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Transcriptional characterization of Wnt pathway during sequential hepatic differentiation of human embryonic stem cells and adipose tissue-derived stem cells

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ABSTRACT

Human embryonic stem cells (hESs) and adipose-derived stem cells (hADSCs) are able to differentiate into hepatocytes. However, a role of Wnt signaling in hepatic differentiation of stem cells is unclear. This study characterized the transcriptional expression pattern of Wnt signaling genes during the sequential hepatocytes differentiation of hES and hADSC. The sequential hepatocytes differentiation of hES and hADSC was induced by three steps including induction, differentiation and maturation steps with the treatment of cytokines. Hepatocytes differentiation was more efficient in hES than hADSC in terms of the expression of hepatocyte-specific genes and the cellular uptake of ICG. The expression of WNT2B, WNT5A, and WISP1 increased at late hepatic differentiation of hES, but the expression of DKK1 and CCND1 decreased during early hepatic differentiation of hES. During hepatic differentiation of hADSC, the expression of WNT2B and WISP1 decreased, but the expression of WNT5B and DKK1 increased at late hepatic differentiation. These results showed that Wnt signaling appears to be activated in hepatic differentiation of hES, but repressed in hepatic differentiation of hADSC in a time-dependent manner, which suggests the differential regulation of Wnt signaling for hepatic differentiation of hES and hADSC.

1. Introduction

Stem cells are unspecialized cells that renew themselves for long periods and differentiated into the specific functional cells. The unique properties of stem cells have generated great interest for therapeutic use. Stem cells could be classified into embryonic stem cells (ESs) and adult stem cells (ASCs) based on their origin. Human embryonic stem cells (hESs) were isolated from the blastocysts of preimplantation embryos [1], and have a potential to develop into a living organism. hES retain the developmental potency and are able to differentiate into all of three germ layers *in vitro* and *in vivo* [1,2]. ASC are found among differentiated cells in a tissue and play a role in replacement for the lost or injured cells. Human adipose-derived stem cells (hADSCs) are ASC within the adipose stromal compartment similar to bone marrow-derived mesenchymal stem cells (MSCs) [3,4].

For clinical application of stem cells for hepatocyte transplantation, stem cells should be differentiated into homogeneous functional hepatocytes. Many studies have reported the differentiation of human hepatocyte-like cells from hES [5–8] or hADSC [9–12]. The *in vitro* approaches for hepatocyte differentiation from hES or hADSC involve the treatment of the cells with specific cytokines critical for liver development [5,11], the introduction of genes inducing hepatocyte differentiation [13], or the modification of culture environment by co-culturing with other cell types [14]. However, although various approaches to generate homogeneous functional hepatocytes have been developed, the efficiency of transplantable hepatic differentiation from hES or hADSC is still not sufficient. Moreover, the mechanisms underlying *in vitro* hepatic differentiation of hES and/or hADSC remain unclear.

Wnt signaling is involved in self-renewal and differentiation of stem cells, and stimulates intracellular signal transduction cascades [15,16]. Wnt signaling involved the canonical pathway for regulating cell fate determination and/or the non-canonical pathway for regulating planar polarity, cell adhesion and motility [17]. Canonical Wnt signals such as WNT2B, WNT3A and WNT7B induces the assembly of Frizzled receptors and LRP5-6 co-receptor

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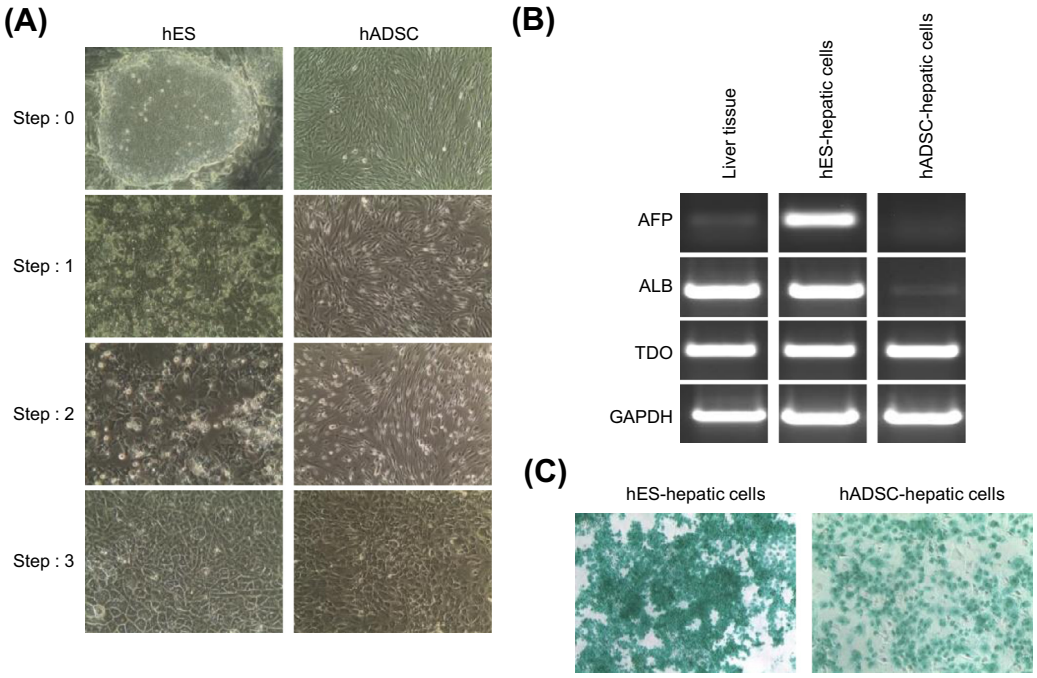

Fig. 1. Hepatic differentiation of hES and hADSC. (A) Morphological changes in three steps during hepatic differentiation (×100 magnification). (B) RT-PCR analysis of hepatocyte-specific gene expression in hES-hepatic cells and hADSC-hepatic cells. (C) Cellular uptake of ICG by hES-hepatic cells and hADSC-hepatic cells. Abbreviations: hES, human embryonic stem cells; hADSC, human adispose-derived stem cells; AFP, alpha-fetoprotein; ALB, albumin; TDO, tryptophan 2,3-dioxygenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

that inhibits the degradation of β-catenin by the glycogen synthase kinase-3β (GSK-3β). The accumulation of the stabilized β-catenin results in its nuclear translocation, where β-catenin binds with the T cell factor/lymphoid enhancer factor (TCF/LEF) family and activates the target genes such as MYC, CCND1, AXIN20, WISP1 and DKK1 [18]. Non-canonical Wnt signals such as WNT5A, WNT5B and WNT11are transduced through Frizzled receptors and ROR2/RYK co-receptors to the Rho family GTPases or the calcium ion-dependent cascades [19].

It has been known that Wnt signaling is involved in animal embryonic development, including the regulation of proliferation and cell fate determination of stem cells [20]. In addition, Wnt signaling regulates the neuronal differentiation of embryonic stem cells [21], the adipocytogenesis of bone marrow stem cells [22], and the osteoblastogenesis of mesenchymal stem cells [23]. Another evidences also showed that Wnt/β-catenin signaling plays a role in liver growth and development [24].

Wnt signaling appears to regulate the proliferation and fate determination of stem cells in a stage-specific and cellular context-dependent manner [25]. Therefore, the aim of this study was to characterize the transcriptional expression of Wnt signaling genes during the sequential hepatic differentiation process of hES and hADSC. The results showed that the transcriptional expression of Wnt signaling genes is associated with hepatocyte differentiation of stem cells in a time and stem cells type-dependent manner.

2. Materials and methods

2.1. Cell culture

The hES line (H9) was provided by the Wisconsin International Stem Cell Bank in the USA. H9 was maintained on mitomycin C (Sigma–Aldrich)-treated MEFs in Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen) supplemented with 20% knock-out serum replacement (Invitrogen), 1 mM nonessential amino acid (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 4 ng/ml FGF2 (PeproTech) and 1% antibiotics. The media were changed every day.

hADSC were isolated from adipose tissue in breast fat pads of a female as approved by the Institution of Review Board. Briefly, the adipose tissue was rinsed with sterile phosphate-buffered saline (PBS) and minced for 5 min with fine scissors, and then incubated with 0.1% collagenase type I (Invitrogen) at 37 °C for 40 min. After adding the same volume of DMEM-10% FBS, the pellet was obtained by centrifugation at 260g for 7 min, resuspended in DMEM/F12 (Invitrogen) supplemented with 10% FBS, 10 ng/ml epidermal growth factor (EGF, Peprotech), 2 ng/ml FGF2 and 1% antibiotics. Non-adherent cells were removed by changing the medium after 24 h. At confluency, the cells were replated using 0.25% trypsin–EDTA (Invitrogen) at a ratio of 1:3. Cells at 3∼5 passages in culture were used for the experiments.

2.2. Induction of hepatocyte differentiation

Hepatic differentiation of hES was induced by three steps. In step 1, hES were cultured on matrigel-coated plates in the induction medium (IM) containing RPMI1640 (Hyclone) supplemented with 0.5% FBS, 100 ng/ml Activin A (PeproTech) and 1% antibiotics for 2 days. In step 2, the cells were cultured in the differentiation medium (DM) containing RPMI1640, 2% KOSR, 10 ng/ml bFGF, 10 ng/ml HGF (PeproTech) for 3 days at 37 °C and 5% CO_2. In step 3, the cells were cultured in the maturation medium (MM) containing William's Medium E (Invitrogen), 1% ITS premix (BD Biosciences), 0.05% bovine serum albumin (BSA, Fisher), 2 mM Ascorbic acid (Sigma), 1 μM hydrocortisone 21-hemisuccinate (StemCell Tech), 10 mM nicotinamide (Sigma), 1 μM dexamethasone (Invitrogen) for 24 days.

Hepatic differentiation of hADSC was induced by the three steps as described in the previous study [10]. In step 1 (induction step), hADSC were pre-cultured in DMEM supplemented with 20 ng/ml EGF and 10 ng/ml bFGF for 2 days. In step 2 (differentiation step), the cells were cultured in DMEM supplemented with 20 ng/ml

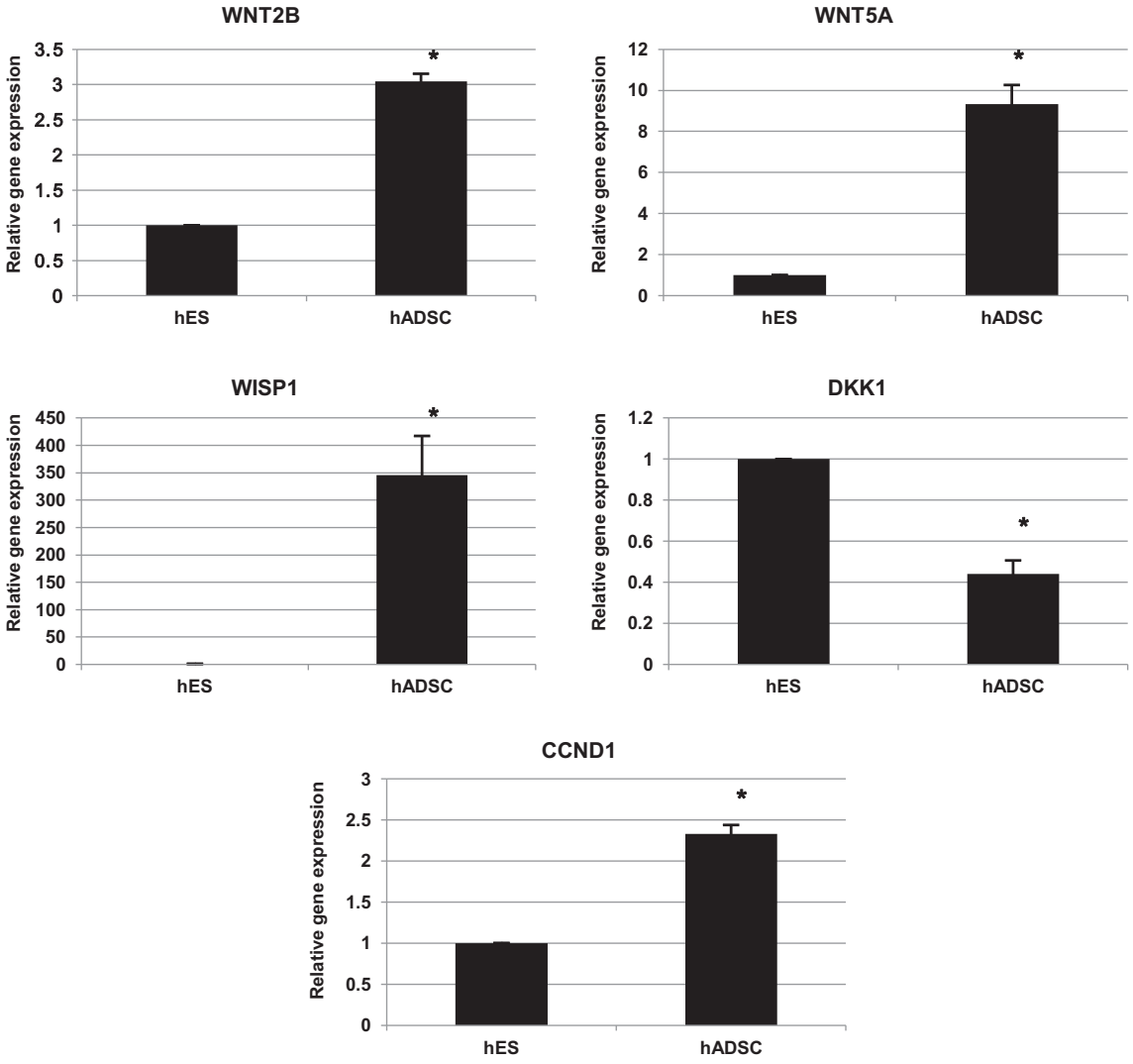

Fig. 2. Basal Wnt signaling gene expression levels in undifferentiated hES and hADSC by real-time PCR analysis. The mRNA level of each gene was normalized to GAPDH and was expressed as a ratio relative to undifferentiated hES (mean ± error deviation). * indicates $p < 0.05$. Abbreviations: hES, human embryonic stem cells; hADSC, human adispose-derived stem cells.

HGF, 10 ng/ml bFGF, and 5 mM nicotinamide for 7 days. To achieve hepatic maturation, in step 3 (maturation step), the cells were cultured in DMEM with 1% ITS premix, 20 ng/ml oncostatin M, and 1 μM dexamethasone for 20 days. Media were changed twice weekly.

2.3. Indocyanine green uptake analysis

The cellular uptake of indocyanine green (ICG) by differentiated cells was determined to identify hepatic differentiation from hES or hADSC. ICG (Sigma) was suspended in DMEM at 100 mg/ml as a stock concentration. The ICG solution was added to the cell culture to final concentration (1 mg/ml) and incubated at 37 °C for 30 min. After the plate was washed three times with PBS, the cellular uptake of ICG was determined under the microscope.

2.4. RNA extraction and PCR

Total RNA was extracted from undifferentiated and differentiated cells at each step using Trizol reagent (Invitrogen) as described in the manufacturer's protocol.

Conventional RT-PCR was performed to determine the hepatic differentiation. 3 μg of total RNA was subjected to cDNA synthesis using the Super Script III First Strand Synthesis Kit (Invitrogen). The cDNA was amplified using GoTaq DNA polymerase (Promega) and 10 pmol of each primer as follows: AFP 5′-CTTTGGGCTGCTCGCT ATGA-3′, 5′-TGGCTTGGAAAGTTCGG GTC-3′; ALB 5′-CCTATGGTGA AATGGCTGAC-3′, 5′-ATTCCGTGTGGACTTTGG TA-3′; TDO 5′-GGCA GCGAAGAAGTACAAATC-3′, 5′-TCGAACAGAATCCAACT CCC-3′; GAPDH 5′-CAGGGCTGCTTTTAACTCTG-3′, 5′-CTGTGGT CATGAG TCCTTCC-3′. Thermal cycling was performed for 30 cycles of 94 °C/30 s, 52 °C/30 s, and 72 °C/30 s in a 2720 Thermal cycler (Applied Biosystems). PCR products were separated by electrophoresis on 1.2% agarose gel.

Quantitative real-time PCR was performed to determine the levels of expression of Wnt signaling genes involved in hepatic differentiation of hES and hADSC. PCR was performed using SYBR Green PCR Core Reagents kit (Applied Biosystems) and 500 nM of each primer as follows: WNT2B 5′-ACGAGTTTGGATGTTGTA-3′, 5′-AGA-AAGGAGAGAAGAGTG-3′; WNT5A 5′-ATCCCATCACAGGTTCTC-3′, 5′-CTCAAATAGGTTGTCTGCTCT-3′; WISP1 5′-CTGTGAGTGCTGTAA-GATGT-3′, 5′-TCCTATTGCGTACCTCGG-3′; DKK1 5′-ACCAAAGGA-CAAGAAGGTT-3′, 5′-TGGACCAGAAGTGTCTAGCA-3′; CCND1 5′-

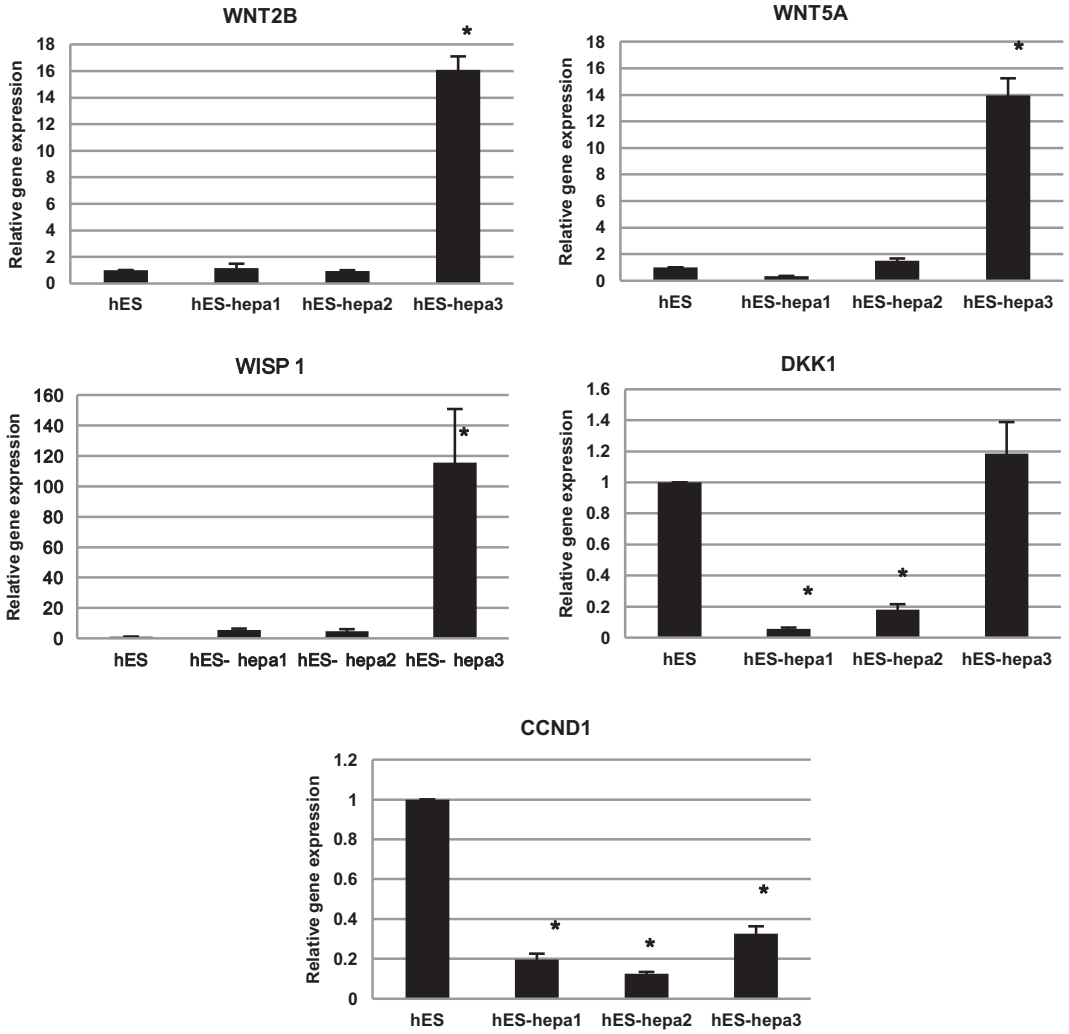

Fig. 3. Wnt signaling gene expression levels during hepatic differentiation of hES for 29 days. The hepatic differentiation of hES was induced for 2 days in step 1 (hES-hepa1), for 1 days in step 2 (hES-hepa2), and for 24 days in step 3 (hES-hepa3). The mRNA level of each gene was normalized to GAPDH and was expressed as a relative ratio to undifferentiated hES (mean ± error deviation). * indicates $p < 0.05$. Abbreviations: hES, human embryonic stem cells; hADSC, human adispose-derived stem cells; hepa1, hepatic cells at step 1; hepa2, hepatic cells at step 1; hepa3, hepatic cells at step 3.

GGTGTCCTACTTCAAATG-3′, 5′-CTCCTCGCACTTCTGTTC-3′. The following conditions were used: 95 °C/15 min, followed by 45 cycles of 95 °C/30 s, 55 °C/30 s, 72 °C/30 s in the ABI PRISM 7700 Sequence Detector (Applied Biosystems). The levels of mRNA expression were normalized by the internal control of housekeeping gene, GAPDH. Relative quantification was performed against the undifferentiated hES or undifferentiated hADSC.

2.5. Statistical analysis

Student t-test was used to estimate differences among samples. All data were presented as mean ± error deviation. All statistical analyses were performed with JMP (Version 4, SAS institute, Cary, NC). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Hepatic differentiation of hES and hADSC

The hepatic differentiation of hES and hADSC was induced by three distinct steps, and the morphological changes were examined under microcopy at each step (Fig.1A). The hepatic differentiation of hES was induced by sequential cultures in IM with the treatment of Activin A, DM with the treatment of bFGF and HGF, and MM with the treatment of dexamethasone. The morphology of hES was significantly changed during three sequential steps. In step 1, the hES were enlarged and flatted. Moreover, many dead cells were observed during the treatment of Activin A. During step 2 differentiation, hES started to develop polygonal shape. At step 3, the differentiated cells displayed typical hepatocyte morphology. For hepatic differentiation of hADSC, hADSC were treated by three sequential steps in the induction step with the treatment of EGF and bFGF, in the differentiation step with the treatment of HGF and bFGF, and then in maturation step with the treatment of oncostatin M and dexamethasone. No significant morphological changes were observed at step 1 and 2 in hADSC. However, the hepatocyte-like morphology appeared in maturation step 3.

To characterize the cells differentiated from hES and hADSC, the expression of hepatocyte specific genes, such as AFP, ALB, and TDO were evaluated by RT-PCR (Fig. 1B). Both of hES-hepatic cells and hADSC-hepatic cells displayed a significant expression of the hepatocyte-specific genes. Especially, the expression of AFP and ALB were much higher in hES-hepatic cells than in hADSC-hepatic cells. Also, the cellular uptake of ICG was much higher in hES-hepatic cells than hADSC-hepatic cells (Fig. 1C).

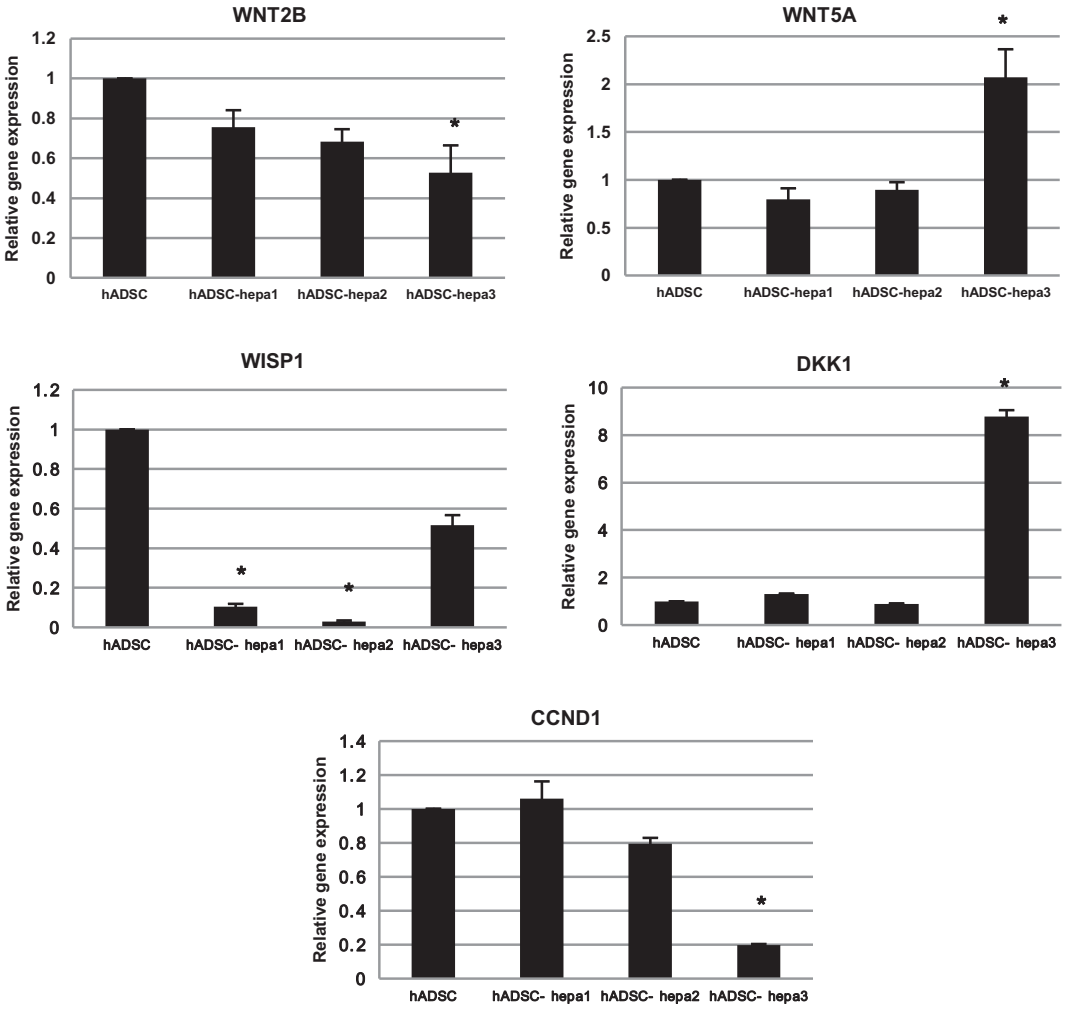

Fig. 4. Wnt signaling gene expression levels during hepatic differentiation of hADSC for 29 days. The hepatic differentiation of hADSC was induced for 2 days in step 1 (hADSC-hepa1), for 7 days in step 2 (hADSC-hepa2), and for 20 days in step 3 (hADSC-hepa3). The mRNA level of each gene was normalized to GAPDH and was expressed as a relative ratio to undifferentiated hADSC (mean ± error deviation). * indicates $p < 0.05$. Abbreviations: hES, human embryonic stem cells; hADSC, human adispose-derived stem cells; hepa1, hepatic cells at step 1; hepa2, hepatic cells at step 1; hepa3, hepatic cells at step 3.

Taken together, these data indicate the hES and hADSC were differentiated into hepatic cells in these differentiation conditions in terms of morphology and gene expression pattern. In addition, the hepatic differentiation was more efficient in hES than in hADSC.

3.2. Basal expression of Wnt signaling genes in hES and hADSC

To determined the basal expression of Wnt signaling genes in hES and hADSC, the expression of Wnt signaling ligands (WNT2B and WNT5A), Wnt signaling targets (WISP1 and CCND1) and Wnt signaling inhibitor (DKK1) were compared in undifferentiated hES and undifferentiated hADSC using quantitative real-time PCR (Fig. 2).

The expression of the canonical (WNT2B) and non-canonical (WNT5A) Wnt signaling genes were detected in both of the hES and hADSC. The expression of Wnt ligands (WNT2B and WNT5A) and Wnt targets (WISP1 and CCND1) were significantly higher in hADSC than in hES. However, the expression of DKK1 was lower in hADSC. These results indicate that the Wnt signaling is more active in undifferentiated hADSC than in undifferentiated hES.

3.3. Transcriptional expression of Wnt signaling genes during hepatic differentiation of hES

To evaluate the involvement of Wnt signaling pathway in hepatic differentiation of hES, we examined the transcriptional expression of Wnt signaling genes during hepatic differentiation of hES (Fig. 3). The hepatic differentiation of hADSC was induced by three sequential steps. At each step, the transcriptional expression levels of Wnt signaling genes were measured using quantitative real-time PCR. The expression of WNT2B, WNT5A and WISP1 in hES were not changed during the induction step 1 and the differentiation step 2, but increased significantly during the maturation step 3. However, the expression of DKK1 decreased during the induction step 1 and the differentiation step 2. During the overall hepatic differentiation process of hES, the expression of CCND1 decreased significantly. Therefore, these results indicate that Wnt signaling pathway in hepatic differentiation of hES is down-regulated at early induction stage, but up-regulated at late maturation stage.

3.4. Transcriptional expression of Wnt signaling genes during hepatic differentiation of hADSC

The involvement of Wnt signaling pathway in hepatic differentiation of hADSC were evaluated by quantitative real-time PCR in three sequential differentiation steps (Fig. 4). The expression of WNT2B and CCND1 decreased significantly at the maturation step 3 in hepatic differentiation of hADSC. However, the expression of WNT5A and DKK1 significantly increased at the maturation step 3. Moreover, WISP1 showed a significantly decreased expression during the overall hepatic differentiation of hADSC. Taken together,

these results indicate that the expression of Wnt signaling genes was differentially regulated during hepatic differentiation of hADSC.

4. Discussion

Liver growth and development is a complicate and well-organized biological process. Previous studies have focused on the development of efficient hepatic differentiation techniques to generate a sufficient transplantable stem cells-derived hepatocytes for clinical therapy [5–9,11]. However, the sufficient hepatic differentiation method is still not available. If the molecular mechanism underlying hepatocyte differentiation from stem cells is elucidated, it may become possible to establish the protocol of sufficient hepatic differentiation. Therefore, this study was performed to characterize the transcriptional expression profiles of Wnt signaling that is involved in the sequential hepatic differentiation process of hES and hADSC. We found that Wnt signaling is involved differentially in the sequential hepatic differentiation of hES and hADSC.

In this study, we used the well-established sequential differentiation protocols for the efficient hepatic differentiation of hES or hADSC that were modified from the previous studies [11,26]. Although hADSC-derived hepatic cells expressed the hepatocyte-specific genes, the hepatocyte-specific gene expression profile of hES-hepatic cells was more similar to that of liver tissue. Moreover, the morphology of hES-hepatic cells displayed a dramatic change with typical hepatocytes morphology during the sequential hepatic differentiation. These results suggest that hES have more efficient potential for hepatocyte differentiation than hADSC.

It has been reported that Wnt signaling is associated with the differentiation of stem cells into various cell types. Activation of Wnt signaling in mesenchymal stem cells inhibits adipocyte differentiation [22] and stimulates osteoblast differentiation [23]. On the other hand, the inhibition of Wnt signaling in human induced pluripotent cells increased the production of cardiomyocytes [27]. The previous studies have also reported that Wnt signaling is directly or indirectly associated with hepatic differentiation of stem cells [28–30]. We demonstrated that Wnt signaling regulates differentially the hepatic differentiation in hES and hADSC. During the sequential hepatic differentiation of hES, the expression of Wnt ligands (WNT2B, WNT5A) and Wnt target (WISP1) were up-regulated at the late step of hepatic differentiation whereas the expression of DKK1 known as a Wnt inhibitor was down-regulated at early step of hepatic differentiation. The previous study has also reported that Wnt/beta-catenin signals are essential for hepatocyte differentiation from hepatoblast during liver development [30]. Therefore, Wnt signaling appears to stimulate the hepatic differentiation of hES at hepatocyte maturation step in a time-dependent manner. Unlike the hepatic differentiation of hES, the expression of Wnt ligand (WNT2B) and target (WISP1) was down-regulated during hepatic differentiation of hADSC. The expression of DKK1 known as Wnt inhibitor also increased at the late hepatic differentiation step. However, the expression of WNT5A known as non-canonical Wnt ligand was up-regulated at the late hepatic differentiation step of hADSC. Like this result, it has been reported that the hepatocyte differentiation of bone marrow-derived mesenchymal stem cells was promoted by down-regulation of Wnt signaling. Therefore, the hepatocyte differentiation of mesenchymal stem cells might be promoted by the down-regulation of Wnt signaling.

In conclusion, the Wnt signaling appears to be up-regulated in hepatic differentiation of hES but down-regulated in hepatic differentiation of hADSC, which suggests that the effect of Wnt signaling on hepatic differentiation might be dependent on the types of stem cells. Moreover, Wnt signaling may activate the hepatocyte differentiation of stem cells in a time-dependent manner.

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