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Overexpression of miR-27b Negatively Regulates Expression of Pluripotency-associated Genes and Hepatic Differentiation in Human Induced Pluripotent Stem Cells

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ABSTRACT

Human induced pluripotent stem (hiPS) cells are defined as its ability of self-renewal and pluripotency, and widely used for studies of lineage-specific differentiation *in vitro*. Under appropriate conditions, hiPS cells differentiate into hepatocyte-like cells, recapitulating human embryonic development. Micro RNAs, short non-coding RNAs, are important regulators for biological events via suppressing gene expression, but little is known about how miRNAs modulate hepatic differentiation from pluripotent hiPS cells. In this report, we focused on miR-27b which has been reported to be upregulated during hepatic differentiation. To assess the role of miR-27b, hiPS cell lines with the Doxycycline-inducible miR-27b expression cassette integrated into Adeno-associated virus integration site (AAVS1) locus were generated utilizing CRISPR/Cas9 genome editing technology and they were subjected to the hepatic differentiation *in vitro*. Induced expression of miR-27b in undifferentiated hiPS cells repressed *NANOG* and *OCT3/4*, crucial genes for maintaining self-renewal and pluripotency. During the definitive endoderm differentiation, miR-27b-induction caused changes in expression of genes associated to definitive endodermal and mesodermal lineage specific differentiation, depending on the timing of induction, and consequently inhibited the hepatic differentiation. These results demonstrated that proper suppression of miR-27b expression in undifferentiated hiPS cells and during early stage of hepatic differentiation is required to keep undifferentiated state of hiPS cells and to secure correct differentiation of hepatocyte-like cells via endoderm formation.

Keywords
Human induced pluripotent stem cells, Hepatic differentiation, miR-27b, Doxycycline-inducible expression

Abbreviation: DOX: Doxycycline; AAVS1: Adeno-associated Virus Integration Site 1; αAT: alpha-1 Antitrypsin; HNF: Hepatocyte Nuclear Factor; FGF: Fibroblast Growth Factor; FBS: Fetal Bovine Serum; BMP: Bone Morphogenetic Protein; HCM: Hepatocyte Culture Medium; HGF: Hepatocyte Growth Factor; OsM: Oncostatin M; BSA: Bovine Serum Albumin; FOXA2: Forkhead Box A2; GSC: Goosecoid; MIXL1: Mix Paired-like Homeobox; OCT: Octamer-binding Transcription Factor; AFP: Alpha-fetoprotein; PAX: Paired-Box; DAPI: 4',6-diamidino-2-Phenylind.

INTRODUCTION

Hepatocytes have potential to be a useful tool for drug screening and studying pathological and molecular pathway involving in liver diseases *in vitro*. Human induced pluripotent stem (hiPS) cells and human embryonic stem (hES) cells are defined as its ability of self-renewal and pluripotency. Under appropriate conditions, hiPS cells can be induced to differentiate into hepatocyte-like cells, recapitulating human embryonic development *in vitro*. Therefore, hiPS cells are considered as a novel model for studying fundamental biological pathways that govern hepatic differentiation. To date, established protocols of hepatic differentiation have shown that hepatocyte-like cells are induced via definitive endoderm cells and hepatoblast-

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-like cells [1]. However, the molecular and genetic mechanism involving in hepatic differentiation still remain to be elucidated.

Micro RNAs (miRNA) are short (18~25 nucleotides) non-coding RNAs generated from genomic sequences. MiRNAs mainly work as negative post-transcriptional regulators by binding to 3'UTRs of target mRNAs, even on condition of imperfect matches. Hence, a single miRNA can target multiple mRNAs and miRNAs are involved in various biological pathways [2]. Accumulating studies have revealed that miRNAs are important molecules for modulating pluripotency [3] and differentiation [4] of hES/hiPS cells. For example, neural stem cell proliferation and neural differentiation are regulated by expression of miR-9, an abundant miRNA in brain [5].

MiR-27b, a somatic enriched miRNA, is one of the paralogs of miR-27, miR-27a and miR-27b, with only one nucleotide difference. Both paralogs are produced as polycystronic clusters of miR-23~27~24. MiR-27 functions in various biological events such as adipogenesis, lipid metabolism [6,7] and suppression of tumor progression [8]. As for the earlier differentiation, recent studies have shown that increasing miR-27b expression was detected during hepatic differentiation from hES cells [9]. Double knock-out of miR-23a/b~27a/b~24 clusters in mouse ES cells suppressed differentiation into mesodermal lineage [10]. Other studies have revealed that over-expression of miR-27a in human embryonal carcinoma cells negatively regulated expression of genes associated to self-renewal and pluripotency [11]. However, the role of miR-27b in maintenance of undifferentiated state and the differentiation into endodermal lineage of hiPS cells has not been addressed directly.

In this study, we have generated hiPS cells, in which Doxycycline (DOX)-inducible miR-27b-expression system was integrated by CRISPR/Cas9 technology-mediated knock-in method into AAVS1 locus. Using the hiPS cell line combined with the *in vitro* hepatic differentiation system, we report here the negative roles of miR-27b on undifferentiation state of hiPS cells and early differentiation toward the hepatic lineage.

MATERIALS AND METHODS

iPS cell culture

Human iPS cell lines (Tic, JCRB Cell Bank) were cultured on mitomycin C treated mouse embryonal fibroblast (MEF, Millipore) with ReproStem (ReproCELL), 10ng/μl fibroblast growth factor-2 (FGF-2, KATAYAMA CHEMICAL INDUSTRIES).

Hepatic differentiation

In vitro hepatic differentiation was performed as previously described [12] with some modifications. Briefly, prior to hepatic differentiation, hiPS cells were dissociated into single cells by Accutase (Sigma Aldrich) and plated onto BD Matrigel Matrix Basement Membrance Growth Factor Reduced-coated plates (Becton, Dickinson and Company) by 4.0×10^5 cells/ml and cultured with ReproStem, 10ng/μl FGF-2 and 10μM Rock inhibitor (Y-27632, Sigma). The definitive endoderm cells were induced by L-Wnt3A-expressing cell (CRL2647;ATCC)-conditioned RPMI-B27 media (RPMI 1640 media (Sigma) containing 1 x B27 Supplement Minus Vitamin A(Invitrogen), 4mM GlutaMAX (Invitrogen)) with 100ng/ml Activin A (R&D Systems) for 4 days. The formation of hepatoblasts was driven by RPMI-B27 media containing 20ng/ml each of bone morphogenetic protein 4 (BMP4, R&D Systems) and FGF-4 (R&D Systems) for 5 days. For heptic differentiation, the hepatoblast-like cells were cultured with RPMI-B27 media supplemented with 20ng of hepatocyte growth factor (HGF, R&D Systems) for 5 days, then cultured with hepatic maturation medium (hepatic maturation medium consists of Hepatocyte Culture Medium (HCM; Lonza, without epidermal growth factor (EGF)) containing 20 ng/mL oncostatin M (OsM) and 3% Gluta MAX) for 11 days.

Inducible gene expression plasmid

Construction of the targeting vector is described in **Figure 2A.** The targeting vector was constructed based on AAVS1 donor plasmid [13]. First, miR-27b expressing plasmid was generated by insertion of double-stranded oligonucleotides encompassing miR-27b into pcDNA^{TM}6.2-GW/miR plasmid (Invitrogen) according to the manufacture's instruction. The sequence of miR-27b was described below. Then, restriction fragments of miR-27b cassette with upstream emerald green fluorescent protein (GFP) were inserted into downstream of tetracycline response element (TRE) of pTetOne^{TM} plasmid (Clontech). Resulting fragments of TRE-EGFP-miR-27b cassette were cloned into upstream of elongation factor 1 alpha (EF1α) promoter in AAVS1 donor plasmid in which enhanced green fluorescent protein (EGFP) was replaced by reverse tet-controlled transcriptional activator (rtTA) from pTetOne^{TM} plasmid.

Human miR-27b Top :

5'-ACCTCTCTAACAAGGTGCAGAGCTTAGC

TGATTGGTGAACAGTGATTGGTTTCCGCTTTGTTCA
CAGTGGCTAAGTTCTGCACCTGAAGAGAAGGTG-3'

Human miR-27b Bottom :

5'-CACCTTCTCTTCAGGTGCAGAACTTAGCCA

CTGTGAACAAAGCGGAAACCAATCACTGTTCACCA
ATCAGCTAAGCTCTGCACCTTGTTAGAGAGGT-3'

Generation of Knock-In hiPS cells

The protocol for electroporation was described in the previous report [13]. Briefly, hiPS cells were treated with 10μM valproic acid for 24 hours before electroporation.

Then, hiPS cells were harvested as single cells using Accutase (Sigma Aldrich). Targeting plasmid (5μg), px330-AAVS1 gRNA/Cas9 expressing plasmid (5μg, construction described in previous report [13]) and RAD51 expression plasmid (1μg, construction is described in previous report [13]) were co-electroporated into hiPS cells ($2x10^6$) using NEPA21 electroporator (Nepagene) according to the manufacturer's instructions. Cells were seeded on iMatrix-511 (Nippi) coated plates in the presence of StemFit®AK02N (ReproCELL) and 10μM Rock inhibitor. Fourty-eight hours after electroporation, 10μg/ml puromycin was added for 48 hours to select knocked-in hiPS colonies. Isolated colonies were picked up and expanded for preparation of genomic DNAs. Genomic DNA was subjected to PCR to amplify the targeted genomic regions. PCR reaction was performed using Verti thermal cycler (Applied Biosystems). The primer sequences for PCR are depicted in **Table 1**. The obtained clone was designated as hiPS-AAVS1-27b. For induction of miR-27b expression, cells were cultured in the presence of 1μg/ml DOX.

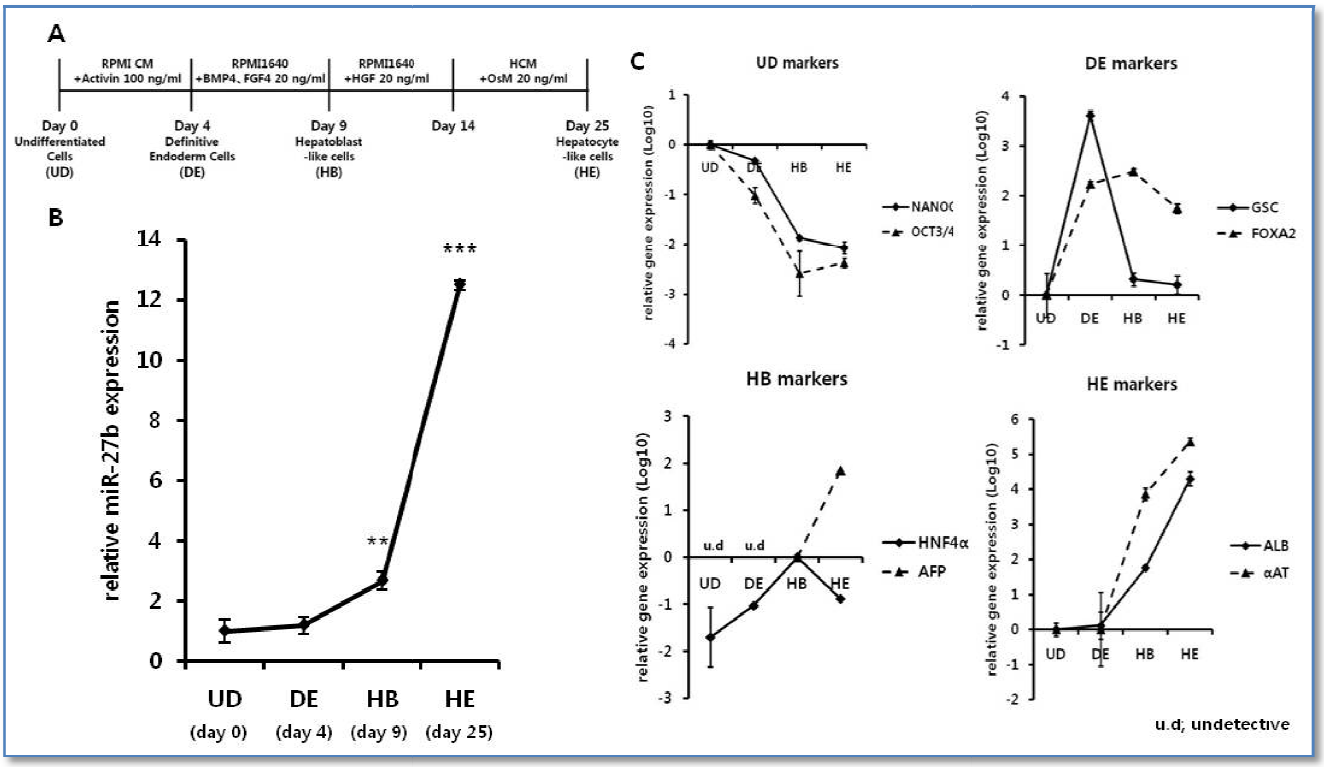

Figure 1. miR-27b expression is induced during *in vitro* hepatic differentiation of hiPS cells. (A) Schematic procedure of the *in vitro* hepatic differentiation. Undifferentiated (UD) hiPS cells were differentiated into hepatocyte-like cells (HE) via definitive endoderm cells (DE) and hepatoblast-like cells (HB). Details of procedure are described in Materials and Methods section. (B) During hepatic differentiation of hiPS cells, RNA was isolated at each stage of DE, HB, HE to determine miR-27b expression. MiR-27b expression level was measured at each stage by qRT-PCR. The data on day0 (undifferentiated hiPS cells) was taken as 1. (C)Stage-specific genes were measured for denoting differentiation stages by qRT-PCR. Except HB markers, the data on UD was taken as 1. The data on HB was taken as 1 in HB markers analysis because Ct values on UD and DE was undetective. (n=3). mean±SD (*p<0.05, **p<0.01, ***p<0.001).

RNA isolation and quantative RT-PCR (qRT-PCR)

Total RNA was isolated using ISOGEN (NIPPON GENE) according to the manufacturer's instruction. 500ng of total RNA was used to synthesize cDNA with a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). qRT-PCR was carried out with StepOnePlus real-time PCR system (Applied Biosystems) using Fast SYPR Green Master Mix (Applied Biosystems). Results were analyzed with ⊿⊿Ct method normalized by internal reference, GAPDH. Student t-test was performed. The primer sequences are described in **Table 2.**

miRNA TaqMan assay

Taqman® MicroRNA Assay Kits (Applied Biosystems) was used for quantification of miR-27b expression according to the manufacturer's instruction. Briefly, 10ng of total RNA were used to perform reverse-transcription (RT) and 1.33μl of RT products out of 7.5μl total reaction mixture was used to qRT-PCR. Results were analyzed with ⊿⊿Ct method normalized by internal control, RNU48 (Applied Biosystems).

Table 1. Primer sets using for genotyping

PCR region	Forward primer	Reverse primer
AAVS1	GGTCCGAGAGCTCAGCTAGT	CACTGAGAACCGGGCAGGT
3' arm	TGACCCGCAAGCCCGGTGCCTGAGATC	CAGCGCTAAAACTAGGCTGTCCTGGGC
5' arm	CACCCCACTTCCGAATTGGAGCGCTTC	TCCCACACCTCCCCCTGAACCTGAAAC

Table 2. Primer sets using for qRT-PCR

Gene	Forward primer	Reverse primer
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTGGTCGTTGAGGGCAATG
NANOG	AGAAGGCCTCAGCACCTAC	GGCCTGATTGTTCCAGGATT
OCT3/4	CTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC
MYC	CCAGCAGCGACTCTGAGGA	GAGCCTGCCTCTTTTCCACAG
TP53	CAGGGCAGCTACGGTTTCC	CAGTTGGCAAAACATCTTGTTGAG
LIN28B	AGCCCCTTGGATATTCCAGTC	AATGTGAATTCCACTGGTTCTCCT
SOX2	GGCAGCTACAGCATGATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG
GSC	TCTCAACCAGCTGCACTGTC	CGTTCTCCGACTCCTCTGAT
FOXA2	GCGACCCCAAGACCTACAG	GGTTCTGCCGGTAGAAGGG
HNF4α	CGTCATCGTTGCCAACACAAT	GGGCCACTCACACATCTGTC
AFP	TGGGACCCGAACTTTCCA	GGCCACATCCAGGACTAGTTTC
PAX6	TGGGCAGGTATTACGAGACTG	ACTCCCGCTTATACTGGGCTA
MIXL1	GGCGTCAGAGTGGGAAATCC	GGCAGGCAGTTCACATCTAC
Brachyury (T)	TATGAGCCTCGAATCCACATAGT	CCTCGTTCTGATAAGCAGTCAC
ALB	GCACAGAATCCTTGGTGAACAG	ATGGAAGGTGAATGTTTTCAGCA
αAT	ACTGTCAACTTCGGGGACAC	CATGCCTAAACGCTTCATCA

STATISTICAL ANALYSIS

Statistical analysis was performed with unpaired two-tailed Student's t-test.

Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde (PFA, Wako) in PBS for 30 min at room temperature. After blocking and permealising cells with PBS containing 0.2% Triton X-100 (Sigma Aldrich) and 2% bovine serum albumin (BSA) for 45 min at 4 °C, the cells were incubated with a primary antibody at 4 °C overnight, and finally, incubated with a secondary antibody at room temperature for 1 hour. All the antibodies are listed in **Table 3**.

Table 3. Antibodies used in immunofluorescence staining

Antigen	Type	Company
NANOG	mouse	Santa Cruz Biotechnology
OCT3/4	goat	Santa Cruz Biotechnology
Alexa Fluor 488 anti-goat IgG	donkey	life technology
Alexa Fluor 594 anti-mouse IgG	donkey	life technology

RESULT

miR-27b expression is induced during *in vitro* hepatic differentiation of hiPS cells

The previous studies have reported that miR-27b was upregulated during hepatic differentiation from ES/iPS cells, suggesting the roles of miR-27b in these stages [9,11]. Therefore, we examined the expression profile of miR-27b through the differentiation according to our procedure (**Figure 1A),** since the time course and the definition of differentiation stages might vary depending on protocols. MiR-27b expression was moderately upregulated at day 4 of culture, which is the definitive endodermal (DE) stage as denoted by the marker genes expression (**Figure 1B, 1C**). Afterward, significant increase in miR-27b expression from definitive endoderm (DE) state to hepatoblast-like cells (HB) and drastic increase to hepatocyte-like cells (HE) were observed, confirming the previous observations with unexpectedly low miR-27b expression during endodermal differentiation.

Generation of hiPS cell lines with inducible miR-27b expression.

In order to examine whether miR-27b regulates the undifferentiated state of hiPS cells and its differentiation towards hepatocyte, we generated hiPS cells carrying DOX-inducible miR-27b expression system to achieve the programmed induction of miR-27b in undifferentiated hiPS cells or during endodermal differentiation. In order to assure stable transgene expression, miR-27b inducible cassette was integrated into AAVS1 loci, known as 'safe-harbor', suitable for constitutive strong transgene expression [14], by using CRISPR/Cas9 [15,16]. Isolated hiPS-clones were subjected to diagnostic PCR (**Figure 2A),** and heterozugously knocked-in clones, designated as hiPS-AAVS1-27b, were successfully obtained. One of the knocked-in clones was further analyzed to confirm inducible expression of miR-27b and its upstream GFP by treatment with DOX **(Figure 2B, 2C).**

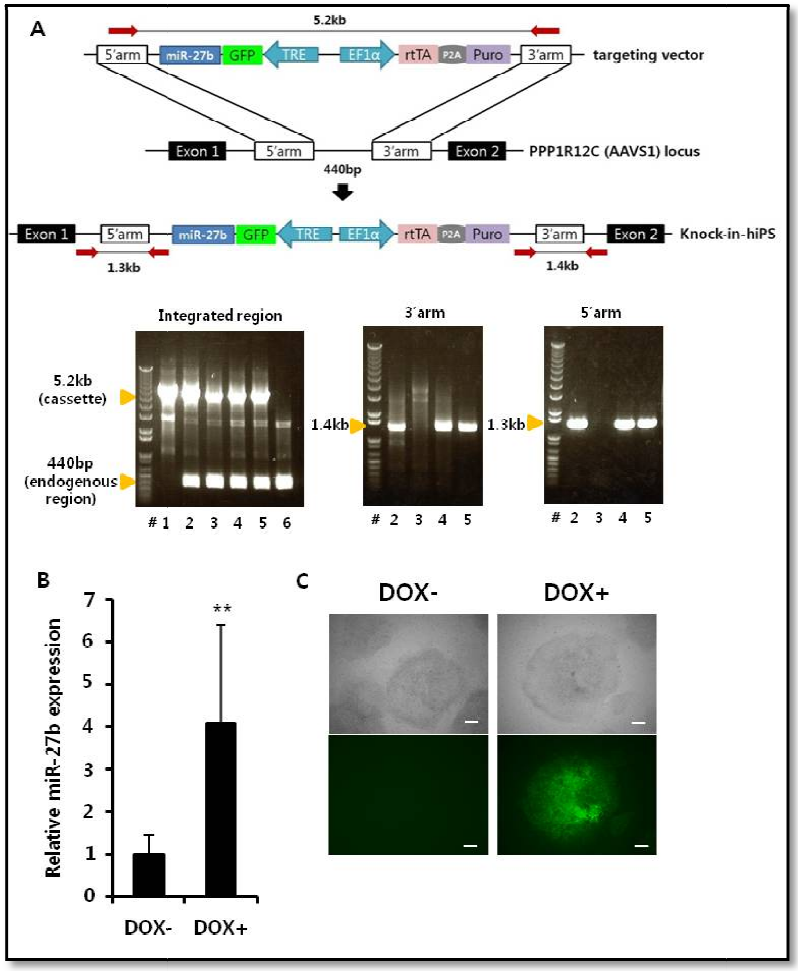

Figure 2. Generation of hiPS cell lines with inducible miR-27b expression. (A) Schematic presentation of knock-in strategy of inducible miR-27b expression cassette into AAVS1 region. Homologous recombination occurred within 1057 bp

of 3' arm and 1165 bp of 5' arm utilizing CRISPR/Cas9. Primer sets for diagnostic genomic PCR of the knocked-in clones are depicted as arrows (red). 5.4kb PCR products and 440bp PCR products indicate expression cassette region and endogenous region, respectively. Loss of 440bp PCR band means that biallelic recombination was occurred (homozygously Knock-in). 1.4kb and 1.3kb PCR product, corresponding to 3' and 5' homologous recombination respectively, indicates correct homologous recombination. (B) Induced expression of miR-27b was measured after DOX treatment for 4 days in the heterozygously clone by qRT-PCR (n=6). We used #4 heterozygously knock-in clone, because the homozygous clone didn't grow well from unknown reasons, mean±SD (*p<0.05, **p<0.01, ***P<0.001). (C) Inducible expression of GFP was examined under the fluorescence microscope (bottom) 1 day after adding DOX. Scale bar, 300μm.

miR-27b negatively regulates the expression of genes associated to pluripotency and self-renewal of hiPS cells.

Previous studies have shown that miR-27a over-expression inhibits expression of pluripotency-associated genes including *OCT3/4* and *LIN28B* in human embryonal carcinoma cells [11] and *Oct3/4* and *Nanog* in mouse ES cells [10]. Therefore, we examined whether miR-27b over-expression also suppressed expression of those genes in hiPS cells. When culturing the hiPS-AAVS1-27bwith or without DOX for 4 days, mRNA expression of *NANOG* and *OCT3/4* was significantly suppressed **(Figure 3A**).

Immunofluorescent staining also indicated decrease of *NANOG* and *OCT3/4* expression (**Figure 3B).** Furthermore, the morphological change with ambiguous edges also indicated that hiPS cells started differentiation (**Figure 3C).** Moreover, expression of *PAX6*, a gene representing ectoderm differentiation, was induced, while expression of genes associated to mesendoderm (*GSC*,*MIXL1*)/mesoderm (*T*)/endoderm (*FOXA2*) differentiation was strongly suppressed (**Figure 3D**). In addition, expression of *SOX2* gene, known as not only for pluripotency factor but also as specific modulator to induce neural differentiation [17] was also increased (**Figure 3A**). Since the neuroectodermal fate is considered to be the default direction of ES/iPS differentiation, these results are well-consistent to the notion that miR-27b acts negatively to maintain the undifferentiated state of hiPS cells.

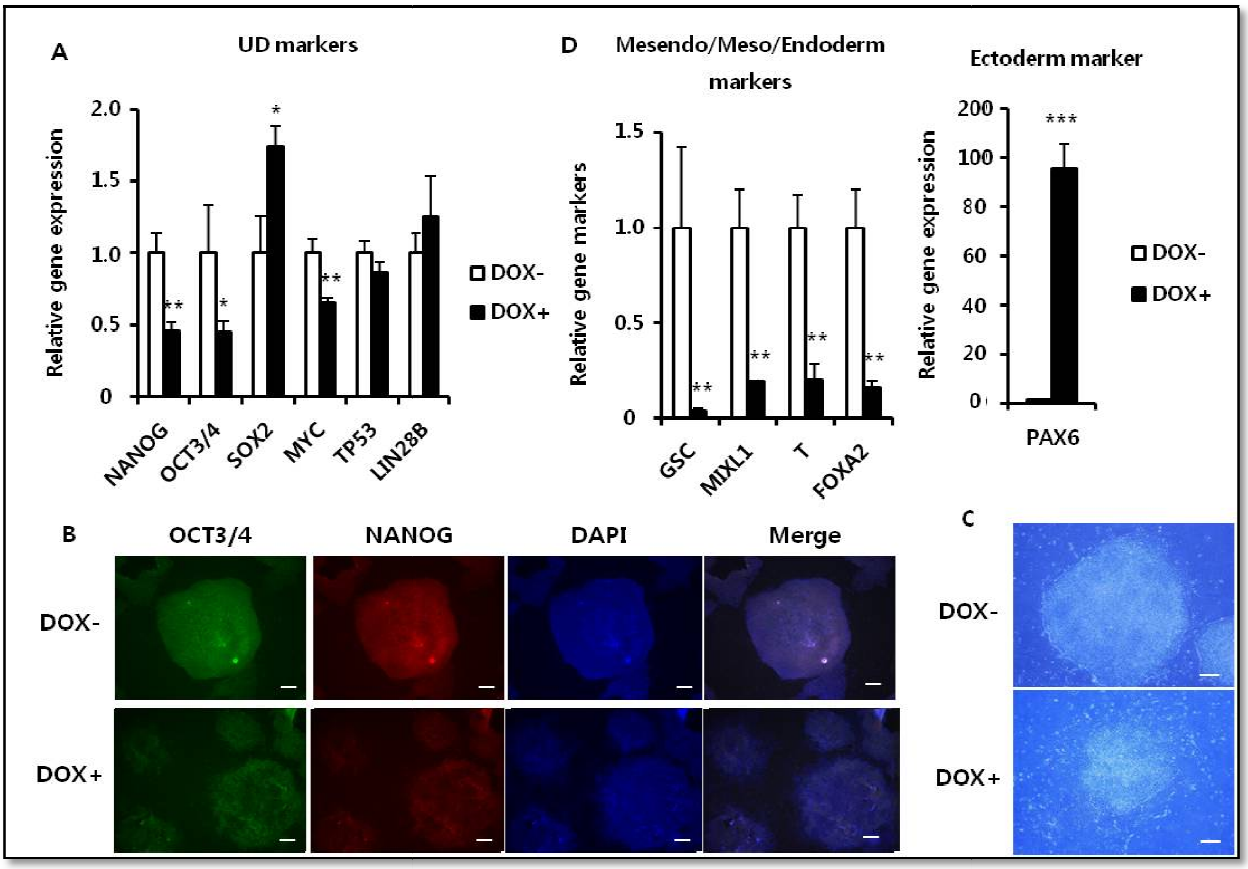

Figure 3. miR-27b negatively regulates expression of genes associated to pluripotency and self-renewal of hiPS cells. Undifferentiated hiPS-AAVS1-27b was cultured with or without DOX for 4 days. (A and D) After 4 days culturing with

DOX, expression of genes promoting pluripotency and self-renewal (A) and lineage-specific genes (D) of hiPS cells was measured by qRT-PCR (n=3).The data on hiPS cells culturing without DOX was taken as 1. (B) Immunofluorescence analysis of *OCT3/4* (green) and *NANOG* (red), after 4 days DOX treatment. Nuclei were counterstained with DAPI (blue). Scale bar, 300μm. (C) Morphology of hiPS cell colony, which were cultured with DOX for 4 days, under bright field microscopy. Scale bar, 300μm. mean±SD (*p<0.05, **p<0.01).

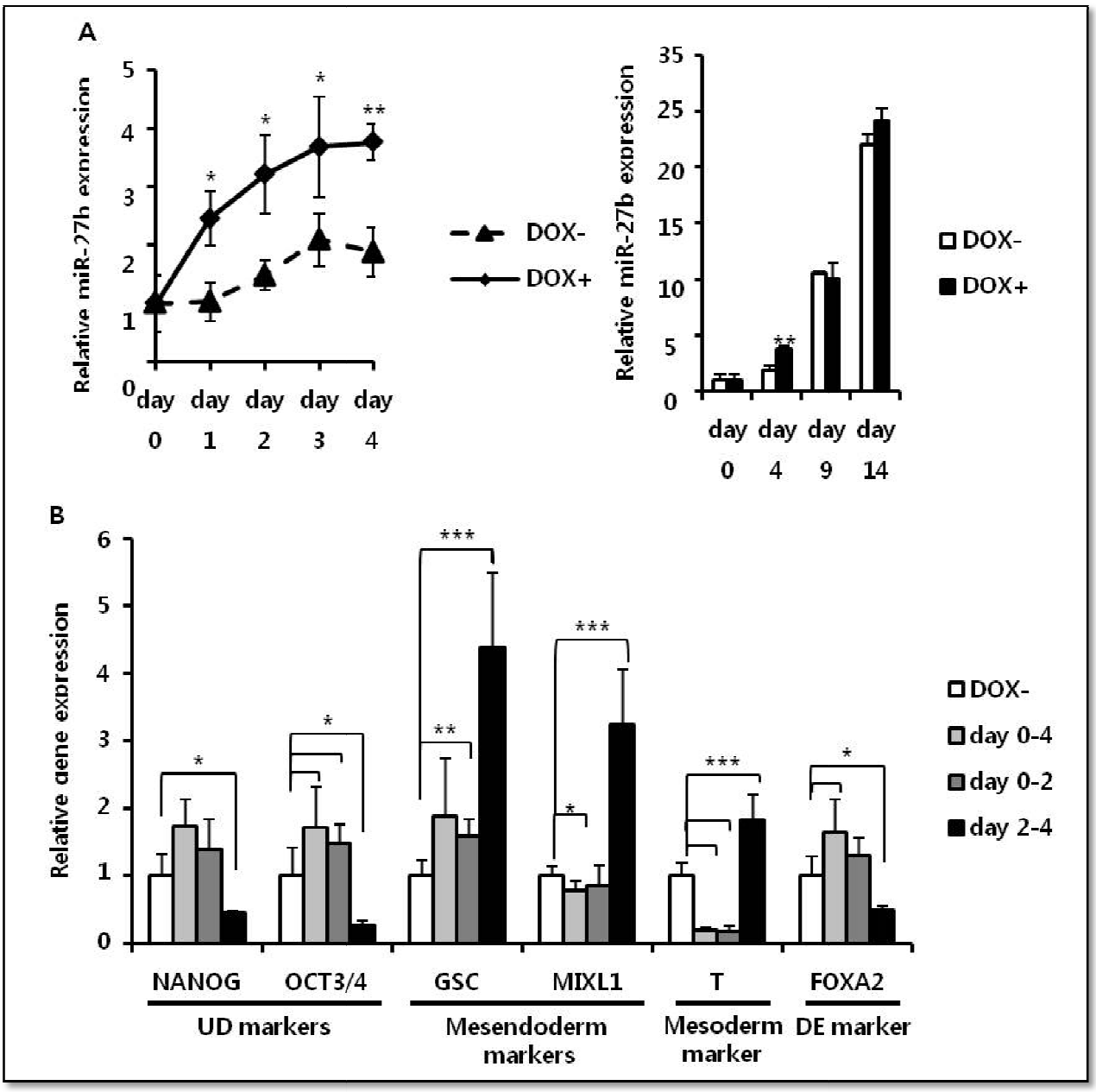

Figure 4. Excessive miR-27b expression impaired the definitive endodermal and mesodermal differentiation. hiPS-AAVS1-27b was differentiated into hepatocyte-like cells as described in Figure. 1A. (A) Doxycycline-induced miR-27b expression during DE (left) and hepatic (right) differentiation was measured by qRT-PCR (n=3). DOX was added during differentiation. (B) MiR-27b expression was induced on different time period (day0-4, day0-2 and day2-4) by DOX treatment. On day 4 (DE), expression of genes associated with undifferentiated (UD) state (*NANOG*, *OCT3/4*), mesendoderm (*GSC*, *MIXL1*), mesoderm (*T*) and DE(*FOXA2*) was analyzed by qRT-PCR (n=6). The data of cells without DOX treatment was taken as 1. mean±SD (*p<0.05, **p<0.01, ***p<0.001).

Excessive miR-27b expression impaired definitive endodermal and mesodermal differentiation.

In the previous report, it has been shown that miR-27b expression is upregulated during hepatic differentiation and overexpression of miR-27a in hEC cells leads to upregulated expression of differentiation-related genes [11], which suggested that miR-27b might also positively regulate the directed differentiation of hiPS cells to the hepatic lineage. During the DE differentiation, expression of miR-27b was kept relatively low, but significantly increased afterward (**Figure 1B**). DOX successfully induced more than 2-fold higher expression of miR-27b compared to the endogenous expression during the DE differentiation. However, additive increase of miR-27b expression from the integrated cassette was not detectable after day9 (**Figure 4A).** It was probably because of silencing in AAVS1 locus during hepatic differentiation as recently reported [18]. Therefore, we decided to examine whether higher expression of miR-27b has some stimulatory or inhibitory effects on the DE differentiation and eventually on hepatic differentiation. When miR-27b was induced for the entire period of the DE differentiation (day0-4), undiffrentiation marker expression (*OCT3/4*) was not suppressed in contrast to the result in undifferentiated hiPS cell-culture (**Figure 3A, 4B**), suggesting that activin-directed initiation of differentiation was inhibited to some extent. Importantly, mesendoderm (*GSC*) and endoderm (*FOXA2*) markers were significantly increased, while mesoderm marker (brachyury (*T*)) was strongly suppressed. These results suggested that increased expression of miR-27b promotes the DE differentiation by suppressing mesoderm differentiation.

Next, we divided the miR-27b induction period into two (day0-2 and day2-4) and examined which period represents the effects of miR-27b induction for day0-4 on the DE and mesoderm differentiation. When miR-27b was induced for the earlier period (day0-2), the mesoderm marker (*T*) was suppressed, while mesendoderm marker (*GSC*) was increased similarly to day0-4. On the other hand, when miR-27b was induced for the later period (day2-4), the DE-specific marker (*FOXA2*) was suppressed, while mesendoderm (*GSC*, *MIXL1*) and mesoderm (*T*) markers were increased (**Figure 4B**). These results revealed that miR-27b induction during endoderm formation, particularly during earlier period of formation, contributes positively to the DE and negatively to mesoderm differentiation, and that the later induction caused the opposite effects.

Excessive miR-27b expression during endodermal differentiation suppressed hepatoblast and hepatic differentiation.

MiR-27b induction during earlier endodermal differentiation caused somewhat increasing of endoderm marker expression and significant suppression of mesoderm marker expression, while later induction caused increase in mesodermal marker (**Figure 4B).** We next examined the influences of these changes in the DE and mesodermal differentiation on later developmental stages. Both hepatoblast (*HNF4α*, *AFP*) and hepatocyte (*ALB*) gene expression were suppressed by miR-27b induction for day2-4 (**Figure 5A, 5B),** indicating that suppression of the DE differentiation and stimulation of mesodermal differentiation at day4 lead to the inefficient hepatic differentiation. In case of miR-27b induction for day0-4 and day0-2, we predicted stimulation of hepatic differentiation because expression of DE markers was upregulated. However, hepatic differentiation was clearly suppressed in both cases as marker gene expression (*αAT*, *ALB*) was decreased (**Figure 5B**). Taken altogether, these results suggested that properly restricted expression of miR-27b during endoderm differentiation was crucial for *in vitro* hepatic differentiation of hiPS cells.

DISCUSSION

In this report, we generated DOX-inducible miR-27b-expressing hiPS cell lines, and uncovered the role of miR-27b for maintaining pluripotency and for proper regulation of the endodermal differentiation in hiPS cells. We found that induced miR-27b expression caused strong suppression of pluripotency-associated gene expression in hiPS cells with morphological changes of hiPS colonies (**Figure 3A, 3C).** In addition, genes associated to ectoderm, the default direction of iPS differentiation, were significantly increased, while mesendoderm/mesoderm/endoderm marker expression was suppressed **(Figure 3D).** These results supported the previous studies, in which overexpression of miR-27b in hEC cells suppressed undifferentiation-related markers, even more convincingly as we employed hiPS [11]. Also, deletion of miR-24a/b~27a/b~24 clusters in mouse ES cells was reported to have no obvious effects on gene expression and colony formation but impaired differentiation of embryoid bodies [10]. Thus, our studies along with others' established the role of miR-27b as a negative regulator against the undifferentiated state of cells and, therefore, miR-27b should be suppressed to maintain ES/iPS cells in undifferentiated state.

MiR-27 has been reported to repress expression of SMAD2/3, a downstream component of activin [11]. As activin was used as a critical cytokine to direct endodermal differentiation in *in vitro* hepatic differentiation, the impaired differentiation might be explained by inhibitory effect of miR-27b on NODAL signal. MiR-27b induction at the beginning of differentiation might inhibit NODAL signal required for the initiation of differentiation program leading to increase in undifferentiation marker-expression (**Figure 4B).** Similarly, induced expression of miR-27b in later period (day 2-4) might weaken the NODAL signal. As the high level of NODAL signal is the inducer of endoderm differentiation and the low level stimulates mesoderm differentiation [19,20], the NODAL signal, weakened by miR-27b induction, might stimulate mesoderm

differentiation but suppress the DE differentiation as we observed (**Figure 4B).**

We found that miR-27b expression was increasing during hepatic differentiation, which was consistent to the previous findings [9,11], but unexpectedly, stayed relatively low level in formation of DE (**Figure 1B).** We also found that the induced miR-27b expression during endoderm differentiation, regardless the inducing periods, eventually caused suppression of hepatoblast- and hepatic-differentiation (**Figure 5A).** In total, these findings uncovered the importance of secured low expression of miR-27b for both endodermal and mesodermal differentiation and consequently formation of hepatoblast-like cells, the progenitor of hepatic differentiation, and hepatocyte-like cells, which further confirms the importance of suppression of miR-27b expression in endodermal differentiation to ensure the hepatocyte development.

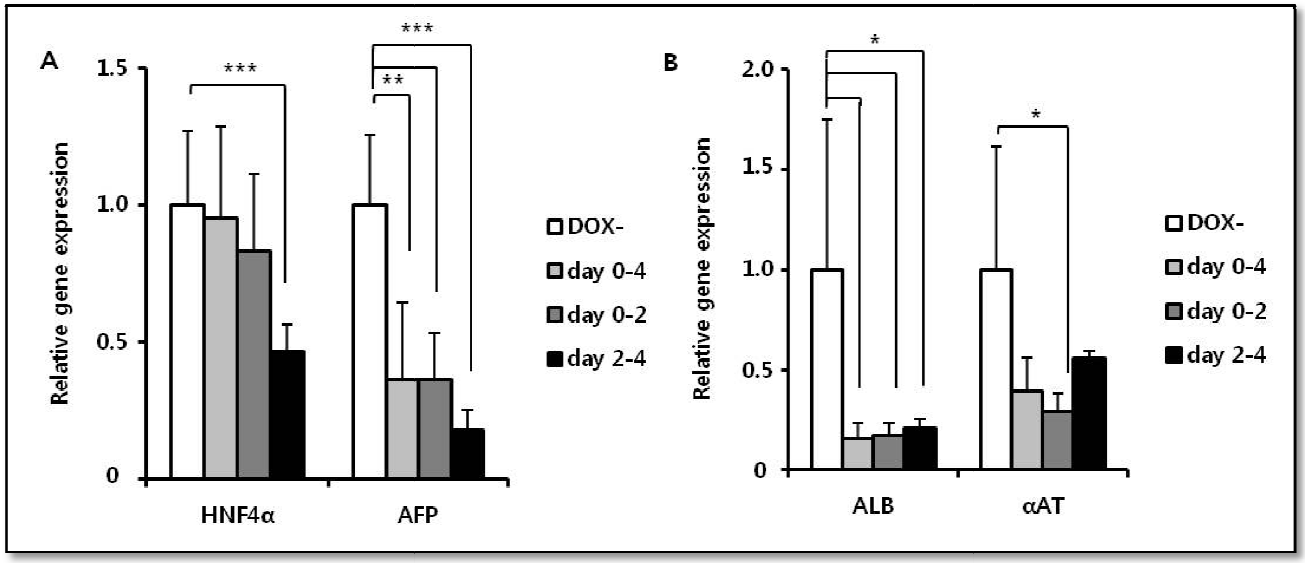

Figure 5. Excessive miR-27b expression during endodermal differentiation suppressed hepatoblast and hepatic differentiation. hiPS-AAVS1-27b was differentiated into hepatocyte-like cells as described in Figure. 1A culturing with DOX on different time period (day0-4, day0-2 and day2-4). Then, expression of marker genes was measured at day9 (hepatoblast-like cells) (A) and day25 (hepatocyte-like cells) (B) by qRT-PCR (n=6). The data of cells without DOX treatment was taken as 1. (*p<0.05, **p<0.01, ***p<0.001)

CONCLUSION

In summary, we revealed the role of miR-27b involving in endodermal differentiation as well as in maintenance of undifferentiation state in hiPS cells. Suppression of miR-27b in early stages is required to keep pluripotency and self-renewal, and to secure correct differentiation of hepatocytes via endoderm formation. The specific role of miR-27b in late differentiation stages still remains to be elucidated. Additional study is necessary to identify how miR-27b regulates hepatic differentiation and clarification of its molecular pathway would contribute to understand underlying mechanism of embryonic development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J.L. performed the experiments, analyzed data, and wrote the manuscript. E.S. designed the experiments and wrote the manuscript. K.T. assisted to design and perform the experiments. F.S. supervised the project. H.M. supervised the project and wrote the manuscript.

REFERENCES

1. Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, et al. (2010) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells Hepatology. 51 (2010) 297–305.

2. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297.

3. Mallannaa SK, Rizzino A (2010) Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. Developmental Biology 334: 16-25.

4. Yao S (2016) MicroRNA biogenesis and their functions in regulating stem cell potency and differentiation. Biol Proced Online 18. 8. s12575-016-0037-y.

5. Zhao C, Sun G, Li S, Shi Y (2009) A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat Struct Mol Biol 16: 365-371.

6. Michael K, Fischer C, Nowitsch S, Opriessnig P, Papak C, et al. (2009) microRNA miR-27b impairs human adipocyte differentiation and targets *PPARγ*. Biochem Biophys Res Commun 390: 247-251.

7. Kasey VC, Shoucri BM, Levin MG, Wu H, Pearson DS, et al. (2013) MicroRNA-27b is a regulatory hub in lipid metabolism and is altered in dyslipidemia. Hepatology 57: 533-542.

8. Lee JJ , Drakaki A, Iliopoulos D, Struhl K (2011) MiR-27b targets *PPARγ* to inhibit growth, tumor progression and the inflammatory response in neuroblastoma cells. Oncogene 31: 3818-3825.

9. Kim N, Kim H, Jung I, Kim Y, Kim D, et al. (2011) Expression profiles of miRNAs in human embryonic stem cells during hepatocyte differentiation. Hepatol Res 41: 170-183.

10. Ma Y, Yao N, Liu G, Dong L, Liu Y, et al. (2015) Functional screen reveals essential roles of miR-27a/24 in differentiation of embryonic stem cells. EMBO J 34: 361-378.

11. Fuchs H, Theuser M, Wruck W, Adjaye J (2014) MIR-27 Negatively Regulates Pluripotency-Associated Genes in Human Embryonal Carcinoma Cells. PLoS One 9: e111627.

12. Takayama K, Morisaki Y, Kuno S, Nagamoto Y, Harada K, et al. (2014) Prediction of interindividual differences in hepatic functions and drug sensitivity by using human iPS-derived hepatocytes. Proc Natl Acad Sci USA 111: 16772-16777.

13. Takayama K, Igai K, Hagihara Y, Hashimoto R, Hanawa M, et al. (2017) Highly efficient biallelic genome editing of human ES/iPS cells using a CRISPR/Cas9 or TALEN system. Nucleic Acids Res: 1-10. doi:10.1093/nar/gkx130.

14. Smith JR, Maguire S, Davis LA, Alexander M, Yang F, et al. (2008) Robust, Persistent Transgene Expression in Human Embryonic Stem Cells Is Achieved with AAVS1-Targeted Integration. Stem Cells 26: 496-504.

15. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157: 1262-1278.

16. Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 339: 819-23.

17. Zhang S, Cui W (2014) Sox2, a key factor in the regulation of pluripotency and neural differentiation. World J Stem Cells 6: 305-311.

18. Ordovás L, Boon R, Pistoni M, Chen Y, Wolfs E, et al. (2015) Efficient recombinase-mediated cassette exchange in hPSCs to study the hepatocyte lineage reveals AAVS1 locus-mediated transgene inhibition. Stem Cell Reports 5: 918–931.

19. Takenaga M, Fukumoto M, Hori Y (2007) Regulated Nodal signaling promotes differentiation of the definitive endoderm and mesoderm from ES cells. J Cell Sci 120: 2078-2090.

20. S.D. Vincent, Dunn NR, Hayashi S, Norris DP, Robertson EJ (2003) Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev 17: 1646–1662.