Wif-1 is expressed at cartilage-mesenchyme interfaces and impedes Wnt3a-mediated inhibition of chondrogenesis

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Summary

Wnt factors are involved in the regulation of all steps of cartilage development. The activity of Wnt factors is generally regulated at the extracellular level by factors like the Dkk family, sFRPs, Cerberus and Wnt inhibitory factor 1 (Wif-1). Here we report that Wif-1 is highly expressed at cartilage-mesenchyme interfaces of the early developing skeleton. In fetal and postnatal skeletal development, Wif-1 is expressed in a sharply restricted zone in the upper hyaline layer of epiphyseal and articular cartilage and in trabecular bone. Coimmunoprecipitation and pull-down assays using recombinant Wif-1 and Wnt factors show specific binding of Wif-1 to Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11. Moreover, Wif-1 was able to block Wnt3a-mediated activation of the canonical Wnt signalling pathway. Consequently, Wif-1 impaired growth of mesenchymal precursor cells and neutralised Wnt3a-mediated inhibition of chondrogenesis in micromass cultures of embryonic chick limb-bud cells. These results identify Wif-1 as a novel extracellular Wnt modulator in cartilage biology.

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Key words: Wif-1, Wnt signalling, β-catenin, Chondrocyte, Chondrogenesis

Introduction

The vertebrate skeleton is a result of precisely coordinated developmental processes. These comprise condensation and chondrogenesis of limb mesenchyme, joint formation, chondrocyte proliferation, differentiation, maturation to hypertrophic cells in the growth plate and endochondral ossification. All these processes are tightly regulated by a plethora of signalling molecules including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), transforming growth factor-β (TGFβ), hedgehog proteins (Shh, Ihh), parathyroid hormone-related peptide (PTHrP) and Wnt factors (for reviews, see Ballock and O'Keefe, 2003; DeLise et al., 2000; Goldring et al., 2006; Provot and Schipani, 2005). Together, these factors form a dense regulatory network with extensive crosstalk between the pathways. Control of the individual signals ranges from transcriptional or post-transcriptional regulation of the respective signalling ligands or receptors to the extracellular control of their biological activity by soluble or membrane-bound modulator proteins (de Crombrugghe et al., 2001). Such modulators have been described for BMP and Wnt signalling (for reviews, see Kawano and Kypta, 2003; Miller, 2002; Piters et al., 2008; Rosen, 2006; Wan and Cao, 2005).

Wnt signalling pathways are important for several processes during skeletal development (DeLise et al., 2000; Hartmann, 2006). Different Wnt factors regulate these events in a specific positive or negative regulatory manner. For example, during early limb-bud development Wnt3a induces the expression of FGF8 in the apical ectodermal ridge (AER), which in turn is responsible for the proximo-distal outgrowth of the limb bud (DeLise et al., 2000; Kengaku et al., 1998). Furthermore, Wnt3a and Wnt7a are expressed in nonchondrogenic limb tissue and, similarly to Wnt1, prevent chondrogenesis in micromass culture (Church and Francis-West, 2002; Hwang et al., 2005; Rudnicki and Brown, 1997; Tufan and Tuan, 2001). By contrast, Wnt5a and Wnt5b support formation of cartilage nodules in micromass cultures. Yet, they impair further maturation of chondrocytes and can induce chondrocyte dedifferentiation associated with loss of type II collagen expression (Church et al., 2002; Kawakami et al., 1999; Ryu and Chun, 2006). Wnt4 and Wnt9a have important roles during joint formation by suppressing the chondrogenic potential of joint interzone cells, and Wnt9a has been shown to induce dedifferentiation of chondrocytes (Guo et al., 2004; Hartmann and Tabin, 2001; Spater et al., 2006a). Certain Wnt-induced activities such as inhibition of chondrogenesis by Wnt3a or Wnt7a, or joint induction by Wnt9a involve the β-catenin-dependent canonical pathway, whereas the same Wnt factors might use noncanonical pathways for other regulatory functions (Guo et al., 2004; Hartmann and Tabin, 2001; Hwang et al., 2005) (for reviews, see Macsai et al., 2008; Yates et al., 2005). At least six different Wnt factors (Wnt2b, Wnt4, Wnt5a, Wnt5b, Wnt10b and Wnt11) are still expressed in the postnatal growth plate where they modulate chondrocyte

proliferation and differentiation to hypertrophic cells (Andrade et al., 2007).

The key role of Wnt signalling in skeletal development is underlined in transgenic and knockout mouse models. Thus, permanent ectopic activation of the canonical Wnt pathway in nascent chondrocytes leads to severe skeletal defects, including defective chondrocyte maturation and endochondral ossification (Tamamura et al., 2005). Therefore, an effective regulatory machinery of Wnt signalling is indispensable for normal skeletal development. Secreted antagonists of Wnt signalling, such as Dkk1 and sFRP1, have been identified as important factors that control the activity of Wnt proteins in skeletal development and maintenance of articular cartilage (Diarra et al., 2007; Gaur et al., 2006; Mukhopadhyay et al., 2001). These antagonists can be grouped into two functional classes. The first class comprises the Dickkopf (Dkk) family of Wnt antagonists, which interact with Wnt co-receptors of the LRP class and thereby interfere with Wnt signalling. Members of the other group consisting of the secreted frizzled-related receptors (sFRP), Cerberus and Wnt inhibitory factor 1 (Wif-1) directly interact with Wnt ligands abrogating their biological function (Kawano and Kypta, 2003; Piters et al., 2008). Animal models demonstrate the importance of several of these factors for normal skeletal development and indicate associations with a number of skeletal diseases. Thus, Dkk1-deficient mice exhibit abnormalities in skeletal development such as duplication and fusion of limb digits (Mukhopadhyay et al., 2001). Furthermore, inhibiting antibodies against Dkk1 reduced the severity of bone destruction in a rheumatoid arthritis mouse model (Diarra et al., 2007). By contrast, targeted mutation of the gene encoding secreted frizzled-related protein 1 (*Sfrp1*) in the mouse leads to a rather mild skeletal phenotype characterised by enhanced endochondral ossification. Chondrogenesis of murine embryonic fibroblasts isolated from *Sfrp1*-deficient mice is also accelerated (Gaur et al., 2006).

The secreted Wnt-binding protein Wnt-inhibitory factor 1 (Wif-1) is another candidate for such a Wnt antagonist involved in skeletal development. Wif-1 was first detected in the human retina, and highly conserved homologues have been described in various vertebrates (Hsieh et al., 1999). Subsequently, *Wif1* mRNA expression was found in many murine and human tissues, being most abundant in brain, lung, retina and cartilage (Hsieh et al., 1999; Hu et al., 2008; Hunter et al., 2004). First evidence for a Wnt-inhibiting function of Wif-1 was obtained after coinjection of human WIF1 and *Xenopus wnt8* or *wnt3a* mRNAs into *Xenopus laevis* blastomeres, which revealed an antagonising effect of Wif-1 on Wnt-induced secondary axis formation. In accordance with these findings, Wif-1 was shown to physically interact with *Xenopus* Wnt8 and Wnt4 (Hsieh et al., 1999; Hunter et al., 2004).

Although most of the recent studies on Wif-1 focus on its epigenetic silencing in a variety of malignancies, little information on a role for Wif-1 during skeletal development is currently available (Batra et al., 2006; Chim et al., 2006; Clement et al., 2008). Nevertheless, *Wif1* has been reported to be upregulated during osteoblast differentiation in vitro, and overexpression of *WIF1* was observed in calvarial sutures of human craniosynostosis patients (Coussens et al., 2007; Vaes et al., 2005). These latter two observations point to a role for Wif-1 during osteoblast differentiation. By contrast, there is little information on a role for Wif-1 during cartilage development (Witte et al., 2009).

The work presented here provides a detailed overview on Wif-1 gene expression in developing chicken and mouse cartilage. In situ hybridisation analysis demonstrates Wif-1 expression in the mesenchyme surrounding cartilage elements forming in the limb during early embryogenesis. In late embryonic and postnatal development, a pronounced expression was observed in a restricted upper zone of articular cartilage. Pull-down assays and coimmunoprecipitations were carried out to identify potential Wif-1-binding Wnt factors, providing evidence for a physical interaction of Wif-1 with cartilage-related Wnt ligands. Moreover, we show that Wif-1 effectively blocks Wnt3a-dependent activation of the canonical Wnt-signalling pathway in chondrogenic cells and interferes with Wnt3a-mediated inhibition of chondrogenesis in micromass cultures of chicken limb-bud cells. These data identify Wif-1 as a potent modulator of Wnt activities during cartilage development.

Results

Wif-1 is expressed at sites of future joint formation and at the borders of cartilage elements during chicken embryogenesis

In a microarray screen we identified *WIF1* as a gene that was strongly downregulated in chondrocytes after their retinoic acid-induced dedifferentiation. This prompted us to analyze the spatial and temporal expression pattern of *WIF1* during vertebrate embryogenesis with emphasis on skeletal elements. To assess the expression of *WIF1* mRNA in the developing limb bud, chicken embryos were analysed by whole-mount in situ hybridisation, starting at embryonic HH (Hamburger-Hamilton) stage 25. At this early stage, the antisense probe detected *WIF1* primarily at the proximal bases of the limb buds in the areas of future shoulder and hip joints (Fig. 1A). Control hybridisations with a sense riboprobe obtained no signals in the respective areas (Fig. 1B). Between HH27 and HH37 (Fig. 1C-L), the *WIF1* signal moved from more proximal limb structures, along the margin of the forming cartilage model, to the very tips of each digit, confirming our initial finding of cartilage-associated expression. Strong *WIF1* expression was also detected in the interdigital mesenchyme in embryos of stages ranging from HH27 to HH33, withdrawing more and more from the centre of the interdigital mesenchyme to the lining of the cartilage anlagen as embryonic development proceeded (Fig. 1C-J). These analyses demonstrate that *WIF1* is most abundantly expressed in cartilage-mesenchyme interfaces of cartilage models during early limb development.

Wif-1 is predominantly expressed in upper zones of epihyseal cartilage during late embryonic and postnatal development of the mouse

In order to determine *Wif1* expression in later stages of skeletal development, RNA in situ hybridisation analyses for murine *Wif1* was performed on paraffin sections of mouse embryonic and postnatal limbs. In perinatal and postnatal mouse limbs *Wif1* mRNA localised most prominently to the uppermost hyaline chondrocyte layers of epiphyseal cartilage, underneath the very superficial layer of flattened cells, as demonstrated for elbow (Fig. 2A,D, arrow head) and knee joints (Fig. 2B,C,E), respectively. All *Wif1*-positive chondrocytes also expressed *Col2a1* (Fig. 2D, arrowhead; Fig. 2E). By contrast, *Wif1* mRNA was absent from the growth plate (Fig. 2D) and *Wif1* expression was not detectable in *Col10a1*-positive hypertrophic chondrocytes (Fig. 2D,E). Furthermore, *Wif1* was also expressed in meniscal cartilage, where *Wif1*-positive cells were detected predominantly in the peripheral cell layers of the meniscus (Fig. 2Bc,Ca,b, arrowheads), in cells of the tendon-cartilage junction (Fig. 2Aa,b,Cc, arrows) and also in

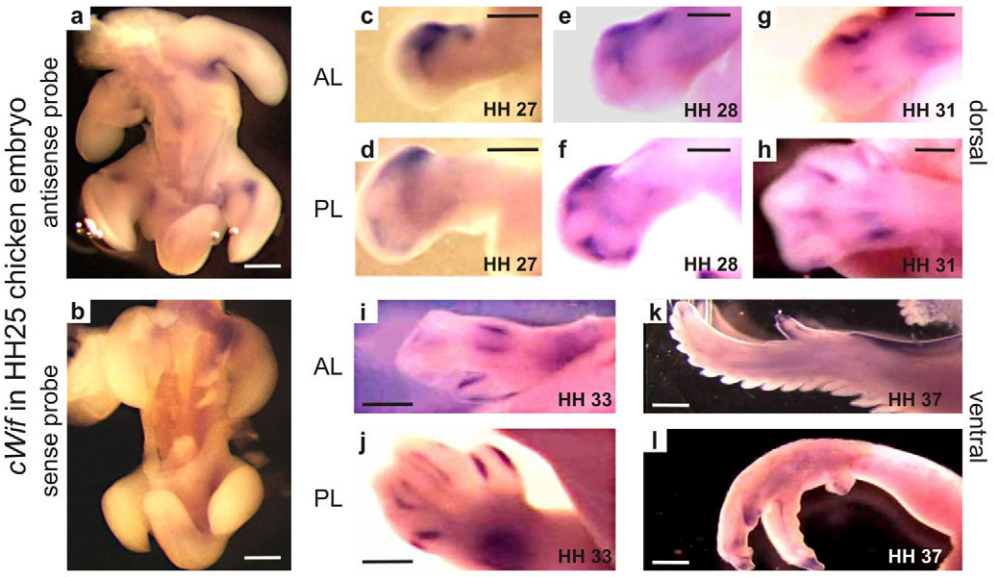

Fig. 1. *WIF1* is predominantly expressed in forming limbs at the borders of the cartilage anlagen during chicken embryonic development. Spatial and temporal expression of *WIF1* in chicken embryos of stages from HH25 to HH37 was analysed by whole-mount RNA in situ hybridisation using *WIF1* antisense riboprobes (A,C-L). Hybridisation signals were most abundant in the forming limbs at HH25 (A). Control hybridisation with a sense riboprobe did not obtain any signal (B). In later stages (C-L) the observations were focused on anterior (AL) and posterior (PL) limbs. Images are of dorsal (C-H) or ventral (A,B,I-L) views. Scale bars: 1 mm (A-J) and 2 mm (K,L).

trabecular bone and periosteum (Fig. 2Ba,D, arrows). During later postnatal development of the mouse, expression of *Wif1* persisted in tendon (Fig. 2Cc, arrow), meniscal cartilage (Fig. 2Ca,b, arrowheads) and in the upper hyaline zone of articular cartilage, where it was still detectable in adult mice (Fig. 2Bc,Cab, arrows; Fig. 2E). By contrast, expression in bone ceased with time and could not be detected by in situ hybridisation in sections of young or adult mice (Fig. 2Ac,Bc,Ca,b).

Wif-1 physically interacts with different cartilage-associated Wnt factors

To identify cartilage-related Wnt factors potentially binding to Wif-1, we generated a polyclonal anti-Wif-1 antibody, murine recombinant His-FLAG-tagged Wif-1 (Fig. 3A) and several cartilage-associated murine recombinant HA-tagged Wnt factors (Fig. 3B). The affinity-purified anti-Wif-1 antibody detected recombinant Wif-1 in western blots (Fig. 3A,C).

For identification of Wif-1-interacting Wnt factors, recombinant Wif-1-His-FLAG and concentrated Wnt-HA-conditioned medium were mixed and immunoprecipitated using an anti-FLAG antibody. Coprecipitated Wnt proteins were detected by western blotting with an anti-HA antibody (Fig. 3C, upper panel). Immunodetection of Wif-1 in the precipitate by the anti-Wif-1 antibody demonstrated similar precipitation efficiencies in all reactions (Fig. 3C, lower panel). The results indicate significant protein-protein interactions between Wif-1 and Wnt3a, Wnt4, Wnt5a and Wnt9a (previously known as Wnt14), whereas binding of Wnt7b and Wnt9b to Wif-1 was barely detectable (Fig. 3C).

To confirm these interactions, recombinant Wif-1 was coupled to Sepharose beads that were used to precipitate recombinant Wnt proteins in pull-down assays. The beads were mixed with recombinant Wnt protein, precipitated and washed extensively. Control experiments with BSA-coupled Sepharose beads were performed analogously. In control precipitates with BSA-Sepharose, Wnt proteins were not detected by western blotting with an anti-HA antibody (data not shown). By contrast, in Wif-1-Sepharose precipitated Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11 were abundantly detected and Wnt5b was also present in small amounts. However, Wnt7b and Wnt9b also did not coprecipitate with Wif-1 in this experiment (Fig. 3D).

Thus, protein-protein interactions of Wif-1 with Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt7a, Wnt9a and Wnt11 could be demonstrated by at least one in vitro approach. For Wnt3a, Wnt4, Wnt5a and Wnt9a, physical interactions with Wif-1 in vitro were verified in both approaches. The failure of Wnt7b and Wnt9b to bind to Wif-1 might indicate a specificity of Wif-1 for a subset of Wnt ligands.

Wnt3a-mediated activation of the canonical Wnt pathway is attenuated by Wif-1

Although our data demonstrate physical interaction of Wif-1 with Wnt factors, the question remained, whether Wif-1 is able to block Wnt-mediated signalling in a chondrocytic environment. For this reason, the ability of Wif-1 to modulate Wnt3a-dependent activation of the canonical Wnt signalling pathway was assessed by investigating intracellular distribution of β-catenin and transcriptional activity of T-cell factor/lymphoid enhancer factor (TCF/LEF)-like transcription factors.

As expected, treatment of the MC615 subclone 4C3 with Wnt3a lead to β-catenin accumulation in the nucleus, as shown by immunofluorescence (Fig. 4A, upper panel) and immunodetection of nuclear β-catenin levels (Fig. 4B). Wif-1 efficiently blocked Wnt3a-mediated nuclear accumulation of β-catenin, whereas Wif-1 alone did not alter subcellular β-catenin distribution in comparison to control cells (Fig. 4A, lower panel; Fig. 4B). Similar results were obtained from two immunofluorescence and three western blotting experiments.

To confirm the functional relevance of Wif-1-blocking Wnt3a-mediated nuclear β-catenin accumulation, β-catenin-dependent activation of TCF/LEF transcription factors was assessed in human HEK293 cells (Fig. 5A) and in 4C3 cells (Fig. 5B). For this purpose the TOPglow-FOPglow reporter assay system was applied, which uses a luciferase gene driven by synthetic promoters containing TCF/LEF-binding sites in the TOPglow reporter vector that are mutated in the FOPglow vector. The ratios of TOPglow- and FOPglow-derived luciferase activities of equally treated cells reflect the specific activation of β-catenin-dependent Wnt signalling via factors of the TCF/LEF family. As expected, Wnt3a-conditioned medium strongly increased luciferase activities of TOPglow-transfected cells in comparison with FOPglow-transfected cells, indicating efficient activation of the canonical Wnt-signalling

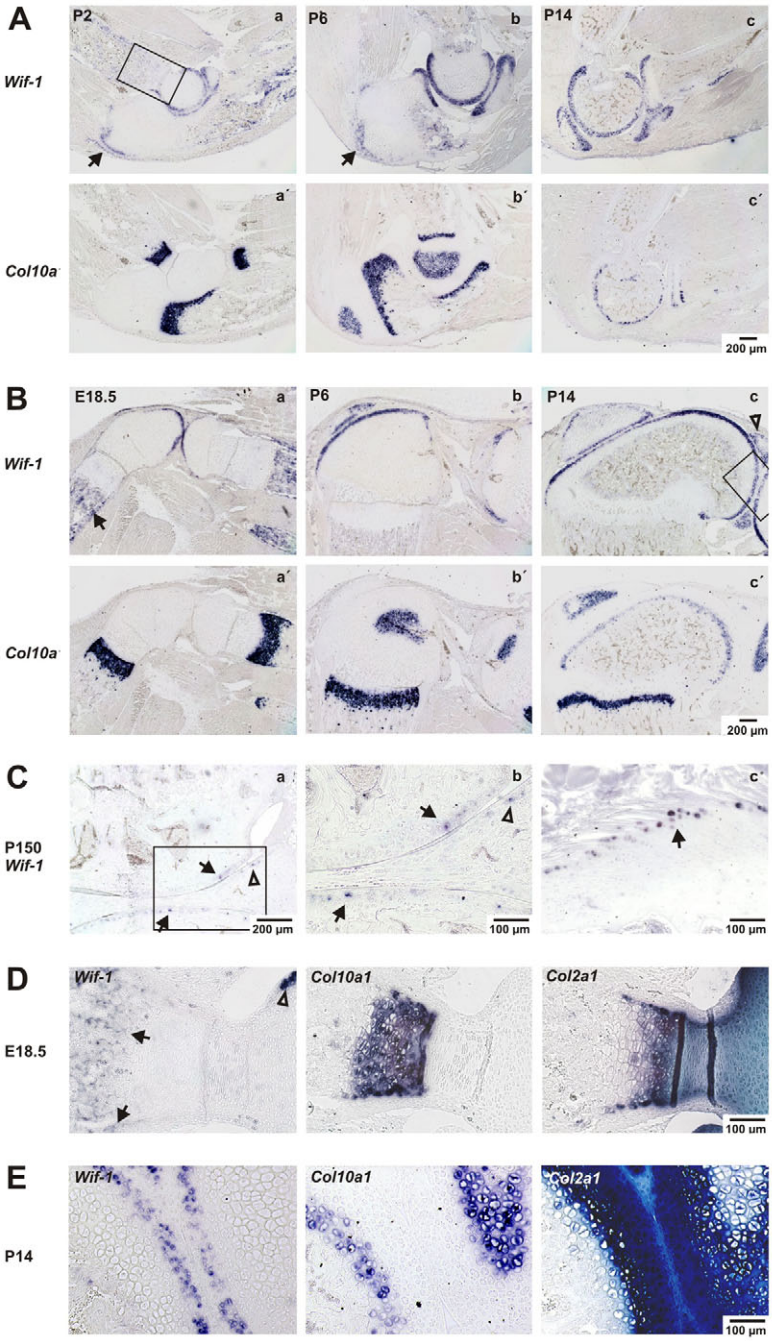

Fig. 2. Abundant expression of *Wif1* is observed in the superficial layers of epiphyseal and articular cartilage of perinatal and postnatal mouse limbs. Expression of *Wif1* in mouse limbs of late embryonic and postnatal developmental stages was assessed by RNA in situ hybridisation on paraffin-embedded sections of elbow (Aa-c and higher magnification of boxed region in Aa in D) and knee joints (Ba-c and higher magnification of boxed region in Bc in E) of the respective developmental stages. Hybridisations with a *Col10a1* (Aa′-c′,Ba′-c′,D,E) and *Col2a1* (D,E) antisense riboprobes were performed on parallel consecutive sections to indicate differentiated (*Col2a1*) and hypertrophic (*Col10a1*) chondrocytes. *Wif1* expression is predominantly observed in the superficial layers of epiphyseal and articular cartilage but absent from growth plate cartilage. In perinatal sections *Wif1* can also be detected in bone (Aa and Ba), whereas this expression is not detectable at later developmental stages (Ac and Bc). (C) Sections of knee joints from adult mice were also analysed for *Wif1* expression *Wif1* transcripts are seen in articular (arrows) and meniscal (arrowheads) chondrocytes (Ca, and higher magnification of boxed region in Cb). Wif-1 expression was also observed in adult cartilage-tendon interfaces, as shown here for patellar tendon (Cc, arrow). E18.5, 18.5 days post coitus embryo; P2, P6, P14, P150, postnatal day 2, 6, 14, 150, respectively.

pathway. Wif-1 alone did not significantly alter canonical Wnt signalling as demonstrated by similar TOPglow:FOPglow ratios as in control reactions in both cell types (Fig. 5A,B). By contrast, Wif-1 significantly impaired Wnt3a-dependent activation of TCF/LEF transcriptional activity (Fig. 5A,B). Reporter assays were performed in triplicate and data shown are representative for two (HEK293 cells) and three (4C3 cells) independent experiments, respectively. Together these results demonstrate that Wif-1 interferes with both Wnt3a-dependent nuclear accumulation of β-catenin and activation of TCF/LEF transcriptional activity, indicating efficient interference with Wnt3a-dependent canonical Wnt signalling in chondrogenic cells.

The finding that Wif-1 interacts with numerous Wnt ligands, including members of the noncanonical Wnt proteins (e.g. Wnt5a), raised the question whether Wif-1 was also able to antagonise noncanonical Wnt signalling. Therefore, JNK activation in response to Wnt5a was investigated in murine chondrogenic cell systems including the cell line 4C3, primary mouse limb-bud cells and primary murine rib chondrocytes. However, induction of JNK kinase or c-Jun phosphorylation in response to Wnt5a was not detected in any of these cells (data not shown).

Wif-1 impairs growth of mesenchymal precursor cells and blocks Wnt3a-mediated stimulation of cell growth

Numerous studies have demonstrated that the activation of canonical Wnt signalling results in increased proliferation and cell growth of many tumour cells and mesenchymal cells (for reviews, see Behrens and Lustig, 2004; Logan and Nusse, 2004; Macsai et al., 2008; Stock and Otto, 2005). A recent study has demonstrated that Wif-1 can

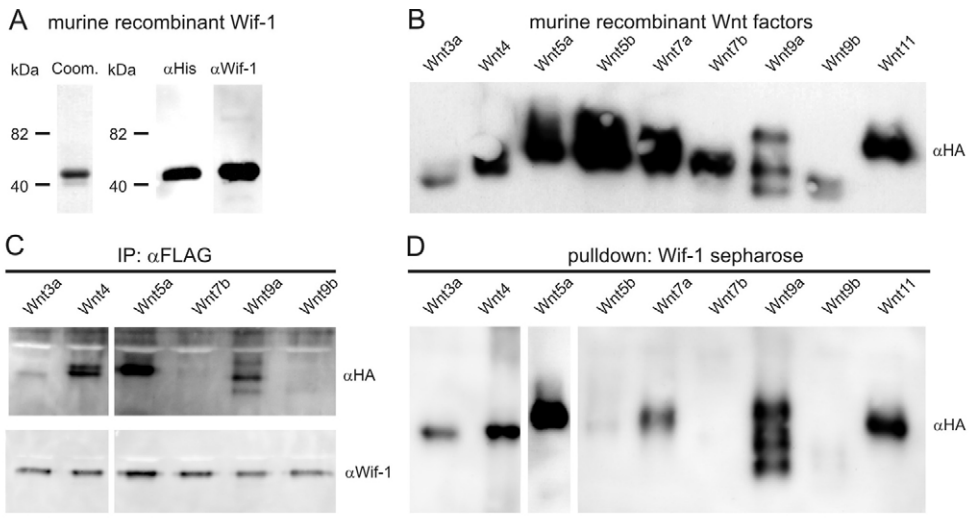

Fig. 3. Wif-1 physically interacts with various cartilage-associated Wnt ligands. (A) Murine His-FLAG-tagged Wif-1 was recombinantly expressed in HEK293-EBNA cells, purified via its His-tag and subsequently used to raise a rabbit antibody against Wif-1. Purity, size and integrity of recombinant Wif-1 was confirmed by Coomassie blue stained PAGE (Coom.) and western blotting using anti-His or anti-Wif-1 antibodies. (B) Murine recombinant HA-tagged Wnt proteins were expressed in HEK293-EBNA cells. Expression and integrity of HA-tagged Wnt ligands in concentrated Wnt-conditioned medium was demonstrated by immunoblotting with an anti-HA antibody. (C) Coimmunoprecipitation of Wif-1 and Wnt proteins was performed with an anti-FLAG antibody. Western blotting with an anti-HA antibody detected respective Wnt ligands in the precipitate (upper panel). Immunodetection of Wif-1 with the anti-Wif-1 antibody indicates equal precipitation efficiencies in each approach (lower panel). (D) Wif-1-coupled Sepharose beads were mixed with HA-tagged Wnt ligands, extensively washed and precipitated by centrifugation. Coprecipitated Wnt proteins were detected by western blotting using an anti-HA antibody.

inhibit cell growth of bladder cancer cells (Tang et al., 2009). Therefore, we addressed the question of whether Wif-1 can also modulate the cell growth of mesenchymal precursor cells and its regulation by Wnt signals. Cell growth of the murine mesenchymal progenitor cell line C3H10T1/2 in response to Wif-1 was determined colorimetrically after 3 and 6 days. Three days after seeding, a dramatic reduction in cell growth was detected when cells were treated with Wif-1 (10 μg/ml). After 6 days, untreated cells had reached confluency, whereas Wif-1-treated cell counts were still lower (Fig. 6A). To confirm these results in primary cells, growth behaviour of mouse limb-bud cells in response to various doses of Wif-1 was analysed. Similarly to C3H10T1/2 cells, mouse limb-bud cells exhibited a markedly decreased growth rate when treated with Wif-1. Augmented growth inhibition was observed with increasing doses of Wif-1 (Fig. 6B). By contrast, growth of mouse limb-bud cells modestly increased after treatment with Wnt3a-conditioned medium. This enhanced cell growth was significantly blocked by Wif-1 (Fig. 6C).

Wif-1 restores Wnt 3a-impeded chondrogenesis in chicken and mouse limb-bud micromass cultures

Wif-1 has been shown to interact with certain Wnt factors and to impair their biological activities (Hsieh et al., 1999; Hunter et al., 2004). As some of these factors, such as Wnt3a, are potent inhibitors of early chondrogenesis, the influence of Wif-1 on this process was analysed in mesenchymal cells from chicken limb buds (Hwang et al., 2005). Micromass cultures of chicken limb-bud cells were treated for 6 days with Wnt3a-conditioned or control medium in the presence and absence of recombinant murine Wif-1. As expected, Wnt3a-conditioned medium blocked the formation of Alcian-blue-positive cartilage nodules, indicating impairment of chondrogenic differentiation (Fig. 7A,B).

Wif-1 interfered with Wnt3a-dependent inhibition of cartilage nodule formation. Increasing doses of Wif-1 restored the number

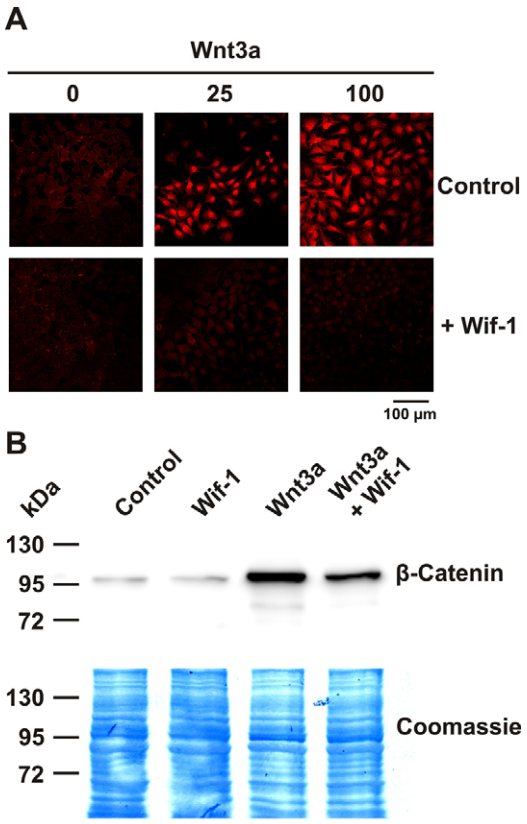

Fig. 4. Wif-1 blocks Wnt3a-mediated nuclear accumulation of β-catenin in 4C3 cells. 4C3 cells were stimulated as indicated with 25 μl/ml (A) or 100 μl/ml (A,B) Wnt3a-conditioned medium and 10 μg/ml recombinant Wif-1, or the respective control medium. (A) Immunofluorescence detection of β-catenin in 4C3 cells 5 hours after stimulation. (B) Nuclear extracts of 4C3 cells were prepared 16 hours after stimulation, and nuclear β-catenin protein was detected by immunoblotting.

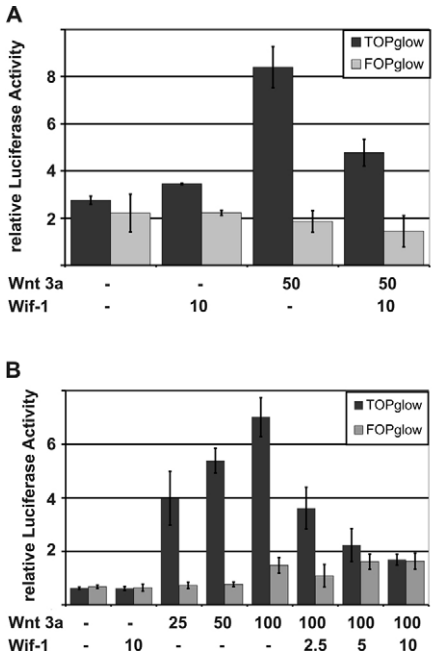

Fig. 5. Wnt3a-mediated induction of TCF/LEF-dependent transcription is attenuated by Wif-1 in HEK293 and 4C3 cells. HEK293 (A) or 4C3 (B) cells were seeded in 24-well plates and cotransfected with reporter vectors TOPglow or FOPglow and a β-galactosidase expression vector for normalisation. 24 hours after transfection, growth medium was replaced by serum-free medium containing indicated doses of Wnt3a- or control-conditioned medium (in µl/ml) and murine recombinant Wif-1 (in µg/ml). Reporter assays were carried out 24 hours after stimulation. Normalised TOPglow-derived (black bars) and FOPglow-derived (grey bars) luciferase activities are shown. Means (n=3) are plotted as bars and error bars indicate s.d.

of cartilage nodules (Fig. 7B), but cartilage nodules formed in the presence of Wnt3a and Wif-1 remained smaller than in control experiments (Fig. 7A). Interestingly, Wif-1 also slightly promoted the formation of cartilage nodules in the absence of exogenous Wnt3a (Fig. 7A,B), indicating that Wif-1 might also modulate the activity of Wnt proteins such as Wnt3a that are endogenously expressed by chicken limb-bud cells (Fig. 7C). Similar results were obtained in three independent experiments.

These findings were verified by expression analyses of the chondrocyte master regulators *Sox9* and *Sox6* and the cartilage-specific collagen *Col2a1* (Fig. 7C,D). In parallel experiments, RNA was isolated from micromass cultures treated analogously and expression levels of *Col2a1*, *Sox9* and *Sox6* were detected by RT-PCR (Fig. 7C) and real-time RT-PCR (Fig. 7D). A strong reduction in expression of these genes was observed after treatment with Wnt3a, which was restored by Wif-1 in a dose-dependent manner. Moreover, Wif-1 alone slightly increased *Col2a1*, *Sox9* and *Sox6* mRNA levels in comparison with control cells (Fig. 7C,D). In addition to Wnt3a, mRNAs of the cartilage-related Wnt proteins Wnt2b, Wnt4, Wnt5a, Wnt5b, Wnt7a, Wnt7b, Wnt9a, Wnt 9b and Wnt11 were identified in chicken limb-bud cells (supplementary material Fig. S1). This proposes potential interactions of Wif-1 with these endogenously expressed Wnt ligands, of which Wnt3a, Wnt4, Wnt7a and Wnt9a have been described to inhibit chondrogenesis (Yates et al., 2005).

The effect of Wif-1 on *Col2a1* expression and on its repression through Wnt3a was also demonstrated in micromass cultures of murine limb-bud cells, confirming the results obtained from chicken micromass cultures (Fig. 7E). Together, these results indicate that Wif-1 interacts with and inhibits Wnt ligands and thus might modulate Wnt-regulated events in chondrogenic differentiation.

Discussion

In this study, we introduce Wif-1 as a novel modulator of Wnt signalling in chondrogenesis. Extensive expression pattern analysis by RNA in situ hybridisation revealed areas of joint formation and cartilage-mesenchymal interfaces as the major sites of *Wif1* expression in early limb development, the surface of epiphyseal cartilage in later stages of skeletal development, and a sharply delineated band of expression in the upper zone of articular cartilage in postnatal development. Wif-1 physically interacts with several cartilage-associated Wnt ligands, and the interaction with Wnt3a was shown to inhibit the induction of the β-catenin-dependent signalling pathway in HEK293 cells and a chondrogenic cell line. Consequently, Wif-1 interfered with Wnt3a-enhanced growth of mesenchymal precursor cells and with Wnt3a-mediated inhibition of chondrogenesis in limb-bud micromass cultures.

The early expression of *Wif1* in the limb bud, particularly at the proximal base (Fig. 1A), suggests a role for Wif-1 in the modulation or fine-tuning of Wnt signals essential for patterning of the limb as well as for a tightly regulated chondrogenesis in the appendicular skeletal elements.

Wif1 expression in cartilage-mesenchyme interfaces during early limb developmental stages is consistent with the well documented

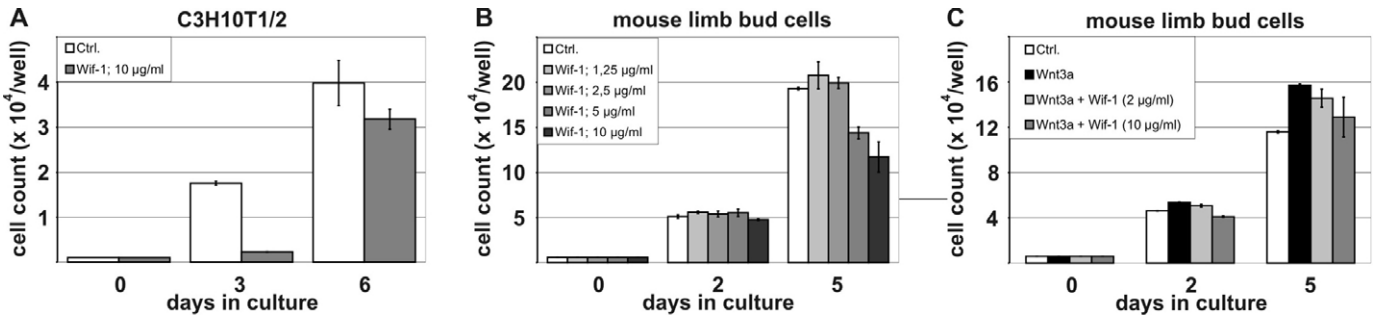

Fig. 6. Wif-1 inhibits growth of mesenchymal precursor cells and blocks Wnt3a-induced cell growth. (A) At day 0, C3H10T1/2 murine embryonic fibroblasts were seeded in 96-well plates (1000 cells per well) and treated with or without 10 µg/ml recombinant Wif-1. Cell growth was monitored by colorimetric determination of cell numbers at the indicated time points. (B,C) Primary murine limb-bud cells were seeded in 96-well plates at 6000 cells per well at day 0. These cells were treated at day 1 with indicated doses of Wif-1 (B) or 100 µl/ml Wnt3a-conditioned medium and indicated doses of Wif-1 (C). Cell counts were determined at days 2 and 5. Bars represent mean values (n=3) and error bars indicate the respective s.d.

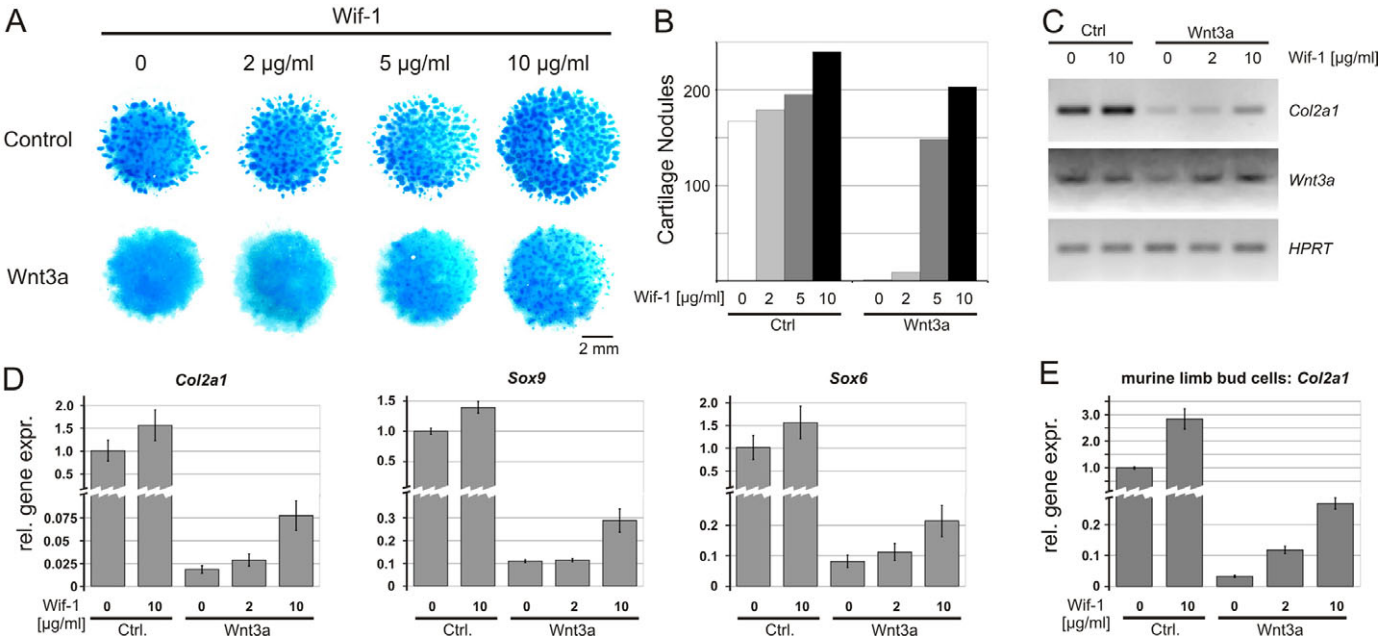

Fig. 7. Wif-1 antagonises Wnt3a-dependent inhibition of chondrogenesis. Micromass cultures of chicken limb-bud cells were incubated for 4 days in the presence of 50 µl/ml Wnt3a or control conditioned medium and indicated doses of Wif-1. (A) The formation of cartilage nodules in Wnt3a- and Wif-1-treated cultures was observed by Alcian blue staining, indicating Wnt3a-dependent repression of nodule formation, which could be antagonised by Wif-1. (B) Cartilage nodule counts in micromass cultures from A. (C) Total RNA isolated from analogously treated cultures. *Col2a1* and *Wnt3a* expression was analysed by RT-PCR. *HPRT* mRNA levels were detected as control. (D) Quantitative analyses of *Col2a1*, *Sox9* and *Sox6* mRNA levels were performed by real-time PCR from the same RNA preparations as in C. Reactions were run in triplicate. *HPRT* mRNA levels were assessed for normalisation. (E) Mouse limb-bud cells were grown in micromass cultures and treated with Wnt3a and Wif-1 as indicated. After RNA extraction, Col2a1 mRNA levels were determined by real-time PCR using cyclophilin A levels for normalisation. Values are means ± s.d.

inhibition of chondrogenic differentiation of limb mesenchyme by Wnt3a, Wnt4 and Wnt7a outside the cartilage blastema and might shield the cartilage from the inhibitory influence of the Wnt factors (Church and Francis-West, 2002; Rudnicki and Brown, 1997; Tufan and Tuan, 2001). The inhibitory effect of Wnt3a, Wnt4 and Wnt7a on chondrogenesis has also been demonstrated in micromass cultures of chick limb-bud mesenchymal cells – a finding that was confirmed for Wnt3a during this study (Church and Francis-West, 2002; Hwang et al., 2005; Rudnicki and Brown, 1997). The addition of Wif-1 neutralised the inhibitory effect of Wnt3a on chondrogenesis in a dose-dependent manner (Fig. 7). These data imply the possible modulation of Wnt3a signalling by Wif-1 in a physiological environment, and support the notion that timely and spatially fine-tuned Wnt3a signalling is important for proper limb development.

The high doses (10 µg/ml) of Wif-1 needed for effective inhibition of Wnt3a activity raise the question of whether the recombinant Wif-1 protein used for these experiments is fully biologically active. Although protein integrity was confirmed by Coomassie Blue staining of SDS gels and western blotting, insufficient post-translational protein modifications cannot be excluded, because recombinant Wif-1 was produced in the nonchondrogenic human cell line HEK293EBNA (Fig. 3A). However, expression in vivo occurs in highly discrete areas, which might lead to high local concentrations of Wif-1 protein (Figs 1 and 2).

After submission of this study, two reports on the expression pattern and function of Wif-1 in early mouse skeletal development were published, which supported and complemented our finding on the expression in early chicken embryos. Moreover, *Wif-1* expression in early mouse limb buds was also demonstrated in the AER (Kansara et al., 2009; Witte et al., 2009). Together, these findings show that during early limb formation *Wif1* expression overlaps with that of several Wnt ligands, including Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9a and Wnt11 (Fig. 8). In limb-bud mesenchyme *Wif1* mRNA overlaps with those of Wnt4, Wnt5a, Wnt9a (predominantly in areas of future joint formation) and Wnt11. Ectodermal expression in the limb bud was reported for Wnt3a (AER in chicken limb buds), Wnt4, Wnt5a (AER) and Wnt7a (dorsal ectoderm) (Gavin et al., 1990; Guo et al., 2004; Kengaku et al., 1998; Kispert et al., 1996; Summerhurst et al., 2008; Witte et al., 2009). These Wnt proteins have been described to control patterning and differentiation in the developing limb (Church and Francis-West, 2002; Hartmann and Tabin, 2001; Kengaku et al., 1998; Loganathan et al., 2005) (for a review, see DeLise et al., 2000).

Here, we demonstrate direct physical interactions of Wif-1 with the above-listed Wnt proteins by coimmunoprecipitation and pull-down assays (Fig. 3). Recently, Malinauskas proposed a possible fatty-acid binding site for palmitoylated Wnt proteins inside the Wif domain in the human Wif-1 (Malinauskas, 2008). Whether palmitoyl residues are involved in Wif-1 binding to all the Wnt proteins shown here, and which signalling pathways elicited by other Wnt factors are affected by Wif-1, remain to be established.

In addition to controlling cell differentiation, canonical Wnt signals are well established inducers of mesenchymal cell proliferation (for reviews, see Behrens and Lustig, 2004; Chien et al., 2009; Logan and Nusse, 2004; Macsai et al., 2008; Stock and Otto, 2005). Consistently, Wnt3a increased growth of mouse limb-bud cells. In line with an inhibitory effect of Wif-1 on canonical Wnt3a signalling, Wif-1 could completely abrogate Wnt3a-

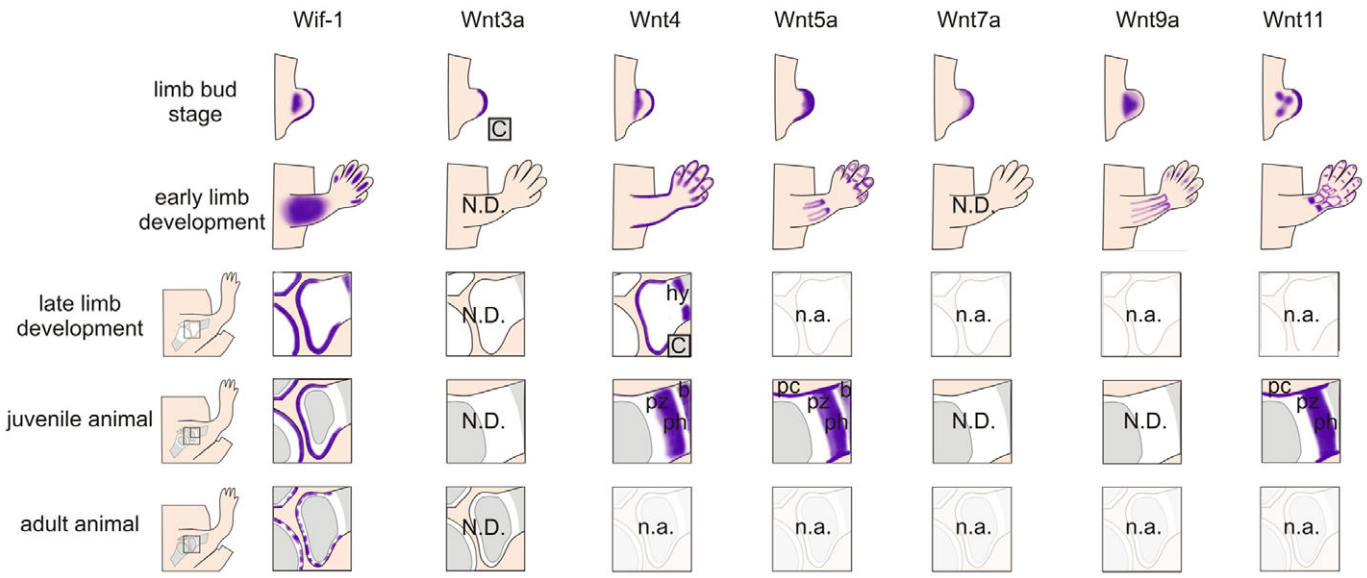

Fig. 8. mRNA expression domains of Wif-1 and Wnt proteins during vertebrate limb development. Limb-bud stage (dorsal view) represents ~E10.5-11.5 (mouse) or HH stage 19-25 (chicken). Early limb development, E14.5-15.5 (mouse) or HH30-33 (chicken). Late limb development, E18.5 newborn (mouse) or HH36-37 (chicken). Drawings summarise Wif-1 expression data demonstrated in this study and elsewhere (Kansara et al., 2009; Witte et al., 2009; Andrade et al., 2007; Church et al., 2002; Guo et al., 2004; Kengaku et al., 1998; Loomis et al., 1998; Spater et al., 2006b; Summerhurst et al., 2008; Visel et al., 2007; Witte et al., 2009; Yamaguchi et al., 1999; Yang et al., 2003). Juvenile animal, 1-2 weeks postnatal (mouse); B, bone; C, chicken data only; n.a., data not available; N.D., expression not detectable; pc, perichondrium; ph, prehypertrophic cartilage; pz, proliferative zone. Regions of mRNA expression are indicated in purple.

dependent acceleration of cell growth. Moreover, Wif-1 impaired cell growth of a mesenchymal precursor cell line (C3H10T1/2) and of mouse limb-bud cells, even in the absence of exogenous Wnt proteins. This might reflect the ability of Wif-1 to inhibit endogenous proproliferative Wnt proteins. Therefore, these findings are consistent with recent studies demonstrating an antiproliferative effect of Wif-1 on tumour cells such as bladder cancer cells and osteosarcoma cell lines (Kansara et al., 2009; Tang et al., 2009). These observations could be explained by Wif-1-dependent inhibition of canonical Wnt signalling. In support of this, activation of β-catenin-dependent signalling by Wnt3a was impaired by Wif-1 in HEK293 and chondrocytic cells (Figs 4 and 5).

In contrast to Wnt3a signalling, which is transduced by the canonical pathway via frizzled-LRP5/6 complexes, Wnt5a has been reported to induce the JNK signalling pathway in rabbit articular chondrocytes (Ryu and Chun, 2006). However, we could not recapitulate Wnt5a-dependent induction of the JNK pathway in the chondrogenic systems used in this study (4C3 cells, primary mouse limb-bud cells, primary murine rib chondrocytes). The fact that Wif-1 nevertheless binds to Wnt5a (Fig. 3) warrants further study in other cell systems to evaluate, whether Wif-1 also affects noncanonical Wnt signals. Also, whether Wif-1-Wnt interactions necessarily result in antagonising Wnt activities, or whether this depends on Wnt-receptor context, is yet to be determined. For example, Dkk1, another Wnt antagonist, has been shown to interfere only with canonical Wnt signalling; noncanonical signalling is not affected by Dkk1 (Torii et al., 2008). In the case of Dkk1, however, the target of Wnt antagonism is the Wnt co-receptor LRP6, which is essential for canonical Wnt signalling but irrelevant for β-catenin-independent Wnt signalling. Thus, Dkk1 binds to LRP6 and prevents Wnt-dependent activation of the β-catenin signalling pathway through the frizzled-LRP6 complex (Mao et al., 2001) (for a review, see Chien et al., 2009). The mechanism and receptor complexes involved in Wif-1-dependent interference with Wnt signalling remain, however, to be determined.

In later embryonic and postnatal development, *Wif1* expression was largely confined to the uppermost layers of cartilage, as shown by RNA in situ hybridisation on murine limb sections (Fig. 2). This expression at the joint surfaces points to a role for Wif-1 in joint formation and/or homeostasis. Wif-1 physically interacts with Wnt4 and Wnt9a, which are expressed in interzone cells of forming joints (Hartmann and Tabin, 2001; Spater et al., 2006b) (Fig. 3; Fig. 8). Although Wnt9a deficiency in the mouse does not affect joint induction, Wnt9a-knockout mice exhibit synovial chondroid metaplasia in some joints, a phenotype that is enhanced in Wnt4 and Wnt9a double knockouts (Spater et al., 2006b). This is in line with the finding that depletion of β-catenin in mesenchymal precursor cells enhances chondrogenic differentiation (Guo et al., 2004; Hartmann and Tabin, 2001; Spater et al., 2006a). Moreover, ectopic expression of a constitutively active form of β-catenin or Wnt9a in young differentiating chondrocytes leads to ectopic joint formation. These findings indicate that canonical Wnt signalling has a major role in joint formation. Furthermore, Wnt9a was reported to induce dedifferentiation of chondrocytes, and Wnt3a was shown to induce expression of catabolic genes in articular chondrocytes, causing osteoarthritis-like degeneration (Hartmann and Tabin, 2001; Spater et al., 2006a; Yuasa et al., 2008). However, inhibition of β-catenin signalling in articular chondrocytes has also been demonstrated to promote articular cartilage destruction, indicating that other Wnt activities are also important for the maintenance of an intact articular surface (Zhu et al., 2008). Thus, *Wif1* expression at the uppermost layer of epiphyseal cartilage and the later articular cartilage surface suggests a control function in the formation and maintenance of articular cartilage by attenuating Wnt signalling.

In vivo loss-of-function models for Wif-1 have not so far revealed any further information on the biological function of this new Wnt modulator. Preliminary investigations of Wif-1-deficient mice did not reveal any obvious abnormalities in skeletal development (data not shown). Our observations are consistent with a study that was published by Kansara et al. during the revision of this work, which demonstrated normal skeletal development of Wif-1-deficient mice (Kansara et al., 2009). Similarly, knockdown of *wif1* using splice-blocking morpholinos did not cause any overt skeletal phenotype in developing zebrafish (data not shown and supplementary material Fig. S2). This might be the result of redundancy of several extracellular Wnt inhibitors. Interestingly, a major site of *Sfrp1* expression is the joint region of the digits, which potentially overlaps with *Wif1* expression (Kawano and Kypta, 2003). Thus, sFrp-1 might substitute for Wif-1 function in these regions, thereby attenuating the deletion phenotype. Coexpression of Wnt antagonists might be a general problem for functional analyses applying gene-knockout strategies. The Wnt antagonists *Dkk1* and *Sfrp2* are both expressed in the lateral plate mesoderm where they might limit the limb- and AER-inducing activity of Wnt2b, Wnt8c and Wnt3a. Along with *Wif-1*, *Dkk1* and *Sfrp2* are also expressed in the developing limb bud (Ladher et al., 2000). Similarly, the secreted Wnt antagonists *Dkk2* and *Dkk3*, similarly to *Wif-1* (Fig. 1E-J), are expressed in the interdigital mesenchyme of developing limbs where they have been proposed to modulate Wnt controlled cell death and survival (Monaghan et al., 1999). Thus, in vitro models to study Wnt signals and their regulation can serve as valuable tools to understand the functions of the individual players of Wnt signalling during skeletal development.

Although under normal conditions, *Wif1*-deficient mice appear unaffected, pivotal roles for Wif-1 might become apparent under pathological conditions, such as malignancies. Thus, several studies have demonstrated epigenetic silencing of *Wif1* in a number of malignancies, and very recently *Wif1*-deficient mice have been shown to be more susceptible to osteosarcomagenesis than control mice (Batra et al., 2006; Chim et al., 2006; Clement et al., 2008; Kansara et al., 2009). Our finding of persistent *Wif1* expression in articular cartilage in adult mice raises the speculation that Wif-1 might also influence degenerative cartilage diseases such as osteoarthritis or rheumatoid arthritis. These diseases result in progressive joint erosions, and recent studies have provided evidence for the involvement of Wnt signals in the progression of associated bone and cartilage destruction (Diarra et al., 2007; Luyten et al., 2009) (for a review, see Sen, 2005).

Although expression of *Wif1* has previously been described in many tissues, this study shows that the major site of *Wif1* expression during vertebrate development is found at margins of forming and persisting cartilage. In these regions Wif-1 might be involved in the fine tuning of Wnt signals essential for normal development and appropriate turnover of cartilage surfaces, including the articular joints. This study gives a concise overview of Wif-1 interactions with cartilage-related Wnt ligands, and furthermore provides evidence for the involvement of Wif-1 in the control of chondrogenesis. Together, this work introduces Wif-1 as a novel player in the network of Wnt signalling controlling skeletal development.

Materials and Methods

Cell lines

Human HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 supplemented with 0.5 U/ml penicillin-streptomycin and 10% fetal calf serum (FCS). 4C3 cells, a subclone of the murine chondrocytic cell line MC615, were cultured in DMEM/Ham's F12 with penicillin-streptomycin and 10% FCS and passaged before reaching confluency (Surmann-Schmitt et al., 2009). HEK293EBNA cells stably transfected with pCEP-Pu derivatives were cultured in DMEM/Ham's F12 with penicillin-streptomycin and 10% FCS containing 250 μg/ml G418 and 0.5 μg/ml puromycin. Murine L cells were cultured in DMEM with 4.5 g/ml glucose, 2 mM L-glutamine, penicillin-streptomycin and 10% FCS. Wnt3a-transfected L cells were cultured in the same medium with the addition of 700 μg/ml G418. L cells and Wnt3a-transfected L cells were kindly provided by Jürgen Behrens (Department of Experimental Medicine II, University of Erlangen, Germany).

Micromass culture of chicken and mouse limb-bud cells

Chicken limb buds were dissected from embryos of Hamburger-Hamilton (HH) stage 23-24 and cells were isolated by sequential digestion at 37°C for 5 minutes in trypsin solution (1 mg/ml trypsin, 0.66 mM EDTA) and for 30 minutes in collagenase P solution [1 mg/ml in DMEM/F-12 with 10% FCS, penicillin-streptomycin, 40 μg/ml insulin, 20 μg/ml holotransferrin, 27 ng/ml sodium selenite (ITS)]. Cells from mouse limb buds were isolated from E11.5 embryos. Limb buds were dissected, and cells were isolated by sequential digestion in trypsin solution on ice for 15 minutes and in collagenase P solution (1 mg/ml in DMEM/F-12 with 10% FCS) at 37°C for 30 minutes.

2.5×10^7 chicken or mouse cells were seeded within a 10 μl drop of medium and incubated at 37°C for 1 hour. After attachment medium (DMEM/F-12, 10% FCS, penicillin-streptomycin, ITS for chicken cells; DMEM/F-12, 10% FCS, 100 μg/ml ascorbate for murine cells) was added and cells were cultured as usual.

Determination of cell growth

Cell growth was measured using a colorimetric assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into its formazan. The protocol used during this work was adapted from Mosmann (Mosmann, 1983). At day 0, 1000 (C3H10T1/2) or 6000 (mouse limb bud) cells per well were seeded in triplicate into 96-well plates. After 2 hours (C3H10T1/2), or at day 1 (mouse limb-bud cells), cells were stimulated with Wif-1 and/or Wnt3a-conditioned medium. At each time point, 10 μl MTT solution (5 mg/ml MTT in PBS) was added to 50 μl medium per well. After incubation for 2 hours at 37°C, cells were lysed in 80 μl DMSO per well and formazan-specific absorption at 550 nm was measured as means of viable cell counts. Reference absorbance was determined at 670 nm.

Preparation of recombinant proteins

For episomal expression of murine recombinant Wif-1, the cDNA sequence (NM_011915) including the complete open reading frame together with C-terminally fused His and FLAG tags was cloned into pCEP-Pu and stably transfected into HEK293EBNA cells. Secreted recombinant Wif-1 was collected from culture supernatants. Recombinant His-tagged Wif-1 was purified by affinity chromatography on nickel-nitriloacetic acid Sepharose (Qiagen) as previously reported (Surmann-Schmitt et al., 2008). Recombinant murine Wnt factors were similarly expressed in HEK293EBNA cells. The ORFs of the following murine Wnt cDNAs, C-terminally fused with hemagglutinin (HA) tags were cloned into pCEP-Pu: Wnt3a (NM_009522), Wnt4 (NM_009523), Wnt5a (NM_009524), Wnt5b (NM_009525), Wnt7a (NM_009527), Wnt7b (NM_009528), Wnt9a (NM_139298), Wnt9b (NM_011719) and Wnt11 (NM_009519). After expression in HEK293EBNA cells, conditioned serum-free medium was collected and Wnt proteins were concentrated by ultrafiltration using an Amicon Ultra 15 centrifugal filter device. Size, integrity and purity of recombinant proteins were analysed by SDS-PAGE followed by Coomassie brilliant blue staining and by immunoblotting with anti-His or anti-HA antibodies for detection of recombinant Wif-1-His-FLAG and Wnt-HA, respectively.

Wnt3a-conditioned medium was collected from L cells stably transfected with Wnt3a (ATCC number CRL-2647) as proposed by ATCC or kindly provided by Kristina Tanneberger (Department of Experimental Medicine II, University of Erlangen, Germany).

Generation of polyclonal rabbit antibodies against Wif-1

Antibodies against Wif-1 were raised by immunisation of a rabbit with 50 μg purified recombinant Wif-1 in complete Freund's adjuvant followed by two booster injections in incomplete adjuvant. The antiserum was purified by chromatography with Wif-1-coupled CNBr Sepharose and tested for specificity by western blotting as previously described (Kirsch and von der Mark, 1991; Schmidl et al., 2006).

RNA in situ hybridisations

Whole-mount RNA in situ hybridisations (WISH) on whole chicken embryos and RNA in situ hybridisation (ISH) on paraffin sections of mouse tissues with digoxigenin-labeled antisense riboprobes for *Wif1* and *Col2a1* and *Col10a1* were performed as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993; Schmidl et al., 2006; Vesque et al., 2000). Specific cDNA fragments for chicken and murine Wif-1 riboprobes including nucleotides 683-941 of the chicken (XM_416072) and nucleotides 1437-1925 of the murine (NM_011915) mRNA sequence, respectively, were obtained by RT-PCR and cloned into the pCRII-TOPO vector

(Invitrogen). Riboprobes for *Wif1* and for murine *Col2a1* and *Col10a1* were prepared as previously reported (Schmidl et al., 2006).

Binding assays and immunoblotting

For coimmunoprecipitation, 2 μg purified recombinant Wif-1 (His-FLAG-tagged) was added to 1 ml concentrated Wnt-HA-conditioned medium and incubated at room temperature for 1 hour. Wif-1-Wnt complexes were immunoprecipitated with 1 μg mouse monoclonal anti-FLAG antibody (Sigma) and Protein-A-Sepharose. precipitates were analysed by western blotting to detect coprecipitated Wnt proteins. For pull-down assays, 50 μl Wif-1-Sepharose (1 mg/ml Wif-1), saturated with 1% BSA, was incubated with 1 ml concentrated Wnt-HA-conditioned medium containing 0.5% BSA. The suspension was incubated overnight at 4°C. After centrifugation, beads were washed three times with 0.1% Tween 20 in PBS. Beads were extracted with PAGE sample buffer and resolved by SDS-PAGE.

Nuclear extracts for investigation of nuclear accumulation of β-catenin were prepared as previously described (Andrews and Faller, 1991; Stock et al., 2004). Briefly, cells were washed in PBS and lysed by incubation for 10 minutes on ice in hypotonic buffer A (10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF, pH 7.9). Afterwards nuclei were spun down and disrupted by incubation in high-salt buffer C (20 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF, pH 7.9) for 20 minutes on ice. Lysates were centrifuged for 2 minutes, and supernatants containing nucleoplasmic proteins were used for western blotting.

For western blot analysis, protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Protein immunodetection was carried out as outlined before using anti-His (Cell Signaling Technology, Danvers, MA), anti-HA (Sigma), anti-Wif-1 and anti-β-catenin (H-102 Santa Cruz Biotechnology) antibodies at a dilution of 1:1000 (Schmidl et al., 2006; Surmann-Schmitt et al., 2008).

Immunofluorescence

Intracellular distribution of β-catenin in 4C3 cells was determined by immunofluorescence using an anti-β-catenin antibody (H-102 Santa Cruz Biotechnology) at a dilution of 1:300. Treated cells were washed with PBS, fixed for 15 minutes at room temperature in 4% paraformaldehyde and permeabilised for 10 minutes at –20°C in methanol. Subsequently, cells were blocked for 1 hour at room temperature in 5% BSA in PBS with 0.3% Triton X-100 (PBS-T) and incubated in primary antibody diluted in PBS-T with 5% BSA overnight at 4°C. After washing with PBS, cells were incubated in secondary antibody (Fluorolink Cy3-labeled goat α-rabbit IgG, dilution 1:800; GE Healthcare, Munich, Germany) in PBS-T with 5% BSA for 1 hour. Fluorescence was detected with a Leica DMRE confocal laser microscope.

Transfection and reporter assays

HEK293 cells were transfected in 24-well plates using 1 μg DNA and 6 μl PEI solution (25 kDa branched polyethylenimine: 7.5 mM monomer, pH 7.3) as reported for linear PEI (Reed et al., 2006). 4C3 cells in 24-well plates were transfected with 1 μg DNA and 4.3 μl ExGen500 (Fermentas) according to the manufacturer's protocol.

Canonical Wnt signalling was analysed using the TOPglow-FOPglow reporter vector system (Millipore, Schwalbach, Germany). Cells were transfected with either TOPglow or FOPglow vector and pL51 (Gebhard et al., 2004) or pCMVβ (BD Clontech) for β-galactosidase-dependent normalisation. The day after transfection, medium was replaced with serum-free medium supplemented with indicated amounts of Wnt3a-conditioned or control-conditioned medium and indicated doses of Wif-1. After 24 hours, cells were lysed in 250 μl lysis buffer [25 mM glycilglycine (Gly-Gly), pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100, 1 mM DTT] by gentle rocking for 15 minutes at room temperature. Luciferase and β-galactosidase assays were performed in triplicate for each sample. Luciferase activities were measured in a plate luminometer at room temperature for 10 seconds after mixing 30 μl of cell lysate with 50 μl of luciferase assay buffer (5.8 mM Gly-Gly, pH 7.8, 9.48 mM $MgSO_4$, 2.52 mM EGTA, 33.6 mM K-phosphate buffer, pH 7.8, 2.8 mM DTT, 7.5 mM ATP) and injection of 40 μl luciferin solution (15 mM Gly-Gly pH 7.8, 9 mM $MgSO_4$, 2.4 mM EGTA, 2 mM DTT, 0.1 mM D-luciferin). β-galactosidase values were determined colorimetrically using ONPG: 30 μl cell lysate was incubated at 37°C with 50 μl β-galactosidase assay buffer (100 mM sodium phosphate buffer, pH 7.5, 1 mM $MgCl_2$, 10 mM KCl, 0.28 % β-mercaptoethanol, 1 mg/ml ONPG) until the reactions appeared yellow. The intensity was quantified using an ELISA reader at 420 nm. Luciferase values were normalised against the respective β-galactosidase values. Means and s.d. of triplicate reactions were plotted.

RT-PCR

For mRNA expression analyses, RT-PCR and real-time RT-PCR were carried out as previously reported (Schmidl et al., 2006; Surmann-Schmitt et al., 2008). Briefly, total RNA from limb-bud micromass cultures was isolated using the RNeasy Kit (Qiagen) including the optional DNase digestion step. cDNA synthesis was performed using the Superscript II reverse transcription system (Invitrogen). The *Taq* PCR Core Kit (Qiagen) was used for PCR and real-time PCR was carried out using the SYBR-Green PCR assay (Thermo Scientific, Hamburg, Germany). Primer sequences are shown in supplementary material Table S1.

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