Wnt9a signaling is required for joint integrity and regulation of 
Ihh during chondrogenesis

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Joints, which separate skeletal elements, serve as important signaling centers that regulate the growth of adjacent cartilage elements by controlling proliferation and maturation of chondrocytes. Accurate chondrocyte maturation is crucial for endochondral ossification and for the ultimate size of skeletal elements, as premature or delayed maturation results predominantly in shortened elements. Wnt9a has previously been implicated as being a player in joint induction, based on gain-of-function experiments in chicken and mouse. We show that loss of Wnt9a does not affect joint induction, but results to synovial chondroid metaplasia in some joints. This phenotype can be enhanced by removal of an additional Wnt gene, Wnt4, suggesting that Wnts are playing a crucial role in directing bi-potential chondro-synovioprogenitors to become synovial connective tissue, by actively suppressing their chondrogenic potential. Furthermore, we show that Wnt9a is a temporal and spatial regulator of Indian hedgehog (Ihh), a central player of skeletogenesis. Loss of Wnt9a activity results in transient downregulation of Ihh and reduced Ihh-signaling activity at E12.5-E13.5. The canonical Wnt/β-catenin pathway probably mediates regulation of Ihh expression in prehypertrophic chondrocytes by Wnt9a, because embryos double-heterozygous for Wnt9a and β-catenin show reduced Ihh expression, and in vivo chromatin immunoprecipitation demonstrates a direct interaction between the β-catenin/Lef1 complex and the Ihh promoter.

KEY WORDS: Wnt, Synovial joint, Chondrocyte maturation, Ihh, Mouse

INTRODUCTION

Joints, which separate adjacent skeletal elements from each other, are important signaling centers that control chondrocyte maturation within the opposing skeletal elements (Francis-West et al., 1999). Three different types, synovial (e.g. joints in the limb), fibrous (e.g. sutures in the skull) and cartilaginous (e.g. joints between vertebral bodies) joints can be distinguished. In the limb, the process of joint formation and differentiation of skeletal elements are tightly linked. The limb skeletal elements are formed by endochondral ossification, a process starting with the condensation of mesenchymal cells forming pre-cartilaginous condensations. It has been proposed that the first step in joint formation is to inhibit cells within the prospective joint region from differentiating into chondrocytes, while neighboring cells can take on this fate and contribute to the cartilage elements. Cells within the prospective joint region form the so-called interzone, which is densely packed and contains flattened cells. Joint interzone cells produce different types of collagens, type I and III, compared with chondrocytes, which produce collagen type II (Ralphs and Benjamin, 1994). The interzone also expresses molecules such as Wnt9a (formerly called Wnt14) and the BMP antagonist Noggin, which are implicated in regulating the non-chondrogenic nature of these cells (Brunet et al., 1998; Debeer et al., 2005; Gong et al., 1999; Guo et al., 2004; Hartmann and Tabin, 2001). In addition, the interzone expresses factors such as parathyroid-hormone related peptide (Pthrp; Phlh – Mouse Genome Informatics), Wnt4, Fgf18, growth differentiation factors (Gdf5, Gdf6 and Gdf7), and various members of the bone morphogenetic proteins (BMPs) that regulate growth and differentiation of the adjacent cartilage elements (Francis-West et al., 1996; Hartmann and Tabin, 2000; Merino et al., 1999; Obbayashi et al., 2002; Storm et al., 1994; Storm and Kingsley, 1996). Recent data suggest that the diverse cell types present in the mature synovial joints, such as synovial cells, articular permanent chondrocytes and cells of the joint capsule, originate from the interzone region (Archer et al., 2003; Rountree et al., 2004).

The molecular mechanisms underlying joint formation are not yet well understood. Various signaling molecules, such as Gdf5, Gdf6 and Noggin have been implicated in joint formation (Brunet et al., 1998; Settle et al., 2003; Storm and Kingsley, 1996). However, none of those factors is sufficient to induce joint formation (Capdevila and Johnson, 1998; Merino et al., 1999; Pathi et al., 1999; Pizette and Niswander, 2000; Storm and Kingsley, 1999; Tsunaki et al., 2002). By contrast, disruption of integrin signaling and ectopic activation of Wnt9a signaling leads to the induction of molecular markers characteristic for the joint-interzone and the formation of joint-like regions (GarciaDiego-Cazares et al., 2004; Guo et al., 2004; Hartmann and Tabin, 2001). Based on this, it has been proposed, that Wnt9a signaling is involved in joint induction.

Factors secreted by cells adjacent to the joint, such as Pthrp, Fgf18 and others, are important regulators for the maturation of chondrocytes, from proliferative, to postmitotic prehypertrophic, to hypertrophic chondrocytes, which mature further and eventually undergo apoptosis. Accurate control of chondrocyte proliferation and maturation is crucial for determining the future size of the skeletal element. A key regulator for these processes is the signaling molecule Ihh, which is produced by prehypertrophic/early hypertrophic chondrocytes and plays essential roles in skeletogenesis coordinating cartilage growth and osteoblastogenesis: Ihh signaling controls the expression of another secreted molecule, Pthrp, that negatively regulates chondrocyte

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maturation. Furthermore, Ihh has additional Pthrp-independent roles; it stimulates chondrocyte proliferation and osteoblast differentiation (Kronenberg, 2003). Ihh expression is under transcriptional control by Runx2 and Runx3 (Yoshida et al., 2004), and its expression levels are regulated in an antagonistic manner by Fgf and Bmp signaling (Minina et al., 2002). Modulation in the expression of secreted factors controlling the central regulator Ihh affect growth and differentiation of the skeletal elements, resulting primarily in a shortening of the skeletal elements.

In order to address whether Wnt9a is necessary for joint induction, we have targeted the Wnt9a locus and generated two alleles, a conditional allele and a lacZ knock-in allele. Wnt9a loss-of-function mutants die at birth. They display partial joint fusions of carpal and tarsal elements and chondroid metaplasia in synovial and fibrous joints. The phenotypes associated with synovial joints are augmented in Wnt9a;Wnt4 double mutants. Our data demonstrate that Wnts are essential to maintain joint integrity, but that they are probably not required for inducing joint formation. In addition, we found that Wnt9a mutants have shortened appendicular long bones. The shortening is due to a temporary downregulation of Ihh expression in Wnt9a mutants at embryonic days E12.5-13.5. Furthermore, we show by genetic interaction and by in vivo chromatin immunoprecipitation that the regulation of Ihh expression and chondrocyte maturation by Wnt9a is mediated through β-catenin.

MATERIALS AND METHODS

Generation of Wnt9a mutant alleles

The targeting construct of the conditional Wnt9a allele was generated as follows: a FRT-flanked neomycin resistance gene (neo) driven by the PGK promoter with an 3′ located loxP site (Sun et al., 2000) was inserted into an Spel restriction site 357 bp upstream of exon 2. A double-stranded loxP oligo was ligated into an ApaLI site 67 bp downstream of exon 2. The lacZ targeting construct was generated by introducing an SA-ires-lacZ-SV40pA-FRT-PGK-neo-FRT cassette into the SmaI site of exon 2. Positively targeted ES cells were identified by Southern blot analyses using external 5′ and 3′ probes on EcoRV-digested genomic DNA (frequency: 1 in 25 in the C1 ES cell line) and introduced into mouse blastocysts (Hendrickson et al., 1995). Four independently targeted ES-cell clones were used to generate chimeras (two for each allele), three of which transmitted the recombinant alleles (only one for the conditional allele). Exon 2 was deleted in the germline using PrxiCre females. Genotyping was performed by PCR (primer sequences available upon request). Phenotypes for embryos homozygous for either of the two Wnt9a alleles (Δ or lacZ) were identical on mixed, random bred (Swiss-Webster), F1 (129/Sv; C57Bl6/J) and inbred (129/Sv) backgrounds.

Mouse strains

Wnt4 heterozygous mice were purchased from Jackson laboratory. Genotyping of Wnt4 and β-catenin alleles (lacZ and floxed) was performed by PCR as previously described (Huelsken et al., 2000; Huelsken et al., 2001; Stark et al., 1994). Limbs lacking β-catenin activity in the mesenchyme were generated as described by Hill et al. (Hill et al., 2005).

Skeletal analysis

Newborn pups (P0) and embryos were skinned, eviscerated and fixed in 95% ethanol. Alizarin Red/Alician Blue or Alician Blue staining of the skeletons were performed as described previously (McLeod, 1980).

β-Galactosidase staining, histology, in situ hybridization and BrdU incorporation

For β-galactosidase staining, embryos E9.5-E13.5 were fixed for 15-30 minutes and skinned newborns were fixed for 1 hour in 0.1 M phosphate buffer containing 0.2% glutaraldehyde, 2 mM MgCl2, 5 mM EGTA on ice, washed three times in 0.1 M phosphate buffer containing 0.01% deoxycholate. 0.02% NP-40 and 2 mM MgCl2 at room temperature, and stained overnight at 37°C in staining solution [1 mg/ml X-gal, 4% diethyl formamide, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6].

For histology and section in situ hybridization, tissue was treated as previously described (Hill et al., 2005). For analysis of Brdu incorporation, 50 μg Brdu/g body weight was injected intra-peritoneally into pregnant mice 2 hours before sacrifice. Brdu incorporation was detected on sections by immunohistochemistry (Zymed Laboratories). For each analysis and developmental stage, at least three independent samples were analyzed.

Western blot analysis

For Western blot analysis, protein was extracted from cultured chicken sternum chondrocytes infected with different RCAS viruses. Extracts of 50 μg per lane were loaded. Luminal detection was performed using an antibody against chicken β-catenin (1:800, Sigma C7027), followed by incubation with a HRP-conjugated secondary antibody (1:2500; Promega).

Limbal explant cultures

Forelimbs were skinned and removed from E12.5 and E13.5 embryos. One limb of a forelimb pair was cultured in the presence of 25 μM SU5402 (Calbiochem)/0.2% DMSO, while the other one was cultured in 0.2% DMSO in DMEM:F12 (Invitrogen) supplemented with 10% FCS and L-Glutamine. Limbs were cultured for 24 hours in 24-well dishes floating on top of Nucleopore Track-ETch Membranes (Whatman) in a humidified tissue culture incubator at 37°C and 5% CO2.

Immunohistochemical staining

For immunohistochemical staining of cultured caudal chondrocytes, cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde in PBS, washed twice with PBS. Endogenous peroxidase activity was inhibited by incubating the cells for 30 minutes in 1% H2O2 in PBS. Cells were subsequently washed three times with PBS; blocked for 30 minutes with PBS, 10% FCS and 0.1% Triton-X100; and incubated with the primary antibodies against collagen type II (II-II6B3 supernatant, 1:30) and collagen type III (3B2 supernatant 1:30) from the Developmental Hybridoma Bank (Iowa). The signal was detected using a biotinylated anti-mouse secondary antibody (dilution 1 in 250; Vector Laboratories) in combination with the ABC kit (Vector labs) and DAB (Sigma) as a substrate. β-Catenin immunohistochemical staining on paraffin sections was performed using the anti-β-catenin (BD Transduction Laboratories, 1 in 250) after heat-induced citrate buffer antigen retrieval. Signal detection was performed as described above.

Chromatin immunoprecipitation (ChiP)

For in vivo cartilage lysates, humeri were dissected from 13.5 dpc limbs (FVB/N mice: litters with 10-13 embryos). Humeri from one litter were dissociated in 500 μl of 0.3% collagenase IV/0.1% Trypsin/2% FCS/DMEM for 15 minutes at 37°C and by additional usage of a bouncer. After a PBS wash, cells were crosslinked with 1% formaldehyde for 10 minutes, followed by quenching with 125 mM glycine. Whole-cell extracts were prepared for ChiP as described (Martens et al., 2005). Approximately
200 µg of fragmented chromatin was used in immunoprecipitation with 4 µg β-catenin (St. Cruz, sc-1496), 4 µg Lef1 (St. Cruz, sc-8592) and 4 µg H3-K4 methylation (Upstate Biotechnology) antibodies. Purified DNA from immunoprecipitates, as well as of the input material was analyzed by real-time PCR using the Roche Sybr green quantitation method on a MJ research Lightcycler (n=3). Results were normalized and presented as percentage of input DNA. For amplification of the three potential TCF/Lef1 sites, the following primer pairs were used: site 1 forward, 5'TCCGGCTGCACTGGTGTCG 3'; site 1 reverse, 5'CGGGCGCGG-GACTGAGG 3'; site 2 forward, 5'ACTCCCCGCACTCC-CCACGACTCC 3'; site 2 reverse, 5'GACGGGACTGCTGGGAAT- CAC 3'; site 3 forward, 5'TGAATCCGGAGCAAGGCTAG 3'; site 3 reverse, 5'TGGGATGGCAGGGGATAGTA 3'.

RESULTS
Generation of Wnt9a mutant alleles
Two Wnt9a alleles were generated by targeting the genomic Wnt9a locus in embryonic stem cells: a conditional allele with loxP sites flanking exon 2 (Fig. 1A) and a lacZ-knock-in allele with an IRES-lacZ-FRT-neo cassette inserted into exon 2 (Fig. 1A). Germline transmission and proper targeting of mutant alleles was verified by Southern blot (Fig. 1B). Mice heterozygous for the deleted (Δ) allele were obtained by germline deletion of the floxed exon 2 (Fig. 1B). A truncated transcript, resulting from aberrant splicing of exon 1 to exon 3, could be amplified by RT-PCR from RNA of Wnt9aΔΔ mutant embryos (Fig. 1C). Sequencing revealed a frame-shift and a premature Stop. Any protein made from this transcript would be a truncated transcript, resulting from aberrant splicing of exon 1 to exon 3, and therefore we will refer to them as –allele, unless translated from this allele. We consider both alleles as being null alleles of the Wnt9a protein. In the lacZ allele, the open reading frame of Wnt9a was disrupted and thus no functional protein can be translated from this allele. We consider both alleles as being null alleles and therefore we will refer to them as –allele, unless otherwise noted. No pups homozygous for either of the mutant alleles were recovered at weaning from heterozygous intercrosses of Wnt9aΔΔ or Wnt9aΔlacZ mice. Homozygous mutant pups died within 12 hours of birth, for so far unknown reasons. Histological analyses of their major organs, heart, lung, liver, intestinal tract, kidney and brain, did not reveal any obvious abnormalities. They are slightly smaller than their littermates and can be readily identified by the absence of milk in their stomach (Fig. 1D).

Homozygous Wnt9a mutants display skeletal abnormalities
Comparative skeletal analyses of Wnt9aΔΔ newborns with their heterozygous and wild-type littermates revealed no gross joint defects but a number of skeletal abnormalities (Fig. 2A and data not shown). In all of the Wnt9a mutants, there was an ectopic Alcian Blue-positive nodule present in the elbow region (Fig. 2A, Fig. 3A, part a). In addition, the appendicular long bones were slightly reduced in length and showed an even greater reduction in the size of the mineralized regions (Fig. 2A). These reductions were more prominent in the proximal bones, such as scapula and humerus, and ileum and femur (Fig. 2B; data not shown). The hyoid bone and atlas were hypoplastic (Fig. 2A). In the skull, the supraoccipital bone showed reduced mineralization and the frontal bones were further apart (Fig. 2C, part a). The basioccipital bone was abnormally shaped and reduced in size (Fig. 2C, part b). Furthermore, the cartilaginous base was extended dorsally, particularly noticeable at the base of the parietal bones (Fig. 2C, part c,e-f'). In addition ectopic cartilage nodules were present within the midline sutures (Fig. 2C, part d-f').

Chondroid metaplasia of fibrous and synovial joint cells in Wnt9a mutants
Wnt9aΔΔ newborns display no obvious defects with respect to fusions of major joints. However, ectopic cartilaginous material was detected by Alcian Blue staining in the interfrental and sagittal

Fig. 1. Construction of Wnt9a alleles and analysis of mutants. (A) Schematic of the two targeting constructs, which were introduced into the Wnt9a locus on mouse chromosome 11. Floxed allele (fl): exon 2 (Ex2) flanked by loxP sites. lacZ allele: insertion of an IRES-lacZ cassette into exon 2. The 5' and 3' external probes are indicated below the genomic locus map. Restriction enzymes: B, BamHI; N, NheI; Ri, EcoRI; RV, EcoRV. (B) Southern-blot on EcoRV-digested genomic DNA from mutant (ΔΔ or lacZ/lacZ), heterozygous (+/Δ or +/lacZ) and wild-type (+/+). 10.5 littersmates from intercrosses of +/Δ and +/lacZ heterozygous mice, respectively, hybridized with the 3' genomic external probe. (C) RT-PCR using RNA obtained from E10.5 wild-type and Wnt9aΔΔ embryos, showing the presence of a 1.1 kb transcript in wild type and a shorter 900 bp transcript in Wnt9aΔΔ mutants. (D) Wild-type (WT) and Wnt9aΔΔ mutant newborn littermates. Mutants are slightly smaller (indicated by the shorter black line on the right side) and that they do not have milk in the stomach (arrow).
suture regions, separating frontal and parietal bones, respectively (Fig. 2C, parts d,e,f; data not shown) and in the elbow joint (Fig. 3). Sutures are fibrous joints between the flat bones of the cranial vault, which serve as major sites for bone expansion during postnatal skull growth (Opperman, 2000). Van Kossa staining showed that the mineralized regions were further apart in mutant than wild-type skulls (Fig. 2C, part e). In situ hybridization of coronal skull sections revealed that the chondrocyte markers Col2a1 and Sox9 were ectopically expressed in cells within the frontal and sagittal sutures (Fig. 2C, parts e,f,g; data not shown). In addition, their normal expression domains at the base were expanded dorsally (Fig. 2C, parts f,f,g). An ectopic Alcian Blue-stained nodule was observed in the humeral-radial space of all Wnt9a mutant newborns (n=24; Fig. 3A, part a'). Histological analyses on sections of P0 Wnt9a–/– forelimbs revealed that cells within the synovial fold had a chondrocyte-like appearance (Fig. 3A, part b') and expressed Sox9 and Col2a1 (Fig. 3A, part c'; data not shown). In humans, this phenotype of synovial cells differentiating into chondrocytes forming loose ectopic cartilaginous nodules is referred to as synovial chondroid metaplasia or synovial chondromatosis. The onset of synovial chondroid metaplasia in the humeral-radial joint (HRJ) of Wnt9a mutants was detectable as early as E15.5 by in situ hybridization [showing expression of the chondrogenic marker Sox9 in a broad domain within the HRJ region (Fig. 3A, part d')], while Col2a1 expression was restricted to a few cells within this region (Fig. 3A, part e'). Additional joint abnormalities were observed in Wnt9a mutants, such as partial joint fusions between the navicular and intermediate cuneiform tarsal elements in the foot and between the carpal elements c and 3 in the wrist (Fig. 3A, parts f,g). These data suggest that Wnt9a signaling is required in some joints to maintain the identity of joint cells.

**Wnt9a misexpression in chondrocytes leads to dedifferentiation associated with stabilization of β-catenin**

Loss of Wnt9a signaling in HRJ and midline suture cells led to their ectopic differentiation into chondrocytes. Based on this, we hypothesized that Wnt9a signaling in synovial and fibrous joint cells is required to suppress their chondrogenic potential. To test this hypothesis further, we asked whether ectopic Wnt9a signaling in chondrocytes would lead to alterations of the cells. We used primary chicken sternal chondrocytes and infected them with replication competent avian retroviruses (RCAS) expressing either Wnt9a, the canonical ligand Wnt3a, the non-canonical ligand Wnt5a, a constitutively active form of β-catenin (caβ-cat) or alkaline
phosphatase (AP) as control. In AP and Wnt5a-infected cultures, the chondrocytes retained their typical cuboidal shape and stained positive for collagen type II (Col2; Col2 staining is shown in Fig. 3B). By contrast, cells infected with either Wnt9a, Wnt5a or caβ-cat viruses had a fibroblastic appearance and ceased to produce Col2 (Fig. 3B, and data not shown). These cells stained positively for collagen type III (Col3) instead, which was not produced by the control cells (Fig. 3B). Western blots from whole-cell extracts revealed that the β-catenin levels were increased in chondrocytes infected with Wnt9a virus compared with AP or Wnt5a-infected cell extracts (Fig. 3C).

These data show that gain of Wnt9a signaling leads to increased β-catenin levels, and that this increase can transform chondrocytes into fibroblast-like cells producing a different type of collagen.

**Wnt4 and Wnt9a act cooperatively in maintaining joint integrity**

Surprisingly, only a few synovial joints were affected in Wnt9a mutants, despite the fact that Wnt9a is expressed in all joints (see Fig. S1 in the supplementary material). At least two other Wnt genes, Wnt4 and Wnt16, are expressed in joints (Guo et al., 2004; Hartmann and Tabin, 2000; Hartmann and Tabin, 2001). Wnt4 mutants do not have any joint abnormalities (Stark et al., 1994). However, mice double mutant for Wnt9a and Wnt4 (Wnt9a−/−;Wnt4−/−) developed synovial chondroid metaplasia in two additional major joints, the ankle and knee joint (n=4; Fig. 4A, parts d,e). In addition, fusions of tarsal and carpal elements were observed in the foot (calcaneus and cuboid, and navicular and intermediate cuneiform) (Fig. 4A, parts f,g) and wrist (carpal elements 2, c and 3) (n=4/4; Fig. 4B, part e). In one specimen, we observed the presence of an ectopic cartilaginous piece in a ligament (see asterisk in Fig. 4A, part g).

In order to address whether joint formation or maintenance were affected by the loss of Wnt9a and Wnt4, we analyzed the expression of various markers Col2a1, Col3, Gdf5, Gli3 and Wnt4 in E13.5 and E15.5 wrists, focusing primarily at the carpal elements 2, c and 3. Those can be distinguished as separate elements in wild-type and mutant wrists at E14.5, showing identical marker expression (Fig. 4A, parts f,g) and wrist (mutant elements 2, c and 3) (n=4/4; Fig. 4B, part e). In one specimen, we observed the presence of an ectopic cartilage piece in a ligament (see asterisk in Fig. 4A, part g).

Alteration of chondrocyte maturation in Wnt9a mutants

The reduction of the mineralization/ossification centers in skeletal elements formed by endochondral ossification (Fig. 2A,B) suggested a possible delay of chondrocyte maturation in Wnt9a
In order to determine the nature and onset, we performed histological and molecular marker analyses on the developing long bones at different embryonic stages. Whole-mount and section in situ analyses at E11.5 using the early chondrogenic markers Sox9 and Col2a1 revealed no difference in the overall size of the long bone anlagen between Wnt9a mutants and littermate controls (data not shown). Proliferation rate (BrdU) and apoptosis (TUNEL) were also not altered (data not shown). Furthermore, the chondrocyte differentiation marker Ihh was expressed in Wnt9a mutant humeri in a domain of similar size and at levels equal to those detected in wild-type or heterozygous littermates (Fig. 5A; data not shown). At E12.5, however, Ihh expression levels were slightly downregulated in mutants (Fig. 5B) and this downregulation was even more pronounced at E13.5 (Fig. 5C). Downregulation of Ihh was independently confirmed by real-time PCR analysis on cDNA generated from E12.5 and E13.5 wild-type and mutant humeri (see Fig. S2A in the supplementary material).

Surprisingly, at E14.5 Ihh expression levels were similar to those in wild-type humeri. Nevertheless, the Ihh domain was not yet separated by a population of more mature, Ihh-negative chondrocytes (Fig. 5D) and resembled wild-type expression at E13.5 (compare Fig. 5D with Fig. 6C). This suggests that Wnt9a signaling temporally and spatially regulates Ihh in proximal long bones.

By contrast, the expression domain of the gene encoding parathyroid hormone receptor 1 (Ppr; Pthr1 – Mouse Genome Informatics), which overlaps with Ihh, was reduced only in extent not in magnitude at E13.5 and E14.5, reflecting a delay in chondrocyte maturation (Fig. 5E; data not shown). The collagen 10a1 (Col10a1) expression domain, which marks hypertrophic chondrocytes, was slightly expanded in Wnt9a mutant humeri at E12.5 (Fig. 5F), while it was either reduced or not detectable at E13.5 (Fig. 5G; data not shown). At E14.5 the Col10a1 domain in mutants resembled that of E13.5 wild-type humeri (Fig. 5H). This marker analysis showed that Wnt9a signaling is crucial for chondrocyte maturation around E12.5-E13.5.

Temporal downregulation of Ihh signaling in Wnt9a mutant long bones

Because Ihh expression was temporarily downregulated, we analyzed whether Ihh signaling and Ihh regulated processes were also affected. Analysis of the Ihh target genes patched 1 (Ptch1), which functions also as a receptor for Ihh, and Pthrp (St-Jacques et al., 1999; Vortkamp et al., 1996), showed a reduced expression in Wnt9a mutant humeri at E12.5 (Fig. 5F), while it was either reduced or not detectable at E13.5 (Fig. 5G; data not shown). At E14.5 the Col10a1 domain in mutants resembled that of E13.5 wild-type humeri (Fig. 5H). This marker analysis showed that Wnt9a signaling is crucial for chondrocyte maturation around E12.5-E13.5.
Wnt9a signaling in joints and chondrocytes

Wnt9a signals through β-catenin regulating chondrocyte maturation

Gain-of-function experiments suggested that Wnt9a signals through the canonical β-catenin pathway (Day et al., 2005; Guo et al., 2004) (this work). To assess this further, we looked at genetic interaction between Wnt9a and β-catenin with regard to chondrocyte maturation at E12.5-E15.5 in embryos double heterozygous for Wnt9a and β-catenin. At E12.5, but not at E13.5, Ihh expression levels were reduced in double-heterozygous embryos compared with wild-type or Wnt9a+/− littermates (Fig. 5A, and data not shown). Treatment with SU5402 did not notably increase the size reduction of the Col10a1 domain already present in Wnt9a+/− humeri, but as in the wild-type and heterozygous humeri, Col10a1 expression was slightly decreased (see Fig. S3G in the supplementary material). Based on our analysis, we would conclude that Fgf signaling at early developmental stages is a positive regulator of chondrocyte maturation. Most importantly, our data suggest that Fgfs do not contribute to the observed downregulation of Ihh in Wnt9a mutants.

for altered Fgf expression of 19 Fgf genes (Fgf1-13, Fgf15, 17, 18 and Fgf20-22) using real-time PCR at E12.5 and E13.5. With the exception of Fgf4, which was downregulated, none of the Fgf genes analyzed showed any significant increase or decrease in relative expression levels at E12.5 (see Fig. S3A in the supplementary material; data not shown). Interestingly at E13.5 the relative expression levels of five Fgf genes (Fgf1, Fgf6, Fgf15, Fgf20 and Fgf21) were increased (see Fig. S3B in the supplementary material). This increase could potentially contribute to downregulation of Ihh at E13.5. To further investigate this possibility, we analyzed the expression of Ihh and Col10a1 in humeri of embryos with constitutive active Fgfr3 signaling in chondrocytes (Fgfr3ach G380R) (Naski et al., 1998). However, no downregulation of Ihh expression was observed in humeri of Fgfr3ach mice at E12.5 (n=5) or E13.5 (n=10) (see Fig. S3C,D in the supplementary material). By contrast, we observed on average a slight expansion of the expression domains of Ihh (by ~8%) and Coll10 (by approx. 6%) at E13.5 compared with wild-type littermates (see Fig. S3D in the supplementary material). This suggests that activation of Fgfr3 signaling might promote chondrocyte maturation around E13.5.

As the upregulated Fgfs might not necessarily signal through Fgfr3, we performed limb explants cultures using E12.5 and E13.5 forelimbs from Wnt9a mutant, heterozygous and wild-type littermates to test whether inhibiting Fgf signaling in Wnt9a mutants would rescue Ihh expression levels. Limbs of corresponding limb pairs were cultured in the presence of DMSO or SU5402, a kinase inhibitor specific to Fgfrs (Mohammadi et al., 1997). Treatment with SU5402 resulted in a strong reduction in the domain size and expression levels of Ihh in wild-type and heterozygous E12.5 cultured humeri (n=5; see Fig. S3E in the supplementary material), while in E13.5 limbs only a size reduction was visible (see Fig. S4F in the supplementary material). In Wnt9a−/− E12.5 and E13.5 humeri, Ihh expression was even further downregulated (n=3; see Fig. S3E,F in the supplementary material). No Col10a1 expression was detected at E12.5 in control and inhibitor-treated limbs (data not shown), while the Col10a1 expression domain was not significantly affected at E13.5 (see Fig. S3G in the supplementary material). Treatment with SU5402 did not notably increase the size reduction of the Col10a1 domain already present in Wnt9a+/− humeri, but as in the wild-type and heterozygous humeri, Col10a1 expression was slightly decreased (see Fig. S3G in the supplementary material).

Based on our analysis, we would conclude that Fgf signaling at early developmental stages is a positive regulator of chondrocyte maturation. Most importantly, our data suggest that Fgfs do not contribute to the observed downregulation of Ihh in Wnt9a mutants.

not shown). No significant difference in proliferation rate could be detected in mutant compared with wild-type humeri at E14.5 (data not shown).

Ihh plays a third role during endochondral bone formation; it is required for osteoblastogenesis in the perichondrium/periosteum (Long et al., 2004; St-Jacques et al., 1999). Analyses of various periosteal markers, such as Runx2, osterix (Osx; Sp7 – Mouse Genome Informatics), osteopontin (Op; Spp1 – Mouse Genome Informatics) and Bmp3, at E13.5 revealed that the expression of all four markers was reduced (Fig. 5K,L; data not shown). However, at E14.5, expression of these markers was comparable with wild type at E13.5 (data not shown). These data demonstrate that loss of Wnt9a activity results in a temporary reduction of Ihh signaling. This ultimately leads to a delay by ~1 day in chondrocyte and osteoblast maturation. Although Ihh levels are back to normal at around E14.5, the maturation delay cannot be compensated and thereby remains noticeable even at birth, reflected in the shortening of proximal skeletal elements.

Fgf signaling has also been shown to negatively regulate Ihh (Chen et al., 2001; Li et al., 1999; Minina et al., 2002; Naski et al., 1998). Therefore, we addressed whether Fgf signaling is involved in the temporary downregulation of Ihh in Wnt9a mutants by assaying
Fig. 6. Genetic interaction of Wnt9a and β-catenin in chondrocyte maturation. (A) In situ hybridization for Ihh, Ptc1 and Col10a1 on alternating sections of humeri from wild type, Wnt9a+/–; β-catenin+/– and Wnt9a+/–; β-catenin+/– mutant littersates. Arrowheads indicate reduced expression of Ptc1. (B) In situ hybridization on E15.5 wild-type and Wnt9a+/–; β-catenin+/– humeri, showing that the Ihh and Col10a1 expression domains are closer together in Wnt9a+/–; β-catenin+/– humeri compared with WT. (C) Immunohistochemical staining at E13.5, showing reduced β-catenin levels in flattened and prehypertrophic chondrocytes (bracket) in Wnt9a mutants in comparison with wild type above. Similar β-catenin levels are found in the periosteum (arrowheads). (D) Alcian Blue/Alizarin Red stained forelimbs of E15.5 wild type, Wnt9a+/–; β-catenin+/– and Wnt9a–/– embryos, showing a similar size reduction of the mineralized regions of scapula and humerus in double heterozygous mutants compared with Wnt9a single mutants.

primarily noticeable in the periosteum, where Ptc1 was less strongly expressed (arrowheads in Fig. 6A). The Col10a1 expression domain was also reduced in size in the double heterozygous at E13.5 and even further reduced in Wnt9a+/–; β-catenin+/– mutant humeri (Fig. 6A). At E15.5, the expression domains of Ihh and Col10a1 were closer together in Wnt9a+/–; β-catenin+/– mutant humeri compared with wild type (Fig. 6B), demonstrating a delay in chondrocyte maturation. This delay in chondrocyte maturation and mineralization was also noticeable in skeletal preparations of E15.5 embryos, where the mineralized region in the long bones, particularly scapula and humerus, was reduced in size in Wnt9a–/–; β-catenin–/– to a similar extent as in Wnt9a–/– embryos (Fig. 6D). Consistent with the observation that Wnt9a signaling led to stabilization of β-catenin, nuclear β-catenin levels were reduced in prehypertrophic chondrocytes and in articular chondrocytes in Wnt9a+/– skeletal elements (Fig. 6C). Thus, Wnt9a is probably regulating chondrocyte maturation and Ihh expression through the canonical Wnt/β-catenin pathway.

**Regulation of Ihh expression by β-catenin/Lef-1**

Our data suggested that Ihh could be a direct target for the β-catenin/TCF-complex. This is supported by the presence of three potential TCF-binding sites within the Ihh promoter (Fig. 7A). Binding of a β-catenin/TCF-complex to the Ihh promoter was shown by in vivo chromatin immunoprecipitation (ChIP) using cell lysates from E13.5 wild-type skeletal elements. Antibodies against β-catenin and Lef1 could immunoprecipitate three different regions of the Ihh promoter, each containing one of the potential binding sites (Fig. 7B). Performing ChIP assays with an antibody against H3K4 confirmed that the chromatin was in a transcriptionally active state. Real-time PCR analysis revealed that the TCF/Lef site closest to the putative transcriptional start site showed the strongest interaction with β-catenin and Lef1 (Fig. 7B). Therefore, we propose that the canonical Wnt pathway directly regulates Ihh transcription and that Wnt9a is the relevant ligand.

**DISCUSSION**

Wnt signaling is required to suppress the chondrogenic potential of joint cells

All joints are initially formed in Wnt9a mutants; however, loss of Wnt9a leads to ectopic differentiation of cartilage in the sagittal suture, to partial joint fusions of carpal and tarsal elements, and to synovial chondroid metaplasia in the HRJ. Double mutant analyses showed that Wnt9a and Wnt4 signaling is cooperatively required to suppress the chondrogenic potential of synovial and other joint cells in different types of joints. Nevertheless, not all joints are affected. This could be due to the presence of another Wnt gene, Wnt11. Our analysis suggests that Wnts might not be required for joint induction as previously proposed (Guo et al., 2004; Hartmann and Tabin, 2001). This is further supported by the observation that even the fusion of the three carpal elements (2, c and 3) present in Wnt9a; Wnt4 double mutant newborns was not due to a defect in the initial joint formation. Both Wnts might use the β-catenin pathway (Guo et al., 2004; Hartmann and Tabin, 2000), therefore we examined the expression of joint markers during early stages of limb development in mice lacking β-catenin in the limb mesenchyme (Hill et al., 2005). This revealed that early joint interzone markers such as Gdf5, Wnt4 and Gli1 were still expressed in the presumptive joint regions at E11.5 (see Fig S4 in the supplementary material), suggesting that canonical Wnt-signaling is not required for induction of those joint markers. However, as previously reported, their expression was lost and the joints never properly formed, resulting in partial fusions at later stages (Guo et al., 2004) (data not shown). Previous work has shown that synovial tissue contains chondroprogenitors (Nalin et al., 1995; Nishimura et al., 1999). Thus, it is likely that, analogous to its function in osteochondroprogenitors (Day et al., 2005; Hill et al., 2005), canonical Wnt/β-catenin signaling is required in an autocrine fashion to suppress the chondrogenic potential of cells in the presumptive joint region, thereby enabling joint formation and maintaining the integrity of the joint at later stages. Based on our analyses we would postulate that in the synovium and joint capsule there are still bi-potential synovioprogenitors present at later stages of joint development, and that continuous canonical Wnt signaling is essential to suppress their chondrogenic potential thereby maintaining joint integrity. This strong pro-synovial anti-
**Wnt9a signaling in joints and chondrocytes**

**Fig. 7. β-Catenin and Lef1 physically interact with the Ihh promoter.** (A) Schematic view of the 2.0 kb Ihh promoter region, showing the position of the three potential TCF/Lef1-binding sites relative to the translational start site (+1). (B) Chromatin immunoprecipitation for β-catenin, H3-K4 tri-methylation and Lef1 from E13.5 dissociated wild-type humeri, showing immunoprecipitation for all three sites in the Ihh promoter, but none of the tubulin promoter. Real-time PCR quantification (normalized to input and indicated by the numbers below) revealed that site 1 is bound with the highest affinity and site 3 with the lowest (n=3).

The development of all three sites in the Ihh from E13.5 dissociated wild-type humeri, showing immunoprecipitation numbers below) revealed that site 1 is bound with the highest affinity and ultimately in osteoblastogenesis by approximately 1 day and ultimately in shortening of the proximal long bones. Fgf signaling has also been implicated in negatively regulating Ihh expression and could potentially mediate the effect seen in Wnt9a mutants (Ornitz and Marie, 2002). However, Ihh expression was not downregulated in E12.5 and E13.5 limbs of Fgf3/3 mice and no significant increase in the expression levels of Fgf/s was observed in Wnt9a mutants at E12.5. Our Fgf signaling analyses, instead, suggest that Fgfs might actually contribute to restoring normal chondrocyte maturation in Wnt9a mutants from E14.5 onwards, concomitant with an upregulation of some Fgfs. Thus, Ihh expression is probably negatively regulated by Wnt signaling and Fgf signaling at different developmental time points. Therefore, it is tempting to speculate that sequential signaling input through different pathways, converging on the regulation of Ihh, which is at the heart of the endochondral bone formation process, could be a crucial mechanism to control the size of individual appendicular skeletal elements.

**Wnt9a regulates Ihh expression through the canonical β-catenin pathway**

The levels of β-catenin are reduced in prehypertrophic chondrocytes in Wnt9a mutants, while Wnt9a overexpression leads to an increase of β-catenin in vitro. This, together with the fact that β-catenin and Lef1 associate with the Ihh promoter in vivo, suggests that Wnt9a-dependent regulation of Ihh is probably mediated via the canonical/β-catenin pathway. This is further supported by the observations that Ihh expression levels in humeri of Wnt9a/β-catenin double heterozygous animals were slightly reduced and that Ihh expression varies from downregulation to temporary loss or delayed expression in skeletal elements of mice lacking β-catenin activity depending on the Cre-deleter line (T.P.H. and C.H., unpublished) (Akiyama et al., 2004; Hu et al., 2005). Ihh expression in the humerus and femur is also transcriptionally dependent on Runx2, and in more distal bones it requires the activity of Runx2 and Runx3 (Inada et al., 1999; Kim et al., 1999; Yoshida et al., 2004). Runx2 has been recently suggested to be a direct target for canonical Wnt/β-catenin signaling in osteoblasts (Gaur et al., 2005); however, chondrogenic Runx2 expression levels were not significantly changed at the onset of Ihh downregulation at E12.5 in Wnt9a mutants (data not shown). The slight decrease in Runx2 levels at later stages is probably due to the downregulation of Ihh, as Runx2 expression is dependent on Ihh signaling (Hu et al., 2005; Long et al., 2004). Furthermore, Runx2 expression was also not significantly altered in β-catenin mutant limbs (Day et al., 2005; Hill et al., 2005; Hu et al., 2005). Interestingly, Runx2 activity in chondrocytes is modulated by interaction with co-repressors such as HDAC4 and co-activators such as groucho 5 (Vega et al., 2004; Wang et al., 2004). Groucho 5 (Grg5; Aes – Mouse Genome Informatics) acts as a de-repressor in β-catenin/TCF signaling, unlike groucho 1-4, which act as co-repressors (Brantjes et al., 2001). Intriguingly, Grg5+/- mice display a postnatal, temporary reduction in Ihh expression, which is further dependent on Runx2 levels (Wang et al., 2004; Wang et al., 2002). Thus, it is conceivable that the β-catenin/Lef1 complex and Runx2 could cooperatively regulate Ihh. Our data, together with published observations, suggest that Wnt signaling regulates the level of Ihh expression temporally during embryonic and postnatal development, thereby controlling chondrocyte maturation and the growth of skeletal elements.

**Wnt9a a temporal regulator of Ihh controlling the size of skeletal elements**

In addition, we discovered a role for Wnt9a signaling in chondrogenesis. Skeletal elements differ in size and shape; however, this is regulated is largely unknown, but there is evidence that signals from the joint might be involved in the size regulation. One way to control size would be through modulation of Ihh signaling, a central regulator of endochondral bone formation (Kronenberg, 2003). Mutations affecting chondrocyte maturation or proliferation predominantly lead to size reduction of all or sometimes only individual skeletal elements. Here, we show that Wnt9a signaling temporally and spatially regulates Ihh expression in the appendicular skeleton. It is required around E12.5-E13.5 to maintain high levels of Ihh expression, but seems to be dispensable from E14.5 onwards. Temporary downregulation of Ihh results in reduced chondrocyte proliferation, and a delay of chondrocyte maturation and osteoblastogenesis by approximately 1 day and ultimately in...
Interestingly, when we analyzed the expression levels of various Wnt genes (Wnt4, Wnt5a, Wnt5b and Wnt6) by semi-quantitative and real-time RT-PCR, we found that Wnt4 expression was elevated at E12.5 and E13.5 in Wnt9a mutant compared with wild-type humeri (see Fig. S5C in the supplementary material; data not shown). Concomitantly, chondrocyte maturation was further delayed in Wnt9a/Wnt4 double mutant humeri (see Fig. S5A in the supplementary material); however, this did not result in further reduction of Ihh expression levels. Wnt4 has been suggested to be a positive regulator of chondrocyte maturation (Hartmann and Tabin, 2000). Although Wnt4 mutant mice have no reported skeletal phenotype (Stark et al., 1994), we did observe a slight delay in chondrocyte maturation in the appendicular skeleton of single mutants (see Fig. S5B in the supplementary material). Our loss-of-function analysis, together with previous gain-of-function data (Guo et al., 2004; Hartmann and Tabin, 2000), suggests that both Wnt9a and Wnt4 are positive regulators of chondrocyte maturation.

Therefore, we propose the following model for Wnts and chondrogenesis: Wnt9a signals from the perichondrium or joints and regulates Ihh levels, probably via the canonical pathway, thereby regulating the pace of chondrocyte proliferation and maturation. In addition, Wnt9a signaling seems to negatively control Wnt4, which is expressed in prehypertrophic chondrocytes from E12.5 onwards (see Fig. SSD in the supplementary material). Wnt4 signaling regulates chondrocyte maturation at the transition from prehypertrophic to hypertrophic chondrocytes, but does not alter Ihh levels. Similarly, Wnt5a, which is expressed in the perichondrium and prehypertrophic chondrocytes, controls chondrocyte proliferation and maturation independently of Ihh (Yang et al., 2003). Part of the Wnt5a loss-of-function phenotype might be due to attenuation of β-catenin-mediated activities (Topol et al., 2003). Surprisingly, the phenotypes of the Wnt5a loss- and gain-of-function mice are very similar (Yang et al., 2003). Therefore, it will be interesting to test whether those phenotypes depend in part on β-catenin.

In addition, our work shows that Wnt signaling is required to maintain joint integrity by actively suppressing chondrocyte differentiation. Interestingly, Ihh mutant mice also display joint defects (St-Jacques et al., 1999) and recent work has shown that the canonical Wnt pathway is affected in Ihh mutants (Hu et al., 2005). Pathological alterations of the joint with spontaneous formation of cartilaginous nodules are known in humans as synovial chondromatosis. The etiology of this disease is largely unknown. However, deregulation of hedgehog signaling has recently been implicated in disease predisposition (Hopyan et al., 2005). It originates from chondroid metaplasia of connective tissue of the synovial membrane, causing pain, joint dysfunction and ultimately joint destruction. Thus, it will be important to examine whether the canonical Wnt/β-catenin pathway is altered in affected individuals.

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