

ROLE OF CANONICAL WNT-SIGNALLING IN JOINT FORMATION

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Abstract

The individual elements of the vertebrate skeleton are separated by three different types of joints, fibrous, cartilaginous and synovial joints. Synovial joint formation in the limbs is coupled to the formation of the prechondrogenic condensations, which precede the formation of the joint interzone. We are beginning to understand the signals involved in the formation of prechondrogenic condensations and the subsequent differentiation of cells within the condensations into chondrocytes. However, relatively little is known about the molecules and molecular pathways involved in induction of the early joint interzone and the subsequent formation of the synovial joints. Based on gain-of function studies Wnt-signalling, in particular the canonical pathway, has been implicated in the joint induction process. Here we provide genetic evidence from loss-of function analysis of embryos lacking either the central player of the canonical Wnt-pathway, β -catenin, in the limb mesenchyme or the two ligands, Wnt9a and Wnt4, demonstrating that canonical Wnt-signalling plays an important role in suppressing the chondrogenic potential of cells in the joint thereby actively allowing joint formation. Furthermore our data show that the β -catenin activity is not essential for the induction of molecular markers expressed in the joint interzone. Thus, suggesting that canonical Wnt-signalling is not required for the induction, but for the subsequent maintenance of the fate of the joint interzone cells.

Key words: Wnt, joint, synovial chondroid metaplasia, β -catenin

Introduction

The formation of synovial joints in the appendicular skeleton is a multistep process, which is tightly connected to the formation of the prechondrogenic anlagen. The latter arise by condensation of mesenchymal cells at the sites of the future elements. In the case of the appendicular skeleton, the early condensations can form branched structures, like in the forelimb where the humerus branches into radius and ulna. In contrast, the carpal elements in the wrist arise as separate condensations (Archer *et al.*, 2003). The induction of a joint occurs at the presumptive joint location within the branched condensations. The location of the future joint is probably determined by a combination of genes involved in patterning of the limb. The induction process involves changes in the cell shape from round to more flattened, elongated cells and the induction of genes more or less specific for the early interzone, among others *Gdf5*, *Wnt4*, *Wnt9a* (formerly known as *Wnt14*), *Gli1*, *Gli3*, *CD44*, *autotaxin*, and *chordin* (Francis-West *et al.*, 1999a; Guo *et al.*, 2004; Hartmann and Tabin, 2001). The expression of these markers precedes or coincides with the down-regulation of type II Collagen (*Col2 α 1*) and *Sox9*. The down-regulation of these chondrogenic markers during joint formation suggested that an essential step in joint formation is to block the further differentiation of these cells into chondrocytes. This notion is also supported by the finding that mice lacking *noggin*, which acts as an antagonist of the activity of Bone morphogenic proteins (BMP), have no signs of joint formation (Brunet *et al.*, 1998; Tylzanowski *et al.*, 2006). *Noggin*, is expressed initially throughout the prechondrogenic region including the joint interzone, and becomes later upregulated at the distal ends of the skeletal elements adjacent to the joints. Recent studies in the chicken have shown that retroviral over expression of Wnt9a (formerly known as Wnt14), one of the 19 ligands of the Wnt-signalling pathway results in a blockage of chondrogenic differentiation of the infected prechondrogenic region, and morphological and molecular changes resembling the joint interzone (Hartmann and Tabin, 2001). Wnt9a is expressed in the early joint interzone in chick and mouse. Phenotypic changes similar to those observed in chicken have also been seen in the mouse using a transgenic approach, whereby *Wnt9a* was misexpressed under the control of the *Col2 α 1* promoter (Guo *et al.*, 2004). The mouse gain-of function studies by Guo *et al.* (2004) suggested, in addition, that Wnt9a could be signalling through the canonical Wnt-pathway. This pathway is activated by the binding of a ligand to a receptor complex composed out of a member of the Frizzled family of serpentine receptors and one of the LRP5/6 co-receptors leading to an

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intracellular stabilization of the key component, β -catenin. Upon stabilization, β -catenin is translocated into the nucleus where it interacts with transcription factors of the TCF/Lef family and activates transcription of various target genes (Logan and Nusse, 2004; Wnt-homepage: <http://www.stanford.edu/~rnusse/wntwindow.html>). Together the misexpression experiments performed in chicken and mouse suggested that Wnt9a-signalling mediated by β -catenin is involved in joint induction. Furthermore, Guo *and colleagues* showed that loss of β -catenin in *Col2 α 1* expressing cells results in fusion of some tarsal joints observed at E15.5 (Guo *et al.*, 2004). Based on the studies in mouse, it was postulated that canonical Wnt-signaling is required for the induction of *Gdf5* in the joint interzone, possibly acting as the joint inducer (Guo *et al.*, 2004).

In order to address whether signaling by Wnt9a is indeed necessary for joint formation we had generated mice lacking Wnt9a activity and performed morphological, histological and marker analysis focusing on the joints of these animals. Since at least two other Wnt-genes, *Wnt4* and *Wnt16*, are expressed in the joints we also generated double mutants for *Wnt9a* and *Wnt4*. In addition, we re-examined the joint phenotypes in mice lacking β -catenin in the entire limb mesenchyme (Später *et al.*, 2006). Here we show a more extensive marker analysis supporting our previously published loss-of function analysis of the Wnt mutants, which suggested that Wnt9a and Wnt4 act redundantly in joint maintenance actively repressing the chondrogenic potential of synovial and fibrous joint cells, but that they are probably not required for the formation of the early joint interzone. This is supported by our accompanying analysis of limbs lacking mesenchymal β -catenin activity at early stages, when the interzone is being formed, which revealed that early joint markers, in particularly *Gdf5* are still expressed, suggesting that *Gdf5* expression is not dependent on canonical Wnt-signalling.

Material and Methods

Mouse mutants

Wnt9a and Wnt4 mutants were genotyped by PCR (Später *et al.*, 2006; Stark *et al.*, 1994). Mice lacking β -catenin activity in the limb mesenchyme were generated as previously described (Hill *et al.*, 2005).

Histology and *in situ* hybridizations

For histology and non-radioactive section *in situ* hybridization, tissue was treated as previously described (Hill *et al.*, 2005).

Retroviral work, micromass and chondrocyte cultures

The RCAS-AP, RCAS-Wnt5a, RCAS-Wnt9a, RCAS-Wnt4, RCAS-Wnt3a and RCAS- Δ N β -cat viruses were previously described (Hartmann and Tabin, 2000; Hartmann and Tabin, 2001; Kengaku *et al.*, 1998). Micromass cultures using chicken HH22-23 limb buds were performed as previously described (Hartmann and Tabin, 2001), cartilage nodule formation was visualized at day 4 by Alcian blue staining. Chondrocytes isolated

from the caudal part of day 18 chick sternae (Koyama *et al.*, 1999) were cultured for one day, collected and plated at 5×10^5 cells/well density in a six well plate. The following day chondrocytes were infected using 5μ l viral supernatant per well (titers: $6-8 \times 10^8$ pfu/ml) and cultured for 3-4 days in DMEM:F12 (Invitrogen). All experiments were done in triplicates.

Western blot analysis

50 μ g of protein extract, isolated from cultured chicken sternal chondrocytes infected with different RCAS viruses, were loaded per lane. 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).

Immunohistochemistry

For immunohistochemical staining of cultured chondrocytes, cells were fixed for 15 min at RT with 4% paraformaldehyde in PBS, washed twice with PBS. Endogenous peroxidase activity was inactivated by 30 min incubation in 1% H₂O₂ in PBS. Cells were subsequently washed three times with PBS, blocked for 30 min with PBS; 10% FCS, 0.1% Triton-X100, incubated with primary antibodies against Collagen type II (II-II6B3 supernatant, 1:30) or Collagen type III (3B2 supernatant 1:30) from the Developmental Hybridoma Bank (Iowa). Signaldetection was done using a biotinylated anti-mouse secondary antibody (dilution 1 in 250; Vector labs) in combination with the ABC kit (Vector labs) and DAB (Sigma) as a substrate.

Results

Mice lacking Wnt9a activity displayed a shortening of the proximal long bones and mild joint abnormalities at birth (Später *et al.*, 2006). The most severely affected joint was the humeral-radial joint (HRJ) in the elbow region, in which synovial cells differentiated in 100% of the mutants into an ectopic cartilage element (Figure 1A,B). This phenotype is known in humans as synovial chondroid metaplasia. We examined earlier developmental stages to determine the onset of the phenotype. No obvious differences were evident in the expression of *Col2 α 1*, *Gdf5*, *Gli3* and *Wnt4* in the elbow joints from *Wnt9a^{-/-}* and wild-type embryos at E13.5 (Figure 1E-H). The first signs of ectopic cartilage formation in the HRJ were observed at E15.5, visible as a small ectopic cluster of *Col2 α 1* positive cells and a broad ectopic *Sox9* domain in the outer joint region (Figure 1C',D'). In addition, partial fusions of the carpal elements c/3 and of the intermediate cuneiform and navicular tarsal elements were observed in approx. 80% of the mutants at birth (Später *et al.*, 2006). Not only synovial joints were affected in the *Wnt9a* mutants, ectopic cartilage formation was also observed in the sutures, which are fibrous joints separating the bony plates in the skull (Später *et al.*, 2006).

As only a few synovial joints were affected in the *Wnt9a* mutants and since two other Wnt-genes, *Wnt4* and *Wnt16*, are known to be expressed overlapping with *Wnt9a*

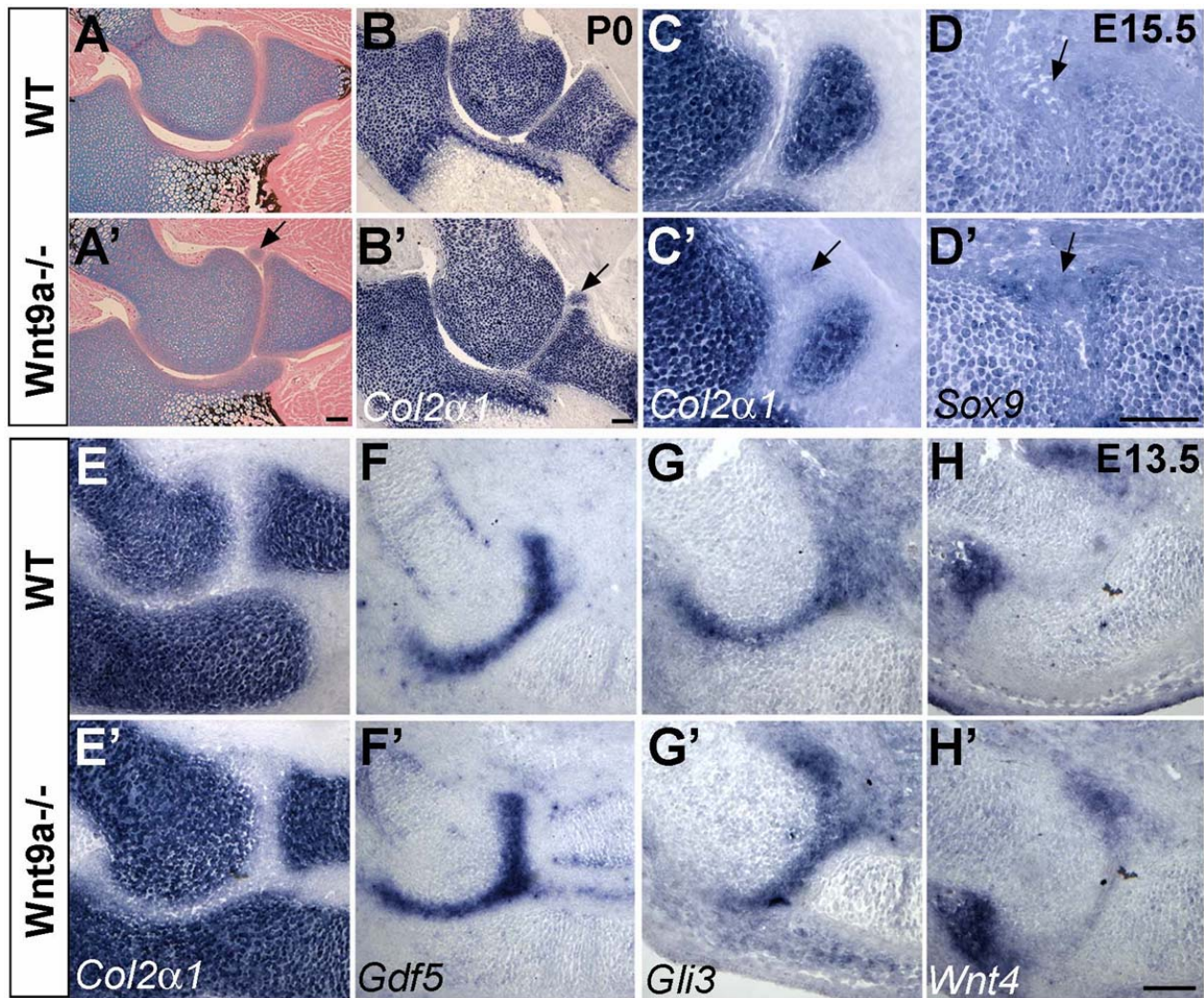


Figure 1. Synovial chondroid metaplasia in the humeral-radial joint (HRJ) of *Wnt9a* mutants. (A-B') Sections through newborn wild-type and *Wnt9a* mutant forelimbs: normal HRJ in (A, B) ectopic cartilage nodule visualized by Alcian blue staining (arrow in A') and *in situ* hybridization for *Col2α1* transcripts (arrow in B'). (C-D') *In situ* hybridizations on sections through E15.5 wild-type and *Wnt9a* mutant forelimbs: (C) no *Col2α1* staining in the wild-type, (C'') ectopic *Col2α1* expression in the HRJ (arrow). (D) No *Sox9* expression in the wild-type HRJ, (D'') ectopic expression of *Sox9* in the mutant (arrow). (E-H') *In situ* hybridizations on sections through E13.5 wild-type and *Wnt9a* mutant forelimbs: no difference in the expression patterns of *Col2α1* (E, E''), *Gdf5* (F, F''), *Gli3* (G, G''), *Wnt4* (H, H'') between wild-type and mutant. Scale bars are 100µm.

in the joints we generated double mutants for *Wnt9a* and *Wnt4* to test for possible functional redundancy. Notably *Wnt4* mutants have no reported skeletal genotypes (Stark *et al.*, 1994). *Wnt9a* and *Wnt4* mutants displayed synovial chondroid metaplasia in two additional major joints, the knee and the ankle joint (Figure 2A-B'). In addition, 100% of the double mutant newborns displayed complete fusions of carpal elements 2, c, and 3 (Figure 2C-C''), which resulted in an abnormal flexure of the forelimb autopod. Closer examination of the articular regions revealed that *Wnt4*^{-/-}; *Wnt9a*^{-/-} articular chondrocytes are stained more intensely by alcian blue, when compared to wild-type, suggesting that their extracellular matrix was altered (Figure 2D-E'). The nature of this articular chondrocyte phenotype will be examined in the future using molecular markers.

The onset of the fusion phenotype of the carpal elements observed in newborns was analyzed further at

earlier stages using a variety of molecular markers. This analysis revealed that the carpal elements were not yet fused in the double mutants at E13.5 (Figure 3 A), but they were fused at E15.5 (Figure 3B). In the foot the intermediate cuneiform and navicular, as well as the calcaneus and cuboid tarsal elements were fused in newborns (Figure 4B', B''). Occasionally we observed the formation of an ectopic cartilage piece in ligaments of the digits (asterisk in Figure 4B''). Similarly to the situation in the carpal region individual tarsal elements could be identified by *Col2α1* staining as separate condensations, surrounded by cells expressing *Gdf5* at E13.5 (Figure 4C', D'), but were fused at E15.5 (data not shown).

The phenotype of the double mutants suggested that early Wnt-signalling is not necessary for the formation of the joint interzone. However, in addition to *Wnt9a* and *Wnt4*, *Wnt16* is also expressed in the joint region and could, in principle, still compensate for the loss of the other two

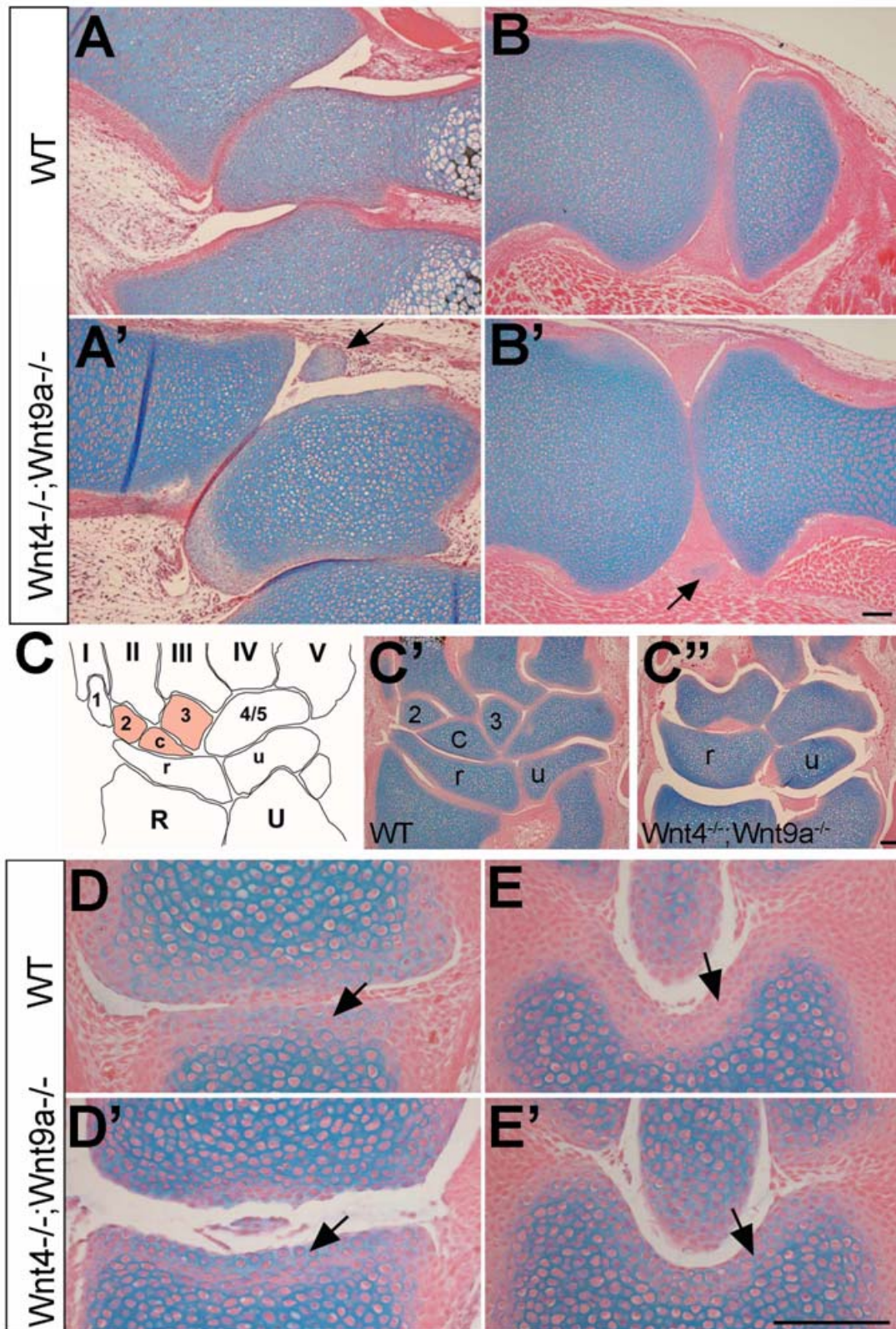


Figure 2. Joint alterations in newborn *Wnt4;Wnt9a* double mutant mice. (A-E') Alcian blue staining on sections through the ankle, knee, wrist and digit regions of newborn wild-type and *Wnt4^{-/-};Wnt9a^{-/-}* mice. (A) Normal ankle joint. (A') Ectopic cartilage nodule instead of the synovial tissue in the double mutants (arrow). (B) Normal knee joint. (B') Ectopic cartilage nodule in the fibrous tissue of the knee capsule (arrow). (C) Schematic diagram showing the arrangement of carpal elements, with carpal 2, c, 3 highlighted in red. (C') Normal arrangement of carpal bones in the wild-type. (C'') Fusion of carpal 2, c, 3 in the double mutants. R: radius; U: ulna; I-V: digits; r: radial element; u: ulnar element. (D, E) Sections through a normal metacarpal-phalangeal joint showing low intensity alcian blue staining in the articular cell layers (arrows). (D', E') Sections through a mutant metacarpal-phalangeal joint showing more intense alcian blue staining in the articular cell layers compared to wild-type (arrows). Scale bars are 100 μ m.

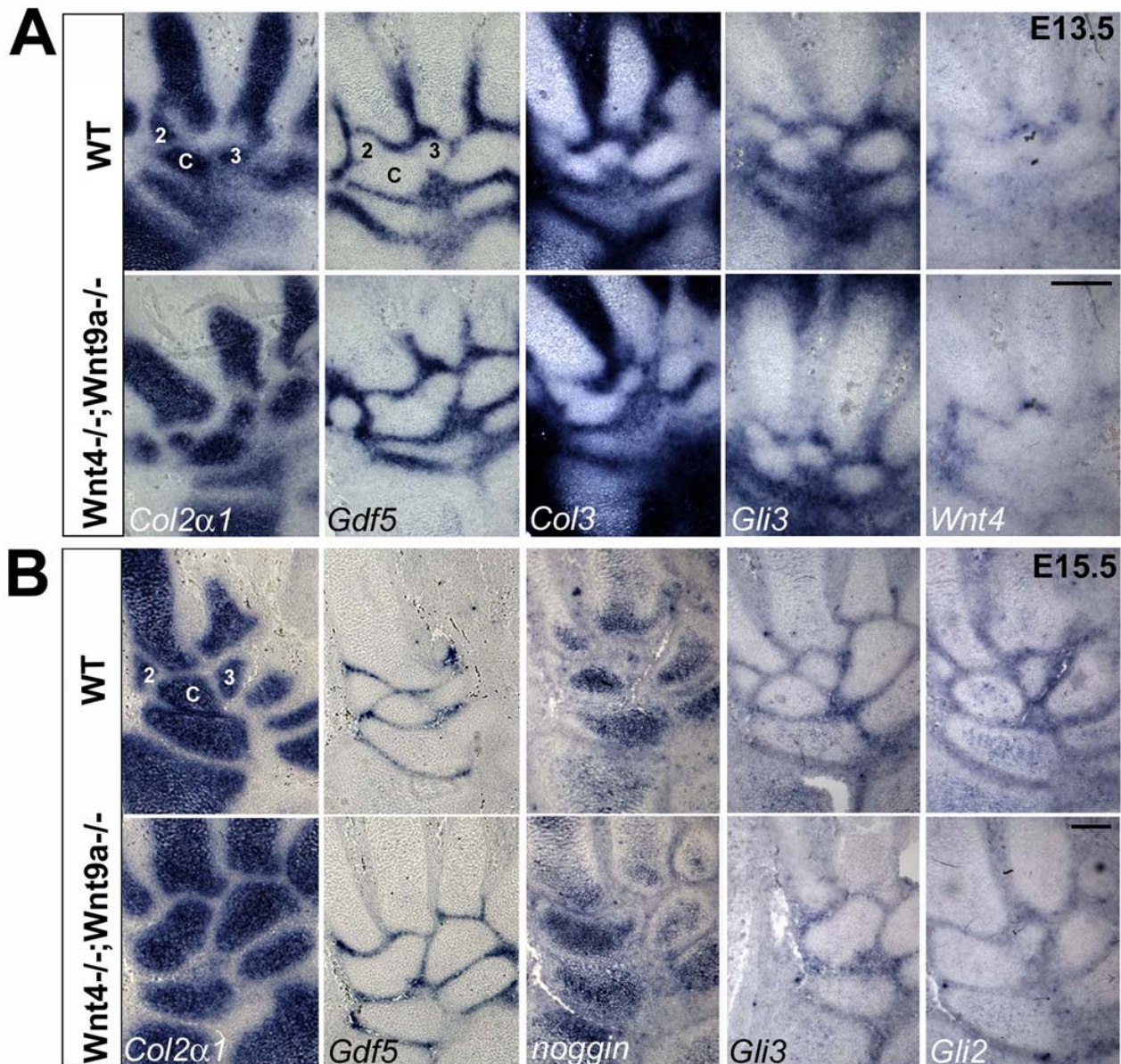


Figure 3. Comparison of molecular marker expression between wild-type and *Wnt4;Wnt9a* double mutants during carpal development. (A) *In situ* hybridizations on sections through the carpal region of wild-type (upper panel) and *Wnt4*^{-/-};*Wnt9a*^{-/-} embryos (lower panel) at E13.5. No difference is visible in the expression patterns of the markers: *Col2α1*, *Gdf5*, *Col3*, *Gli3*, *Wnt4*. (B) *In situ* hybridizations on sections through the region of the carpals of wild-type (upper panel) and *Wnt4*^{-/-};*Wnt9a*^{-/-} embryos (lower panel) at E15.5. No difference is visible in the expression patterns of the markers: *Col2α1*, *Gdf5*, *noggin*, *Gli3*, *Gli2*. Scale bars are 100µm.

Wnt-genes. Retroviral mediated misexpression of *Wnt4* and *Wnt9a* both inhibited chondrogenesis in chick micromass cultures, as did ectopic expression of a stabilized form of β -catenin ($\Delta N\beta$ -cat) (Figure 5B-D) see also (Guo *et al.*, 2004; Hartmann and Tabin, 2001), suggesting that both Wnts are probably signalling through the β -catenin pathway (Guo *et al.*, 2004; Hartmann and Tabin, 2000). Therefore, we asked the question, if the formation of the early joint interzone is affected in mice lacking mesenchymal β -catenin activity in the limbs (β -cat^{*APrx*}^{-/-}). These mice were generated as previously described (Hill *et al.*, 2005). The early condensations of the skeletal elements visualized by *Col2α1* *in situ* hybridization are in principle formed, but are slightly

abnormal, since a part of the scapula anlage is missing and the digits are not formed normally (Hill *et al.*, 2006) (Figure 6A'). Analysis of early joint interzone markers such as *Gdf5*, *Wnt4* and *Gli1* revealed that all three markers were still expressed in the prospective joint region (Figure 6B'-D'), although their expression domains were somewhat broader compared to heterozygous littermates (Figure 6B-D). In wild-type limbs *Col2α1* expression starts to be down-regulated in the joint region around E12.5 (arrowhead in Figure 6E), however, this down-regulation was not clearly visible in β -cat^{*APrx*}^{-/-} mice (arrowheads in Figure 6E'). At E12.5 *Gdf5* expression was similarly expressed comparing the joints of wild-type and mutant limbs (Figure 6F, F'). In contrast to the broadened *Gdf5*

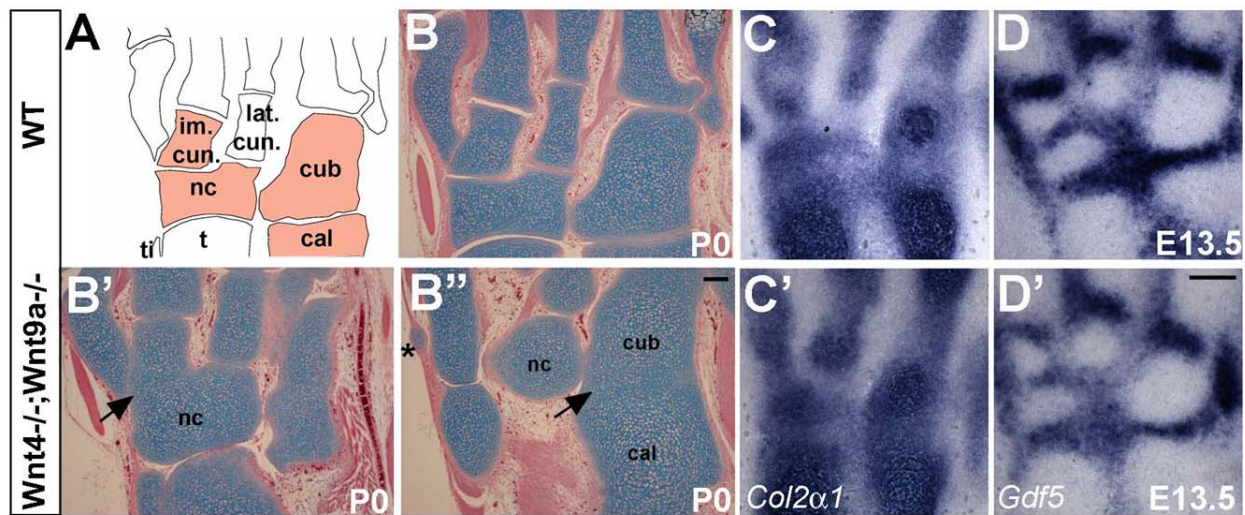


Figure 4. Joint fusions between tarsal elements in *Wnt4;Wnt9a* double mutants. (A-B'') Sections through the tarsal region of wild-type and *Wnt4^{-/-};Wnt9a^{-/-}* newborns. (A) Schematic diagram showing the arrangement of tarsal elements: cal: calcaneus; cub: cuboid; l.c.: lateral cuneiform; i.c.: intermediate cuneiform; nc: navicular; t: tarsal. (B) Alcian blue staining of a wild-type tarsal region. (B', B'') Alcian blue staining of a *Wnt4^{-/-};Wnt9a^{-/-}* mutant showing fusion between the intermediate cuneiform and navicular tarsal elements (arrow in B'') and between the calcaneus and cuboid tarsal elements (arrow in B'') and synovial chondroid metaplasia in the joint capsule ligament of digit I (asterisk in B'). Pattern of condensation of tarsal elements visualized by *Col2α1* expression is similar between wild-type (C) and mutant (C'). *Gdf5* expression pattern is similar between wild-type (D) and mutant (D'). Scale bars are 100µm.

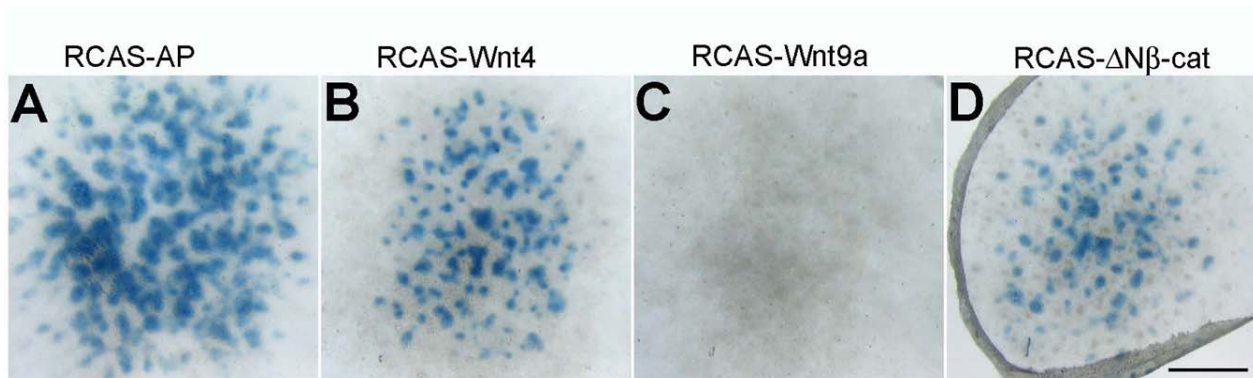


Figure 5. Inhibitory effects of Wnt4, Wnt9a and stabilized β -cat on cartilage formation. (A-D) Alcian blue staining visualizing cartilage nodule formation in micromass cultures at day 4. (A) RCAS-AP control infected cultures. (B) RCAS-Wnt4 infected cultures show a reduced number of cartilage nodules. (C) RCAS-Wnt9a infected cultures show a complete block in cartilage nodule formation. (D) RCAS- Δ N β -catenin show a reduced number of alcian blue stained cartilage nodules. Scale bar is 1mm.

domain in the mutant at E11.5, the *Gdf5* expression domain was much more refined at E12.5, but the expression level was slightly reduced compared to the wild-type (Figure 6F'). At E14.5, when *Col2α1* expression is normally absent from the wild-type joint (Figure 6G), *Col2α1* was still expressed in the "joint-like" region of the mutant, but at slightly lower levels compared to the neighbouring cartilage element (arrowheads in Figure 6G'). *Gdf5* was like in the wild-type at E14.5 still expressed in the mutant, but more restricted to the lateral edges (arrowheads Figure 6H'). The presence of the early joint markers suggests that the process of joint induction is not affected by the loss of β -catenin. On the other hand, what seems to be affected is the down-regulation of *Col2α1* expression and suppression of chondrogenesis in the joint region fails.

Since certain Wnts can interfere with chondrogenesis in micromass cultures (see above) we wanted to address the effect of ectopic activation of Wnt-signalling on proliferating chondrocytes. For that reason, chondrocytes isolated from the caudal part of day 18 chick sterna were infected with retroviruses expressing either alkaline phosphatase (AP), Wnt5a, Wnt9a, Wnt3a or the stabilized form of β -catenin. Chondrocytes were cultured for 4 days and stained for Collagen II and Collagen III. While control infected chondrocytes (RCAS-AP) and RCAS-Wnt5a infected chondrocytes still produced a Collagen II positive matrix and retained a cuboidal shape, chondrocytes infected either with RCAS-Wnt9a, RCAS-Wnt3a or RCAS- Δ N β -cat down-regulated Collagen II production. Furthermore, the morphology of the cells in RCAS-Wnt9a,

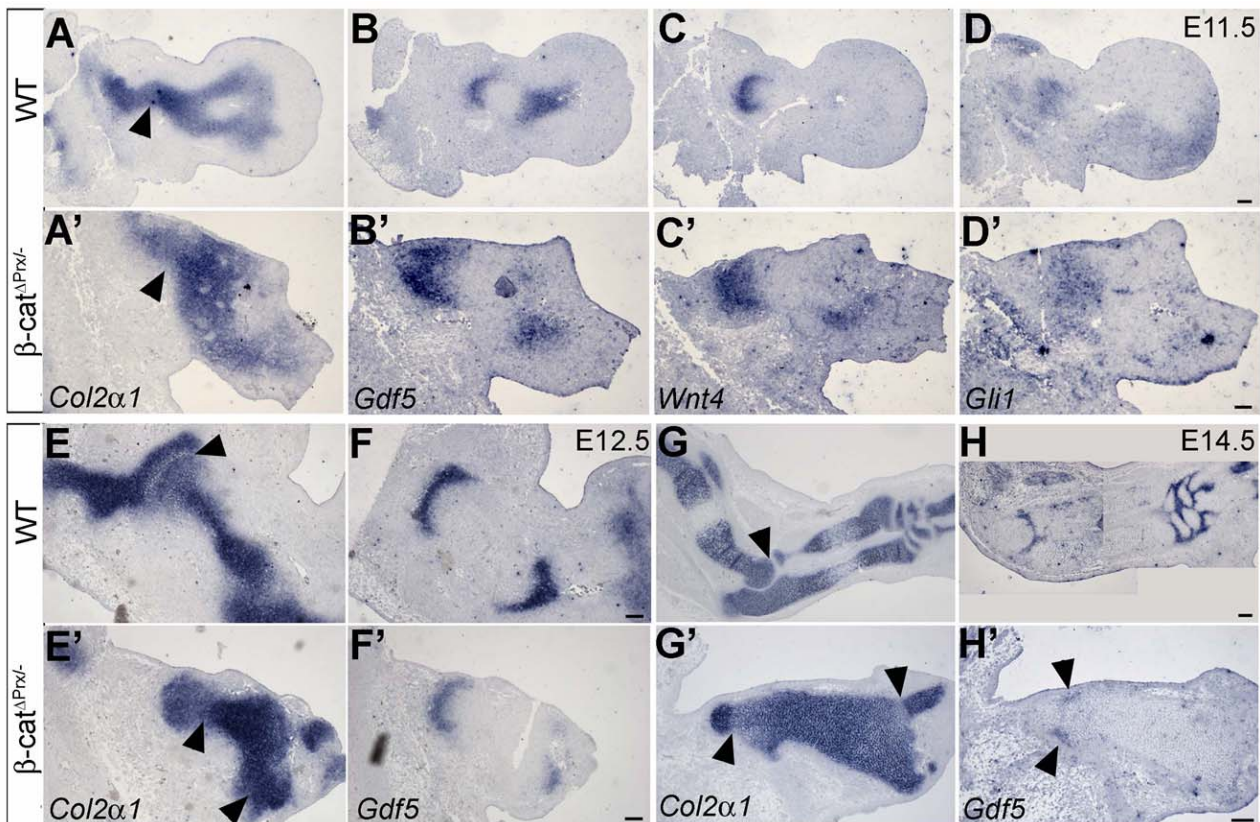


Figure 6. Analysis of joint induction and formation in mouse limbs lacking mesenchymal β -catenin (β -cat ^{Δ Prx⁻}) activity. (A-H') Marker analysis by *in situ* hybridization of wild-type (A-H) and β -cat ^{Δ Prx⁻} mutant (A'-H') forelimbs at E11.5 (A-D'), E12.5 (E-F') and E14.5 (G-H'). (A) *Col2 α 1* expression showing prechondrogenic condensations of the scapula, humerus, radius and ulna (from left to right; arrowhead marks the position of the joint between the scapula and humerus) in wild-type (A) and mutant (A'). *Gdf5* expression in the shoulder joint is similar between the wild-type (B) and the mutant (B'); note: *Gdf5* is also expressed in elbow joints as well as the space between radius and ulna in the section of the wild-type limb (B). (B') *Wnt4* expression in the shoulder joint is similar between wild-type (C) and mutant (C'). *Gli1* expression is similar between wild-type (D) and mutant (D'). Note that in the mutant the expression domain of the early joint interzone markers, *Gdf5*, *Wnt4* and *Gli1* is broader compared to the wild-type. (E) Down-regulation of *Col2 α 1* expression in the shoulder joint in the wildtype (arrowhead). (E') Incomplete down-regulation of *Col2 α 1* expression in joints of the mutant (arrowheads). (F) *Gdf5* expression in the wild-type joints. (F') *Gdf5* is still expressed in the joints of the mutant. (G) Complete absence of *Col2 α 1* expression in the wild-type joints (see arrowhead in elbow joint). (G') Incomplete down-regulation of *Col2 α 1* expression in mutant joint-like areas (arrowheads). (H) *Gdf5* expression in wild-type joints. (H') Weak remaining *Gdf5* expression in joint-like area of the mutant (arrowheads). Scale bars are 100 μ m.

Wnt3a and Δ N β -cat infected cultures changed to a more fibroblast-like appearance and the cells produced a Collagen III rich matrix (Figure 7A). Interestingly, western blot analysis revealed that infection of chondrocytes with RCAS viruses expressing either the canonical Wnt-ligand, Wnt3a, or Wnt9a resulted in increased levels of β -catenin protein, in contrast to RCAS-AP control infected or cultures infected with an RCAS-virus expressing the non-canonical Wnt-ligand, Wnt5a (Figure 7B). Thus, the phenotypic changes in the chondrocyte cultures infected with RCAS-Wnt9a or Wnt3a are probably due to the increase in β -catenin levels. Particularly, since this effect can be mimicked by infecting the cells with an RCAS expressing a stable form of β -catenin.

Discussion

All joints are initially formed normally in double mutants for Wnt9a and Wnt4, this suggests that those two Wnt genes are not required for the initiation of joint formation. However, around E15.5 cells in the joint region of various joints seem to fail to maintain their joint cell identity in the absence of these two ligands. Therefore, we propose that Wnt-signalling, probably through the canonical Wnt-pathway, is actively involved in maintaining joint identity by repressing the chondrogenic potential of cells within the joint region. Some of these cells might still remain as precursor cells, which are still bipotential, meaning that they can give rise to chondrocytes as well as synoviocytes or joint capsule cells. This hypothesis is supported by previous findings showing that synovial tissue contains chondro-progenitors (Nalin *et al.*, 1995; Nishimura *et al.*,

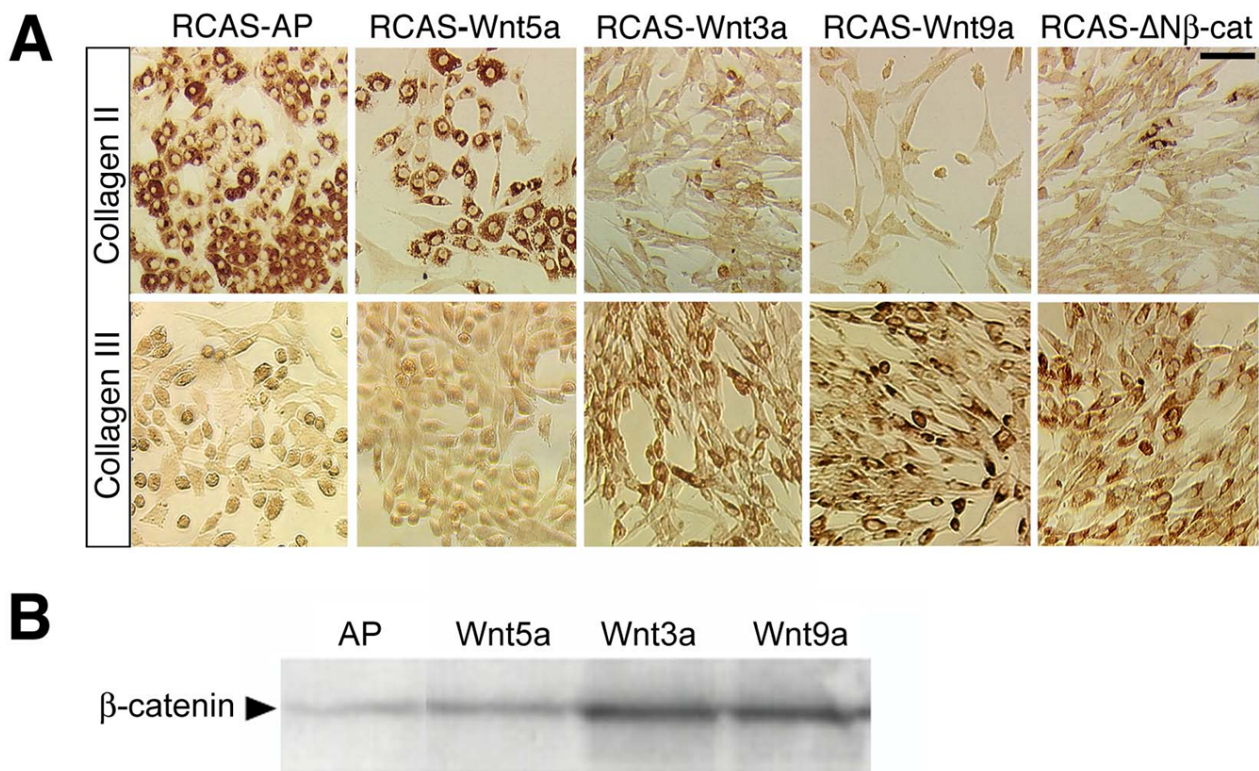


Figure 7. Changes in cultured chondrocytes upon retroviral misexpression of various Wnts and stabilized β -catenin. (A) Immunohistochemical staining for extracellular matrix proteins Collagen II and Collagen III on chondrocytes infected with RCAS-AP (control), RCAS-Wnt5a (non-canonical Wnt), RCAS-Wnt3a (canonical Wnt), RCAS-Wnt9a and RCAS- Δ N β -cat. Collagen II is not down-regulated in Wnt5a infected cells, but is down-regulated in RCAS-Wnt3a, Wnt9a and Δ N β -cat infected cells. RCAS-AP and Wnt5a infected cells do not stain positive for Collagen III, while RCAS-Wnt3a, Wnt9a and Δ N β -cat infected cells do. (B) Western-blot showing increased β -catenin protein levels in chondrocytes infected with RCAS-Wnt3a and Wnt9a. Scale bars are 20 μ m.

1999). Not all of the joints are affected in the Wnt9a; Wnt4 double mutants. This could be due to the fact that a third Wnt, *Wnt16*, is expressed in the joints as well (Guo *et al.*, 2004). Therefore, it remains possible that Wnts might play a more important role in joint initiation and formation than what we propose on the basis of the analysis of the double mutants. However, our molecular marker analysis in the limbs, which lack β -catenin activity in the mesenchyme, demonstrated that the joint interzone markers *Gdf5*, *Wnt4* and *Gli1* are initially expressed (E11.5) and remain to be expressed even at later stages. These observations favour our hypothesis that canonical Wnt signalling is not required for the initiation of joints and also show that it is not required for *Gdf5* expression as previously proposed (Guo *et al.*, 2004). Furthermore, we never observed ectopic activation of *Gdf5* expression in micromass cultures infected either with Wnt9a, Wnt4 or Δ N- β -catenin expressing retroviruses (C. Hartmann, unpublished observation). Thus, in analogy to the previously identified role of β -catenin in osteo-chondroprogenitors (Day *et al.*, 2005; Hill *et al.*, 2005) the canonical Wnt-pathway is likely to be required to repress the chondrogenic potential of what might be a synovio-chondroprogenitor. This strong anti-chondrogenic activity of the canonical Wnt-signalling pathway is also reflected by the observation that increased levels of β -catenin can revert chondrocytes *in vitro* into

more fibroblast-like cells expressing different extracellular matrix molecules. What the nature of these cells is remains to be determined in the future.

In conclusion the canonical Wnt-pathway seems to be important to allow the differentiation of the joint lineage by actively suppressing the chondrogenic potential in precursor cells (see Model Figure 8). The data presented here together with the previously identified role of β -catenin in the osteoblast lineage (Day *et al.*, 2005, Hill *et al.*, 2005, Rodda and McMahon, 2006) suggests that during skeletal lineage differentiation, chondrogenesis might be the default state and that inhibition of chondrogenesis by a β -catenin mediated Wnt-signal is required for differentiation of the other two cell lineages. While the Wnt signals involved in osteoblast lineage differentiation remain to be identified, our data suggest that Wnt9a together with Wnt4 and probably Wnt16 are the signals essential during joint lineage formation.

From a medical point of view, the observed synovial chondroid metaplasia phenotype in the elbow in case of loss-of Wnt9a activity alone or in the knee and ankle joints in mutants lacking both Wnt9a and Wnt4 activity is very interesting. Similar pathological alterations of the joint with spontaneous formation of cartilaginous nodules are known in humans as well. In their more severe form they are referred to as synovial chondromatosis. The etiology of

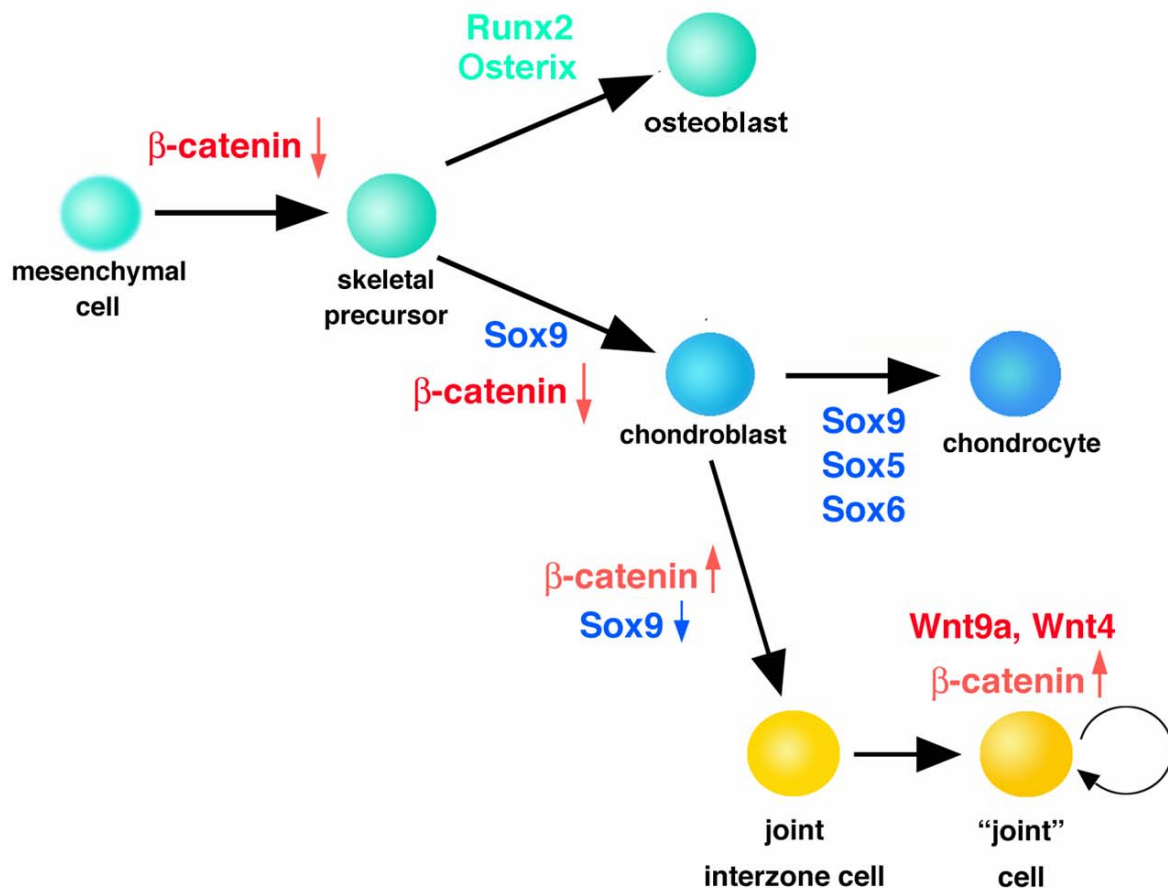


Figure 8. Model for the requirement of canonical Wnt-signalling during joint lineage differentiation. Low levels of β -catenin are required for cells to enter the skeletal lineage. For the further differentiation of skeletal precursors into chondroblasts / chondrocytes β -catenin levels need to be reduced. For the establishment of a true joint interzone β -catenin levels need to be elevated to suppress the chondrogenic potential of interzone cells and levels of β -catenin need to stay elevated in joint cells to maintain joint integrity.

this disease is still largely unknown. Therefore, it will be interesting to examine whether the canonical Wnt-signalling pathway is in some way altered in affected individuals.

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Discussion with Reviewers

I Kahn: Why does ectopic expression of Wnt9a induce *gdf5* expression in (chick) limbs but not *in vitro* micromass cultures using limb mesenchyme?

Authors: In the previous gain-of function studies in chicken pre-cartilaginous digit anlagen were infected with Wnt9a expressing retroviral particles. Although a broader region was infected with Wnt9a retrovirus only a part of the infected area would express Gdf5 and other early joint interzone markers. These areas were always localized immediately adjacent to an uninfected cartilaginous element and probably originated from the pre-cartilaginous anlagen. Like in the *in vivo* situation Wnt9a in the *in vitro* studies inhibited chondrogenesis, but no induction of Gdf5 or any other joint interzone markers was observed. Similarly, infection of the non-chondrogenic mesenchyme never did lead to ectopic expression of Gdf5 and other joint interzone markers. This suggests that Wnt-signaling is not sufficient to induce Gdf5, but probably requires additional signals originating from the adjacent cartilage element.

I Kahn: On the basis of the authors' conclusion that Wnts are not responsible for initiating joint interzone formation, could they speculate as to the identity of the factors that may perform this function?

Authors: The conclusion is that canonical Wnt-signaling alone is not required for the initiation of the joint interzone, based on the presence of molecular markers such as Gdf5, Wnt4 and Gli1. However, canonical Wnt-signaling or at least the function of β -catenin is required for the formation of a true interzone, which is characterized by the down-regulation of Collagen II and *Sox9*. Which factors are required for the initiation of interzone markers remains an open question. The initiation of the joint interzone characterized by the expression of a variety of markers is likely to depend on signals and transcription factors involved in pre-patterning the limb along its proximo-distal axis, an aspect of limb patterning which is only poorly understood. Future experiments addressing the transcriptional regulation of the early interzone markers might lead to the identification of common elements which are responsible for the expression in the joint regions. On the basis of such common elements it might be possible to identify the key transcription factors, which are necessary and sufficient to induce restrict the expression of these markers to the joint interzone.