

# Oct3/4 is potentially useful for the suppression of the proliferation and motility of hepatocellular carcinoma cells

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**Abstract.** Hepatocellular carcinoma (HCC) cells are immature compared with healthy mature hepatocytes. Transcription factors serve a role in hepatocyte differentiation. The expression levels of transcription factors in HCC cell lines have been investigated to determine potential therapeutic targets. In the present study, the HLE, HLF, PLC/PRF/5, Huh-7, Hep3B, Huh-6 and HepG2 HCC cell lines were subjected to reverse-transcription polymerase chain reaction (RT-PCR) of transcription factors, including NANOG, Oct3/4, GATA binding protein 4 (GATA4), GATA6 and hematopoietically expressed homeobox (HHEX). In addition, these cell lines were analyzed using RT-quantitative PCR (RT-qPCR) of NANOG and Oct3/4. The 201B7 human induced pluripotent stem cells were evaluated as a model of pluripotent cells. The HLF cells were transfected with Oct3/4 small interfering RNA (siRNA) and used in an MTS colorimetric assay and a scratch assay. NANOG was not expressed in any of the cell lines. However, GATA4, GATA6 and HHEX were expressed in the majority of the HCC cell lines. In addition, NANOG and Oct3/4 were expressed in 201B7 cells. Oct3/4 was expressed in HLE, HLF and Hep3B cells; however, its expression levels were significantly reduced compared with those in 201B7 cells. RT-qPCR demonstrated that the expression of Oct3/4 siRNA suppressed the proliferation and motility of HLF cells. Oct3/4 siRNA may be a potentially effective therapy for the suppression of the proliferation and motility of HCC cells.

## Introduction

Hepatocellular carcinoma (HCC) develops in the liver following long-term infection with the hepatitis B or C virus (1).

One of the biological characteristics of HCC is that the cancer cells are similar to immature hepatocytes (2). Additionally, HCC cells are positive for cluster of differentiation (CD)133, a cancer stem cell marker (3), and for biliary epithelial cell markers (3). These results suggest that HCC cells have the potential to differentiate into hepatocytes and biliary epithelial cells (4).

Transcription factors serve an important role in hepatocyte differentiation. For example, CCAAT/enhancer-binding protein (C/EBP) $\alpha$  promotes the maturation of immature hepatocytes (5), but it is downregulated in HCC (6). Similarly, GATA binding protein (GATA)4 and GATA6 are involved in hepatocyte differentiation. Whilst GATA4 is associated with endoderm differentiation (7), GATA6 is required for liver bud formation (8). In addition, hematopoietically-expressed homeobox (HHEX) is essential for the differentiation of human induced pluripotent stem (iPS) cells into hepatocytes (9). These studies indicate that GATA4, GATA6 and HHEX may be involved in the carcinogenesis of HCC.

iPS cells are generated from adult cells upon the introduction of reprogramming factors, including NANOG, sex determining region Y box 2 (Sox2), Oct3/4 and Krüppel-like factor (KLF)4 (10). The expression patterns of these genes in HCC cells have yet to be elucidated. Therefore, the present study investigated the expression patterns of these transcription factors in order to identify potential novel treatments for HCC. Human iPS cells were used as a model for pluripotent stem cells to compare the expression patterns of these genes.

## Materials and methods

**Cell culture.** The 201B7 iPS cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in ReproFF (ReproCELL, Yokohama, Japan) in 6-well plates (Asahi Techno Glass Corporation, Funabashi, Japan) coated with Matrigel™ (BD Biosciences, Franklin Lakes, NJ, USA), in feeder-cell-free conditions at 37°C in an incubator with 5% CO<sub>2</sub>. ReproFF was a complete medium and ready to use. The cells were harvested with Accutase® (Innovative Cell Technologies, Inc., San Diego, CA, USA) and seeded onto 6-well plates or 96-well plates coated with Matrigel™ at a density of 10<sup>6</sup> cells/well. The HLE, HLF, PLC/PRF/5, Huh-7, Hep3B, Huh-6 and HepG2 HCC cell lines were also purchased from RIKEN BioResource Center.

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The HCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an incubator with 5% CO<sub>2</sub>. The HCC cells were trypsinized, harvested and plated onto 6-well plates or 96-well plates. The cells were observed under a light microscope (CKX41N-31PHP; Olympus Corporation, Tokyo, Japan).

**Transfection and cell proliferation assay.** The HLF cells were trypsinized, harvested and plated onto 96-well flat-bottom plates (Asahi Techno Glass Corporation) at a density of 1,000 cells/well, and then incubated for 24 h in DMEM supplemented with 10% FBS at 37°C in an incubator with 5% CO<sub>2</sub>. The cells were transfected with negative control small interfering RNA (siRNA) of random sequences or with Oct3/4 siRNA at 20 or 200 nM using Lipofectamine® 2000 (all from Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO<sub>2</sub>. The sequence of the Oct3/4 siRNA was 5'-CACCCUUGUGUCCCAAUCCUUC-3'. The negative control siRNA was Stealth RNAi™ siRNA Negative Control, MedGC (cat. no. 12935-300; Thermo Fisher Scientific, Inc.). Transfection with Lipofectamine® 2000 and no nucleic acid material was used as a mock transfection control. Furthermore, the siRNA-treated cells were cultured for 72 h and an MTS colorimetric assay was performed according to the manufacturer's protocol (CellTiter 96® Aqueous One Solution Cell Proliferation Assay; Promega Corporation, Madison, WI, USA). MTS was ready to use (11). Cells reduce MTS into formazan, a colored product, which has a maximum absorbance at a wavelength of 490 nm; this was measured using an iMark Microplate Absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Scratch assay.** HLF cells were plated onto 4-well chamber slides (BD Biosciences) at a density of 5x10<sup>4</sup> cells/well. When the cells were confluent, the cell sheets were scratched with a sterile razor. The cells were immediately transfected with Oct3/4 siRNA or the mock transfection control as aforementioned. After 2 days of culture, the cells were stained with hematoxylin and eosin, observed using an AX80 light microscope (magnification, x100; Olympus Corporation) and five distinct fields were imaged; the five distinct fields were chosen randomly. The distance between the edge of the cell sheets and the scratched line was measured. The experiment was repeated three times.

**Reverse transcription polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR).** Total RNA (5 µg) was isolated using Isogen® (Nippon Gene Co., Ltd., Tokyo, Japan) from cells cultured in 6-well plates, was utilized for first-strand complementary (c)DNA synthesis, using SuperScript® III First Strand Synthesis and oligo(dT) and following the manufacturer's protocol (Thermo Fisher Scientific, Inc.). RNA from human fetal and adult liver was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). PCR was performed using Taq DNA polymerase (Thermo Fisher Scientific, Inc.) and products were separated using gel electrophoresis in 2% agarose in 1X TAE (40 mM Tris-acetate/1 mM EDTA). PCR primer (Thermo Fisher Scientific, Inc.) sequences,

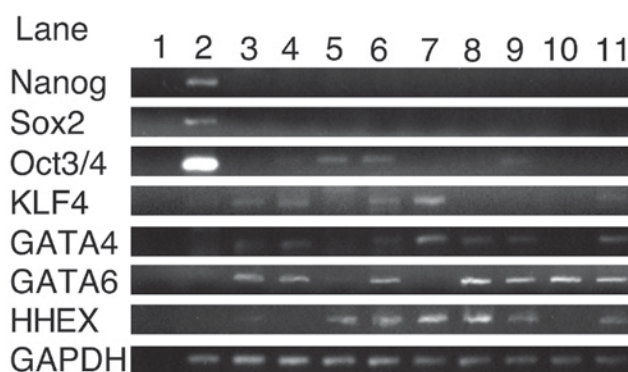


Figure 1. Expression levels of NANOG, Sox2, Oct3/4, KLF4, GATA4, GATA6 and HHEX in control and HCC cell lines as measured by reverse transcription polymerase chain reaction. Lane 1, H<sub>2</sub>O; 2, 201B7; 3, fetal liver; 4, adult liver; 5, HLE; 6, HLF; 7, PLC/PRF/5; 8, Huh-7; 9, Hep3B; 10, Huh-6 and 11, HepG2 cells. HCC, hepatocellular carcinoma; Sox2, sex determining region Y box 2; KLF4, Krüppel-like factor 4; GATA, GATA binding protein; HHEX, hematopoietically expressed homeobox.

annealing temperatures, reaction cycle numbers and amplicon lengths for RT-PCR are presented in Table I. RT-qPCR was performed using the Fast SYBR-Green Master Mix (Thermo Fisher Scientific, Inc.) and analyzed with the MiniOpticon™ Detection System (Bio-Rad Laboratories, Inc.). RT-qPCR was performed for 40 cycles of two steps consisting of 5 sec denaturation at 95°C and 5 sec annealing-extension at 60°C. PCR primers (Thermo Fisher Scientific, Inc.), annealing temperatures, reaction cycle numbers and amplicon lengths for RT-qPCR are listed in Table II. GAPDH and ribosomal protein L19 (RPL19) were used as internal controls for RT-PCR and RT-qPCR, respectively. RPL19 was used as an endogenous control to monitor the quantity of mRNA as a constitutively expressed housekeeping gene (12). The gene expression levels were analyzed using the automated MiniOpticon™ system based on the  $\Delta\Delta$  cycle threshold ( $\Delta\Delta C_q$ ) method (13). The relative expression was calculated as the expression level of a specific gene divided by the expression level of RPL19. The experiments were performed three times, and triplicates were used in each experiment.

**Statistical analysis.** One-way analysis of variance was applied for statistical analysis. JMP 10.0.2 software (SAS Institute Inc., Cary NC, USA) was used for statistical analysis.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**RT-PCR indicates the expression levels of transcription factors in HCC cells.** To reveal the expression patterns of transcription factors in HCC cells, RT-PCR was performed (Fig. 1). Total RNA from fetal and adult liver cells was analyzed to search for genes expressed in HCC cells but not in fetal and adult liver. These genes may be involved in proliferation of HCC. Whilst NANOG and Sox2 were not expressed in any of the HCC cell lines, Oct3/4 was expressed in HLE, HLF and Hep3B cells (Lanes 5, 6 and 9, respectively), and GATA4, GATA6 was expressed in HLF, Huh-7, Hep3B, Huh-6 and HepG2 cells. HHEX was expressed in HLE, HLF, PLC/PRF/5, Hep3B, Huh-7 and HepG2 cells. KLF4 was expressed in fetal and

Primer name	Sequence	Description	Product size, bp	Annealing temperature, °C	No. of cycles	GenBank accession number
OMC21	5'-ACCTGACCTGCCGTCTAGAA-3'	GAPDH, forward	350	63	40	BC025925
OMC22	5'-TCCACCACCCCTGTTGCTGTA-3'	GAPDH, reverse				
OMC271	5'-CTTGCTGCAGAAAGTGGTGGAGGAA-3'	Oct3/4, forward	187	60	40	NM_002701
OMC272	5'-CTGCAGTGTGGGTTTCGGGCA-3'	Oct3/4, reverse				
OMC273	5'-AAAAAAGGAAGACAAAGTCCCCG-3'	NANOG, forward	299	56	40	NM_024865
OMC274	5'-ATCCCTGGTAGGAAAGATAAAG-3'	NANOG, reverse				
OMC277	5'-TTCCTCCAACGACCAACCATCG-3'	HHEX, forward	348	56	40	NM_002729
OMC278	5'-TTTTATCGCCCTCAATGTCCAC-3'	HHEX, reverse				
OMC279	5'-TTCATCACGGCGCTTGGATTGTC-3'	GATA6, forward	299	56	40	NM_005257
OMC280	5'-GTGTTGTGGGGAAGTATTTTGC-3'	GATA6, reverse				
OMC281	5'-GAAAACGGAAAGCCCAAGAAC-3'	GATA4, forward	218	56	40	NM_002052
OMC282	5'-AGACATCGCACTGACTGAGAACG-3'	GATA4, reverse				
OMC283	5'-TGCGGCAAAACCTACACAAAG-3'	KLF4, forward	379	57	40	NM_004235
OMC284	5'-CACTCACAAGATGACTCAGTTGGG-3'	KLF4, reverse				
OMC285	5'-AGAAAACGAGGGGAAATGGGAG-3'	Sox2, forward	161	53	40	NM_003106
OMC286	5'-TTGCGTGAGTGTGGATGGGATTGG-3'	Sox2, reverse	187	65	40	

HHEX, hematopoietically expressed homeobox; GATA, GATA binding protein; KLF4, Kruppel-like factor 4; Sox2, sex determining region Y box 2.

Table II. Primer sequences and conditions for reverse transcription-quantitative polymerase chain reaction.

Primer name	Sequence	Description	Product size, bp	Annealing temperature, °C	No. of cycles	GenBank accession number
OMC311	5'-CCGTTTGGCTCTGTTTG-3'	NANOG, forward	187	60	40	NM_024865
OMC312	5'-TCATCGAAACACTCGGTGAA-3'	NANOG, reverse				
OMC321	5'-CGAATGCCAGAGAAGTCAAC-3'	RPL19, forward	157	60	40	BC095445
OMC322	5'-CCATGAGAAATCCGCTTGTTT-3'	RPL19, reverse				

Primer sequences for Oct3/4 are the same as those in Table I. RPL19, ribosomal protein L19.

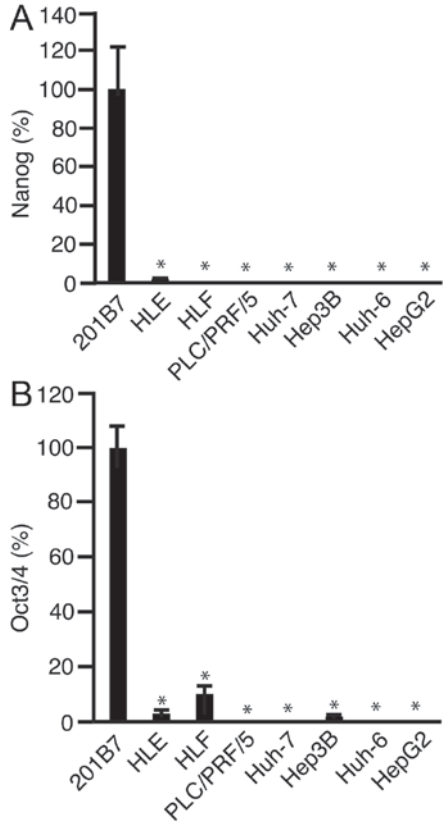


Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis of NANOG and Oct3/4 expression. (A) NANOG mRNA expression. (B) Oct3/4 expression. Expression levels were normalized against that of 201B7 cells. Data are presented as mean  $\pm$  standard deviation, \* $P<0.05$ , compared with the expression levels in the control 201B7 cells. Experiments were repeated three times.

adult liver, and not in 201B7 cells. Oct3/4 was expressed in HLE and HLF. Furthermore, NANOG, Sox2 and Oct3/4 were highly expressed in 201B7 cells while not in fetal and adult liver cells (Lane 2). Nanog and Sox2 were not expressed in HCC cells analyzed in the present study; therefore Nanog and Sox2 may not be associated with HCC carcinogenesis. Oct3/4 was expressed in HLE, HLF and Hep3B cells. Therefore, Oct3/4 may have a role in the proliferation of the HLE, HLF and Hep3B cell lines.

*RT-qPCR indicates the expression levels of NANOG and Oct3/4 in HCC cells.* RT-qPCR was performed to analyze the expression levels of Oct3/4. The expression levels of NANOG (Fig. 2A) and Oct3/4 (Fig. 2B) were significantly lower in all the HCC cell lines, as compared with in the 201B7 iPS cells ( $P<0.05$ ). The expression levels of Oct3/4 were significantly lower in HLE, HLF and Hep3B cells, compared with those in 201B7 cells ( $P<0.05$ ). In addition, the expression level of Oct3/4 was significantly higher in HLF cells, compared with in HLE ( $P<0.05$ ) and Hep3B cells ( $P<0.05$ ). These results suggest that Oct3/4 has a role in carcinogenesis in HLF cells. Therefore, HLF cells were used for further investigation.

*Oct3/4 siRNA transfection reduces the expression levels Oct3/4 and cell proliferation.* HLF cells were transfected



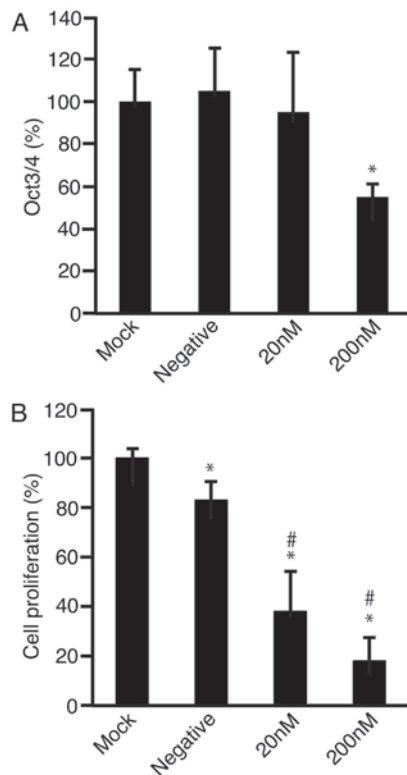


Figure 3. HLF cell proliferation significantly decreases following transfection with Oct3/4 siRNA. Oct3/4 mRNA expression levels in untreated (mock) HLF cells or HLF cells transfected with negative control or Oct3/4 siRNA. (B) HLF cell proliferation following transfection. After 3 days of culturing, the transfected cells were subjected to an MTS colorimetric assay. Data are presented as mean  $\pm$  standard deviation, \* $P$ <0.05, compared with the mock transfection control, # $P$ <0.01, compared with negative control. Experiments were repeated three times.

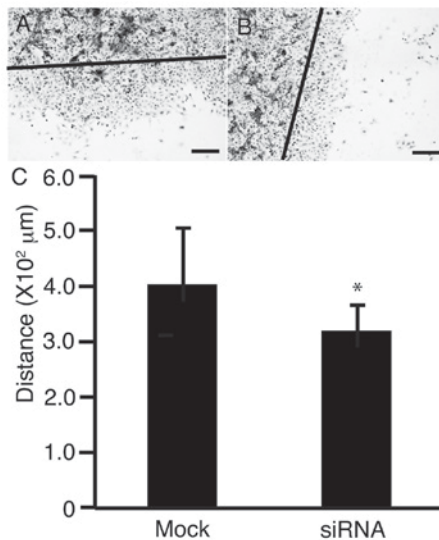


Figure 4. Oct3/4 siRNA significantly inhibits the ability of HLF cells to migrate. Scratch wound assay for (A) untreated (mock) HLF cells and (B) HLF cells treated with Oct3/4 siRNA. The cells were stained with hematoxylin and eosin. Distances from the edge of the cell sheet to the scratched line (solid line) were measured at five individual points. (C) Quantification of the distance migrated by untreated and Oct3/4 siRNA-treated HLF cells in three independent experiments. Distances from the edge of the cell sheet to the scratched line (solid line) were measured at five individual points. Data are presented as mean  $\pm$  standard deviation. Magnification,  $\times 100$ ; scale bar, 200  $\mu$ m. \* $P$ <0.05, compared with mock transfection control. siRNA, small interfering RNA.

with Oct3/4 siRNA and after 2 days of culture, RNA was isolated and subjected to RT-qPCR to analyze the expression levels of Oct3/4 (Fig. 3A). It was observed that the expression levels of Oct3/4 were significantly downregulated following treatment with 200 nM targeted-siRNA ( $P$ <0.05). The MTS assay demonstrated that cell proliferation was suppressed with Oct3/4 siRNA at 20 and 200 nM compared with the mock control (both  $P$ <0.01; Fig. 3B). Negative control siRNA also decreased cell proliferation compared with mock ( $P$ <0.05); however, Oct3/4 siRNA significantly decreased cell proliferation compared with the negative control siRNA at 20 and 200 nM (both  $P$ <0.01).

*Oct3/4 siRNA reduces the ability of HLF cells to migrate.* To clarify the effect of Oct3/4 siRNA on cell motility, a scratch assay was performed. HLF cells were scratched with a sterile razor and transfected with Oct3/4 siRNA. After 2 days of culture, the transfected cells were imaged (Fig. 4A). The distance between the edge of the cell sheet and the scratched line was measured (Fig. 4B). It was observed that the distance significantly decreased in the layer of cells transfected with Oct3/4 siRNA, as compared with the mock transfection control ( $P$ <0.05).

## Discussion

HCC cells are hypo-methylated in the promoter region of NANOG (14). Cancer stem cells are rare and a population may be enriched for cancer stem cells using serum-free media (15). In the present study, NANOG and Sox2 were not expressed in any of the HCC cell lines. Therefore, it is hypothesized that the promoter region of NANOG may be methylated. However, it remains to be elucidated as to why Sox2 is not expressed in HCC cells.

Oct3/4 is involved in pluripotency along with NANOG, Sox2 and KLF4 (16). OCT3/4 is expressed in HCC cells (17), as well as being highly expressed in the cancer stem cells of HCC (18). Oct3/4 is upregulated by growth factors, including the insulin-like growth factor-1 via protein kinase B (19,20). These previous studies suggest that Oct3/4 may affect the stemness of HCC cells, and also their phenotype. Higher expression levels of Oct3/4 are predictive of poor prognosis in patients with HCC (21). This previous study suggests that Oct3/4 has a role in carcinogenesis or cancer progression (21). In the present study, Oct3/4 siRNA significantly suppressed the proliferation and motility of HLF cells. These results indicate that Oct3/4 may be a potential therapeutic target for the treatment of HCC. This hypothesis was supported by Murakami *et al* (22), who demonstrated that Oct3/4 was a potentially effective target for differentiation therapy.

One limitation of the present study is that stemness markers were not analyzed in HLF cells following transfection with Oct3/4 siRNA. Therefore, the differentiation state of the cells was not determined. An investigation into the differentiation state of HCC cells transfected with Oct3/4 siRNA may facilitate further understanding. In conclusion, Oct3/4 is expressed in HLE, HLF and Hep3B cells, and Oct3/4 siRNA suppresses the proliferation and motility of HLF cells.

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