Wnt/β-catenin signaling is sufficient and necessary for synovial joint formation

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A critical step in skeletal morphogenesis is the formation of synovial joints, which define the relative size of discrete skeletal elements and are required for the mobility of vertebrates. We have found that several *Wnt* genes, including *Wnt4*, *Wnt14*, and *Wnt16*, were expressed in overlapping and complementary patterns in the developing synovial joints, where β-catenin protein levels and transcription activity were up-regulated. Removal of β-*catenin* early in mesenchymal progenitor cells promoted chondrocyte differentiation and blocked the activity of *Wnt14* in joint formation. Ectopic expression of an activated form of β-*catenin* or *Wnt14* in early differentiating chondrocytes induced ectopic joint formation both morphologically and molecularly. In contrast, genetic removal of β-*catenin* in chondrocytes led to joint fusion. These results demonstrate that the Wnt/β-catenin signaling pathway is necessary and sufficient to induce early steps of synovial joint formation. *Wnt4*, *Wnt14*, and *Wnt16* may play redundant roles in synovial joint induction by signaling through the β-catenin-mediated canonical Wnt pathway.

[*Keywords*: Wnt; β-catenin; joint formation; skeletal development]

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Formation of synovial joints between different skeletal elements is essential for the mobility of vertebrates. The number and position of joints also determine characteristic skeletal patterns in each vertebrate species by defining the size and shape of skeletal elements. As alterations of early patterning signals often lead to changes in the position and number of joints in the developing limb (Dahn and Fallon 2000; Suzuki et al. 2004), understanding the regulation of joint formation in the limb will also provide critical insights into how early-limb patterning is linked to later skeletal morphogenesis at the molecular level.

In the developing limb, studies of descriptive embryology have shown that skeletal elements form through temporally and spatially regulated processes that include mesenchymal condensation, elongation, branching, and/or segmentation (Shubin and Alberch 1986). Most of the synovial joints in the limb form through segmentation of a pre-existing cartilage rod. For instance, in the developing forelimb, the initial de novo mesenchymal condensation forms the cartilage anlagen of the humerus, the growth and branching of which then produce a Y-shaped bifurcation. It is the segmentation of this Y-shaped cartilage primordium that forms the elbow joint that separates the radius and ulna from the humerus (Shubin and Alberch 1986).

Synovial joint formation starts from the differentiation of newly differentiated chondrocytes into flattened and densely packed interzone cells (for review, see Archer et al. 2003), which express joint-specific markers such as *Gdf5* and lose the expression of chondrocyte-specific markers such as *ColII* (Craig et al. 1987; Nalin et al. 1995; Morrison et al. 1996; Storm and Kingsley 1996). Later in development, the interzone cells differentiate and form three layers. The middle layer eventually cavitates to form a fluid-filled joint space. Synthesis of hyaluronan (HA), expression of its principle receptor CD44 in the interzone cells, and movement of the embryo have all been suggested to play an essential role in joint cavitation (Pitsillides et al. 1999). Meanwhile, the two lateral layers of the interzone participate in the formation of articular cartilage of the two opposing skeletal elements. In the mature joint, the opposing articular cartilages are wrapped in the joint capsule, which is enforced by ligaments and tendons outside and lined by synovial membrane inside (Archer et al. 2003). In adult life, the mature joint structures need to be properly maintained, as dis-

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ruption of articular cartilage leads to pathological conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA), which are common diseases.

The molecular mechanisms regulating joint formation are just beginning to be elucidated. It has been shown that cell–cell signaling mediated by Gdf5, Noggin, and Wnt14 plays a critical role in controlling synovial joint formation (Storm et al. 1994; Brunet et al. 1998; Hartmann and Tabin 2001). However, none of these signaling molecules are both necessary and sufficient for synovial joint induction. First, bone morphogenetic protein (Bmp) family members Gdf5 and Gdf6 are essential for joint formation in certain regions of the limb, but they are not sufficient for inducing joint formation. *Gdf5* and *Gdf6* null mutant mice exhibit joint development defects in digits, wrists, and ankles (Storm and Kingsley 1996; Settle et al. 2003). Yet, overexpression of *Gdf5* in both chick and mouse does not induce joint formation (Francis-West et al. 1999a; Merino et al. 1999; Storm and Kingsley 1999; Tsumaki et al. 1999). In contrast, *Gdf5* overexpression resulted in extensive cartilage overgrowth and complete absence of joints (Tsumaki et al. 1999). Second, joint formation is inhibited in the *Noggin* mutant mice (Brunet et al. 1998), but overexpression of *Noggin* only inhibited cartilage formation (Capdevila and Johnson 1998; Pathi et al. 1999; Pizette and Niswander 2000). Lastly, ectopic expression of *Wnt14* in the chick limb is sufficient to induce joint formation (Hartmann and Tabin 2001), but it is not clear whether *Wnt14* is also required in the mouse for joint formation. Furthermore, it was unknown prior to this study how Wnt14 transduces its signal in joint induction.

There are 19 Wnt family members and they can transduce their signals through several different pathways (Veeman et al. 2003; Yang 2003; Nelson and Nusse 2004 and references therein). Among them, the canonical Wnt pathway plays pivotal roles in controlling cell proliferation and cell-fate determination during many embryonic development processes. Central to the canonical Wnt pathway is the stabilization and nuclear translocation of β-catenin after Wnt ligands bind to their receptors Frizzled and LRP5/6. β-Catenin then activates downstream gene expression through binding to the LEF/TCF transcription factors.

Here, we found that *Wnt4*, *Wnt 14*, and *Wnt16* were expressed in the forming joints in overlapping and complementary patterns. These Wnts can transduce their signals through the canonical Wnt pathway both in vitro and in vivo. The activity of *Wnt14* was blocked when β-*catenin* was inactivated in the forming cartilage. Overexpression of a constitutively active β-*catenin* or *Wnt14* in early differentiating chondrocytes induced ectopic joint formation both morphologically and molecularly. Conversely, genetic ablation of β-catenin in early differentiating chondrocytes led to fusion of skeletal elements. Our data demonstrate that the canonical Wnt signaling is not only sufficient, but also necessary for inducing at least early steps of synovial joint formation. Our results also indicate that *Wnt4*, *Wnt 14*, and *Wnt16* may play redundant roles in inducing joint formation by signaling through the canonical Wnt pathway.

Results

Expression of Wnt4, Wnt14, Wnt16 *and accumulation of* β-*catenin protein in the developing synovial joints*

To test the role of Wnt signaling in synovial joint induction, we first examined the expression of *Wnt* genes in the forming synovial joints of the developing mouse limb. We found that *Wnt4*, *Wnt14*, and *Wnt16* were already expressed in the future joint region, as indicated by *Gdf5* expression, at the same time when chondrogenic mesenchymal condensations form at 11.5 days postcoitum (dpc) (Fig. 1A,B). However, the expression patterns of the *Wnt*s were distinct. *Wnt14* was expressed at higher levels in the mesenchyme surrounding the cartilage primordium than in the presumptive joint (Fig. 1A,B). Interestingly, *Wnt14* was also expressed in the presumptive joint region between the scapula and humerus (Fig. 1A) that does not form by segmentation of a pre-existing chondrogenic condensation. *Wnt4* was also expressed in the future elbow joint, but its expression in the flanking mesenchyme, which will give rise to the joint capsule, was stronger (Fig. 1B,D). *Wnt16* was initially expressed as a stripe that marks the future metatarsophalangeal (MTP) area (Fig. 1A). At 11.5 dpc, *Gdf5* expression was broad and *Wnt4* expression was stronger than *Wnt14* (Fig. 1B). At 12.5 dpc, *Gdf5* expression marks future joints in the digit rays. *Wnt14* expression was still observed around the forming cartilage with stronger expression in the future joint (Fig. 1A). Interestingly, the initial stripe-like expression pattern of *Wnt16* was expanded at 12.5 dpc. *Wnt16* was expressed almost exclusively in the MTP joints and the proximal interdigital area (Fig. 1A). At 13.5 dpc, *Wnt4*, *Wnt14*, and *Wnt16* were all expressed in the developing digit joints, but *Wnt4* expression was the strongest (Fig. 1C). In the MTP joint region, however, *Wnt16* expression was the strongest (Fig. 1C). In the elbow region, where the joint was more mature and *Gdf5* was expressed as a narrow stripe, *Wnt14* expression was stronger than *Wnt4* in the joint interzone. *Wnt14* was also expressed strongly in the mesenchyme surrounding the cartilage and in the cells that will form tendons (Fig. 1D). *Wnt4* was expressed at much higher levels in the mesenchyme that will form the joint capsule (Fig. 1D). Taken together, the dynamic expression of *Wnt4*, *Wnt14*, and *Wnt16* in the forming joints of the limb was both overlapping and complementary. These *Wnt* genes were also expressed in noncartilaginous mesenchymal cells that will form fibrous tissues, such as the joint capsule, ligaments, and tendons.

As the first attempt to identify the pathway through which these Wnts transduce their signaling in the developing joint, we examined β-catenin protein levels. Upregulation of β-catenin protein levels due to stabilization

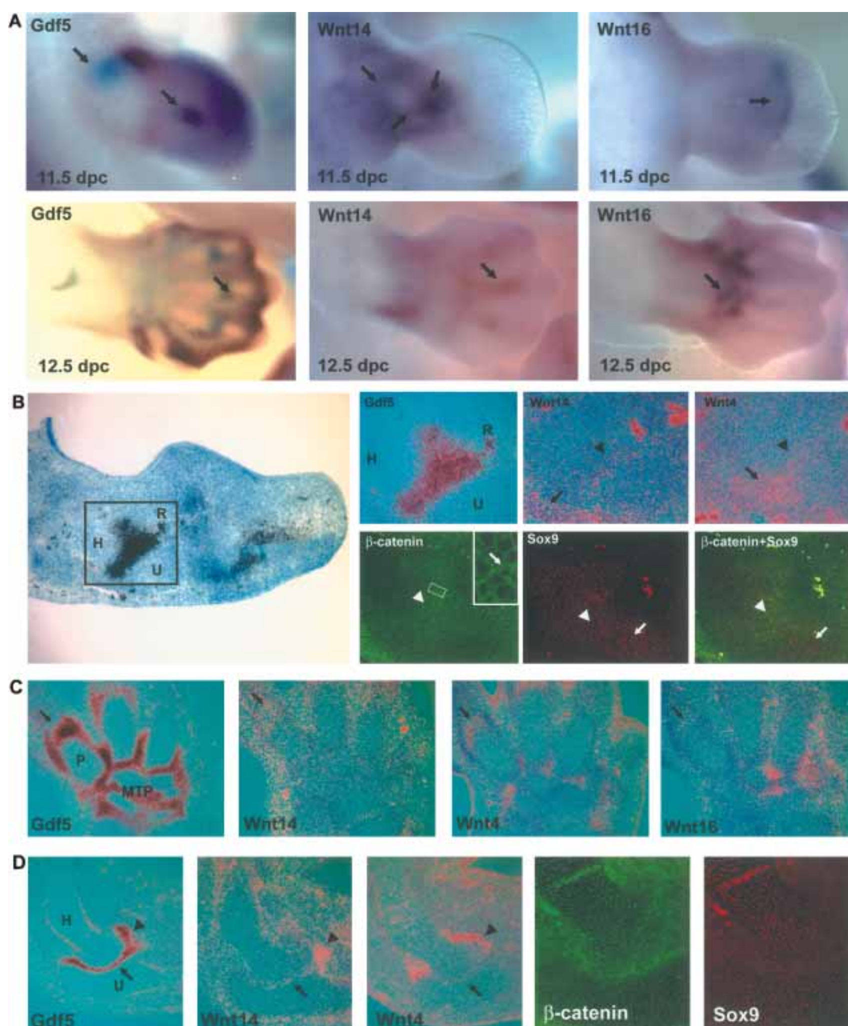

Figure 1. Expression of *Wnt* genes correlates with the expression of the joint marker *Gdf5*, up-regulation of β-catenin protein, and down-regulation of Sox9 expression. (*A*) Whole-mount in situ hybridizations. At 11.5 dpc, *Wnt14* and *Gdf5* were expressed in the future shoulder and elbow joints (arrows). *Gdf5* was also expressed in the distal limb bud, whereas *Wnt14* was also expressed in the mesenchymal cells surrounding the forming cartilage. *Wnt16* was expressed in the future MTP joints (arrow). At 12.5 dpc, *Wnt14* was still expressed around the cartilage, but stronger expression was detected in the forming joints (arrows). *Gdf5* was expressed in all developing joints (arrow) and the interdigital area. The MTP region was expanded and *Wnt16* was expressed in the forming MTP joints (arrow). Joint-specific *Wnt4* expression detected by whole-mount in situ hybridization was obscure at 11.5 or 12.5 dpc due to its expression in the surface ectoderm and tissues around the cartilage. (*B*) A limb bud section at 11.5 dpc hybridized with a ^{35}S-labeled *Gdf5* probe is shown as a bright-field image. The boxed elbow region was enlarged, and expression of *Gdf5*, *Wnt14*, and *Wnt4* (arrowheads) was detected in consecutive sections. *Wnt14* and *Wnt4* were also expressed at stronger levels in the mesenchyme surrounding the forming cartilage (arrows) than in the presumptive joint (arrowheads). Immunohistochemistry of β-catenin and Sox9 was performed on a consecutive section. β-Catenin was up-regulated in the future joint region (arrowhead). The boxed region was enlarged in an *inset* to show a cell with nuclear localized β-catenin protein (arrow). Sox9 was detected in the forming cartilage (arrow), but down-regulated in the forming joint (arrowhead). (H) Humerus; (R) radius; (U) ulna. (*C*) Expression of *Gdf5*, *Wnt14*, *Wnt4*, and *Wnt16* was all detected in the developing joints between phalanges (P) at 13.5 dpc (arrows). Expression of *Wnt16* in the MTP joints was much stronger than *Wnt14* and *Wnt4*. (*D*) Only *Gdf5* and *Wnt14* expression was detected in the elbow joint at 13.5 dpc (arrows). *Gdf5* and *Wnt4* were also expressed in the future joint capsule region (arrowhead). *Wnt14* was also expressed in the future tendons (arrowhead). Complementary patterns of β-catenin and Sox9 protein levels in the joint were similar to those in *B*.

of β-catenin protein is an important outcome of the canonical Wnt signaling activation. We found that β-catenin protein levels were low in newly differentiated chondrocytes that express Sox9 at11.5 dpc (Fig. 1B). However, in the developing joint region, β-catenin protein levels were up-regulated and nuclear localization of β-catenin protein was observed (Fig. 1B). In the same region, Sox9 expression was down-regulated (Fig. 1B). This reciprocal relationship between β-catenin and Sox9 protein levels was also observed later in development at 13.5 dpc in more mature joints (Fig. 1D). The correlation between *Wnt* gene expression and β-catenin protein accumulation during synovial joint formation suggested that at least some of the *Wnt*s expressed in the forming synovial joint may signal through the canonical Wnt pathway to increase β-catenin protein levels and its subsequent nuclear localization.

The canonical Wnt signaling pathway was up-regulated in the developing joint and activated by Wnt4/14/16

We next tested whether the canonical Wnt signaling activity was activated in the developing synovial joints using the *TOPGAL* mouse embryo (DasGupta and Fuchs 1999; Topol et al. 2003), in which *LacZ* expression is under the control of LEF/TCF-binding sites and can be activated by β-catenin. Consistent with the increased β-catenin protein levels, the canonical Wnt signaling activity was up-regulated in the developing joints in vivo, indicated by the *LacZ*-expressing cells (Fig. 2B). To further test whether Wnt4, Wnt14, and Wnt16 signal through the canonical Wnt pathway, *Wnt 4*, *Wnt14*, and *Wnt16* were expressed in a rat chondrocyte cell line RCS (Mukhopadhyay et al. 1995). In the in vitro LEF/TCF

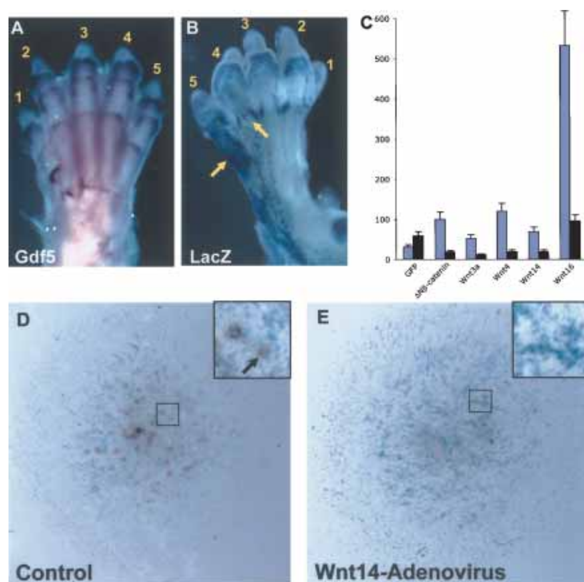

Figure 2. The canonical Wnt pathway was activated by *Wnt4/14/16* and ectopic Wnt14 signaling inhibited chondrogenesis. (*A*) Whole-mount in situ hybridization showing that *Gdf5* expression was detected in the forming digit joints of the right foot of a *TOPGAL* embryo at 14.5 dpc. (*B*) *LacZ* staining showing that the canonical Wnt signaling activity was up-regulated in the forming digit joint and joint capsule (arrows) of the left foot of the same embryo shown in *A*. Skin was peeled off in most areas to facilitate *LacZ* staining of the cartilage. (*C*). Expression of *Wnt4*, *Wnt14*, and *Wnt16*, like *Wnt3a* and *ΔN*β*-catenin*, activated TOPFLASH reporter (blue bars), but not the control FOPFLASH reporter (black bars), in RCS cells. (*D*,*E*) Micromass cultures using the limb mesenchymal cells from the *TOPGAL* mice. A comparable representative area was enlarged and shown in the *inset*. (*D*) *LacZ* staining was absent in cartilage nodules (brown color, arrow). (*E*) After infected by a *Wnt14*-adenovirus, *LacZ* staining was up-regulated and cartilage nodule formation was completely inhibited.

reporter (TOPFLASH) assay for the canonical Wnt activity (Korinek et al. 1997), expression of *Wnt4*, *Wnt14* and *Wnt16*, like *Wnt3a* or *ΔN*β*-catenin*, which encodes a consistitutively active β-catenin, led to the up-regulation of TOPFLASH activity (Fig. 2C). In addition, we performed micromass culture with the limb mesenchymal cells from the *TOPGAL* mouse embryo to mimic the in vivo process of cartilage formation in vitro. We found that *Gdf5*, *Wnt 4*, *Wnt14*, and *Wnt16* were expressed around the cartilage nodule in the micromass culture (data not shown). The *LacZ* staining was found only in the cells that surrounded the cartilage nodules, but not within the nodules (Fig. 2D). Ectopic expression of *Wnt14*, by infecting micromass cultures with a *Wnt14*-adenovirus, completely inhibited cartilage nodule formation (Fig. 2E), consistent with the previous observation in the chick micromass culture infected by a RCAS-*Wnt-14* virus (Hartmann and Tabin 2001). Importantly, we found that *LacZ* expression was activated to a higher level throughout the micromass infected with the *Wnt14*-adenovirus (Fig. 2E). All these data indicate that Wnt14 transduces its signal through β-catenin in regulating downstream gene expression.

Wnt14 *activity is blocked by inactivation of* β-catenin *or* β-catenin *transcription activity*

We further tested whether the activity of *Wnt14* in inducing joint formation requires β*-catenin* by performing micromass assays where the *Wnt14*-adenovirus was added to limb mesenchymal cells, in which β*-catenin* was inactivated by the Cre recombinase. We generated a conditional targeted allele of β*-catenin*, $Catnby^{c/c}$ (Supplementary Fig. S1). Because the joint-inducing activity of *Wnt14* is reflected in part in its ability to inhibit chondrocyte differentiation, we performed micromass cultures with the mesenchymal cells from the $Catnby^{c/c}$ limb bud at 12.5 dpc to determine whether Wnt14-mediated inhibition of chondrogenesis requires β*-catenin*. The dissociated limb mesenchymal cells were first infected with a *Cre*-adenovirus before plated at high density to achieve efficient virus infection. We found that chondrogenesis was unaffected or slightly reduced by the *Cre*-adenovirus in the $Catnby^{+/+}$ micromass culture (Fig. 3A,B) and Cre-mediated deletion of β*-catenin* occurred in most cells of the $Catnby^{c/c}$ culture (Fig. 3M). However, when β*-catenin* was inactivated in the $Catnby^{c/c}$ culture by the *Cre*-adenovirus infection, cartilage nodule formation was greatly enhanced and the nodules were bigger in size when compared with the uninfected $Catnby^{c/c}$ micromass culture (Fig. 3E,F). *Wnt14*-adenovirus blocked chondrogenesis completely in both $Catnby^{+/+}$ and $Catnby^{c/c}$ micromass cultures when β*-catenin* was functional (Fig. 3C,G). Significantly, only when the $Catnby^{c/c}$ micromass culture (Fig. 3H), not the $Catnby^{+/+}$ one (Fig. 3D), was sequentially infected by the *Cre*-adenovirus and the *Wnt14*-adenovirus, cartilage nodule formation was not blocked. Cartilage nodule formation between the *Wnt14*-adenovirus-infected and noninfected micromass cultures was similar to each other when β*-catenin* was inactivated (Fig. 3F,H). In addition, *Wnt14*-adenovirus was not able to up-regulate *LacZ* expression in the *Cre*-adenovirus infected $Catnby^{c/c}$;*TOPGAL* micromass cultures (Fig. 3O,P). These results demonstrated that the activity of *Wnt14* in inhibiting chondrogenesis requires β*-catenin*. We further found that expression of a dominant-negative *Lef1* (*ΔNLef1*), which blocks the transcription activity of β-catenin (Niemann et al. 2002) resulted in increased cartilage nodule formation (Fig. 3J) and blocked Wnt14-mediated inhibition of chondrogenesis (Fig. 3L) in the $Catnby^{+/+}$ micromass culture. Because β-catenin is involved in both cell adhesion and activation of downstream gene transcription in the canonical Wnt pathway, these data indicate that activation of LEF/TCF-mediated transcription by β-catenin is required for *Wnt14* to execute chondrogenic inhibitory effect.

Ectopic expression of a constitutively active β-catenin *or* Wnt14-*induced ectopic joint formation*

To test whether the β-catenin-mediated canonical Wnt signaling plays a pivotal role in inducing joint formation in vivo, we generated *Col2a1*-*ΔN*β*-catenin* transgenic

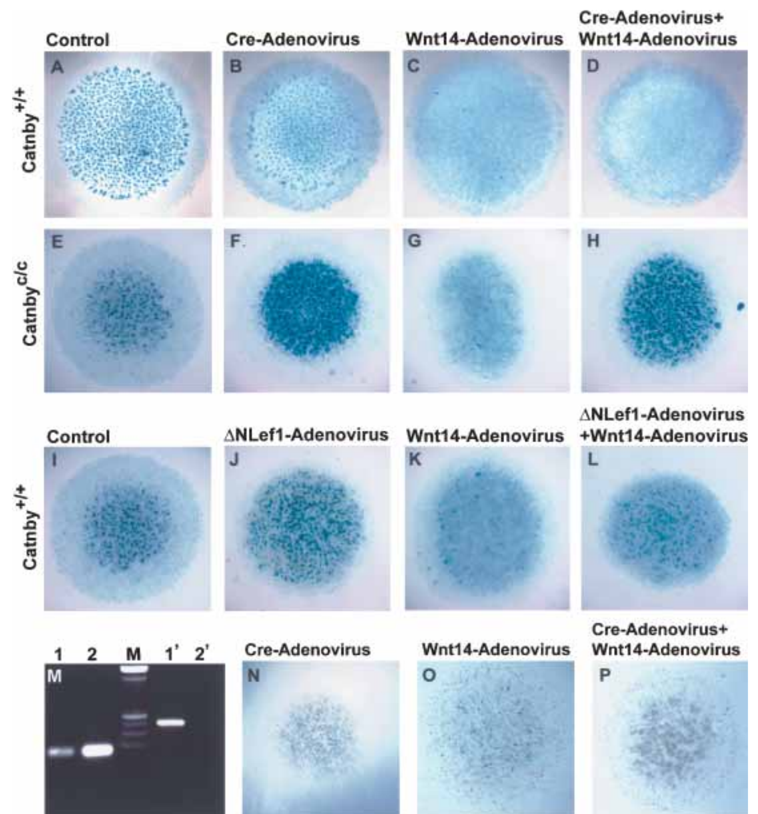

Figure 3. β-catenin transcription activity is required for the function of *Wnt14* in inhibiting chondrocyte differentiation and activating *TOPGAL* expression. Micromass cultures were stained by Alcian blue to show cartilage nodules. (*A–D*) Wild-type ($Catnby^{+/+}$) micromass cultures. (*B*) The *Cre*-adenovirus had no or little effect on cartilage nodule formation. (*C*) The *Wnt14*-adenovirus completely inhibited cartilage nodule formation. (*D*) The *Cre*-adenovirus infection did not block the chondrogenic inhibitory effect of *Wnt14*. (*E–H*) Homozygous conditional ($Catnby^{c/c}$) micromass cultures. The *Cre*-adenovirus significantly increased cartilage nodule formation (*F*) compared with the uninfected $Catnby^{c/c}$ control culture (*E*). The *Wnt14*-adenovirus completely inhibited cartilage nodule formation (*G*), but such inhibition was completely blocked by the *Cre*-adenovirus, which inactivates *β-catenin* (*H*). (*I–L*) Wild-type ($Catnby^{+/+}$) micromass cultures. The *ΔNLef1*-adenovirus increased cartilage nodule formation (*J*) compared with the uninfected control culture (*I*). The *Wnt14*-adenovirus completely inhibited cartilage nodule formation (*K*), but such inhibition was significantly blocked by the *ΔNLef1*-adenovirus (*L*). (*M*) Genotyping the cells in the micromass cultures for the conditional allele (lanes *1*,*2*) and the null allele (lanes *1′*,*2′*). (Lanes *1*,*1′*) *Cre*-adenovirus-infected $Catnby^{c/c}$ micromass cultures. Most $Catnby^{c/c}$ cells infected by the *Cre*-adenovirus had undergone deletion of the *β-catenin* gene (cf. lanes *1* and *1′*). (Lanes *2*,*2′*) $Catnby^{c/c}$ micromass cultures without virus infection. (Lane *2′*) No deletion occurred in the absence of *Cre*. (*N–P*) Micromass cultures performed using limb mesenchymal cells from the homozygous conditional embryos that also carry the *TOPGAL* transgene ($Catnby^{c/c}$; *TOPGAL*). (*O*) The *Wnt14*-adenovirus inhibited cartilage formation and up-regulated *LacZ* expression. (*P*) Both effects of the *Wnt14*-adenovirus were blocked by the *Cre*-adenovirus.

mice, in which a constitutively active, N-terminally truncated form of *β-catenin* (*ΔNβ-catenin*) (Topol et al. 2003) was expressed in chondrocytes under the *Col2a1* promoter/enhancer (Yang et al. 2003). We also generated *Col2a1-Wnt14* transgenic mice to confirm that Wnt14 signals through the canonical Wnt pathway. The *Col2a1-ΔNβ-catenin* mouse was perinatal lethal and the *Col2a1-Wnt14* mouse embryos died around 16.5 dpc. Both the *Col2a1-ΔNβ-catenin* and the *Col2a1-Wnt14* mouse embryos had dome-shaped heads and shorter limbs, tails, and snouts as compared with their wild-type litter mates (Fig. 4B,D). When skeletal preparations were examined, we found that cartilage formation in both transgenic mice was greatly reduced, as indicated by much weaker Alcian blue staining (Fig. 4B,D). In addition, endochondral ossification, but not intramembranous ossification, was significantly reduced in both transgenic mice (Fig. 4B,D), possibly caused by the impaired cartilage formation. We also observed joint fusions in both *Col2a1-ΔNβ-catenin* and the *Col2a1-Wnt14* transgenic mice (Fig. 4D,H). The similar phenotypes in the *Col2a1-ΔNβ-catenin* and the *Col2a1-Wnt14* mice supported our conclusion that Wnt14 signals through stabilizing β-catenin in controlling skeletal development.

To analyze chondrocyte differentiation and synovial joint formation in the *Col2a1–ΔNβ-catenin* and *Col2a1-Wnt14* transgenic mouse embryos in more detail, we first examined histological sections of the developing limb at 15.5 and 18.5 dpc. We found that in many areas of the cartilage, chondrocyte-specific staining was lost. For instance, we observed regions inside the cartilage where chondrocyte-specific staining was greatly diminished in both *Col2a1-ΔNβ-catenin* and *Col2a1-Wnt14* mice (Fig. 4H,I). Loss of cartilage tissue was more severe at 18.5 dpc than at 15.5 dpc (Fig. 4J). We concluded that chondrocytes had lost their characteristic cellular phenotypes and may have adopted other cell fates. To test this, we examined alterations of cell morphology and gene expression by immunohistochemistry and in situ hybridization. Chondrocytes have a characteristic small, cobble stone-like shape, whereas other cells, including the joint-forming interzone cells, osteoblasts, and cells that will form the tendon and ligament are elongated. At the molecular level, chondrocytes express very low levels of β-catenin, but high levels of Sox9 (Fig. 1B,D). We found that *Wnt14* ectopic expression leads to strong β-catenin accumulation and nuclear translocation (Fig. 5A″), again confirming that Wnt14 signals through stabilizing β-catenin protein. In addition, we found that ectopic expression of *ΔNβ-catenin* or *Wnt14* leads to loss of Sox9 expression and the cell morphology change from cobble stone-like to elongated (Fig. 5, cf. insets in A′,B′,C′,A″,B″,C″ and A,B,C). Chondrocytes also express

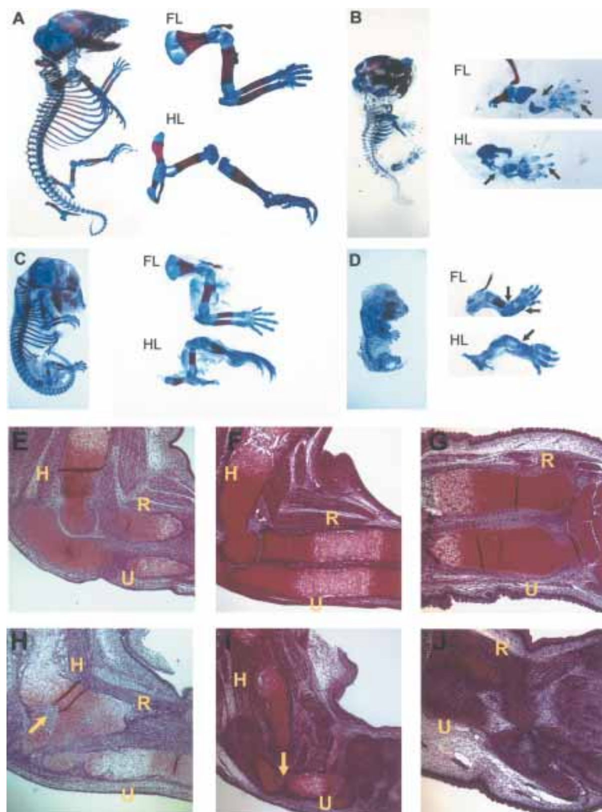

Figure 4. Expression of a constitutively active β-*catenin* or *Wnt14* under the control of the *Col2a1* promoter and enhancer-disrupted skeletal morphogenesis. (*A–D*) Skeletal preparations in which cartilage was stained by Alcian blue and ossified bone was stained by Alizarin red. Forelimbs (FL) and hindlimbs (HL) are shown in higher magnification. (*A*) Wild-type embryo at 18.5 dpc. (*B*) *Col2a1*-Δ*N*β-*catenin* transgenic littermate. Cartilage and endochondral bone formation was greatly reduced (arrows). (*C*) Wild-type embryo at 15.5 dpc. (*D*) *Col2a1-Wnt14* transgenic littermate. Cartilage and endochondral bone formation was also greatly reduced and the joint between the humerus and radius was fused (arrows). (*E–J*) A 5-µm section of the limb stained according to the Weigert-Safranin staining procedure. Chondrocytes were stained red by Safranin O. (H) Humerus; (R) radius; (U) ulna. (*E*,*F*) Wild-type limb at 15.5 dpc. (*G*) Wild-type limb at 18.5 dpc. (*H*) Limb from *Col2a1*-Δ*N*β-*catenin* transgenic littermate of *E*. Some areas in the cartilage had lost cartilage-specific staining (arrow). The joint between humerus and radius was fused. (*I*) Limb from the *Col2a1-Wnt14* transgenic littermate of *F*. Cartilage-specific staining was greatly reduced, and some areas had lost cartilage-specific staining (arrow). (*J*) Limb from the *Col2a1*-Δ*N*β-*catenin* transgenic littermate of *G*. Loss of cartilage specific staining was more extensive.

specific extracellular matrix protein such as ColII (Fig. 5D,F), but not ColIII (Craig et al. 1987; Nalin et al. 1995; Fig. 5E,G). In both *Col2a1*-Δ*N*β-*catenin* and *Col2a1-Wnt14* embryos, ColIII was ectopically expressed (Fig. 5E′,G′) at the expense of ColII expression (Fig. 5D′,F′). As the *Col2a1* promoter and enhancer are directly activated by Sox9, which is expressed in differentiating chondrogenic mesenchymal condensations (Wright et al. 1995; Lefebvre et al. 1996), our results indicate that Wnt14 signals though β-catenin in differentiating chondrocytes in vivo to reverse chondrocyte differentiation, the first step in synovial joint formation.

We next tested whether up-regulation of the Wnt14/β-catenin signaling specifically leads to ectopic joint formation by examining the expression of joint-specific markers. We found that *Gdf5* expression was ectopically activated in the cartilage by ectopic expression of Δ*N*β-*catenin* or *Wnt14*, indicating that ectopic joint formation was induced by the canonical Wnt signaling (Fig. 6A,B). The domain of ectopic *Wnt14* expression overlaps with that of *Gdf5* induction (Fig. 6A,B). But, in the *Col2a1*-Δ*N*β-*catenin* mice, ectopic *Gdf5* expression was detected at the periphery of the regions where chondrocyte phenotypes were absent (Fig. 6A). In the adjacent section of the same *Col2a1*-Δ*N*β-*catenin* embryo (Fig. 5A′,B′,C′), nuclear β-catenin colocalized with Sox9 in the peripheral cells of the same region where chondrocyte phenotypes were absent, and it is likely that these peripheral cells ectopically expressed *Gdf5*. Because the *Col2a1* promoter was activated by Sox9, absence of *Gdf5* expression in the cells that had lost chondrocyte phenotypes and Sox9 expression in the *Col2a1*-Δ*N*β-*catenin* embryo (Fig. 6A) suggested that Wnt/β-catenin not only activates *Gdf5* expression, it is also required to maintain it. The expression of other joint markers such as *Chordin* and *Fgf18* was also found to be ectopically expressed in the *Col2a1*-Δ*N*β-*catenin* and the *Col2a1-Wnt14* mouse embryos (Fig. 6A,B; data not shown). As *Fgf18* was not expressed in early condensed mesenchyme (Maruoka et al. 1998), the Wnt/β-catenin signaling did not simply block chondrocyte differentiation or reverse the differentiating chondrocytes to the earlier mesenchymal condensation stage, it ectopically activated early joint formation process in what was previously cartilage proper.

To further examine the loss of chondrocyte phenotype in the *Col2a1*-Δ*N*β-*catenin* and the *Col2a1-Wnt14* mouse embryo, we examined the expression of additional markers for chondrocyte differentiation including *Noggin* and *ColX* in the *Col2a1*-Δ*N*β-*catenin* or *Col2a1-Wnt14 embryos*. *Noggin* was expressed in early proliferative chondrocytes and its expression was significantly decreased in both *Col2a1*-Δ*N*β-*catenin* and *Col2a1-Wnt14* embryos (Fig. 6B; data not shown). In addition, *ColX* was expressed in hypertrophic chondrocytes that did not express *ColII*. In both *Col2a1*-Δ*N*β-*catenin* and *Col2a1-Wnt14* embryos, *ColX* expression was delayed, because its expression was absent in the digit (Fig. 6B; Supplementary Fig. S2B), but normal in the humerus (Supplementary Fig. S2A). These data indicate that loss of early chondrocyte characteristics occurs before chondrocyte hypertrophy in the *Col2a1*-Δ*N*β-*catenin* and *Col2a1-Wnt14* embryos.

We then examined the differentiation of other cell types such as osteoblasts and cells that form the tendons and ligaments, because these cells express *ColI* and/or *ColIII* (Niederreither et al. 1995; Rossert et al. 1995), both of which were ectopically expressed in the *Col2a1*-Δ*N*β-*catenin* and *Col2a1-Wnt14* embryos (Figs. 6A, 5E′,G′). However, neither Δ*N*β-*catenin* nor *Wnt14* in-

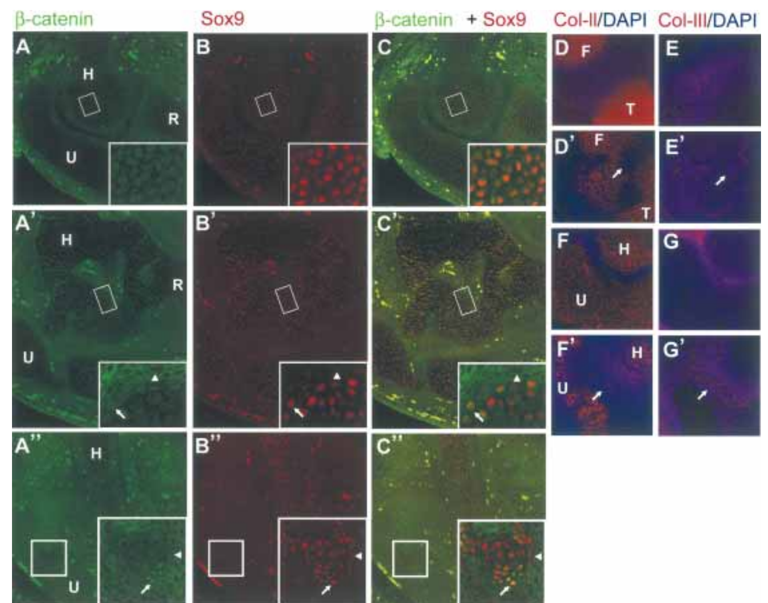

Figure 5. Ectopic Wnt14/β-catenin signaling in chondrocytes resulted in alteration of cell morphology and gene expression. Forelimb sections at the elbow region of a wild-type embryo (*A*,*B*), a *Col2a1-*Δ*N*β*-catenin* transgenic embryo (*A*′,*B*′) and a *Col2a1-Wnt14* transgenic embryo (*A*″,*B*″) at 15.5 dpc were costained for β-catenin and Sox9. Merged images of *A* and *B*, *A*′ and *B*′, and *A*″ and *B*″ are shown in *C*, *C*′, and *C*″, respectively. Boxed regions inside Humerus or Ulna were enlarged and shown in *insets*. Cells with higher levels of β-catenin and low levels of Sox9 were elongated, similar to fibroblasts (arrowheads). Chondrocytes containing both nuclear β-catenin and Sox9 were indicated by arrows. (*D*,*D*′,*E*,*E*′) Adjacent sections of a limb at the knee joint region at 15.5 dpc stained for ColII or ColIII. The nucleus was stained by DAPI. (*D*,*E*) Wild-type controls. (*D*′,*E*′) Sections of the *Col2a1-*Δ*N*β*-catenin* transgenic embryo. (*F*,*F*′,*G*,*G*′) Adjacent sections of a limb at the elbow joint region at 15.5 dpc stained for ColII or ColIII. The nucleus was stained by DAPI. (*F*,*G*) Wild-type controls. (*F*′,*G*′) Sections of the *Col2a1-Wnt14* transgenic embryo at 15.5 dpc. Arrows indicate the area where ColIII was ectopically expressed and ColII expression was absent. (H) Humerus; (R) radius; (U) ulna; (F) femur; (T) tibia

duced ectopic osteoblast formation, as *Cbfa1* expression was not ectopically activated (Fig. 6B; Supplementary Fig. S2B). It has been reported that many genes that are expressed in the interzone are also expressed in cells that form the tendons and ligaments. For instance, *Cd44* and *Autotaxin* are expressed in the developing joints (Edwards et al. 1994; Noonan et al. 1998; Bachner et al. 1999). However, both are also strongly expressed in some of the connective tissue sheets (Noonan et al. 1996; Bachner et al. 1999), where *Scleraxis* is highly expressed (Cserjesi et al. 1995; Schweitzer et al. 2001). *Scleraxis* was also expressed in the joints at a weaker level compared with its expression in the tendon and ligament (Fig. 6B; Supplementary Fig. S2A,B). We found that in the early stages of *Wnt14* ectopic expression in the digit cartilage, ectopic expression of *Autotaxin* and *Scleraxis* was detected in the cartilage, but stronger ectopic expression of these genes was only detected at the peripheral of the digits where ligament and tendon tissues normally form (Fig. 6B). These results suggested that ectopic Wnt/β-catenin signaling did not transdifferentiate chondrocytes into ligaments or tendons, as the ectopically activated *Autotaxin* and *Scleraxis* expression in cartilage was at the level typical of its normal expression in the joints, weaker than its expression in the normal ligament and tendons.

We also found that *Cd44* expression was not activated at the same time as *Autotaxin* or *Scleraxis* in the digits at 15.5 dpc in the *Col2a1-Wnt14* embryo (Fig. 6B). However, after *Wnt14* or Δ*N*β*-catenin* had been expressed for a longer period of time, for example, in the humerus at 15.5 dpc, ectopic expression of *Cd44* in the cartilage was detected (Supplementary Fig. S2A). This is consistent with the normal temporal expression order of *Autotaxin*, *Scleraxis*, and *Cd44* in the developing joint and the previous studies that showed that CD44 acts at later stages of joint development to promote cavitation (Pitsillides 2003). During normal synovial joint development, *Autotaxin* and *Scleraxis* expression was strong, but the expression of *Cd44* was weak in the newly formed digit joints at 15.5 dpc (Fig. 6B). In contrast, in more mature joints, for instance, the elbow joint at 15.5 dpc or the digit joint at 18.5 dpc, *Autotaxin* and *Scleraxis* expression was weaker and *Cd44* expression was stronger (Supplementary Fig. S2A,B). It appears that the joint markers were ectopically induced by the Wnt/β-catenin signaling in the same temporal order as that of the endogenous one.

Loss of β*-catenin in the early differentiating chondrocytes leads to joint fusion*

To test whether the β-catenin mediated Wnt signaling is also required for synovial joint formation, we crossed mice containing the β*-catenin* conditional allele *Catnby*$^{c/c}$ with the *prion-Cre* mice (Scheel et al. 2003) and *Col2a1-Cre* mice (Ovchinnikov et al. 2000) to inactivate β*-catenin* in the early differentiated chondrocytes. We found that the *Catnby*$^{c/-}$;*Col2a1-Cre* and *Catnby*$^{c/c}$;*Col2a1-Cre* embryos exhibited similar skeletal development defects and they died shortly after birth. The limbs were shortened and the heads were dome shaped (Y. Yang, unpubl.). When synovial joint formation in the limb was examined, we found that some joints between the future tarsal bones in the ankle region were either missing or incompletely formed (Fig. 7A′). The calcaneus was fused to the cuboid and the navicular was partially fused to the intermedial cuneiforms at 15.5 dpc (Fig. 7A′). The joints between these tarsal bones had just formed at 15.5 dpc in the *Catnby* $^{c/+}$;*Col2a1-Cre* mouse

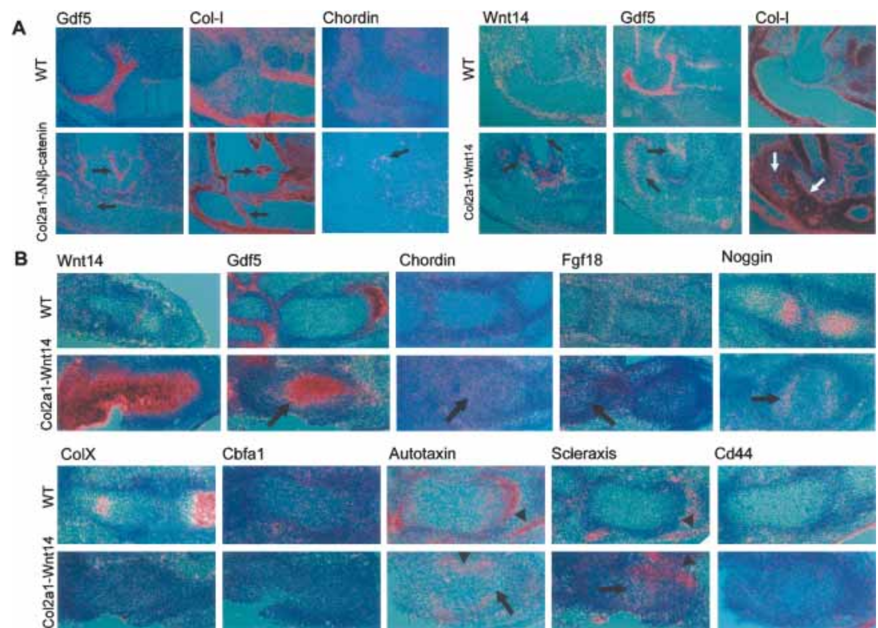

Figure 6. Induction of joint markers by ectopic expression of *ΔNβ-catenin* and *Wnt14* in chondrocytes. In situ hybridization with ^{35}S-labeled riboprobes was performed on adjacent limb sections. (*A*) In the elbow region, ectopic expression of *Gdf5*, *ColI*, and *Chordin* (arrows) was induced at 15.5 dpc in the *Col2a1-ΔNβ-catenin* transgenic mouse embryo (same embryo as in Fig. 5A′) and correlated with ectopic *β-catenin* expression shown in Figure 5A′. Ectopic expression of *Gdf5*, *ColI* (arrows) in the *Col2a1-Wnt14* embryos at 15.5 dpc correlated with the ectopic expression of *Wnt14* (arrows). (*B*) In the digit region of a *Col2a1-Wnt14* transgenic embryo at 15.5 dpc, ectopic expression of *Wnt14* correlated with ectopic expression of *Gdf5*, *Chordin*, and *Fgf18* (arrows). *Noggin* expression was suppressed (arrow). No ectopic expression was detected for *ColX*, *Cbfa1*, and *Cd44*. Ectopic expression was detected for *Autotaxin* and *Scleraxis* in the cartilage (arrows) in a pattern similar to *Gdf5* ectopic expression, but stronger expression was detected in the cells around the cartilage (arrowheads). In the wild-type (WT) samples, expression of *Autotaxin* and *Scleraxis* in ligaments were indicated (arrowheads).

embryos (Fig. 7A). Furthermore, we found that lack of joint formation at 15.5 dpc was not due to the delay in joint formation, because no joint between the fused tarsal bones was found at 18.5 dpc in the mutant embryo (Fig. 7B′). We then examined gene expression in these fused joints. We found that *ColII* expression was not decreased (Fig. 7D′) and *Gdf5* expression was missing or greatly reduced in the fused or partially fused joints, respectively (Fig. 7C′). BrdU labeling showed that chondrocyte proliferation was reduced in the fused joint as compared with the normal joint (data not shown). These data indicate that joint fusion was not a result of extensive cell proliferation and that β-catenin activity is required for inducing synovial joint formation.

The lack of joint phenotypes in most synovial joints of the *Catnby*$^{c/c}$*;Col2a1-Cre* embryos may be due to the timing of *Cre* expression. As *Col2a1* promoter is inactivated in the interzone that forms soon after chondrocyte differentiation, the joint interzone cells only express *Cre* briefly in the *Col2a1-Cre* mice. The brief expression of *Cre* driven by the *Col2a1* promoter may not be enough to inactivate *β-catenin* to stop the progress of joint formation in most joints. Because joint formation in the ankle region is completed relatively late (at 15.5 dpc), we reasoned that the time window of *Cre* expression may be wide enough only in the ankle region to affect the joints between the tarsal bones. To test whether expressing *Cre* earlier in skeletal formation would result in more efficient *β-catenin* deletion and lead to more profound joint formation, we used the *Dermo1-Cre* mice (Yu et al. 2003) to inactivate *β-catenin in* early mesenchymal condensations. Joint fusions were observed in the elbow, hip, knee, ankle, and digit regions of the *Catnby*$^{c/c}$*;Dermo1-Cre* embryos (Fig. 7E′,F′G′; data not shown). In addition, in the fused knee joint of a *Catnby*$^{c/c}$*;Dermo1-Cre* embryo (Fig. 7G′), *Gdf5* expression was missing (Fig. 7H′), demonstrating that β-catenin is required for joint formation.

Discussion

Here, we report the identification of the β-catenin-mediated canonical Wnt pathway as the first signaling pathway that is both sufficient and necessary for the induction of synovial joints in the limb. This indicates that the Wnt/β-catenin may act on the top of the regulatory hierarchy in inducing synovial joint formation. We also showed that *Wnt4*, *Wnt14*, and *Wnt16* were expressed in the forming joint in overlapping and complementary patterns, and they may play redundant roles in activating β-catenin and joint formation. Taken together, we propose a model for the molecular control of synovial joint induction (Fig. 8). Expression of Wnt genes such as *Wnt4*, *Wnt14*, and*Wnt16* in the presumptive joint results in up-regulation of β-catenin protein levels and inhibition of Sox9 expression. This leads to the induction of synovial joint formation, the first step of which is the formation of interzone cells by inhibiting or reversing chondrocyte differentiation and inducing *Gdf5* expression (Fig. 8). Our study is consistent with the previous studies in chick that show that *Wnt14* induces early steps of joint formation and that β-catenin protein levels decrease dramatically upon chondrocyte differentiation, and that ectopic accumulation of β-catenin-reversed chondrocyte differentiation (Hartmann and Tabin 2001; Ryu et al. 2002). Our results further indicate that the canonical Wnt signaling pathway has at least two physiological functions in synovial joint formation, suppression of chondrocyte differentiation and the induction of *Gdf5* expression. Reversion of chondrocyte differentia-

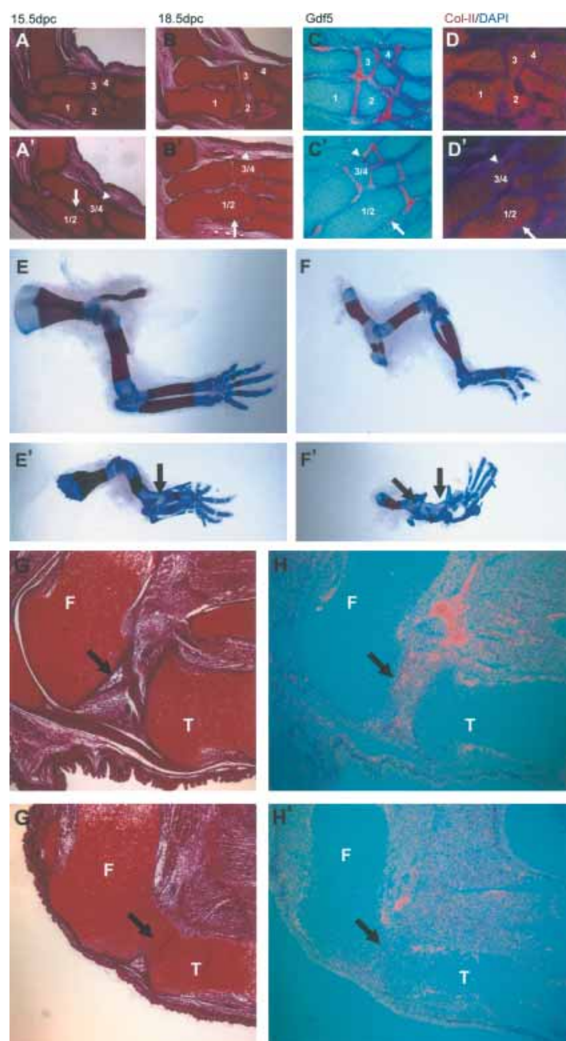

Figure 7. Loss of β-*catenin* in the early cartilage caused joint fusions. (*A*,*B*,*A*′,*B*′) Limb sections in the ankle region were stained according to the Weigert-Safranin staining procedure. (*A*,*B*) *Catnby*$^{c/+}$;*Col2a1-Cre* embryo. (*A*′,*B*′) The conditional mutant embryo (*Catnby*$^{c/c}$;*Col2a1-Cre*). The joint between the calcaneus (1) and cuboid (2) was fused (arrow), and the joint between the navicular (3) and the intermediate cuneiform (4) was partially fused (arrowhead) in *A*′, and completely fused in *B*′ in the ankle region of the conditional mutant embryo. (*C*,*C*′) At 15.5 dpc, *Gdf5* expression was missing in the *Catnby*$^{c/c}$; *Col2a1-Cre* embryo in the fused joint and reduced in the partially fused joint (arrowhead). (*D*,*D*′) Immunohistochemistry showing that at 15.5 dpc in the *Catnby*$^{c/c}$;*Col2a1-Cre* embryo, ColII expression was not down-regulated in the fused joint (arrow), partially down-regulated in the partially fused joint (arrowhead). (*E*,*F*) Forelimb (*E*) and hindlimb (*F*) of a *Catnby*$^{c/+}$; *Dermo1-Cre* embryo at 15.5 dpc. (*E*′,*F*″) Forelimb (*E*′) and hindlimb (*F*′) of a *Catnby*$^{c/c}$;*Dermo1-Cre* littermate. The joints in the elbow, pelvic, and knee regions were fused (arrow). (*G*,*G*′,*H*,*H*′) Weigert-Safranin staining procedure (*G*,*G*′) or in situ hybridization showing the expression of *Gdf5* (*H*,*H*′) were performed on 5-µm hind limb sections of a *Catnby*$^{c/+}$; *Dermo1-Cre* embryo (*G*,*H*) and a *Catnby*$^{c/c}$;*Dermo1-Cre* embryo (*G*′,*H*′) at 15.5 dpc. The knee joint (arrows) between femur (F) and tibia (T) was fused (*G*′) and *Gdf5* expression was missing in the fused knee joint in the *Catnby*$^{c/c}$; *Dermo1-Cre* embryo (*H*′).

tion itself is not sufficient for joint formation. Chondrocyte differentiation is reversed in *Sox9*$^{c/c}$;*Col2a1-Cre* mice, but *Gdf5* expression is not up-regulated or ectopically expressed (Akiyama et al. 2002). Conversely, induction of *Gdf5* alone by the Wnt/β-catenin signaling cannot reverse chondrocyte differentiation, because overexpression of *Gdf5* instead caused excessive chondrocyte differentiation and cartilage overgrowth (Francis-West et al. 1999a; Tsumaki et al. 1999).

We have observed joint fusions when ectopic joint formation was detected in both the *Col2a1-ΔN* β-*catenin* and *Col2a1-Wnt14* transgenic embryos (Fig. 4D,H). This is consistent with what has been shown in the chick limb bud that ectopically expresses *Wnt14*, and confirms that the spacing of joint can be regulated by a previously formed joint (Hartmann and Tabin 2001). We speculate that *Noggin* expressed in chondrocytes may play a critical role in regulating joint spacing by regulating the responsiveness of the differentiating chondrocytes to joint-inducing signals such as Wnt4, Wnt14, and Wnt16 (Fig. 8). It has been proposed that *Chordin* might act downstream of Wnt14 signaling in regulating joint spacing (Hartmann and Tabin 2001) on the basis of the observation that exogenous application of Noggin in the interdigit area resulted in alteration of *Wnt14* expression and decrease in the number of phalanges in the altered digit (Dahn and Fallon 2000; Hartmann and Tabin 2001). However, in the developing joint region, both *Chordin* and *Bmp* family members including *Bmp2* and *Bmp4* are expressed (Francis-West et al. 1999b). Moreover, there is no direct genetic evidence for the function of *Chordin* in regulating joint spacing. In contrast, *Noggin* is required for joint formation (Brunet et al. 1998) and *Noggin* expression was down-regulated by Wnt/β-catenin signaling. In addition, Noggin protein activity might be neutralized by Bmp family members including Gdf5 when joint formation is induced, because GDF5 can also bind Noggin (Merino et al. 1999). Therefore, one mechanism of regulating joint spacing could be through regulating the active Noggin protein level, which is low in a chondrogenic condensation near a developing joint. It is possible that a new joint could only form at a distance from the previously formed joint where the accumulation of active Noggin protein above a certain threshold could occur. Thus, the positions of synovial joints may be regulated at two levels. First, the early patterning signals including Bmps determine the expression pattern of key inductive signaling molecules, which may include *Wnt4*, *Wnt14*, and *Wnt16* at the presumptive joint region at the beginning of skeletal development. Second, later in the developing skeletal system, Wnt/β-catenin signaling regulates the expression of *Noggin* and *Gdf5* in the developing cartilage to further modify the program of joint spacing.

The β-catenin-mediated canonical Wnt pathway plays a pivotal role in synovial joint formation

In our study, we found that ectopic expression of a constitutively active β-*catenin* (*ΔN*β-*catenin*) or *Wnt14* in

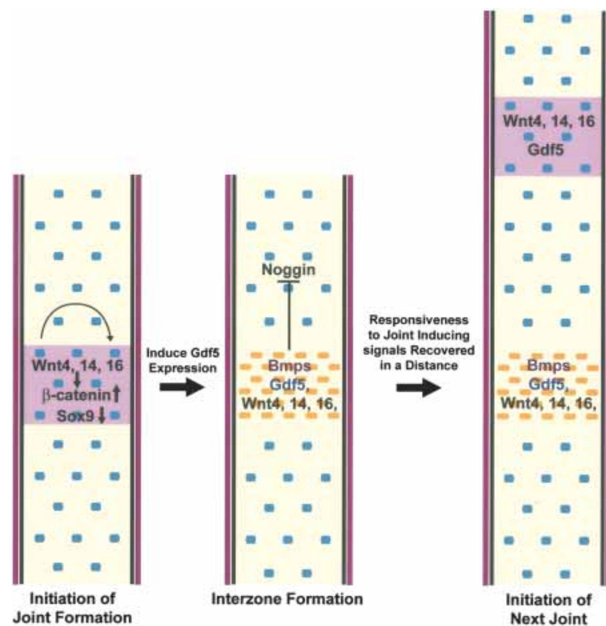

Figure 8. Model for the induction of joint formation by the Wnt/β-catenin signaling. Expression of *Wnt4*, *Wnt14*, and *Wnt16* (shown in pink) in the presumptive joint region of the newly formed cartilage may be the first step in initiation of joint formation. These Wnts signal through the canonical pathway, leading to up-regulation of β-catenin protein level and down-regulation of Sox9 protein in the presumptive joint region, the result of which is the induction of *Gdf5* expression and interzone formation. Wnt signaling at this stage suppressed *Noggin* expression, whereas Gdf5 and other Bmp family members may antagonize Noggin protein function by sequestering it. As Noggin is required for the chondrocytes to sense the joint-inducing signaling, new joint would only form in a distance where active Noggin protein concentration recovers from the inhibitory signals (Wnts, Gdf5, and Bmps) from the previously formed interzone.

newly differentiated chondrocytes under the control of *Col2a1* promoter and enhancer both induced early steps of joint formation morphologically and molecularly. Just as in endogenous joint formation, chondrocyte characteristics, such as the expression of the master controlling transcription factor Sox9 were lost, whereas joint markers were ectopically induced in both *Col2a1-ΔN β-catenin* and *Col2a1-Wnt14* transgenic embryos. In addition, the induced joint marker expression has the same temporal order as the endogenous one, which indicates that there are at least two types of genes associated with joint induction. The ones that were induced early such as *Gdf5*, *Fgf18*, *Autotaxin*, and *Scleraxis* are candidates for being direct targets of Wnt signaling and are likely to act more upstream in the regulatory hierarchy of joint or even tendon formation. Interestingly, *Fgf18* has been reported to be a direct transcription target of the canonical Wnt signaling in colon cancer cells (Shimokawa et al. 2003) and joint fusions between the tarsal bones have been observed in the mice in which *Fgf receptor 2* (*FgfR2*) is inactivated in the early condensing mesenchyme (Yu et al. 2003). The joint markers that were induced later, such as *Cd44*, are likely to be expressed as a result of the differentiation of interzone cells, and they may act at later stages of joint formation. Indeed, CD44-hyaluronan signaling has been implicated in synovial joint cavitation (Pitsillides 2003), a later step in joint morphogenesis. Therefore, both the Wnt14 and β-catenin signaling activate not only the early regulatory gene expression, but also expression of genes important in later joint maturation.

Prior to this work, it was unknown how Wnt14 transduces its signal in joint induction (Hartmann and Tabin 2001). Here, we found that *Wnt14* activated LEF/TCF-mediated transcription in vivo and in vitro. In addition, *Wnt14* and the activated form of *β-catenin* not only had the same activity in inducing joint formation, *β-catenin* was also required for the activity of *Wnt14* and joint formation, demonstrating that Wnt14 transduces its signal through the canonical Wnt pathway in joint induction. Because *Wnt4*, *Wnt14*, and *Wnt16* are expressed in the developing joint region, and they all can activate β-catenin transcription activity, it is not surprising that the function of each individual Wnt ligand is redundant when they all signal through the same canonical pathway in inducing joint formation. Interestingly, we found that *Wnt14* was also expressed in the developing joint between the future scapular and humerus, even though this joint does not form through segmenting a pre-existing chondrogenic condensation. It is likely that Wnt14/β-catenin signaling regulates the first step in different kinds of joint formation by either preventing chondrocyte differentiation from mesenchymal precursors or reversing chondrocyte differentiation that has just started.

Relationship of Wnt/β-catenin and Bmp signaling in skeletal morphogenesis

Unlike the β-catenin-mediated canonical Wnt signaling pathway, previously identified signaling molecules such as *Gdf5*, *Noggin*, and *Wnt14* are either necessary or sufficient, but not both, in joint formation. *Gdf5* appears to be a transcription target of the Wnt/β-catenin signaling in our study, it is likely that the Gdf5 signaling pathway is one of those immediately downstream of Wnt/β-catenin signaling, and these pathways need to act together in joint induction. As a potent antagonist of Bmp signaling, Noggin plays a critical role in regulating the strength of Bmp activity. Because joint formation was inhibited in the Noggin mutant mouse limb or in the chick limb that misexpresses an activated Bmp receptor IB (BmpRIB) (Zou et al. 1997), up-regulation of Bmp signaling generates a similar phenotype to that of the loss of the canonical Wnt signaling activity in synovial joint formation. This raises an interesting question as to whether Wnt and BMP signalings are antagonistic to each other, and that their relative signaling strength may determine a particular biological outcome such as joint formation. Interestingly, mutually exclusive expression patterns and functional antagonism between *wingless* (*Wg*) and *decapentaplegic* (*dpp*), the *Drosophila* orthologs of vertebrate Wnt and Bmps, respectively, have

been observed in several embryonic developmental processes in *Drosophila* (Jiang and Struhl 1996; Lee and Treisman 2001). Testing whether *Wnt4*, *Wnt14*, and *Wnt16* are still expressed, and whether ectopic Wnt14/β-catenin signaling can still induce joint formation in the *Noggin* mutant embryo, will provide more insight into the functional relationship of Wnt and Bmps in joint formation.

Apart from their expression in the forming joints, *Wnt4*, *Wnt14*, and *Wnt16* were also expressed in mesenchymal cells that surround the newly formed cartilage (Fig. 1). This pattern of expression may lead to inhibition of cartilage appositional growth to limit cartilage lateral expansion, which is critical for maintaining the spacing between each individual skeletal element. Because Wnt antagonists such as *SFRP-2* and *SFRP-3* are expressed in the prechondrogenic mesenchymal condensations (Lescher et al. 1998; Duprez et al. 1999), it is likely that the complementary expression patterns of Wnt antagonists and Wnt ligands in chondrogenic mesenchymal condensations and their surrounding mesenchyme are functionally important in defining the position and spacing of parallel skeletal elements such as the digit rays in the limb. Indeed, implanting cell pellets that ectopically express *SFRP-2* in the interdigit area induced ectopic cartilage formation (Topol et al. 2003). Moreover, we have observed partial fusion of digit 4 with digit 5 in the *Catnby$^{c/c}$;Dermo1-Cre* embryo (Y. Yang, unpubl.). Interestingly, formed cartilages expanded laterally, fused with each other, and interdigital tissue was lost in the limb of *Noggin$^{-/-}$* embryos. This phenotype further indicates that the Wnt and BMP signalings exhibit opposite activities not only in maintaining chondrocyte phenotype and inducing joint formation, but also in recruiting chondrocytes from mesenchymal progenitors. This is supported by our observation that deletion of β-catenin resulted in significantly more cartilage nodule formation in micromass culture (Fig. 3F), a similar phenotype has been observed when exogenous GDF5 or BMP4 proteins were applied to wild-type micromass cultures (Hatakeyama et al. 2004).

Maintenance of joint cartilage

After the formation of a mature joint, the two opposing articular cartilages must be properly maintained. Because the Wnt/β-catenin signaling can reverse chondrocyte differentiation, it would be important to keep the Wnt/β-catenin signaling at an appropriate level in the articular chondrocytes. Abnormal expression of *Wnt*s in articular chondrocytes or the fibroblast-like synoviocytes that cover the articular cartilage would be likely to disrupt normal joint structure in pathological conditions such as rheumatoid arthritis and osteoarthritis. It appears that Wnt signaling level is kept low at least in part through the expression of Wnt antagonists. *SFRP-3* is expressed in the articular chondrocytes and *SFRP-2* is expressed in the perichondrium including the articular surface (Y. Yang, unpubl.). In addition, it was recently shown that Sox9 in differentiated chondrocytes promotes the degradation of β-catenin (Akiyama et al. 2004). Because constitutive activation of the canonical Wnt signaling pathway has been found in the fibroblast-like synoviocytes in rheumatoid arthritis and up-regulated Wnt signaling is associated with fibronectin and pro-matrix metalloproteinase 3 expression (Sen et al. 2002), further understanding of the Wnt/β-catenin signaling in joint formation will be important for elucidating a fundamental developmental process and identifying therapeutic targets for treating cartilage and joint diseases.

Materials and methods

Generation of transgenic and targeted mouse lines

The Col2a1 transgenic vector used to generate the *Col2a1-ΔNβ-catenin* and *Col2a1-Wnt14* mice has been described (Yang et al. 2003). Transgenic mice were generated by pronuclear injection and G0 embryos at 15.5 and 18.5 dpc were analyzed. All transgenic embryos that expressed the transgenes showed phenotypes and the severity of phenotypes correlated with the level of transgene expression. The results on representative ones (more than 50% of the transgenic embryos) were shown. Genomic DNA prepared from embryonic liver was genotyped by PCR. Oligos pWnt14 5′-GGTTCACTGCCTGTTAGC-3′ and pColII 5′-GCAACGTGCTGGTTGTTGTG-3′ were used to genotype *Col2a1-Wnt14* mice. Oligos pcatnb 5′-CAGCATCAAACTGT GTAGATG-3′ and pColII were used to genotype the *Col2a1-ΔNβ-catenin* mice. *Col2a1-Cre* transgenic mice that have been described before were kindly provided by Dr. Richard Behringer (Department of Molecular Genetics, M.D. Anderson Cancer Center, Houston, TX) (Ovchinnikov et al. 2000).

For the conditional *β-catenin* allele, a complete description of the targeting vector construct, chimera production, and allele identification is provided in the Supplemental Material.

LacZ *staining, histology, in situ hybridization, and immunohistochemistry*

Wild-type and mutant embryos were dissected in PBS. For *LacZ* staining, embryos were fixed in 0.5% formaldehyde and 0.5% glutaraldehyde for 10 min at room temperature. *LacZ* staining was performed as previously described (Topol et al. 2003). For in situ hybridizations, embryos were fixed in 4% paraformaldehyde at 4°C overnight. Some fixed samples were embedded in paraffin and sectioned at 5-µm thickness. Histological analysis, BrdU labeling, immunohistochemistry, whole mount and radioactive ^{35}S RNA in situ hybridization were performed as described before (Yang et al. 2003). Primary antibodies included anti-Sox9 (Santa Cruz) at 1:100, anti-ColII (Santa Cruz) at 1:200, Anti-ColIII (Santa Cruz) at 1:50, and anti-β-catenin (Transduction laboratories) at 1:50. Signals were detected using Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Full-length cDNAs of *Wnt14*, *Wnt16*, and *Fgf18* were generated by PCR and subcloned into the pBluescript vector. The cDNAs were verified by sequencing and used to generate probes for in situ hybridization. EST clones for *Autotaxin* (Clone ID: 5043391), *Scleraxis* (Clone ID: 5056 300), *Noggin* (UI-M-AM0-adr-a-03-0-UI), *Cd44* (Clone ID: 4911674), and *Chordin* (UI-M-BH3-bsf-u-05-0-UI) were ordered from Invitrogen and used to generate RNA probes. *Lef1* cDNA was ordered from ATCC. Other RNA probes have been described previously: *ColX*, *ColII*, and *Cbfa1* (Yang et al. 2003); *Wnt4* (Parr et al. 1993); and *Gdf5* (Storm and Kingsley 1999).

Skeletal analysis

Embryos at 15.5 dpc or 18.5 dpc were dissected in PBS. The embryos were then skinned, eviscerated, and fixed in 95% ethanol. Skeletal preparations were performed as described (McLeod 1980).

Plasmids and virus

The coding regions of *Wnt4*, *Wnt14*, and *Wnt16* were inserted into pCS-2, pIRES-hrGFP-1a expression vector (Stratagene), and pCDNA3 (Invitrogen), respectively. The *Cre*-adenenovirus was provided by Dr. Francis Collins (National Genome Research Institute, National Institutes of Health, Bethesda, MD), the *Wnt14* and *ΔNLef1*-adenovirus were generated using a kit purchased from Clontech according to manufacturer's instruction.

Cell culture, transfection, and reporter analysis

Rat chondrosarcoma cells were obtained from Dr. Yoshi Yamada (National Institutes of Health, Bethesda, MD). Cells were seeded the day before transfection and electroporation was done with the Nucleofector technology (Amaxa GmbH). The TOPFLASH assay was performed as described before (Topol et al. 2003). Luciferase activity was measured 24 h after transfection according to the Dual-Luciferase Reporter Assay System (Promega). The results are shown as relative luciferase activity. The histograms are presented as the average ± S.D. from three independent transfections.

Micromass cultures

Micromass cultures were performed according to a procedure described previously (Akiyama et al. 2002). The *Cre*-adenovirus was added to the limb mesenchymal cell suspension (~2×10^6 cells/mL) at 5×10^9 pfu/mL, and the cell suspension was gently rocked at 4°C for 2 h. Then, the cell suspension was precipitated and resuspended to 2×10^7 cells/mL with medium (DMEM, 10% FCS) containing the *Cre*-adenovirus (5×10^9 pfu/mL) and 20-µL drops were plated. The *Wnt14*-adenovirus at 1×10^8 pfu/mL was added to the micromass culture 24 h after the cells were plated in high density.

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