe shortening of the infected cartilage elements due to a delay in chondrocyte maturation, indicating that an endogenous Wnt signal does indeed function to promote chondrogenic differentiation.

Key words: Chondrocyte differentiation, Bone collar, Wnt, Frizzled, β-catenin, Chick

INTRODUCTION

Skeletal elements of vertebrate limbs are derived from mesenchymal cells, which condense to form initial prechondrogenic cell aggregates. These early condensations, by their position, shape and size, prefigure the future skeletal elements (Hinchcliffe and Johnson, 1990). Cells within the core of these condensations differentiate into chondrocytes, synthesizing and secreting an extensive extracellular matrix (Pechak et al., 1986; Hall, 1988), while fibroblast-like cells in the periphery go on to form a perichondrial sheath surrounding the cartilage element (Rooney and Archer, 1992). Initial growth of these cartilage elements is due to proliferation of chondrocytes and perichondrial cells, as well as deposition of new matrix. Subsequently, chondrocytes in the middle of the cartilage element (diaphysis) exit the cell cycle and mature into hypertrophic chondrocytes, characterized by an increase in cell size and by secretion of a distinct extracellular matrix, which eventually becomes calcified (Poole, 1991). As a result, immature proliferating chondrocytes are only found at each end (epiphysis) of developing bones, appearing as two morphologically distinct populations. Proliferating chondrocytes in the articular region are small, round and closely packed, while adjacent to them is a zone of radially flattened proliferating chondrocytes (Fig. 8). The different populations of chondrocytes are morphologically distinguishable in cartilage elements of the stylopod and zeugopod from day 7.5 onwards, and express distinct molecular markers. For example, proliferating chondrocytes as well as a subset of the non-proliferating chondrocytes express the marker Col-II (Nah et al., 1988), while the non-proliferating hypertrophic cells express the marker Col-X (Linsenmayer et al., 1991). The population of non-proliferating Col-II expressing chondrocytes also expresses the marker Ihh and is referred to as the prehypertrophic chondrocytes (Vortkamp et al., 1996).

The maturation process of the chondrocytes is accompanied by characteristic morphological changes within the surrounding perichondrium. Initially the cells in the perichondrium are small and round chondroblasts, which are morphologically indistinguishable from the proliferating chondrocytes. As the cartilage element begins to mature, the chondroblasts in the articular perichondrium maintain this morphology, while these surrounding the diaphysis become flattend. As the adjacent chondrocytes hypertrophy the perichondrium of the diaphysis differentiates into a structure

known as the periosteum. The innermost periosteal cells adjacent to the region of hypertrophied chondrocytes differentiate into osteoblasts, which secrete a bone matrix (osteoid) that becomes progressively calcified, forming a membranous bone collar around the diaphysis of the cartilage element (Caplan and Pechak, 1987). The outer layer of the periosteum consists of several layers of longitudinally elongated cells, and elongated perichondrial cells also surround the zone of flattened chondrocytes (Rooney and Archer, 1992) (Fig. 8). Several growth factors and/or their receptors have been reported to be expressed in cells of the perichondrium, including members of the TGF-β superfamily and their receptors (Thorp et al., 1992; Macias et al., 1997; Zou et al., 1997), the hedgehog receptor Patched-1 (Ptc-1) (Vortkamp et al., 1996), Parathyroid hormone-related peptide (PTHrP) and the PTH/PTHrP-receptor (Vortkamp et al., 1996), retinoid acid and one of its receptors (Koyama et al., 1999) and Fgf receptors (Delezoide et al., 1998; Szebenyi et al., 1995). In contrast to molecular markers expressed in chondrocytes, which have been assigned to different subpopulations, the expression of perichondrial markers has not been as well defined.

The molecular mechanisms controlling the differentiation program of chondrocytes and surrounding perichondrial/periosteal cells are just starting to be elucidated (for reviews see Reddi, 1994; Erlebacher et al., 1995; Wallis, 1996; Rodan and Harada, 1997). Several lines of evidence suggest that signals from the perichondrium negatively regulate chondrocyte differentiation (Vortkamp et al., 1996; Zou et al., 1997; Long and Linsenmayer, 1998). One of the key negative regulators identified so far is the secreted molecule PTHrP, which is normally expressed in the periarticular region (Lee et al., 1995). The expression of PTHrP in the periarticular region is regulated by Ihh signaling (Vortkamp et al., 1996; St-Jacques et al., 1999). The current model is that Ihh, secreted by the prehypertrophic chondrocytes, signals to its receptor Ptc, which is expressed in cells of the surrounding perichondrium (Vortkamp et al., 1996). Ihh signaling has been proposed to act on the expression of BMPs in the perichondrium (Zou et al., 1997; Pathi et al., 1999), which in turn signal through BMPRIA, expressed in the periarticular region and in prehypertrophic chondrocytes (Zou et al., 1997). Ihh and BMP signaling ultimately regulate the level of periarticular PTHrP, which signals to its receptor expressed in chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997; Chung et al., 1998; St-Jacques et al., 1999). This signaling loop forms a feedback mechanism, which ultimately negatively regulates the number of cells committing to a prehypertrophic fate.

Another group of secreted signaling molecules implicated in limb development are encoded by members of the *Wnt* gene family (Dealy et al., 1993; Parr and McMahon, 1995; Riddle et al., 1995; Vogel et al., 1995; Yang and Niswander, 1995; Kengaku et al., 1998; Kawakami et al., 1999; Yamaguchi et al., 1999). *Wnt* genes are defined by sequence similarity to the founding members *Wnt-1* in mouse (originally called int-1; Nusse and Varmus, 1982; van Ooyen and Nusse, 1984) and *wingless* (*wg*) in *Drosophila* (Cabrera et al., 1987; Rijsewijk et al., 1987). To date, 19 members of the *Wnt* family have been identified in higher vertebrates (Wnt-homepage: http://www.stanford.edu/~rnusse/wntwindow.html), and they have been implicated in regulating a variety of processes during development (for reviews see Nusse and Varmus, 1992; Cadigan and Nusse, 1997; Moon et al., 1997). For example, recent gain-of-function studies in mouse (Zakany and Duboule, 1993) and chick (Rudnicki and Brown, 1997; Kawakami et al., 1999) have suggested that Wnt molecules exert negative effects on chondrogenesis; however, the cellular mechanism by which they act is unclear.

Wg/Wnt proteins bind to seven-transmembrane receptors encoded by the *Frizzled* (*Fz*) gene family (Bhanot et al., 1996; Wang et al., 1996). There is evidence suggesting that several intracellular pathways can be activated in response to stimulation by a Wnt signal (Kengaku et al., 1998; Lee et al., 1999; Peters et al., 1999; Sakanaka et al., 1999; for review see Cox and Peifer, 1998; Bejsovec, 1999; Arias et al., 1999); however, only one of these signaling pathways has been well characterized using genetic and biochemical tools. In this classical pathway, Wg/Wnt signaling eventually leads to the stabilization of an effector protein, known as armadillo (arm) in flies and β-catenin (β-cat) in vertebrates, resulting in the activation of Wg/Wnt target genes through a complex containing arm/β-cat and DNA-binding transcription factors encoded by the *TCF/LEF* gene family (for reviews see Cadigan and Nusse, 1997; Moon et al., 1997; Cox and Peifer, 1998). Recent studies have led to the conclusion that the diversity of the signal transduction pathways downstream of the Wnt signals is mediated at the level of the receptors, which can be subdivided into at least two classes on the basis of their intracellular response: one class, which functions through the β-cat/TCF-LEF complex, and a second class, which functions by stimulating an increase in intracellular Ca^{2+} and the subsequent activation of protein kinase C (Sheldahl et al., 1999; for review see Miller et al., 1999).

In this study, we show that three *Wnt* genes and various components of the Wnt signaling pathway are expressed in particular chondrogenic regions of the chick limb. Furthermore we use the replication-competent avian retrovirus system to demonstrate that misexpression of two of the *Wnt* genes, *Wnt-5a* and *Wnt-4*, result in opposite effects on the maturation process of chondrocytes: *Wnt-5a* misexpression delays chondrocyte maturation, while *Wnt-4* misexpression accelerates both chondrocyte and perichondrium maturation. In addition, we present evidence that the acceleration of chondrogenesis in response to Wnt signaling is mediated by β-catenin. Misexpression of dominant-negative Frizzleds belonging to the β-cat/TCF-mediated class results in a delay in chondrocyte maturation, supporting an endogenous role for β-catenin-mediated Wnt signaling in positively regulating chondrogenesis.

MATERIALS AND METHODS

RNA probes

Antisense riboprobes radiolabeled with [^{33}P]UTP were prepared as described: *Chfz1*, *Chfz7* (Kengaku et al., 1997), *Lef-1* (Kengaku et al., 1998), *Ihh*, *PTHrP* and *PTH/PTHrP-receptor* (Vortkamp et al., 1996), *Col-X* (LuValle et al., 1988) and *Bmp-4* (Francis et al., 1994). Riboprobes for cWnt-5a and cWnt-5b were kindly provided by A. McMahon. A cDNA for chicken *Patched-2* (*Ptc-2*) was kindly provided by R.V. Pearse. Radiolabeled riboprobes for chick *Wnt-4* (containing the full-length open reading frame of *Wnt-4* in pGEM-T (Tanda et al., 1995) amplified by RT-PCR), chick *Tcf-4*

(corresponding to amino acids 163-290 of mouse TCF-4; amplified by RT-PCR using degenerate primers and cloned into pGEM-T), chick *bone sialoprotein II* (*cBspII*; RT-PCR product corresponding to amino acids 79-257 (Yang et al., 1995) cloned into pGEM-T) and chick *osteopontin* (*cOp*; RT-PCR product corresponding to amino acids 34-249 (Castagnola et al., 1991) cloned into pGEM-T) were generated by transcription reactions using Sp6-RNA polymerase from templates linearized with the restriction enzyme *Nco*I. A radiolabeled riboprobe for *β-catenin* (Lu et al., 1997; RT-PCR product corresponding to amino acids 64-272 cloned into pGEM-T) was generated by a transcription reaction using T7-RNA polymerase from a *Spe*I-linearized template.

Processing of chick embryos and in situ hybridization

White Leghorn chicken eggs were obtained from SPAFAS (Norwich, CT, USA) and incubated at 37.5°C for the desired length of time and staged according to Hamburger and Hamilton (1951) (HH). Embryos were fixed in 4% paraformaldehyde/PBS, pH 7.4, overnight at 4°C and processed either for whole embryo skeletal staining or paraffin sectioning. Limbs for paraffin sectioning were dehydrated through an ethanol series and embedded in paraffin according to standard protocols (Prophet, 1994). Uninjected contralateral (left) wing and virally infected (right) wing were treated in parallel and embedded into the same mold. For serial sections, 2-3 continuous microtome sections of 5-6 μm were successively collected on 5-9 alternating slides. Radioactive RNA-section in situ hybridization on paraffin sections was carried out as described by Tessarollo et al., (1992), with the following modifications. Slides were hybridized at 70°C in a humidified chamber for 18 hours under polypropylene coverslips with a solution containing 50% formamide, 10% dextran sulfate, 300 mM NaCl, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 10 mM $NaPO_4$ buffer, pH 8.0, 1× Denhardt's, 0.5 mg ml^{-1} yeast total RNA and radiolabeled probe. Posthybridization steps included RNAse A (25 μg ml^{-1}, Boehringer) treatment for 60 minutes at 37% followed by a high-stringency wash in 50% formamide/2× SSC at 55°C. The slides were dehydrated, dipped in NTB-2 emulsion (Kodak), exposed for 3-7 days, developed and counterstained with 0.1% Toluidine Blue.

Construction of retroviral constructs and viral misexpression

The retroviral construct RCASBP(A)/stabilized β-catenin has been described previously (Kengaku et al., 1998). The RCASBP(A) construct carrying the full-length open reading frame of mWnt-5a construct was kindly provided by Y. Yang and A. McMahon. The RCASBP(A) constructs carrying the full-length open reading frames of *mWnt-4* (5′ *Bsa*I-primer: GGTCTCCATGAGCCCCCGTTCGT-GC; 3′ *Eco*RI-primer: CTGAATTCACCGGCACGTGTGCATCTCC), *Chfz-1* (5′ *Nco*I-primer: TGACCATGGCCGAGCGGCGCGGG; 3′ *Eco*RI-primer: CTGAATTCTCACACGGTGGTCTCAC), *Chfz-7* (5′ *Bsa*I-primer: CAGGTCTCCCATGCGGCCCGCGGC; 3′ *Eco*RI-primer: TGGAATTCTCATACCGCCGTCTCGC), and the constructs carrying C-terminal truncated coding regions of *Chfz-1* (5′ *Nco*I-primer: TGACCATGGCCGAGCGGCGCGGG; 3′ *Eco*RI-primer: GCGAATTCTTACCCCGACCAGATCC) and Chfz-7 (5′ *Bsa*I-primer: CAGGTCTCCCATGCGGCCCGCGGC; 3′ *Eco*RI-primer: GCGAATTCTTAGCCAGACCAGATCC) were engineered as outlined in Logan and Tabin (1998). Transfection and growth of RCAS viruses were performed as described by Morgan and Fekete (1996). Concentrated virus with a titer of $1\text{-}6\times10^8$ pfu/ml was injected into the posterior half of the right wing bud of HH 21-23 embryos. This targeted injection resulted in localized infection of the posterior cartilage elements such as ulna and posterior digits, and a more widespread infection within the soft tissues, as assayed by expression of exogenous or viral transcripts (not shown). In most cases, only the infected cartilage elements showed phenotypes, whereas uninfected elements served as an internal control and were similar to the elements of the uninjected contralateral limbs. Injections into the limb field at day 1.5 (HH 9-10) resulted in an infection of the whole limb, but often influenced the survival of the infected embryos, for unknown reasons. However, the early infection did not produce a different or more pronounced phenotype in the cartilage elements, and therefore all of the injections were carried out at HH 21-23, allowing for a more controlled infection of only the posterior region of the wing bud.

Skeletal and histological staining

Whole-mount Alcian Blue staining of cartilage of day 9.5 chick embryos was done as previously described in Goff and Tabin (1997). Whole-mount Alcian Blue/Alizarin Red staining of day 9.5 chick embryos was carried according to McLeod (1980). Weigert-Safranin O staining of paraffin sections was done according to the protocol described by Gaffney (1994).

RESULTS

Expression of *Wnts* and components of the Wnt-signaling pathway during chondrogenesis

To determine if Wnt signaling is involved in regulating chondrogenesis, we screened a number of *Wnt* genes and components of the Wnt signaling pathway to see whether any were expressed in the chondrogenic regions of the developing chick limb. We found that three *Wnt* genes, *Wnt-5a*, *Wnt-5b* and *Wnt-4*, are expressed in limb cartilage elements. As previously reported (Dealy et al., 1993; Kawakami et al., 1999), in situ hybridizations using a *Wnt-5a* anti-sense riboprobe to sections of chicken wing buds at different developmental stages revealed that *Wnt-5a* is initially expressed in the distal mesenchyme of early chick limb buds (data not shown). As the limb bud grows out, *Wnt-5a* continues to be expressed in the mesenchyme in a distal to proximal gradient, but as the skeletal elements start to form, *Wnt-5a* expression becomes excluded from the condensing core mesenchyme (Dealy et al., 1993; Kawakami et al., 1999). *Wnt-5a* continues to be expressed in mesenchymal cells immediately adjacent to the early cartilage elements, however, as previously noted (Dealy et al., 1993; data not shown). These cells will eventually contribute to the perichondrium. *Wnt-5a* expression in the perichondrium/periosteum is spatially restricted to the middle region of the cartilage elements (diaphysis) from HH 30 onwards; its expression does not extend to the articular region (Fig. 1A).

The highly related *Wnt* gene, *Wnt-5b*, is expressed in the apical ectodermal ridge (AER) of the early limb bud (Kengaku et al., 1997; data not shown). On sections, we detected a strong expression of *Wnt-5b* at HH 27 in the mesenchyme surrounding cartilage elements of the wing (data not shown). From HH 28 onwards, *Wnt-5b* expression can be detected in a subpopulation of chondrocytes in the middle region of the cartilage elements (data not shown). At HH 29, the mesenchymal expression of *Wnt-5b* becomes refined to the outer cell layer of the perichondrium and the expression in chondrocytes of the diaphysis is more intense (Fig. 1B). In contrast to *Wnt-5a*, *Wnt-5b* expression in the perichondrium is not restricted to the diaphysis; rather *Wnt-5b* is expressed in perichondrial cells surrounding the entire cartilage element and in chondrocytes of the diaphysis (HH 32; Figs 1B, 5D).

Recently, *Wnt-4* has been reported to be expressed in regions of developing joints in the chick (Kawakami et al., 1999). In whole-mount in situ hybridizations, *Wnt-4*

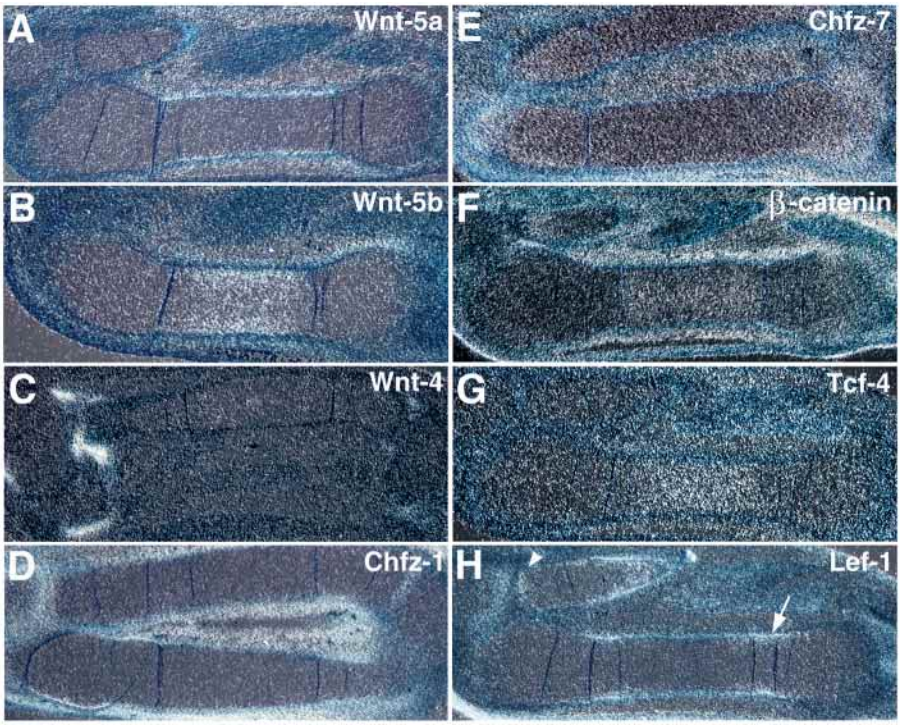

Fig. 1. Endogenous expression patterns of *Wnt-5a*, *Wnt-5b*, *Wnt-4*, *Chfz-1*, *Chfz-7*, *β-catenin*, *Tcf-4* and *Lef-1* in the ulna of a chick wing at HH 32 (day 7.5 of development). Radioactive in situ hybridizations on 5 μm microtome sections. (A,B,C,F,G,H) Alternate sections of one HH 32 forearm skeletal elemen; (D,E) alternate sections of another HH 32 forearm skeletal element. (A) *Wnt-5a* is expressed in the perichondrium of the diaphysis. (B) *Wnt-5b* is expressed in chondrocytes of the diaphysis and in cells of the outer layers of the perichondrium surrounding the skeletal element. (C) *Wnt-4* is expressed in chondroblasts flanking the joint regions. (D) *Chfz-1* is expressed in perichondrial cells surrounding the skeletal element. (E) *Chfz-7* is expressed in chondrocytes at the ends of the skeletal elements in the articular region and at lower levers in cells of the perichondrium. (F) *β-catenin* is expressed at low levels throughout the wing and at high levels in cells of the perichondrium. *β-catenin* is also expressed in high levels in chondrocytes of the diaphysis and to a lesser extent in chondrocytes of the articular region. (G) *Tcf-4* is expressed at low levels throughout the limb mesenchyme and at high levels in chondrocytes of the diaphysis. (H) *Lef-1* is expressed in cells of the perichondrium surrounding the diaphysis of the skeletal element and at a higher levels in perichondrial chondroblasts in a region abutting prehypertrophic chondrocytes (arrow). In addition, *Lef-1* is expressed in chondrocytes of the articular region (arrowhead).

expression was reported to be first visible at HH 27 in the joint of the elbow region and from stage 30 onwards in joint regions of the digits (Kawakami et al., 1999); however, we detect *Wnt-4* expression in joint regions of the digits in section in situ hybridizations from as early as HH 27 onwards (data not shown), where *Wnt-4* is expressed at high levels in populations of mesenchymal cells flanking the joint regions (Fig. 1C). In addition, we detect expression of *Wnt-4* in the limb bud ectoderm (data not shown).

We further examined the expression patterns of two members of the Wnt receptor family, the *Fz* genes. Both *Chfz-1* and *Chfz-7* show dynamic expression patterns in cartilaginous elements of the wings. At HH 27, *Chfz-1* is expressed at low levels in chondrocytes of the diaphysis but is excluded from epiphyseal chondrocytes (data not shown). *Chfz-1* is additionally expressed throughout the adjacent mesenchymal cells. From HH 29 onwards, *Chfz-1* expression becomes restricted to cells of the perichondrium surrounding the cartilage elements and can no longer be detected in the chondrocytes themselves (Fig. 1D). At stages later than HH 32, *Chfz-1* expression within the perichondrium becomes upregulated in a region surrounding the zone where flattened proliferating chondrocytes abut prehypertrophic chondrocytes (data not shown). At HH 27, the expression pattern of *Chfz-7* is similar to *Chfz-1* (data not shown); however, *Chfz-7* is expressed in a broader domain than *Chfz-1* in chondrocytes of the cartilage elements. This becomes more obvious at HH 28, when *Chfz-7* expression can be detected in chondrocytes of the articular region, which do not express *Chfz-1* (data not shown). From HH 29 onwards, *Chfz-7* expression becomes restricted to the proliferating chondrocytes in the articular region of the cartilage element; lower levels of expression can also be detected in cells of the perichondrium (Fig. 1E).

We further analyzed the expression pattern of several downstream effector molecules of the Wnt signaling pathway. *β-catenin* is expressed at low levels throughout the developing limb, including the cartilage elements. Interestingly, *β-catenin* is expressed at high levels in the cells of the perichondrium, in more mature prehypertrophic as well as hypertrophic chondrocytes, and at slightly lower levels in chondrocytes of the articular region at HH 32 (Fig. 1F). *Tcf-4* is expressed in a domain encompassing prehypertrophic and hypertrophic chondrocytes from HH 30 onwards (Fig. 1G), overlapping with the *β-catenin* expression domain. Another member of the LEF/TCF-family, *Lef-1*, is expressed throughout the perichondrium of the developing cartilage elements at HH 29 (data not shown). From HH 32 onwards, we observed increased levels of *Lef-1* expression in a region of the perichondrium surrounding those prehypertropic chondrocytes expressing high levels of *Ihh* (Fig. 1H and data not shown). The *Lef-1* expression pattern at HH 32 resembles the expression of the Wnt receptor, *Chfz-1*, in the perichondrium at later stages. In addition, *Lef-1* is expressed in chondrocytes of the articular region (Fig. 1H).

Misexpression of Wnt-5a or Wnt-4 results in shortening of the long bones

Since we observed that the expression patterns of *Wnt-5a*, *Wnt-5b* and *Wnt-4* are temporally regulated and spatially restricted within the differentiating cartilage elements, we next asked whether these *Wnt* genes might play a functional role during chondrogenesis. In the current study we focused our attention on two of these genes, *Wnt-5a* and *Wnt-4*. The role of *Wnt-5b*

will be addressed in future studies. In order to address the function of *Wnt-5a* and *Wnt-4* we conducted retroviral misexpression experiments using replication competent avian retroviruses (RCAS). Misexpression of either *Wnt-5a* or *Wnt-4* in the posterior half of HH 21-23 chicken wing buds resulted in a statistically significant shortening of posterior skeletal elements of the zeugopod and the autopod in the infected limb (Fig. 2A,B). The difference in length (Fig. 2G,H) of affected skeletal elements compared to elements of the contralateral wing was determined at day 9.5 of development (HH 35-36). Although individual embryos were affected to varying degrees (indicated by the error bars in Fig. 2G,H), shortening of the skeletal elements was observed in 9 out of 10 limbs infected with the *Wnt-5a* expressing virus, and in 8 out of 10 limbs infected with the *Wnt-4* expressing virus. In limbs infected with *Wnt-5a*, shortening of the ulna in the infected limb was first detectable at day 7 (HH 30-31). In contrast, shortening of the cartilage elements in limbs infected with *Wnt-4* was not detectable before day 9.5 of development. While a slight shortening of the humerus was also observed in 50% of the *Wnt-5a* infected wings (n=10), no shortening of the humerus was detected in any of the *Wnt-4* affected wings (n=8).

Wnt-5a misexpression delays chondrocyte differentiation

Shortening of the long bones can result from a variety of different underlying mechanisms, including either a delay (Vortkamp et al., 1996; Zou et al., 1997; Crowe et al., 1999), an acceleration of chondrocyte differentiation (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996) or a decrease in chondrocyte proliferation (St-Jacques et al., 1999; Karp et al., 2000). In addition, defects in the differentiation of osteoblasts also result in shortening of cartilage elements (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). We therefore examined the status of chondrocyte differentiation in cartilage elements infected with *Wnt-5a*. From day 7.5 (HH 31-32) onwards, three morphologically distinct zones of

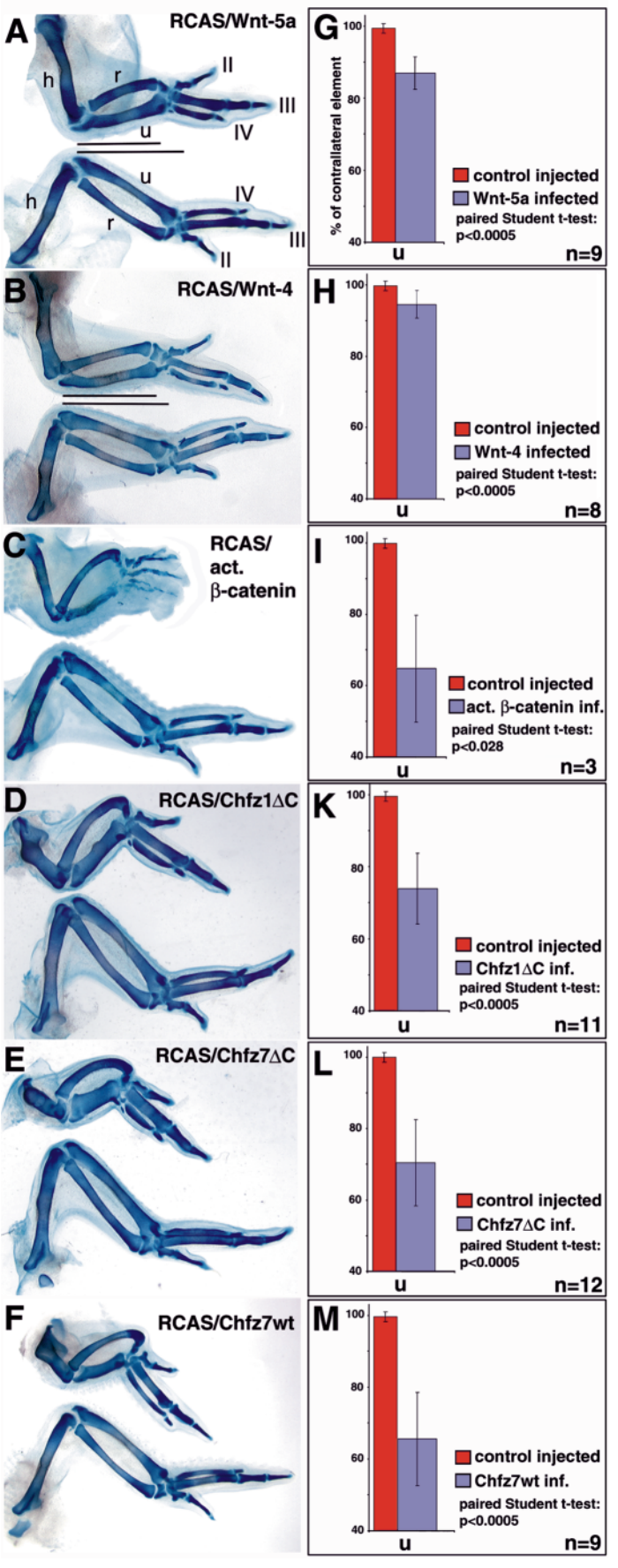

Fig. 2. (A-F) Shortening of skeletal elements caused by misexpression of various retroviral constructs visualized by whole-mount Alcian Blue staining at day 9.5. All wing pairs are orientated proximal towards the left, distal towards the right. Injected right wings are on top of the uninjected contralateral control left wing. (G-M) Blue bars represent the mean length of the infected ulnae, red bars, the mean length of control injected ulnae (n=21) as a percentage of the contralateral control element. Values are means ± s.d. Large deviations in the individual experimental data sets result from variable levels of infections in the individual embryos, which result in variable degrees of shortening of the affected cartilage element. Due to the nature of the injection into the posterior half of HH 22-23 wing buds, the ulna is always more affected than the radius. The number of specimens analyzed (n) for each retroviral misexpression experiment is indicated in the bottom left corner of the diagrams (G-M). h, humerus; u, ulna; r, radius; II-IV, digits. (A,G) RCASBP/mWnt-5a infected; (B, H) RCASBP/mWnt-4 infected limbs show minor reduction in the size of posterior skeletal elements, such as the ulna; (C,I) RCASBP/activated β-catenin infected limbs show a severe shortening of the ulna. Note the polydactyly phenotype in the infected limb. (D,K) RCASBP/Chfz1ΔC infected; (E,L) RCASBP/Chfz7ΔC infected; (F,M) RCASBP/Chfz7wt infected. Note that the humeri in these limbs are severely shortened and bent, and that the radii are also bent due to the shortening of the infected ulna.

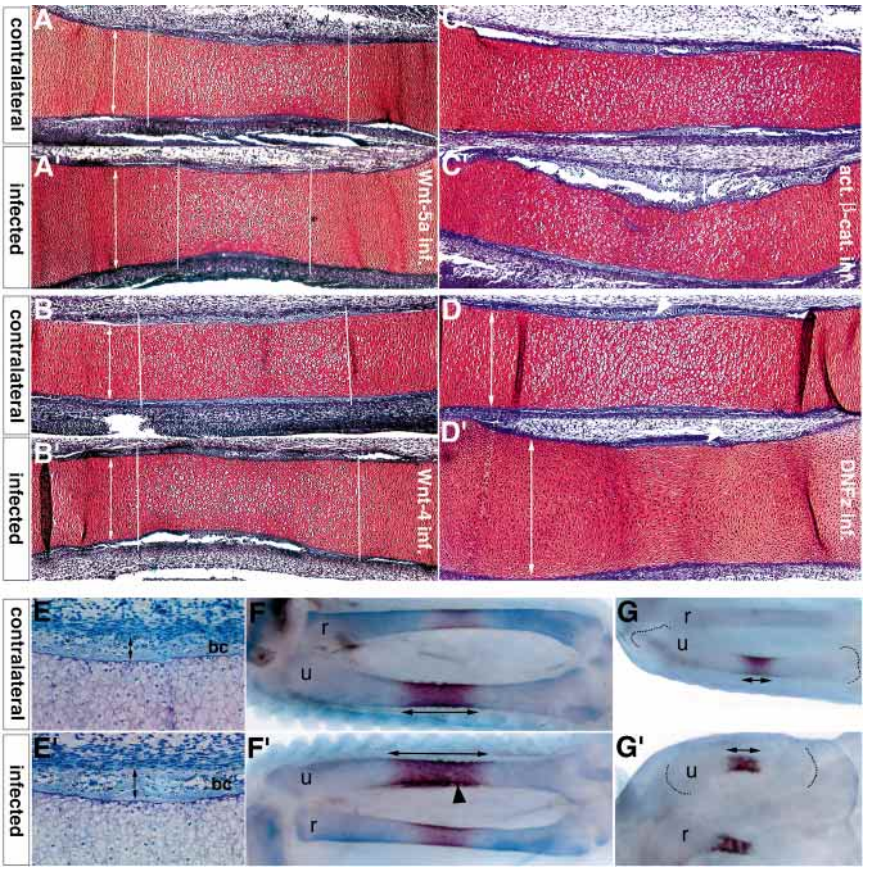

Fig. 3. (A-D′) Histological analysis on sections of ulnas infected with various viruses at HH 32 (day 7.5) by Weigert-Safranin O staining: the chondromucin-rich extracellular matrix of chondrocytes is stained red, nuclei are stained black, and mesenchymal cells are stained dark purple. The osteoid matrix of the bone collar is stained light purple (see arrowheads in D,D′). (A) Contralateral uninfected ulna showing the region of hypertrophic chondrocytes, which with their enlarged cytoplasm appear white. (A′) Corresponding *Wnt-5a* infected ulna showing a reduced number of hypertrophic chondrocytes within the domain demarcated by the white lines. The double-headed arrow indicates the radial expansion of the cartilage element compared to the contralateral element. (B) Contralateral uninfected ulna and (B′) corresponding *Wnt-4* infected ulna showing a slight expansion of the hypertrophic chondrocyte region (demarcated by the white lines) but no significant difference in the diameter of the cartilage element (double-headed arrow). (C) Contralateral uninfected ulna and (C′) corresponding activated *β-catenin* infected ulna showing an increased diameter of the bone collar (double-headed arrow). (D) Contralateral uninfected ulna and (D′) corresponding Chfz7wt infected ulna showing absence of hypertrophic chondrocytes, retarded development of the bone collar (arrowhead), and a radial expansion of the cartilage element (double-headed arrow). (E,E′) High magnification brightfield images of Toluidine Blue-stained sections of the ulna of the contralateral ulna (E) and *Wnt-4* injected ulna (E′) showing the increase in thickness of the bone collar (bc). (F-G′) Whole-mount Alcian Blue/Alizarin Red stained wing pairs at day 9.5. (F) Contralateral uninjected wing and (F′) corresponding *Wnt-4* infected wing, showing the increase in the calcified regions in *Wnt-4* injected limbs (red). Arrowhead points to calcified nodules of the bone collar. (G) Contralateral uninjected wing and (G′) corresponding activated *β-catenin* infected wing, showing a relative increase in the calcified regions (red) of the ulna and radius. The ends of the ulnae are marked by the dotted lines (in G,G′). Due to the viral spread, the calcified region of the radius is also slightly advanced, reflected in the more intense Alizarin Red staining, in the infected wings shown here for *Wnt-4* (F′) and the activated *β-catenin* (G′). All limbs are orientated proximal towards the left, distal towards the right. U, ulna; r, radius.

chondrocytes can be distinguished in a wild-type cartilage element of the stylopod or zeugopod. Small, densely packed proliferating chondrocytes are found at the ends of the cartilage elements. In an adjacent domain, the chondrocytes are flattened but are still proliferating. This region is followed by postmitotic, enlarged hypertrophic chondrocytes. Histological analysis of cartilage elements infected with *Wnt-5a* revealed that there are very few or no hypertrophic chondrocytes present at HH 32 (data not shown). A day later (HH 34-35), while maturation of the uninfected cartilage elements in the contralateral wing has progressed, reflected in the increased number of hypertrophic cells (Fig. 3A), fewer hypertrophic chondrocytes can be detected in *Wnt-5a* infected cartilage elements (Fig. 3A′). This observation suggests that the differentiation of hypertrophic chondrocytes is delayed in limbs infected with a *Wnt-5a* expressing virus.

Next, we examined *Wnt-5a* infected limbs with molecular markers to determine the level at which the delay in chondrocyte maturation occurs. Molecular analysis of cartilage elements infected with *Wnt-5a* revealed a delay in the onset of *Col-X* expression (a marker for hypertrophic chondrocytes). *Col-X* expression is absent at HH stages 31-32 in the infected ulnae (Fig. 4A′), while normal levels of *Col-X* expression can be detected in the uninfected ulnae of the contralateral wings (Fig. 4A, *n*=3). A day later in development, at HH 35, a few cells expressing *Col-X* are detectable in the *Wnt-5a* infected ulna (Fig. 5A′, *n*=3), while the ulna of the contralateral wings expresses normal levels (Fig. 5A).

To determine whether this lag in chondrocyte differentiation occurs before or after the differentiation of prehypertrophic chondrocytes, a population of postmitotic chondrocytes with no distinct morphological appearance, we analyzed the expression of the two molecular markers for prehypertrophic chondrocytes, *Ihh* and *PTH/PTHrP-receptor*. *Ihh* is expressed in *Wnt-5a* infected limbs, but the entire *Ihh* domain (measured from the distal ends of the Ihh expression domains, and encompassing the region in between, where *Ihh* is being downregulated and *Col-X* is being activated) is smaller in the infected ulna (Figs 4B′, 5B′) than in the ulna of the uninfected limb (Figs 4B, 5B). Nevertheless, the proportion of the entire *Ihh* domain relative to the length of the cartilage element is not altered (proportional size), spanning approximately 50% of the length of the cartilage element in both uninfected and *Wnt-5a* infected wings. Even before chondrocytes express the hypertrophic marker *Col-X*, they begin to downregulate *Ihh* expression during the normal differentiation process. Whereas

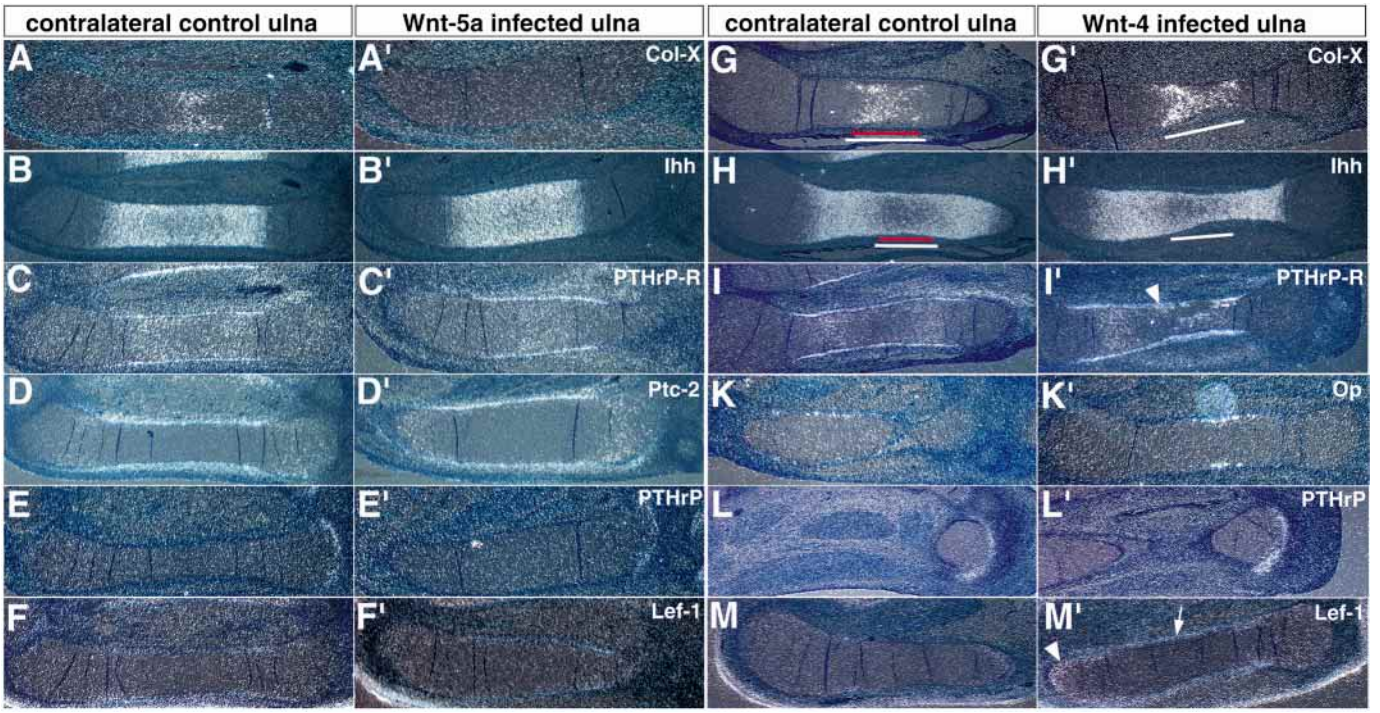

Fig. 4. Analysis of molecular markers in *Wnt-5a* and *Wnt-4* infected ulnae and their corresponding contralateral ulnae at HH 32 (day 7.5) by radioactive section in situ hybridization. (A-M) Alternate sections of the corresponding contralateral uninfected ulnae showing the endogenous expression pattern and level of the various molecular markers. (A′-F′) Alternate sections of a *Wnt-5a* infected ulna. (A′) No *Col-X* expression in the Wnt-5a infected ulna. (B′) Smaller *Ihh* expression domain (note that the central region, where *Ihh*-expression is downregulated, is also smaller in the *Wnt-5a* infected ulna compared to the endogenous expression pattern in the contralateral ulna). (C′) Expression domain of *PTH/PTHrP-receptor* is not split into two domains in the *Wnt-5a* infected ulna. (D′-F′) No alterations in the expression pattern or level of the molecular markers *Ptc-2*, *PTHrP* and *Lef-1* in *Wnt-5a* infected ulna. (G′-M′) Alternate sections of *Wnt-4* infected ulna. (G′) Shows the expanded *Col-X* expression domain (indicated by the white bar). (H′) Shows the expanded domain of more mature chondrocytes, which have downregulated *Ihh* expression (white bar). To directly compare the expanded domains of *Col-X* as well as the domain where *Ihh*-expression is downregulated in the *Wnt-4* infected ulna (white bars), the white bars have been projected into G and H adjacent to orange bars demarcating the endogenous expression domains. (I′) Expanded expression of *PTH/PTHrP-receptor* in the periosteum (arrowhead). (K′) Shows increased and expanded expression of the osteoblast marker *osteopontin* (Op). (L′) Shows that the periarticular *PTHrP*-expression is not altered. (M′) Increased expression of perichondrial/periosteal (arrow) and articular *Lef-1* expression (arrowhead).

differentiated chondrocytes in the diaphysis of the contralateral ulna show a broad domain where *Ihh* is downregulated (Figs 4B, 5B), this domain is smaller in the ulna infected with *Wnt-5a* (Figs 4B′, 5B′). Since the proportional size of the entire *Ihh* domain does not appear to be altered, we interpret the observed difference in the absolute size of the entire *Ihh* domain, and in the domain where *Ihh* is downregulated, as a consequence of a delayed maturation of hypertrophic chondrocytes.

PTH/PTHrP-receptor has been reported to be expressed in prehypertrophic cells distal to and partially overlapping with *Ihh* expressing cells in the epiphyseal growth plate of a 3-week-old chicken (Vortkamp et al., 1996). During embryonic stages of chick development, we observed strong expression of the *PTH/PTHrP-receptor* in the innermost layer of the perichondrium/periosteum flanking the *Ihh* expression domain in chondrocytes. In addition, we observed a weaker expression of *PTH/PTHrP-receptor* in a subpopulation of chondrocytes. These chondrocytes correspond to a region of prehypertrophic chondrocytes that have begun to downregulate *Ihh* but do not yet express *Col-X*. Thus in the embryo, chondrocytes expressing *PTH/PTHrP-receptor*, in contrast to those in the growth plate of a postnatal chicken, are likely to mark a distinct population of prehypertrophic chondrocytes that are more mature than *Ihh* expressing chondrocytes. We therefore analyzed the expression of *PTH/PTHrP-receptor* in *Wnt-5a* infected limbs to address whether the differentiation of this more mature population of prehypertrophic chondrocytes occurs normally in these limbs. The perichondrial expression of *PTH/PTHrP-receptor*, flanking the domain of *Ihh* expressing chondrocytes, is similar in cartilage elements of injected (Figs 4C′, 5C′) and contralateral control limbs (Figs 4C, 5C). In contrast, the expression pattern of the *PTH/PTHrP-receptor* in chondrocytes is altered by *Wnt-5a*. At HH 32, while the chondrogenic *PTH/PTHrP-receptor* expression domain is split into two domains in the contralateral ulna (Fig. 4C), we see a continuous domain of *PTH/PTHrP-receptor* expression in the prehypertrophic chondrocytes in the *Wnt-5a* infected ulna (Fig. 4C′). Together, the smaller domain of *Ihh* expression, the absence of *Col-X* positive chondrocytes, and the observation that the *PTH/PTHrP-receptor* expression domain is continuous in *Wnt-5a* infected cartilage elements at HH 32, are all likely to reflect an earlier developmental state. Likewise, the expression domains of *Col-X*, *Ihh* and *PTH/PTHrP-receptor* in the *Wnt-5a* infected limb at HH 35 (Fig. 5A′-C′) resemble more the differentiation state of an uninfected cartilage element at HH 32 (see Fig. 4A-C). These results

support the model that Wnt-5a blocks or delays chondrocyte differentiation prior to the hypertrophic stage.

As shown in Fig. 1B, *Wnt-5b* is also expressed in a subpopulation of prehypertrophic chondrocytes, overlapping with the expression of *PTH/PTHrP-receptor* (see Fig. 5C,D). In contrast to the relatively minor changes in the *PTH/PTHrP-receptor* expression following *Wnt-5a* misexpression, however, we observed that *Wnt-5b* expression in chondrocytes is upregulated and expanded in *Wnt-5a* infected limbs (Fig. 5D′). This could imply that *Wnt-5b* expressing cells reflect a distinct population of prehypertrophic cells prior to the Wnt-5a induced block in maturation, which are less differentiated than *Col-X* expressing cells but slightly more differentiated than *PTH/PTHrP-receptor* expressing cells. Alternatively, *Wnt-5b* could be specifically upregulated in response to Wnt-5a signaling. Since the perichondrial expression of *Wnt-5b* is also expanded in *Wnt-5a* infected limbs, we favor the latter model.

Ihh protein produced by prehypertrophic chondrocytes has been shown to be involved in negatively regulating the progression of cells from proliferating to postmitotic prehypertrophic chondrocytes (Vortkamp et al., 1996). In our analysis of *Ihh* expression we detected no significant difference in the level of *Ihh* expression in cartilage elements infected with *Wnt-5a* expressing virus (Figs 4B′, 5B′, *n*=6), but slight alterations in the expression level of *Ihh* might be difficult to detect using radioactive in situ hybridizations on sections. We therefore asked whether the expression of *Ihh* target genes was altered in the injected limbs. *Ihh* has been shown to regulate the expression of its receptor *Ptc-1* and *PTHrP* in chick (Vortkamp et al., 1996) and mouse (St-Jacques et al., 1999). In most vertebrates, two hedgehog-receptors, *Ptc-1* and *Ptc-2*, have been isolated (Goodrich et al., 1996; Hahn et al., 1996; Johnson et al., 1996; Marigo et al., 1996; Carpenter et al., 1998; Motoyama et al., 1998; Lewis et al., 1999; Smyth et al., 1999; Zaphiropoulos et al., 1999). Likewise, we have identified a second *Patched* gene in chick (*Ptc-2*), which is expressed at very high levels and is strongly upregulated by Shh and Ihh (K. J. Vogan, R. V. Pearse II and C. J. T., unpublished). While *Ptc-2* is not expressed in developing long bones of the mouse (St-Jacques et al., 1999), *Ptc-2* in the chick displays an expression pattern similar to *Ptc-1* but is expressed at a higher level in response to hedgehog signaling than *Ptc-1*. We therefore chose to analyze the expression of *Ptc-2* as a sensitive readout for Ihh signaling in *Wnt-5a* infected limbs. We detected no differences in *Ptc-2* expression in ulnae infected with *Wnt-5a* (Fig. 4D′) and the corresponding contralateral ulnae (Fig. 4D). In addition, expression of *PTHrP* in the periarticular region appears normal (Fig. 4E′,E). Therefore, *Wnt-5a* misexpression does not appear to interfere with the Ihh/PTHrP regulatory loop, which is involved in controlling the progression from the proliferating to the prehypertrophic fate. Instead, our results indicate that Wnt-5a signaling from the perichondrium negatively regulates the progression of chondrocytes from the prehypertrophic to the hypertrophic cell fate independent of the Ihh/PTHrP loop, and that this event is associated with an upregulation of *Wnt-5b* expression in prehypertrophic cells.

Wnt-4 misexpression accelerates chondrocyte maturation and bone collar formation

Misexpression of *Wnt-5a* and *Wnt-4* both result in a shortening of the long bones as assayed by whole-mount Alcian Blue staining. We next asked whether *Wnt-4* misexpression, like *Wnt-5a*, causes a delay in chondrogenesis. Surprisingly, histological analysis of *Wnt-4* infected limbs revealed a different phenotype from limbs infected with *Wnt-5a*. Specifically, cartilage elements of limbs infected with *Wnt-4* show a slightly expanded zone of hypertrophic chondrocytes compared to the contralateral wing (Fig. 3B,B′). In addition, the osteoid layer of the bone collar is thicker compared to the bone collar of the contralateral wing (Fig. 3E,E′), suggesting that, in contrast to Wnt-5a, Wnt-4 signaling is accelerating chondrogenesis. The expansion of the zone of hypertrophic chondrocytes is very subtle and difficult to demarcate on sections, where slight variations in the plane of sectioning can affect the precise size of this zone. To verify this conclusion, we examined the state of mineralization in infected cartilage elements in whole-mount preparations. As hypertrophic chondrocytes begin to degenerate, they resorb their surrounding matrix. This process is associated with the deposition of calcium, resulting in a mineralization of the cartilage, and takes place prior to the invasion of blood vessels, osteoblasts and osteoclasts and the replacement of hypertrophic chondrocytes by trabecular bone (Poole, 1991). We therefore stained *Wnt-4* infected embryos at day 9.5 using Alcian Blue/Alizarin Red, which highlights the unmineralized (Alcian Blue) and the mineralized regions (Alizarin Red) of the cartilage. We than compared the mineralized regions between *Wnt-4* injected right wings and their corresponding uninjected left wings. Although extents varied, the calcified region within ulnae elements was expanded by 11-27% in 90% of the *Wnt-4* infected limbs (*n*=10; Fig. 3F). The Alizarin Red stained region encompasses the calcified cartilage region as well as the calcified regions within the bone collar, the latter of which display a nodular like appearance. In *Wnt-4* infected limbs, we observe more nodules (arrowhead in Fig. 3F′), supporting the idea that *Wnt-4* misexpression also affects maturation of the bone collar.

The histological analysis suggested that chondrogenesis is advanced in *Wnt-4* infected limbs, but did not allow us to determine which step of chondrocyte differentiation was accelerated. We therefore analyzed *Wnt-4* infected cartilage elements at HH 31-32 (*n*=3) and HH 34-35 (*n*=3) by section in situ hybridization using various molecular differentiation markers. This molecular analysis revealed an expanded expression domain of the hypertrophic marker *Col-X* in *Wnt-4* infected ulnae (Figs 4G′, 5H′). In addition, the region where *Ihh* is downregulated, encompassing more mature prehypertrophic and hypertrophic chondrocytes, is expanded in *Wnt-4* infected ulnae (Figs 4H′, 5I′), indicating an acceleration of chondrocyte maturation. The expression level and pattern of *PTH/PTHrP-receptor,* which is expressed in more mature prehypertrophic chondrocytes, was generally unaffected in *Wnt-4* infected ulnae (Figs 4I′, 5K′), except that the two expression domains are slightly further apart from each other in *Wnt-4* infected ulnae. This observation is consistent with the observed expansions of the *Col-X* domain and of the region where *Ihh* is downregulated in *Wnt-4* infected limbs.

In the periosteum surrounding the region of prehypertrophic and hypertrophic chondrocytes, we detected a broadened and longitudinally expanded expression domain of *PTH/PTHrP-receptor* (arrowheads and bars, respectively in Figs 4I′, 5K′). *PTH/PTHrP-receptor* has been reported to be expressed in

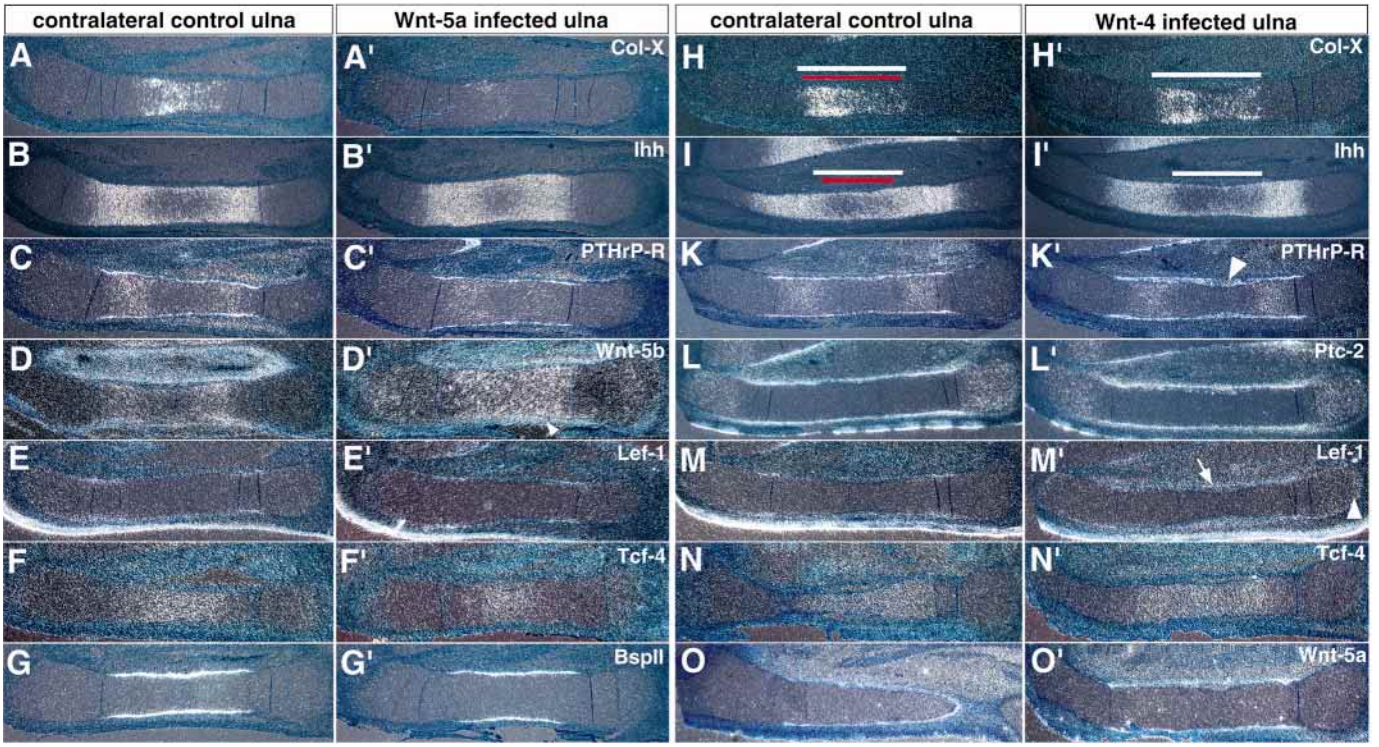

Fig. 5. Analysis of molecular markers in *Wnt-5a* and *Wnt-4* infected ulnae in comparison to the wild-type pattern in the corresponding contralateral ulnae at HH 35 (day 8.5) by radioactive section in situ hybridization. (A-O) Alternate sections of the corresponding contralateral uninfected ulnae showing the endogenous expression pattern and level of the various molecular markers. (A′-G′) Alternate sections of a *Wnt-5a* infected ulna. (A′) Fewer cells express the hypertrophic marker *Col-X*. (B′) Smaller *Ihh* expression domain. Note that the central region, where *Ihh*-expression is downregulated, is also smaller in the *Wnt-5a* infected ulna compared to the endogenous expression pattern in the contralateral ulna. (C′) The two *PTH/PTHrP-receptor* expression domains marking a subpopulation of more mature hypertrophic chondrocytes are closer together compared to the contralateral ulna. (D′) Upregulation of *Wnt-5b* expression in prehypertrophic chondrocytes and in chondrocytes of the articular region. Note the continuous expression domain in the *Wnt-5a* infected limb compared to the two separated expression domains in the contralateral control ulna (see D). In addition, we see a slight expansion of the perichondrial *Wnt-5b* expression (arrowhead). (E′) *Lef-1* expression is not altered. (F′) The expression domain of *Tcf-4* is smaller, due to a delay in differentiation of more mature chondrocytes. (G′) The expression of the osteoblast marker *BspII* is slightly reduced in *Wnt-5a* infected limbs due to a delay in the differentiation of the periosteum. (H′-O′) Alternate sections of a *Wnt-4* infected ulna: (H′) Expanded *Col-X* expression domain (white bar). (I′) Expanded domain of more mature chondrocytes, which have downregulated *Ihh* expression (white bar). For direct comparison of the expansion of the endogenous *Col-X* domain and the domain where *Ihh*-expression is downregulated, the white bars representing the expansion of the corresponding domains in the *Wnt-4* infected ulna have been projected into H and I adjacent to orange bars demarcating the wild-type expression domains. (K′) Radially (arrowhead) and longitudinally (white bars) expanded expression of *PTH/PTHrP-receptor* in the periosteum. For direct comparison of the longitudinal expansion of the periosteal *PTH/PTHrP-receptor* expression, the white bars have been projected into K adjacent to the orange bars demarcating normal expansion in the uninjected limb. (L′) *Ptc-2* expression is not altered in the *Wnt-4* infected ulna. (M′) Increased expression of the perichondrial/periosteal (arrow) and articular expression of *Lef-1* (arrowhead). (N′) *Tcf-4* expression is not altered in *Wnt-4* infected limbs. (O′) Perichondrial *Wnt-5a* expression is not altered in *Wnt-4* infected limbs.

immature osteoblasts in the mouse and rat (Lee et al., 1995). The radially and longitudinally expanded expression of *PTH/PTHrP-receptor* is probably due to the presence of more immature osteoblasts, reflecting the advanced developmental state of the bone collar, which is histologically visible (Fig. 3E′). To confirm this result, we analyzed the expression of two other osteoblast markers, *bone sialoprotein II* and *osteopontin*. *Bone sialoprotein II* is also a marker for immature osteoblasts, while *osteopontin* is expressed in more mature osteoblasts (for review see Gerstenfeld and Shapiro, 1996). As expected, we observed an expansion in the expression of *bone sialoprotein II* (data not shown) and *osteopontin* in *Wnt-4* infected ulnae (shown at HH 32; Fig. 4K′) in comparison to the uninfected contralateral wings (Fig. 4K). The expansion in the periosteal expression of the various osteoblast markers together with the morphologically detectable increase in the osteoid layer support the hypothesis that Wnt-4 signaling positively regulates periosteum differentiation.

Wnt-4 misexpression affects the expression of the transcription factor Lef-1

Lef-1, one of the transcriptional downstream effector genes of the Wnt signaling pathway, is expressed in the perichondrium (Fig. 1H). When we analyzed *Lef-1* expression in *Wnt-4* infected limbs, we detected an increase in *Lef-1* expression throughout the periosteum and perichondrium (Figs 4M′, 5M′) relative to contralateral limbs (Figs 4M, 5M). In addition, *Lef-1* expression is upregulated in the articular chondrocytes (arrowheads in Figs 4M′, 5M′). These data suggest that *Lef-1* itself could be a target of a *Wnt-4* stimulated signaling pathway.

Similarly, experiments by Kengaku et al., (1998) have shown that *Lef-1* expression in the early limb bud is upregulated in response Wnt-3a signaling. *Wnt-5a* misexpression, however, had no effect on *Lef-1* expression in the perichondrium or chondrocytes (Figs 4F′, 5E′). Moreover, the expression of the related gene, *Tcf-4*, was not altered in either *Wnt-4* or *Wnt-5a* infected limbs (Fig. 5F′,N′). The restricted upregulation of *Lef-1* expression in perichondrial/periosteal cells and in chondrocytes of the articular region suggests that only these cells may be competent to respond to Wnt-4 signaling. We cannot, however, rule out the possibility that cells do respond to Wnt-4 signaling without upregulating *Lef-1* expression.

To rule out the possibility that the increased *Lef-1* expression in *Wnt-4* infected limbs is due to a general expansion of the perichondrium/periosteum, we analyzed the expression of other perichondrial markers such as *Bmp-4*, *Ptc-2* and *Wnt-5a*. The expression of *Bmp-4* (data not shown) and *Ptc-2* (Fig. 5L′) are not altered in *Wnt-4* infected limbs. Since both genes have been shown to be regulated by *Ihh* (Pathi et al., 1999 and data not shown), these findings further suggest that Ihh signaling is not affected in *Wnt-4* infected limbs. The expression of *Wnt-5a* in the perichondrium is also not affected in *Wnt-4* infected limbs (Fig. 5O′), ruling out the possibility that *Wnt-4* acts positively on chondrocyte maturation through downregulating the negatively acting Wnt-5a signal.

We further analyzed the expression of the Ihh responsive gene *PTHrP*. Downregulation of the PTHrP signal in the articular region would result in an acceleration of chondrocyte differentiation, based on the phenotype of *PTHrP* loss-of-function mutants (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996); however, *PTHrP* expression in the periarticular region appeared normal in *Wnt-4* infected elements (Fig. 4L′). This result rules out the possibility that Wnt-4 signaling affects chondrocyte differentiation by downregulating *PTHrP* in the periarticular region. Instead, our results suggest that Wnt-4 signaling positively regulates maturation of the perichondrium/periosteum and chondrocytes, independently of Ihh/PTHrP-mediated signals.

Misexpression of stabilized β-catenin accelerates chondrocyte maturation

To investigate whether either the Wnt-5a triggered delay in chondrogenesis or the Wnt-4 stimulated acceleration of chondrocyte maturation is transduced by a β-catenin-mediated pathway, we performed RCAS mediated misexpression of a stabilized mutant form of β-catenin in the posterior half of HH 21-23 chicken limb buds. This amino-terminal deleted, mutant form of β-catenin acts as a constitutively activated intracellular signal, simulating Wnt signaling (Funayama et al., 1995; Yost et al., 1996; Morin et al., 1997; Rubinfeld et al., 1997; Kengaku et al., 1998). As with the Wnt-5a and Wnt-4 viruses, limbs infected with the activated *β-catenin* virus show shortening of the cartilage elements (Fig. 2C,I), although the degree of shortening was markedly greater than following misexpression of either Wnt-5a or Wnt-4. In addition, these limbs show an increase in muscle mass, ectopic ectodermal outgrowth and, often, polydactyly. The ectopic ectodermal outgrowth and the polydactyly of the infected limb are likely to be due to activation and maintenance of ectopic *Fgf-8* expression together with disruptions in *Fgf-8* expression within the normal AER, both of which have been shown to occur following misexpression of activated *β-catenin* (Kengaku et al., 1998). In principle, the shortening of the cartilage elements could be a secondary effect due to mechanical constraints caused by the overproliferation of the muscle fibers. However, histological examination revealed a phenotype similar to Wnt-4 infected limbs: the zone of hypertrophic chondrocytes in cartilage elements infected with the activated *β-catenin* virus is expanded in relation to the total length of the cartilage element (Fig. 3C′ and data not shown), suggesting an accelerated maturation of chondrocytes, and the osteoid layer of the bone collar is also thickened (see Fig. 3C,C′). The accelerated maturation of chondrocytes was also confirmed by Alcian Blue/Alizarin Red staining, which showed an expanded and more intense mineralized region in the infected ulna (Fig. 3G′; n=3).

A molecular analysis of the infected cartilage elements revealed an increase in expression of the hypertrophic marker *Col-X* at HH 31 and 35 in comparison to *Col-X* expression in the contralateral uninfected limb (Fig. 6 and data not shown). In addition, the *Col-X* expression domain was expanded in infected limbs. Misexpression of activated *β-catenin* also led to an upregulation of *Lef-1* expression throughout the mesenchyme surrounding the cartilage elements (Fig. 6B′); however, we did not detect ectopic *Lef-1* expression in chondrocytes of the infected cartilage elements, with the exception of articular chondrocytes, where we observed an expansion of the *Lef-1* expression domain (Fig. 6B′, double-headed arrow).

Similar to *Wnt-4* misexpressing limbs, we observed an upregulated and expanded expression of *PTHrP-receptor* and *bone sialoprotein II* in the periosteum of limbs infected with the activated *β-catenin* expressing virus, reflecting an advanced differentiation of the periosteum (Fig. 6E′,F′ and data not shown), but misexpression of activated *β-catenin* did not affect the perichondrial expression of *Wnt-5a*, *Wnt-5b* or *Ptc-2* (data not shown and Fig. 6D′). The expression of *PTHrP* in the periarticular region was also not affected (data not shown). Furthermore, we did not detect any change in the expression levels of *Ihh*, *Wnt-5b* and *Tcf-4* in prehypertrophic and prehypertrophic/hypertrophic chondrocytes, respectively (Fig. 6C′ and data not shown).

The histological and molecular phenotypes observed in limbs with *Wnt-4* or activated *β-catenin* are very similar, suggesting that the Wnt-4 signal could be mediated by β-catenin. Nevertheless, the phenotypic effects are much stronger in the case of viral infections using the activated *β-catenin*. This could be explained by a difference in competence of the responding cells: in order to transduce a Wnt-4 signal the responding cells have to express the relevant receptor, while almost all cells that virally express the activated *β-catenin* virus will express the stabilized form of β-catenin and should therefore be capable of activating target gene transcription as long as they express a member of the TCF/LEF family.

Misexpression of putative dominant-negative Frizzleds delay chondrogenesis

Having shown that ectopic expression of either *Wnt-4* or an activated form of *β-catenin* is capable of accelerating the maturation of cartilage elements, we wanted to investigate whether an endogenous β-catenin-mediated Wnt signaling pathway is involved in positively regulating chondrogenesis. The two *Frizzled* genes, *Chfz-1* and *Chfz-7*, both belong to the

class of Wnt-receptors that can act through the β-catenin/TCF-LEF mediated pathway (Sheldahl et al., 1999). Since both genes are expressed in the chondrogenic regions, which have been shown to be affected by misexpression of either *Wnt-4* or activated *β-catenin*, they are likely candidates to be involved in the Wnt-4 signal transduction. In order to test this hypothesis, we constructed potential dominant-negative forms of the two Frizzled proteins by deleting their intracellular carboxy-terminal domains of 24 amino acids, based on the report that a similar C-terminally truncated Frizzled protein acts as a dominant-negative molecule in zebrafish (Nasevicius et al., 1998).

Retroviral misexpression of either the C-terminally truncated version of *Chfz-1* (Chfz1ΔC) or *Chfz-7* (Chfz7ΔC) in the posterior half of HH 21-23 wing buds resulted in shortening of the posterior cartilage elements in the infected wings at HH 35-36 (Fig. 2D,E). Although individual embryos were affected to varying extents (indicated by the error bars in Fig. 2K,L), shortening of skeletal elements was observed in >90% of limbs infected with either Chfz1ΔC (*n*=12) or Chfz7ΔC (*n*=13). In both cases, shortening of Alcian Blue stained cartilage elements was already detectable at day 7 of development (HH 30). We also misexpressed the full-length Chfz-1 and Chfz-7 proteins using the retrovirus system. Retroviral misexpression of either a full-length protein of Chfz-1 (Chfz1wt) or Chfz-7 (Chfz7wt) also resulted in shortening of the posterior cartilage elements in the infected wings (shown for Chfz7wt in Fig. 2F,M). Similar to Chfz1ΔC or Chfz7ΔC misexpression, shortening of Alcian Blue stained cartilage elements was already detectable at day 7 of development (HH 30).

Histological analysis of wings infected with a virus expressing either the potential dominant-negative Chfz1ΔC or Chfz7ΔC proteins or wild-type proteins of Chfz-1 or Chfz-7 revealed that shortening of the cartilage elements is, in all cases, likely to be due to a delay or block in chondrocyte maturation. Chondrocytes in the diaphysis are less organized and no hypertrophic chondrocytes can be detected in limbs infected with viruses expressing the various *Frizzled* constructs (Fig. 3D,D′ and data not shown). In order to address whether chondrocyte maturation was delayed or blocked, we examined infected limbs histologically at various developmental stages (days 7, 8.5 and 11). Even as late as day 11 of development, we could not detect hypertrophic chondrocytes in heavily infected limbs (data not shown). Nevertheless, even in the absence of hypertrophic chondrocytes, we did detect the formation of a bone collar in the central region of the affected cartilage element (Fig. 3D′ and data not shown). However, the onset of bone collar formation and its maturation were developmentally delayed in limbs infected with the various *Frizzled* viruses (Fig. 3D′ and data not shown). The histological analysis suggests that the different *Frizzled* viruses might all have dominant-negative effects. This conclusion is consistent with the finding that in *Drosophila* overexpression of *Dfrizzled* can disrupt intracellular signaling (Tomlinson et al., 1997; Zhang and Carthew, 1998; Tomlinson and Struhl, 1999).

In order to test this assumption further, we analyzed cartilage elements of wings infected with the different *Frizzled* viruses at HH 32 and 35 using a variety of molecular markers. No significant differences were observed at the level of the molecular markers when we compared limbs infected with the different *Frizzled* viruses with each other (*n*≥3 for each retroviral construct at HH 32 and 35). These findings support our interpretation that all four *Frizzled* viruses have a dominant-negative effect. We will therefore refer to all of them here as dominant-negative effective *Frizzled* viruses (*DNFz*). Consistent with the histological observations (absence of hypertrophic cells) we could not detect the expression of *Col-X* in *DNFz* infected cartilage elements at HH stages 32 (day 7.5; Fig. 7F′ and data not shown) and 35 (day 8.5, see Fig. 7M′ and data not shown). Expression of *Ihh*, a marker for early prehypertrophic chondrocytes, was also downregulated in cartilage elements infected with *DNFz* (Fig. 7A′,G′ and data not shown). In addition to the reduced level of *Ihh* expression, we observed that the entire *Ihh* domain was smaller in relation to the length of the cartilage element (25-28% of the length of the cartilage element in the infected wings versus 50% in the uninfected contralateral wings). Since the *Ihh* domain normally extents over 50% of the length of a cartilage element even as early as HH 26, the observation of the severely reduced size of the *Ihh* domain in *DNFz* infected cartilage elements suggests that misexpression of *DNFz* viruses delays chondrocyte maturation at the transition from proliferating to postmitotic, prehypertrophic chondrocytes, resulting in fewer cells expressing the prehypertrophic marker *Ihh*.

The expression of *PTH/PTHrP-receptor*, a marker for more mature prehypertrophic chondrocytes, is absent or severely reduced in cartilage elements infected with *DNFz*-virus (Fig. 7D′,K′ and data not shown). *PTH/PTHrP-receptor* is also expressed in the perichondrium. As in wild-type limbs, the expression of the *PTH/PTHrP-receptor* in the perichondrium of *DNFz* infected limbs is restricted to the region adjacent to *Ihh* expressing chondrocytes at HH 32 (data not shown) and 35 (Fig. 7K′ and data not shown). Although the perichondrial expression domain of *PTH/PTHrP-receptor* is significantly smaller in *DNFz* infected limbs, we did not detect an obvious difference in the level of *PTH/PTHrP-receptor* expression as long as significant *Ihh* expression was detectable. However, in heavily infected limbs at HH 30, where we detect only very low levels of *Ihh* expression (Fig. 7A′ and data not shown), we did not detect any expression of *PTH/PTHrP-receptor* (Fig. 7D′ and data not shown). Since *PTH/PTHrP-receptor* is expressed in immature osteoblasts, this observation is likely to reflect a delay in the differentiation of osteoblasts.

We also analyzed the expression of two Ihh-dependent genes (*Ptc-2* and *Bmp-4*) in cartilage elements infected with *DNFz*-viruses to assess the consequences of the decreased *Ihh* expression observed in these limbs. The perichondrial expression of both *Ptc-2* and *Bmp-4* was dramatically reduced in limbs at HH 30 and HH 35 (Fig. 7B′,C′,H′ and data not shown). The expression of *Ptc-2* in chondrocytes of the articular region was also weaker (Fig. 7H′ and data not shown) than in the contralateral ulna (Fig. 7H). These results suggest that Ihh signaling is significantly reduced in cartilage elements infected with *DNFz*-viruses. However, when we analyzed the expression of *PTHrP*, which has been shown to be dependent on Ihh signaling (Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999) as well as BMP signaling (Zou et al., 1997), we did not detect any significant difference in the periarticular expression of *PTHrP* between *DNFz* injected (Fig. 7E′ and data not shown) and uninjected control wings (Fig. 7E). The analysis of the *Ihh* knockout mouse clearly demonstrates that Ihh is required for *PTHrP* expression (St-

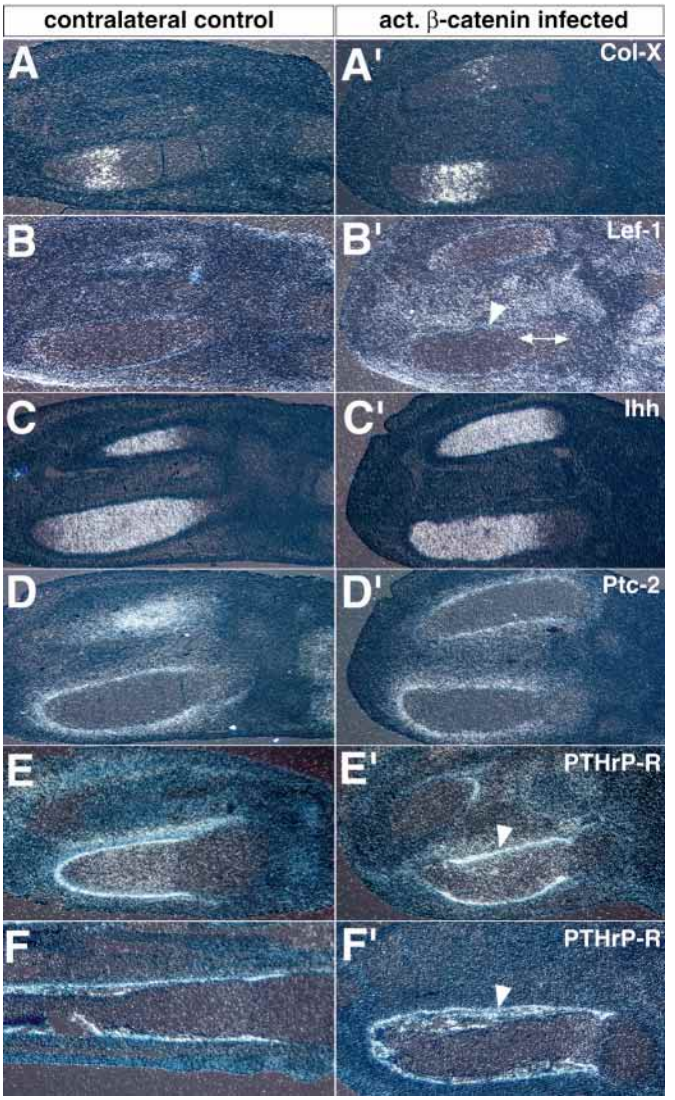

Fig. 6. Analysis of molecular markers in activated *β-catenin* infected ulnae and their corresponding contralateral ulnae at HH 32 (A-E′) and at HH 35 (F-F′) by radioactive section in situ hybridization. (A-F) Endogenous expression patterns and levels of the molecular markers *Col-X*, *Lef-1*, *Ihh*, *Ptc-2* and *PTH/PTHrP-receptor* in the uninfected control ulna. (A′) Increased and expanded expression of *Col-X* in the infected ulna. (B′) Ectopic expression of *Lef-1* in soft tissue of the infected ulna, upregulation of *Lef-1* expression in the perichondrium (arrowhead), and expansions of the articular expression domain (double-headed arrow). (C′) Unaffected expression of *Ihh*. (D′) Unaffected expression of *Ptc-2*. (E′,F′) Increased and expanded expression of *PTH/PTHrP-receptor* in the periosteum (arrowheads).

Jacques et al., 1999); however, the observation that *PTHrP* expression is not affected in limbs showing a significant downregulation in *Ihh* expression and signaling suggests that low levels of Ihh signaling might be sufficient for maintaining periarticular *PTHrP* expression, nevertheless.

We also analyzed the effect of *DNFz* misexpression on the expression of *Lef-1*, which is likely to be a target gene of a Wnt-4 activated, β-catenin-mediated pathway. Expression of *Lef-1* was still detectable in *DNFz* infected limbs; however, the perichondrial *Lef-1* expression domain was smaller and was restricted to perichondrial cells surrounding the *Ihh* expression domain (Fig. 7I′ and data not shown). In the articular region, the level of *Lef-1* expression is almost indistinguishable from the background signal in limbs infected with *DNFz* expressing virus (Fig. 7I′ and data not shown). Similarly, we detected no *Tcf-4* expression in heavily infected cartilage elements (Fig. 7L′ and data not shown), consistent with the absence of more mature prehypertrophic and hypertrophic chondrocytes.

These experiments show that the *Frizzled* viruses act in a manner opposite to the activated *β-catenin* virus, delaying maturation of the chondrocytes. This observation is consistent with the hypothesis that the *Frizzled* constructs all function as dominant-negative effectors, implying that an endogenous Wnt pathway operates within the embryo as a positive regulator of chondrocyte maturation.

DISCUSSION

Wnt-4, Wnt-5a and Wnt-5b, as well as various components of the Wnt signaling pathway, show very localized expression patterns in differentiating perichondrium and in chondrocytes, suggesting that their expression has to be tightly regulated in order to coordinate differentiation of chondrogenic elements. The fact that their developmental expression patterns are complex and dynamic implies that they could be involved in controlling various differentiation steps during chondrogenesis. Our misexpression experiments using replication-competent retroviruses have shown that two different Wnt signaling pathways regulate differentiation of chondrocytes independently of the recently described cascade involving Ihh, BMPs and PTHrP (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997). Other recent studies have implicated Wnt molecules in the negative control of chondrocyte differentiation (Zakany and Duboule, 1993; Rudnicki and Brown, 1997; Kawakami et al., 1999). Here we present evidence that a β-catenin-mediated Wnt signal accelerates the maturation process of chondrocytes and periosteum, while a β-catenin independent Wnt-signaling pathway delays chondrogenesis.

Wnt-5a negatively regulates the maturation of prehypertrophic chondrocytes

Cartilage elements in which *Wnt-5a* is misexpressed are smaller in size and show histologically and molecularly a delay in the maturation of hypertrophic chondrocytes. Earlier stages of chondrocyte differentiation do not seem to be affected, since injected and uninjected limbs display normal expression levels of the prehypertrophic markers *Ihh* and *PTH/PTHrP-receptor*. *Wnt-5a* misexpression did not affect the periarticular expression of *PTHrP*, suggesting that the phenotype is not due to a disturbance of the *Ihh-PTHrP* feedback loop. In contrast, *Wnt-5a* infected ulnae show an upregulation of *Wnt-5b* expression in prehypertrophic chondrocytes and in the surrounding perichondrium. Comparison of *Wnt-5b* expression with *PTH/PTHrP-receptor* expression on adjacent sections suggested that both genes are expressed in the same population of prehypertrophic chondrocytes. Since we do not detect an alteration in the expression level of the *PTH/PTHrP-receptor* or any other morphological or molecular indication that the size

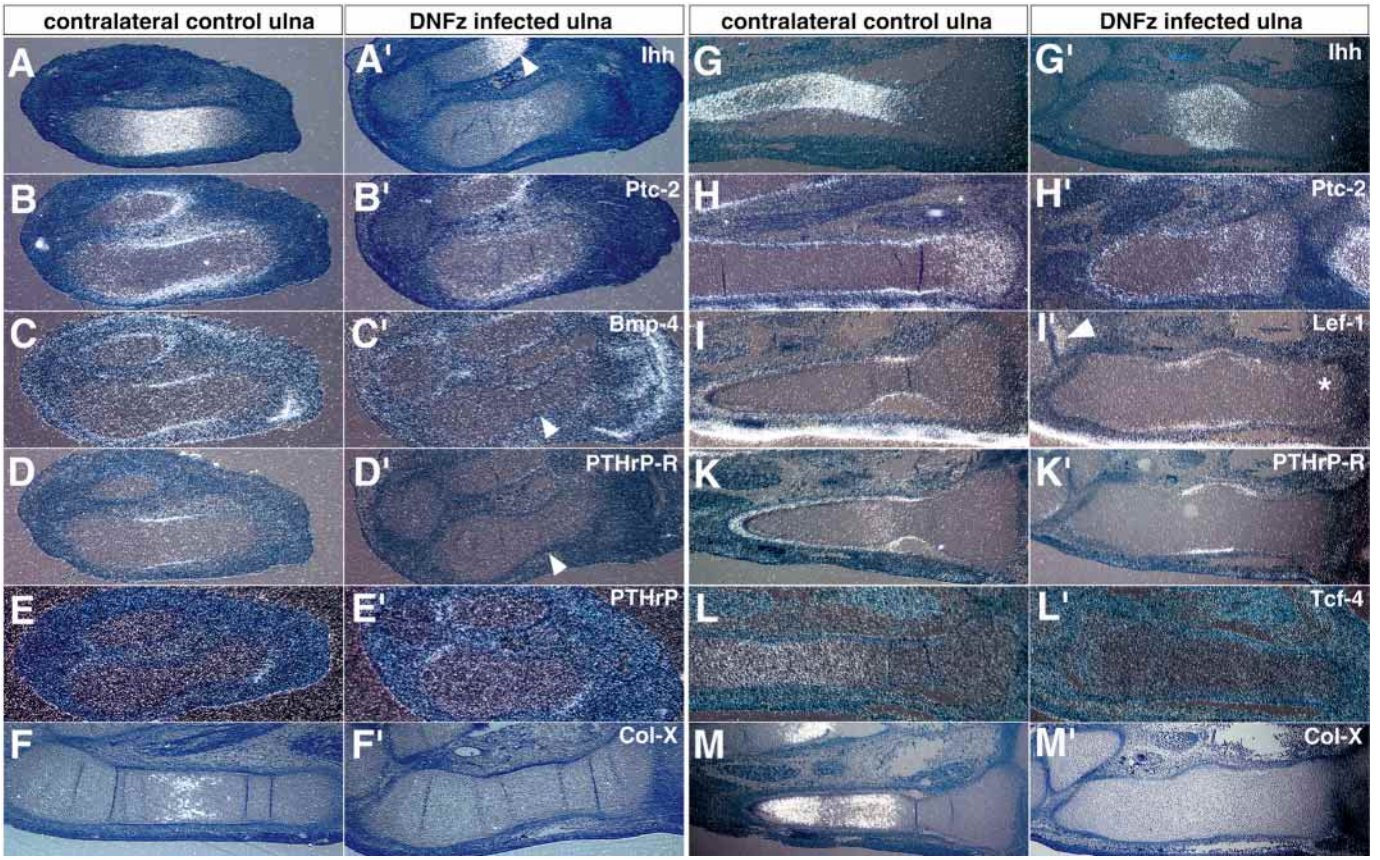

Fig. 7. Analysis of molecular markers in *DNFz*-infected ulnae and their corresponding contralateral ulnae at HH 30 (A-E′), at HH 32 (F-F′), and at HH 35 (G-M′) by radioactive section in situ hybridization. These particular examples were infected with the Chfz7wt virus. (A-M) Endogenous expression of the various molecular markers in the contralateral uninjected ulnae. (A′) Severely downregulated *Ihh* expression in the infected ulna, while the uninfected radius expresses normal levels of *Ihh* (arrowhead). (B') Reduced *Ptc-2* expression. (C′) Reduced *Bmp-4* expression in the perichondrium of the diaphysis (arrowhead). (D′) Absence of *PTH/PTHrP-receptor* expression in the perichondrium (arrowhead). (E′) Normal *PTHrP* expresssion in the periarticular region. (F') Absence of *Col-X* expression. (G′) Reduction in both the size of the *Ihh* domain and in the expression levels. (H′) Reduced *Ptc-2* expression in articular chondrocytes. (I′) Reduced expression of *Lef-1* in the articular chondrocytes (asterisk) compared to the normal expression in the unaffected radius (arrowhead). (K′) Absence of *PTH/PTHrP-receptor* in prehypertrophic chondrocytes and a reduced expression domain in the periosteum. (L′) Absence of *Tcf-4* expression. (M′) Absence of *Col-X* expression.

of this subpopulation of prehypertrophic chondrocytes is increased, the observation that *Wnt-5b* is upregulated implies that *Wnt-5b* itself could be a target of Wnt-5a signaling. We therefore propose that, in the wild-type limb, Wnt-5a signaling from the perichondrium negatively controls the progression from prehypertrophic to hypertrophic chondrocytes, and that this negative signal might be mediated through *Wnt-5b*. The Wnt-5b level in the prehypertrophic chondrocytes could serve as a secondary signal to adjacent, less mature prehypertrophic chondrocytes, thereby controlling their maturation along with Wnt-5a. Whether *Wnt-5b* is indeed involved in modulating chondrocyte differentiation at the prehypertrophic/hypertrophic checkpoint and/or is playing additional roles at other stages of chondrogenesis will be the subject of future studies.

Interestingly, Delta/Notch signaling has been proposed to be involved in regulating this checkpoint as well (Crowe et al., 1999). Specifically, misexpression of Delta-1 causes a block in chondrocyte maturation at the level of the progression of prehypertrophic to hypertrophic chondrocytes, but does not affect earlier steps in the differentiation process (Crowe et al., 1999). This resembles what we observe in *Wnt-5a* infected limbs, where the maturation process of hypertrophic chondrocytes is delayed. Wnt/Fz signaling and Notch signaling have been shown to intersect during various developmental processes, including differentiation of the wing margin in *Drosophila* (Hing et al., 1994), ommatidial polarity in the *Drosophila* eye (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999) and formation of the AER in the chick limb (Kengaku et al., 1998). In each of these cases, it has been suggested that the Wnt/Fz signaling pathway is acting upstream of the Notch pathway. Recent data have shown that Wnt and Notch signaling can interact in multiple ways (Axelrod et al., 1996; Wesley, 1999). In the light of these results, it tempting to speculate that Delta/Notch signaling may be modified by the perichondrial Wnt-5a signal, either directly or indirectly through Wnt-5b signaling.

Recent experiments by Long and Linsenmayer (1998) have shown that the perichondrium flanking the diaphysis of the cartilage element is involved in negatively regulating the maturation of hypertrophic chondrocytes. *Wnt-5a*, which is expressed in the perichondrium/periosteum and has been shown to delay maturation of the hypertrophic chondrocytes

(this study and Kawakami et al., 1999), could be one of the factors involved in this regulatory role of the perichondrium. Experiments by Lanske et al., (1996) and Vortkamp et al., (1996) have shown that the periarticular expression of PTHrP is also involved in a negative regulation of chondrocyte regulation. They have shown that a negative feedback loop between Ihh and PTHrP regulates the progression of chondrocytes from the proliferative to the prehypertrophic state (Fig. 8). Since *Wnt-5a* misexpression does not influence the Ihh/PTHrP loop and seems to control a different checkpoint in the chondrocyte maturation process, we propose that Wnt-5a secreted by the perichondrium acts as a negative regulator, independent of the PTHrP-signal (Fig. 8). Significantly, the Wnt-5a signaling pathway in the cartilage does not appear to be mediated by β-catenin, since misexpressing a stabilized form of β-catenin results in a phenotype which is opposite to that produced by Wnt-5a. Wnt-5a has been shown to signal through a G-protein-linked phosphatidylinositol pathway in zebrafish (Slusarski et al., 1997), suggesting that this might be the pathway involved in negatively regulating the maturation of hypertrophic chondrocytes by the perichondrial Wnt-5a signal.

Wnt-4 triggers a β-catenin-mediated pathway that positively regulates chondrogenesis

Retroviral misexpression of *Wnt-4* or an activated form of *β-catenin* both result in an acceleration of chondrocyte maturation. The phenotypic consequences are more pronounced in the case of activated *β-catenin* misexpression than misexpressing *Wnt-4*. Specifically, misexpression of stabilized *β-catenin* results in a more dramatic shortening of the infected cartilage element and in premature expression of *Col-X*, while *Wnt-4* misexpression results in a more subtle phenotype, including less dramatic effects on the length of the infected cartilage element, a slight expansion of the *Col-X* expression domain, as well as an expansion of the domain in which *Ihh* is downregulated. As a result, these limbs show an increased mineralization of the diaphysis, as assessed by whole-mount Alizarin Red staining. The difference in the strength of the phenotypic consequences could be explained as following: in one case, where we misexpress the ligand, not all cells might express the relevant receptor or some other downstream components necessary to transduce the signal, whereas in the other case, we misexpress the activated form of a factor at the end of the signal transduction pathway, which can potentially activate the signaling pathway in every cell expressing the relevant transcriptional cofactor(s).

In both cases, we detected an upregulation of *Lef-1* expression in cells of the perichondrium and in articular chondrocytes, suggesting that *Lef-1* could be a direct target of Wnt-4 and activated β-catenin signaling. Nevertheless, it is surprising that the responding region is so restricted, especially in the case of the ectopic misexpression of the stabilized form of β-catenin. While we see upregulation of *Lef-1* in infected muscle cells, we do not detect any ectopic expression of *Lef-1* in more mature chondrocytes. Since there is endogenous *Lef-1* expression in muscle cells (see Fig. 1H), but none in more mature chondrocytes, it is likely that *Lef-1* itself is required for the observed upregulation in response to activated *β-catenin* misexpression. Similarly, Kengaku et al. (1998) reported that *Lef-1* expression is required for and upregulated in response to β-catenin-mediated Wnt-3a signaling during early limb bud development. In contrast, the expression level of *Tcf-4*, which is expressed in a domain encompassing both prehypertrophic and hypertrophic chondrocytes, is not altered in limbs infected with *Wnt-4* or activated *β-catenin* expressing viruses. Although TCF-4 has been shown to bind constitutively to β-catenin (Korinek et al., 1997; Cho and Dressler, 1998), it has not been shown to be directly regulated by Wnt signaling. It is interesting to note that the high-level expression domain of *β-catenin* coincides with the *Tcf-4* expression domain in a normal cartilage element. Whether this overlap in their expression domains is functionally relevant remains an open question and subject for future investigations.

Since *Lef-1* is upregulated in the perichondrium, one possibility would be that Wnt-4 signaling downregulates a negative signal originating from the perichondrium. However, neither *Wnt-5a* nor *PTHrP* expression levels are affected in limbs infected with the *Wnt-4* or activated *β-catenin* viruses. This observation makes it unlikely that the β-catenin-mediated Wnt-4 signal accelerates chondrogenesis simply by downregulating the known negative regulatory pathways.

Within the cartilage element, we detected an upregulation of *Lef-1* expression only in chondrocytes of the articular region, and this expression of *Lef-1* in articular chondrocytes was downregulated in limbs infected with the various dominante-negative *Frizzled*-viruses. In addition, misexpression of the various dominant-negative *Frizzled*-viruses seems to block chondrocyte differentiation at the transition from proliferating to non-proliferating prehypertrophic chondrocytes, as reflected in the smaller *Ihh* expression domain as well as the lower expression level of *Ihh*, due to a decrease in the number of *Ihh* expressing cells, and the absence of *Wnt-5b* and *PTHrP-receptor* expression. On the basis of these observations, we propose a model in which *Wnt-4*, expressed adjacent to the joint region, signals through a β-catenin/Lef-1 complex to the articular chondrocytes and positively controls chondrocyte differentiation by promoting progression of chondrocytes from the proliferating to the postmitotic, prehypertrophic state (Fig. 8). This could be explained through a mechanism whereby proliferating cells exit the cell cycle faster, and are therefore pushed out of the proliferating zone and become postmitotic. They would then undergo the normal maturation program, and as a result, we would see an increase in the number of terminally differentiated chondrocytes. This explanation would also account for the overall shortening of the cartilage elements. Importantly, none of the subsequent regulatory steps involved in chondrocyte maturation seem to be affected by Wnt-4 misexpression. This is reflected in the normal size of the prehypertrophic zone and of the expression domains of prehypertrophic markers such as *Ihh*, *PTHrP-receptor* and *Wnt-5b*.

Frizzled-7 a potential receptor for the endogenous Wnt-4 signal

Chfz-7 is expressed in chondrocytes of the articular region, and recent data by Sheldahl et al., (1999) suggest that it transduces signals through the β-catenin/TCF-LEF pathway, making Chfz-7 a candidate for the receptor of the Wnt-4 signal. In some other systems, overexpression of *Frizzled* genes has been shown to result in an activation of *Wnt* signaling (Yang-Snyder et al., 1996; Slusarski et al., 1997; Cadigan et al., 1998; Itoh

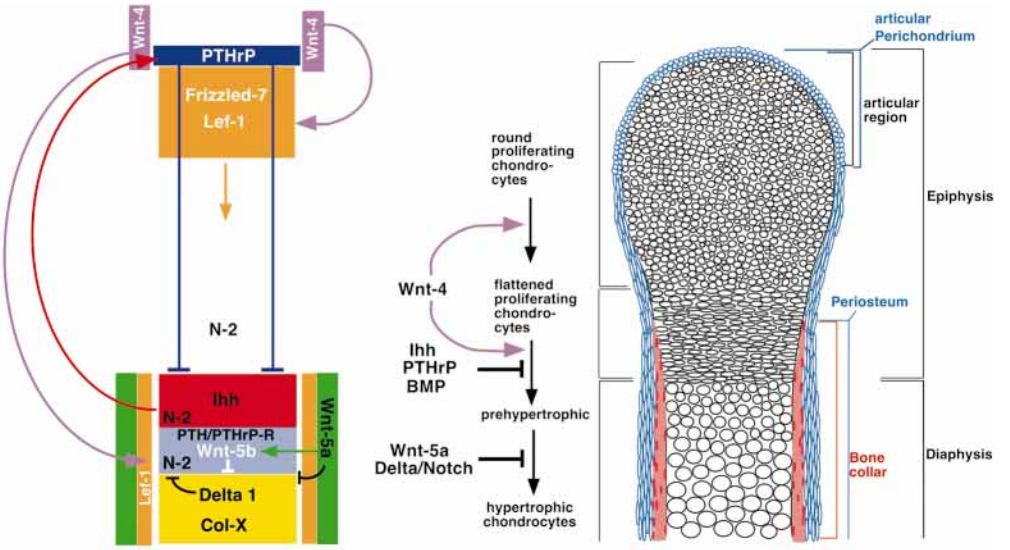

Fig. 8. Molecular regulation of chondrocyte maturation. Schematic representation of the different zones of differentiation within the cartilage element and the expression domains of corresponding molecular markers. Cartilage differentiation occurs progressively as chondrocytes mature from a population of highly proliferative cells to postmitotic prehypertrophic chondrocytes to hypertrophic chondrocytes. Previous studies indicated that the PTHrP signal from the periarticular region (dark blue) negatively regulates the progression of proliferating to prehypertrophic chondrocytes, and that this negative signal is controlled by a feedback mechanism involving Ihh (red), which is expressed in prehypertrophic chondrocytes. A second checkpoint in chondrocyte maturation, the progression of prehypertrophic to hypertrophic chondrocytes, has previously been shown to be regulated by Delta 1, which is coexpressed with Col-X in hypertrophic chondrocytes (yellow). The studies described here show that Wnt-5a (green) expressed in the perichondrium signals to a subpopulation of more mature prehypertrophic chondrocytes to prevent their progression to the hypertrophic fate, either directly or indirectly through Wnt-5b (light blue), which is upregulated in response to Wnt-5a signaling. A second Wnt molecule, Wnt-4, is expressed in cells flanking the periarticular region (purple) and signals to the articular region, accelerating the progression of proliferating to postmitotic, prehypertrophic chondrocytes. This signal is mediated through the Chfz-7 receptor (orange) and a β-catenin/Lef-1 (orange) complex. In addition, Wnt-4 signals to the perichondrium and either directly through Lef-1 (orange) or indirectly exerts a positive effect on the maturation of the periosteum.

et al., 1998); however, misexpression of Chfz7wt in our system, like misexpression of Chfz1wt or the truncated Chfz7ΔC and Chfz1ΔC constructs, resulted in a phenotype opposite to that obtained by misexpression of activated *β-catenin* and *Wnt-4*, delaying chondrocyte maturation instead of accelerating it. Therefore, it seems unlikely that misexpression of Chfz-7 protein mimics an activated Wnt-4 signaling pathway; our data rather imply that in our misexpression experiment, Chfz-7 has a dominant-negative effect. This can be explained through a possible interference with the endogenous Wnt signaling. The ectopically expressed Chfz-7 protein, for example, could bind intracellular components and thus antogonize with the endogenous signaling by depleting the system. A precedent for this is seen in the *Drosophila* eye, where both loss of *frizzled* activity in clones and ectopic expresssion of *Dfrizzled* have been shown to cause a similar tissue polarity phenotype (Tomlinson et al., 1997; Zhang and Carthew, 1998; Tomlinson and Struhl, 1999), while ectopic expression of *Dfrizzled* in the embryo (Tomlinson et al., 1997) or of *Dfrizzled-2* in the wing imaginal disc mimic weak *wg* gain-of function phenotypes (Cadigan et al., 1998; Zhang and Carthew, 1998; Lin and Perrimon, 1999). The data from the fly suggest that in a tissue-specific context, the signaling pathway has to be finely balanced and can easily be disturbed by either reduction or excess of *Frizzled* expression. The interpretation that the various *Frizzled* constructs all act as dominant negative molecules is consistent with our proposed model that Wnt-4 signals through Chfz-7 to the articular chondrocytes (Fig. 8). Like Wnt-4, the dominant-negative *Frizzled* constructs act at the level of the transition from proliferating to non-proliferating chondrocytes; however, their misexpression results in an opposite phenotype from Wnt-4 delaying this transition. This is reflected in a consequent decrease or absence of markers for more differentiated chondrocytes such as *Ihh* and *PTH/PTHrP-receptor*, even though the Wnt-4 pathway acts independently of the Ihh/PTHrP loop.

In our proposed model (Fig. 8), the joint region serves as an important signaling center from which positive and negative signals emanate (Wnt-4 and PTHrP respectively), controlling the number of cells progressing from the proliferative to the prehypertrophic state. In order to coordinate growth of the cartilage element, it is likely that these positive and negative signals themselves are under a feedback control mechanism originating from postmitotic differentiated chondrocytes. Recent data by Vortkamp et al., (1996) and St-Jacques et al., (1999) have shown that this is indeed the case for the negatively acting PTHrP signal, which is controlled by Ihh. Therefore, we suggest that the positive acting Wnt-4 signal may also be regulated by a feedback loop originating from more differentiated chondrocytes. The nature of the signal(s) controlling *Wnt-4* expression in the joint region, however, remains to be determined.

Accelerated maturation of the periosteum in response to Wnt-4 signaling

In addition to the accelerated maturation of chondrocytes, we observed a positive effect of *Wnt-4* or activated *β-catenin* misexpression on the maturation of the periosteum. This is reflected in the expanded expression domains of osteoblast markers such as *PTH/PTHrP-receptor*, *bone sialoprotein II* and *osteopontin*, and by the histology of the infected cartilage elements. The upregulation in the expression of the effector gene *Lef-1* in the periosteum implies that Wnt-4 / β-catenin signaling could be directly involved in controlling the differentiation of osteoblasts in the periosteum, although we cannot rule out the possibility that it is an indirect effect,

mediated through a secondary signal. Misexpression of the various *DNFz*-viruses not only delays chondrocyte maturation, but also retards differentiation of the periosteum. This observation supports the notion that osteoblast differentiation could be under the positive control of Wnt-4 signaling. However, since perichondrial *Lef-1* expression is not downregulated in limbs infected with the *DNFz*-viruses, we propose that the observed delay in periosteum maturation is likely to be an indirect effect due to decreased Ihh expression. Recent observations support the idea that hedgehog signaling is involved in osteoblast differentiation. Specifically, Ihh mutant mice do not form a bone collar due to an absence of osteoblast precursors (St-Jacques et al., 1999), and both Shh and Ihh have been shown to promote differentiation of osteoblasts in in vitro assay systems (Kinto et al., 1997; Nakamura et al., 1997).

Wnt-4 and Wnt-5a belong to the same class of Wnt genes

Wnt molecules have previously been classified into three subgroups, based on their activities in various assays such as axis duplication in Xenopus (Moon et al., 1993; Du et al., 1995) or transformation of mouse mammary cell lines (Wong et al., 1994). Interestingly, Wnt-4 has previously been grouped together with Wnt-5a into the same subclass of Wnt molecules, the Wnt-5a class (Moon et al., 1993; Wong et al., 1994; Du et al., 1995; Ungar et al., 1995). Strikingly, in our system, these two Wnt molecules show opposite effects on the chondrocyte maturation process and seem to utilize different intracellular signaling pathways. Recent gain-of-function studies by Kengaku et al. (1998) have similarly shown that two Wnt molecules (Wnt-7a and Wnt-3a) belonging to the same class, in this case the highly transforming Wnt-1 class, also act through divergent signaling pathways, resulting in different patterning events during early limb bud development.

Our hypothesis that Wnt-4 signals through β-catenin is contrary to a finding by Shimizu et al. (1997), showing that Wnt-4 signaling in a cell culture assay system does not increase cytosolic β-catenin levels, and implying that Wnt-4 in this system does not signal through β-catenin. In another experimental system, however, Wnt-4 has been shown to have similar activities to Wnt-1 and Wnt-3 (Münsterberg et al., 1995), two Wnt molecules which are thought to act through a β-catenin/TCF-LEF mediated pathway. Studies in zebrafish (Slusarski et al., 1997) and Xenopus (He et al., 1997) on Wnt-5a signaling ability have similarly shown that Wnt-5a can utilize two different signaling pathways depending on which Fz-receptor is present. Since we still know very little about the specificity of ligand-receptor interactions and whether the relevant receptors are expressed in the various systems used, it remains to be determined which Wnt molecules signal through which intracellular pathway within any given experimental system.

Excitingly, Wnt-4 is to our knowledge the first secreted signaling molecule accelerating chondrocyte maturation in in vivo overexpression studies. Misexpression studies of many other signaling molecules, including retinoid acid (DeSimone and Reddi, 1983), Fgf proteins (Coffin et al., 1995; Vogel et al., 1996), BMP proteins (Duprez et al., 1996a), TGF-β molecules (Serra et al., 1997, 1999), Ihh (Vortkamp et al., 1996) and other Wnt molecules (Zakany and Duboule, 1993; Rudnicki and Brown, 1995; Kawakami et al., 1999; this study) in mouse and chick result in a delay of chondrocyte maturation, while some of these molecules promote in vitro cartilage formation in micromass culture assays (Kulyk et al., 1989; Luyten et al., 1994; Roark and Greer, 1994; Duprez et al., 1996b). Thus, these studies have identified a novel mechanism for positively controlling the rate of growth of developing long bones within the avian limb.

We are very thankful to Y. Yang and A. McMahon for providing unpublished reagents and for critical comments on the manuscript. We would like to thank M. Kengaku, T.A. Heanue, A. Vortkamp, D. Smith and S. Rowan for their technical advice and R. Schweitzer for invaluable discussions. We would furthermore like to thank S. M. Dymecki, R. V. Pearse, G. Kardon and K. J. Vogan for comments on the manuscript. C.H. was supported by a postdoctoral fellowship from the Human Frontiers Science Program. This work was supported by a program project grant from the NIH and a grant from March of Dimes.

REFERENCES

Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman, D. and Karaplis, A. C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol.* **126**, 1611-1623.

Arias, A. M., Brown, A. M. and Brennan, K. (1999). Wnt signalling: pathway or network? *Curr. Opin. Genet. Dev.* **9**, 447-454.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science* **271**, 1826-1832.

Bejsovec, A. (1999). Wnt signalling shows its versatility. *Curr. Biol.* **9**, R684-R687.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* **382**, 225-230.

Cabrera, C. V., Alonso, M. C., Johnston, P., Phillips, R. G. and Lawrence, P. A. (1987). Phenocopies induced with antisense RNA identify the wingless gene. *Cell* **50**, 659-663.

Cadigan, K. M., Fish, M. P., Rulifson, E. J. and Nusse, R. (1998). Wingless repression of Drosophila frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93**, 767-777.

Cadigan, K. M. and Nusse, R. (1997). Wnt signaling: a common theme in animal development. *Genes Dev.* **11**, 3286-3305.

Caplan, A. I. and Pechak, D. G. (1987). The cellular and molecular embryology of bone formation. In *Bone and Mineral Research* (ed. W. A. Peck), pp. 117-183. New York, NY: Elsevier.

Carpenter, D., Stone, D. M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A. and de Sauvage, F. J. (1998). Characterization of two patched receptors for the vertebrate hedgehog protein family. *Proc. Natl. Acad. Sci. USA* **95**, 13630-13634.

Castagnola, P., Bet, P., Quarto, R., Gennari, M. and Cancedda, R. (1991). cDNA cloning and gene expression of chicken osteopontin. Expression of osteopontin mRNA in chondrocytes is enhanced by trypsin treatment of cells. *J. Biol. Chem.* **266**, 9944-9949.

Cho, E. A. and Dressler, G. R. (1998). TCF-4 binds beta-catenin and is expressed in distinct regions of the embryonic brain and limbs. *Mech. Dev.* **77**, 9-18.

Chung, U., Lanske, B., Lee, K., Li, E. and Kronenberg, H. (1998). The parathyroid hormon/parathyroid hormone-related peptide receptor coordinates endrochondral bone development by directly controlling chondrocyte differentiation. *Proc. Natl. Acad. Sci. USA* **95**, 13030-13035.

Coffin, J. D., Florkiewicz, R. Z., Neumann, J., Mort-Hopkins, T., Dorn, G. W. n., Lightfoot, P., German, R., Howles, P. N., Kier, A., O'Toole, B. A. et al. (1995). Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol. Biol. Cell* **6**, 1861-1873.

Cooper, M. T. and Bray, S. J. (1999). Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. *Nature* **397**, 526-530.

Cox, R. T. and Peifer, M. (1998). Wingless signaling: the inconvenient complexities of life. *Curr. Biol*. **8**, R140-R144.

Crowe, R., Zikherman, J. and Niswander, L. (1999). Delta-1 negatively regulates the transition from prehypertrophic to hypertrophic chondrocytes during cartilage formation. *Development* **126**, 987-998.

Dealy, C. N., Roth, A., Ferrari, D., Brown, A. M. and Kosher, R. A. (1993). Wnt-5a and Wnt-7a are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsoventral axes. *Mech. Dev*. **43**, 175-186.

Delezoide, A. L., Benoist-Lasselin, C., Legeai-Mallet, L., Le Merrer, M., Munnich, A., Vekemans, M. and Bonaventure, J. (1998). Spatio-temporal expression of FGFR 1, 2 and 3 genes during human embryo- fetal ossification. *Mech. Dev.* **77**, 19-30.

DeSimone, D. P. and Reddi, A. H. (1983). Influence of vitamin A on matrix-induced endochondral bone formation. *Calcif. Tissue Int.* **35**, 732-739.

Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L. and Moon, R. T. (1995). Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos. *Mol. Cell. Biol.* **15**, 2625-2634.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89**, 747-754.

Duprez, D., Bell, E. J., Richardson, M. K., Archer, C. W., Wolpert, L., Brickell, P. M. and Francis-West, P. H. (1996a). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57**, 145-157.

Duprez, D. M., Coltey, M., Amthor, H., Brickell, P. M. and Tickle, C. (1996b). Bone morphogenetic protein-2 (BMP-2) inhibits muscle development and promotes cartilage formation in chick limb bud cultures. *Dev. Biol.* **174**, 448-452.

Erlebacher, A., Filvaroff, E. H., Gitelman, S. E. and Derynck, R. (1995). Toward a molecular understanding of skeletal development. *Cell* **80**, 371-378.

Fanto, M. and Mlodzik, M. (1999). Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. *Nature* **397**, 523-526.

Francis, P. H., Richardson, M. K., Brickell, P. M. and Tickle, C. (1994). Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. *Development* **120**, 209-218.

Funayama, N., Fagotto, F., McCrea, P. and Gumbiner, B. M. (1995). Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. *J. Cell Biol.* **128**, 959-968.

Gaffney, E. (1994). Carbohydrates. In *Laboratory Methods in Histotechnology (Armed Forces Institute of Pathology)* (ed. E. B. Prophet, B. Mills, J. B. Arrington and L. H. Sobin). Washington, D.C.: American Registry of Pathology.

Gavin B. J., McMahon, J. A. and McMahon, A. P. (1990). Expression of multiple novel Wnt-1/int-1-related genes during fetal and adult mouse development. *Genes Dev.* **4**, 2319-2332.

Gerstenfeld, L. C. and Shapiro, F. D. (1996). Expression of bone-specific genes by hypertrophic chondrocytes: implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J. Cell Biochem.* **62**, 1-9.

Goff, D. J. and Tabin, C. J. (1997). Analysis of Hoxd-13 and Hoxd-11 misexpression in chick limb buds reveals that Hox genes affect both bone condensation and growth. *Development* **124**, 627-636.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. and Scott, M. P. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. *Genes Dev.* **10**, 301-312.

Hahn, H., Christiansen, J., Wicking, C., Zaphiropoulos, P. G., Chidambaram, A., Gerrard, B., Vorechovsky, I., Bale, A. E., Toftgard, R., Dean, M. and Wainwright, B. (1996). A mammalian patched homolog is expressed in target tissues of sonic hedgehog and maps to a region associated with developmental abnormalities. *J. Biol. Chem.* **271**, 12125-12128.

Hall, B. K. (1988). The embryonic development of bone. *Am. Sci.* **76**, 174-181.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

He, X., Saint-Jeannet, J.P., Wang, Y., Nathans, J., Dawid, I. and Varmus, H. (1997). A member of the frizzled protein family mediating axis induction by Wnt-5A. *Science* **275**, 1652-1654.

Hinchcliffe, J. R. and Johnson, D. R. (1990). *The Development of the Vertebrate Limb.* Oxford, UK: Claredon Press.

Hing, H. K., Sun, X. and Artavanis-Tsakonas, S. (1994). Modulation of wingless signaling by Notch in Drosophila. *Mech. Dev.* **47**, 261-268.

Itoh, K., Jacob, J. and Y Sokol, S. (1998). A role for Xenopus Frizzled 8 in dorsal development. *Mech. Dev.* **74**, 145-157.

Johnson, R. L., Rothman, A. L., Xie, J., Goodrich, L. V., Bare, J. W., Bonifas, J. M., Quinn, A. G., Myers, R. M., Cox, D. R., Epstein, E. H., Jr. and Scott, M. P. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* **8**, 277-289.

Karp, S., Schipani, E., St-Jaques, B., Hunzelman, J., Kronenberg, H. and McMahon A.P. (2000). Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. *Development* **127**, 543-548.

Kawakami, Y., Wada, N., Nishimatsu, S. I., Ishikawa, T., Noji, S. and Nohno, T. (1999). Involvement of Wnt-5a in chondrogenic pattern formation in the chick limb bud. *Dev. Growth Differ.* **41**, 29-40.

Kengaku, M., Twombly, V. and Tabin, C. (1997). Expression of Wnt and Frizzled genes during chick limb bud development. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 421-429.

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Pena, J., Johnson, R. L., Belmonte, J. C. I. and Tabin, C. J. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* **280**, 1274-1277.

Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Noji, S., Ohuchi, H., Yoshioka, H., Kataoka, H., Wada, Y., Yuhao, G., Takahashi, H. E., Yoshiki, S. and Yamaguchi, A. (1997). Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. *FEBS Lett.* **404**, 319-323.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. and Kishimoto, T. (1997). Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89**, 755-764.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**, 1784-1787.

Koyama, E., Golden, E. B., Kirsch, T., Adams, S. L., Chandraratna, R. A., Michaille, J. J. and Pacifici, M. (1999). Retinoid signaling is required for chondrocyte maturation and endochondral bone formation during limb skeletogenesis. *Dev. Biol.* **208**, 375-391.

Kulyk, W. M., Rodgers, B. J., Greer, K. and Kosher, R. A. (1989). Promotion of embryonic chick limb cartilage differentiation by transforming growth factor-beta. *Dev. Biol.* **135**, 424-430.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Juppner, H., Segre, G. V. and Kronenberg, H. M. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**, 663-666.

Lee, J. S., Ishimoto, A. and Yanagawa, S. (1999). Characterization of mouse dishevelled (Dvl) proteins in Wnt/Wingless signaling pathway. *J. Biol. Chem.* **274**, 21464-21470.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**, 453-463.

Lewis, K. E., Concordet, J. P. and Ingham, P. W. (1999). Characterisation of a second patched gene in the zebrafish *Danio rerio* and the differential response of patched genes to Hedgehog signalling. *Dev. Biol.* **208**, 14-29.

Lin, X. and Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. *Nature* **400**, 281-284.

Linsenmayer, T. F., Chen, Q. A., Gibney, E., Gordon, M. K., Marchant, J. K., Mayne, R. and Schmid, T. M. (1991). Collagen types IX and X in the developing chick tibiotarsus: analyses of mRNAs and proteins. *Development* **111**, 191-196.

Logan, M. and Tabin, C. (1998). Targeted gene misexpression in chick limb buds using avian replication-competent retroviruses. *Methods* **14**, 407-420.

Long, F. and Linsenmayer, T. F. (1998). Regulation of growth region cartilage proliferation and differentiation by perichondrium. *Development* **125**, 1067-1073.

Lu, J., Chuong, C. M. and Widelitz, R. B. (1997). Isolation and characterization of chicken beta-catenin. *Gene* **196**, 201-207.

LuValle, P., Ninomiya, Y., Rosenblum, N. D. and Olsen, B. R. (1988). The type X collagen gene. Intron sequences split the 5′-untranslated region and separate the coding regions for the non-collagenous amino- terminal and triple-helical domains. *J. Biol. Chem.* **263**, 18378-18385.

Luyten, F. P., Chen, P., Paralkar, V. and Reddi, A. H. (1994). Recombinant bone morphogenetic protein-4, transforming growth factor- beta 1, and activin A enhance the cartilage phenotype of articular chondrocytes in vitro. *Exp. Cell Res.* **210**, 224-229.

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A. and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* **124**, 1109-1117.

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J. (1996). Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233.

McLeod, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by Alcian Blue and Alizarin Red S. *Teratology* **22**, 299-301.

Miller, J. R., Hocking, A. M., Brown, J. D. and Moon, R. T. (1999). Mechanism and function of signal transduction by the Wnt/β-catenin and Wnt/Ca^{2+} pathways. *Oncogene* **18**, 7860-7872.

Moon, R. T., Campbell, R. M., Christian, J. L., McGrew, L. L., Shih, J. and Fraser, S. (1993). Xwnt-5A: a maternal Wnt that affects morphogenetic movements after overexpression in embryos of *Xenopus laevis*. *Development* **119**, 97-111.

Moon, R. T., Brown, J. D. and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.

Morgan, B. A. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. *Methods Cell Biol.* **51**, 185-218.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790.

Motoyama, J., Takabatake, T., Takeshima, K. and Hui, C. (1998). Ptch2, a second mouse Patched gene is co-expressed with Sonic hedgehog. *Nat. Genet.* **18**, 104-106.

Münsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassar, A. B. (1995). Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9**, 2911-2922.

Nah, H. D., Rodgers, B. J., Kulyk, W. M., Kream, B. E., Kosher, R. A. and Upholt, W. B. (1988). In situ hybridization analysis of the expression of the type II collagen gene in the developing chicken limb bud. *Coll. Relat. Res.* **8**, 277-294.

Nakamura, T., Aikawa, T., Iwamoto-Enomoto, M., Iwamoto, M., Higuchi, Y., Pacifici, M., Kinto, N., Yamaguchi, A., Noji, S., Kurisu, K. and Matsuya, T. (1997). Induction of osteogenic differentiation by hedgehog proteins. *Biochem. Biophys. Res. Commun.* **237**, 465-469.

Nasevicius, A., Hyatt, T., Kim, H., Guttman, J., Walsh, E., Sumanas, S., Wang, Y. and Ekker, S. C. (1998). Evidence for a frizzled-mediated wnt pathway required for zebrafish dorsal mesoderm formation. *Development* **125**, 4283-4292.

Nusse, R. and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**, 99-109.

Nusse, R. and Varmus, H. E. (1992). Wnt genes. *Cell* **69**, 1073-1087.

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. B. and Owen, M. J. (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89**, 765-771.

Parr, B. A. and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* **374**, 350-353.

Pathi, S., Rutenberg, J. B., Johnson, R. L. and Vortkamp, A. (1999). Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. *Dev. Biol.* **209**, 239-253.

Pechak, D. G., Kujawa, M. J. and Caplan, A. I. (1986). Morphology of bone development and bone remodeling in embryonic chick limbs. *Bone* **7**, 459-472.

Peters, J. M., McKay, R. M., McKay, J. P. and Graff, J. M. (1999). Casein kinase I transduces Wnt signals. *Nature* **401**, 345-350.

Poole, A. R. (1991). The growth plate: Cellular physiology, cartilage assembly and mineralization. In *Cartilage: Molecular Aspects* (ed. B. K. Hall and S. A. Newman), pp. 179-211. Boca Raton, FL: CRC Press.

Prophet, E. B. (1994). Tissue processing: Dehydration, clearing, and Infiltration. In *Laboratory Methods in Histotechnology (Armed Forces Institute of Pathology)* (ed. E. B. Prophet, B. Mills, J. B. Arrington and L. H. Sobin). Washington, D.C.: American Registry of Pathology.

Reddi, A. H. (1994). Bone and cartilage differentiation. *Curr. Opin. Genet. Dev.* **4**, 737-744.

Riddle, R. D., Ensini, M., Nelson, C., Tsuchida, T., Jessell, T. M. and Tabin, C. (1995). Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* **83**, 631-640.

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell* **50**, 649-657.

Roark, E. F. and Greer, K. (1994). Transforming growth factor-beta and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. *Dev. Dyn.* **200**, 103-116.

Rodan, G. A. and Harada, S. (1997). The missing bone. *Cell* **89**, 677-680.

Rooney, P. and Archer, C. W. (1992). The development of the perichondrium in the avian ulna. *J. Anat.* **181**, 393-401.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* **275**, 1790-1792.

Rudnicki, J. A. and Brown, A. M. (1997). Inhibition of chondrogenesis by Wnt gene expression in vivo and in vitro. *Dev. Biol.* **185**, 104-118.

Sakanaka, C., Leong, P., Xu, L., Harrison, S. D. and Williams, L. T. (1999). Casein kinase iepsilon in the wnt pathway: regulation of beta-catenin function. *Proc. Natl. Acad. Sci. USA* **96**, 12548-12552.

Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, D. M., Derynck, R. and Moses, H. L. (1997). Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J. Cell Biol.* **139**, 541-552.

Serra, R., Karaplis, A. and Sohn, P. (1999). Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor beta (TGF-beta) on endochondral bone formation. *J. Cell Biol.* **145**, 783-794.

Sheldahl, L. C., Park, M., Malbon, C. C. and Moon, R. T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Curr. Biol.* **9**, 695-698.

Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A. M. and Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of beta-catenin. *Cell Growth Diff.* **8**, 1349-1358.

Slusarski, D. C., Corces, V. G. and Moon, R. T. (1997). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* **390**, 410-413.

Smyth, I., Narang, M. A., Evans, T., Heimann, C., Nakamura, Y., Chenevix-Trench, G., Pietsch, T., Wicking, C. and Wainwright, B. J. (1999). Isolation and characterization of human patched 2 (PTCH2), a putative tumour suppressor gene inbasal cell carcinoma and medulloblastoma on chromosome 1p32. *Hum. Mol. Genet.* **8**, 291-297.

St-Jacques, B., Hammerschmidt, M. and McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13**, 2072-2086.

Szebenyi, G., Savage, M. P., Olwin, B. B. and Fallon, J. F. (1995). Changes in the expression of fibroblast growth factor receptors mark distinct stages of chondrogenesis in vitro and during chick limb skeletal patterning. *Dev. Dyn.* **204**, 446-456.

Tanda, N., Kawakami, Y., Saito, T., Noji, S. and Nohno, T. (1995). Cloning and characterization of Wnt-4 and Wnt-11 cDNAs from chick embryo. *DNA Seq.* **5**, 277-281.

Tessarollo, L., Nagarajan, L. and Parada, L. F. (1992). c-ros: the vertebrate homolog of the sevenless tyrosine kinase receptor is tightly regulated during organogenesis in mouse embryonic development. *Development* **115**, 11-20.

Thorp, B. H., Anderson, I. and Jakowlew, S. B. (1992). Transforming growth factor-beta 1, –beta 2 and –beta 3 in cartilage and bone cells during endochondral ossification in the chick. *Development* **114**, 907-911.

Tomlinson, A., Strapps, W. R. and Heemskerk, J. (1997). Linking Frizzled and Wnt signaling in *Drosophila* development. *Development* **124**, 4515-4521.

Tomlinson, A. and Struhl, G. (1999). Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in

establishing planar polarity in the *Drosophila* eye. *Development* **126**, 5725-5738.

Ungar, A. R., Kelly, G. M. and Moon, R. T. (1995). Wnt4 affects morphogenesis when misexpressed in the zebrafish embryo. *Mech. Dev.* **52**, 153-164.

van Ooyen, A. and Nusse, R. (1984). Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact. *Cell* **39**, 233-240.

Vogel, A., Rodriguez, C., Warnken, W. and Izpisua-Belmonte, J. C. (1995). Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature* **378**, 716-720.

Vogel, A., Rodriguez, C. and Izpisua-Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737-1750.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Wallis, G. A. (1996). Bone growth: coordinating chondrocyte differentiation. *Curr. Biol.* **6**, 1577-1580.

Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1996). A large family of putative transmembrane receptors homologous to the product of the Drosophila tissue polarity gene frizzled. *J. Biol. Chem.* **271**, 4468-4476.

Wesley, C. S. (1999). Notch and wingless regulate expression of cuticle patterning genes. *Mol. Cell. Biol.* **19**, 5743-5758.

Wong, G. T., Gavin, B. J. and McMahon, A. P. (1994). Differential transformation of mammary epithelial cells by Wnt genes. *Mol. Cell. Biol.* **14**, 6278-6286.

Yamaguchi, T. P., Bradley, A., McMahon, A. P. and Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development* **126**, 1211-1223.

Yang, R., Gotoh, Y., Moore, M. A., Rafidi, K. and Gerstenfeld, L. C. (1995). Characterization of an avian bone sialoprotein (BSP) cDNA: comparisons to mammalian BSP and identification of conserved structural domains. *J. Bone Min. Res.* **10**, 632-640.

Yang, Y. and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* **80**, 939-947.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J. and Moon, R. T. (1996). A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Curr. Biol.* **6**, 1302-1306.

Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D. and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.

Zakany, J. and Duboule, D. (1993). Correlation of expression of Wnt-1 in developing limbs with abnormalities in growth and skeletal patterning. *Nature* **362**, 546-549.

Zaphiropoulos, P. G., Unden, A. B., Rahnama, F., Hollingsworth, R. E. and Toftgard, R. (1999). PTCH2, a novel human patched gene, undergoing alternative splicing and up-regulated in basal cell carcinomas. *Cancer Res.* **59**, 787-792.

Zhang, J. and Carthew, R. W. (1998). Interactions between Wingless and DFz2 during *Drosophila* wing development. *Development* **125**, 3075-3085.

Zou, H., Wieser, R., Massague, J. and Niswander, L. (1997). Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11**, 2191-2203.