Chapter 4

Apc bridges Wnt/β-catenin and BMP signaling during osteoblast differentiation of KS483 cells

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Abstract

The canonical Wnt signaling pathway influences the differentiation of mesenchymal cell lineages in a quantitative and qualitative fashion depending on the dose of β-catenin signaling. Adenomatous polyposis coli (Apc) is the critical intracellular regulator of β-catenin turnover.

To better understand the molecular mechanisms underlying the role of Apc in regulating the differentiation capacity of skeletal progenitor cells, we have knocked down Apc in the murine mesenchymal stem cell-like KS483 cells by stable expression of Apc-specific small interfering RNA. In routine culture, KSFrt-Apc<sub>i</sub> cells displayed a mesenchymal-like spindle shape morphology, exhibited markedly decreased proliferation and increased apoptosis. Apc knockdown resulted in upregulation of the Wnt/β-catenin and the BMP/Smad signaling pathways, but osteogenic differentiation was completely inhibited. This effect could be rescued by adding high concentrations of BMP-7 to the differentiation medium. Furthermore, KSFrt-Apc<sub>i</sub> cells showed no potential to differentiate into chondrocytes or adipocytes.

These results demonstrate that Apc is essential for the proliferation, survival and differentiation of KS483 cells. Apc knockdown blocks the osteogenic differentiation of skeletal progenitor cells, a process that can be overruled by high BMP signaling.
INTRODUCTION

During endochondral bone formation, skeletal progenitor cells (SPC) arise from mesenchymal cells, transit several differentiation steps to ultimately develop into bone or cartilage (1). Their commitment to one of the two lineages requires a very intricate and tightly controlled crosstalk between transcription factors, cytokines, and growth factors (2). However, the precise molecular interactions that control their lineage commitment and differentiation to mature skeletal cells are not fully understood.

Increasing evidence suggests an important role of the canonical Wnt signaling pathway in the regulation of lineage commitment of SPC (3). In this pathway, in the absence of the Wnt signal, cytoplasmic β-catenin is degraded in the proteasome upon its phosphorylation at specific Ser-Thr residues by a destruction complex consisting of Axin, Adenomatous Polyposis Coli (APC), Glycogen synthase kinase 3β (GSK3β) and Casein-kinase 1α (CK1α). Wnt growth factors bind to the receptor Frizzled and low-density lipoprotein receptor-related protein-5 or 6 (LRP-5/6) to inactivate this destruction complex, via Dishevelled (DVL). This leads to accumulation of unphosphorylated β-catenin and subsequent translocation into the nucleus. Together with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family, nuclear β-catenin stimulates transcription of Wnt target genes (4). Up-regulation of β-catenin in bi-potential SPC leads to osteoblast formation, whereas down-regulation favors their commitment to the chondrogenic lineage (5).

Another signaling cascade equally important in the differentiation of SPC is the bone morphogenetic protein (BMP)/Smad pathway which promotes both osteo- and chondrogenesis (6). In this pathway, BMPs bind to and activate BMP type I or II receptors thereby initiating phosphorylation of receptor-regulated Smads (R-Smads) 1, 5, and 8. Phosphorylated active R-Smads form heteromeric complexes with common-partner Smad4 that translocate to the nucleus to regulate the transcription of target genes in cooperation with other transcription factors (7).

Due to the great importance of the Wnt/β-catenin and BMP pathway during both osteogenic and chondrogenic differentiation of SPC, the interaction between these two powerful regulatory pathways has received much attention. For example, it has been shown that BMP-2 upregulates expression of Wnt-3a and β-catenin and that β-catenin is crucial for BMP-induced new bone formation (8,9). However, the BMP signal can also antagonize Wnt in SPC by promoting an interaction between Smad1 and Dvl that restricts β-catenin accumulation (10). These and other data suggest that Wnt and BMP signaling can alternatively synergize or antagonize one another in differentiation of SPC (11,12).

We have recently shown that, by downregulating the canonical Wnt/β-catenin signal, Apc is essential for the commitment of SPC to the chondrogenic and osteogenic lineage (13). Moreover, distinct Apc mutations unevenly affect the differentiation potential of mouse embryonic stem cells (ES): whereas Apc alleles entirely deficient in β-catenin downregulation domains block the differentiation potential of ES, more
hypomorphic alleles which are still able to partially downregulate β-catenin impair the differentiation of ES only to some tissues, e.g. bone and cartilage (14). In cells carrying a hypomorphic Apc mutation, the levels of β-catenin are upregulated only when Apc activity levels are below 2% of normal (14).

To further unravel the subtle role of Apc in the regulation of SPC differentiation, we have knocked down the mouse Apc gene using RNA interference (RNAi) in the murine mesenchymal stem cell-like KS483 cell line. This cell line shows SPC-like characteristics, since it can form osteoblasts, chondrocytes, and adipocytes (15). Our data suggest that Apc knockdown in KS483 cells leads to up-regulation not only of the Wnt/β-catenin, but also of the BMP signaling pathway, further sustaining the interaction of these biological routes during various steps of SPC differentiation. Low levels of Apc inhibited osteoblast, chondrocyte and adipocyte differentiation. Interestingly, the inhibitory effects of Apc knockdown on osteogenic differentiation could be rescued by high levels of BMP-7.

**MATERIALS AND METHODS**

**Generation of the KS483 cell lines with stable expression of Apc**<sub>si</sub>**constructs**

To obtain the KSFrt-Apc<sub>si</sub> stable cell line, the shRNA plasmid pSH1-Apc<sub>si</sub> designed to express shRNA targeting the mouse Apc gene, was constructed as described previously (15). To obtain the control, KSFrt-mtApc<sub>si</sub> stable cell line, the shRNA plasmid pSH1-mtApc<sub>si</sub> was generated by introducing mismatches at position 7 and 15 of the Apc target sequence. To demonstrate the biological reproducibility of our results, the KSFrt-Apc<sup>*</sup><sub>si</sub> and the KSFrt-mtApc<sup>*</sup><sub>si</sub> cell lines were also generated using the pSH1-Apc<sup>*</sup><sub>si</sub> and the pSH1-mtApc<sup>*</sup><sub>si</sub> plasmid (carrying mismatches at position 5 and 16 of the Apc<sup>*</sup><sub>si</sub> construct), respectively. The target sequences used to specifically silence Apc (Apc<sub>si</sub>, Apc<sup>*</sup><sub>si</sub>) and their corresponding mutant (control) sequences (mtApc<sub>si</sub>, mtApc<sup>*</sup><sub>si</sub>) are shown in Figure 1A. Stable transfections of the 4C3 Frt clone of the KS483 murine host cell line were performed as previously described (15). In this clone, a unique Flp recombinase target (FRT) sequence is introduced in the genome. This site is subsequently used for targeted insertion of the short hair pin vector using Flp-mediated homologous recombination (15).

**Cell culture**

KS483 cells were routinely cultured in T75 culture flasks (Greiner Bio-One) as described previously (16). For the KSFrt 4C3 host cell line the medium was supplemented with blasticidin S HCl (2 μg/ml; Invitrogen). All stably transfected cell lines were cultured in the presence of hygromycin B (100 μg/ml; Invitrogen).

**Immunofluorescence**

Immunofluorescence for Apc and β-catenin was performed as described previously with minor modifications (16). In brief, cells were seeded on glass slides (BD Falcon) and either left untreated or treated with Wnt3a (30ng/ml; R&D Systems) for
3hrs. The primary antibodies were rabbit polyclonal anti-Apc (1:500 in NETGEL; Abcam) and rabbit polyclonal anti-β-catenin (1:500 in NETGEL; Abcam). The second antibody used was goat anti-rabbit FITC-conjugate (1:250; Sigma). The F-actin cytoskeleton was counterstained using Phalloidin-TRITC (0.33 mg/ml; Sigma). Cells were imaged using the 63× objective of an inverted Leica SP2 confocal microscope.

Western Blot
Approximately 2 x 10^7 cells were either cultured in the control conditions for 24 hrs or with 30ng/ml Wnt3a, rinsed twice with PBS and lysed for 5 min on ice in 400 μl of Cell Lysis Buffer (Cell Signaling) and a cocktail of protease inhibitors (Roche). For detection of Apc and β-catenin proteins by Western blot, whole cell lysates were loaded on a 4–20% linear gradient Tris-HCl Gel (BIO-RAD), and transferred onto PVDF membranes (Millipore) by 1 hr electroblotting at 300 mA constant current at RT in blotting buffer (BIO-RAD). Following transfer, the membranes were blocked with 5% nonfat dry milk in TPBS (0.05% Tween 20 in PBS) for 1 hr. Incubation with primary antibodies was performed overnight at 4°C using rabbit polyclonal anti-Apc (1:100; Abcam) or mouse monoclonal anti-β-catenin (1:2000; Epitomics) antibodies. Blots were washed 3 times with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. The peroxidase was visualized and quantified by enhanced chemiluminescence using the Molecular Imager Gel Doc XR+ System (BIO-RAD).

Real-time quantitative PCR
Real-time quantitative PCR was performed using QuantiTect real-time PCR primers (Qiagen) for the detection of the mouse Apc, Ctnnb1, Axin2, Smad1, Smad3, Smad4, and Bmp7 genes and analyzed as described previously (16).

Proliferation assay
For proliferation assays, the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) was used. Cells were seeded at a density of 2,500 cells/cm². After 24, 48, 72 and 96 hours, 20 μl of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)] was added to the medium and the mitochondrial activity was measured at 490 nm after 2 hr incubation at 37°C.

Apoptosis assay
For detection of apoptotic cells, Annexin V staining was performed using Annexin V-FITC (1:250; Bender Med Systems), which specifically binds phosphatidyl serine (PS) residues on the cell membrane, and propidium iodide (PI; Bender Med Systems) at 1μg/ml which binds to DNA once the cell membrane has become permeable. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson) using the CellQuest program (FACS-Calibur, Becton Dickinson).

Luciferase transient transfection assays
The KSFrt-Apcsi and KSFrt-mtApcsi stable cells were seeded at a density of 19,000 cells/cm² and 9,500 cells/cm², respectively, in 24-well plates, and transiently trans-
fected with 2 μg of the reporter construct (pGL3-(BRE)$_2$-Luc, BAT-Luc or pSAR-MT-APC) using Fugene HD transfection reagent (Roche), according to the manufacturer’s protocol. To correct for transfection efficiency, 25 ng of Renilla luciferase (pGL4-CAAGS; Promega) was co-transfected. Twenty-four hours after transfection, transfected cells were either left non-stimulated or stimulated for an additional 24 hrs. Luciferase assays were performed as described previously (16).

**Differentiation assays**

To induce osteogenic differentiation, the KSFrt-Apc$_{si}$ and KSFrt-mtApc$_{si}$ stable cells were seeded at a density of 24,000 cells/cm$^2$ and 12,000 cells/cm$^2$, respectively, and cultured in the presence or absence of BMP-7 at the concentrations indicated. The medium was changed every 3 to 4 days. At confluence (from Day 4 of culture onward), ascorbic acid (50 μg/ml; Merck) and, when nodules appeared (from Day 11 of culture onward), β-glycerol phosphate (5 mM; Sigma) were added to the culture medium. Analysis of the Alkaline Phosphatase activity (at Day 11) and the degree of mineralization (at Day 21) was performed as previously described (17).

To induce chondrogenic differentiation, 300,000 cells were pelleted by centrifugation in a round-bottom-well of a 96-well-plate (Corning) and cultured in 250 μl high-glucose DMEM (Gibco), supplemented with 100 U/ml Pen/Strep, 50 μg/ml ascorbic acid (Merck), 40 μg/ml proline (Sigma), 1 mM Pyruvate, 1:100 ITS + Premix (BD Biosciences). During the first 2 weeks of culture, medium was further enriched with 10 ng/ml TGFβ3 (R&D Systems) and $10^{-7}$ M Dexamethasone (Sigma), while beginning with week 3, 500 ng/ml BMP-6 and 5mM β-glycerol-phosphate (Merck) was added to the medium. The medium was replaced every 3 to 4 days. After 6 weeks of culture, pellets were fixed, embedded in paraffin and sectioned. Sections were stained with Toluidine Blue or immunostained for collagen II as previously described (13). Glycosaminoglycan quantification corrected for DNA after 2, 4 and 6 weeks of culture was performed as previously described (16).

To induce adipogenic differentiation, the KSFrt-Apc$_{si}$ and KSFrt-mtApc$_{si}$ stable cells were seeded at a density of 24,000 cells/cm$^2$ and 12,000 cells/cm$^2$, respectively, and cultured in the presence of 25 μM Indomethacin after confluence (from Day 4 of culture onward). After 3 weeks of culture, cells were stained with Oil Red O as described previously (17). Quantification of adipocytes was performed by counting adipocytes, defined by the presence of at least 3 lipid droplets per cell from 9 randomly selected fields (3 fields/well) for each group.

**Statistics**

All values represent mean ± SEM of two or three independent triplicate experiments. Differences were examined by one way analysis of variance (ANOVA). Results were considered significant at p < 0.05.
RESULTS

The KSFrt-Apcsi cell line is a valid model for studying the role of Apc in SPC differentiation

To study the role of the Apc gene in regulating lineage commitment and differentiation of SPC, we generated a cell line with decreased Apc expression by RNA interference (RNAi) using the 4C3 Frt clone of the KS483 murine host cell line (18).

Overexpression of Apcsi but not of mtApcsi decreased wild-type Apc protein levels with approximately 50%, suggesting an efficient gene knockdown at the protein level (Figure 1B). KSFrt-Apcsi cells also showed less total β-catenin protein expression in comparison to control mtApcsi cells in whole cell extracts (Figure 1C). Nevertheless, total β-catenin levels were reduced in both cytoplasmic and nuclear cell fractions (data not shown). Treatment with Wnt3a did not affect the Apc expression, but upregulated β-catenin in both KSFrt-Apcsi and KSFrt-mtApcsi cells.

Figure 1. Morphological and structural characterization of the KSFrt-Apcsi cell line. (A) The Apc target sequence of Apcsi, mtApcsi, Apcsi, and mtApcsi RNAi vectors driven by the hRNAseH1 promoter used to obtain the KSFrt-Apcsi, KSFrt-mtApcsi, KSFrt-Apcsi, and KSFrt-mtApcsi cell line, respectively. (B, C) Quantified western blot analysis, performed on total protein lysates, demonstrated that KSFrt-Apcsi cells (black bars) express less Apc and β-catenin protein in comparison to control KSFrt-mtApcsi cells (white bars). (D) Bright field (upper row), IF for Apc (green; middle row) and for β-catenin (green; lower row) pictures of the control KSFrt-mtApcsi and of the KSFrt-Apcsi cells coupled with Phalloidin staining for the F-actin cytoskeleton (red). Note the spindle shape mesenchymal-like morphology of the KSFrt-Apcsi cells, expressing less Apc and mostly nuclear β-catenin. Wnt3a (30ng/ml) induced neither morphological nor structural changes in the KSFrt-Apcsi cells. Bar represents 50 μm (bright field pictures) or 20 μm (IF pictures). *p < 0.05.
The morphology of the KSFrt-\textit{Apc}\textsubscript{si} cells was considerably changed into thin, elongated, spindle shape mesenchymal-like cells in contrast to control cells that maintained the polygonal, cuboidal shape of the parental 4C3 cell line (Figure 1D, upper row). Morphology was not influenced by treatment with Wnt3a in neither of the cell lines. To investigate the cellular level and distribution of Apc and β-catenin in the KSFrt-\textit{Apc}\textsubscript{si} cells, we next performed immunofluorescence (IF) analysis coupled with Phalloidin staining for visualizing the F-actin cytoskeleton in non-confluent cultures. IF for Apc confirmed the WB results, indicating overall less Apc expression in KSFrt-\textit{Apc}\textsubscript{si} cells in comparison to control cells (Figure 1D, middle row). Wnt3a affected neither the level of Apc nor its cellular distribution in both cell lines. In control cells, β-catenin was mainly membrane-bound and cytoplasmic, while stimulation with Wnt3a induced β-catenin nuclear translocation (Figure 1D, lower row). In contrast, in the KSFrt-\textit{Apc}\textsubscript{si} cells, β-catenin was mainly present in the nucleus in both non- and Wnt3a-stimulated conditions. Similar results were obtained on confluent cultures of both cell lines (data not shown).

\textbf{Functional characterization of the KSFrt-\textit{Apc}\textsubscript{si} cell line}

Proliferation of both KSFrt-\textit{Apc}\textsubscript{si} and KSFrt-\textit{Apc}\textsuperscript{+}\textsubscript{si} cells was significantly reduced after 24, 48, 72 and 96 hrs of culture in comparison to control cells, as confirmed by MTS proliferation assay (\(p < 0.01\) at all time points; Figure 2A and data not shown). The percentage of apoptotic cells detected by Annexin V staining was significantly increased in the KSFrt-\textit{Apc}\textsubscript{si} cells as compared to control cells (18.02\% and 2.73\%, respectively, \(p < 0.05\); Figure 2B).

We next used the Wnt responsive BAT-Luc reporter construct to evaluate the effect of Apc knockdown on Wnt responsiveness (19). In basal conditions, the reporter activity was significantly increased in the KSFrt-\textit{Apc}\textsubscript{si} cells in comparison to control cells (\(p < 0.01\); Figure 2C), suggestive for increased endogenous canonical Wnt signaling. Remarkably, the response to Wnt3a was blunted in the KSFrt-\textit{Apc}\textsubscript{si} cell line. This could be due to the lower total β-catenin levels (Figure 1C) and relatively higher percentage of active β-catenin over total β-catenin which already resides in the nucleus of the KSFrt-\textit{Apc}\textsubscript{si} cells even in basal conditions (Figure 1D). We next examined whether Apc knockdown could be rescued by transient transfection of an \textit{APC} expression vector, which induces the expression of wild-type APC in the presence of \textit{ZnCl}\textsubscript{2} (20). As expected, pSAR-MT-APC induced a dose-dependent decrease in BAT-Luc reporter activity in Wnt3a-, but not in non-stimulated control cells. Wild-type APC expression in the KSFrt-\textit{Apc}\textsubscript{si} cells decreased the high basal Wnt-reporter activity dose-dependently (0\(\mu\text{M}\) vs. 100\(\mu\text{M} \textit{ZnCl}\textsubscript{2}, \(p < 0.05\)) and rescued the ability of Wnt3a to activate the BAT-Luc reporter indicative for a partial rescue of the knock down phenotype. Upregulation of the established Wnt/β-catenin target gene \textit{Axin2} at the mRNA level further confirmed the increased canonical Wnt signaling in the KSFrt-\textit{Apc}\textsubscript{si} cells (\(p < 0.01\); Figure 2D) in line with β-catenin immunofluorescence and BAT-LUC reporter assays (21).
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KSFrt-Apc<sub>si</sub> cells display an altered differentiation potential to the chondrogenic, adipogenic and osteogenic lineage

We next examined the multipotency of the KSFrt-Apc<sub>si</sub> cells. To determine the potential of KSFrt-Apc<sub>si</sub> cells to differentiate into chondrocytes, we cultured them as pellets for 6 weeks. Throughout the chondrogenic differentiation experiment, all KSFrt-mtApc<sub>si</sub> pellets remained compact spheres, whereas some of KSFrt-Apc<sub>si</sub> gradually lost their spherical shape and others disintegrated. At the end of the culture period, KSFrt-mtApc<sub>si</sub> pellets displayed a matrix rich in both Toluidine Blue-positive glycosaminoglycans (GAGs) and Collagen II protein (Figure 3A). In marked contrast, KSFrt-Apc<sub>si</sub> cells did not form a cartilage matrix and did not express Collagen II. GAG quantification corrected for DNA in pellets after 2, 4 and 6 weeks of culture confirmed these observations (Figure 3B). At all time points, we detected significantly lower GAG contents in
the KSFrt-Apc\textsubscript{m} pellets in comparison to controls (p < 0.05 at 2 weeks, p < 0.01 at 4 and 6 weeks).

The adipogenic differentiation potential of the KSFrt-Apc\textsubscript{m} cells was investigated by performing Oil Red O staining on cells cultured for 1, 2 and 3 weeks in adipogenic medium. After 3 weeks of culture, many of the KSFrt-mtApc\textsubscript{m} cells differentiated into adipocytes containing lipid droplets that positively stained with Oil Red O (Figure 3C). In contrast, differentiation of KSFrt-Apc\textsubscript{m} cells into adipocytes was severely impaired. Quantification of the number of adipocytes indicated that after 1, 2 and 3 weeks the number of Oil Red O-positive cells was significantly lower in the KSFrt-Apc\textsubscript{m} cells in comparison to controls (p < 0.05 at 2 weeks, p < 0.01 at 3 weeks; Figure 3D).
Figure 4. KSFrt-Apc<sub>si</sub> cells display an impaired osteogenic differentiation potential that can be counteracted by high concentrations of BMP-7. (A) Alp activity after 11-day-long osteoblast differentiation protocol. In the absence and at low concentrations of BMP-7, KSFrt-Apc<sub>si</sub> cells (black bars) display significantly less Alp activity in comparison to control KSFrt-mtApc<sub>si</sub> cells (white bars). When stimulated with 100 ng/ml BMP-7, KSFrt-Apc<sub>si</sub> cells show significantly increased osteogenic differentiation potential in comparison to control KSFrt-mtApc<sub>si</sub> cells. Values are expressed as fold induction of Alp activity corrected for DNA of untreated control KSFrt-mtApc<sub>si</sub> cells. (B) Representative images of Alizarin Red S-stained cultures after 21-day-long osteoblast differentiation protocol. BMP-7 stimulates the formation of mineralized nodules dose-dependently in control KSFrt-mtApc<sub>si</sub> cells, whereas KSFrt-Apc<sub>si</sub> cells show mineralized nodules only in the presence of high BMP-7 concentrations. (C) Quantification of the amount of Alizarin Red corrected for DNA. When treated with 100 ng/ml BMP-7, KSFrt-Apc<sub>si</sub> cells (black bars) show significantly increased mineral deposition in comparison to control condition. Values are expressed as fold induction of Alizarin Red S content corrected for DNA of untreated control KSFrt-mtApc<sub>si</sub> cells (white bars). *p < 0.05, **p < 0.01.
To determine the osteogenic potential of KSFrt-Apc<sub>i</sub> cells, we performed short-term osteoblast differentiation experiments. Alkaline Phosphatase (ALP) staining and its consequent quantification indicated that, in comparison to control cells, both KSFrt-Apc<sub>i</sub> and KSFrt-Apc*<sub>i</sub> cells display a significantly decreased potential to differentiate into osteoblasts (Figure 4A and data not shown). We next tested whether the inhibition of osteoblastogenesis in the KSFrt-Apc<sub>i</sub> cells could be rescued by the addition of pro-osteogenic growth factors like Basic fibroblast growth factor (bFGF), Transforming growth factor, beta 3 (TGF-β3), Parathyroid hormone-related peptide (PTHrP), Insulin-like growth factor 1 (IGF-1), and 2 members of the BMP family, BMP-6 and BMP-7. Of these, only BMP-7 (and, to a lesser extent, BMP-6) could rescue the Apc<sub>i</sub>-mediated inhibition of osteogenic differentiation (Figure 4A and data not shown). Osteoblast maturation of KSFrt-Apc<sub>i</sub> cells was investigated by alizarin Red S staining after long-term cultures to depict mineralization of the osteoblast nodules. Similar to their controls, neither KSFrt-Apc<sub>i</sub> nor KSFrt-Apc*<sub>i</sub> cells displayed mineralized nodules in the absence of BMP-7 (Figure 4B and data not shown). In contrast to KSFrt-Apc<sub>i</sub> cells, low concentrations of BMP-7 (10 and 30 ng/ml) were sufficient to induce matrix mineralization in control cells. Interestingly, high concentrations of BMP-7 (100ng/ml) efficiently induced the formation of alizarin Red S-positive nodules in the KSFrt-Apc<sub>i</sub> cells. No statistically significant difference was found when the alizarin Red S staining was quantified between KSFrt-Apc<sub>i</sub> and control cells cultured in the presence of 100ng/ml BMP-7 (Figure 4C). However, the osteoblast nodules formed by the KSFrt-Apc<sub>i</sub> cells were bigger in comparison to those formed by control cells.

Increased BMP signaling in the KSFrt-Apc<sub>i</sub> cells

We next assessed the level of BMP signaling in the KSFrt-Apc<sub>i</sub> cells by performing transient transfection assays using the BMP-responsive pGL3-(BRE)<sub>2</sub>-Luc reporter construct (22). KSFrt-Apc<sub>i</sub> cells displayed significantly increased endogenous levels of BMP signaling in comparison to control KSFrt-mtApc<sub>i</sub> cells (p < 0.01; Figure 5A). BMP-7 activated the (BRE)<sub>2</sub>-Luc reporter dose-dependently in control cells in contrast to KSFrt-Apc<sub>i</sub> cells. In these latter cells, only a high BMP-7 concentration activated the reporter compared to the control condition. The response was blunted in the KSFrt-Apc<sub>i</sub> cells compared to KSFrt-mtApc<sub>i</sub> cells (0.5-fold vs. 4-fold increase, respectively). Noggin, a potent inhibitor of the BMP signaling pathway (7), managed to decrease both the endogenous and the BMP-7-induced activity of the (BRE)<sub>2</sub>-Luc reporter in the KSFrt-Apc<sub>i</sub> cells, suggestive for autocrine stimulation of the BMP signaling pathway for example by increased expression of BMPs. Upregulation of the BMP signaling pathway in the KSFrt-Apc<sub>i</sub> cells was further confirmed at the mRNA level by quantitative RT-PCR. Smad1, Smad3, and Smad4 were significantly increased in the KSFrt-Apc<sub>i</sub> cells (p < 0.05; Figure 5B). Interestingly, Bmp7 showed a 4.4-fold higher expression at the mRNA level in the KSFrt-Apc<sub>i</sub> cells in comparison to KSFrt-mtApc<sub>i</sub> cells (p < 0.01).
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DISCUSSION

APC is a multifunctional protein involved in cell adhesion, mitosis, apoptosis, cytoskeletal organization, microtubule assembly, cell fate determination and chromosomal stability, yet it remains mostly investigated as the key intracellular gate-keeper of the canonical Wnt/β-catenin signaling pathway (23-25). In our present study, we demonstrate that Apc is required for proliferation, suppression of apoptosis and differentiation of murine mesenchymal stem cell-like KS483 cells into the osteogenic, chondrogenic and adipogenic lineage. We obtained similar results by using 2 different shRNA sequences targeting Apc, while stable transfection of the respective control mutant shRNA plasmids (containing 2 nucleotide mismatches) did not alter the proliferation, survival and differentiation capacity of KS483 cells. This clearly indicates that

Figure 5. Increased BMP signaling in the KSFrt-Apc<sub>si</sub> cells. (A) KSFrt-mtApc<sub>si</sub> (white bars) and KSFrt-Apc<sub>si</sub> cells (black bars) were transiently transfected with pGL3-(BRE)<sub>2</sub>-Luc. KSFrt-Apc<sub>si</sub> cells display increased endogenous (BRE)<sub>2</sub>-Luc activity that is enhanced by treatment with only high concentrations of BMP-7. Noggin inhibits both the endogenous and the BMP-7-induced (BRE)<sub>2</sub>-Luc activity in the KSFrt-Apc<sub>si</sub> cells. Values are expressed as fold induction of firefly luciferase activity of untreated control KSFrt-mtApc<sub>si</sub> cells. (B) Significantly increased relative mRNA expression of Smad1, Smad3, Smad4, and Bmp7 in KSFrt-Apc<sub>si</sub> cells (black bars) in comparison to KSFrt-mtApc<sub>si</sub> cells (white bars). *p < 0.05, **p < 0.01.
our results were the consequence of a bona-fide and specific siRNA effect lowering wild-type Apc expression. This was further confirmed by the partial rescue of BAT-Luc reporter activity by transient transfection of a human APC expression vector. Interestingly, KSFrt-Apc<sub>i</sub> cells displayed not only high levels of the canonical Wnt/β-catenin pathway, but also augmented BMP signaling, further sustaining the multifaceted interaction between these two signaling pathways during the differentiation of SPC.

RNAi is a complex biological mechanism during which shRNAs act either by cleavage ("slicing") or by translational repression of their target mRNA (26). KSFrt-Apc<sub>i</sub> cells showed decreased Apc expression at the protein level, thereby documenting an efficient Apc knockdown by RNAi (27;28). B-catenin protein expression was also lower in comparison to control cells, suggesting, as has been reported in other cell lines, that low levels of Apc are sufficient to downregulate β-catenin (29). Lower β-catenin expression due to Apc knockdown contrasts observations in tumors, in which Apc inactivation due to deletion or mutation is linked to increased β-catenin expression (24). In contrast to these models, KSFrt-Apc<sub>i</sub> still expresses wild type Apc albeit at lower levels. Furthermore, cells carrying hypomorphic Apc mutations show up-regulation of β-catenin levels only when the Apc activity is reduced below 2% of the normal levels (14). Interestingly, the increased activity of the BAT-Luc Wnt responsive construct in the KSFrt-Apc<sub>i</sub> cells implies a shift of the inactive/active β-catenin balance in favor of the active fraction. The partial rescue of the Apc<sub>i</sub>-induced Wnt activation after transfection with an APC expression vector demonstrates that the upregulation of the Wnt signal in the KSFrt-Apc<sub>i</sub> cells is due to Apc knockdown. We recently described that the 4C3 Frt clone of the parental KS483 murine mesenchymal progenitor line can differentiate into osteoblasts, chondrocytes and adipocytes, when cultured in the appropriate conditions and represents a valuable biological tool for the evaluation of gene function both in vivo and in vitro (15;18;30). Thus, the KSFrt-Apc<sub>i</sub> cell line is a reliable model to study the role of Apc in regulating differentiation of SPC (18). It is well established that APC modulates cell shape by organizing the cytoskeleton in particular through stabilization of microtubules (31). The KSFrt-Apc<sub>i</sub> cell line developed elongated cellular protrusions, thereby displaying a clearly distinct morphology from the control cells. In agreement with this, upregulation of the canonical Wnt signal has been shown to promote a spindle-like cell morphology (13;32;33).

It is generally accepted that Apc inhibits cell proliferation via β-catenin-dependent and -independent actions, and that inactivation of APC represents the early, initiating event in several malignant diseases (34-36). However, evidence is also available suggesting that APC is essential for cell proliferation (29). Likewise, no consensus regarding the effect of APC on apoptosis has been reached since both stimulation and inhibition of apoptosis by APC have been described (20;37-39). The role of APC in apoptosis, such as observed in the KSFrt-Apc<sub>i</sub> can be either β-catenin dependent or independent (23;38). Based on these results, we currently favor the hypothesis that Apc plays opposing roles during development and malignant transformation, by modulating cell shape, proliferation, and survival in a context dependent manner, with distinct consequences in different cell types and at different developmental stages.

The canonical Wnt/β-catenin signaling pathway governs the lineage commitment of bi-potential SPC into osteoblasts or chondrocytes (40). Roughly, it is proposed that
upregulation of this pathway induces the differentiation of SPC into precursors of the osteogenic lineage, whereas its downregulation is needed for chondrogenic differentiation (5). Data available from in vivo and ex vivo studies indicate that the osteogenic differentiation potential is altered when Apc is lacking or mutated, even if the resulting levels of β-catenin are high (13;14). Although being exposed to higher levels of transcriptionally active Wnt and BMP signaling, KSFrt-Apc<sub>si</sub> cells display a diminished osteogenic differentiation potential. Similar findings were made in conditional Apc knockout mice, in which inactivation of Apc in SPCs completely blocked osteoblast and chondrocyte differentiation particular in early stages of skeletogenesis (13). The latter study has also shown that the inhibitory phase in some skeletal elements is followed by accelerated osteoblast formation in later developmental stages (13). Complete inhibition of osteogenesis by knock down of Apc appears in contrast with increased BMD and high incidence of osteoma in FAP patients carrying a heterozygous inactivating mutation of APC (41). In addition, conditional Apc knock out using Cre expression under the influence of the Osteocalcin promoter, a late marker of osteoblast differentiation, results in increased bone formation and lack of osteoclast formation (42). Therefore we hypothesized that the inhibitory effect on osteoblast differentiation in the KSFrt-Apc<sub>si</sub> cells is cell type dependent and may be reversed by environmental factors like exposure to exogenous growth factors.

Interestingly, when the KSFrt-Apc<sub>si</sub> cells were exposed to additional high concentrations of BMP-7 and to a lesser extent BMP-6, both potent stimulators of osteogenesis (43), they displayed an increased potential to form osteoblasts in comparison to control cells. Such rescue effect was not observed when using other pro-osteogenic growth factors like bFGF, TGF-β3, PTHrP, IGF-1. One of the potential interpretations is that BMP signaling further activates canonical Wnt signaling, thus it synergistically induces the osteoblast differentiation in KSFrt-Apc<sub>si</sub> cells. Our results indicate that Apc is essential for the osteogenic differentiation of the KS483 cell line and that the noxious effect of Apc knockdown on osteogenesis can be overruled by high BMP signaling induced by BMP-7. Consistently, in vitro observations made in C3H10T1/2 cells demonstrate that canonical Wnt signaling itself is not sufficient, but in synergy with BMP signaling it can promote osteoblast differentiation (44).

Both the canonical Wnt and the BMP signaling pathway have been shown to promote osteoblast differentiation, maturation and mineralization (45). However, the complexity of the interactions between these regulatory pathways and the abundance of in vitro reports investigating this interrelation in different osteogenic experimental set-ups, complicate its understanding (9;10;44;46-48). The most probable explanation for the wide variety of effects arising upon this interaction is that they represent different aspects of Wnt and BMP functions that are only visible in certain cell types, at specific developmental stages and under particular experimental conditions. Our results add insight to the complexity of interactions between Wnt/β-catenin and BMP signaling during the differentiation of SPC. In vitro, BMPs induce Wnt expression (8;49), whereas Wnt signaling induces BMP expression (46;50), suggesting that both Wnt and BMP signaling may jointly regulate each other in osteoblasts. In the KS483 cells, Apc knockdown upregulated not only transduction of the Wnt signal, but also the BMP signaling pathway, most likely via up-regulation of Bmp7 expression. APC can
shuttle into and out of the nucleus (51;52), and thus a possible Apc-mediated interaction between Wnt and BMP may occur in any of these two subcellular locations. While in the nucleus the Smad/β-catenin/Lef protein complex regulates many shared target genes (53-55), in the cytoplasm, BMP can either impede or stimulate the canonical Wnt signal via Axin (10;56). Since Apc comprises both Axin and β-catenin binding domains, we speculate that Apc might link the Wnt/β-catenin to BMP signaling pathways during osteoblast differentiation of KS483 cells.

Our present results indicate that Apc is essential for osteogenic, chondrogenic and adipogenic differentiation of the murine mesenchymal-like KS483 cell line which has SPC-like characteristics. Our approach has provided a valuable model in which we demonstrate that levels of functional Apc must be tightly controlled for proper modulation of the transcriptionally active β-catenin and BMP-signaling dosage required for multilineage SPC-differentiation in vitro.

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