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Canonical Wnt signaling maintains the quiescent stage of hepatic stellate cells

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Abstract

It is well known that hepatic stellate cells (HSC) develop into cells, which are thought to contribute to liver fibrogenesis. Recent data suggest that HSC are progenitor cells with the capacity to differentiate into cells of endothelial and hepatocyte lineages. The present study shows that β-catenin-dependent canonical Wnt signaling is active in freshly isolated HSC of rats. Mimicking of the canonical Wnt pathway in cultured HSC by TWS119, an inhibitor of the glycogen synthase kinase 3β, led to reduced β-catenin phosphorylation, induced nuclear translocation of β-catenin, elevated glutamine synthetase production, impeded synthesis of α-smooth muscle actin and Wnt5a, but promoted the expression of glial fibrillary acidic protein, Wnt10b, and paired-like homeodomain transcription factor 2c. In addition, canonical Wnt signaling lowered DNA synthesis and hindered HSC from entering the cell cycle. The findings demonstrate that β-catenin-dependent Wnt signaling maintains the quiescent state of HSC and, similar to stem and progenitor cells, influences their developmental fate.

Keywords: HSC; Ki-67; Lef1; Pitx2; Tcf1; TWS119; Wnt

Hepatic stellate cells (HSC) carrying CD133 are undifferentiated cells capable to develop cells of endothelial and hepatocyte lineages [1]. The β-catenin-dependent or canonical Wnt signaling pathway is of functional relevance for stem cells by preventing cell differentiation and maintaining pluripotency [2,3]. Thus canonical Wnt signaling should be active in quiescent HSC. In their quiescent stage, HSC store retinoids and synthesize glial fibrillary acidic protein (GFAP). A gradual loss of retinoids and GFAP accompanies their development into myofibroblast-like cells with increased synthesis of α-smooth muscle actin (α-SMA) and extracellular matrix proteins. Due to these properties HSC are thought to play an important role in liver fibrogenesis. In fact, some elements of Wnt signaling were implicated in wound healing and fibrosis [4,5] and found to be up-regulated in HSC-derived myofibroblast-like cells [6]. However, little is known about β-catenin-dependent Wnt signaling in quiescent HSC thus far.

In absence of canonical Wnt signaling, cytoplasmic β-catenin is recruited into a protein destruction complex that facilitates phosphorylation of β-catenin by glycogen synthase kinase 3β (Gsk3β) and its proteasomal degradation (Supplemental Fig. S1A). Some Wnt ligands are capable to stimulate the canonical signaling by binding to their receptors frizzled (Fz) and co-receptors lipoprotein receptor-related protein 5/6 (Lrp5/6). Wnt binding leads to phosphorylation of dishevelled, a downstream scaffold protein of Wnt signaling, and disruption of the β-catenin destruction complex. Wnt signaling finally results in inhibition of Gsk3β activity, reduced proteolysis of β-catenin, and translocation of β-catenin into the nucleus. Nuclear β-catenin alters expression of Wnt target genes by binding to the transcription factors lymphoid enhancer binding factor (Lef) and T-cell specific transcription factors (Tcf) (Supplemental Fig. S1B) [7]. To analyze the existence and

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function of the canonical Wnt pathway in HSC, the synthetic substance TWS119 was used in the present study to inhibit Gsk3β activity and mimic canonical Wnt signaling [8].

Materials and methods

Isolation, culture, and experimental treatment of HSC. Stellate cells from the liver of male Wistar rats (500–600 g body weight) were isolated by serial perfusions with enzymes and enriched by Nycodenz density gradient centrifugation (Nycomed Pharma, Oslo, Norway) essentially as described earlier [9]. Due to their high lipid contents, HSC were enriched at the upper layer of the gradient. The HSC were cultured in Dulbeccos's modified Eagle's Medium (DMEM, Gibco, Invitrogen, Karlsruhe, Germany) containing 10% FCS (fetal calf serum; PAA Laboratories, Coelbe, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). The cells were seeded at a density of approximately 1000,000 (HSC 7d) and 2000,000 (HSC 1d) cells per culture dish (diameter 6 cm). HSC were cultured for 1 and 7 days prior treatment with TWS119 (Calbiochem, Merck Biosciences, Schwalbach, Germany). A 10 mM stock solution of TWS119 was prepared with dimethyl sulfoxide (DMSO; Sigma, Sigma–Aldrich). Control cells were treated with DMSO alone. TWS119 was applied for 4 h and 48 h under serum free conditions. Serum was only added to the experimental media to determine the DNA synthesis of HSC.

Western blot analysis. Western blot analysis of protein lysates was performed using the semidry Western blot technique according to standard protocols. To obtain nuclear protein fractions, the CNM Compartment Protein Isolation Kit (BioCat, Heidelberg, Germany) was used according to the manufacturer's recommendations. The primary antibodies against Wnt4, Wnt5a, Wnt7a/b, Wnt10b (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-SMA, actin, γ-tubulin (Sigma, Sigma–Aldrich, Taufkirchen, Germany), β-actin, β-catenin (Abcam, Cambridge, UK), phospho-β-catenin (Cell Signaling, Beverly, MA, USA), GFAP, glutamine synthetase, Ki-67 (Chemicon International, Hampshire, UK), and Pitx2a/b/c (CeMines, Golden, CO, USA) as well as appropriate secondary antibodies coupled with horseradish peroxidase (Bio-Rad, München, Germany) were used to label protein bands.

Immunofluorescence staining. HSC were cultured for 1 day on glass coverslips. After fixation with ice-cold methanol the cells were incubated with the primary antibody against β-catenin (Abcam). The purity of HSC was determined by immunofluorescence staining of GFAP (Chemicon International) and octamer binding factor 4 (Oct4; Abcam) versus DAPI staining (4′,6-diamino-2-phenylindole; ProLonged Gold; Molecular Probes, Invitrogen, Karlsruhe, Germany). The cells were subsequently incubated with anti-rabbit or anti-mouse antibodies labeled with Cy3 (Jackson Immunoresearch, West Grove, PA, USA). The fluorescence images were taken with a confocal laser scanning microscope (LSM510 META, Zeiss, Göttingen, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR). The first strand cDNA was made from 5 ng purified mRNA (Oligotex kit; Qiagen, Hilden, Germany) per 20 μl reaction volume using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The 2xPCR Master Mix (Fermentas) were used for the PCR reaction mixture. The primers (Supplemental Table S1) were applied at a final concentration of 200 nmol/l and 1 or 5 μl template cDNA was added per 25 μl reaction volume. The PCR was performed according to standard protocols. All PCR products were sequenced to verify the specificity of primer sets.

Measurement of DNA synthesis. Synthesis of DNA in response to TWS119 treatment was measured using a colorimetric BrdU cell proliferation assay (5-bromo-2′-deoxyuridine enzyme-linked immunosorbent assay; Roche, Mannheim, Germany) according to the manufacturer's recommendations. HSC were seeded into flat-bottomed 96-well culture plates (20,000 cells/well) and cultured for 1 day. The culture medium was then removed and replaced by medium containing 10% FCS, 10 μM BrdU, and 5 μM TWS119. Control cells were treated with 10% FCS and 10 μM BrdU alone. HSC were also cultured for 6 days, trypsinized, and plated into 96-well culture plates (20,000 cells/well). The cells were allowed to recover for 1 day and finally treated with the experimental media as described above. To investigate the effects of FCS on DNA synthesis, the BrdU uptake was measured after addition of 10% FCS and compared with serum free conditions. The cells were incubated with all experimental media for 48 h.

Statistics. The data were analyzed using the Student's *t*-test and considered significant at $p < 0.05$. The results of at least three independent experiments were expressed as mean values in percent relative to untreated controls and their variance was specified as standard error of mean (±SEM).

Results

Canonical Wnt signaling is active in freshly isolated HSC

The purity of HSC obtained by density gradient centrifugation was greater than 98% as analyzed by their typical stellate-like cell morphology with perinuclear lipid droplets and immunostaining of the HSC marker protein GFAP and the stem/progenitor cell marker Oct4 (Supplemental Fig. S2). Freshly isolated HSC displayed nuclear immunofluorescence staining of β-catenin (Fig. 1A and B), indicating active canonical Wnt signaling. The nuclear localization of β-catenin was further confirmed by Western blot analysis of nuclear protein fractions (Fig. 1C). During formation of myofibroblast-like cells the β-catenin synthesis was elevated in whole cell lysates (Fig. 1D), but decreased in the cell nuclei (Fig. 1C). Apart from cellular β-catenin distribution the expression of the Wnt target gene paired-like homeodomain transcription factor 2 (Pitx2) was analyzed by RT-PCR and Western blot. During formation of myofibroblast-like cells the isoform c of Pitx2 (exon 4–6 of Pitx2), decreased sharply at the protein level and a switch to another isoform of Pitx2 was detected at day 7 of culture. RT-PCR revealed that only the mRNA of the Pitx2c isoform (EF519321) was present in freshly isolated HSC, whereas the Pitx2a isoform (exon 1–2 and 5–6 of Pitx2) appeared later during culture (Fig. 1E and F). Axin2 is another Wnt target gene primarily expressed by freshly isolated HSC (Supplemental Fig. S3) that indicated active canonical Wnt signaling.

Elements of the canonical Wnt signaling pathway in HSC

Essential elements of canonical Wnt signaling comprising Wnt ligands, frizzled receptors, co-receptors and proteins involved in signal transduction and regulation of gene transcription were identified in HSC by RT-PCR (Supplemental Fig. S3). With the exception of Wnt8b all known Wnt ligands were identified in primary cultures of HSC. Interestingly, canonical Wnt ligands such as Wnt7a/b and Wnt10b were primarily expressed by freshly isolated HSC and their synthesis decreased during formation of myofibroblasts (Fig. 1E and G; Supplemental Fig. S3). In contrast to this, Wnt ligands known to stimulate β-catenin-independent or noncanonical Wnt signaling like Wnt4, Wnt5a, and Wnt11 were mainly synthesized

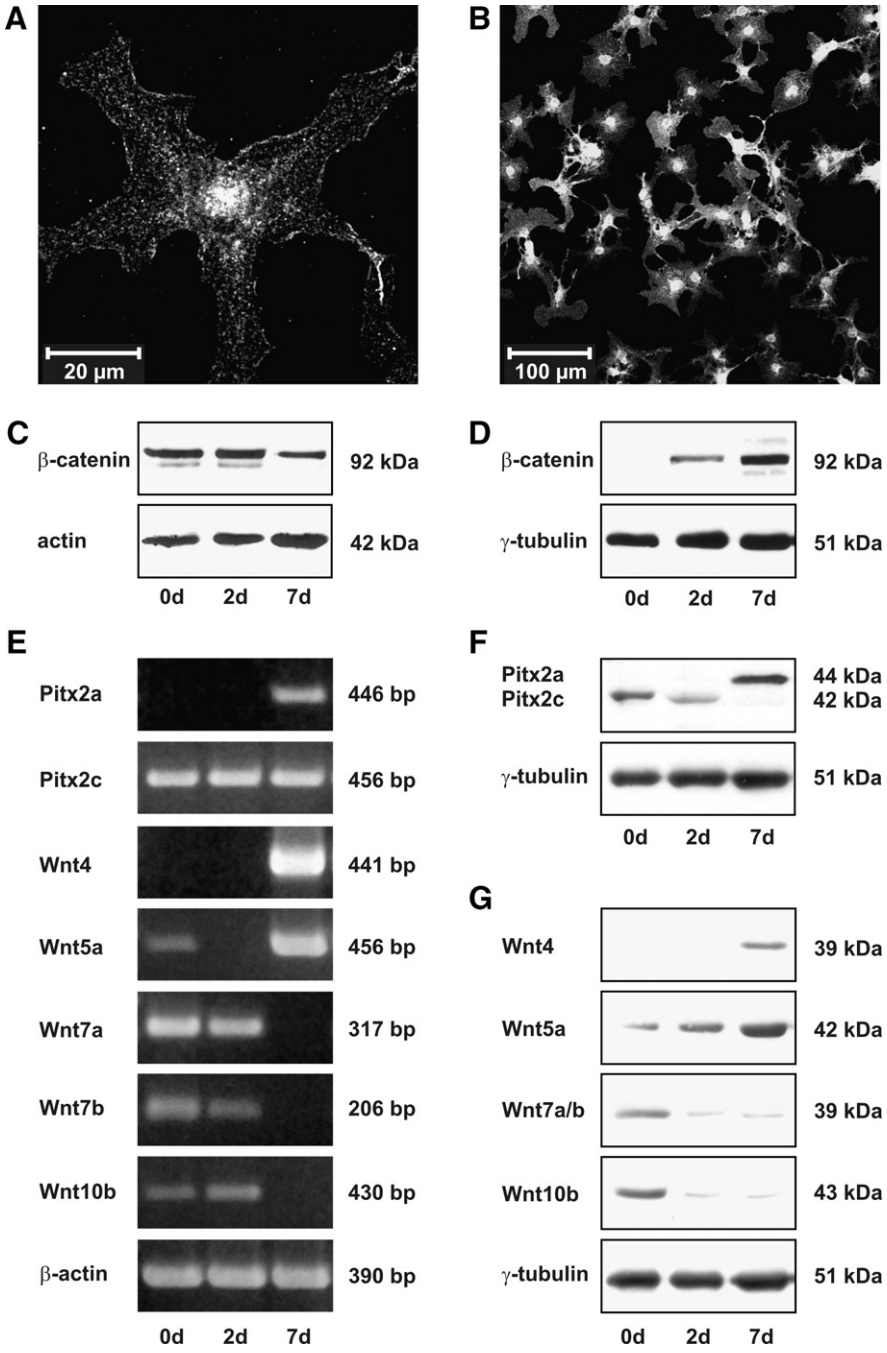

Fig. 1. Nuclear localization of β-catenin in freshly isolated HSC and analysis of Pitx2 and Wnt ligand expression in time series of cultured HSC. (A) The protein β-catenin was detected by immunofluorescence staining in the cell nucleus of freshly isolated HSC. (B) Nuclear localization of β-catenin was observed in the majority of HSC cultured for 1 day. (C) The presence of β-catenin in the cell nuclei was confirmed by Western blot of nuclear protein fractions (10 µg protein). Total actin served as a control. (D) The synthesis of β-catenin was elevated during culture of HSC as investigated in whole cell lysates (40 µg protein). The protein γ-tubulin was used as a control. (E) The expression of Pitx2 isoforms and Wnt ligands of cultured HSC was investigated by RT-PCR. The mRNA of β-actin served as a control for RT-PCR. (F) The Pitx2 isoforms (30 µg protein) and (G) Wnt ligand precursors Wnt4, Wnt5, Wnt7a/b, and Wnt10b (40 µg protein) were analyzed by Western blot.

by myofibroblast-like cells (Fig. 1E and G; Supplemental Fig. S3). RT-PCR revealed also the expression of all known Wnt receptors (Fz1-10; Fz8, EF519320) and co-receptors (Lrp5/6, Ror2, Ryk) in cultured HSC. Moreover, proteins of the β-catenin destruction complex like adenomatous polyposis coli, Gsk3β, and axin as wells as proteins involved in Wnt signal transduction like dishevelled 1–3 (Dvl2, EF613276) were detected (Supplemental Fig. S3). The expression of transcription factors of Wnt signaling such as Tcf1, Tcf3, Tcf4, and Lef1 was also detected in HSC. Tcf1 (EF519318) and Lef1 (EF519319) displayed mRNA splicing variants lacking nucleotides in the central domain responsible for binding of proteins involved in repression and activation of gene transcription. Notewor-

thy, the short Lef1 isoform (EF519319) was mainly expressed by freshly isolated HSC. This Lef1 isoform was also detected by RT-PCR in lysates of whole fetal rat brain and liver, but its expression was, if at all, only weakly detectable in liver lysates of adult rats (Supplemental Figs. S3 and S4). Inhibitory elements of Wnt signaling such as dickkopf (Dkk1–4) were detected in cultured HSC. The Dkk1 and Dkk2 expression was higher in myofibroblast-

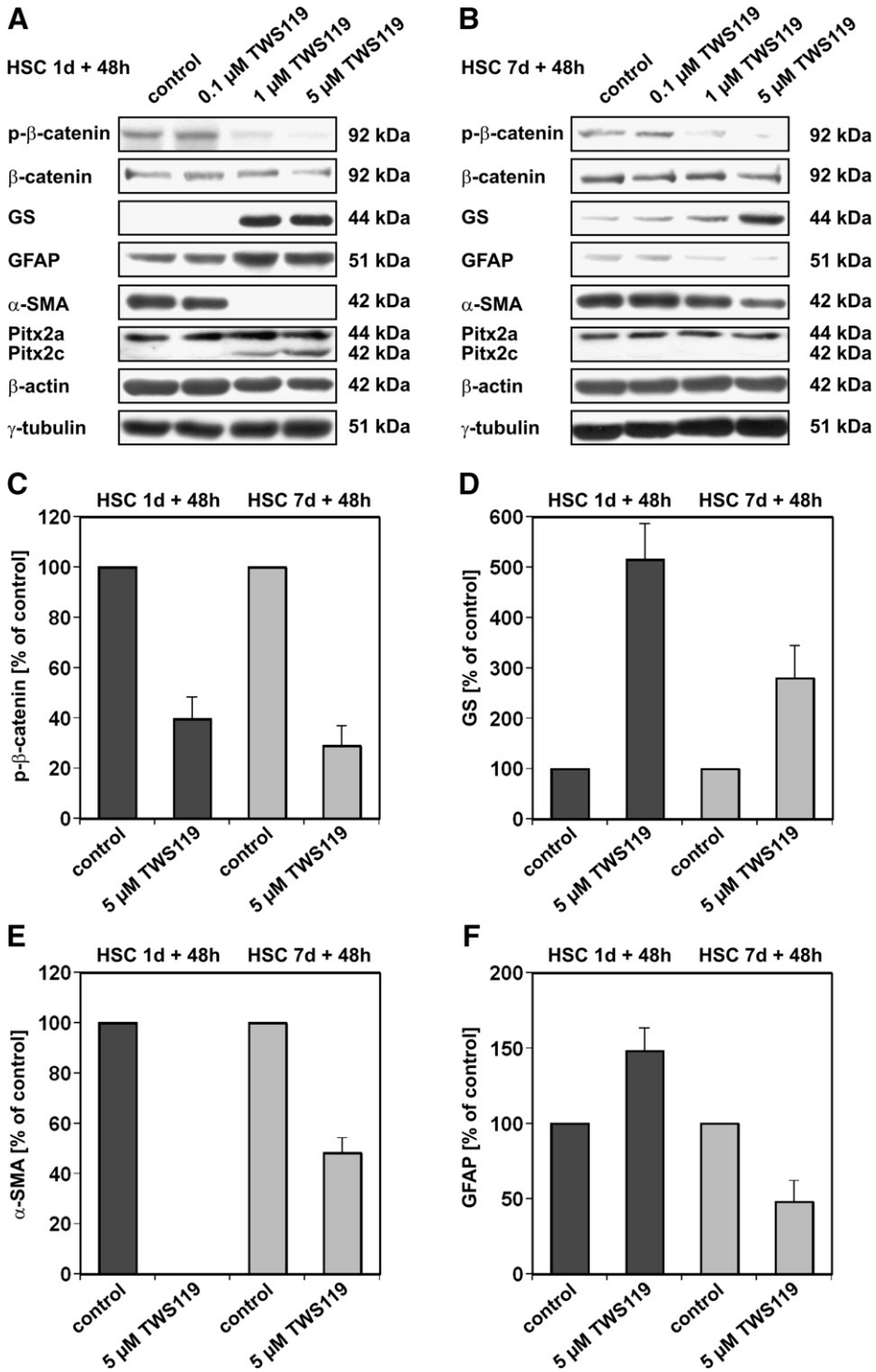

Fig. 2. Mimicking of canonical Wnt signaling by TWS119 in freshly isolated HSC and myofibroblast-like cells (HSC 7d). HSC were cultured for (A) 1 and (B) 7 days and subsequently exposed to 0.1, 1, and 5 μM TWS119 for 48 h. The proteins phospho-β-catenin (p-β-catenin; 40 μg protein), total β-catenin (10 μg protein), glutamine synthase (GS; 10 μg protein), GFAP (10 μg protein), α-SMA (5 μg protein), Pitx2 isoforms (30 μg protein), β-actin (5 μg protein), and γ-tubulin (20 μg protein) were analyzed by Western blot. Graphic illustration of changes in (C) p-β-catenin levels ($n = 6$) as well as protein synthesis of (D) GS ($n = 6$), (E) α-SMA ($n = 7$), and (F) GFAP ($n = 7$) as investigated by Western blot after treatment of freshly isolated HSC (1d) and myofibroblast-like cells (HSC 7d) with 5 μM TWS119 for 48 h.

like cells than in freshly isolated HSC, whereas Dkk4 mRNA occurred predominantly in HSC cultured for 1 day. In addition, the mRNAs of Wnt inhibitory factor 1 (Wif1) and secreted frizzled-related protein (Sfrp1–5) were found in HSC. The Wnt inhibitors Sfrp5 and Wif1 were highly expressed in myofibroblast-like cells (Supplemental Fig. S3).

Canonical Wnt signaling is mimicked by TWS119

The β-catenin-dependent signaling was mimicked by treatment of HSC with 0.1, 1, and 5 μM TWS119, an inhibitor of Gsk3β. Increased translocation of β-catenin into the cell nucleus was visible 4 h after application of 5 μM TWS119 as investigated by Western blot of nuclear β-catenin (Supplemental Fig. S5). The inhibitory effect of TWS119 on Gsk3β activity was further verified by decreased phosphorylation of β-catenin and increased glutamine synthetase production (Fig. 2A–D). β-Catenin phosphorylation decreased by 61 ± 9% in freshly isolated HSC and by 71 ± 8% in myofibroblast-like cells after application of 5 μM TWS119 for 48 h compared to untreated controls (Fig. 2C). The same TWS119 concentration increased synthesis of glutamine synthetase by 415 ± 72% in HSC cultured for 1 day and 180 ± 64% in myofibroblast-like cells above levels of control cells within 48 h (Fig. 2D). Interestingly, TWS119-induced β-catenin-dependent signaling prevented accumulation of total β-catenin in HSC. Levels of total β-catenin declined by 53 ± 11% and also β-actin displayed a slight decrease (21 ± 6%) in HSC cultured for 1 day (Fig. 2A). Impairment of synthesis of total β-catenin and β-actin was not significant in myofibroblast-like cells (Fig. 2B). In addition to decreased production of these cytoskeletal elements, the morphology of HSC (1d) changed after application of 5 μM TWS119. Freshly isolated HSC lost their flattened shape and got rotund after TWS119 treatment (Fig. 3A and B), whereas myofibroblast-like cells displayed minor changes in their cell morphology only (Fig. 3C and D).

Effects of TWS119-induced canonical Wnt signaling

Western blot analysis revealed that synthesis of the myofibroblast marker α-SMA was prevented in freshly isolated HSC after treatment with 5 μM TWS119 for 48 h. In myofibroblast-like cells, TWS119 lowered α-SMA levels by 52 ± 6% (Fig. 2A, B, and E). In contrast to α-SMA, the GFAP synthesis was elevated by 48 ± 15% in HSC cultured for 1 day after treatment with 5 μM TWS119 for 48 h, but was lowered by 52 ± 14% in myofibroblast-like cells compared to GFAP levels of untreated control cells (Fig. 2A, B, and F). Treatment of freshly isolated HSC with 5 μM TWS119 for 48 h elevated Pitx2c synthesis by 29 ± 16% compared to untreated controls. The synthesis of Pitx2a

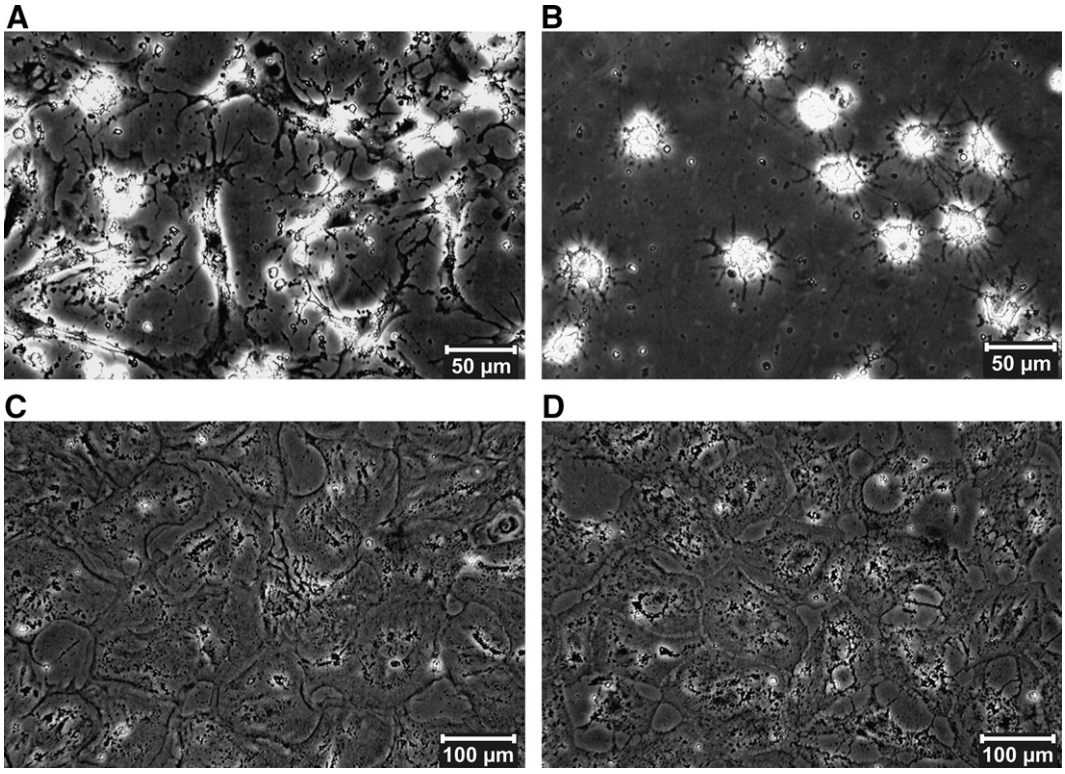

Fig. 3. Effects of TWS119 on morphology of freshly isolated HSC and myofibroblast-like cells (HSC 7d). (A) Phase contrast microscopic image of freshly isolated HSC (1d), which were cultured for additional 48 h without TWS119 treatment. (B) Freshly isolated HSC, which were treated with 5 μM TWS119 for 48 h, changed their morphology compared to untreated cells. (C) Myofibroblast-like cells were cultured for additional 48 h without TWS119 treatment. (D) Exposure of myofibroblast-like cells to 5 μM TWS119 for 48 h displayed minor effects on the cell morphology only.

was only slightly affected under these experimental conditions. No significant effects of TWS119 treatment on Pitx2 isoform synthesis were observed in HSC after myofibroblast formation (Fig. 2A and B). Further effects of TWS119 were seen on Wnt ligand expression. Exposure of freshly isolated HSC to 5 μM TWS119 for 48 h declined Wnt5a precursor protein synthesis by 56 ± 5%, but Wnt5a protein levels in myofibroblast-like cells were only weakly affected. The synthesis of Wnt10b was regulated in an opposite manner after mimicking of β-catenin-dependent Wnt signaling. Applica-

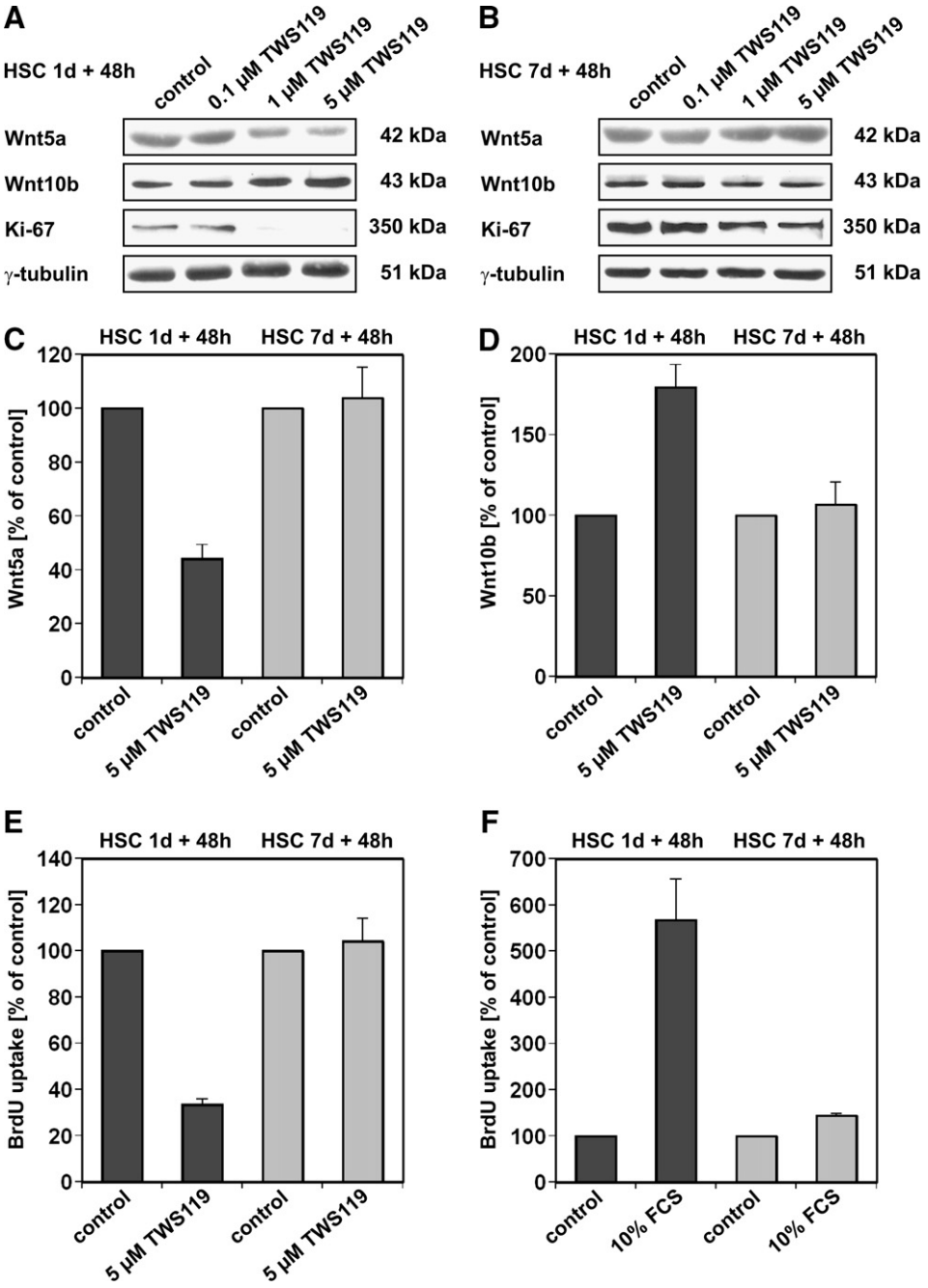

Fig. 4. Effects of TWS119 on Wnt precursor proteins, Ki-67, and DNA synthesis of freshly isolated HSC (1d) and myofibroblast-like cells (HSC 7d). (A) Western blot analysis of the Wnt5a and Wnt10b precursor proteins (30 μg protein) as well as Ki-67 (40 μg protein) after treatment of freshly isolated HSC with 0.1, 1, and 5 μM TWS119 for 48 h. The protein γ-tubulin served as a control. (B) The synthesis of these Wnt precursor proteins and Ki-67 was also investigated in myofibroblast-like cells after incubation with TWS119 for 48 h. Graphic illustration of changes in (C) Wnt5a ($n = 4$) and (D) Wnt10b ($n = 4$) precursor levels as investigated by Western blot after treatment of freshly isolated HSC and myofibroblast-like cells with 5 μM TWS119 for 48 h. (E) The BrdU uptake in response to TWS119 treatment was measured by an enzyme-linked immunosorbent assay after 48 h ($n = 5$). To stimulate an overall DNA synthesis of HSC and myofibroblast-like cells, 10% FCS was added to the culture medium containing 5 μM TWS119 and to the control medium without TWS119. (F) Also the effect of 10% FCS on DNA synthesis in comparison with serum free conditions (control) was analyzed by this method in HSC cultured for 1 and 7 days ($n = 5$).

tion 5 μM TWS119 increased Wnt10b precursor levels by 79 ± 14% within 48 h (Fig. 4A–D). Mimicking of the canonical Wnt signaling by 5 μM TWS119 lowered also the DNA synthesis of freshly isolated HSC by 67 ± 2% as investigated by their BrdU uptake over a period of 48 h. The BrdU incorporation of myofibroblast-like cells was not significantly changed by TWS119 (Fig. 4E). The addition of 10% FCS elevated the DNA synthesis of freshly isolated HSC by 467 ± 89% and of myofibroblast-like cells by 44 ± 4% above levels of control cells, which were cultured under serum free conditions (Fig. 4F). The lowered DNA synthesis in response to TWS119 treatment was accompanied by declined protein levels of Ki-67, which decreased by 99 ± 1% in freshly isolated HSC and about 48 ± 16% in myofibroblast-like cells (Fig. 4A and B). Ki-67 was barely detectable in freshly isolated HSC and up-regulated in myofibroblast-like cells (Supplemental Fig. S6), indicating that quiescent HSC stayed in G0 of the cell cycle.

Discussion

Wnt signaling via β-catenin plays a critical role in maintaining self-renewal and pluripotency of stem cells [2,3]. HSC from rat liver were recently identified as undifferentiated cells, related to stem/progenitor cells derived from the hematopoietic system [1]. Therefore, canonical Wnt signaling should be active in HSC. Indeed, nuclear β-catenin and the expression of the Wnt target genes Pitx2 [10] and axin2 [11] indicate active canonical Wnt signaling in freshly isolated HSC. Quiescent HSC expressed also Wnt ligands known to initiate β-catenin-dependent Wnt signaling like Wnt1, Wnt2, Wnt3/3a, Wnt7a/b, Wnt8a, and Wnt10b. During culture-induced myofibroblast formation a remarkable change from canonical to noncanonical Wnt ligands (i.e. Wnt4, Wnt5a, and Wnt11) was observed. This change was accompanied by increased expression of inhibitors of Wnt signaling such as Dkk1/2, Sfrp5, and Wif1 as well as decreased nuclear β-catenin. These findings indicate that β-catenin-dependent Wnt signaling persists in myofibroblast-like cells, but at a lower level compared to freshly isolated HSC. Ongoing canonical Wnt signaling in myofibroblast-like cells is further indicated by their expression of glutamine synthetase [12]. This enzyme is controlled by β-catenin-dependent Wnt signaling [13] and was used in the present study as a marker to demonstrate stimulation of this signaling pathway by TWS119.

Canonical Wnt signaling seems to be required for prevention of HSC differentiation as indicated by the maintenance of their quiescent state. Typical indicators for a formation of myofibroblast-like cells from HSC are the cell morphology, which changes from stellate-like morphology with lipid inclusions to a flat and heightened cell type without lipids, and the onset of α-SMA synthesis. Also the amount of GFAP is typically lowered during development of HSC into myofibroblast-like cells [14]. Stimulation of β-catenin-dependent Wnt signaling by TWS119 counteracts this process as indicated by prevention of α-SMA and elevation of GFAP synthesis. The alteration of cell morphology to a rotund cell with fine processes is another aspect suggestive for the induction of the quiescent stage of HSC. An additional indicator for the induction of quiescent HSC by TWS119 is the differential expression of Wnt5a and Wnt10b. Mimicking of canonical Wnt signaling by TWS119 induced a decline of Wnt5a, but elevation of Wnt10b protein levels as observed in freshly isolated HSC. Maintenance of the quiescent stage of HSC was further shown by decreased BrdU uptake and Ki-67 levels when TWS119 was applied. Declined Ki-67 suggests that HSC were hindered from entering the cell cycle by canonical Wnt signaling. Extremely low Ki-67 levels without nuclear immunostaining of Ki-67 are typical for freshly isolated HSC. Another example is the high synthesis of the stem/progenitor cell marker Pitx2c that was found in quiescent HSC. Pitx2c synthesis was sustained after mimicking β-catenin-dependent signaling. However, for yet unknown reasons many effects described above were largely limited to freshly isolated HSC.

In addition to maintaining characteristics of undifferentiated cells, canonical Wnt signaling is involved in embryogenesis. During embryonic development of mice Wnt3a, Wnt6, Wnt7b, Wnt9a, and Wnt10b expression is significantly detectable at the blastocyst stage [15]. Also the expression of Wnt3 is mainly restricted to embryogenesis, but was also observed in adult brain [16]. HSC express Wnt ligands involved in early embryogenesis and a fetal Lef1 isoform indicating that HSC possess properties of immature or undifferentiated cells. This expression pattern and the presence of the canonical Wnt signaling strengthen our previous finding that stellate cells are undifferentiated cells of the vertebrate liver. However, the complexity of Wnt signaling with high diversity of Wnt ligands and receptors holds a great challenge for future studies on HSC biology.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.085.

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