Journal of Cellular Biochemistry 84:816–831 (2002)
DOI 10.1002/jcb.10091

Wnt Signaling During BMP-2 Stimulation of Mesenchymal Chondrogenesis

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Abstract Members of both the Wnt and bone morphogenetic protein (BMP) families of signaling molecules have been implicated in the regulation of cartilage development. A key component of the Wnt signaling pathway is the cytosolic protein, β-catenin. We have recently shown that the chondrogenic activity of BMP-2 in vitro involves the action of the cell–cell adhesion protein, N-cadherin, which functionally complexes with β-catenin. The aim of this study is to test the hypothesis that Wnts may be involved in BMP-2 induced chondrogenesis, using an in vitro model of high-density micromass cultures of the murine multipotent mesenchymal cell line, C3H10T1/2. Expression of a number of Wnt members was detected in these cultures, including Wnt-3A and Wnt-7A, whose levels were up- and downregulated, respectively, by BMP-2. To assess the functional involvement of Wnt signaling in BMP-2 induced chondrogenesis, cultures were treated with lithium chloride, a Wnt-7A mimetic that acts by inhibiting the serine/threonine phosphorylation activity of glycogen synthase kinase-3β (GSK-3β). Lithium treatment significantly inhibited BMP-2 stimulation of chondrogenesis as well as GSK-3β enzymatic activity, and decreased the levels of N-cadherin protein and mRNA. Furthermore, lithium decreased BMP-2 upregulation of total and nuclear levels of LEF-1 and β-catenin as well as their interaction during later chondrogenesis; similarly, the interaction of β-catenin with N-cadherin was also decreased. Interestingly, lithium treatment did not affect the ability of BMP-2 to decrease ubiquitination of β-catenin, although it did reduce the interaction of β-catenin with GSK-3β during late chondrogenesis (days 9–13). We suggest that the chondro-inhibitory effect of lithium on BMP-2 induced chondrogenesis indicates antagonism between lithium-like Wnts and BMP-2 during mesenchymal condensation. J. Cell. Biochem. 84: 816–831, 2002. © 2002 Wiley-Liss, Inc.

Key words: cartilage development; micromass culture; N-cadherin; Wnt signaling; GSK-3β; lithium

Mesenchymal cell condensation is a prerequisite for chondrogenesis, e.g., in the embryonic limb bud, and has been shown to be a key step regulated by chondrogenesis-stimulating factors, including members of the transforming growth factor-β (TGF-β) and BMP families [see review by DeLise et al., 2000]. A key cell–cell adhesion molecule involved in the initiation of mesenchymal condensation is N-cadherin, a calcium-dependent homophilic transmembrane adhesion protein. The expression pattern of N-cadherin during cell aggregation and compaction of the developing limb is consistent with its demonstrated functional requirement in chondrifying mesenchyme in vivo and in vitro [Oberlender and Tuan, 1994a,b]. Recent studies from our laboratory have shown that TGF-β1 and BMP-2 stimulation of mesenchymal chondrogenesis involves the upregulation of N-cadherin expression and associated activities [Tyndall and Tuan, 1994a,b; Denker et al., 1999; Haas and Tuan, 1999; Fischer et al., 2001].

At a subcellular level, N-cadherin interacts with the actin cytoskeleton through the cadherin associated catenins, α-catenin, β-catenin, γ-catenin, and p120ctn [Aberle et al., 1996]. Either γ-catenin or β-catenin first binds to the N-cadherin cytoplasmic domain, and α-catenin

Grant sponsor: NIH; Grant numbers: ES07005, DE12864, AR39740, AR45181; Grant sponsor: Foerderer Fellowship of Thomas Jefferson University (to L.F. and G.B.).

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Received 16 August 2001; Accepted 14 November 2001

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then binds to the N-terminus of β-catenin and further binds to the actin skeleton [Hinck et al., 1994a]. As well as bound to the cytoplasmic domain of N-cadherin, β-catenin is also found in the cytoplasm, where it interacts with the adenomatous polyposis coli (APC) tumor suppressor protein [Rubinfeld et al., 1993], and in a nuclear pool in association with the transcription factors, lymphoid enhancing factor and T-cell factors (LEF-1/TCF) [Behrens et al., 1996], originally identified in mouse thymus [Oosterwegel et al., 1993].

The Wnt family of signaling proteins consist of a large number of cysteine rich secreted glycoproteins involved in tissue induction and axis determination in early embryos [Cadigan and Nusse, 1997]. In the current model of canonical Wnt signaling, in the absence of Wnt protein, glycogen synthase kinase-3β (GSK-3β) is active and phosphorylates APC, causing its increased affinity for β-catenin [Rubinfeld et al., 1996]. This interaction of β-catenin with APC/GSK-3β is quickly followed by N-terminal serine/threonine phosphorylation of β-catenin via GSK-3β and rapid destruction of β-catenin by the ubiquitin/proteasome pathway [reviewed in Seidensticker and Behrens, 2000]. In the presence of Wnt, GSK-3β is inactive, β-catenin accumulates in the cytoplasm, and translocates to the nucleus where it interacts with LEF-1/TCF in a transcription complex to regulate gene expression [Behrens et al., 1996]. Additional regulation of the Wnt signaling pathway is indicated by recent evidence that protein kinase C (PKC) can regulate GSK-3β activity and ubiquitination [Orford et al., 1997; Chen et al., 2000].

The Wnt-1 transforming group consists of Wnt-1, -3A, -7A, -2, -5B, and -7B, and the Wnt-5A non-transforming group contains Wnt-5A, -4, and -6. These two groups have been shown to have antagonistic effects toward each other regarding cadherin mediated adhesion [Hinck et al., 1994b]. Furthermore, depending on the Frizzled (Frz) receptor specificity, the immediate cytosolic component of Wnt signal, Disheveled (Dsh), can act as a switch and direct Wnt away from β-catenin regulation to Jun N-terminal Kinase (JNK) regulation [reviewed in McEwen and Peifer, 2000; Pandur and Kuhl, 2000]. Various members of the Wnt family are expressed in the developing limb, and have been implicated in mesenchymal chondrogenesis [Gavin et al., 1990; Hartmann and Tabin, 2000; Tufan and Tuan, 2001]. Specifically, Wnt-7A has been shown to inhibit chondrogenesis in high-density chick limb mesenchyme cultures [Rudnicki and Brown, 1997; Tufan and Tuan, 2001], and in chick limb bud, Wnt-4 signals through LEF-1/β-catenin, whereas, Wnt-5A does not. In fact, Wnt-4 and Wnt-5A exert opposing effects on chondrocyte differentiation [Hartmann and Tabin, 2000].

A widely used in vitro model system for the study of chondrogenesis has been the high-density micromass culture of chick embryonic limb bud mesenchymal cells [Ahrens et al., 1977]. We have recently established a similar high-density micromass culture system using multipotent C3H10T1/2 murine cells [Reznikoff et al., 1973], plated at high-density to mimic the cell–cell contacts of developing limb mesenchyme, which are induced to chondrify exclusively upon treatment with BMP-2 and TGF-β1 [Denker et al., 1995, 1999; Haas and Tuan, 1999; Carlberg et al., 2001]. Interestingly, TGF-β or BMP-2 mediated stimulation or induction of chondrogenesis in both micromass culture systems involves the upregulation of the expression of N-cadherin and its activities via the modulation of the cadherin–catenin complex [Tyndall and Tuan, 1994a,b; Haas and Tuan, 1999]. Thus, BMP-2 treatment alters distribution, phosphorylation, and protein levels of β-catenin during chondrogenesis in C3H10T1/2 micromass cultures [Fischer et al., 2001].

In view of these effects of BMP-2 on β-catenin and the reported negative effect of Wnt-7A on mesenchymal chondrogenesis [Tufan and Tuan, 2001], this investigation aims to analyze the functional involvement of Wnt signaling during BMP-2 induced chondrogenesis of C3H10T1/2 micromass culture, specifically utilizing lithium as a Wnt mimetic. Our results show that lithium is a chondroinhibitory compound like Wnt-7A [Rudnicki and Brown, 1997; Tufan and Tuan, 2001], causing changes in the characteristics of N-cadherin and Wnt signaling pathway members (β-catenin, GSK-3β, and LEF-1), including their interactions, relative levels, subcellular compartmentalization, and phosphorylation states. Furthermore, BMP-2 treatment downregulates mRNA levels of the chondroinhibitory Wnt-7A while upregulating transcription of Wnt-3A, raising the intriguing possibility that regulation of mesenchymal chondrogenesis involves an intricate balance between the actions of BMP-2 and specific Wnts.

MATERIALS AND METHODS

Cell Culture

For routine passage, C3H10T1/2 cells were plated as monolayer cultures and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone). For micromass culturing, the technique of Ahrens et al. [1977] was modified as described previously [Denker et al., 1995], using 10 μl drops of cells at 10^7 cells/ml. Micromass cultures were maintained in Ham's F12 medium (GIBCO-BRL) containing 10% FBS with or without 100 ng/ml recombinant human BMP-2 (a gift of Genetics Institute, Inc.), and with or without 5–10 mM lithium chloride or sodium chloride as control. Medium was changed every three days. The inositol monophosphatase (IMPase) inhibitor L-690,330 (Tocris Cookson; IC_{50} of 1 μM), which is 1,000 times more potent than lithium in in vitro studies [Atack et al., 1993], was used to treat micromass at a concentration of 1–150 μM in ethanol. Control cultures were treated with ethanol alone.

Examination of Chondrogenesis by Alcian Blue Staining and Metabolic Sulfate Labeling

Metabolic sulfate labeling was carried out by addition of 5.0 μCi/ml sodium [^{35}S]sulfate and 1.0 μCi/ml [^{3}H]leucine (both from DuPont NEN) to micromass cultures for 24 h prior to the desired time point to estimate synthesis of sulfated proteoglycans and total protein, respectively. Radioactivity incorporation was measured by liquid scintillation counting [San Antonio and Tuan, 1986], and [^{35}S]sulfate incorporation normalized to [^{3}H]leucine. Parallel cultures were fixed on culture days 5, 9, and 13 with Kahle's fixative and stained with 1% Alcian blue 8-GX in 0.1 N HCl, pH 1.0 (Sigma). Staining intensity was assessed by imaging using a Kodak Digital Image Station 440 CS.

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 $NaVO_3$, and 1 nM okadaic acid, and extracted at 4°C for 20 min. Extracts were cleared of membranes by centrifugation at top speed in a microfuge and protein concentration determined by Bradford assay. Fifteen microgram aliquots of the total lysates were subjected to Western blot analysis to verify loading (see below). Another 100 μg aliquot of the total lysates was immunoprecipitated with antibody to GSK-3β (see below). The immune complex was then washed in 50 mM Tris-HCl, pH 7.5, with 1 mM DTT and resuspended in 45 μl of kinase reaction buffer containing 200 μM ATP, 4.5 μl 10 × GSK-3β kinase buffer (New England BioLabs), 100 μM [γ-^{33}P]ATP (0.25 μCi/ml) (DuPont NEN), and allowed to autophosphorylate for 15 min at 30°C. Kinase reactions were stopped with either 1.5% phosphoric acid or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and samples were subjected to SDS–PAGE followed by autoradiography for 2 weeks at − 80°C. [^{33}P]Labeled GSK-3β band intensities were determined using Kodak Digital Science Imaging Station 440 CS, and normalized to total GSK-3β levels determined by immunoblotting.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from micromass cultures using TRI-REAGENT RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc), glyoxylated, and processed for Northern blot analysis using the following random-prime [^{32}P]labeled cDNA probes: (1) full length mouse N-cadherin cDNA clone from Dr. M. Takeichi, Kyoto University [Miyatani et al., 1989] and (2) mouse full length Wnt cDNAs (Wnt-7A, 1, 5A, 3, 6, and 7B) from Dr. A. McMahon, Harvard University [Gavin et al., 1990]. To normalize RNA loading the p1B15 probe for rat cyclophilin A was used [Danielson et al., 1988].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The following RT-PCR primers (forward and backward, 5′–3′) were used: mouse Wnt-3A-AACC ACG GGA GCA GGG TTC ATT C and AAG GGG GTC TCC AAA AGT TCC ACC, yielding a 534 bp product. To normalize loading on the basis of β-actin mRNA, the following primer set was used: GTG GGC CGC TCT AGG CAC CAA and CTC TTT GAT GTC ACG CAC GAT TTC, yielding a 540 bp product of the transcript.

Seven micrograms of total RNA isolated from C3H10T1/2 micromass were reverse transcribed in 20 μl using oligo dT primers with the Superscript Amplification System (GIBCO-BRL) as per the manufacturer's suggestions. PCR for Wnt-3A was carried out on 5 μl of the

original reaction mixture using 35 cycles of 1 min at 94°C, 2 min at 62.5°C, and 3 min at 72°C. PCR for β-actin was carried out on 2 μl of the original reaction mixture using 18 cycles of 1 min at 94°C, 2 min at 60.5°C, and 3 min at 72°C. Products of the RT-PCR were analyzed by electrophoresis and stained with ethidium bromide.

Immunofluorescent Localization of β-Catenin

Micromass cultures were fixed with HISTO-CHOICE MB (Amresco), permeablized with PBS/0.2% Triton X-100, and incubated with the β-catenin monoclonal antibody (Transduction Labs) at 1:100 dilution in PBS/0.2% gelatin for 1 h at 37°C, followed by incubation with FITC conjugated goat anti-mouse secondary antibodies (Cappel, 1:50). Subsequently, cultures were incubated for 30 min at room temperature with 0.5 μg/ml DAPI and 0.5 U/ml rhodamine phalloidin in PBS/0.2% gelatin for nuclear and cytoskeleton detection, respectively. Cultures were rinsed, coverslipped with Fluoromount-G (Southern Biotechnology Associates), and viewed using an Olympus fluorescence microscope. Controls were treated as above without anti-β-catenin primary antibody.

Protein Isolation and Western Blotting

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 $CaCl_2$, and protease inhibitors (phenylmethylsulfonylflouride (PMSF), benzamidine HCl, amino-caproic acid, and pepstatin A; Sigma). Extracts were cleared by centrifugation and protein concentrations determined (MicroBCA, Pierce). Nuclear and cytoplasmic extracts were prepared by the method of Schreiber et al. [1989].

For Western blotting, 20 μg of each protein sample was separated by SDS–PAGE, and electro-transferred onto nitrocellulose membranes. The blots were blocked with buffer containing Tween 20 (0.05%) and bovine serum albumin (BSA, 3%), and reacted with primary and secondary antibodies (see below): (a) primary antibodies–N-cadherin (13A9) at 1:200, β-catenin at 1:500, LEF-1 at 1:3,000, GSK-3β at 1:3,800, ubiquitin at 1:1,000, or γ-catenin at 1:2,000 and (b) alkaline phosphatase conjugated secondary antibodies (Sigma) at a 1:3,800 dilution. The blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) (Zymed), and reactive bands quantified as described above. Negative controls for all immunoblots consisted of a total cell extract lane reserved for probing with secondary antibody in the absence of primary antibody. Protein loading was confirmed by staining the immunoblots with Ponceau S or by staining parallel SDS–PAGE gels with Coomassie Brilliant Blue.

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. K. Knudsen (Lankenau Medical Research Center) [Peralta Soler et al., 1995]. Monoclonal antibodies to β-, γ-catenin, and GSK-3β were purchased from Transduction Labs. Monoclonal antibody to ubiquitin tagged proteins was obtained from Santa Cruz Biotechnology. The anti-mouse LEF-1 antibody was a generous gift from Dr. R. Grosschedl, Universtiy of Munich [Behrens et al., 1996].

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 $CaCl_2$, 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. Electroblotting and Western analysis were performed as described above. Positive controls for all immunoblots of immunoprecipitates consisted of a lane containing total cell extracts immunoprobed for the protein(s) of interest.

RESULTS

Various Wnts Are Expressed in C3H10T1/2 Micromass Cultures

The expression of various Wnts, whose in vivo expression profiles correlate with condensation and chondrogenesis within the developing limb [Gavin et al., 1990] or have been shown to inhibit chondrogenesis in vitro [Rudnicki and

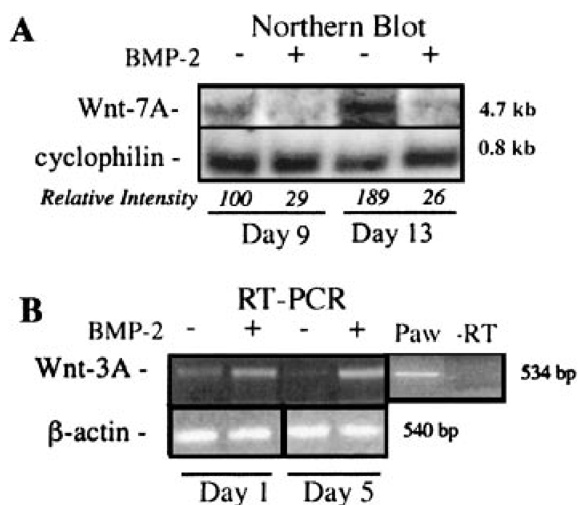


Fig. 1. Analysis of Wnt mRNA expression in C3H10T1/2 micromass cultures. **A**: Northern analysis of Wnt-7A transcript in micromass cultures treated with BMP-2. Wnt-7A is drastically downregulated by BMP-2 on days 9 and 13 of culture, normalized to cyclophilin transcript. Densitometric data presented as percentage of day 9 untreated culture. **B**: RT-PCR analysis of Wnt-3A expression in cultures treated with BMP-2. Wnt-3A is upregulated on days 1 and 5 following BMP-2 stimulation, normalized to β-actin. Total RNA isolated from day 10.5 mouse paw was also analyzed for Wnt-3A expression as a positive control (minus RT as negative control).

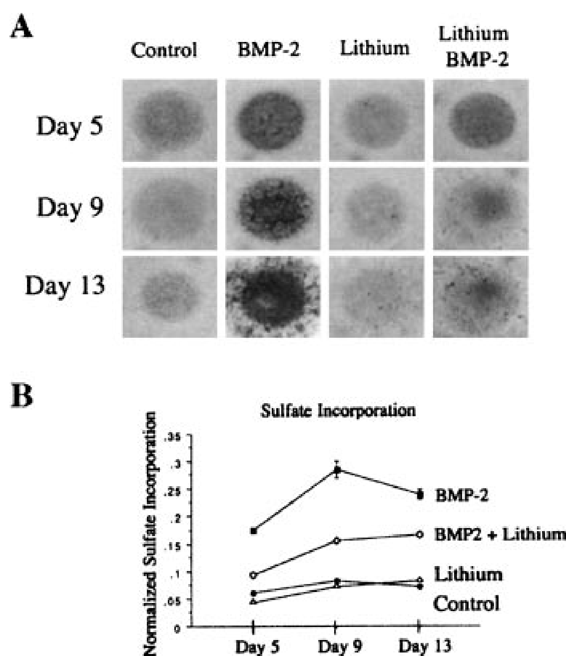


Fig. 2. Lithium inhibits BMP-2 induced chondrogenesis of C3H10T1/2 micromass. Micromass cultures were incubated with combinations of 5 mM LiCl and 100 ng/ml rhBMP-2. **A**: Alcian Blue histochemical staining of micromass. BMP-2 treatment resulted in significantly higher staining than control, untreated cultures. Lithium co-treatment inhibited the stimulatory effect of BMP-2 on chondrogenesis on the basis of Alcian Blue staining on days 5 through 13 of culture. **B**: [^{35}S]sulfate incorporation into micromass cultures. BMP-2-treated cultures displayed significantly higher levels of sulfate incorporation from days 5–13. Co-treatment of these cultures with lithium significantly ($P < 0.0001$) reduced sulfate incorporation into sulfated proteoglycans on all days of culture. Values represent the mean ± SD (n = 4), and significance is assessed by Bonferonni/Dunn Post hoc analysis.

Brown, 1997; Tufan and Tuan, 2001], was analyzed by Northern blotting or RT-PCR. Our findings indicate that Wnt-3, -3A, -5A, -1, and -7A were expressed in micromass during BMP-2 stimulation of chondrogenesis. Among these Wnts, there was no noticable change in Wnt-1, -3, or -5A levels as a function of time or BMP-2 treatment (data not shown). However, Wnt-7A transcription was downregulated by BMP-2 in later culture periods, i.e., days 9 and 13 (Fig. 1A), while Wnt-3A was upregulated by BMP-2 at days 1 and 5 of culture (Fig. 1B).

Lithium Inhibits BMP-2 Stimulation of Chondrogenesis and GSK-3β Kinase Activity

To assess the possible involvement of Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis, we tested the effect of lithium chloride treatment on BMP-2 mediated chondrogenesis of C3H10T1/2 micromass cultures. While lithium had no effect on cultures not treated with BMP-2 (which did not chondrify), lithium treatment of BMP-2 treated cultures significantly decreased chondrogenesis on the basis of Alcian Blue staining (Fig. 2A) and sulfate uptake (Fig. 2B) at all culture time points.

Since lithium has also been shown to inhibit IMPase ($IC_{50} = 0.8$ mM) [Berridge et al., 1989], we ascertained the specificity of the inhibitory effect of lithium observed here in terms of GSK-3β inhibition by addition of L-690,330, a potent and specific inhibitor ($IC_{50} = 1$ μM) of IMPase activity [Atack et al., 1993]. As shown in Figure 3, 1–50 μM final concentrations of L-690,330 in the culture medium did not significantly affect BMP-2 induced chondrogenesis. Furthermore, the chondrogenesis-inhibiting effect of lithium persisted in the presence of 50 μM and higher concentrations of L-690,330 (Fig. 3).

To confirm that the lithium effect on BMP-2 mediated chondrogenesis is mediated via inhibition of GSK-3β, cultures treated with or without BMP-2 and/or lithium were analyzed using an in vitro kinase assay, specifically in terms of GSK-3β autophosphorylation activation [Wang

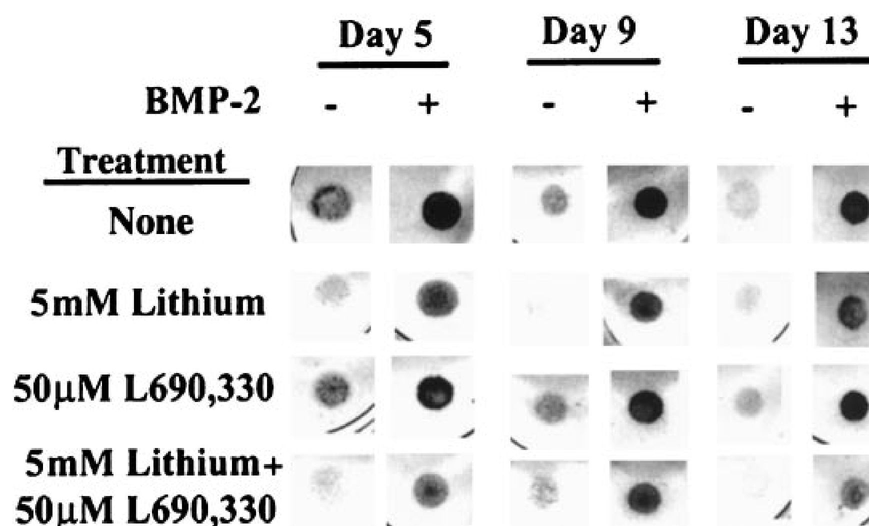


Fig. 3. Lithium does not affect chondrogenesis via the IMPase. Micromass cultures treated with combinations of 50 μM L-690,330, lithium, and 100 ng/ml BMP-2 were harvested at the indicated time points and stained with Alcian Blue, pH 1.0, for 24 h following fixation in Kahle's Fixative. Treatment of micromass with the IMPase inhibitor L-690,330 ($IC_{50} = 1$ μM) at the above concentrations did not affect BMP-2 stimulation of chondrogenesis, while lithium ($IC_{50} = 2$ mM) was chondroinhibitory at all concentrations tested, and a combination of 5 mM lithium and 50 μM L-690,330 inhibits chondrogenesis on all culture days.

et al., 1994]. GSK-3β autophosphorylation capacity was found to be upregulated by BMP-2 treatment and severely inhibited in the presence of lithium, suggesting lithium treatment resulted in specific inactivation of the enzyme (Fig. 4A). Interestingly, BMP-2 treatment resulted in a moderate to large increase in GSK-3β protein during the 13 day culture period that was somewhat enhanced by co-treatment with lithium (Fig. 4B), indicating that lithium inhibited the enzymatic activity, but not the biosynthesis of GSK-3β.

Lithium Affects N-Cadherin and Catenins in C3H10T1/2 Micromass Cultures

Because chondrogenesis occurs following a requisite period of cellular condensation mediated by cell–cell adhesion molecules such as N-cadherin [Oberlender and Tuan, 1994a; Haas and Tuan, 1999; DeLise et al., 2000], we were interested in determining if lithium might exert its chondroinhibitory influence in part through regulation of cellular condensation. As reported previously [Haas and Tuan, 1999], BMP-2 upregulates N-cadherin expression in C3H10T1/2 micromass cultures at both mRNA and protein levels as early as day 1 (Fig. 5A,B). However, in the presence of lithium, the ability of BMP-2 to increase N-cadherin protein levels was severely decreased during days 5–13 of culture (Fig. 5B). We also noted that lithium also reduced the ability of BMP-2 to upregulate N-cadherin mRNA levels as early as Day 1(Fig. 5A).

Since lithium inhibits the kinase activity of GSK-3β, and has been shown to change both the level and phosphorylation state of β-catenin protein [Yost et al., 1996; Hedgepeth et al., 1997], N-cadherin mediated cell–cell interactions may in turn be affected. Therefore, we next examined the effect of lithium co-treatment on catenins during BMP-2 stimulated chondrogenesis in C3H10T1/2 micromass cultures. As reported recently [Fischer et al., 2001], BMP-2 treatment of micromass cultures protects both γ- and β-catenin protein levels beginning on day 5 and continuing to day 13 of culture (Fig. 6A). Interestingly, lithium co-treatment did not cause accumulation of β- or γ-catenin, as would be expected if lithium was acting as a β-catenin protective Wnt signal (Fig. 6A). On the contrary, beginning on culture day 9 and most noticeably by day 13, lithium co-treatment appeared to decrease the level of the largest isoform of both β-and γ-catenin without concomitant accumulation of other isoforms. This lithium-mediated decrease was most noticeable on days when BMP-2 substantially accumulates γ- and β-catenin, suggesting that lithium is functioning to counteract BMP-2 protection of catenins. The association of β-catenin with N-cadherin was reduced upon BMP-2 treatment, but was

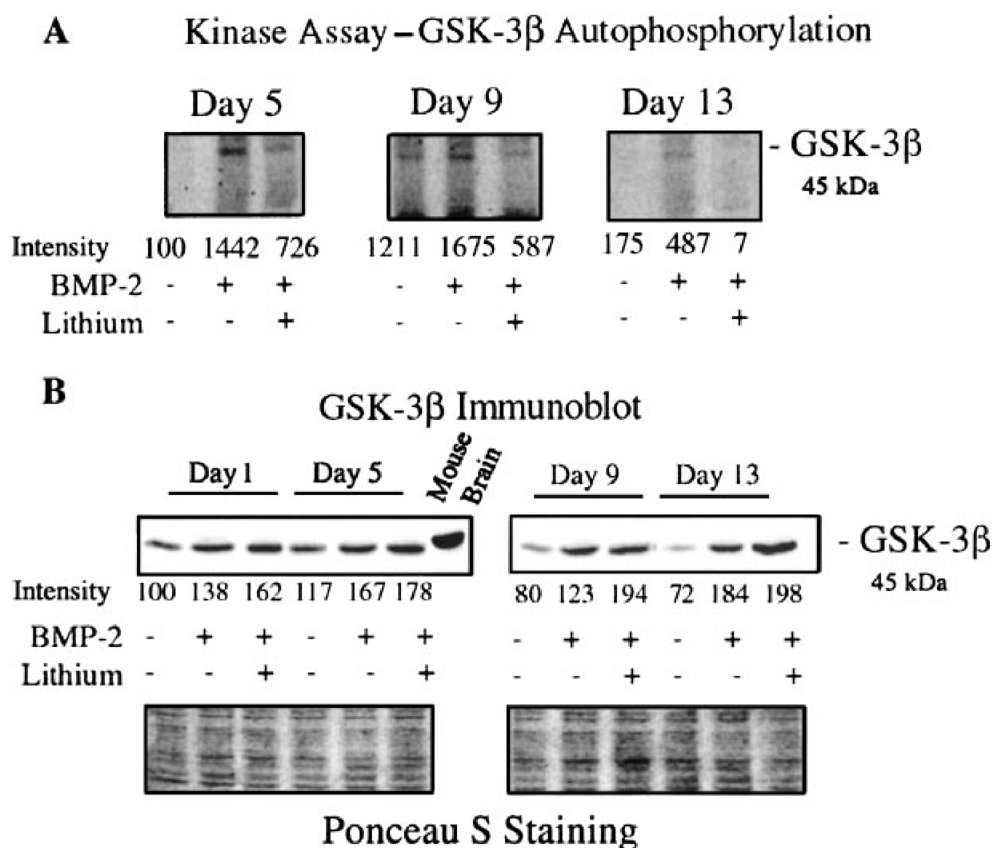


Fig. 4. Lithium co-treatment regulates GSK-3β protein and autophosphorylation levels in C3H10T1/2 micromass cultures. **A**: In vitro kinase assay assessing GSK-3β autophosphorylation capacity in the presence of BMP-2 and/or lithium. BMP-2 increases GSK-3β autophosphorylation, while lithium chloride decreases GSK-3β autophosphorylation activity from days 5–13. Densitometric data are presented as a percentage of day 5 untreated control cultures. **B**: GSK-3β immunoblot analysis of whole cell lysates. Both BMP-2 and lithium co-treatment resulted in increased GSK-3β protein levels on all culture days. BMP-2 upregulation is most dramatic on days 9–13 and lithium co-treatment enhances BMP-2 upregulation of GSK-3β on days 9–13. Ponceau S staining of the immunoblot is included to indicate uniform protein loading. All densitometric data are presented as a percentage of day 1 untreated cultures.

not affected by co-treatment with lithium (Fig. 6B).

Lithium Alters Wnt Signal Transducers During BMP-2 Induction of Chondrogenesis

As presented in Figure 7, Western blot analysis showed that BMP-2 treated cultures consistently displayed elevated whole cell LEF-1 levels compared to untreated controls; this BMP-2 mediated effect was reduced by co-treatment with lithium, the effect being most apparent during late stages, i.e., days 5–13 (Fig. 7A). In control cultures not treated with BMP-2 or lithium, LEF-1 levels remained relatively constant throughout the whole culture period. As shown in Figure 7B, by means of β-catenin immunoprecipitation from total cell lysates followed by LEF-1 immunoblotting, BMP-2 treatment also increased the association of LEF-1 with β-catenin during late culture periods, i.e., days 9 and 13, an effect that was suppressed with lithium co-treatment at these time points. Furthermore, although BMP-2 increased nuclear levels of LEF-1 beginning on day 1 of culture, lithium co-treatment abrogated this increased nuclear localization, yielding profiles similar to control untreated cultures by day 13 (Fig. 7C).

In view of the postulated action of lithium as a suppressor of GSK-3β activity leading to accumulation of β-catenin [Hedgepeth et al., 1997], we next analyzed the level of β-catenin in nuclear extracts of C3H10T1/2 micromass cultures treated with lithium and/or BMP-2. β-Catenin levels were low and equivalent on day 1 in all treatment groups and generally increased as a function of culture time (Fig. 8A). While BMP-2 treatment substantially increased the

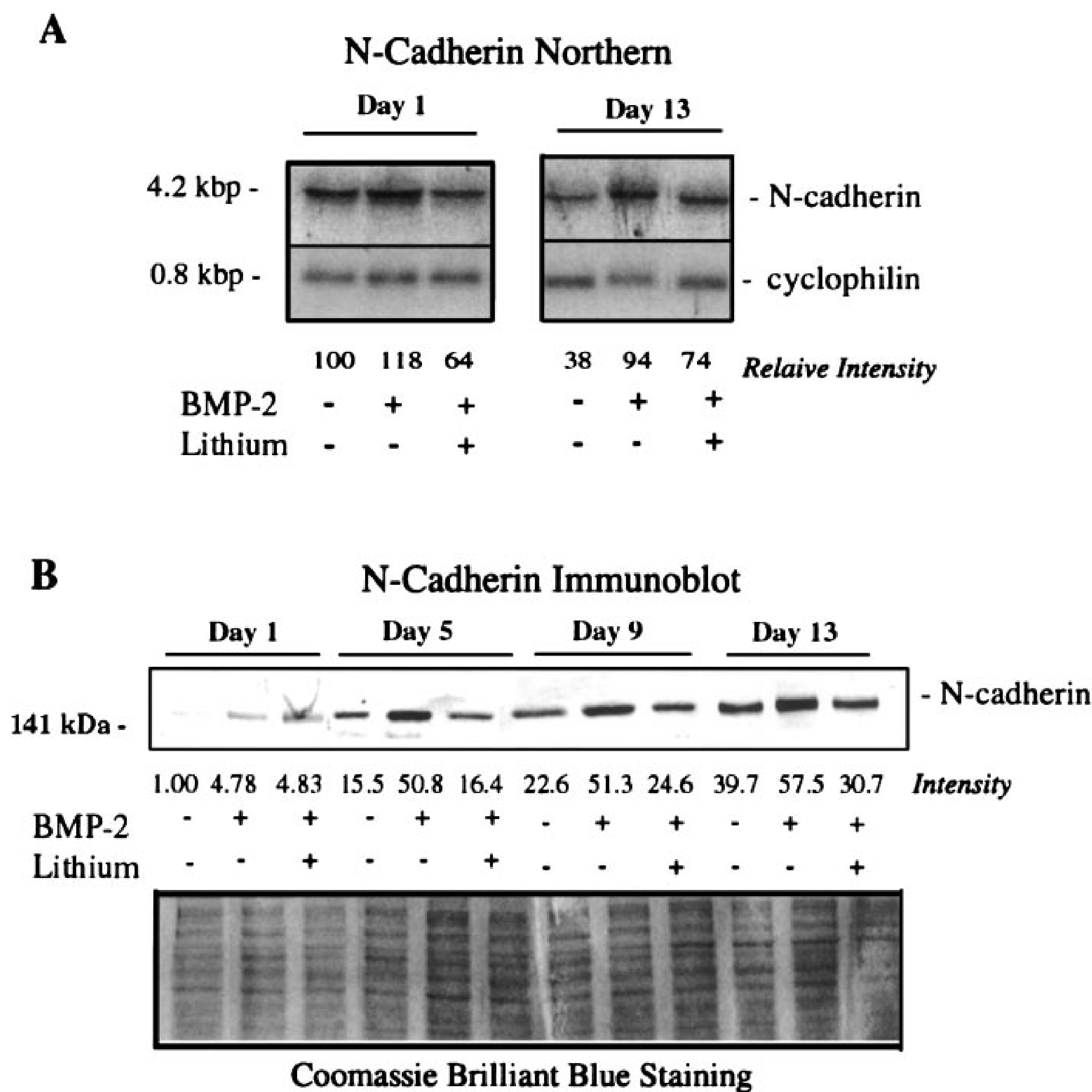


Fig. 5. Lithium co-treatment decreases mRNA and protein levels of N-cadherin in BMP-2 stimulated C3H10T1/2 micromass. **A**: Northern analysis of total mRNA isolated from lithium and BMP-2 treated C3H10T1/2 micromass cultures. Lithium treatment decreases BMP-2 mediated increase in N-cadherin mRNA level as early as day 1 and as late as day 13. mRNA loading is normalized on the basis of cyclophilin mRNA, and N-cadherin transcript signals are presented as percentage of day 1 untreated cultures. **B**: Immunoblot analysis of whole cell extracts immunoprobed for N-cadherin. While BMP-2 increases N-cadherin protein levels over control untreated cultures at all time points, lithium co-treatment downregulates BMP-2 stimulated N-cadherin protein levels on days 5–13. A parallel SDS–PAGE gel was stained with Coomassie Brilliant Blue for loading comparison, and densitometric data are presented as percentage of day 1 untreated cultures.

nuclear level of β-catenin beginning on day 9, lithium co-treatment suppressed the BMP-2 effect, resulting in nuclear levels of β-catenin similar to those in control untreated cultures by day 13. These effects were confirmed by immunoflorescence localization of β-catenin (Fig. 8B). Thus, day 1 cultures displayed almost no β-catenin within DAPI stained nuclei regardless of treatment (Fig. 8B, arrows). However, day 5 cultures treated with BMP-2 showed an early nuclear localization of β-catenin, an effect suppressed upon lithium co-treatment (Fig. 8C, arrows).

Other candidate regulatory agents involved in Wnt signaling include APC and GSK-3β that in complex are both known to decrease levels of β-catenin via tagging the molecule for ubiquitination and degradation [Cook et al., 1996; Aberle et al., 1997]. In Figure 9A, GSK-3β immunoprecipitates probed for β-catenin

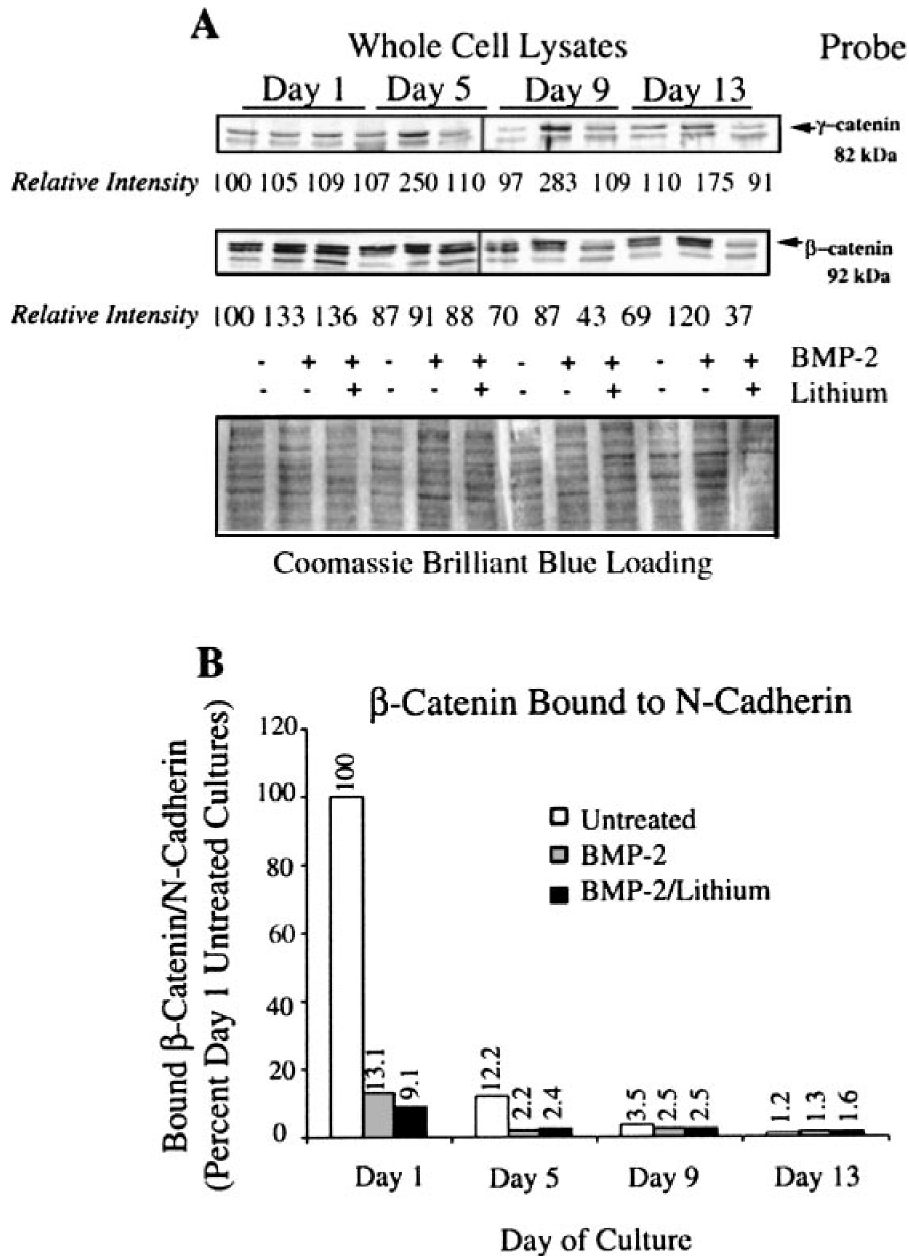


Fig. 6. Lithium co-treatment affects catenin levels in BMP-2 stimulated C3H10T1/2 micromass cultures. **A**: Immunoblot analysis of catenins in whole cell lysates isolated from micromass cultures. BMP-2 treatment increases the level of the largest isoform of both γ- and β-catenin between days 5–13. However, lithium co-treatment abrogates the ability of BMP-2 to protect the levels of both γ- and β-catenin by day 13. A parallel SDS–PAGE gel was stained with Coomassie Brilliant Blue to visualize protein loading. All densitometric data are expressed as percentage of day 1 untreated protein levels and represent the largest isoform of each catenin. **B**: Analysis of N-cadherin bound β-catenin. N-Cadherin immunoprecipitates were immunoprobed for β-catenin and β-catenin levels normalized to total N-cadherin levels. Relative values are indicated as percentage of that in day 1 untreated culture. These results showed that BMP-2 treatment decreases the levels of N-cadherin bound β-catenin, an effect unchanged by lithium co-treatment.

showed that while BMP-2 treatment reduced the interaction of β-catenin with GSK-3β in young cultures (day 5), the cytosolic levels of β-catenin associated with GSK-3β in these cultures appeared to increase during late stages (days 9 and 13) of culture, an effect which was suppressed by lithium co-treatment. However, densitometric analysis, when normalized to increasing levels of GSK-3β used to immunoprecipitate β-catenin, showed that BMP-2

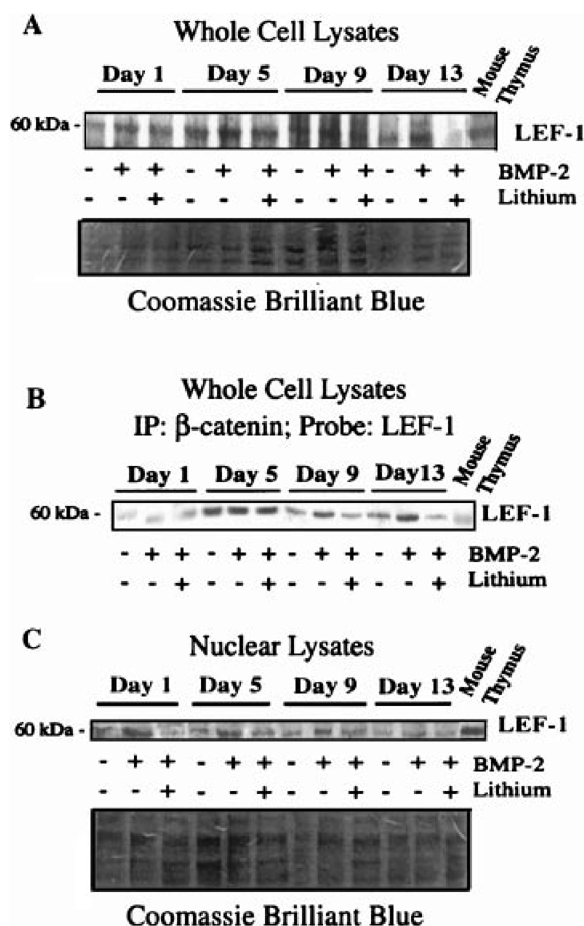


Fig. 7. Lithium co-treatment affects BMP-2 regulation of LEF-1 during chondrogenesis of C3H10T1/2 micromass cultures. **A**: Immunoblot analysis of whole cell lysates. Levels of LEF-1 are higher in all BMP-2 stimulated cultures. Protein levels appear to peak from days 5–13. Lithium co-treatment abrogates the ability of BMP-2 to increase LEF-1, and cultures display LEF-1 protein levels similar to that in control untreated cultures. A parallel SDS–PAGE gel was stained with Coomassie Brilliant Blue to visualize protein loading. **B**: β-Catenin immunoprecipitates from total cell lysates immunoprobed for LEF-1. BMP-2 increases the interaction of β-catenin with LEF-1 in late (days 9 and 13) culture periods, an effect inhibited by lithium co-treatment. **C**: Immunoblot analysis of nuclear lysates. LEF-1 protein levels within the nucleus are increased by BMP-2 treatment from days 1–13, while lithium co-treatment appears to decrease LEF-1 nuclear localization during later chondrogenesis (day 13). A parallel SDS–PAGE gel was stained with Coomassie Brilliant Blue to visualize protein loading. Positive control for LEF-1 consisted of total protein extract of adult mouse thymus, a tissue known to contain high LEF/TCF levels [Oosterwegel et al., 1993].

indeed did decrease the association of GSK-3β with β-catenin on all culture days (Fig. 9B). Since decreased interaction of β-catenin with the GSK-3β/APC complex has been shown to decrease ubiquitination and degradation of β-catenin [Aberle et al., 1997; Orford et al., 1997], the level of ubiqutin tagged β-catenin was examined by ubiquitin immunoprecipitation followed by β-catenin immunoblotting. Alhough BMP-2 treatment remarkably decreased ubiquitination of β-catenin during early stage of culture (i.e., day 5), neither BMP-2 nor lithium co-treatment appeared to further modify ubiquitination of β-catenin in older cultures (Fig. 9C).

DISCUSSION

In this investigation, we have examined the relationship between BMP-2 stimulation of mesenchymal chondrogenesis and activities of the Wnt signaling pathway. Our results show that BMP-2 stimulation of chondrogenesis in high-density micromass cultures of C3H10T1/2 cells results in repression of Wnt-7A mRNA level, specifically during late chondrogenesis (days 9 and 13). That BMP-2 downregulates this Wnt family member is consistent with previous reports that Wnt-7A inhibits chondrogenesis when introduced into micromass cultures of embryonic limb mesenchymal cells [Rudnicki and Brown, 1997; Tufan and Tuan, 2001]. Interestingly, BMP-2 treatment also upregulates the mRNA levels of Wnt-3A during early chondrogenesis (days 1 and 5). Together these results suggest that BMP-2 may act on mesenchymal chondrogenesis in part via the modulation of the Wnt signaling pathway.

To further analyze the importance and specificity of Wnt signal transduction in BMP-2 mediated stimulation of chondrogenesis, we have used lithium chloride as a Wnt mimetic by virtue of its reported non-competitive inhibition of GSK-3β kinase activity [Hedgepeth et al., 1997]. Our results clearly show that lithium co-treatment severely inhibits chondrogenesis beginning on day 1 of culture at levels as low as 5 mM, and appears to do so via inhibition of GSK-3β activity rather than via inositol signaling pathways. GSK-3β enzyme activity has been reported to be regulated as a function of serine and tyrosine phosphorylation [Hughes et al., 1993; Wang et al., 1994], and is likely to be regulated in a similar manner here. In addition, GSK-3β actively autophosphorylates in the presence of BMP-2, and the activity is inhibited in the presence of lithium, most likely due to lithium mediated inactivation of the molecule. These results would suggest that modulation of

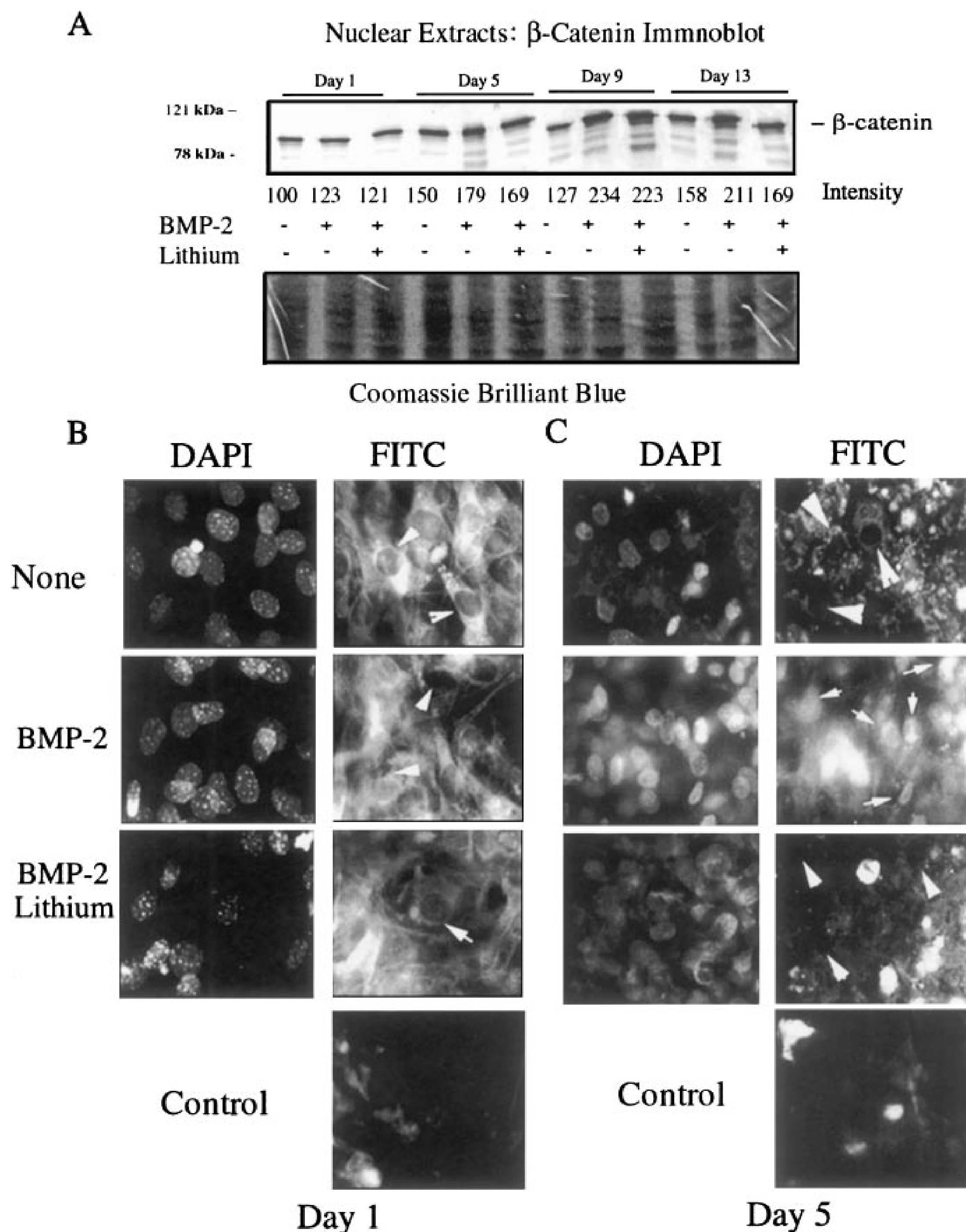


Fig. 8. Lithium does not induce β-catenin nuclearization during chondrogenesis of C3H10T1/2 micromass cultures. **A**: Immunoblot analysis of nuclear lysates from micromass cultures in the presence of BMP-2 and/or lithium. Neither lithium nor BMP-2 regulates nuclear levels of β-catenin on culture day 1. However, the largest isoform of β-catenin within the nucleus is increased upon BMP-2 addition beginning on day 9 and continuing to day 13 of culture. Lithium co-treatment decreases BMP-2 mediated nuclear localization of β-catenin in older (day 13) cultures. A parallel SDS–PAGE gel was stained with Coomassie Brilliant Blue to visualize protein loading. All densitometric data are presented as percentage of untreated day 1 values and represent the largest isoform of β-catenin. **B**, **C**: Immunofluorescence localization of β-catenin within BMP-2 and lithium treated micromass. Cultures were immunostained for β-catenin using FITC-conjugated antibodies (arrows) and nuclei were stained using DAPI (arrowheads). Day 1 cultures lack β-catenin staining (FITC, green) within DAPI stained nuclei (blue) regardless of treatment (B, arrows). However, on day 5 of culture (C, arrows), BMP-2 treatment induces a dramatic increase in nuclear β-catenin (green) compared to untreated or lithium co-treated cultures. Control cultures were treated with secondary antibody (FITC conjugated anti-mouse IgG) in the absence of β-catenin primary antibody.

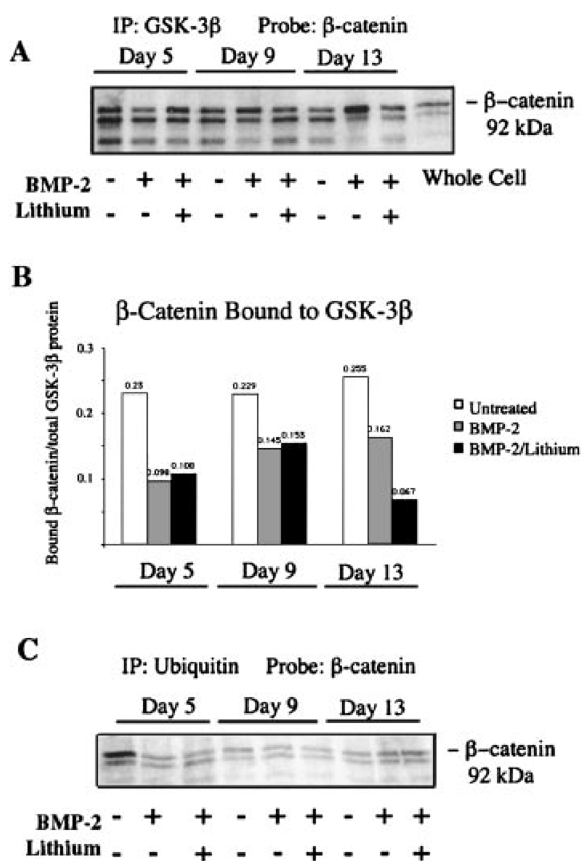


Fig. 9. BMP-2 and lithium co-treatment modifies β-catenin ubiquitination and interaction with GSK-3β in C3H10T1/2 micromass cultures. **A**: Immunoprecipitates of GSK-3β immunoprobed for β-catenin. Control, untreated cultures initially display high level of interaction of β-catenin with GSK-3β that declines as a function of time, while BMP-2 treated cultures initially display low interaction of β-catenin with GSK-3β that appears to increase temporally. Lithium co-treatment modifies the interaction of β-catenin with GSK-3β to levels similar to those found in control cultures. **B**: β-Catenin bound to GSK-3β normalized to GSK-3β protein levels in BMP-2 and lithium co-treated cultures. All densitometric data of bound β-catenin are normalized to total GSK-3β levels (see Fig. 3). Relative to increasing levels of GSK-3β, the association of β-catenin with GSK-3β is reduced by BMP-2 addition to micromass throughout the culture period. Lithium co-treatment only affects β-catenin/GSK-3β interaction on the final day of culture, day 13. **C**: Immunoprecipitates of ubiquitin tagged proteins immunoprobed for β-catenin. Total β-catenin ubiquitination is decreased in BMP-2 treated cultures initially (day 5), but is unaffected in older cultures. Lithium co-treatment does not appear to overtly modify BMP-2's initial reduction in ubiquitination of β-catenin.

β-catenin subcellular compartmentalization, which depends on GSK-3β activity, is crucial in BMP-2 induced chondrogenesis.

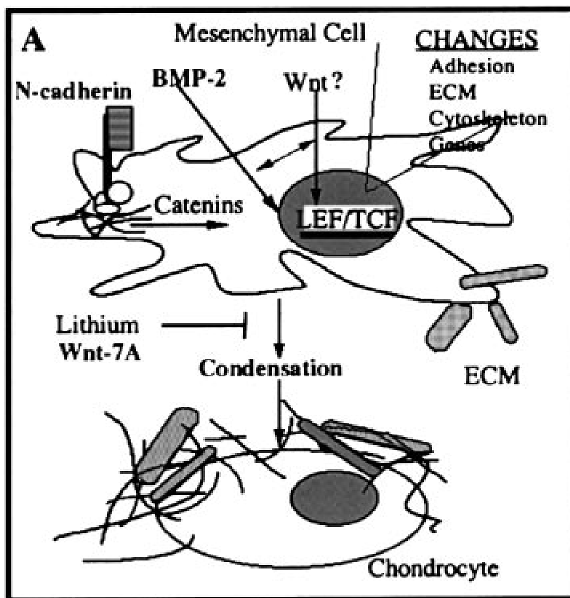
Interestingly, BMP-2 treatment of C3H10T1/2 cultures results in a moderate increase in the protein level of GSK-3β, which is enhanced upon lithium co-treatment. We believe that this phenomenon represents a feedback response in part to overcome lithium-mediated inhibition of the enzyme. Since BMP-2 increases the level of GSK-3β without concomitant reduction in the level of β-catenin protein, BMP-2 induction of chondrogenesis in our system may involve GSK-3β activity in pathways unrelated to β-catenin regulation. There is recent evidence suggesting the selective regulation of GSK-3β; for example, axin-dependent phosphorylation of β-catenin, which is catalyzed by GSK-3β, can be inhibited selectively by peptides having no effect on the ability of GSK-3β to phosphorylate other substrates [Thomas et al., 1999]. Similarly, inhibition of GSK-3β by insulin-stimulated Akt blocks the ability of GSK-3β to phosphorylate glycogen synthase without effecting β-catenin or LEF-1 activation of genes [Yuan et al., 1999]. Also, some substrates of GSK-3β require prephosphorylation [Plyte et al., 1992], whereas β-catenin does not [Yost et al., 1996].

Our previous studies have shown that the expression and activity of N-cadherin is crucially required during the mesenchyaml condensation phase of chondrogenesis [Oberlender and Tuan, 1994a,b, Denker et al., 1999; Haas and Tuan, 1999; Woodward and Tuan, 1999]. Lithium co-treatment of micromass cultures abrogates the ability of BMP-2 to stimulate N-cadherin levels and enhance cellular condensation, thus leading to lack of chondrogenesis. These results suggest that one mode of Wnt inhibition of chondrogenesis may be to decrease cellular adhesion, and that BMP-2 may overcome chondroinhibitory Wnt signal via N-cadherin upregulation and changing the cytosolic availability of catenins. BMP-2 could stimulate chondrogenesis by increasing N-cadherin expression and modifying the cytosolic availabilty of catenins, thereby, overcoming the apparent chondroinhibitory effect of Wnt-like signals such as lithium that act otherwise to reduce cell adhesion. In fact, recent evidence shows that increasing levels of both N- and E-cadherin can efficiently inhibit signal transduction via β-catenin and LEF-1 [Sadot et al., 1998].

Remarkably, lithium co-treatment of micromass cultures severely decreases the ability of BMP-2 to protect γ- and β-catenin protein levels over the course of chondrogenesis. Previous reports have shown that inhibition of GSK-3β

by lithium does not always accumulate β-catenin or activate LEF-1 dependent transcription [Yuan et al., 1999, Schmidt et al., 2001]. Therefore, given that lithium co-treatment does not result in β-catenin accumulation in BMP-2 treated C3H10T1/2 cells, and this does not emanate from changes in its association with N-cadherin, we suggest that GSK-3β inhibition is not the only determinant of β-catenin degradation. In fact, recent studies have shown the necessity of the scaffold protein axin, which bridges GSK-3β and APC to β-catenin, to negatively regulate Wnt-3A signaling [Kishida et al., 1999]. Some Wnt signals may require dissociation of all of these factors along with complete GSK-3β inactivation via several mechanisms [Kishida et al., 1999; Chen et al., 2000].

There is additional evidence supporting the apparently partial Wnt mimetic nature of lithium, i.e., lithium-induced decreased interaction of LEF-1 with β-catenin. Therefore, lithium could mimic Wnts that act to partially inhibit GSK-3β and do not accumulate β-catenin, leading to transcriptional changes that are chondroinhibitory. On the other hand, during later stages of chondrogenesis, lithium co-treatment of BMP-2 treated cultures abrogates cytosolic GSK-3β/β-catenin interaction, an activity associated with canonical Wnt signal. However, since lithium treatment obviously does not accumulate nuclear or total β-catenin, this decreased β-catenin/GSK-3β interaction suggests that in the presence of lithium, there is an alternative mechanism for destruction of β-catenin. Indeed, in support of this hypothesis, ubiqutination of β-catenin, which is decreased in younger cultures by BMP-2 treatment, is unaffected by lithium. We suggest that β-catenin in lithium co-treated cultures still undergoes proteolysis mediated degradation, and recent reports do in fact indicate that β-catenin is still proteasome sensitive regardless of inactivation of GSK-3β and APC [Bonvini et al., 2000].



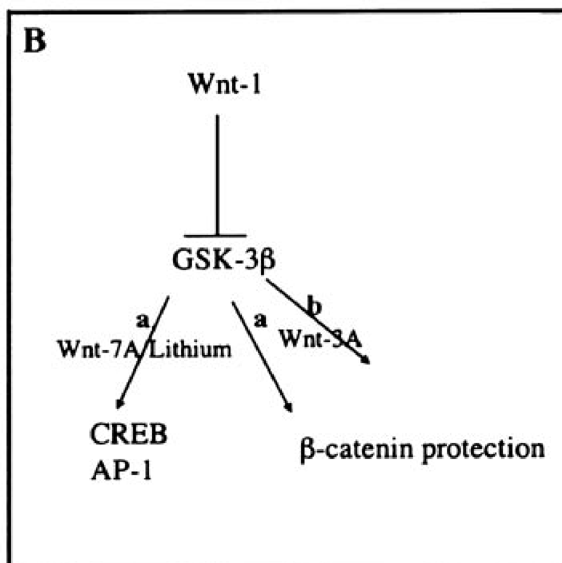


Fig. 10. Model of lithium inhibition of BMP-2 mediated chondrogenesis. **A**: BMP-2 stimulation of mesenchymal cells induces upregulation of adhesion molecules such as N-cadherin, and extracellular matrix molecules such as collagen type II. This effect may act in synergy with β-catenin protective Wnts that allow nuclear accumulation of β-catenin, and in opposition to chondroinhibitory Wnts such as Wnt-7A. The final result is the promotion of mesenchymal condensation and differentiation into the rounded phenotype typical of mature chondrocytes. Lithium may act as a chondroinhibitory Wnt via downregulation of N-cadherin and modification of both the cadherin–catenin complex and gene expression via β-catenin/LEF-1 or other transcription complexes. **B**: Lithium mimics chondroinhibitory Wnt-7A. The Wnt-1 family, which contains the chondroinhibitory Wnt-7A and the chondro-enhancing Wnt-3A (Fischer et al., unpublished results), acts by protecting β-catenin through inhibition of GSK-3β kinase activity. Our results suggest that lithium may mimic chondro-inhibitory Wnt-7A signal and partially inhibit GSK-3β activity (pathway a), but is insufficient for accumulation of β-catenin. We suggest that lithium/Wnt-7A inhibition of GSK-3β represents a category of Wnt-1 signal that does not accumulate β-catenin. Furthermore, β-catenin protection via chondro-enhancing Wnts, such as Wnt-3A, requires a second signal (pathway b) such as dissociation from the APC/axin destruction complex or inhibition of a second negative regulator of β-catenin, in order to protect the molecule to elicit nuclear signaling.

There is mounting evidence that Wnt-7A and lithium signal in the same fashion, e.g., promoting both chondroinhibition in in vitro micromass cultures [Rudnicki and Brown, 1997; Tufan and Tuan, 2001] as well as axonal spreading in nerve cells, effects which appear to be mediated via inhibition of GSK-3β activity [Lucas et al., 1998]. However, in vivo, Wnt-7A, expressed in dorsal limb ectoderm, does not accumulate β-catenin [Kengaku et al., 1998] similar to our results of lithium co-treated C3H10T1/2 cultures. Wnt-3A, however, which is expressed exclusively in mouse AER [Kengaku et al., 1998], signals through β-catenin nuclearization [Kengaku et al., 1998] and accumulates β-catenin in BMP-2 stimulated C3H10T1/2 micromass cultures (Fischer et al., unpublished results). This suggests that Wnt-7A is chondro-inhibitory and that its level is lowered by BMP-2 treatment, while Wnt-3A may be a positive modulator of chondrogenesis upregulated by BMP-2 and leading to β-catenin protection. Therefore, in the C3H10T1/2 micromass culture system, lithium is likely to mimic Wnt-7A type inhibiton of GSK-3β, rather than Wnt-3A, and may be chondroinhibitory via excessive reduction of cell–cell adhesion leading to lack of condensation and chondrocyte differentiation (Fig 10A).

Recent data indicate that inhibition of GSK-3β is insufficient to transmit all Wnt signals [Yuan et al., 1999] and full enzymatic inactivation may require both lithium-like inhibition of the molecule as well as PKC mediated inhibition of GSK-3β [Chen et al., 2000]. It has also been shown that activation of specific Wnt signaling pathways [reviewed in McEwen and Peifer, 2000] are cell type specific or can diverge at GSK-3β [Staal et al., 1999]. Our findings suggest that Wnt signaling through GSK-3β may have roles unrelated to β-catenin accumulation, and that BMP-2 may overcome chondroinhibitory Wnt signal by upregulation of adhesion molecules and enhanced mesenchymal condensation (Fig. 10A). We propose that regulation of GSK-3β activity by BMP-2 is a component in protection of β-catenin and induction of chondrogenesis, but alone is insufficient to accumulate β-catenin; hence BMP-2 also mediates upregulation of Wnt-3A. Wnt signal transduction is likely to involve a second mechanism to ensure β-catenin protection, a function which lithium does not sufficiently fulfill (Fig. 10B). However, that lithium does inhibit BMP-2 induction of chondrogenesis, and has been shown to mimic the activity of Wnt-7A [Lucas et al., 1998], strongly suggests significant interplay between Wnts and BMP-2 signals. We are currently investigating the possible positive regulatory role of Wnt-3A during BMP-2 induction of mesenchymal chondrogenesis.

ACKNOWLEDGMENTS

The authors thank Dr. David Hall, Alyssa Carlberg, and Cynthia Coleman for comments and invaluable discussion; Dr. K. Knudsen for the 13A9 antibody; Dr. M. Takeichi for supplying the mouse N-cadherin cDNA; Dr. A. McMahon for mouse Wnt cDNAs; and Dr. R. Grosschedl for the LEF-1 antibody. This work is supported in part by NIH grants ES07005, DE12864, AR39740, and AR45181. L.F. was supported in part by the Foerderer Fellowship of Thomas Jefferson University.

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