Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 301 (2003) 84–91
BBRC
www.elsevier.com/locate/ybbrc

# Activated β-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction

Gerard Bain,*[,1] Thomas Müller,[1,2] Xin Wang,[3] and Jackie Papkoff[4]

*Aventis Cambridge Genomics Center, 26 Landsdowne Street, Cambridge, MA 02139, USA*

Received 18 November 2002

**Abstract**

Wnt glycoproteins are important regulators of cellular differentiation and embryonic development. Some Wnt proteins induce stabilization of β-catenin which cooperatively regulates gene expression with LEF/Tcf transcription factors. Here we demonstrate a direct role for β-catenin signaling in osteoblast differentiation and in BMP2-mediated signal transduction. Similar to treatment with BMP-2 protein, ectopic expression of stabilized β-catenin in C3H10T1/2 cells or activation of endogenous β-catenin signaling with LiCl induces expression of alkaline phosphatase mRNA and protein, a defined marker of early osteoblast differentiation. Unlike BMP2 protein, stabilized β-catenin does not induce osteocalcin gene expression, a marker of late osteoblast differentiation. BMP2-induced differentiation also leads to activation of endogenous β-catenin signaling thus implicating β-catenin in early steps of BMP2-mediated osteoblast differentiation. Effects of β-catenin and BMP2 on C3H10T1/2 differentiation are not completely overlapping, implying that some aspects of BMP2-induced differentiation may be mediated by β-catenin signaling and that β-catenin can also participate in non-BMP2-dependent differentiation processes.


*Keywords:* Wnt; β-Catenin; Stabilized β-catenin; GSK3; BMP2; Osteoblast differentiation; C3H10T1/2 cells

Secreted Wnt glycoproteins participate in regulation of embryonic development and cellular differentiation [1]. Several Wnt genes are expressed in developing limb and function in pattern formation and chondrogenic differentiation [2]; patterning of the developing brain and control of tissue polarity and lineage segregation are also regulated by Wnt signaling [3,4]. Wnt proteins activate signal transduction through Frizzled and LRP co-receptors and some Wnt family members induce stabilization of cytoplasmic β-catenin protein [5]. In addition to participating in cadherin mediated cell adhesion, β-catenin regulates gene expression together with LEF/Tcf transcription factors [6–8]. Mutations of β-catenin or other regulatory components of this signaling pathway have been identified in many tumor types leading to constitutively activated β-catenin signaling, an important driving force in malignant transformation of epithelial cells [9]. β-Catenin signal transduction can also be activated by growth factors such as EGF and HGF which play a role in differentiation and malignant transformation [10,11]. At the cellular level, β-catenin signaling can modulate apoptosis, migration, proliferation, and epithelial–mesenchymal transition [12–16].

TGFβ superfamily members exhibit a complex array of activities in embryogenesis, cell and tissue differentiation and proliferation [17]. The bone morphogenetic proteins BMP2 and BMP4 promote formation of bone and cartilage by stimulating differentiation of osteoblasts and cartilage cells, respectively, from undifferentiated mesenchymal cells [18]. BMP proteins signal through transmembrane receptors which possess

* Corresponding author. Fax: 1-617-374-8811.
*E-mail address:* Gerard.Bain@aventis.com (G. Bain).
[1] These authors contributed equally to this work.
[2] Present address: Kosan Biosciences, 3832 Bay Center Place, Hayward, CA 94545, USA.
[3] Present address: Biogen, Inc., 14 Cambridge Center, Cambridge, MA 02142, USA.
[4] Present address: DiaDexus, Inc., 343 Oyster Point Blvd, South San Francisco, CA 94080, USA.

0006-291X/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(02)02951-0

intrinsic serine/threonine protein kinase activity and activate a cascade of cytoplasmic and nuclear responses culminating in participation of activated Smad proteins in transcriptional regulation [17]. Both synergistic and antagonistic roles for TGFβ and Wnt signaling have been documented in developmental and genetic systems [19–22]. While these pathways are generally thought to be distinct, some Wnt proteins are implicated in osteoblast differentiation [23,24] and LDL receptor-related protein 5 (LRP5), expressed by osteoblasts, participates in bone mass accrual [25].

Here we characterize a new function for β-catenin signaling in induction of early osteoblast differentiation and provide new evidence showing that BMP2-mediated osteoblast differentiation leads to activation of β-catenin signaling.

## Materials and methods

*Cell culture and retrovirus-mediated gene expression.* C3H10T1/2 cells were grown in MEMα medium containing 10% fetal bovine serum. All experiments were performed with cells passaged less than four times.

A retroviral vector used to express stabilized S37/45Aβ-catenin in C3H10T1/2 cells was previously described [15]. For differentiation studies 14,000 C3H10T1/2 cells/well were seeded in 12-well plates. Approximately 18 h later, culture medium was replaced with retrovirus-containing culture supernatant with polybrene (6 μg/ml). The viral supernatant was replaced 24 h later with MEMα containing 2% fetal bovine serum. Where indicated, a final concentration of 500 ng/ml human recombinant BMP2 was added (provided by S. Roman-Roman and Aventis Protein Production Group). For some experiments various concentrations of LiCl were added. Cells were subsequently cultured for 6 days at 37 °C followed by analysis. Infection efficiency, monitored by FACS analysis, was routinely found to be 70–90%.

*Detection of alkaline phosphatase expression and activity.* Alkaline phosphatase was detected by staining cells using a kit according to manufacturer's directions (Sigma). A quantitative spectrophotometric assay was also used [26]. All measurements were performed on triplicate samples.

*TaqMan analysis of gene expression.* RNA was isolated from cells using a Qiagen kit according to manufacturer's protocol. RNA samples were treated with DNaseI to eliminate contaminating genomic DNA. Fluorogenic primer/probe sets (specific sequences available upon request) to detect mRNAs encoding alkaline phosphatase, osteocalcin, aP2, collagen XIα2, and SM22α were designed using Primer Express software (Applied Biosystems). Relative mRNA levels were measured by quantitative real-time PCR with an ABI 7700 instrument using manufacturers' protocols (Applied Biosystems). All measurements were performed on triplicate samples.

*Luciferase reporter gene assays.* For reporter assays, cells were transfected 24 h after retroviral infection with the β-catenin-responsive firefly luciferase reporter plasmids TopFlash (wild-type promoter) or FopFlash (mutant promoter) (obtained from H. Clevers) using Fugene transfection reagent (Roche). To control for transfection efficiency, a TK-*Renilla* luciferase control plasmid (Promega) was also included. One day after transfection, medium was replaced with MEMα containing 2% fetal bovine serum. Where indicated a final concentration of 500 ng/ml BMP2 was added. Two days later, cells were solubilized and assayed for firefly and *Renilla* luciferase activities according to manufacturer's instructions (Promega). Firefly luciferase activity was normalized to the transfection efficiency using *Renilla* luciferase activity. All measurements were performed on triplicate samples. The efficiency of retroviral infection was 60–90% while the efficiency of the subsequent reporter gene transfection was 50% or greater.

*Western immunoblot analysis.* Following treatments described above C3H10T1/2 cells were solubilized and subjected to Western immunoblot analysis as described [15]. Nitrocellulose membranes (Amersham) were probed with an antibody against β-catenin (BD Transduction Laboratories) or with a monoclonal antibody against the FLAG tag (Sigma) at the N terminus of β-catenin followed by ECL detection (Amersham). To confirm that equivalent amounts of protein were analyzed, blots were stained with Ponceau S.

## Results

### *Expression of stabilized β-catenin induces morphological differentiation of C3H10T1/2 cells*

To investigate a potential role for β-catenin in osteoblast differentiation we employed the pluripotent C3H10T1/2 cell line, a well-characterized model system with potential to differentiate in culture into osteoblast, adipocyte, chondrocyte, and muscle cell types [27,28]. The precise differentiation response depends on the inducing factor, serum concentration, and culture conditions. Under the experimental conditions used here, BMP2 treatment of the C3H10T1/2 cells leads to predominantly osteoblast and chondrocyte differentiation, which can be documented by analysis of marker-specific gene expression.

To determine the effects of β-catenin on the differentiation process, we first expressed a stabilized β-catenin protein (S37/45A) in undifferentiated C3H10T1/2 cells and monitored several lineage-specific differentiation markers. S37/45Aβ-catenin, with serine to alanine substitutions at positions 37 and 45, cannot be phosphorylated by glycogen synthase kinase 3β. Consequently, degradation via a ubiquitin-mediated process is blocked, leading to accumulation of signaling-active β-catenin [29,30]. Cells were infected with a retrovirus expressing S37/45Aβ-catenin or with a control retrovirus and then cultured with or without BMP2 protein. Cells expressing S37/45Aβ-catenin and cultured without BMP2 exhibited distinct morphological changes compared to control vector-infected cells (Fig. 1A). The cells were smaller, more elongated and, upon visual inspection, appeared to be denser. Two-dimensional cell cycle analysis of these cell populations did not reveal differences in the cell cycle distribution (data not shown), suggesting that the apparent increase in cell number could be due to anti-apoptotic or pro-survival effects of β-catenin as well as BMP2 protein [12,14].

### *Expression of stabilized β-catenin induces early, but not late, osteoblast differentiation of C3H10T1/2 cells*

Cells infected with either control retrovirus or S37/45Aβ-catenin expressing retrovirus and cultured with or

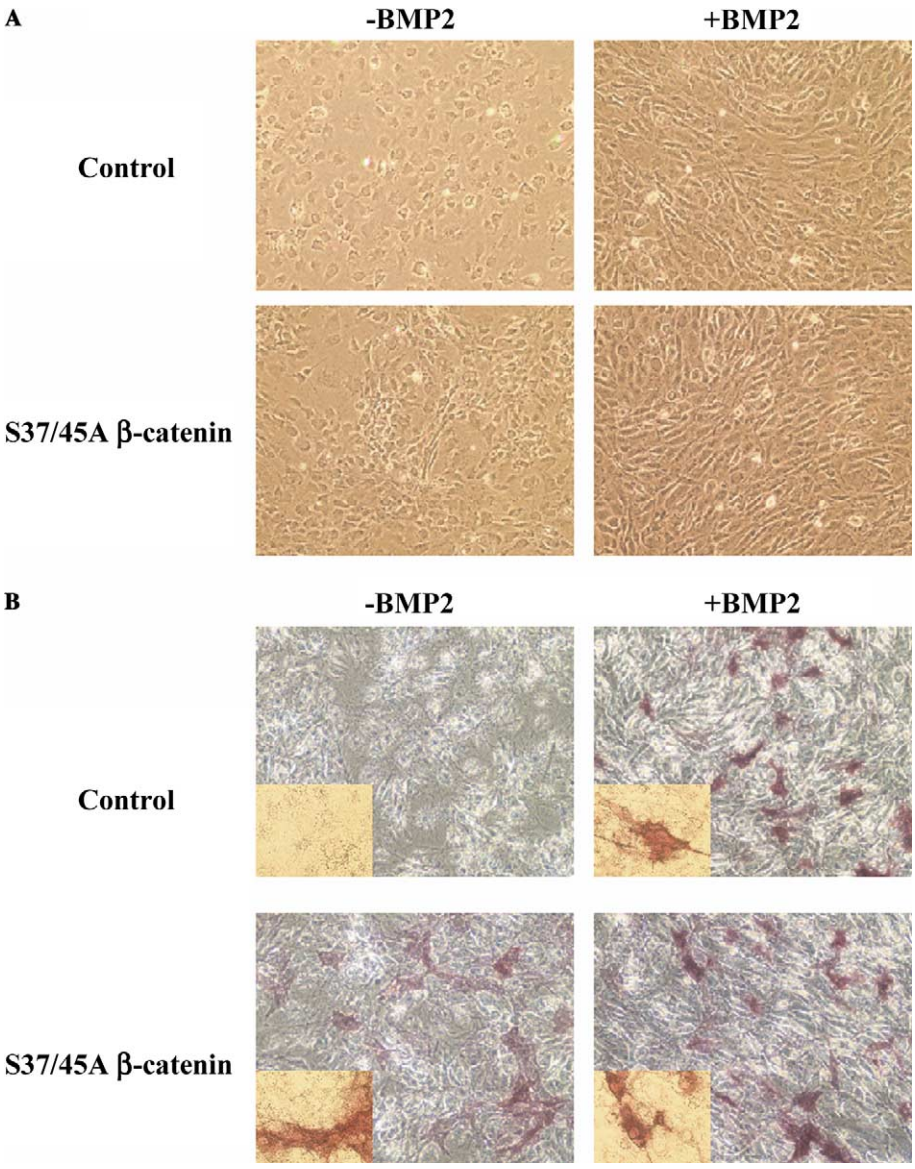


Fig. 1. Expression of stabilized β-catenin in C3H10T1/2 cells alters cell morphology and induces AP enzyme activity. Cells infected with a control retrovirus or a retrovirus expressing S37/45Aβ-catenin were cultured with or without BMP2 for 6 days and viewed by phase contrast microscopy (A) or stained for AP activity and viewed by bright-field microscopy (B). Magnification: 40× (insets: 100×).

without BMP2 were stained for the activity of an early osteoblast marker, alkaline phosphatase (AP) [31]. As expected, cultures treated with BMP2 alone contained many AP-positive cells (Fig. 1B). S37/45Aβ-catenin expression alone strongly stimulated the appearance of AP-positive cells compared to control cultures and treatment of these cells with BMP2 protein did not lead to additional AP-positive cells by visual inspection. Expression of the S37/45Aβ-catenin protein in the retrovirus-infected cells was detected by Western immunoblot analysis of cell extracts (Fig. 2). We consistently observed a decrease in S37/45Aβ-catenin protein expression in cells treated with BMP2 protein, whereas there was no effect on endogenous β-catenin (Fig. 2). Similar inhibition of LTR-driven transgene expression by various growth/differentiation factors has also been observed by others [32–34].

To confirm and quantify the AP staining observations, AP enzyme activity was measured. Cells infected with control vector expressed no detectable AP activity while BMP2 treatment induced AP activity, as expected (Fig. 3A). Expression of S37/45Aβ-catenin in the absence of BMP2 protein induced approximately fourfold more AP activity when compared to BMP2 treatment alone (Fig. 3A). Treatment of S37/45Aβ-catenin-expressing cells with BMP2 slightly reduced the AP activity when compared to S37/45Aβ-catenin alone (Fig. 3A). The reduced AP activity was presumably caused by the BMP2-mediated decrease in S37/45Aβ-catenin protein levels described above (Fig. 2).

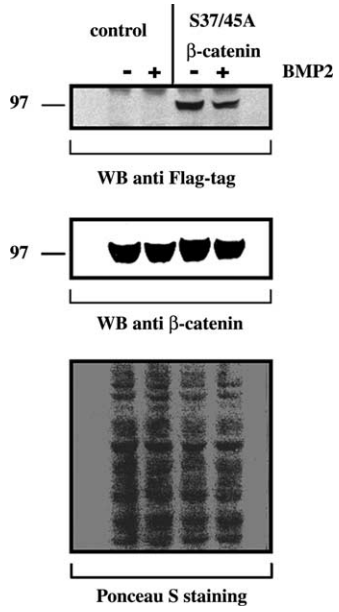


Fig. 2. Detection of stabilized β-catenin protein in C3H10T1/2 cells by Western immunoblot. Protein equivalent extracts from cells infected with control retrovirus or with a retrovirus expressing S37/45Aβ-catenin and cultured with or without BMP2 for 6 days were analyzed by immunoblot with an antibody directed against the Flag epitope tag to detect S37/45Aβ-catenin (top). Total β-catenin levels were detected with an antibody directed against β-catenin (middle). Relative amounts of protein present in aliquots of each cell extract were revealed by staining the nitrocellulose membrane with Ponceau S (bottom).

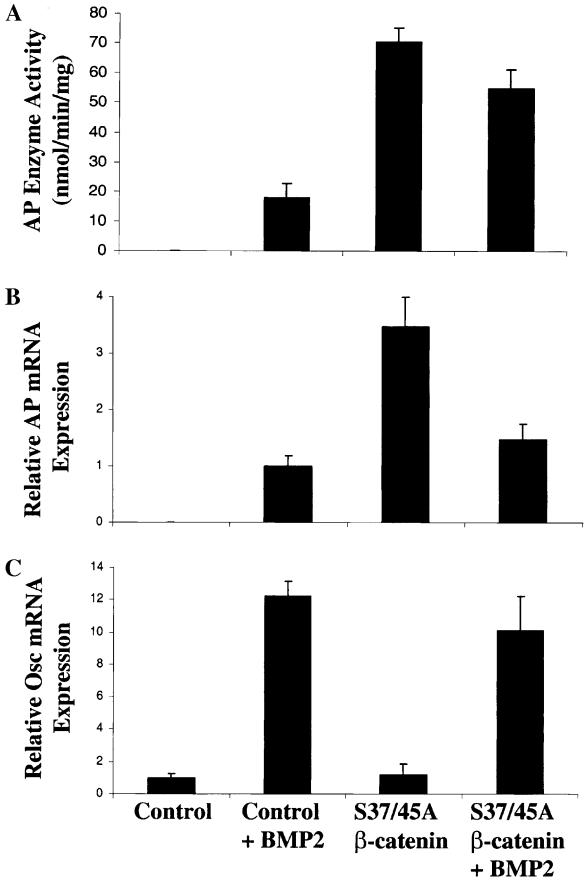


Fig. 3. Expression of stabilized β-catenin in C3H10T1/2 cells induces AP but not osteocalcin expression. Cells infected with a control retrovirus or a retrovirus expressing S37/45Aβ-catenin were cultured with or without BMP2 for 6 days. Protein extracts were prepared and AP enzyme activity was measured (A). Alternatively, RNA was isolated from the cells and relative AP (B) or osteocalcin (C) mRNA levels were measured by qPCR.

AP mRNA levels were also measured by quantitative real-time PCR (qPCR assay), showing a similar pattern to the AP enzyme activity studies. Both BMP2 and to a greater extent S37/45Aβ-catenin induce AP mRNA accumulation (Fig. 3B). Again, the combination of S37/45Aβ-catenin expression with BMP2 treatment increased AP mRNA levels but less than with S37/45Aβ-catenin alone. Taken together these findings suggest that activated β-catenin signaling can induce AP mRNA and protein expression in C3H10T1/2 cells similar to the effect of BMP2, a known inducer of osteoblast differentiation. Examination of the mouse AP promoter region did not reveal the presence of any LEF/Tcf binding sites, indicating that AP is not a direct transcriptional target of β-catenin.

To extend the analysis of bone-specific differentiation markers in C3H10T1/2 cells, qPCR was used to measure expression of osteocalcin mRNA, a bone-specific gene expressed later in the osteoblast lineage than AP [31]. As expected, BMP2 treatment strongly activated osteocalcin gene expression (Fig. 3C). However, osteocalcin mRNA levels were not upregulated in response to S37/45Aβ-catenin expression (Fig. 3C). Combination of S37/45Aβ-catenin with BMP2 treatment led to osteocalcin mRNA levels similar to BMP2 treatment alone (Fig. 3C). Together with the AP results these data indicate that expression of S37/45Aβ-catenin induces early, but not late, osteoblast differentiation markers in C3H10T1/2 cells whereas BMP2 treatment induces both.

### *Activation of endogenous β-catenin signaling induces AP expression in C3H10T1/2 cells*

Inhibition of GSK-3β activity by treatment with $Li^+$ leads to a significant increase in endogenous β-catenin signaling activity [35]. We next investigated the effect of activating endogenous β-catenin signaling with LiCl on AP expression in C3H10T1/2 cells. In preliminary experiments, C3H10T1/2 cells were cultured with various concentrations of LiCl and then stained for AP enzyme activity. While high doses of LiCl (100 mM) were toxic to the cells, lower doses were well tolerated. Staining results indicated that 10 mM LiCl induced weak but detectable AP activity while lower doses had no detectable effect on AP expression (results not shown). In subsequent experiments, RNA was prepared from C3H10T1/2 cells treated with moderate concentrations of LiCl and AP

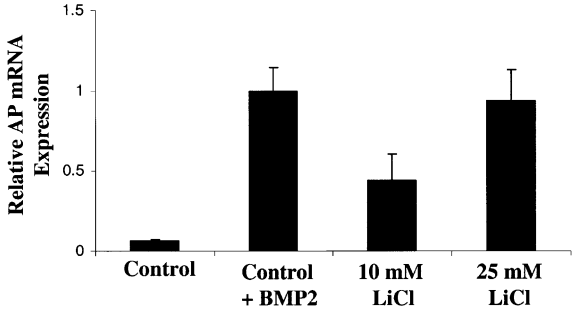


Fig. 4. LiCl treatment of C3H10T1/2 cells leads to induction of AP expression. RNA was extracted from control cells, BMP2 treated cells (positive control) or cells treated with 10 or 25 mM LiCl for 6 days. Relative AP mRNA levels were measured by qPCR.

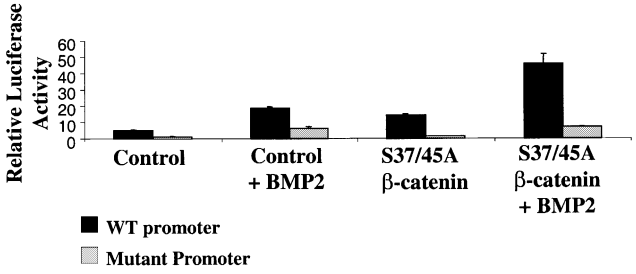


Fig. 5. BMP2 treatment activates endogenous β-catenin signaling activity in C3H10T1/2 cells. Following infection with control retrovirus or with a S37/45Aβ-catenin-expressing retrovirus, cells were transfected with TopFlash (wild-type promoter) or FopFlash (mutant promoter) reporter plasmids and cultured with or without BMP2. Luciferase activity was measured in cell lysates and normalized to a *Renilla* transfection control.

mRNA levels were measured by qPCR assay. Treatment with 10 mM LiCl induced AP mRNA and 25 mM LiCl was as effective as BMP2 in stimulating AP mRNA expression (Fig. 4). These results demonstrate a role for β-catenin signaling in osteoblast differentiation thus confirming the ectopic S37/45Aβ-catenin expression studies described above. Together our findings indicate that β-catenin signaling alone can activate expression of the osteoblast marker AP in C3H10T1/2 cells.

### *β-Catenin signaling is activated during BMP2-induced differentiation of C3H10T1/2 cells*

Based on our observations that either ectopic expression of S37/45Aβ-catenin or activation of endogenous β-catenin signaling induced early osteoblast marker expression in C3H10T1/2 cells, we hypothesized that endogenous β-catenin signaling would be activated during BMP2-induced differentiation. To address this question, a β-catenin responsive reporter gene was employed to detect activation of β-catenin signaling in BMP2-treated C3H10T1/2 cells. The reporter consists of a β-catenin-responsive promoter (TopFlash) or a mutated, nonfunctional version (FopFlash) linked to a luciferase reporter gene. Upon transfection of these constructs into cells, the levels of β-catenin signaling can be quantified by measuring the activity of the luciferase reporter gene product.

Cells infected with either control retrovirus or the S37/45Aβ-catenin expressing retrovirus (positive control) were transiently transfected with the TopFlash or FopFlash reporter plasmids and then cultured with or without BMP2 protein. To avoid gradual loss of the reporter plasmid due to cell proliferation, cell extracts were prepared 2 days after transfection and assayed for luciferase activity. As predicted, BMP2 treatment of control cells led to a threefold increase in endogenous β-catenin signaling, similar to the stimulation observed in cells infected with the S37/45Aβ-catenin retrovirus (Fig. 5). BMP2 treatment combined with S37/45Aβ-catenin expression resulted in an approximately ninefold increase in β-catenin signaling, greater than either stimulus alone (Fig. 5). These results indicate that endogenous β-catenin signaling is activated during BMP2-induced differentiation of C3H10T1/2 cells.

### *Expression of stabilized β-catenin induces muscle but not adipocyte or chondrocyte markers in C3H10T1/2 cells*

Our results show that β-catenin can activate expression of an early osteoblast marker in C3H10T1/2 cells. To investigate whether β-catenin can also induce other lineage-specific markers, expression of aP2 (adipocytes), collagen XIα2 (chondrocytes), and SM22α (muscle) was measured using gene-specific qPCR assays [36–38]. Under standard culture conditions expression of the adipocyte marker gene aP2 was detectable in untreated C3H10T1/2 cells infected with control vector (Fig. 6A). BMP2 treatment had no significant effect on aP2 expression. However, expression of S37/45Aβ-catenin strongly inhibited the basal expression of aP2. Combination of S37/45Aβ-catenin with BMP2 had no significant effect on aP2 expression compared to the control cells. The chondrocyte differentiation marker, collagen XIα2, was significantly upregulated in control cells treated with BMP2 (Fig. 6B). However, S37/45Aβ-catenin alone did not induce this marker and the combination of S37/45Aβ-catenin and BMP2 led to increased collagen XIα2 expression similar to that seen with BMP2 alone (Fig. 6B). Finally, the muscle differentiation marker SM22α was not induced by BMP2 treatment but was significantly upregulated by S37/45Aβ-catenin expression (Fig. 6C). Consistent with its known inhibitory effect on muscle differentiation [39,40], BMP2 treatment blocked the S37/45Aβ-catenin-induced upregulation of SM22α (Fig. 6C). Taken together, the differentiation marker results indicate that activated β-catenin signaling in C3H10T1/2 cells leads to early, but not late, osteoblast differentiation as well as muscle differentiation but not adipocyte or chondrocyte differentiation. On the other hand, BMP2 treatment of

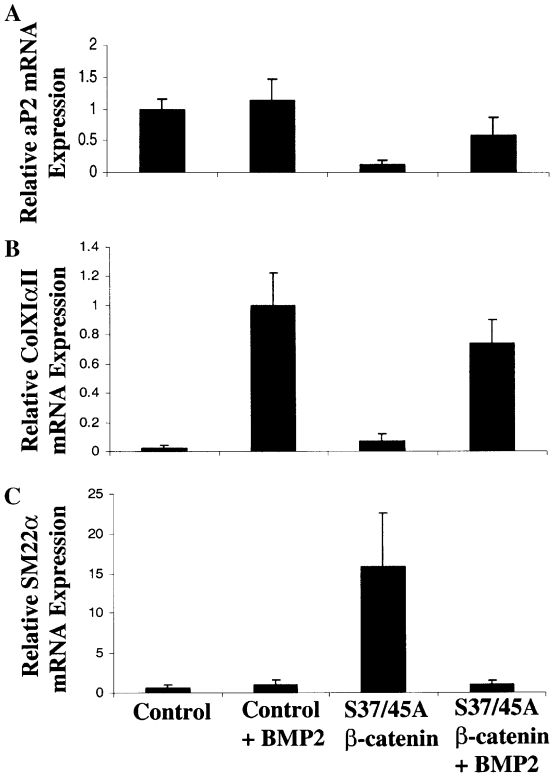


Fig. 6. Effect of stabilized β-catenin expression on lineage markers in C3H10T1/2 cells. Cells infected with a control retrovirus or a retrovirus-expressing S37/45Aβ-catenin were cultured with or without BMP2. Following RNA isolation, relative mRNA levels for aP2 (adipocyte marker) (A), collagen XIα2 (chondrocyte marker) (B), or SM22α (muscle marker) (C) were measured by qPCR.

C3H10T1/2 cells, under similar conditions, leads to both early and late osteoblast differentiation as well as chondrocyte differentiation but not adipocyte or muscle differentiation.

## Discussion

We have used the C3H10T1/2 cell culture model system to study the effects of β-catenin signaling on differentiation in comparison to BMP2 protein which induces these cells to differentiate along an osteoblast lineage. Here we provide direct evidence that BMP2 induced differentiation of C3H10T1/2 cells leads to activation of β-catenin signaling, measured by a β-catenin-dependent reporter gene assay. Furthermore, we show that ectopic expression of activated β-catenin or activation of endogenous β-catenin signaling by LiCl treatment leads to morphological changes characteristic of BMP2 induced differentiation as well as induction of AP mRNA and protein, an early marker of osteoblast differentiation [31]. These data show first that β-catenin itself can activate early osteoblast differentiation of mesodermal precursor cells and second, implicate β-catenin as a key signaling effector in BMP2-induced osteoblast differentiation. However, while BMP2 protein induced osteocalcin gene expression, a marker of late osteoblast differentiation, activated β-catenin signaling did not. These findings suggest that while β-catenin is a component of the BMP2-induced signal leading to osteoblast differentiation, BMP2 activates other non-β-catenin regulated pathways to promote the full differentiation process.

BMP2, like other TGFβ superfamily members, binds to a serine/threonine kinase receptor which activates a pathway that includes Smad protein phosphorylation and nuclear translocation [17]. While these signaling components are distinct from the known Wnt/β-catenin signal transduction pathway they may stimulate processes that cooperate with activated β-catenin to promote osteoblast differentiation. In another system, *Xenopus* β-catenin and LEF/Tcf form a complex with Smad4 and participate in synergistic activation of the twin gene during formation of the Spemann organizer [22]. Here we present an example of activated β-catenin signaling as a consequence of BMP2 activated signal transduction during bone differentiation. Based on our data we propose that β-catenin alone can activate early gene expression during osteoblast differentiation but both β-catenin and Smad proteins are needed to activate late gene expression and promote full differentiation. It is interesting to note that Smad4/DPC4 was shown to be a tumor suppressor in pancreatic cancer and colon cancer, a majority of which have constitutively activated β-catenin signaling [41]. Knockout mice lacking DPC4, when crossed with mice harboring an APC mutation leading to elevated β-catenin signaling, show enhanced tumorigenesis [42]. These and other findings support both synergistic and antagonistic roles for Wnt and TGFβ superfamily member signaling depending on context [19–22].

An early study examining effects of Wnt gene expression on C3H10T1/2 cell transformation showed that Wnt-1, Wnt-6, and Wnt-7b, but not Wnt-4 or Wnt-5b, induced morphological changes and increased cell density at confluence in 5% serum [43]. Since these Wnt proteins did not induce cell growth in soft agar the study concluded that this mesenchymal cell line responded to the Wnt gene products but was not transformed. Our data agree with these findings and now provide an explanation for the observed morphological changes. Interestingly, Wnt-1, Wnt-6, and Wnt-7b activate β-catenin signaling whereas Wnt-4 and Wnt-5B do not, again in agreement with our finding that β-catenin signaling induces early osteoblast differentiation of C3H10T1/2 cells.

We found that β-catenin signaling in C3H10T1/2 cells inhibited basal expression of aP2, a marker of adipocyte differentiation, whereas BMP2 had no effect. This is consistent with another study showing that overexpression of Wnt-1 inhibited adipocyte differentiation of the

3T3L1 cell line by inhibiting expression of the pro-adipogenic transcription factors C/EBPα and PPARγ [44]. With the C3H10T1/2 cells we also showed that activated β-catenin, but not BMP2, stimulated expression of SM22α RNA, a marker of muscle differentiation. This agrees with the documented roles of Frizzled, FRZB, and Wnt family proteins in somitogenesis and myogenesis [45]. Notably, Wnt-1 acting through the β-catenin pathway can provide a Myf5 inducing activity in the myogenic process [46].

The work presented here supports a new role for β-catenin signaling in osteoblast differentiation and in the BMP2 activated signal transduction pathway. Our findings also contribute to the emerging participation of β-catenin signaling in stem cell function and differentiation [47].

## Acknowledgments

We are grateful to Dr. Hans Clevers for providing the TopFlash/FopFlash reporter system and to Dr. Sergio Roman-Roman for generously sharing recombinant human BMP2 protein. We thank J. Bowers for FACS analysis of retrovirus-infected cells.

## References

[1] R.T. Moon, J.D. Brown, M. Torres, WNTs modulate cell fate and behavior during vertebrate development, Trends Genet. 13 (1997) 157–162.
[2] C. Hartmann, C.J. Tabin, Dual roles of Wnt signaling during chondrogenesis in the chicken limb, Development 127 (2000) 3141–3159.
[3] U. Nordstrom, T.M. Jessell, T. Edlund, Progressive induction of caudal neural character by graded Wnt signaling, Nature Neurosci. 5 (2002) 525–532.
[4] E.J. Jin, C.A. Erickson, S. Takada, L.W. Burrus, Wnt and BMP signaling govern lineage segregation of melanocytes in the avian embryo, Dev. Biol. 233 (2001) 22–37.
[5] J. Papkoff, B. Rubinfeld, B. Schryver, P. Polakis, Wnt-1 regulates free pools of catenins and stabilizes APC–catenin complexes, Mol. Cell. Biol. 16 (1996) 2128–2134.
[6] J. Behrens, J.P. Vonkries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, W. Birchmeier, Functional interaction of β-catenin with the transcription factor LEFR-1, Nature 382 (1996) 638–642.
[7] O. Huber, R. Korn, J. McLaughlin, M. Ohsugi, B.G. Hermann, R. Kemler, Nuclear localization of β-catenin by interaction with transcription factor LEF-1, Mech. Dev. 59 (1996) 3–10.
[8] M. Molenaar, M. Vandewetering, M. Oosterwegel, J. Petersonmaduro, S. Godsave, V. Korinek, J. Roose, O. Destree, H. Clevers, XTcf-3 transcription factor mediates β-catenin induced axis formation in *Xenopus* embryos, Cell 86 (1996) 391–399.
[9] M. Bienz, H. Clevers, Linking colorectal cancer to Wnt signaling, Cell 103 (2000) 311–320.
[10] T. Mueller, A. Choidas, E. Reichmann, A. Ullrich, Phosphorylation and free pool of β-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration, J. Biol. Chem. 274 (1999) 10173–10183.
[11] J. Papkoff, H. Aikawa, Wnt-1 and HGF regulate GSK3 β activity and β-catenin signaling in mammary epithelial cells, Biochem. Biophys. Res. Commun. 247 (1998) 851–858.
[12] R. Orford, C.C. Orford, S.W. Byers, Exogenous expression of β-catenin regulates contact inhibition, ancorage-independent growth, anoikis, and radiation-induced cell cycle arrest, J. Cell Biol. 146 (1999) 855–868.
[13] P. Savagner, Leaving the neighborhood: molecular mechanisms involved during epithelial–mesenchymal transition, Bioessays 23 (2001) 912–923.
[14] Z. Weng, M. Xin, L. Pablo, D. Grueneberg, M. Hagel, G. Bain, T. Mueller, J. Papkoff, Protection against anoikis and down-regulation of cadherin expression by a regulatable β-catenin protein, J. Biol. Chem. 277 (2002) 18677–18686.
[15] T. Mueller, G. Bain, X. Wang, J. Papkoff, Regulation of epithelial cell migration and tumor formation by β-catenin signaling, Exp. Cell Res. 280 (2002) 119–133.
[16] S.S. Cheon, A.Y. Cheah, S. Turley, P. Nadesan, R. Poon, H. Clevers, B. Alman, β-Catenin stabilization dysregulates mesenchymal cell proliferation, motility and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds, Proc. Natl. Acad. Sci. USA 99 (2002) 6973–6978.
[17] L. Attisano, J.L. Wrana, Signal transduction by the TGF-β superfamily, Science 296 (2002) 1646–1647.
[18] P. Ducy, G. Karsenty, The family of bone morphogenetic proteins, Kidney Int. 57 (2000) 2207–2214.
[19] S. Hoppler, R.T. Moon, BMP-2/-4 and Wnt-8 cooperatively pattern the *Xenopus* mesoderm, Mech. Dev. 71 (1998) 119–129.
[20] J.C. Baker, R.S.P. Beddington, R.M. Harland, Wnt signaling in *Xenopus* embryos inhibits BMP4 expression and activates neural development, Genes Dev. 13 (1999) 3149–3159.
[21] E. Labbe, A. Letamendia, L. Attisano, Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-β and wnt pathways, Proc. Natl. Acad. Sci. USA 97 (2000) 8358–8363.
[22] M. Nishita, M.K. Hashimoto, S. Ogata, M.N. Laurent, N. Ueno, H. Shibuya, K.W.Y. Cho, Interaction between Wnt and TGF-β signaling pathways during formation of Spemann's organizer, Nature 403 (2000) 781–785.
[23] C. Bergwitz, T. Wendlandt, A. Kispert, G. Brabant, Wnts differentially regulate colony growth and differentiation of chondrogenic rat calvaria cells, Biochim. Biophys. Acta 1538 (2001) 129–140.
[24] L. Fischer, G. Boland, R.S. Tuan, Wnt-3A enhances bone morphogenetic protein-2-mediated chondrogenesis of murine C3H10T1/2 mesenchymal cells, J. Biol. Chem. 277 (2002) 30870–30878.
[25] Y. Gong, R. Slee, N. Fykai, et al., LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development, Cell 107 (2001) 513–523.
[26] H. Harada, S. Tagashira, M. Fujiwara, S. Ogawa, T. Katsumata, A. Yamaguchi, T. Komori, M. Nakatsuka, Cbfa1 isoforms exert functional differences in osteoblast differentiation, J. Cell Biol. 274 (1999) 6972–6978.
[27] S.M. Taylor, P.A. Jones, Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine, Cell 17 (1979) 771–779.
[28] E.A. Wang, D.I. Israel, S. Kelly, D.P. Luxenberg, Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells, Growth Factors 9 (1993) 57–71.
[29] P. Morin, A. Sparks, V. Korinek, N. Barker, H. Clevers, B. Vogelstein, K. Kinzler, Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC, Science 275 (1997) 1787–1790.
[30] B. Rubinfeld, P. Robbins, M. El-Gamil, I. Albert, E. Porfiri, P. Polakis, Stabilization of β-catenin by genetic defects in melanoma cell lines, Science 275 (1997) 1790–1792.
[31] G.A. Candeliere, Y. Rao, A. Floh, S.D. Sandler, J.E. Aubin, cDNA fingerprinting of osteoprogenitor cells to isolate differentiation stage-specific genes, Nucl. Acids Res. 27 (1999) 1079–1083.

[32] R.J. Germinario, S.P. Colby-Germinario, A. Acel, R. Chandok, K. Davison, J. Mak, L. Kleiman, E. Faust, M.A. Wainberg, Effect of insulin-like growth factor I on HIV type 1 long terminal repeat-driven chloramphenicol acetyltransferase expression, AIDS Res. Hum. Retroviruses 15 (1999) 829–836.
[33] S. Ghazizadeh, J.M. Carroll, L.B. Taichman, Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy, J. Virol. 71 (1997) 9163–9169.
[34] M. Kitamura, Bystander macrophages silence transgene expression driven by the retroviral long terminal repeat, Biochem. Biophys. Res. Commun. 257 (1999) 74–78.
[35] V. Stambolic, L. Ruel, J.R. Woodgett, Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signaling in intact cells, Curr. Biol. 6 (1996) 1664–1668.
[36] D. Bernlohr, C.W. Angus, M.D. Lane, M.A. Bolanowski, T.J. Kelly, Expression of specific mRNAs during adipose differentiation: identification of an mRNA encoding a homologue of myelin P2 protein, Proc. Natl. Acad. Sci. USA 81 (1984) 5468–5472.
[37] Y. Liu, H. Li, K. Tanaka, N. Tsumaki, Y. Yamada, Identification of an enhancer sequence within the first intron required for cartilage-specific transcription of the α2(XI) collagen gene, J. Biol. Chem. 275 (2000) 12712–12718.
[38] L. Li, J.M. Miano, P. Cserjesi, E.N. Olson, SM22α, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis, Circ. Res. 78 (1996) 188–195.
[39] A. Yamaguchi, T. Katagiri, T. Ikeda, J.M. Wozney, V. Rosen, E.A. Wang, A.J. Kahn, T. Suda, S. Yoshiki, Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro, J. Cell Biol. 113 (1991) 681–687.
[40] T. Katagiri, S. Akiyama, M. Namiki, M. Komaki, A. Yamaguchi, V. Rosen, J.M. Wozney, S. Fujisawa, T. Suda, Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin, Exp. Cell Res. 230 (1997) 342–351.
[41] P. Polakis, Wnt signaling and cancer, Genes Dev. 14 (2000) 1837–1851.
[42] K. Takaku, M. Oshima, H. Miyoshi, M. Matsui, M.F. Seldin, M.M. Taketo, Intestinal tumorigenesis in compound mutant mice of both DPC4 (Smad4) and APC genes, Cell 92 (1998) 645–656.
[43] J.M. Bradbury, C.C. Niemeyer, T.C. Dale, P.A. Edwards, Alterations of the growth characteristics of the fibroblast cell line C3H10T1/2 by members of the Wnt gene family, Oncogene 9 (1994) 2597–2603.
[44] S.E. Ross, N. Hemati, K.A. Longo, C.N. Bennett, P.C. Lucas, R.L. Erickson, O.A. MacDougald, Inhibition of adipogenesis by Wnt signaling, Science 289 (2000) 950–953.
[45] G. Cossu, U. Borello, Wnt signaling and the activation of myogenesis in mammals, EMBO J. 18 (1999) 6867–6872.
[46] D.L. Shi, A. Bourdelas, M. Umbhauer, J.C. Boucaut, Zygotic Wnt/β-catenin signaling preferentially regulates the expression of Myf5 gene in the mesoderm of *Xenopus*, Dev. Biol. 245 (2002) 124–135.
[47] E. Fuchs, J.A. Segre, Stem cells: a new lease on life, Cell 100 (2000) 143–155.