The Wnt-inducible Transcription Factor Twist1 Inhibits Chondrogenesis*

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Wnt signaling is essential for many developmental processes, including skeletogenesis. To investigate the effects of Wnt signaling during skeletogenesis we studied the effects of Wnt on cultured chondrocytic cells and differentiating limb-bud mesenchyme. We showed that Wnt3a strongly repressed chondrogenesis and chondrocyte gene expression. Canonical Wnt signaling was responsible for the repression of differentiation, as evidenced by results showing that inhibition of glycogen synthase kinase 3 or expression of β-catenin caused similar repression of differentiation. Significantly, we showed that the transcription repressor Twist1 is induced by canonical Wnt signaling. Expression of Twist1 strongly inhibited chondrocyte gene expression and short hairpin RNA knockdown of Twist1 transcript levels caused increased expression of the chondrocyte-specific genes aggrecan and type II collagen. Interestingly, Twist1 interfered with BMP2-induced expression of aggrecan and type II collagen expression and knockdown of Twist1 augmented BMP2-induced aggrecan and type II collagen expression. These data support the conclusions that Twist1 contributes to the repression of chondrogenesis and chondrocyte gene expression resulting from canonical Wnt signaling and that Twist1 interferes with BMP-dependent signaling.

Wnt signaling is divided into canonical and non-canonical pathways. Canonical Wnt signaling regulates the protein levels of β-catenin (βcat). The binding of Wnt ligands to cell-surface receptors leads to the activation of the intracellular protein Dishevelled. When activated, Dishevelled is released from the receptor complex at the cell surface and interacts with the multiprotein complex that controls βcat levels. This complex includes axin, the adenomatous polyposis coli gene product, glycogen synthase kinase 3 (GSK3), βcat, and other proteins (1). Within the complex, phosphorylation of βcat by GSK3 leads to its ubiquitination and degradation by the proteasome. GSK3 is inhibited by activated Dishevelled. This in turn stabilizes βcat, thereby increasing the amount of βcat, which accumulates in the nucleus and regulates gene expression in conjunction with the TCF/LeF family of transcription factors. Thus the canonical Wnt signaling pathway is in part a transcription control pathway that regulates the levels of the transcription co-activator βcat. Non-canonical Wnt signaling pathways are subdivided into the Wnt-calcium and planar cell polarity pathways (2–4). The Wnt-calcium pathway increases intracellular calcium levels in response to Wnt signaling. Like canonical Wnt signaling, this pathway also requires Dishevelled (5). The release of calcium stimulates calcium-dependent kinases and transcription factors, like the nuclear factor of activated T-cells family of transcription factors (6, 7). The planar cell polarity pathway, also linked to Dishevelled activation, requires the Rho family GTPases and Jun kinase. Through this pathway Wnt signaling regulates polarized cell movements.

A hierarchy of Wnt signaling regulates diverse developmental processes. During skeletal development, Wnt signaling regulates a series of developmental steps. The earliest stages of limb initiation require Wnt signaling operating through a regulatory loop with FGF10 (8). This directs the anatomical site for initiation of the limb bud. Later during limb-bud development Wnt signaling initiates and maintains the apical ectodermal ridge and regulates formation of the dorsal-ventral axis (9, 10). As the bone rudiments of the skeleton are formed, Wnt signaling is also essential. Chondrogenesis, one of the earliest steps of bone development, begins as mesenchymal cells aggregate forming the earliest outline of nascent bones. The cell condensations differentiate into chondrocytes thereby forming the template of the endochondral skeleton. Wnt signaling has a vital role during chondrogenesis and chondrocyte differentiation. The effects of Wnt signaling on chondrocytes and chondroprogenitor cells are complex, as demonstrated by results showing both stimulation and inhibition of differentiation. In assays of chondrogenesis using limb-bud mesenchyme, the application of Wnts both stimulates and inhibits differentiation (11–14). Ablation of βcat, and thereby canonical Wnt signaling, in cultured limb mesenchyme stimulates cartilage development (15). Interestingly, however, targeted disruption of βcat signaling in the developing skeleton of mice causes a skeletal dysplasia with repressed chondrocyte differentiation (15–17). In addition, mice expressing a stabilized form of βcat, thereby augmenting canonical Wnt signaling, surprisingly also exhibit a skeletal dysplasia with diminished chondrocyte differentiation (16). The findings indicate that 1) Wnts can both stimulate and inhibit chondrogenesis and 2) overexpression or targeted deletion of βcat has similar consequences on chondrocyte differentiation. This suggests that Wnt signaling regulates different target genes or proteins depending on the context of the cell or stage of differentiation. These targets may also differ depending on whether canonical or non-canonical Wnt signaling is operative. This suggests that understanding the effects of Wnts on chondrocytes will require greater knowledge of the contribution of canonical or non-canonical Wnt pathways and the specific targets of Wnt signaling that regulate differentiation.

To gain insight into the Wnt signaling pathways and targets of Wnt signaling that control chondrogenesis and chondrocyte differentiation, we studied chondrogenesis using cultured limb-bud mesenchyme and the chondrogenic cells ATDC5. We find that Wnt3a represses chondrogenesis and chondrocyte gene expression. The repression of chondrogenesis by Wnt signaling is through canonical Wnt signaling as evidenced by repression resulting from inhibition of GSK3 with the pharmacological antagonist SB216763 or expression of a stabilized form...
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of βcat. Significantly, we identified a transcriptional target of canonical Wnt signaling, Twist1, that repressed chondrocyte gene expression. Accordingly, knockdown of Twist1 expression with shRNA augmented chondrocyte gene expression. Also, expression of Twist1 inhibited chondrocyte gene expression following stimulation with BMP2 and conversely, shRNA knockdown of Twist1 augmented chondrocyte gene expression at low concentrations of BMP2. These data support our conclusion that Twist1 is an important target of Wnt signaling that contributes to the repression of chondrogenesis by canonical Wnt signaling. These data also suggest that Twist1 regulates chondrocyte gene expression through interactions with BMP-dependent pathways.

MATERIALS AND METHODS

Growth Factors—Growth factors and inhibitors were obtained from the following suppliers: SB216763 (Tocris, Ellisville, MO), Wnt3a and BMP2 (R&D Systems, Minneapolis, MN). These materials were solubilized according to the manufacturer’s instructions.

Cell Culture—ATDC5 (RIKEN cell bank) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO2 incubator. Limb bud mesenchyme was prepared from C57Bl/6 mouse embryos at 11.5 days post-coitius and grown as described previously (18).

Western Blotting—ATDC5 cells treated with Wnt3a or SB216763 were harvested in Laemmli sample buffer, sheared by passage through a 25-gauge needle, and 15 μg of total protein was fractionated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 1% BSA (Tropix, Bedford, MA), probed with either anti-β-catenin (Santa Cruz Biotechnology) or anti-actin (Chemicon) followed by a goat anti-mouse-horseradish peroxidase (ICN/Cappel, Aurora, OH) and developed by chemiluminescence (West Dura-extended, Pierce).

Real-time Quantitative PCR—Total RNA was prepared from cells using RNeasy according to the manufacturer’s instructions (Qiagen). 1 μg of total RNA was reverse transcribed as described previously (19). Real-time PCR was done using a SYBR green PCR mix (Applied Biosystems) in an ABI 7000 sequence detection system (Applied Biosystems). The primers used are as follows: ubiquitin, 5’-CGGTCTTCTTCTGTGA-GGTTGTG-3’ and 5’- TCATTGGGCTCCACCTCTA-3’; Twist1, 5’-TCGCGTCCACTAGCA-3’ and 5’-TCTCTGGGAACAATGTAC-ATCTAGT-3’; aggrecan, 5’-GGAGATCCCTAAGCTGCTTG-3’ and 5’-ACTGCAAGATGACACCT-3’; type II collagen, 5’-GAGGATG-GAAAGCAAAGTGTA-3’ and 5’-CATGATACCAGAGGTGCCA-3’; conductin, 5’-AAACGGATCTAAGTCCCAA-3’ and GCTA-GTGGCTGCGTGGATAAC-3’; Twist1, 5’-GACATGCTCA-GCTTGTGGA-3’ and 5’-CTTGTCCACGTGCTCT-3’. The sequence of other primers is available on request.

Transient Transfections—Transient transfections were done using a modified calcium phosphate precipitation method as described previously (20). Cells were transfected in triplicate with a luciferase reporter. Transfection efficiencies were normalized to β-galactosidase activity derived from a co-transfected, constitutively expressed β-galactosidase plasmid. Lysates were assayed as described (21).

Adenovirus—Adenoviral constructs were made as described previously (18). Briefly, myc-epitope-tagged mouse Twist1 cDNA (generously supplied by A. Firulli, Indianapolis, IN) and β-catenin S45A cDNA (generously supplied by D. Rimm, New Haven, CT) were subcloned into the pShuttle vector (BD Bioscience) and then transferred into a replication-deficient, Ad5 genome via a set of unique restriction sites (I-CeuI and PI-SceI) according to the manufacturer’s instructions (BD Bioscience, Palo Alto, CA). Adenoviral supernatants were prepared from infected HEK293 producer cells as described previously (18).

Advection of ATDC5 Cells—5 × 105 ATDC5 cells were plated in a 6-cm dish. The next day, the volume of virus necessary for >90% infection was added to 1 ml of serum-free Dulbecco’s modified Eagle’s medium, and the mixture was briefly vortexed. After addition of 25 μl of 1 M CaCl2 the mixture was briefly vortexed and incubated at room temperature for 20 min. Following removal of the media from the cells, the 1 ml of Dulbecco’s modified Eagle’s medium containing the Ad-calcium phosphate precipitate was added to the dish and incubated for 30 min at 37 °C. Cells were then washed twice with phosphate-buffered saline and Dulbecco’s modified Eagle’s medium, 10% fetal calf serum was added, and cells were incubated at 37 °C for the indicated time period.

RNA Interference—The small interference RNA oligonucleotide against mouse Twist1 was designed as previously described (22). The target sequence is AAGCTGAGCAAGATTACACC. A negative control shRNA oligonucleotide from the BD Knock-out RNAi System, unrelated to Twist1, was used as control. The BD Knock-out RNAi System (BD Biosciences) was used for generating small interference RNAs. Briefly, a double-stranded DNA oligonucleotide containing the shRNA and BamHI and EcoRI overhangs was cloned into the BamHI- and EcoRI-digested RNAi-Ready pSIREN-Shuttle vector, an expression vector designed to express shRNAs using the U6 promoter. The construct was verified by sequencing. ATDC5 cells wherein steady-state Twist1 transcripts were reduced by the shRNA were prepared by stable transfection of pSIREN containing control or Twist1 shRNA and pcDNA3.1 at 1:14 ratio. Stable transfectants were selected in growth medium containing 450 μg/ml G418 (Invitrogen). Individual clones were isolated and assayed for Twist1 expression by real-time quantitative PCR. ATDC5 cells were transiently transfected with shRNA plasmids using Nucleofector (Amaza Inc., Gaithersburg, MD). Briefly, 106 cells were resuspended in 100 μl of Nucleofector solution T and transfected with 5 μg of DNA using Program T-20. Transfection efficiency using these conditions was shown to be >80%. 24 h post transfection cells were treated with BMP2 and harvested 16 h later. RNA was prepared from cells using an RNeasy (Qiagen) kit according to the manufacturer’s instructions.

Statistical Methods—Statistical significance was determined using the Student’s t test. The null hypothesis was rejected for p < 0.05.

RESULTS

To better understand the regulation of chondrogenesis by Wnt signaling, we investigated early steps of chondrocyte differentiation as determined by the regulation of the cartilage-specific genes aggrecan and type II collagen in the chondrogenic cell line ATDC5. Wnt signaling was initiated by adding Wnt3a to the medium or by inhibiting glycogen synthase kinase 3 (GSK3) activity with the pharmacological antagonist SB216763. Fig. 1 (A and B) shows that aggrecan and type II collagen expression was dramatically inhibited by either Wnt3a or the inhibitor of GSK3. Both Wnt3a and the GSK3 inhibitor SB216763 activate canonical Wnt signaling in ATDC5 cells as evidenced by increased levels of βcat (Fig. 1, C and D). Wnt signaling also inhibited chondrogenesis in a limb-bud mesenchyme differentiation assay. Mesenchyme from mouse embryos e11.5 days post-coitus was prepared and plated at high density to initiate differentiation into cartilage. Fig. 1E shows that chondrogenesis is dramatically inhibited by canonical Wnt signaling. Inhibition of GSK3 with SB216763 repressed the formation of cartilage nodules. The inhibition evidenced by the culture morphology was corroborated by gene expression studies showing that aggrecan and collagen type II gene
expression was strongly repressed (Fig. 1F). As expected, the βcat-inducible gene conductin (23) was strongly induced. The expression of type I collagen was unchanged. Significantly, the expression of Wnt5a, Wnt7a, Wnt7b, and Wnt11 diminished as chondrocyte differentiation ensued (Fig. 1G). The expression of Wnt2, -4, -5b, -9a, and -10a was largely unchanged, whereas Wnt8b, -9b, and -10b increased during the time course of the assay (data not shown). The expression levels of Wnt1, -3, -3a, and -8a was not detectable. These data support the view that certain Wnt ligands act to repress differentiation. In particular, Wnt7a, -7b, and -11 have been shown to activate canonical Wnt signaling (24–27). Therefore, the diminished expression of these Wnts may contribute to increased differentiation.

These data showed that inhibition of GSK3 with Wnt3a or the direct-acting antagonist repressed chondrocyte gene expression and chondrogenesis. This suggests that canonical Wnt signaling is primarily responsible for the inhibition. To test this directly, we investigated the effects of a stabilized form of βcat. ATDC5 cells were infected with a recombinant adenovirus containing either a protease-resistant form of βcat (S45A) or β-galactosidase as a control. Consistent with canonical Wnt signaling repressing chondrogenesis, transduction of βcat inhibited aggrecan and
type II collagen expression (Fig. 2A). Additionally, the stabilized form of βcat inhibited cartilage development in the limb-bud chondrogenesis assay (Fig. 2B). From these results we conclude that Wnt signaling represses chondrogenesis through a βcat-dependent pathway.

Canonical Wnt signaling stabilizes βcat resulting in nuclear accumulation of βcat and induction of βcat-responsive genes. Because we observed strong inhibition of aggrecan and collagen expression, we hypothesized that canonical Wnt signaling induced a transcriptional repressor that contributes to the inhibition of chondrogenesis and chondrocyte gene expression. Therefore we searched for transcription repressors that are induced by canonical Wnt signaling in ATDC5 cells. The transcription repressors snail, slug, or engrailed1 were not induced by Wnt signaling, and engrailed2 was only slightly induced (data not shown). Significantly, the transcriptional repressor Twist1 was induced in response to Wnt signaling. Fig. 3A shows that Twist1 was induced following inhibition of GSK3 with SB216763 or Wnt3a. Twist1 expression was also induced following inhibition of GSK3 in limb-bud mesenchyme (Fig. 3B) or following adenoviral transduction of βcat in ATDC5 cells (Fig. 3C). Lastly, we observed diminished expression of Twist1 as cartilage developed in the limb-bud differentiation assay (Fig. 3D). As shown in Fig. 1G, the expression of Wnt7a, -7b, and -11 decreased as differentiation occurred. Because these Wnt can activate canonical Wnt signaling cascades, diminished expression may contribute to the decrease in Twist1 expression. These data support our conclusion that canonical Wnt signaling induces Twist1 and that Twist1 contributes to the repression of chondrogenesis.

We showed that canonical Wnt signaling induces the transcriptional repressor Twist1 and represses chondrogenesis and chondrocyte gene expression. We then hypothesized that Twist1 is responsible for the repression of chondrocyte gene expression produced by canonical Wnt signaling. To examine this, we asked if Twist1 repressed chondrocyte gene expression. ATDC5 cells were infected with a Twist1 adenovirus, and the expression of aggrecan and type II collagen was measured by real-time quantitative PCR. Fig. 4 (A and B) show that Twist1 caused strong repression of both aggrecan and type II collagen, respectively. To further examine the effects of Twist1 on chondrocyte gene expression, we studied the effects of Twist1 on aggrecan or the type II collagen promoters. Co-transfection of Twist1 with luciferase reporters containing promoters from the type II collagen or aggrecan genes caused repression of both promoters (Fig. 4, C and D). The expression of aggrecan and type II collagen is synonymous with the chondrocyte cell lineage. Because Twist1 repressed the expression of both genes this suggested that Twist1 interacts with regulatory pathways that are central to chondrocyte differentiation. BMP signaling is essential for chondrogenesis (28) and the expression of aggrecan and type II collagen. Therefore, we asked if Twist1 interferes with aggrecan and type II collagen expression in response to BMP signaling. Twist1 or β-galactosidase as a control was virally transduced into ATDC5 cells, and thereafter cells were stimulated with 100 ng/ml BMP2. Significantly, we found that Twist1 strongly inhibited the induction of aggrecan and type II collagen by BMP2 (Fig. 4, E and F). Moreover, inhibition of GSK3 with SB216763, and thereby induction of Twist1, also repressed the induction of aggrecan and type II collagen by BMP2 (Fig. 4, G and H).

These data showed that Twist1 is induced by canonical Wnt signaling and that, like canonical Wnt signaling, Twist1 represses chondrocyte gene expression. Because we showed that forced expression of Twist1 repressed chondrocyte gene expression, we hypothesized that knockdown of Twist1 expression by RNA interference would increase chondrocyte gene expression. To knockdown Twist1 expression we stably transfected ATDC5 cells with a Twist1 shRNA expression plasmid. This hairpin RNA effectively reduced Twist1 expression without altering expression of the related bHLH protein Twist2 (data not shown). We obtained clones wherein steady-state levels of Twist1 miRNA were suppressed as determined by quantitative real-time PCR (Fig. 5A). Signifi-
cantly, the expression of aggrecan and type II collagen was dramatically increased in these cells (Fig. 5, B and C). To further study the effects of Twist1 knockdown on aggrecan and type II collagen, we co-transfected ATDC5 cells with luciferase reporter plasmids containing the collagen II or aggrecan promoters together with the Twist1 shRNA expression plasmid. Consistent with Twist1 acting to repress chondrocyte gene expression, we observed that Twist1 knockdown augments the activity of both the aggrecan and collagen II promoters (Fig. 5, D and E).

Knockdown of Twist1 expression in stably transfected cells resulted in increased expression of aggrecan and type II collagen (Fig. 5, B and C). Interestingly, transient knockdown of Twist1 by transient transfection of the shRNA expression vector did not alter the steady-state mRNA levels of type II collagen or aggrecan expression (data not shown). We therefore reasoned that the effects of Twist1 on aggrecan and collagen
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FIGURE 6. Increased response to BMP2 following knockdown of Twist1. Real-time quantitative PCR determination of aggrecan (A) or type II collagen (B) with RNA prepared from ATDC5 cells transfected with control or Twist1 shRNA expression plasmid and treated with 25 ng/ml BMP2 for 16 h. C. Quantitative PCR determination of Twist1 after transfection with control or Twist1 shRNA expression plasmid. Expression levels determined by quantitative PCR were normalized to an unaffected control, ubiquitin. Error bars represent ± S.E. The results show representative results of three independent experiments.

expression are indirect and require a longer period of time to integrate the effects of Twist1 knockdown (as expected following stable knockdown of Twist1). Consistent with an indirect effect, we showed that Twist1 interfered with the induction of aggrecan and type II collagen by BMP2. We then hypothesized that transient knockdown of Twist1 expression with the shRNA may augment aggrecan and type II collagen expression at a submaximal dose of BMP2. To test this, we transiently knocked down Twist1 levels by transient transfection of the shRNA expression plasmids and stimulated the cells with 25 ng/ml BMP2. We observed that this concentration of BMP2 only slightly affected aggrecan and type II collagen expression in control cells (Fig. 6, A and B). However, treatment of cells with this concentration of BMP following transient knockdown of Twist1 caused significant increases in both aggrecan and collagen II expression (Fig. 6, A and B). The knockdown of Twist1 expression was validated by real-time quantitative PCR (Fig. 6C).

DISCUSSION

Our data show that Wnt signaling represses chondrogenesis and chondrocyte gene expression. These data corroborate other results showing that Wnt signaling represses chondrocyte differentiation (14, 15, 29, 30). Also, while the manuscript for this report was under review additional reports were published showing that Wnt signaling has inhibitory effects on chondrocyte development from immature precursors (31, 32). Additional data however show that Wnt signaling stimulates chondrocyte differentiation (13). For instance, Wnt5a and -5b stimulate chondrogenesis in limb-bud chondrogenesis assays (11) and undifferentiated mesenchymal cells adopt the chondrocyte phenotype when stimulated with Wnt3a (33). In addition, Wnt5a null mice exhibit delayed chondrocyte differentiation, suggesting that Wnt signaling stimulates differentiation. Because of the complexity of Wnt signaling, it is possible that conflicting effects of Wnt signaling are a consequence of canonical versus non-canonical Wnt signaling. To investigate this we performed experiments that only stimulate canonical Wnt signaling. Non-canonical Wnt signaling pathways require activation of Dishevelled (34). Therefore, experiments activating Wnt signaling downstream of Dishevelled will only activate canonical Wnt signaling. GSK3 is biochemically and genetically downstream of Dishevelled in the Wnt signaling cascade, thus our experiments using the GSK3 inhibitor stimulate canonical (βcat), but not non-canonical Wnt signaling pathways. Additionally, we showed that βcat fully repressed the repression of chondrogenesis caused by Wnt signaling. These effects of βcat are observed in both confluent and non-confluent cultures, indicating that the effects of βcat are not due to augmented assembly of intercellular junctions. Therefore, we conclude that canonical Wnt signaling strongly represses chondrogenesis and speculate that stimulation of chondrogenesis by Wnt signaling observed in other studies is a consequence of non-canonical signaling pathways. In support of this are data showing that Wnt5α can activate non-canonical signaling and repress canonical Wnt signaling (35, 36).

We showed that βcat repressed chondrogenesis in limb-bud mesenchyme and that expression of aggrecan and type II collagen in ATDC5 cells was repressed by βcat. Recent data shows that βcat binds the chondrogenic transcription factor Sox9 and facilitates Sox9 degradation (16). Based on these data we predict that βcat directly represses the type II collagen promoter, because this promoter is directly regulated by Sox9 (37, 38). However, cotransfection of βcat did not repress the transcriptional activity of the type II collagen or aggrecan promoters (data not shown). Given that βcat stimulates transcription in conjunction with several transcription regulators (39–41), this result is not wholly unexpected. We therefore concluded that βcat indirectly inhibited chondrocyte gene expression and sought to identify targets of canonical Wnt signaling that contribute to the repression of chondrocyte gene expression. Because the expression of type II collagen and aggrecan was strongly suppressed when βcat was transduced into ATDC5 cells, we hypothesized that canonical Wnt signaling induced the expression of a transcription repressor. Significantly, the repressor Twist1 was induced by canonical Wnt signaling and Twist1 expression diminished in parallel with the expression of certain Wnt family members. In fact, recent results show that the Twist1 gene promoter is directly activated by canonical Wnt signaling and the TCF/Lef transcription factors (42). Twist1 is an important regulator of skeletal development (43, 44). Consequently, we considered Twist1 a likely mediator of the inhibitory activity of Wnt signaling on chondrogenesis and chondrocyte gene expression. During skeletal development Twist1 is expressed in regions of the limb-bud mesenchyme overlapping sites of Wnt signaling (45). Thus, Twist1 is induced by Wnt signaling and is expressed in limb-bud chondroprogenitor cells where chondrocyte differentiation is actively repressed.

Expression of Twist1 in ATDC5 cells caused dramatic repression of aggrecan and collagen II expression. Also, knockdown of Twist1 expression using shRNA targeted to Twist1 up-regulated aggrecan and collagen II expression. These data support our conclusion that Twist1 is an essential inhibitor of chondrocyte development. We then wished to determine if Twist1 is essential for the inhibition of chondrocyte gene expression caused by canonical Wnt signaling. Using cells wherein Twist1 levels were diminished by shRNA, we asked if the inhibition of aggrecan and collagen II resulting from GSK3 inhibition is blocked. That is, when Twist1 levels are diminished, is the repression of aggrecan and collagen II expression caused by Wnt signaling similarly dimin-
ished? When the GSK3 inhibitor was applied to cells with reduced levels of Twist1, a similar degree of aggrecan and collagen II repression was observed as in the control ATDC5 cells (data not shown). These results suggest that Twist1 is not required for the repression of aggrecan and collagen II resulting from the inhibition of GSK3. However, we observed that Twist1 was induced equivalently following GSK3 inhibition, regardless of the presence of the Twist1 shRNA (data not shown). Thus, the knockdown of Twist1 expression effectively reduced steady-state levels of Twist1 transcript but did not reduce Twist1 induction. We hypothesize that the induction of Twist1 by canonical Wnt signaling is sufficient to inhibit aggrecan and collagen II expression. However, there may be other transcription regulators that are induced by Wnt signaling that also contribute to the inhibition of chondrocyte gene expression. Further understanding of the relationship of Twist1 to the repression of aggrecan and type II collagen expression following Wnt signaling will require cells such as Twist1 null cells, where Twist1 cannot be induced. Twist1 null mice die early during development, prior to the development of cartilage (46). Hence, chondrocytes that lack Twist1 are currently unavailable.

How does Twist1 repress chondrocyte gene expression? Our data showed that Twist1 strongly suppressed chondrocyte gene expression. There are several possible ways that Twist1 may inhibit chondrocyte gene expression. These possibilities are non-exclusive and include inhibition of transcription factors that promote chondrocyte gene expression or inhibition of co-factors that support chondrocyte gene expression. Interestingly, published data show that Twist1 binds to and inhibits the histone acetylase activity of p300 (47). p300 is an essential co-activator of transcription, and therefore it is possible that repression of p300 by Twist1 contributes to the inhibition of chondrocyte genes like collagen II and aggrecan. A recent report shows that p300 associates with Sox9 and promotes type II collagen expression (48). In addition, the histone deacetylase Hdac4 represses chondrocyte differentiation (49). These results implicate the histone acetylation/deacetylation cycle as essential for chondrocyte differentiation. If Twist1 inhibited type II collagen expression solely by repressing p300, then we predict that overexpression of p300 would reverse the effects of Twist1. However, we could not completely reverse the inhibition of the type II collagen promoter by Twist1 when 1) p300 was overexpressed or 2) histone deacetylases were inhibited with trichostatin A (data not shown). Also, trichostatin A did not prevent the inhibition of aggrecan and type II collagen expression resulting from transduction of a Twist1 into ATDC5 cells. From this we conclude that Twist1 does not repress chondrogenesis entirely by inhibiting histone acetyltransferase activity.

In addition to inhibiting the co-activator p300, Twist1 can target and repress specific transcription factors. Twist1 interacts with the p65 subunit of NFκB and thereby represses the activation of an NFκB reporter construct (50). Certain studies demonstrate that NFκB represses chondrogenesis or chondrocyte gene expression (51). In this circumstance we expect that the repression of NFκB by Twist will lead to the stimulation rather than repression of chondrogenesis. However, other data show that NFκB stimulates BMP2 expression through NFκB binding sites in the BMP2 promoter (52). Because BMP2 is a powerful promoter of chondrocyte gene expression, NFκB may promote chondrogenesis by stimulating BMP2 gene expression. If Twist1 blocks type II collagen and aggrecan gene expression by blocking NFκB-dependent expression of BMP2, then addition of BMP2 should complement or reverse the effects of Twist1. This was not observed. The expression of type II collagen and aggrecan was repressed by Twist1 even following addition of BMP2. In addition, expression of a protease-resistant form of NFκB inhibitor, 1κB, in ATDC5 cells did not inhibit aggrecan or type II collagen expression (data not shown). These data suggest that NFκB is not a principle regulator of chondrocyte gene expression in these culture systems.

Other targets of Twist1 include Runx2 and Mef2. The C-terminal domain of Twist1 binds to and inhibits 1) the osteoblast specific transcription factor Runx2, resulting in impaired osteoblast differentiation (43) and 2) the myogenic transcription factor Mef2, resulting in impaired myoblast differentiation (53). Runx2 also contributes to chondrocyte differentiation and perhaps repression of Runx2 by Twist1 inhibits chondrocyte differentiation; however, Runx2 null mice develop an endochondral skeleton, suggesting that chondrogenesis occurs normally in the absence of Runx2. Therefore, if Twist1 regulates chondrocyte differentiation through Runx2 repression, it probably determines late rather than early stages of chondrocyte differentiation. Twist1 may also regulate chondrogenesis and chondrocyte gene expression by dimerizing with other bHLH transcription factors. Interestingly, recent data show that Twist1 forms heterodimers with the bHLH transcription factors Hand2 or Hand1 in response to specific phosphorylation events (54). These data show that Hand and Twist1 have compensatory interactions that contribute to skeletal patterning during limb development. Induction of Twist1 expression by canonical Wnt signaling may alter the dimer equilibrium for certain bHLH transcription factors that are necessary for chondrogenesis.

Twist1 has the intriguing capacity to bind to co-activators and transcription factors both within and outside the bHLH family. This suggests that Twist1 is structured to perform different tasks depending on the demands of the cell. This is supported by the various events, including cell survival (55–57), cell death (50, 58), epithelial-mesenchymal transition (22), and mesenchymal cell differentiation (43, 45, 46, 53, 59) that are ascribed to Twist1 function. It is interesting to note that Twist1 targets different transcription factors in different cell lineages; Runx2 in osteoblasts and Mef2 in myoblasts. Our data show that Twist1 strongly suppresses the chondrocyte lineage. We speculate that this repression is exerted through a transcriptional pathway that is central to chondrocyte differentiation. Twist1 repressed the effect of BMP2 on chondrocyte gene expression and conversely knockdown of Twist1 facilitated the stimulation of chondrocyte gene expression by BMP2. This suggests that Twist1 inhibits transmission of BMP signaling in or to the nucleus. Further experiments are required to determine how Twist1 interferes with BMP action during chondrocyte differentiation.

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