Wnt-3A Enhances BMP-2 Mediated Chondrogenesis of Murine C3H10T1/2 Mesenchymal Cells

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SUMMARY

We have recently reported the chondrogenic effect of bone morphogenetic protein–2 (BMP-2) in high-density cultures of the mouse multipotent mesenchymal C3H10T1/2 cell line, and have shown functional requirement of the cell-cell adhesion molecule, N-cadherin, in BMP-2 induced chondrogenesis in vitro (Denker et al., Differentiation 59, 25-34, 1995; Haas and Tuan, Differentiation 64, 77-89, 1999). Furthermore, BMP-2 treatment also results in increased protein level of β-catenin, a known N-cadherin associated, Wnt signal transducer (Fischer et al., Signal Transd. 2: 66-78, 2001), suggesting functional crosstalk between the BMP-2 and Wnt signaling pathways. We have previously observed that BMP-2 treatment up-regulates expression of Wnt-3A in high-density cultures of C3H10T1/2 cells. To assess the contribution of Wnt-3A to BMP-2 mediated chondrogenesis, we have generated C3H10T1/2 cell lines over-expressing Wnt-3A and various forms of glycogen synthase kinase–3β (GSK-3β), an immediate cytosolic component of the Wnt signaling pathway, and examined their response to BMP-2. We show that over-expression of either Wnt-3A or kinase-dead GSK–3β enhances BMP-2 mediated chondrogenesis. Furthermore, Wnt-3A over-expression results in decreases in both N-cadherin and GSK-3β protein levels, while Wnt-3A as well as kinase-dead GSK-3β over-expression increase total and nuclear levels of both β-catenin and LEF-1. Direct cross-talk between Wnts and BMP-2 was also indicated by the up-regulated interaction between β-catenin and SMAD-4 in response to BMP-2. These results suggest that Wnt-3A acts in a manner opposite to that of other
Wnts, such as Wnt-7A, which were previously identified as inhibitory to chondrogenesis, and is the first BMP-2 regulated, chondrogenesis-enhancing member of the Wnt family.
INTRODUCTION

Cellular condensation is a requisite step of mesenchymal chondrogenesis, the first differentiation event of endochondral skeletal development, since perturbation of cell-cell contact abrogates chondrogenesis both in vitro and in vivo (1, 2). We have previously shown that in the developing limb bud, N-cadherin, a calcium-dependent homotypic adhesion molecule, is expressed in a manner that correlates spatiotemporally with cellular condensation (3, 4). Furthermore, N-cadherin is functionally required in developmental chondrogenesis (3, 4) as well as the induction of chondrogenesis by bone morphogenetic protein-2 (BMP-2) (5). Finally, we have shown that the chondrogenic modulation of N-cadherin is accompanied by protection of β-catenin protein levels (6).

N-Cadherin mediates cell-cell adhesion via homotypic interactions and cell-cytoskeletal adhesion via regulation of the catenin cytosolic complex (7, 8). Catenins interact hierarchically with N-cadherin: β-catenin binds the cytoplasmic tail of N-cadherin, and is subsequently bound by α-catenin which anchors the complex to the actin cytoskeleton (9, 10). β-Catenin is also found cytoplasmically interacting with several molecules including glycogen synthase kinase-3β (GSK-3β), the adenomatous polyposis tumor suppressor protein (APC), lymphoid enhancing factor/T-cell factor (LEF-1/TCF), subunits of protein phosphatase 2A (PP2A), and the product of the mouse fused locus, axin (11 - 16).

The Wnt family consists of a number of small, cysteine-rich, secreted glycoproteins involved in embryonic development, tissue induction, and axial polarity (17, 18). According to the current mechanistic model of canonical Wnt action, in cells
lacking Wnt signal, GSK-3β phosphorylates β-catenin inducing rapid degradation of β-catenin via the ubiquitin/proteasome pathway (19, 20). In the presence of Wnt, Disheveled (Dsh) through a PKC mechanism inhibits GSK-3β kinase activity, β-catenin is protected from proteasome-mediated degradation. β-Catenin instead translocates to the nucleus, where it functions in a transcription complex in association with LEF-1/TCFs (reviewed in 21). In recent studies, we have observed increased β-catenin and LEF-1 protein levels as well as their interaction during BMP-2 stimulated chondrogenesis of mesenchymal cells in vitro (22), suggesting a possible regulatory role for Wnts during chondrogenesis.

A number of Wnt genes are expressed in the developing limb (18, 23), including Wnt-5A in distal mesenchyme, Wnt-7A in dorsal ectoderm, and Wnt-3A in mouse apical ectodermal ridge (AER) (24, 25). Wnt-7A has been shown to inhibit chondrogenesis in vitro (25, 26), and Wnt-4, localized to developing joint regions (28), has been implicated in chondrocyte differentiation (29). Wnt-3A has been shown to enhance bmp-2 expression (25), and wnt-3A and lef1 deficient mice both display similar phenotypes, with the latter developing limb deformities (30). In addition, Wnt-3A is able to induce cytoskeletal reorganization (31), an event previously shown to be critical for the requisite cell shape change that occurs during mesenchymal chondrogenesis (32, 33). It is noteworthy that in the developing limb, Wnt-7A and Wnt-5A have been found to act through a pathway other than β-catenin/LEF-1, whereas Wnt-3A and Wnt-4 appear to specifically utilize β-catenin and LEF-1 in their signal (25, 29). Interestingly, recent evidence has shown that a combination of Frizzled (Frz) receptor specificity and Dsh acting as a molecular switch can transduce a Wnt signal into both β-catenin pathways and
Jun N-terminal kinase (JNK) regulation separately or concomitantly (34-36). Wnts have been classified into the Wnt-1 class, which functions through β-catenin/LEF, and the Wnt-5A class, which acts via the modulation of intracellular calcium levels (14). It has been postulated that the two classes of Wnts may act antagonistically when present together (37).

Our laboratory has established high density micromass cultures of the murine multipotent cell line, C3H10T1/2, as an experimental model of mesenchymal chondrogenesis under the stimulation of the chondro-inductive factor, BMP-2 (5, 38). We have observed that BMP-2 induced chondrogenesis in C3H10T1/2 cells is accompanied by downregulation of Wnt-7A and upregulation of Wnt-3A, while the level of other Wnts, including Wnt-3 and Wnt-5A, remains unchanged (22). This observation raises the intriguing possibility that BMP-2 mediated mesenchymal chondrogenesis results from the regulated, antagonistic relationship between the chondro-inhibitory Wnts, such as Wnt-7A (26, 27), and chondro-enhancing Wnts. Such a scenario in the developing vertebrate limb would thus bear some analogy to the interactive relationship between the actions of decapentaplegic (Dpp; a BMP homolog) and Wingless (Wg; a Wnt homolog) that synergistically regulate a number of developmental and patterning events in Drosophila (39, 44). Consistent with this mechanistic scheme is the recent evidence suggesting direct crosstalk between TGF-βs and Wnts through interaction of SMAD-4 and β-catenin (44), as well as SMAD-3 and LEF-1 (46).

The central hypothesis of this study is that Wnt-3A is a candidate as a BMP-2 up-regulated chondro-enhancing Wnt. To test this hypothesis, we have investigated whether BMP-2 induced chondrogenesis in high-density cultures of C3H10T1/2 cells can be
altered by changes in Wnt-3A expression and signaling. Specifically, we tested the effects of stable over-expression of Wnt-3A, wild-type GSK-3β, or kinase-dead GSK-3β on the level of chondrogenesis, N-cadherin expression, and the subcellular distribution of β-catenin and LEF-1. Our results suggest that Wnt-3A plays a positive role in mesenchymal chondrogenic differentiation, specifically by enhancing BMP-2 induced chondrogenesis through regulation of events important for both cellular adhesion and nuclear transcriptional modification.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*

For routine cellular maintenance, C3H10T1/2 cells were plated as monolayer cultures and maintained in Dulbecco's Modified Eagle’s Medium (DMEM; Gibco BRL) containing 10% fetal bovine serum (FBS; Hyclone). For micromass culturing, the technique of Ahrens et al. (2) was modified as described previously (47), using 10 µl drops of cells at 10⁷ cells/ml. Micromass cultures of both clonally derived Wnt-3A and GSK-3β over-expressors and parental C3H10T1/2 cells were maintained in Ham's F12 medium (GIBCO BRL) containing 10% FBS with or without 100 ng/ml recombinant human BMP-2 (generous gift of Genetics Institute, Inc.). Medium was changed every three days.

*Assays for Chondrogenesis*

Sodium [³⁵S]sulfate (5 µCi/ml) and [³H]leucine (1 µCi/ml), both from DuPont NEN, were added to micromass cultures for 24 hours prior to the desired time point to
estimate sulfate incorporation into newly synthesized proteoglycans and total protein synthesis, respectively. Radioactivity incorporation was measured by liquid scintillation counting (48). Parallel cultures were fixed with Kahle's fixative and stained with 1% Alcian blue 8-GX, pH 1.0 overnight (Sigma).

Production of Stably Transfected C3H10T1/2 Clone Cell Lines:

cDNA constructs included: 1) mouse GSK-3β full-length cDNA and mouse kinase-dead GSK-3β (ΔGSK-3β) cDNA, both under the control of CMV promoter (gifts from Dr. Paul Polakis, Onyx Pharmaceuticals, CA) (15); and 2) the 3.1 kb mouse Wnt-3A full-length cDNA in pGEM 3ZF (gift from Dr. H. Roelink, Stanford University, San Francisco, CA) (24) subcloned 5’ EcoR1 to 3’ Xba1 into pCANMyc (pCDNA3 with an added myc tag, developed by Dr. Polakis, Onyx Pharmaceuticals CA), under the control of the CMV promoter.

Stable Transfections Passage 10 C3H10T1/2 cells obtained from ATTC were plated one day prior to transfection in complete DMEM at a density of 1.5 x 10^5 cells per 30 mm dish. Transfection was carried out per manufacturer’s specifications using Superfect Transfection reagent (Qiagen). The Wnt-3A, wild-type GSK-3β or ΔGSK-3β, or for control, empty pCMV expression constructs were added with the carrier plasmid, pSV2NEO, at a ratio of 4:1. Two days following transfection, cells were split into 100 mm dishes at a density of 10^5 cells/dish and fed with 10 ml of complete DMEM containing Neomycin/Geneticin (G418) at 50 µg/ml. After approximately 14 days in selective medium, cloning cylinders dipped in sterile vacuum grease were placed around
individual colonies, and 0.25 ml of 0.1% trypsin/EDTA was added to dissociate colonies from the plates. Cells were plated in 75 cm$^2$ flasks for expansion and fed with DMEM containing G418 at 50 µg/ml.

**RNA Isolation and Reverse Transcription –Polymerase Chain Reaction (RT-PCR)**

The following RT-PCR primers (forward and backward, 5’ to 3’) were used: 1) mouse Wnt-3A - AACC ACG GGA GCA GGG TTC ATT C 3 and AAG GGG GTC TCC AAA AGT TCC ACC to amplify a 534 bp region within the 3’ untranslated region of mouse Wnt-3A; and for the purpose of normalizing mRNA load 2) mouse GAPDH - CCA CCC ATG GCA AAT TCC ATG GCA and TCT AGA CGG CAG GTC AGG TCC ACC to amplify a 600 bp region of mouse GAPDH.

Total cellular RNA was isolated from micromass cultures or Day 10.5 mouse limb buds using TRI-REAGENT RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc.), and reverse transcribed using oligo dT primers with the Superscript Amplification System (Gibco BRL) as per the manufacturer’s instructions. PCR was carried out in a volume of 50 µl using 25 cycles of 1 minute at 94°C, 2 minutes at 60.5°C, and 3 minutes at 72°C. Products of the RT-PCR were analyzed by electrophoresis on a 1.5 % TAE agarose gel stained with ethidium bromide, and band intensities quantified using a Kodak Digital Science Image Station (Model 440 CF).

**Protein Isolation and Immunoblot Analysis**

Cultures were harvested at Days 1, 5, 9, and 13 and extracted at 4°C in Tris-buffered saline (50 mM Tris-HCl, pH 7.6, 275 mM NaCl, and 5.5 mM KCl) containing
1% Triton X-100, 1% Nonidet P-40, 1.0 mM CaCl₂, and protease inhibitors [phenylmethylsulfonylfluoride (PMSF), benzamidine HCl, amino-caproic acid, and pepstatin A; Sigma]. Extracts were cleared by centrifugation and protein concentrations determined (MicroBCA, Pierce). Mouse thymus, used as a control, was similarly extracted. Nuclear and cytoplasmic extracts were prepared by the method of Schreiber et al. (49).

For Western analysis, 20 µg of each protein sample was separated by (SDS-PAGE) (6-10%), and electro-transferred onto nitrocellulose membranes (0.2 µm, Schleicher & Schuell). The blots were blocked with buffer containing Tween 20 (0.05%) and bovine serum albumin (BSA, 3%), and reacted sequentially with primary and secondary antibodies (see below): a) primary antibodies - 13A9 at 1:200, β-catenin and SMAD 4 at 1:500, LEF-1 at 1:3000, GSK-3β at 1:3800, for normalization of protein loading, β-actin at 1:10,000 and b) alkaline phosphatase conjugated secondary antibodies (Sigma) at 1:3800 dilution. Blots were developed with BCIP/NBT (Zymed), and densitometrically scanned as described above. Negative controls for all immunoblots consisted of a lane of total cell extracts immunoprobed with secondary antibodies in the absence of primary antibody. Either Ponceau S staining of immunoblots or parallel Coomassie Brilliant Blue stained gels confirmed equal protein loading.

Immunoprecipitation  Protein extracts were incubated with the indicated antibodies in 1 ml of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM CaCl₂, 1% Triton X-100, 0.5% Nonidet P-40, containing protease inhibitors) for 1 hr at 4°C with agitation. Immunoprecipitation was done by adding 30 µl of Protein G Agarose beads (50% suspension; Gibco BRL) followed by a 45 min
incubation at 4°C. After washing, the resin was resuspended into SDS-PAGE electrophoresis buffer, boiled, centrifuged, and the supernatant fractionated by SDS-PAGE. Electro-blotting and Western analysis were performed as described above. Positive controls for all immunoprecipitation experiments consisted of a lane of total cell extract immunoprobed for the protein(s) of interest.

**Monoclonal Antibodies** The N-cadherin monoclonal antibody, 13A9, which recognizes the intracellular C-terminal domain of human N-cadherin, was the generous gift of Dr. Karen Knudsen (Lankenau Medical Research Center, Wynnewood, PA; 49). Monoclonal antibody to β-catenin and GSK-3β were purchased from Transduction Labs. Rabbit antibodies to mouse LEF-1 was a generous gift from Dr. R. Grosschedl, University of Munich, Germany (16). Monoclonal antibodies to SMAD-4 and anti-myc clone 9E10 were purchased from Santa Cruz Biochemicals. Monoclonal antibody to mouse β-actin was purchased from Sigma Chemicals.

**In vitro GSK-3β Kinase Assay**

Micromass cultures were harvested in 20 mM Tris-HCl, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.5% Triton-X 100 with protease inhibitors as well as 0.5 mM NaVO₃ and 1 nM okadaic acid, and extracted at 4°C for 20 minutes. Extracts were cleared of membranes by centrifugation at top speed in a microfuge and protein concentration determined. Fifteen µg aliquots of the total lysates were subjected to Western blot analysis and Ponceau S staining to verify loading. Another 100 µg aliquot of the total lysates was immunoprecipitated with antibody to GSK-3β. The immune complex was then washed in 50 mM Tris-HCl pH 7.5 with 1 mM dithiothreitol and
resuspended in 45 µl of kinase reaction buffer containing 200 µM ATP, 4.5 µl 10 X GSK-3β kinase buffer (NEB), 100 µM [γ-32P]ATP (0.25 µCi/ml) (DuPont NEN) with or without (as negative control) 60 µM phospho-CREB peptide or 0.5 Units rabbit GSK-3β (as positive control). Kinase reactions were carried out for 15 minutes at 30°C and stopped with 1.5 % phosphoric acid. 32P-labeled reactions were spotted on p81 Whatman paper and washed 3 x 5 min with 0.5% phosphoric acid. Filters were air-dried, placed right side up in scintillation vials with 400 µl of 0.2 N NaOH overnight at room temperature, and radioactivity of the CREB peptide determined by liquid scintillation counting.

RESULTS

Over-expression of Wnt Signaling Components on BMP-2 Induced Chondrogenesis

Stable, Wnt-3A transfected clones were analyzed for Wnt-3A over-expression. Both Western immunoblot (Fig. 1A) and RT-PCR (Fig. 1B) analyses revealed the presence of Wnt-3A at protein and mRNA levels, respectively. Myc-tagged Wnt-3A protein was detected in monolayer cultures of several clones examined (Fig. 1A), and increased mRNA was also detected in over-expressing Wnt-3A cell lines (clones 2 and 6 shown; Fig. 1B). We next examined over-expression of GSK-3β in C3H10T1/2 micromass at the protein level using the myc-tag as an identifier of exogenously introduced GSK-3β. A significant level of expression of both exogenously introduced wild-type and kinase-dead ΔGSK-3β was detected, with myc-reactive proteins migrating
at the expected 45 kDa size, similar to the endogenous product, detected using anti-GSK-3β antibody, in untransfected parental cells (Fig. 1C).

Effect of Over-expression of Wnt Signaling Components on BMP-2 Induced Chondrogenesis

We next examined the effect of stable over-expression of Wnt-3A and GSK-3β on BMP-2 induction of chondrogenesis in micromass cultures of C3H10T1/2 cells. On the basis of Alcian blue staining (Fig. 2A) and metabolic sulfate incorporation into sulfated proteoglycans (Fig. 2B), Wnt-3A over-expressing cultures were found to be significantly more responsive to BMP-2 in terms of chondrogenesis at all time points examined (Days 5, 9, and 13). It is interesting to note that Wnt-3A over-expression alone was insufficient to induce chondrogenesis in the micromass cultures, i.e. BMP-2 treatment was still required for the induction of chondrogenesis. In the case of GSK-3β, it appeared that increased levels of wild-type GSK-3β negatively affected BMP-2 induced chondrogenesis, while expression of ΔGSK-3β contributed positively to the effect of BMP-2 on C3H10T1/2 micromass cultures (Fig. 3A, B), during early culture period (before Day 9). As controls, C3H10T1/2 cells were transfected with empty pCMV vector, and showed no difference from non-transfected parental micromass cultures with respect to BMP-2 treatment.

Wnt-3A Over-expression Inhibits GSK-3β Kinase Activity

To assess whether Wnt-3A over-expression regulates GSK-3β enzymatic activity in C3H10T1/2 micromass cultures, we employed a kinase assay using GSK-3β
immunoprecipitates from both parental and Wnt-3A over-expressing micromass cultures stimulated by BMP-2. As shown in Figure 4, GSK-3β kinase activity was lower in Wnt-3A over-expressing micromass cultures at all time points. Inhibition of GSK-3β activity in Wnt-3A expressing cultures was greatest at Day 5 of culture (20.8% of parental micromass GSK-3β activity), compared to 41.1% at Day 9 and 58.6% at Day 13. When GSK-3β enzyme activity was normalized to levels of GSK-3β protein in parental and Wnt-3A expressing micromass cultures (Fig. 4B), it is clear that over-expression of Wnt-3A decreased the specific activity of GSK-3β, suggesting that GSK-3β biosynthesis was similarly affected (also see Fig. 5).

**Wnt-3A Over-expression Affects N-Cadherin, β-Catenin, LEF-1 and GSK-3β**

Both GSK-3β and N-cadherin protein levels (Fig. 5A) were decreased in Wnt-3A-over-expressing cultures regardless of BMP-2 treatment at all time points in comparison to parental cultures. On the other hand, both parental and Wnt-3A transfected micromass cultures displayed increased β-catenin protein (total and nuclear) in response to BMP-2 treatment on Days 5 through 13 (Figs. 5A,B). However, in Wnt-3A over-expressing micromass cultures from Days 5 through 13, both total β-catenin level (Fig. 5A) and the degree of nuclearization of β-catenin (Fig. 5B) were greater than in non-transfected parental cultures.

In view of our recent observation that BMP-2 treatment of C3H10T1/2 micromass cultures upregulates LEF-1 (21a), we examined whether the effect of Wnt-3A over-expression functioned through regulation of LEF-1. As shown in Figure 6, BMP-2 treatment of clonally derived Wnt-3A-over-expressing micromass cultures increased the
nuclear levels of LEF-1 protein over that of non-transfected parental cultures on Days 5-13 (Fig. 6A). By means of co-immunoprecipitation analysis, the level of BMP-2 induced association of β-catenin with LEF-1 was slightly decreased in Wnt-3A over-expressing cultures during Days 9-13, compared to parental controls (Fig. 6B); however, the effects of Wnt-3A were not remarkable.

*Over-expression of Wild-Type and ΔGSK-3β Regulates N-Cadherin and β−Catenin Levels*

Since both β-catenin and N-cadherin were responsive to Wnt-3A over-expression, suggesting Wnt regulation, we analyzed changes in the protein levels of these molecules in response to wild-type and ΔGSK-3β over-expression. As shown in Figure 7, BMP-2 increases N-cadherin and β-catenin protein levels on Days 5-13 in parental non-transfected cultures. In comparison, over-expression of ΔGSK-3β in the presence of BMP-2 dramatically increased the levels of both N-cadherin and β-catenin early (up to Day 5) with little effect thereafter (Figure 7). It is noteworthy that over-expression of ΔGSK-3β also resulted in increased protein levels of N-cadherin and β-catenin in the absence of BMP-2, while parental non-transfected cultures required BMP-2 treatment for such increases. Furthermore, over-expression of wild-type GSK-3β, while not remarkably affecting levels of N-cadherin or β-catenin during early (Days 1-5) culture, decreased the levels of both molecules during later time points of chondrogenesis (Days 9-13).

*Over-expression of Wild-type and ΔGSK-3β Regulates Nuclear LEF-1*
Since inhibition of GSK-3β activity has been shown to result in increased levels of LEF-1 and β-catenin protein (51, 52, 53), and we have shown that Wnt-3A increases LEF-1 nuclearization, we analyzed the effect of over-expression of both wild-type and kinase-dead GSK-3β on nuclear LEF-1 and β-catenin levels in BMP-2 stimulated micromass cultures. As shown in Figure 8A, while wild-type GSK-3β expression had little effect on nuclear levels of β-catenin, over-expression of ΔGSK-3β increased BMP-2 induced nuclearization of β-catenin on Days 1-9; on Day 13, levels of nuclear β-catenin were similar in both parental and ΔGSK-3β expressing micromass (data not shown). Interestingly, levels of nuclear LEF-1, similar to nuclear β-catenin, also did not markedly respond to wild-type GSK-3β over-expression in micromass (Fig. 8B). On the other hand, over-expression of ΔGSK-3β dramatically enhanced nuclearization of LEF-1 throughout the entire culture period in both control and BMP-2 treated micromass (Fig. 8B).

**BMP-2 Treatment of C3H10T1/2 Micromass Cultures Alters SMAD-4 Profiles**

Since the preferred signal transducer of BMP-2 is SMAD-4, which has recently been shown to interact directly with β-catenin (45), we examined the effect of BMP-2 on the spatiotemporal distribution of SMAD-4 as well as its interaction with β-catenin in C3H10T1/2 micromass cultures in response to BMP-2. Nuclear extracts showed elevated SMAD-4 signal after BMP-2 treatment throughout the entire culture period, while control cultures showed only a slight temporal increase in SMAD-4 (Fig. 9A). To analyze the interaction between β-catenin and SMAD-4, β-catenin immunoprecipitates from C3H10T1/2 micromass cultures were probed for SMAD-4. SMAD-4 interaction with
β-catenin was indeed detected in these cultures and was strongly enhanced by BMP-2 treatment from Day 5 to Day 13 (Fig. 9B). For comparison, immunoprecipitates of β-catenin from micromass cultures were immunoprobod for LEF-1 (Fig. 9C). Interestingly, increased association between β-catenin and LEF-1 did not appear in response to BMP-2 until Day 9 of culture, suggesting that β-catenin interaction with SMAD-4 was an early response that preceded the association of LEF-1 with the complex.

DISCUSSION

Our previous studies (5,6) have suggested a role for β-catenin, LEF-1, and GSK-3β in BMP-2 induced chondrogenesis in high-density micromass cultures of the mesenchymal cell line C3H10T1/2. Interestingly, we have also recently observed upregulation of Wnt-3A in response to BMP-2 (22). In this study we have determined that overexpression of Wnt-3A and kinase-dead GSK-3β both significantly enhance, while that of wild-type GSK-3β inhibits, BMP-2 mediated chondrogenesis of micromass cultures. Interestingly, these molecules could not regulate chondrogenesis in the absence of BMP-2, suggesting the necessity of crosstalk between BMPs and Wnts. It is also noteworthy that while Wnt-3A enhances BMP-2 induced chondrogenesis as early as Day 5 and continuing to Day 13, over-expression of either form of GSK-3β did not exert a significant effect post Day 9, i.e. during late stages of mesenchymal differentiation into chondrocytes. Since Wnt-3A affects chondrogenesis throughout the culture period, these results imply that both early and late availability of Wnt signal can enhance BMP-2 mediated chondrogenesis, while introduction of kinase-dead GSK-3β may mimic only
the aspect of Wnt signal related to the acceleration of chondrocyte differentiation. Thus, it is possible that Wnt-3A induces changes in C3H10T1/2 micromass cultures via pathways in addition to β-catenin protection.

In view of the fact that some Wnts do not act via the inhibition of the serine/threonine kinase activity of GSK-3β (14), we have analyzed GSK-3β enzymatic activity in BMP-2 stimulated micromass stably transfected with Wnt-3A. Indeed, Wnt-3A over-expression inhibits GSK-3β enzyme activity during the entire culture period, with inhibition most dramatic at earlier time points. This finding also suggests that GSK-3β inhibition is more important during early chondrogenesis, supporting our results that introduction of both wild-type and ΔGSK-3β affect younger rather than older cultures during BMP-2 mediated chondrogenesis. Wnt-3A over-expression in C3H10T1/2 micromass cultures also regulates the level of N-cadherin protein, supporting the postulate that Wnts act, at least in part, via regulating cadherin levels (27, 37, 54). Previously we have observed that over-expression of both wild-type and mutant N-cadherin disrupts BMP-2 induced chondrogenesis (5), indicating that tight regulation of N-cadherin is required for the correct series of event to move mesenchymal cells through condensation to differentiation. Our data suggest that one function of Wnt-3A may be optimization of N-cadherin levels during BMP-2 stimulation of chondrogenesis.

The BMP-2 mediated increase of both total and nuclear levels of β-catenin, as described previously (6), is further enhanced by Wnt-3A over-expression. Increases in β-catenin levels are indicative of Wnt canonical inhibition of GSK-3β, and are presumed to result in increased β-catenin nuclearization and interaction with LEF-1 in a transcription complex (55 - 57). It is likely that Wnt-3A protection of β-catenin in BMP-2 stimulated
C3H10T1/2 micromass cultures results in translocation of β-catenin to the nucleus and subsequent regulation of chondrogenesis-enhancing gene expression, such as those involved in adhesion and cytoskeletal rearrangement (31). There is also evidence that cadherins can sequester catenins away from Wnt signal (58, 59). Therefore, dowregulation of N-cadherin by Wnt-3A may serve a dual function in our system, i.e. regulation of adhesion, and increased β-catenin signal transduction. In further support of this hypothesis, we have consistently observed a Wnt-3A induced decrease in β-catenin association with N-cadherin (data not shown), another possible mechanism to modulate adhesion, and strongly suggestive of β-catenin being utilized in a non-membrane associated function.

We have recently observed that the consequence of BMP-2 treatment of C3H10T1/2 micromass cultures are remarkably similar to those known for Wnt signaling, i.e. both total and nuclear LEF-1 and β-catenin levels are increased in response to BMP-2, most likely as a result of BMP-2 upregulation of Wnt-3A (22). In this report, we have shown that there is a remarkable decrease in levels of GSK-3β protein in response to Wnt signal, as has been reported in other systems (60), as well as an increase in total and nuclear LEF-1 protein in response to Wnt-3A, again suggesting inhibition of GSK-3β. Of remarkable interest is our observation that Wnt-3A induced increases in β-catenin and LEF-1 nuclearization are not accompanied by increased association between LEF-1 and β-catenin. This suggests that β-catenin in BMP-2 stimulated C3H10T1/2 micromass, in response to Wnt-3A, may interact with another HMG transcription partner, such as TCF, or may increase interaction with factors not directly involved in Wnt signal (see below).
Since GSK-3β is a ubiquitous and multifunctional kinase (61-64), its contribution to chondrogenesis could be through various pathways. Our results of GSK-3β over-expression, along with the Wnt-3A data, suggest that early nuclearization of β-catenin and LEF-1 can accelerate BMP-2 induction of chondrogenesis. However, why would both ΔGSK-3β and Wnt-3A enhance chondrogenesis while exerting opposite effects on N-cadherin levels during early condensation? Since ΔGSK-3β over-expression increases N-cadherin levels in cultures with or without BMP-2 treatment but does not enhance chondrogenesis in untreated cultures, we suggest that it is not the induced N-cadherin upregulation that is chondrogenesis-enhancing, but rather nuclearization of LEF-1 and β-catenin. Thus, on Day 13, when ΔGSK-3β over-expression no longer dramatically affects nuclear β-catenin or LEF-1, compared to parental non-transfected micromass, its chondrogenesis-enhancing activity is also abrogated. We suggest that Wnt-3A indeed inhibits GSK-3β activity, as does expression of ΔGSK-3β, by virtue of competition, and results in nuclearization of LEF-1 and β-catenin, but that in addition, Wnt-3A acts through a separate pathway to control N-cadherin mediated adhesion, possibly via Dsh activation of JNK (35). Since GSK-3β acts downstream of Dsh in Wnt signal, over-expression of ΔGSK-3β only partially mimics Wnt-3A introduction.

Of particular interest here is the recent discovery that TGF-β signaling results in SMAD-4 and SMAD-3 directly interacting with β-catenin/LEF-1 and the transcriptional activation of LEF-1 responsive promoters (45, 46). We observe here that, although total levels of SMAD-4 do not increase in response to BMP-2 (data not shown), both nuclear levels of SMAD-4 as well as the interaction between SMAD-4 and β-catenin are enhanced upon BMP-2 treatment. The temporal profiles show that, by Day 5, there is a
remarkable increase between SMAD-4 and β-catenin in response to BMP-2, likely due to the increasing levels of nuclear β-catenin. Then as LEF-1 begins to accumulate in the nucleus in response to BMP-2 during mid- to late-chondrogenesis, increased interaction between β-catenin and LEF-1, presumably in complex with SMAD-4, is observed. Though previous reports have shown that SMADs can interact with LEF and/or β-catenin in response to TGF-β (45, 46), this is the first evidence that BMP-2 induction of chondrogenesis facilitates SMAD/catenin interaction, reminiscent of the well documented coordination of Wg and Dpp in Drosophila (65, 41 - 45).

In conclusion, our results strongly suggest that Wnt-3A has the capacity to enhance BMP-2 mediated chondrogenesis of mesenchymal micromass cultures through (1) the regulation of N-cadherin mediated adhesion, (2) the inhibition of GSK-3β kinase activity, and (3) the nuclear signaling of β-catenin and LEF-1. Given the fact that BMP-2 upregulates Wnt-3A during the early period of culture, and expression of exogenous Wnt-3A regulates N-cadherin during this same time period, Wnt-3A is likely to act primarily during mesenchymal condensation rather than during chondrocyte maturation. Furthermore, introduction of an inactive GSK-3β can mimic some but not all of the activities of Wnt-3A, suggesting that Wnt-3A may function in a second yet-to-be-defined pathway, not involving GSK-3β, to modify cadherin-mediated adhesion. To our knowledge, Wnt-3A is the first described Wnt member that positively affects chondrogenesis in coordination with BMP-2 during early mesenchymal induction, and may do so via SMAD/catenin mediated nuclear signal. Our continuing studies focus on transcriptional regulation of cartilage specific genes by β-catenin and LEF-1,
coordination of SMAD/β-catenin, and the functional contribution of GSK-3β to BMP-2 mediated chondrogenesis.

ACKNOWLEDGMENT

The authors wish to thank Dr. David Hall, Alyssa Carlberg, and Cynthia Coleman for comments and invaluable discussion; Dr. K. Knudsen for the 13A9 antibody, Dr. P. Polakis for the β-catenin and GSK-3β cDNAs, Dr. H. Roelink for the Wnt-3A cDNA, Dr. M. Takeichi for the N-cadherin cDNA, Dr. R. Grosschedl for the LEF-1 antibody, and Genetics Institute for recombinant human BMP-2. This work was supported in part by NIH grants ES07005, DE12864, AR39740, and AR45181. L.F. and G.B. were supported in part by the Foerderer Fellowship of Thomas Jefferson University.

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65. Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.-C., Grosschedl, R.,

FIGURE LEGENDS

Fig. 1. Over-expression of Wnt-3A, ΔGSK-3β, and wild-type GSK-3β in C3H10T1/2 cells. Immunoblot and RT-PCR analysis of C3H10T1/2 cells transfected with Wnt signal constructs. (A) Immunoblot analysis of anti-myc immunoprecipitates from whole cell extracts of clonally derived Wnt-3A transfected C3H10T1/2 monolayer cultures (clones 2, 3, 4, 6 & 7). Myc-tagged mouse Wnt-3A protein is detected migrating at approximately 41 kDa in all clone lysates. Negative control consists of whole cell lysates immunoprobed with secondary antibody in the absence of primary antibody (2o only).

(B) RT-PCR analysis of Wnt-3A mRNA expression within clonally derived C3H10T1/2 micromass cultures. Two clonal lines (clone 2 and clone 6) that express exogenous Wnt-3A protein were chosen and compared to untransfected parental C3H10T1/2 cells. The level of expression was normalized to GAPDH message and ranged from 1.95 to 2.25 times over-expression compared to control. Positive control consists of RT-PCR analysis of Day 10.5 mouse embryonic limb buds (limb), and negative control was performed on Wnt-3A overexpressing cells without reverse transcriptase (-RT).

(C) Immunoblot analysis of total protein isolated from clonally derived C3H10T1/2 monolayer cultures transfected with either myc-tagged wild-type GSK-3β (lanes 1 – 3) or kinase-dead GSK-3β (lanes 4 – 7). Clonal isolates express myc-tagged GSK-3β or ΔGSK-3β, both
migrating at approximately 45 kDa, detected using anti-myc antibodies. The production of exogenous GSK-3β protein by the transfected cells was shown by immunoblot analysis of protein extracts from WT- GSK-3β clone 2 using anti- GSK-3β antibodies, showing higher immunoreactive band intensity than that of the positive control (Parental lane) that consists of similarly probed extract from parental non-transfected cultures.

Fig. 2. **Effect of Wnt-3A over-expression on BMP-2 induced chondrogenesis in C3H10T1/2 micromass cultures.** (A) Whole mount alcian blue histochemistry. Wnt-3A over-expressing micromass cultures treated with (+) and without (-) BMP-2 display increased Alcian Blue staining, compared to the respective untransfected parental cultures. (B) Metabolic sulfate incorporation in BMP-2 stimulated micromass cultures over-expressing Wnt-3A (Clone 2 and Clone 6), compared to parental cultures. While both cell types respond to BMP-2, Wnt-3A micromass cultures display significantly (*, p < 0.0001) increased (2-3 fold) [35S]sulfate incorporation (normalized to [3H]leucine incorporation) upon BMP-2 treatment from Days 5 through 13 of culture. Values represent the mean ± s.d. (n=4) and are expressed as percentage of Day 5 untreated cultures. Statistical significance was assessed with Bonferonni Dunn Post Hoc analysis.

Fig. 3. **Effect of over-expression of wild-type GSK-3β and ΔGSK-3β on BMP-2 induced chondrogenesis of C3H10T1/2 cultures measured by metabolic sulfate incorporation.** (A) Over-expression of wild-type GSK-3β significantly decreased (30.1 % – 34.5 % of non-transfected) BMP-2 mediated [35S]sulfate incorporation (normalized to [3H]leucine incorporation) on Days 5 and 9 of culture (*, p < 0.0438). No significant
effect was seen on Day 13. (B) Over-expression of kinase dead GSK-3β significantly increased (108.9% - 247.6% of non-transfected) BMP-2 mediated $[^{35}S]$sulfate incorporation (normalized to $[^{3}H]$leucine incorporation) from Days 3 through 9 of culture (*, p <0.0319). No significant effect was seen on Day 13. All values represent the mean ± s.d. (n=4) and are expressed as percentage of Day 5 untreated cultures. Statistical significance was assessed with Bonferonni Dunn Post Hoc analysis.

Fig. 4. Effect of Wnt-3A over-expression on GSK-3β kinase activity in BMP-2 stimulated C3H10T1/2 micromass cultures. In vitro GSK-3β kinase assay was carried out using a phospho-CREB substrate and γ–$[^{32}P]$ATP. (A) Level of GSK-3β activity per 100 µg of cell lysate. BMP-2 treated non-transfected parental C3H10T1/2 micromass cultures display GSK-3β kinase activity that remains relatively unchanged through Days 5 - 13. However, over-expression of Wnt-3A in BMP-2 stimulated micromass severely inhibits the enzymatic activity of GSK-3β by Day 5 (20.8% of parental activity) as well as on Day 9 (41.1%) and Day 13 (58.6%). (B) GSK-3β kinase activity normalized to GSK-3β protein levels. In cultures overexpressing Wnt-3A, the specific activity of GSK-3β remains reduced relative to that in non-transfected parental cultures. Specific activity levels are presented as percentage of that in day 5 non-transfected cultures.

Fig. 5. Effect of Wnt-3A over-expression on β-catenin, N-cadherin, and GSK-3β protein levels during BMP-2 induced chondrogenesis in C3H10T1/2 micromass cultures. (A) Immunoblot analysis of whole cell extracts from micromass. Parental non-transfected C3H10T1/2 cultures display increased levels of β-catenin, N-cadherin, and
GSK-3β in response to BMP-2 throughout the entire period of culture. Wnt-3A overexpression enhances the ability of BMP-2 to protect levels of β-catenin, as well as suppresses the ability of BMP-2 to stimulate N-cadherin and GSK-3β protein levels, throughout the entire culture period. The negative control (2°) consists of cell extracts probed with secondary antibody in the absence of primary antibody. (B) Immunoblot analysis of β-catenin in nuclear extracts. Parental C3H10T1/2 non-transfected cultures display increased levels of the largest isoform of β-catenin in response to BMP-2 throughout the entire culture period, while Wnt-3A over-expression increases β-catenin nuclearization from Days 1 through 13, as compared to BMP-2 treated parental cells. For comparison, a lane of whole cell extract (Whole Cell) was probed with anti-β-catenin and displays all three isoforms of β-catenin characteristic of C3H10T1/2 cultures. A negative control lane (2°) consists of nuclear extracts immunoprobed with secondary antibody in the absence of β-catenin primary antibody. All densitometric intensities are expressed as a percentage of Day 1 control signal. Equal protein loading was confirmed by either Coomassie Blue staining of parallel gels or Ponceau S staining of the immunoblot.

Fig. 6. Wnt-3A over-expression modifies BMP-2 regulation of LEF-1 in C3H10T1/2 micromass cultures. (A) Immunoblot analysis of LEF-1 in nuclear extracts. Parental non-transfected cultures show increased nuclearization of LEF-1 in response to BMP-2 in later periods (Days 5 – 13) of chondrogenesis. Wnt-3A over-expression enhances the ability of BMP-2 to stimulate nuclearization of LEF-1 throughout the entire culture period, most dramatic from Days 9 to 13. (B) Co-immunoprecipitation of β-catenin immunoprobed for LEF-1. Parental non-transfected cultures show a slight increased
interaction of β-catenin with LEF-1 in response to BMP-2 in older (Days 9-13) cultures, while Wnt-3A over-expression does not remarkably modify β-catenin/LEF-1 interaction during chondrogenesis. Positive controls for these immunoblots consist of a lane (thymus) containing total cell extract from mouse thymus, a tissue with high endogenous LEF content. Negative controls consist of a lane immunoprobed with secondary antibody in the absence of LEF-1 primary antibody (2°). Equal protein loading was confirmed by Ponceau S staining of the immunoblot.

Fig. 7. Effect of GSK-3β over-expression on N-cadherin and β-catenin levels in C3H10T1/2 micromass cultures. (A) Immunoblot analysis of whole cell extracts from micromass cultures. Parental non-transfected cultures display increased levels of both N-cadherin and β-catenin in response to BMP-2 on all days of culture. Over-expression of ΔGSK-3β enhances the ability of BMP-2 to stimulate both N-cadherin and β-catenin levels on Day 1 only, while levels of both molecules remain similar between parental and ΔGSK-3β over-expressors in response to BMP-2 on all other Days. It is noteworthy that in Day 9 and Day 13 cultures, β-catenin and N-cadherin levels remain elevated in non-BMP-2 treated, ΔGSK-3β over-expressing cultures. Over-expression of wild-type GSK-3β decreases the ability of BMP-2 to stimulate both N-cadherin and β-catenin levels during later stages of chondrogenesis (Days 9-13) without remarkably affecting the levels in younger (Days 1 and 5) cultures. (B) Ponceau S staining of the above immunoblot to visualize equivalent protein loading.
Fig. 8. **Effect of GSK-3β over-expression on nuclear levels of β-catenin and LEF-1 in C3H10T1/2 micromass cultures.** Immunoblot analysis of nuclear extracts probed for (A) β-catenin, or (B) LEF-1. All densitometric intensities of immunoreactive protein bands are expressed as a percentage of Day 1 control signal. (A) Parental non-transfected cultures display increased nuclearization of the largest form of β-catenin in response to BMP-2 on culture Days 1 – 9. Over-expression of wild-type GSK-3β does not markedly affect nuclear β-catenin levels. However, over-expression of ΔGSK-3β increased nuclear β-catenin in response to BMP-2 throughout the culture period. For comparison, a lane of whole cell extracts (Whole Cell) probed for β-catenin shows the three isoforms of β-catenin found in total cell lysates of C3H10T1/2 cultures. (B) Parental non-transfected cultures display increased nuclearization of LEF-1 in response to BMP-2 in older cultures, while over-expression of wild-type GSK-3β does not remarkably affect nuclear LEF-1 levels. However, over-expression of ΔGSK-3β results in an increase in nuclear LEF-1 throughout the culture period, as compared to parental cultures. There was no remarkable difference on Day 9 between GSK-3β over-expressing cell lines and parental cultures in terms of either β-catenin or LEF-1 nuclear levels. As a positive control, a lane of mouse thymus extract (Mouse Thymus) probed for LEF-1 accompanies the nuclear immunoblots for LEF-1. Equal protein loading was confirmed by Ponceau S staining of the immunoblot.

Fig. 9. **BMP-2 modifies SMAD-4 protein levels and its association with β-catenin in C3H10T1/2 micromass cultures.** (A) Immunoblot analysis of SMAD-4 in nuclear extracts. BMP-2 treatment increases SMAD-4 nuclearization over that of untreated,
control cultures on all culture days. A parallel gel was stained with Coomassie Brilliant Blue to verify equal loading. Densitometric intensities of immunoreactive protein bands are expressed as percentage of Day 1 untreated control signal. (B) Immunoblot analysis of β-catenin immunoprecipitates probed for SMAD-4. SMAD-4 interacts with β-catenin in both treated and untreated cultures and this interaction is remarkably enhanced upon BMP-2 addition on culture Days 5 – 13. Densitometric intensities of immunoreactive bands are expressed as percentage of Day 1 untreated cultures. Smad 4 immunoreactive band derived from total cell extract is shown as control. (C) Immunoblot analysis of β–catenin immunoprecipitates probed for LEF-1. LEF-1 interacts with β-catenin in both treated and untreated cultures and this interaction is enhanced upon BMP-2 addition on culture Days 9 – 13. Positive control consists of a lane containing mouse thymus cell extracts immunoprobed for LEF-1 (Mouse Thymus). Densitometric intensities of immunoreactive protein bands are expressed as percentage of Day 1 untreated control signal. Equal protein loading was confirmed by Coomassie Blue staining of parallel gels.
Fig. 1
Fig 2
Fig. 4
A  Whole Cell Immunoblot  Probe

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| 125 kDa    | -     | -     | -
| 45 kDa     | -     | -     | -

β-catenin
N-cadherin
GSK-3β

Day 9
Day 13

BMP-2 - + - + - + - + 
Parental Wnt-3A Parental Wnt-3A

B  Nuclear Immunoblot

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β-catenin

Day 9
Day 13

BMP-2 - + - + - + - + 
Parental Wnt-3A Parental Wnt-3A

Fig 5
A  Nuclear Immunoblot;  Probe: LEF-1

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- 60 kDa

B  IP: β-catenin;  Probe: LEF-1

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- 60 kDa

Fig. 6
A

Whole Cell Immunoblot

Day 1          Day 5

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Day 9          Day 13

| 141 kDa   | -       | -      | -        | -       | -      |
| 92 kDa    | -       | -      | -        | -       | -      |

B

Ponceau S Staining

---

Fig 7
### A: Nuclear Immunoblot

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Probes: -β-catenin

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Probes: -LEF-1

Mouse Thymus
### A
**Nuclear Extracts**
Probe: SMAD-4

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**Relative Intensity**

**Coomassie Blue**

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### B
**Whole Cell Lysates**
IP: β-catenin; Probe: SMAD-4

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**Relative Intensity**

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### C
**Whole Cell Lysates**
IP: β-catenin; Probe: LEF-1

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**Relative Intensity**

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**Mouse Thymus**

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Fig 9
Wnt-3A enhances BMP-2 mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells
Leslie Fischer, Genevieve Boland and Rocky S. Tuan

J. Biol. Chem. published online June 20, 2002

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