59. Kopan R, Ilagan MX. The canonical notch signaling pathway: unfolding the activation mechanism. Cell. 2009;137:216–33.
60. Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S. Notch signals control the fate of immature progenitor cells in the intestine. Nature. 2005;435:964–8.
61. Apelqvist A, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Curr Biol. 1997;7:801–4.
62. Kodama Y, Hijikata M, Kageyama R, Shimotohno K, Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology. 2004;127:1775–86.
63. Mazur PK, Einwächter H, Lee M, Sipos B, Nakhai H, Rad R, et al. Notch2 is required for progression of pancreatic intraepithelial neoplasia and development of pancreatic ductal adenocarcinoma. Proc Natl Acad Sci USA. 2010;107:13438–43.
64. Sonoshita M, Aoki M, Fuwa H, Aoki K, Hosogi H, Sakai Y, et al. Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell. 2011;19:125–37.
65. Ohashi S, Natsuizaka M, Yashiro-Ohtani Y, Kalman RA, Nakagawa M, Wu L, et al. NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. Gastroenterology. 2010;139:2113–23.
66. Sikandar SS, Pate KT, Anderson S, Dizon D, Edwards RA, Waterman ML, et al. NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. Cancer Res. 2010;70:1469–78.
67. Reya T, Clever H. Wnt signaling in stem cells and cancer. Nature. 2005;434:843–50.
68. van de Wetering M, Molenaar M, Oost-erwegel M, Peterson-Maduro J, Godsave S, Korinek V, et al. The b-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 2002;111:241–50.
69. Abu-Remaileh M, Gerson A, Farago M, Nathan G, Alkalay I, Zins Rousso S, et al. Oct-3/4 regulates stem cell identity and cell fate decisions by modulating Wnt/β-catenin signaling. EMBO J. 2010;29:3236–48.
70. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumors. Nature. 2003;425:846–51.
71. Varnat F, Duquet A, Malerba M, Zbinden M, Mas C, Gervaz P, et al. Human colon cancer epithelial cells harbor active HEDGEHOG-GLI signaling that is essential for tumor growth, recurrence, metastasis and stem cell survival and expansion. EMBO Mol Med. 2009;1:338–51.
72. Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature. 2003;425:851–6.
73. Watabe T, Miyazono K. Roles of TGF-beta family signaling in stem cell renewal and differentiation. Cell Res. 2009;19:103–15.
74. Ikushima H, Miyazono K. TGFβ signalling: a complex web in cancer progression. Nat Rev Cancer. 2010;10:415–24.
75. Takaku M, Oshima M, Miyoshi H, Matsui M, Seldin MF, Taketo MM. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell. 1998;92:645–56.
76. Kabashima A, Higuchi H, Takaishi H, Matsuzaki Y, Suzuki S, Izumiya M, et al. Side population of pancreatic cancer cells predominates in TGF-beta-mediated epithelial to mesenchymal transition and invasion. Int J Cancer. 2009;124:2771–9.
77. Tang B, Yoo N, Vu M, Mamura M, Nam JS, Ooshima A, et al. Transforming growth factor β can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res. 2007;67:8643–52.
78. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128:683–92.
79. Berdasco M, Esteller M. Aberrant epigenetic landscape in cancer: how cellular identity goes awry. Dev Cell. 2010;19:698–711.
80. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.
81. Jenuwein T, Allis CD. Translating the histone code. Science. 2001;293:1074–80.
82. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006;125:315–26.
83. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, et al. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. Cancer Res. 2002;62:6456–61.
84. Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet. 2005;37:391–400.
85. Yasuda H, Soejima K, Watanabe H, Kawada I, Nakachi I, Yoda S, et al. Distinct epigenetic regulation of tumor suppressor genes in putative cancer stem cells of solid tumors. Int J Oncol. 2010;37:1537–46.
86. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, et al. Global histone modification patterns predict risk of prostate cancer recurrence. Nature. 2005;435:1262–6.
87. You H, Ding W, Rountree CB. Epigenetic regulation of cancer stem cell marker CD133 by transforming growth factor-beta. Hepatology. 2010;51:1635–44.
88. Hernandez-Vargas H, Ouzounova M, Calvez-Kelm FL, Lambert MP, McKay-Chopin S, Tavtigian SV, et al. Methylome analysis reveals Jak-STAT pathway deregulation in putative breast cancer stem cells. Epigenetics. 2011;6:428–39.
89. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
90. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature. 2005;435:839–43.
91. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, et al. RAS is regulated by the let-7 microRNA family. Cell. 2005;120:635–47.
92. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, et al. Dicer is essential for mouse development. Nat Genet. 2003;35:215–7.
93. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 2005;19:489–501.
94. McKenna LB, Schug J, Vourekas A, McKenna JB, Bramswig NC, Friedman JR, et al. MicroRNAs control intestinal epithelial differentiation, architecture, and barrier function. Gastroenterology. 2010;139:1654–64.
95. Sekine S, Ogawa R, Ito R, Hiraoka N, McManus MT, Kanai Y, et al. Disruption of Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis. Gastroenterology. 2009;136:2304–15.
96. Suh MR, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, et al. Human embryonic stem cells express a unique set of microRNAs. Dev Biol. 2004;270:488–98.
97. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39:673–7.

98. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
99. Ji J, Yamashita T, Budhu A, Forgues M, Jia HL, Li C, et al. Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM-positive hepatic cancer stem cells. Hepatology. 2009;502:472–80.
100. Wang Y, Yu Y, Tsuyada A, Ren X, Wu X, Stubblefield K, et al. Transforming growth factor-b regulates the sphere-initiating stem cell-like feature in breast cancer through miRNA-181 and ATM. Oncogene. 2011;30:1470–80.
101. Wang B, Hsu SH, Majumder S, Kutay H, Huang W, Jacob ST, et al. TGF beta-mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3. Oncogene. 2010;29:1787–97.
102. Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, et al. miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell. 2010;7:694–707.
103. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med. 2011;17: 211–5.
104. Iliopoulos D, Lindahl-Allen M, Polytarchou C, Hirsch HA, Tsichlis PN, Struhl K. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. Mol Cell. 2010;39: 761–72.
105. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature. 2004;432:332–7.
106. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell. 2008;132:598–611.
107. Moore KA, Lemischka IR. Stem cells and their niches. Science. 2006;311:1880–5.
108. Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, et al. Variable β-catenin expression in colorectal cancer indicates tumor progression driven by tumor environment. Proc Natl Acad Sci USA. 2001;98:10356–61.
109. Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A perivascular niche for brain tumor stem cells. Cancer Cell. 2007;11:69–82.
110. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci USA. 2011;108:1397–402.
111. Mazumdar J, O'Brien WT, Johnson RS, LaManna JC, Chavez JC, Klein PS, et al. O_2 regulates stem cells through Wnt/β-catenin signaling. Nat Cell Biol. 2010;12:1007–13.
112. Yeung TM, Gandhi SC, Bodmer WF. Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells. Proc Natl Acad Sci USA. 2011;108:4382–7.
113. Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, McKay RD, et al. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. Oncogene. 2009;28:3949–59.
114. Heddleston JM, Li Z, McLendon RE, Hjelmeland AB, Rich JN. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. Cell Cycle. 2009;8:3274–84.
115. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell. 2009;138:645–59.
116. Dong TT, Zhou HM, Wang LL, Feng B, Lv B, Zheng MH. Salinomycin selectively targets 'CD133+' cell subpopulations and decreases malignant traits in colorectal cancer lines. Ann Surg Oncol. 2011;18:1797–804.
117. Mueller MT, Hermann PC, Witthauer J, Rubio-Viqueira B, Leicht SF, Huber S, et al. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. Gastroenterology. 2009;137:1102–13.
118. Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med. 2006;12:1167–74.
119. Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res. 2009;69:3382–9.
120. Zhu Z, Hao X, Yan M, Yao M, Ge C, Gu J, et al. Cancer stem/progenitor cells are highly enriched in CD133+ CD44+ population in hepatocellular carcinoma. Int J Cancer. 2010;126: 2067–78.
121. Hwang-Verslues WW, Chang PH, Wei PC, Yang CY, Huang CK, Kuo WH, et al. miR-495 is upregulated by E12/E47 in breast cancer stem cells, and promotes oncogenesis and hypoxia resistance via downregulation of E-cadherin and REDD1. Oncogene. 2011;30:2463–74.
122. Henry JC, Park JK, Jiang J, Kim JH, Nagorney DM, Roberts LR, et al. miR-199a–3p targets CD44 and reduces proliferation of CD44 positive hepatocellular carcinoma cell lines. Biochem Biophys Res Commun. 2010;403:120–5.
123. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007;131:1109–23.
124. Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, et al. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell. 2009;138:592–603.
125. Yu F, Deng H, Yao H, Liu Q, Su F, Song E. Mir-30 reduction maintains self-renewal and inhibits apoptosis in breast tumor-initiating cells. Oncogene. 2010;29:4194–204.

Ann Surg Oncol (2011) 18:3005–3012
DOI 10.1245/s10434-011-1653-z

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

The Significance of *PITX2* Overexpression in Human Colorectal Cancer

Hajime Hirose, MD[1], Hideshi Ishii, MD, PhD[1,2], Koshi Mimori, MD, PhD[2], Fumiaki Tanaka, MD, PhD[2], Ichiro Takemasa, MD, PhD[1], Tsunekazu Mizushima, MD, PhD[1], Masataka Ikeda, MD, PhD[1], Hirofumi Yamamoto, MD, PhD[1], Mitsugu Sekimoto, MD, PhD[1], Yuichiro Doki, MD, PhD[1], and Masaki Mori, MD, PhD, FACS[1]

[1]Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Osaka, Japan; [2]Division of Molecular and Surgical Oncology, Department of Molecular and Cellular Biology, Kyushu University, Medical Institute of Bioregulation, Beppu, Oita, Japan

ABSTRACT

Purpose. The paired-like homeodomain transcription factor 2 (*PITX2*) gene encodes a transcription factor controlled by the *WNT/Dvl/CTNNB1* and Hedgehog/*TGFB* pathways in the pathogenesis of colorectal cancer (CRC). Although *PITX2* is reportedly involved in various functions, including tissue development by controlling cell growth, its significance in CRC remains unclear. We report our findings regarding abnormal *PITX2* expression in human CRC.

Methods. *PITX2* expression was evaluated in 5 human CRC cell lines and 92 primary CRC samples. Cell growth was evaluated after inhibition of *PITX2* expression or after exogenous introduction of *PITX2*.

Results. *PITX2* expression was seen in all the five CRC cell lines. The study of tissue samples indicated that *PITX2* expression was significantly higher in cancerous tissue than in paired control tissue ($P = 0.0471$). Patients with lower *PITX2* expression showed a poorer overall survival rate than those with higher *PITX2* expression ($P = 0.0481$). Multivariate analysis demonstrated that *PITX2* expression was an independent prognostic factor. Experimental knockdown and introduction of *PITX2* also demonstrated that the level of *PITX2* expression is inversely associated with cell growth and invasion in vitro.

Conclusions. *PITX2* expression is significantly related to the biological behavior of CRC cells and appears to be correlated with clinical survival. Thus, this study revealed a previously uncharacterized unique role and significance of *PITX2* expression in CRC.

In developed countries, cancer is a serious health issue. The prevalence of colorectal cancer (CRC) has increased in Japan and is becoming similar to that in Western countries, probably due to changes in lifestyle.[1] To date, the majority of studies on CRC have focused on molecular characterization of its tumor biology with the aim of establishing an adequate treatment algorithm for CRC.[2,3]

Several genes, including critical transcription factors, are located in chromosome 4, and several are reportedly involved in tumor development.[4–15] Among these, the paired-like homeodomain transcription factor 2 (*PITX2*) gene, located in chromosome 4q25–q27, encodes a transcription factor of the paired-like homeodomain family that functions as a hormone; in particular, it regulates prolactin and growth hormone in the pituitary gland.[16] *PITX2* is known to be involved in the embryological asymmetric development of anteroposterior alignment in the pituitary gland and heart, and mediates specific morphogenesis in response to left–right asymmetric signals as a transcription factor.[17,18] *PITX2* is also thought to regulate the expression of other oncogenes, such as *MYC*, *CCND1*, and *CCND2*, and is controlled by *WNT/Dvl/CTNNB1*. This is an important pathway in the pathogenesis of CRC.[19,20] It has been reported that hypermethylation of *PITX2*'s promoter is associated with poorer prognosis in patients with hormone receptor-positive breast cancer.[21] *PITX2* has also

Electronic supplementary material The online version of this article (doi:10.1245/s10434-011-1653-z) contains supplementary material, which is available to authorized users.

First Received: 6 August 2010;
Published Online: 9 April 2011

M. Mori, MD, PhD, FACS
e-mail: mmori@gesurg.med.osaka-u.ac.jp

been associated with disease recurrence in prostate cancer.[22] However, its association with gastrointestinal cancer remains to be investigated.

In the present study, we analyzed *PITX2* expression in 5 human CRC cell lines as well as in normal noncancerous colon tissue taken from 92 paired cases of primary human CRC. In addition, we performed an in vitro study where gene-knockdown techniques and the introduction of *PITX2* were used to investigate the relevance of *PITX2* expression and its relation to clinicopathological characteristics.

MATERIALS AND METHODS

Cell Culture

CRC cell lines (SW480, LoVo, KM12SM, and HT29) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and incubated at 37°C. Caco-2 cells were maintained in minimum essential medium containing 20% FBS. For the small interference RNA (siRNA)-knockdown experiment, double-stranded RNA duplexes that targeted the human *PITX2* gene (5′-GC AUGUUAUACAGAAGCGATT-3′/5′-UCGCUUCUGUA UAACAUGCTT-3′) were synthesized (Hs_PITX2_6HP siRNA; QIAGEN, Valencia, CA). Negative control siRNA (Neg) was used (AllStars Neg siRNA, sense sequence: UUCUCCGAACGUGUCACGU; QIAGEN). CRC cell lines were transfected with siRNA at concentration of 5 μmol/L using HiPerFect transfection reagent (QIAGEN). The cell growth rate was studied using the CellTac kit (Nihon Koden, Tokyo, Japan). Plasmids with the human *PITX2* NM_000325 (Origene Inc., Rockville, MD) were transfected in LoVo with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). An empty vector was used as a mock control. Values are presented as mean ± standard deviation (SD) of four independent experiments. The invasion assay was performed using Transwell cell culture chambers (BD Biosciences, Bedford, MA) as described previously.[23] Briefly, 2×10^5 cells were seeded on a Matrigel-coated membrane. After 48 h, cells that had invaded the undersurface of the membrane were fixed with 100% methanol and stained with 1% toluidine blue. Values are presented as mean ± SD of four independent experiments.

Clinical Tissue Samples

Ninety-two patients (56 men, 36 women) diagnosed with CRC underwent surgery at the Medical Institute of Bioregulation at Kyusyu University between 1992 and 2002. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after receiving their informed consent and in accordance with the institutional guidelines. Every patient was definitively diagnosed as having CRC on the basis of clinicopathological findings. The resected surgical specimens were equally divided into two parts; one half of each tumor was frozen in liquid nitrogen and preserved at −80°C for RNA and protein studies, and the other half was fixed in formalin, processed through graded ethanol, and embedded in paraffin. The formalin-fixed sections were stained with hematoxylin and eosin and elastica van Gieson, and the degree of histological differentiation, lymphatic invasion, and venous invasion was microscopically examined. None of the patients received chemotherapy or radiotherapy prior to surgery. Clinicopathological factors were assessed according to the tumor–node–metastasis (TNM) classification criteria as defined by the International Union against Cancer.[24,25] The patients were followed up with blood examination, including levels of tumor markers such as serum carcinoembryonic antigen and cancer antigen, and underwent imaging investigations such as abdominal ultrasonography and/or computed tomography as well as chest X-ray every 3–6 months.

RNA Study

Total RNA was extracted from frozen tissue and prepared by reverse transcription.[26,27] Two human *PITX2* oligonucleotide primers for the PCR were designed as 244-bp *PITX2* fragments [5′-ACTTTACCAGCCAGCAGCTC-3′ (forward) and 5′-GGGTACATGTCGTCGTAGGG-3′ (reverse)]. The forward primer was located in exon 1, and the reverse primer in exon 2. As an internal control, a PCR assay with primers specific to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was performed. These *GAPDH* primers, 5′-TTGGTATCGTGGAAGGACTCA-3′ (forward) and 5′-TGTCATCATATTGGCAGGTT-3′ (reverse), produced a 270-bp amplicon. *MYC* primers, 5′-GAGAGGCAGAG GGAGCGAGCGGGC-3′ (forward) and 5′-TGTCGTTGA-GAGGGTAGGGGAAGA-3′ (reverse), produced a 188-bp amplicon. Real-time monitoring of the PCRs was performed using the LightCycler system (Roche Diagnostics, Tokyo, Japan) for complementary DNA (cDNA) amplification of *PITX2* and *GAPDH*.

Protein Study

Western blot analysis was performed as described previously.[28] The membrane was incubated with anti-PITX2 rabbit polyclonal antibody and anti-actin antibody (Santa Cruz Biotechnology, SantaCruz, CA) (1:200 for PITX2 and 1:1,000 for actin).

Statistical Analysis

For continuous variables used in the in vitro analysis, data are expressed as mean ± SD and were analyzed with the Wilcoxon rank test. The relationship between messenger RNA (mRNA) expression and clinicopathological factors was analyzed by the chi-square test and Student's *t*-test. Kaplan–Meier survival curves were plotted and compared using a generalized log-rank test. Univariate and multivariate analyses for the identification of factors prognostic for overall survival were carried out using the Cox proportional hazard regression model. All tests were analyzed using JMP software (SAS Institute, Cary, NC). P values < 0.05 were considered statistically significant.

RESULTS

PITX2 mRNA Expression in CRC Clinical Tissue Specimens

The expression of *PITX2* was assessed by reverse-transcription polymerase chain reaction (RT-PCR) analysis, which showed that the PCR amplification was specific and produced a single band in all five cell lines as well as in five randomly selected clinical samples of CRC, including one stage 1, two stage 2, and two stage 3 samples (Supplementary Fig. 1). The RT-PCR data were consistent with those determined using real-time quantitative RT-PCR. Therefore, we extended the study. Examination of the 92 paired CRC tissue samples by real-time quantitative RT-PCR showed that, in 73 of 92 cases (79.3%), higher levels of *PITX2* mRNA were seen in tumor tissue compared with matched normal tissue (Fig. 1a). Microscopic analysis did not detect any apparent characteristics in normal mucosa with high *PITX2* expression, compared with cancerous portions (data not shown).

PITX2 Expression and Clinicopathological Characteristics

For clinicopathological evaluation, the tissue samples were divided into two groups according to the level of *PITX2* expression. Patients with tumors that showed more than the median value of calculated *PITX2* expression were assigned to a high-expression group ($n = 46$); the others were assigned to a low-expression group ($n = 46$). Clinicopathological factors related to *PITX2* expression status are summarized in Table 1. In terms of histological grade, there were 32, 56, and 3 cases with well, moderately, and poorly differentiated CRC, respectively. There was one case of mucinous adenocarcinoma. According to the International Union against Cancer stage, the number of cases for TNM stage 0, I, IIA, IIB, IIIA, IIIB, IIIC, and IV was 0, 19, 23, 2, 3, 20, 9, and 16, respectively. Thirty-two patients had stage III disease. Ten of 32 patients received adjuvant chemotherapy. All patients underwent fluorouracil-based chemotherapy. Sixteen patients had stage IV, all of whom underwent macroscopic resection of primary lesion. Twelve of 16 patients had only liver metastasis, 2 of 16 patients had only peritoneal dissemination, and 2 of 16 patients had both metastases.

Our data indicated that *PITX2* expression did not correlate with clinicopathological factors; we only found that the high-expression group exhibited a tendency towards left side location, i.e., descending and sigmoid colon, and rectum ($P < 0.001$).

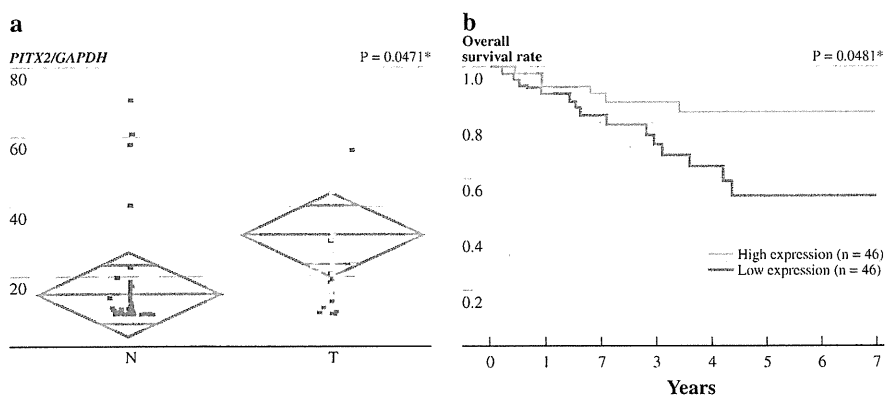

FIG. 1 **a** PITX2 mRNA expression in clinical tissue specimens. Quantitative RT-PCR of 92 clinical samples showed that 73 (79.3%) of the 92 CRC cases had higher levels of PITX2 mRNA compared with matched normal tissue. The mean PITX2 mRNA expression in tumor tissues (normalized by GAPDH gene expression) was significantly higher than that among the corresponding normal tissues ($P = 0.0471$; Student's *t*-test). *Dots in red* show data for the poor prognosis group. **b** Overall survival curves of patients with CRC, based on PITX2 mRNA expression status. The overall survival rate was higher in the PITX2 high-expression group compared with the low-expression group ($P = 0.0481$)

TABLE 1 PITX2 mRNA expression and clinicopathological factors

Factor	Low expression ($n = 46$) Number (%)	High expression ($n = 46$) Number (%)	P value
Age (mean ± SD) (years)	69.2 ± 11.2	65.2 ± 8.6	0.0558
Sex			0.2938
Male	23 (50.0%)	28 (60.8%)	
Female	23 (50.0%)	18 (39.1%)	
Histological grade			0.2960
Well, Moderately	43 (93.5%)	45 (97.8%)	
Others	3 (6.5%)	1 (2.2%)	
Tumor location			≤0.001[a]
Right side	26 (56.5%)	5 (10.9%)	
Left side	20 (43.5%)	41 (89.1%)	
Size			0.2118
<30 mm (small)	4 (14.3%)	8 (17.4%)	
≥31 mm (large)	42 (85.7%)	38 (82.6%)	
Tumor stage			0.1526
T1–2	9 (19.6%)	15 (32.5%)	
T3–4	37 (80.4%)	31 (67.5%)	
Lymph node metastasis			0.6763
Absent	25 (54.4%)	23 (50.0%)	
Present	21 (45.6%)	23 (50.0%)	
Lymphatic invasion			0.4022
Absent	27 (58.7%)	23 (50.0%)	
Present	19 (41.3%)	23 (50.0%)	
Venous invasion			0.3272
Absent	37 (80.4%)	33 (71.7%)	
Present	9 (19.6%)	13 (28.3%)	
Liver metastasis			1.0000
Absent	39 (84.8%)	39 (84.8%)	
Present	7 (15.2%)	7 (15.2%)	
Peritoneal dissemination			0.2960
Absent	43 (93.5%)	45 (97.8%)	
Present	3 (6.5%)	1 (2.2%)	
UICC stage			0.4022
1–2	23 (50.0%)	19 (41.3%)	
3–4	23 (50.0%)	27 (58.7%)	

SD standard deviation, *UICC* International Union against Cancer

Well = well-differentiated adenocarcinoma; Moderately = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocarcinoma and mucinous carcinoma

Right side = cecum, ascending, and transverse colon; Left side = descending, sigmoid, and rectum

[a] Statistically significant

Relationship between PITX2 Expression and Prognosis

The data showed that the overall survival rate was significantly higher in patients with elevated *PITX2* expression ($P = 0.0481$). The median follow-up period was 9.98 ± 2.94 years (Fig. 1b). Table 2 shows univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that the following factors were significantly related to overall survival: tumor size, tumor invasion, lymph node metastasis, lymphatic invasion, venous invasion, and *PITX2* mRNA expression. Multivariate analysis indicated that inclusion in the *PITX2* mRNA low-expression group was an independent predictor of overall survival, as was lymph node metastasis.

In Vitro Assessment of Knockdown and Transfection of PITX2

The *PITX2* expression study indicated that Caco-2 and KM12SM cells had higher levels of expression than other CRC cell lines such as LoVo, HT29, and SW480 (Supplementary Fig. 2). We performed a knockdown experiment of *PITX2* expression using the Caco-2 cell line. RT-PCR and Western blotting were used to confirm the reduction in expression due to siRNA treatment (Fig. 2a, b). A proliferation assay indicated that the knockdown resulted in an increase in the number of Caco-2 cells with high *PITX2* expression at 48 h and 72 h ($P < 0.05$; Fig. 2c). The invasion assay demonstrated that the

TABLE 2 Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

Factor	Univariate analysis			Multivariate analysis		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Age (>68 years/≤68 years)	1.01	0.96–1.05	0.7568			
Sex (female/male)	1.11	0.45–2.56	0.8178			
Histological grade (others/well, moderately)	5.40	0.84–19.63	0.0697			
Tumor location (right side/left side)	1.08	0.38–2.68	0.8735			
Size (<30 mm/≥30 mm)	5.62	1.15–101.2	0.0287[a]	1.26	0.06–11.95	0.8507
Tumor stage (T3–4/T1–2)	6.58	1.86–40.85	0.0016[a]	3.10	0.66–27.41	0.1671
Lymph node metastasis (present/absent)	6.39	2.38–22.16	<0.001[a]	4.54	1.56–16.84	0.0043[a]
Lymphatic invasion (present/absent)	3.88	1.62–10.21	0.0020[a]	1.72	0.68–4.81	0.2587
Venous invasion (present/absent)	2.55	1.00–6.07	0.0486[a]	1.90	1.02–7.55	0.1855
PITX2 expression (low/high)	2.40	1.01–6.21	0.0483[a]	2.63	1.02–7.55	0.0445[a]

RR relative risk, *CI* confidence interval

Well = well-differentiated adenocarcinoma; Moderately = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocarcinoma and mucinous carcinoma

Right side = cecum, ascending, and transverse colon; Left side = descending, sigmoid, and rectum

[a] Statistically significant

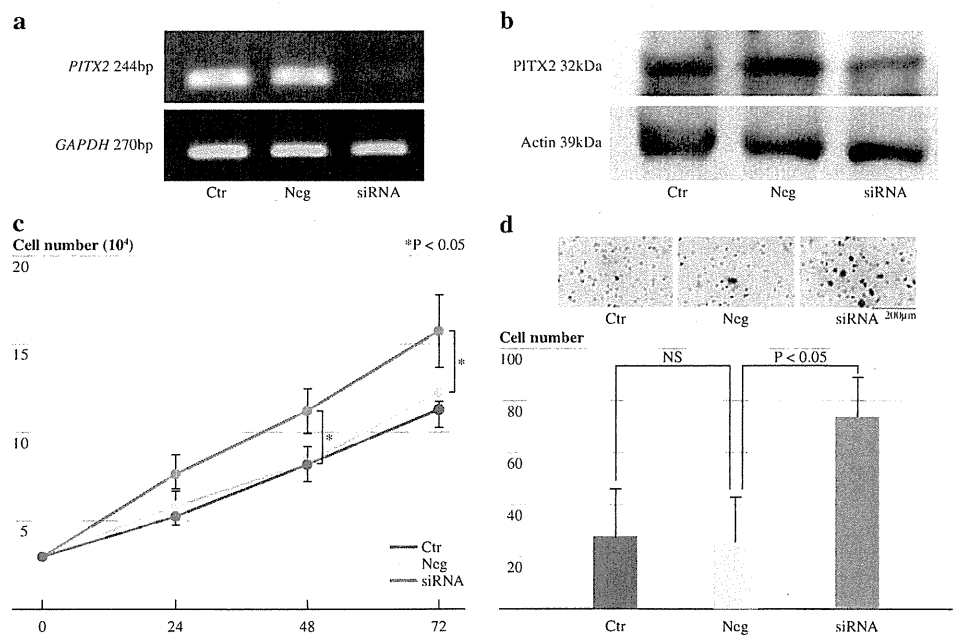

FIG. 2 Knockdown assessment of PITX2 by siRNA in the Caco-2 cell line. Confirmation of reduced PITX2 mRNA levels was by RT-PCR (**a**) and Western blot (**b**) analyses. A proliferation assay showed a difference in growth of the Caco-2 cell line. There were significant differences between Neg and siRNA (**c**). The invasion assay revealed that siRNA was significantly more invasive than Neg (**d**). Values are mean ± SD of four independent experiments. *Ctr* a control without transfection, *Neg* transfection control of scramble sequence, *siRNA* PITX2 small interference RNA

knockdown led to an increase in invasive activity at 48 h ($P < 0.05$; Fig. 2d). We induced *PITX2* expression in the LoVo cell line using a plasmid technique. RT-PCR and Western blotting showed successful induction (Fig. 3a, b). Proliferation assays indicated that high *PITX2* expression reduced cell numbers at 48 h and 72 h in LoVo cells, which have low *PITX2* expression by default ($P < 0.05$; Fig. 3c). An invasion assay demonstrated that the increased *PITX2* expression resulted in a decrease in the invasiveness of LoVo cells at 48 h ($P < 0.05$; Fig. 3d).

DISCUSSION

Cancer is a genetic and epigenetic disorder that involves activation of growth-promoting oncogenes and deactivation of growth-constraining tumor suppressor genes. The present study analyzed the expression of *PITX2* in 92 cases of human CRC and demonstrated that *PITX2* expression is increased in cancerous tissues significantly more than in matched noncancerous control tissue. Furthermore, and importantly, the high *PITX2* expression in cancer was

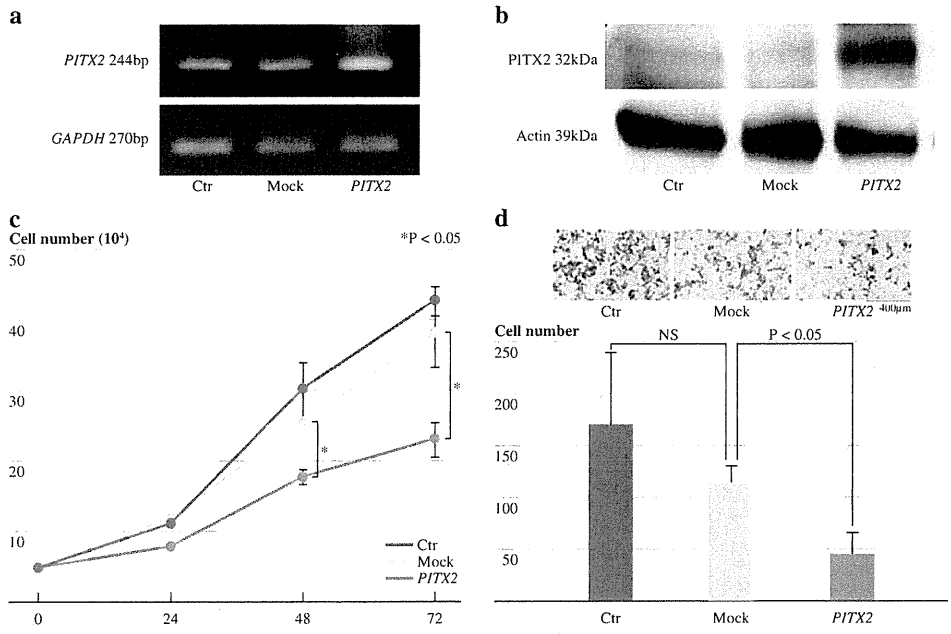

FIG. 3 PITX2 transfection assessment by plasmid in the LoVo cell line. Confirmation of PITX2 mRNA levels was by RT-PCR (**a**) and Western blot (**b**) analyses. The proliferation assay showed a difference in growth of the plasmid-containing LoVo cell line. There were significant differences between Mock and PITX2 (**c**). The invasion assay determined that PITX2-transfected cells became significantly less invasive than Mock cell lines (**d**). Values are mean ± SD of four independent experiments. *Ctr* a control without transfection, *Mock* empty vector transfection control, *PITX2* PITX2 plasmid-transfected cells

associated with improved survival in patients. Considering this observation, one can hypothesize that this gene may function partially to inhibit carcinogenesis through high expression status, but that, through an uncharacterized mechanism, its expression is more suppressed, leading to more invasive and proliferative tumor cell behavior. In this study, we noted the unique expression of this gene, suggesting the involvement of a complex epigenetic mechanism. Other factors, such as size, tumor stage, lymphatic invasion, and venous invasion, disappeared after multivariate analysis. We think this suggests that *PITX2* expression has a strong relationship with invasiveness and cell growth. To assess whether the increase in *PITX2* expression is biologically involved in the development of CRC, or if the expression is a nonfunctional association, we performed knockdown and overexpression experiments in vitro. Our findings indicate that *PITX2* knockdown in cells showing high expression of *PITX2* elicits a reduction in cell growth and invasion, whereas *PITX2* overexpression in cells showing low expression of *PITX2* induces tumor characteristics. This suggests that, in established CRC cell lines, *PITX2* appears to have a tumor-suppressive function. Nevertheless, we have to consider other aspects of *PITX2* function. First, *PITX2* expression might have been induced as a physiological regulatory process to counteract the stimuli of carcinogenesis. Second, the transcription factor function of *PITX2* might regulate different sets of downstream genes during the early stages of carcinogenesis and in established CRC. Further investigations are necessary to clarify these suggestions.

Diverse functions of *PITX2* have been reported in human diseases. *PITX2* is known to be overexpressed in Rieger syndrome, which is associated with congenital glaucoma, dental hypoplasia, and failure of skin closure around the umbilicus.[29] Regarding tumors, involvement of *PITX2* has been reported in nonfunctional pituitary adenomas, desmoid tumor, and Wilms' tumor (35).[30–34] It is also thought to have a role as a cell differentiation determinant in acute leukemia, where it targets the *ALL1* fusion gene.[35] In prostate cancer, hypermethylation within a CpG island of *PITX2*'s promoter has been reported, and is proposed as a marker for tumor recurrence with distant metastasis.[22] A significant association between *PITX2* hypermethylation and recurrence risk was also reported in hormone receptor-positive, lymph node metastasis-negative breast cancer, suggesting that downregulation of *PITX2* could be involved in cancer development, presumably due to hypermethylation of this gene.[21] To the best of our knowledge, this is the first report on the role of *PITX2* in CRC. The present data showed higher levels of *PITX2* expression in tumors (79.3%) compared with paired normal tissue, supporting the hypothesis that high *PITX2* expression is associated with cancer development, as has been suggested by others. An outstanding feature of *PITX2* in CRC appears to be its inverse correlation with the survival of patients, which is supported by the in vitro data on cell growth and invasion. The biological function of *PITX2* observed in CRC cells suggests that *PITX2* plays at least a partial role in the clinicopathological features of patients with CRC.

PITX2 encodes for a homeodomain transcription factor, one of the paired-like homeodomain families, which activates downstream targets, such as *CCND* and *MYC* under the control of the *WNT/Dvl/CTNNB1* and Hedgehog/*TGFB* pathways.[19,20] In the present study, we assessed expression of *CCND* and *MYC* before and after *PITX2* overexpression/knockdown. We found a tendency toward an inverse correlation between expression of *PITX2* and *MYC* (Supplementary Fig. 4a, b), but this did not achieve statistical significance. We also could not find any relation between *PITX2* and *CCND*. This showed discernible alterations (data not shown), further supporting the notion that the transcription factor *PITX2* is finely tuned during cell differentiation, and it might differentially regulate downstream target genes during malignant transformation.[21]

CONCLUSIONS

The present study indicates that *PITX2* is a functional prognostic marker for survival in CRC patients. We hope that the present study will contribute to early detection of carcinogenesis, suppression of transformation, and efficient therapeutic approaches, presumably based on the unique function of the *PITX2* gene.

ACKNOWLEDGMENT We thank Dr. Miyoshi, Dr. Kim, Dr. Okano, and Dr. Uemura for their excellent advice and technical assistance. This work was supported in part by a grant from Core Research for Evolutional Science and Technology (CREST), a Grant-in-Aid for Scientific Research on Priority Areas (20012039), Grants-in-Aid for Scientific Research (S: 21229015 and C: 20590313) from the Ministry of Education, Culture, Sports, Science, and Technology, and a grant from the Tokyo Biochemical Research Foundation, Japan.

REFERENCES

1. Kohno SI, Luo C, Nawa A, et al. Oncolytic virotherapy with an HSV amplicon vector expressing granulocyte-macrophage colony-stimulating factor using the replication-competent HSV type 1 mutant HF10 as a helper virus. *Cancer Gene Ther.* 2007;14(11):918–26.
2. Hermsen M, Postma C, Baak J, et al. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. *Gastroenterology.* 2002;123(4):1109–19.
3. Leslie A, Pratt NR, Gillespie K, et al. Mutations of APC, K-ras, and p53 are associated with specific chromosomal aberrations in colorectal adenocarcinomas. *Cancer Res.* 2003;63(15):4656–61.
4. Xiao XY, Zhou XY, Yan G, et al. Chromosomal alteration in Chinese sporadic colorectal carcinomas detected by comparative genomic hybridization. *Diagn Mol Pathol.* 2007;16(2):96–103.
5. Li P, Maines-Bandiera S, Kuo WL, et al. Multiple roles of the candidate oncogene ZNF217 in ovarian epithelial neoplastic progression. *Int J Cancer.* 2007;120(9):1863–73.
6. Cheng SH, Ng MH, Lau KM, et al. 4q loss is potentially an important genetic event in MM tumorigenesis: identification of a tumor suppressor gene regulated by promoter methylation at 4q13.3, platelet factor, 4 *Blood.* 2007;109(5):2089–99.
7. Chan KY, Lai PB, Squire JA, et al. Positional expression profiling indicates candidate genes in deletion hotspots of hepatocellular carcinoma. *Mod Pathol.* 2006;19(12):1546–54.
8. Kang JU, Kang JJ, Kwon KC, et al. Genetic alterations in primary gastric carcinomas correlated with clinicopathological variables by array comparative genomic hybridization. *J Korean Med Sci.* 2006;21(4):656–65.
9. Sterian A, Kan T, Berki AT, et al. Mutational and LOH analyses of the chromosome 4q region in esophageal adenocarcinoma. *Oncology.* 2006;70(3):168–72.
10. Midorikawa Y, Yamamoto S, Ishikawa S, et al. Molecular karyotyping of human hepatocellular carcinoma using single-nucleotide polymorphism arrays. *Oncogene.* 2006;25(40):5581–90.
11. Dykema KJ, Furge KA. Diminished transcription of chromosome arm 4q is inversely related to local spreading of hepatocellular carcinoma. *Genes Chromosomes Cancer.* 2004;41(4):390–4.
12. Franke S, Wlodarska I, Maes B, et al. Lymphocyte predominance Hodgkin disease is characterized by recurrent genomic imbalances. *Blood.* 2001;97(6):1845–53.
13. Pimkhaokham A, Shimada Y, Fukuda Y, et al. Nonrandom chromosomal imbalances in esophageal squamous cell carcinoma cell lines: possible involvement of the ATF3 and CENPF genes in the 1q32 amplicon. *Jpn J Cancer Res.* 2000;91(11):1126–33.
14. Fujimoto Y, Kohgo Y. Alteration of genomic structure and/or expression of cancer associated genes in hepatocellular carcinoma. *Rinsho Byori.* 1998;46(1):9–14.
15. Tirkkonen M, Johannsson O, Agnarsson BA, et al. Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res.* 1997;57(7):1222–7.
16. Quentien MH, Pitoia F, Gunz G, et al. Regulation of prolactin, GH, and Pit-1 gene expression in anterior pituitary by Pitx2: An approach using Pitx2 mutants. *Endocrinology.* 2002;143(8):2839–51.
17. Logan M, Pagan-Westphal SM, Smith DM, et al. The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell.* 1998;94(3):307–17.
18. Ryan AK, Blumberg B, Rodriguez-Esteban C, et al. Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature.* 1998;394(6693):545–51.
19. Baek SH, Kioussi C, Briata P, et al. Regulated subset of G1 growth-control genes in response to derepression by the Wnt pathway. *Proc Natl Acad Sci USA.* 2003;100(6):3245–50.
20. Kioussi C, Briata P, Baek SH, et al. Identification of a Wnt/Dvl/beta-Catenin → Pitx2 pathway mediating cell-type-specific proliferation during development. *Cell.* 2002;111(5):673–85.
21. Maier S, Nimmrich I, Koenig T, et al. DNA-methylation of the homeodomain transcription factor PITX2 reliably predicts risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients—technical and clinical validation in a multi-centre setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology group. *Eur J Cancer.* 2007;43(11):1679–86.
22. Hampton T. New markers may help predict prostate cancer relapse risk. *JAMA.* 2006;295(19):2234–8.
23. Hoshino H, Miyoshi N, Nagai K, et al. Epithelial-mesenchymal transition with expression of SNAI1-induced chemoresistance in colorectal cancer. *Biochem Biophys Res Commun.* 2009;390(3):1061–5.
24. Sobin LH, Fleming ID, TNM classification of malignant tumors, fifth edition. Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer.* 1997;80(9):1803–4.

25. Miyoshi N, Ishii H, Sekimoto M, et al. RGS16 is a marker for prognosis in colorectal cancer. *Ann Surg Oncol.* 2009;16(12): 3507–14.
26. Mimori K, Mori M, Shiraishi T, et al. Clinical significance of tissue inhibitor of metalloproteinase expression in gastric carcinoma. *Br J Cancer.* 1997;76(4):531–6.
27. Mori M, Staniunas RJ, Barnard GF, et al. The significance of carbonic anhydrase expression in human colorectal cancer. *Gastroenterology.* 1993;105(3):820–6.
28. Yamamoto H, Kondo M, Nakamori S, et al. JTE-522, a cyclooxygenase-2 inhibitor, is an effective chemopreventive agent against rat experimental liver fibrosis1. *Gastroenterology.* 2003;125(2): 556–71.
29. Semina EV, Reiter R, Leysens NJ, et al. Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat Genet.* 1996; 14(4):392–9.
30. Moreno CS, Evans CO, Zhan X, et al. Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. *Cancer Res.* 2005;65(22):10214–22.
31. Muccielli ML, Martinez S, Pattyn A, et al. Otlx2, an Otx-related homeobox gene expressed in the pituitary gland and in a restricted pattern in the forebrain. *Mol Cell Neurosci.* 1996; 8(4):258–71.
32. Pellegrini-Bouiller I, Manrique C, Gunz G, et al. Expression of the members of the Ptx family of transcription factors in human pituitary adenomas. *J Clin Endocrinol Metab.* 1999;84(6):2212–20.
33. Bacac M, Migliavacca E, Stehle JC, et al. A gene expression signature that distinguishes desmoid tumours from nodular fasciitis. *J Pathol.* 2006;208(4):543–53.
34. Zirn B, Samans B, Wittmann S, et al. Target genes of the WNT/beta-catenin pathway in Wilms tumors. *Genes Chromosomes Cancer.* 2006;45(6):565–74.
35. Arakawa H, Nakamura T, Zhadanov AB, et al. Identification and characterization of the ARP1 gene, a target for the human acute leukemia ALL1 gene. *Proc Natl Acad Sci USA.* 1998;95(8): 4573–8.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Review Article

Reprogramming of gastrointestinal cancer cells

DyahLaksmi Dewi,[1,2,4] Hideshi Ishii,[1,4] Naotsugu Haraguchi,[1,4] Shimpei Nishikawa,[1,2] Yoshihiro Kano,[1,2] Takahito Fukusumi,[1,2] Katsuya Ohta,[1,2] Susumu Miyazaki,[2] Miyuki Ozaki,[1,2] Daisuke Sakai,[1] Taroh Satoh,[1] Hiroaki Nagano,[2] Yuichiro Doki[2] and Masaki Mori[2,3]

Departments of [1]Frontier Science for Cancer and Chemotherapy, [2]Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan

(Received October 13, 2011/Revised November 30, 2011/Accepted December 5, 2011/Accepted manuscript online December 12, 2011)

Cell reprogramming reverts cells to multipotent, preprogrammed states by re-establishing epigenetic markers. It can also induce considerable malignant phenotype modification. Because key events in cancer relapse and metastasis, including epithelial–mesenchymal transition phenotypes, are regulated primarily by reversible and transient epigenetic modifications rather than the accumulation of irreversible and stable genetic abnormalities, studying dynamic mechanisms regulating these biological processes is important. Transcription factors for induced pluripotent stem cells and non-coding microRNAs allow pluripotent phenotype induction. We present the current knowledge of the possible applications of cell reprogramming in reducing aggressive phenotype expression, which can induce tumor cell hibernation and maintain appropriate phenotypes, thereby minimizing relapse and metastasis after surgical resection of gastrointestinal cancer. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2011.02184.x, 2012)

During cell reprogramming, mature cells revert to an immature, preprogrammed (undifferentiated) state, and usually acquire multidifferentiated characteristics following the loss and re-establishment of important epigenetic markers including DNA methylation.[1] This deregulation of important genomic and epigenomic factors is commonly associated with the abnormal cell differentiation characteristics of different cancers.[2–7] Emerging data suggest that epigenetic modifications and cell reprogramming-like processes are important for cellular transformation and the development of malignant cancer phenotypes.[8–12] Understanding the underlying process of epigenome reprogramming facilitates the use of regenerative medicine and cancer therapy. Here we discuss whether the reprogramming-like phenomenon observed in normal cells can be adapted for developing novel therapies.

Programming and Reprogramming of Cells

Mammalian tissues develop from a totipotent zygote. During cell differentiation, a less specialized cell (i.e. stem or progenitor cell) continuously produces more specialized cell types through cell division, and thus, a complex tissue system containing increasingly differentiated and specialized cells is established. Subsequently, pluripotent primitive ectodermal cells in the inner cell mass of blastocysts develop from the totipotent zygote.[13,14] Following blastocyst implantation, pluripotent epiblast cells differentiate into somatic cells. Repression of the somatic program and re-expression of pluripotency-specific genes through epigenetic modifications are necessary for germ cell development,[13,15] indicating that differentiated cells retain all the genetic information necessary to generate an entire organism. This was initially tested by cloning animals from differentiated cells by nuclear transfer. Mouse[16,17] and human ES cells[18] are derived from their respective blastocysts. The recently developed iPSCs[19,20] can produce derivatives of each germ layer. Differentiation occurs both during the developmental stages and in adults;[21] multipotent tissue stem cells produce completely differentiated daughter cells during normal cell turnover in adult tissues and during tissue repair.[22] Differentiation is associated with dynamic alterations in cell morphology, cell metabolism, and responsiveness to cell signaling,[21,23] which occur largely because of highly regulated gene expression through mRNA regulation[23] and non-coding miRNA expression.[24–26]

Defined factor-mediated reprogramming. Considering the ethical issues regarding the use of fertilized oocytes for establishing and producing ES cells, and the immunological compatibility that occurs in case of unrelated donors, a great breakthrough was reported by Takahashi and Yamanaka[19], who discovered that complete reprogramming can be achieved by introducing defined biological factors, such as Oct4 (also known as Pou5f1), Sox2, Klf4, and c-Myc, in mouse and human fibroblasts.[20] The initial experimental injection of Fbx15-selected iPSCs into mouse blastocysts revealed that iPSCs alone could not efficiently produce chimeric mice, presumably because of substantial methylation of immature gene (including Nanog and Oct4) promoters.[19] Subsequent studies indicated that modified selection methods of completely reprogrammed cells through expression of endogenous Nanog[27,28] and Oct4[28] allowed the successful generation of viable chimeras and detectable transmission into the germline.

Complete pluripotency. During stable Oct4 and Nanog selection, although the overall appearance of the colonies was similar,[27,28] quantitative differences existed between the two selection strategies. Oct4-selected ES-like colonies provided more stable and homogenous iPSC lines than Nanog-selected ES-like colonies.[28] Eventually, the fraction of ES-like colonies from Oct4-selected MEF cultures was two or threefold higher than that from Nanog-selected cultures, although initially fewer colonies existed with Oct4-selected MEF-derived iPSCs. This suggests that although the Nanog locus was more easily activated, a higher fraction of colonies from Oct4-selected MEF cells was reprogrammed to pluripotency.[28] These studies established that selection for Oct4 and Nanog expression results in germline-competent iPSCs with increased ES cell-like gene expression and DNA methylation patterns compared with Fbx15-iPSCs. Whereas one clone from seven Nanog-iPSC clones was transmitted through the germline to the next generation,[27] Oct4-iPSCs injected into tetraploid blastocysts can generate live late-term embryos.[28] The biological potency and epigenetic state of iPSCs and ES cells are the same. The overall estimated efficiency (0.05–0.10%) to establish iPSC lines from

[3]To whom correspondence should be addressed.
E-mail: mmori@gesurg.med.osaka-u.ac.jp
[4]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2011.02184.x

MEFs was similar between Oct4 and Nanog selection, despite the larger number of total Nanog-iPSC colonies[28] (Table 1).

Reprogramming barriers by tumor suppressors. Several factors can enhance the efficiency of iPSC generation, such as cell cycle checkpoints mediated by the cyclin-dependent kinase inhibitor family. The *CDKN2b–CDKN2a* locus on human chromosome 9p21 (mice chromosome 4) is frequently lost in cancer. The locus encodes three cyclin-dependent kinase inhibitors of the cell cycle: *p15INK4b*, *p16INK4a*, and *p14ARF* (*p19Arf* in mice) encoded by *CDKN2b*, *CDKN2a*, and an alternative reading frame of *CDKN2a*, respectively.[29] These inhibitors are endogenously expressed in differentiated cells and downregulated

Table 1. Summary of current studies of normal somatic cell reprogramming

Method for factor delivery	Factor	Starting material	Efficiency	Ref.
Retroviral vector	OSKM	Mouse embryonic and adult fibroblast	n.d.	(19)
Retroviral vector	OSKM	Human fibroblast	10 colonies/5 × 10^4	(20)
Lentiviral	OSNL	Human fetal fibroblast	198 colonies/0.9 × 10^6	(71)
Plasmid transfection	OSKM	MEF	Lower than the viral delivery method	(41)
Adenovirus (non-integrating vector)	OSKM	MEF and hepatocyte	n.d.	(72)
Retrovirus	OSKM	Adult mouse liver and stomach cells	n.d.	(73)
Retrovirus	OSKM OK OM	Mouse neural stem cell	3.6% 0.11% Slower than OK	(74)
Retrovirus	OSKM+5'Aza OSK+VPA/5'Aza	MEF	0.5% 11.8% 100 × higher than the OSKM method	(75)
Doxycycline-inducible lentiviruses	OSKM	Secondary somatic cells containing Dox-inducible OSKM expression (MEF, intestinal epithelium)	20–50 × higher than the direct infection method	(76)
Retroviral	OSKM	Adult human adipose stem cells	0.2%	(77)
Repeated protein transduction	OSKM + VPA OSK + VPA	MEF	Slower kinetic than the viral delivery method	(49)
Retrovirus	OK + BIX/BayK compound	Neural progenitor cells	12 colonies/3.5 × 10^4	(78)
Doxycycline-inducible transcription factors delivered by PiggyBac transposition	OSKM	Murine and human embryonic fibroblasts	n.d.	(43)
Retrovirus for OSK Transfection of miRNA mimics	OSK+miR-291-3p, miR-294, miR-295	MEF	0.1–0.3%	(79)
2A-peptide linked reprogramming cassette introduced by nucleofection	OSKM	MEF	2.5%	(42)
Sendai virus	OSKM	Human terminally differentiated circulating T cells	0.1%	(45)
Lentivirus	Oct4 + small compound (A-83-01, PD0325901, PS48, NaB)	Neonatal human epidermal keratinocytes, HUVECs, and amniotic fluid-derived cells	4–6 colonies/1 × 106 slower kinetic	(50)
Repeated transfection of synthetic modified messenger RNAs	OSKM	Primary human neonatal epidermal keratinocytes, BJ human neonatal foreskin fibroblasts, human fetal lung fibroblasts, and human fetal skin fibroblasts	1.4%, 36-fold higher than retrovirus	(80)
Lentivirus	miR-302a,b,c,d, miR-367 + VPA miR-302a,b,c,d, miR-367	MEF Human fibroblast	Faster kinetics efficiency 2 × higher compared with OSKM Efficiency 10000 × higher	(39)
Repeated transfection	miR-302s, miR-369-3p, miR-369-5p, miR-200c	Human and mouse adipose stromal cells, dermal fibroblast	5 colonies/5 × 10^4	(51)
Retroviral Repeated transfection of miRNA mimics	OSKM miR-106b, miR-93, miR-106a, miR-17	MEF	Addition of miRNA enhanced efficiency by 4–6-fold (miR-106b, 93), 3–4-fold (miR-106a,17)	(81)
Retrovirus Repeated transfection of miRNA mimics	OSKM OSK miR-302b, miR-372, miR-294	Human fibroblast	Addition of miRNA enhanced efficiency by promoting MET	(82)

BIX, the small molecule BIX-01294, an inhibitor of the G9a histone methyltransferase; K, Klf4; L, Lin28; M, c-Myc; MEF, mouse embryonic fibroblast; MET, mesenchymal–epithelial transition; miR, microRNA; N, Nanog; n.d.: not determined; O, Oct3/4; Ref., reference; S, Sox2; VPA, valproic acid, a HDAC inhibitor.

doi: 10.1111/j.1349-7006.2011.02184.x

by aberrant mitogenic signaling. The study of double KO (*Ink4ab*$^{-/-}$) and triple KO of all three ORFs (*Cdkn2ab*$^{-/-}$) showed that p15^{Ink4b} can act as a critical backup for p16^{Ink4a}, suggesting a rationale for frequent loss of the complete *CDKN2b–CDKN2a* locus in human tumors.[30] Endogenous p19^{Arf},[31] p16^{Ink4a},[32] and *Trp53* (also known as *p53*), all inactivated in several tumors,[33–35] can limit reprogramming and inhibit pathways leading to an increased level of iPSC generation. In mice, *Arf*, rather than *Ink4a*, blocks important reprogramming pathways through p53 and p21 (encoded by *Cdkn1a*) activation. However, in humans, *INK4a* is more important than *ARF*.[32] Loss of replicative potential may prevent cell reprogramming. The acquisition of cell immortality is a rate-limiting step for establishing pluripotency in somatic cells.[31,32] The transient inhibition of these proteins may significantly improve iPSC generation,[31–35] although the ability of the resultant iPSCs to become tumorigenic is not completely understood. During reprogramming, cells increase their intolerance to different types of DNA damage. A p53-mediated DNA damage response limits reprogramming to ensure iPSC genomic integrity and prevent genomic instability.[35] This phenomenon emphasizes the similarities between induced pluripotency and tumorigenesis. Eventually, approximately 20% of the offspring developed tumors attributable to c-Myc transgene reactivation. Retroviral c-Myc introduction should be avoided for clinical application.[27] Studies of the other barriers indicated that increased iPSC generation efficiency is observed after treating cells with butyrate[36] or vitamin C[37] or after exposing them to hypoxia.[38]

Reprogramming using miRNA. Considering the future application of reprogramming technology, two major non-mutually exclusive issues that should be solved are safety and efficiency. The introduction and addition of specific non-coding miRNA[25] can, for example, improve reprogramming efficiency.[9,39,40] Regarding safety, genomic modification, which is critical to carcinogenesis, is an important concern. The introduction of genes involved in reprogramming events is often facilitated by viral vector-mediated transduction, which can involve random insertions of exogenous sequences into the genome.[19,20] iPSCs can be obtained using virus-free, removable PiggyBac transposons or episomal systems,[41–44] but these approaches still use DNA constructs; thus, the possibility of genomic integration of introduced sequences is still a potential problem. Alternatively, the Sendai virus has been used; iPSCs were generated from human terminally differentiated circulating T cells[45] using Sendai RNA virus vectors.[46,47] Reprogramming events using just protein or mRNA has also been reported, but the protocols involved are technically challenging.[48–50]

Recently, two independent studies from the Morrisey group and our group have demonstrated that human and mouse somatic cells can be reprogrammed to iPSCs through forced miRNA expression, completely eliminating the need for ectopic protein expression.[39,51] Morrisey group[39] revealed that lentiviral-mediated transfection of immature miR-302/367 sequences generated reprogrammed cells (miR-302/367 iPSCs) displaying characteristics similar to those of Oct4/Sox2/Klf4/Myc-iPSCs, including pluripotency marker expression, teratoma formation, and chimera contribution and germline contribution for mouse cells. miR-367 expression is required for miR-302/367-mediated reprogramming, activation of *Oct4* expression, and Hdac2 suppression.[39] Conversely, direct transfection of direct mature double-stranded miRNAs (miR-200c + miR-302s + miR-369s) resulted in PSC generation from differentiated adipose-derived stem cells in humans and mice.[51] This reprogramming method does not require vector-based gene transfer, and thus holds significant potential in biomedical research and regenerative medicine.

Other reports have indicated that electroporation of the polycistronic cassette of hsa-miR-302a/b/c/d resulted in the reprogramming of human hair follicle cells.[40] This reprogramming mechanism functioned through miR-302-targeted cosuppression of four epigenetic regulators: AOF2 (also known as KDM1 or LSD1), AOF1, MECP1-p66, and MECP2.[40] Furthermore, retroviral expression of the polycistronic cassette of hsa-miR-302a/b/c/d allowed the development of iPSC-like phenotypes from human skin cancer cells.[9] Because these methods were carried out without transcription factors, the introduction of miRNAs may play critical roles in differentiated cell reprogramming in humans and mice.

The underlying mechanism of miRNA reprogramming is not completely understood. Generally, miRNAs are involved in translation inhibition, mRNA destabilization, and coding mRNA function suppression.[52,53] We hypothesize that miRNA expression fine-tunes cell reprogramming mainly by inhibiting mRNA signaling, although evidence also suggests that miRNAs may have other functions including translation stimulation through an unknown mechanism. For example, miR-369-3p, which was used for reprogramming,[51] acts as a unique switch for regulating translation repression and activation.[54] miR-302,[39,40,51,55] which targets TGFβ receptor 2 and antagonizes EMT,[55] was also reported to suppress AOF2, AOF1, MECP1-p66, and MECP2,[40] indicating that the miR-302 pathway is fundamental for reprogramming. Inhibition or reversion of EMT could be stimulated by miR-302,[39,40,55] miR-367,[39,55] and miR-200c.[51] TGFβ modulates reprogramming by EMT signaling, whereas Klf4 stimulated E-cadherin expression, a hallmark of MET, which is involved in the stimulation of important reprogramming events.[56] When mammary epithelial cells, which express endogenous Klf4 (MET expression is unnecessary), were used as the starting material, iPSCs were successfully developed only by introducing Sox2 and Oct4 without adding Klf4.[56] This suggests that the requirements needed for EMT inhibition may be dependent on cellular context.

Effect of reprogramming on cancer cells. Retrovirus-mediated gene transfer in gastrointestinal cancer cells resulted in the induction of ES-like gene and protein expression (patterns induced from the endoderm of the gastrointestinal tract to the mesoderm and ectoderm).[10] Interestingly, retrovirus-mediated exogenous expression of Oct4/Sox2/Klf4/Myc or Oct4/Sox2/Klf4 sensitized gastrointestinal cancer cells to vitamins and other chemotherapeutic agents.[10] *In vivo* experiments involving short-term cultured reprogrammed cells showed an inhibition of tumorigenicity in DLD-1 colorectal cancer cells.[10] The study also revealed changes in DNA methylation and histone modification and revealed that the epigenome of DLD-1 cells resembled that of ES cells. The promoter region of *p16Ink4a* was demethylated similar to the heavily demethylated state.[10] Long-term cultured reprogrammed cells with gain-of-function mutations, including *TP53*^{R175H} and *KRAS*^{G12D}, elicit a malignant transformation with c-Myc activation in *KRAS* and *TP53*-mutated HuCC-T1 cholangiocellular carcinoma cells, suggesting a role of such oncogenic mutations in malignant phenotype reactivation.[57] Recent studies have indicated that decreasing the p53 expression level enables the development of murine fibroblasts into iPSCs capable of generating germline-transmitting chimeric mice, indicating that p53 may not be necessary for reprogramming. Silencing p53 will significantly increase the reprogramming efficiency of human somatic cells.[33,34,58] Gain-of-function TP53 oncogenic mutations enhance defined factor-mediated cell reprogramming,[59] suggesting that the TP53 mutation context is influenced by the quality and quantity of reprogramming events. Reprogramming efficiency was increased in hypoxia,[38] an effect observed in cancer cells (Masaki Mori, unpublished data, 2011).

Transfection of miR-302 induces ES-like phenotypes of skin cancer.[9] MiR-302 also inhibits tumorigenecity by coordinating suppression of the Cdk2 and Cdk4/6 cell cycle pathways.[60] The study indicated that concurrent silencing of BMI-1, an

Table 2 Summary of current studies of cancer cell reprogramming

Method	Type of cancer	Malignant-related phenotype	Characterization	Ref.
Nuclear transfer	Medulloblastoma (primary culture, mouse, Ptc1 heterozygous)	Suppressed proliferation, restore normal differentiation, normal proliferation in cultured blastocyst	Cloned blastocyst can support postimplantation development, as the embryo appeared normal and showed extensive differentiation, although not viable after E8.5	(83)
Nuclear transfer (two-step cloning)	Melanoma *RAS+/Ink4a/Arf*$^{-/-}$	NT ES-cell chimeric mice developed various types of tumors with shorter latency and higher penetrance compared with the donor mouse model	NT ES cells could form teratoma and generate chimera. Injection into tetraploid blastocyst resulted in a normal embryo viable until E9.5	(84)
Nuclear transfer	EC	Dependent on donor ECs, one NT ES cell chimera suffered from head and neck EC and was inviable, and the other resulting NT ES cells showed a broad differentiation potential into teratomas and broad contribution to normal-appearing mid-gestation embryos	Nuclei from EC can direct preimplantation development, resulting in normal appearing blastocyst, higher efficiency of producing an ES cell line compared with the differentiated cells, although the degree of differentiation depends on the cell line character	(85)
Embryonic microenvironment	Metastatic melanoma, breast cancer	Reduced invasion, tumor growth, increased apoptosis Downregulated Nodal signaling through Lefty activation	n.d.	(86)
microRNA (miR-302a, b, c, d)	Melanoma (Colo), prostate cancer cell line (PC3)	Reduced migration ability, reduced expression of cell cycle-related genes (*CCND1*, *CCND2*, *CDK2*), and DNA methylation facilitator, MeCP2 MECP1-p66, and some melanoma oncogenes	Expression of pluripotency markers Nanog, Oct4, Sox2, SSEA3, SSEA4 Demethylation of *Oct4* Teratoma (+)	(9)
Defined transcription factor (OKM)	Melanoma (R545)	Chimeras were tumor-free at 5 months of age	Teratoma (+), chimera (+), ES cell marker expression, demethylation of *Nanog* and *Oct4*	(87)
Defined transcription factor (OSKM)	Gastrointestinal cancer (colon, liver, pancreatic cancer) DLD-1 completely characterized	Differentiated iPC (post-iPC) showed sensitivity to chemotherapy, reduced invasion, and reduced tumorigenicity, showed higher expression of p16 and p53 as compared to the parental cell	Expression of pluripotency marker, demethylation of *Nanog*, *in vitro* differentiation into adipocyte, epithelial, mesenchymal, and neural lineage, teratoma (–)	(10)
Defined transcription factor (OSKM)	KBM7 cells derived from blast crisis stage chronic myeloid leukemia (CML)	Completely resistant to imatinib, loss of *BCR-ABL*-dependent signaling	ES cell marker expression (+), demethylation of *Oct4* and *Nanog*, teratoma (+)	(88)
Defined transcription factor (OSLN)	A549 lung cancer	Increased tumorigenic properties when transplanted into a NOD/SCID mouse, more aggressive and invasive, teratoma (–)	Demethylation of *Oct4* promoter expressed endogenous Nanog and Oct4 although lower than HES cell ALP(+), teratoma (–) Reprogramming efficiency was higher compared with normal primary lung fibroblast	(89)
Oocyte extract	Breast cancer (MCF7 and HCC1945 cell lines)	Re-expression of tumor suppressor genes *RARB*, *CST6*, *CCND2*, *CDKN2A* through demethylation and remodeling of histone marks to a more euchromatic state No changes in DNA methylation at pluripotency gene promoters *Oct4/Nanog* Reduced colony formation	n.d.	(90)

ALP, alkaline phosphatase (staining); EC, embryonal carcinoma; HES, human embryonic stem; iPC, induced pluripotent stem (iPS)-like cancer cells; K, Klf4; L, Lin28; M, c-Myc; N, Nanog; n.d., not determined; NT ES, nuclear transfer-generated embryonic stem cells; O, Oct3/4; Ref., reference; S, Sox2.

miR-302-targeted CSC marker, further promoted tumor suppressor functions of p16Ink4a and p14/p19Arf directed against Cdk4/6-mediated cell proliferation. Also, miR-302 inhibits human pluripotent stem cell tumorigenicity by enhancing the multiple G_1 phase arrest pathways.[60] Another study of glioma indicated that the miR-302–367 cluster drastically affects the

doi: 10.1111/j.1349-7006.2011.02184.x

self-renewal and infiltration properties of glioma-initiating cells through Cxcr4 repression and consequent disruption of the Shh-Gli-Nanog network.[61] This indicates that the miR-302–367 cluster can efficiently trigger a cascade of inhibitory events leading to the disruption of CSC-like and tumorigenic properties.[61] Taken together, further study of novel reprogramming-based therapeutic approaches that could prove beneficial for treatment of tumors with p53 inactivation[33,34,58] and/or of CSCs, which can survive in a region of hypoxia,[38] is warranted (Table 2; Fig. 1).

Defined Factor-Induced Reprogramming and CSCs

The differential mechanisms between cancer cells, which undergo a mutated form of reprogramming, and naturally occurring CSCs remain unclear.

Gastrointestinal cancer cells. Recently, it has been proposed that two types of stem cells coexist in normal and cancer cells and that these stem cells are transiently regulated by epigenetic controls.[62–65] Emerging evidence indicates that quiescent and active stem cell subpopulations that are in lower metabolic and proliferative states, respectively, may coexist in several tissues.[62] It has been proposed that these stem cell populations have separate but cooperative functional roles, and these adult stem cells are crucial for physiological tissue renewal and regeneration after injury.[62] Generally, a stem cell divides asymmetrically into a new stem cell (self-renewal) and a committed progenitor (differentiation). Whereas the asymmetric architecture of the stem cell niche in *Drosophila* and *Caenorhabditis elegans* is apparent, mammalian adult stem cells are generally detected in a predominantly quiescent state.[63,64] Quiescent stem cells have been proposed to produce transit-amplifying cells in rapidly regenerating tissue, which differentiate into mature cells and provide tissue architecture. Considering that transit-amplifying cells have a short lifespan and limited self-renewal capabilities, recent studies suggest that stem cell populations that are long-lived yet constantly cycling are involved in the maintenance of tissue homeostasis.[62] A new model describes the coexistence of quiescent and active adult stem cell subpopulations in bone marrow, intestinal epithelium, and hair follicles.[62] In contrast to physiological tissues, serial transplantation experiments indicated that liver CSCs are composed of quiescent and active CSCs. This system plays a role in the exertion of resistance against chemoradiotherapy. During the study of CSCs, we identified $CD13^+$ CSCs as a subpopulation of quiescent stem cells of the liver.[65] Our study indicated that TGFβ induced the development of a $CD13^+$ CSC population (Masaki Mori, submitted). $CD13^+$ CSCs express immature genes often connected with a lower differentiation state, an observation that might explain why $CD13^+$ CSCs exhibit aggressive behaviors (Masaki Mori, unpublished data, 2011). Considering TGFβ signaling counteracts the induction of cell reprogramming from normal differentiated cells, the outcome of reprogramming-like stimulation should be investigated.

Reprogramming effect on CSCs. Endogenous expression levels of ES-like genes could be relevant to tumor cell malignancy.[66] The concept that a small population is contained in adult tissues may be relevant to CSCs in a tumor.[67] The involvement of a very small embryonic/epiblast-like stem cell population in carcinogenesis could support century-old concepts involving embryonic rest- or germline-origin hypotheses of cancer development;[67] however, this working hypothesis requires further direct experimental confirmation.[67] Further evidence indicates that tissues contain a unique population of mesenchymal stem cells or Muse cells,[68] and that Muse cells are a primary source of iPSCs in human fibroblasts.[69] By using immunocytochemistry to express Nanog, Oct3/4, and Sox2 and TRA-1–81 to assess reprogramming efficiency, the authors showed that iPSC

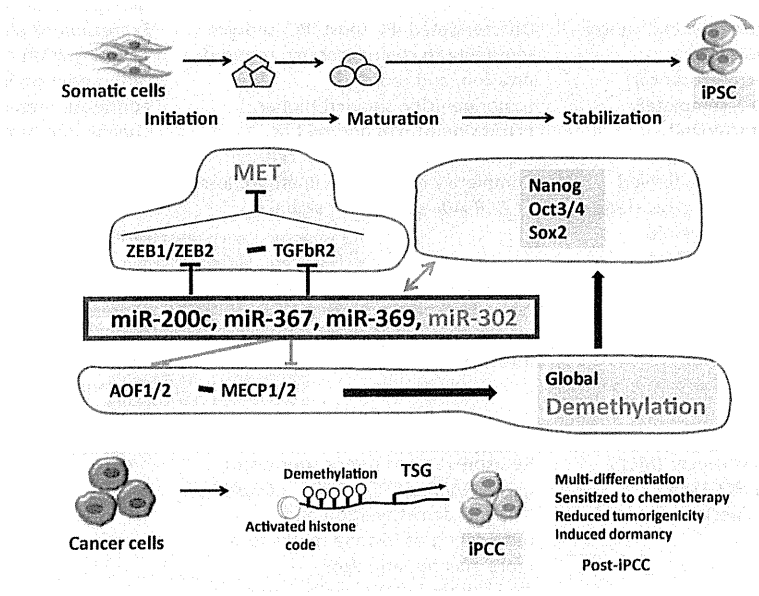

Fig. 1. Cellular reprogramming in normal and cancer cells. Cellular reprogramming in normal and cancer cells can be viewed globally as a mechanism of phenotype reversal of parental cells through the modulation of epigenetic status into a more undifferentiated state. Defined transcription factors (Yamanaka cocktail)-induced reprogramming is involved in the regulation of mesenchymal–epithelial transition (MET), which is controlled by a group of microRNAs (miR) through ZEB1/ZEB2 and TGFβR2. Those miRNAs play a role in global demethylation through AOF1/2 and MECP1/2. In contrast to normal cell reprogramming (upper panel), cancer cell reprogramming (lower panel) remains obscure. The reverse of MET, epithelial–mesenchymal transition (EMT), results in a chemotherapy-resistant phenotype. Thus, reprogramming is supposed to open the silent chromatin through DNA demethylation and activate histone codes, which would elicit re-expression of tumor suppressor genes, pushing cancer cells into a more benign phenotype. Further investigation would provide insight into how much of the tumor phenotype could be reversed through the contribution of reversible epigenetic and irreversible genetic changes in cancer. Reprogramming cancer cells might become a promising method for reversing or attenuating malignancy for therapeutic purposes. iPCC, induced pluripotent stem cell-like cancer cell; iPSC, induced pluripotent stem cell; TGF, transforming growth factor; TSG, tumor suppressor gene.

lines were generated with an efficiency of 0.001% from naive human skin fibroblasts, whereas Muse-iPSCs were formed with an efficiency of 0.03%, indicating that Muse cells generate iPSCs 30-fold more efficiently than naive fibroblasts.[69] This type of subpopulation study elicits a challenging notion that a subset of pre-existing adult stem cells in adult human tissues (or fibroblasts), which are somewhat similar to iPSCs, selectively become iPSCs, whereas the remaining cells make no contribution to iPSC generation.[69] Nevertheless, at least two issues should be considered. First, the efficiency of iPSC generation in this study is much lower than that reported in other studies (≥0.02%; Table 1).[20,70] Although the susceptibility to each cell reprogramming may be presumably based on pre-existing conditions of epigenetic and transcription factor networks, underestimation cannot be excluded without adjusting the complete reprogramming technology. Second, given that higher efficiencies of reprogramming have been reported (up to approximately 10%, see Table 1) than the pre-existing frequency of Muse cells in tissues, (1.1–1.3% of human fibroblasts or bone marrow stromal cells formed Muse cell-derived cell clusters in naive populations without long-term trypsin incubation), cells other than Muse cells may generate iPSCs. Taken together, it may be too early to conclude whether the defined factor-induced reprogramming fits the elite model,[69] rather than the stochastic model of iPSC generation.[70] To reconcile these issues, further investigation is necessary to improve the reprogramming efficiency and understand the mechanism by which cellular reprogramming functions, especially in subpopulations of susceptible clones subjected to defined factor-induced reprogramming. Considering that ES-like genes expressing CSCs and unique populations including very small embryonic/epiblast-like stem cells and Muse cells could be essential in cancer development, further research is necessary to determine the presence of these cell subpopulations in tumor tissues, relevancy to epithelial cancerous cells, and susceptibility of reprogramming events in these cell populations.

Perspective

Tissue homeostasis is a carefully balanced process controlled by epigenome regulation and efficient interplay between stem cells, their progeny, and the microenvironment (e.g. recently reviewed in intestinal stem cells[23]). Epigenome deregulation and malignant stem cell formation lead to tumor cell development. Reprogramming technology or epigenome modification through transfection of iPSC factors can lead to ES-like gene expression patterns and considerable malignant phenotype modification,[10,60] indicating that this technology could be used to create novel therapeutic targets against CSCs by combining small non-coding RNAs with efficient drug delivery systems.

Disclosure Statement

This work was supported in part by: a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (H.I., N.H., H.N., Y.D., M.M.); the Ministry of Health, Labour, and Welfare; the Princess Takamatsu Foundation, Japan (H.I., M.M.); the Takeda Foundation, Japan (H.I.); the Senshin Medical Research Foundation (H.I.); Chugai Pharmaceutical Corporation, Japan (H.I., N.H. D.S., T.S.); and Yakult Corporation, Japan (H.I., N.H. D.S., T.S.). Patents pending on PCT/JP2011/053457, PCT/JP2011/054287, and PCT/JP2010/068406.

Abbreviations

CSC	cancer stem cell
EMT	epithelial–mesenchymal transition
iPSC	induced pluripotent stem cell
MEF	mouse embryonic fibroblast
MET	mesenchymal–epithelial transition
miRNA	microRNA
Muse (cells)	multilineage-differentiating stress-enduring
TGF	transforming growth factor

References

1 Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001; **293**: 1089–93.
2 Vogelstein B, Kinzler KW. The multistep nature of cancer. *Trends Genet* 1993; **9**: 138–41.
3 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
4 Barker N, Ridgway RA, van Es JH *et al.* Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2009; **457**: 608–11.
5 Irons RD, Stillman WS. The process of leukemogenesis. *Environ Health Perspect* 1996; **104** (Suppl 6): 1239–6.
6 Bonnet D, Dick JE. Human acute leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730–37.
7 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105–11.
8 Kasemeier-Kulesa JC, Teddy JM, Postovit LM *et al.* Reprogramming multipotent tumor cells with the embryonic neural crest microenvironment. *Dev Dyn* 2008; **237**: 2657–66.
9 Lin SL, Chang DC, Chang-Lin S *et al.* Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* 2008; **14**: 2115–24.
10 Miyoshi N, Ishii H, Nagai K *et al.* Defined factors induce reprogramming of gastrointestinal cancer cells. *Proc Natl Acad Sci USA* 2010; **107**: 40–5.
11 Wang J, Emadali A, Le Bescont A, Callanan M, Rousseaux S, Khochbin S. Induced malignant genome reprogramming in somatic cells by testis-specific factors. *Biochim Biophys Acta* 2011; **1809**: 221–5.
12 Ben-David U, Benvenisty N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* 2011; **11**: 268–77.
13 Surani MA, Hayashi K, Hajkova P. Genetic and epigenetic regulators of pluripotency. *Cell* 2007; **128**: 747–62.
14 Rasmussen TP, Corry GN. Epigenetic pre-patterning and dynamics during initial stages of mammalian preimplantation development. *J Cell Physiol* 2010; **225**: 333–6.
15 Surani MA, Durcova-Hills G, Hajkova P, Hayashi K, Tee WW. Germ line, stem cells, and epigenetic reprogramming. *Cold Spring Harb Symp Quant Biol* 2008; **73**: 9–15.
16 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154–6.
17 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981; **78**: 7634–8.
18 Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145–7.
19 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663–76.
20 Takahashi K, Tanabe K, Ohnuki M *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861–72.
21 Beers MF, Morrisey EE. The three R's of lung health and disease: repair, remodeling, and regeneration. *J Clin Invest* 2011; **121**: 2065–73.
22 Herdrich BJ, Lind RC, Liechty KW. Multipotent adult progenitor cells: their role in wound healing and the treatment of dermal wounds. *Cytotherapy* 2008; **10**: 543–50.
23 Medema JP, Vermeulen L. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. *Nature* 2011; **474**: 318–26.
24 Houbaviy HB, Murray MF, Sharp PA. Embryonic stem cell-specific MicroRNAs. *Dev Cell* 2003; **5**: 351–8.
25 Mattick JS, Makunin IV. Non-coding RNA. *Hum Mol Genet* 2006; **1**: R17–29.
26 Leung AK, Sharp PA. MicroRNA functions in stress responses. *Mol Cell* 2010; **40**: 205–15.
27 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; **448**: 313–7.
28 Wernig M, Meissner A, Foreman R *et al.* In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007; **448**: 318–24.
29 Gil J, Peters G. Regulation of the INK4b–ARF–INK4a tumour suppressor locus: all for one or one for all. *Nature Rev Mol Cell Biol* 2006; **7**: 667–77.
30 Krimpenfort P, IJpenberg A, Song JY, van der Valk M, Nawijn M, Zevenhoven J. Berns A. p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. *Nature* 2007; **448**: 943–6.
31 Utikal J, Polo JM, Stadtfeld M *et al.* Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature* 2009; **460**: 1145–8.

doi: 10.1111/j.1349-7006.2011.02184.x

32 Li H, Collado M, Villasante A *et al.* The Ink4/Arf locus is a barrier for iPS cell reprogramming. *Nature* 2009; **460**: 1136–9.
33 Hong H, Takahashi K, Ichisaka T *et al.* Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 2009; **460**: 1132–5.
34 Kawamura T, Suzuki J, Wang YV *et al.* Linking the p53 tumour suppressor pathway to somatic cell reprogramming. *Nature* 2009; **460**: 1140–4.
35 Marión RM, Strati K, Li H *et al.* A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature* 2009; **460**: 1149–53.
36 Mali P, Chou B.-K, Yen J *et al.* Butyrate Greatly Enhances Derivation of Human Induced Pluripotent Stem Cells by Promoting Epigenetic Remodeling and the Expression of Pluripotency-Associated Genes. *Stem Cell* 2010; **28**: 713–20.
37 Esteban MA, Wang T, Qin B *et al.* Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells. *Cell Stem Cell* 2010; **6**: 71–9.
38 Yoshida Y, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 2009; **5**: 237–41.
39 Anokye-Danso F, Trivedi CM, Juhr D *et al.* Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376–88.
40 Lin SL, Chang DC, Lin CH, Ying SY, Leu D, Wu DT. Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Res* 2011; **39**: 1054–65.
41 Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008; **322**: 949–53.
42 Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009; **458**: 771–5.
43 Woltjen K, Michael IP, Mohseni P *et al.* piggybac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 2009; **458**: 766–70.
44 Jia F, Wilson KD, Sun N *et al.* A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 2010; **7**: 197–9.
45 Seki T, Yuasa S, Oda M *et al.* Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* 2010; **7**: 11–4.
46 Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; **85**: 348–62.
47 Ban H, Nishishita N, Fusaki N *et al.* Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. *Proc Natl Acad Sci USA* 2011; **108**: 14234–9.
48 Kim D, Kim CH, Moon JI *et al.* Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 2009; **4**: 472–6.
49 Zhou H, Wu S, Joo JY *et al.* Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009; **4**: 381–4.
50 Warren L, Manos PD, Ahfeldt T *et al.* Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 2010; **7**: 618–30.
51 Miyoshi N, Ishii H, Nagano H *et al.* Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell* 2011; **8**: 633–8.
52 Ambros V. The functions of animal microRNAs. *Nature* 2004; **431**: 350–5.
53 John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol* 2004; **2**: e363.
54 Vasudevan S, Tong Y, Steitz JA. Switching from Repression to Activation: microRNAs Can Up-Regulate Translation. *Science* 2007; **318**: 1931–4.
55 Liao B, Bao X, Liu L *et al.* MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J Biol Chem* 2011; **286**: 17359–64.
56 Li R, Liang J, Ni S *et al.* A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 2010; **7**: 51–63.
57 Nagai K-i, Ishii H, Miyoshi N *et al.* Long-term culture following ES-like gene-induced reprogramming elicits an aggressive phenotype in mutated cholangiocellular carcinoma cells. *BiochemBiophysic Res Commun* 2010; **395**: 258–63.
58 Zhao Y, Yin X, Qin H *et al.* Two supporting factors greatly improve the efficiency of human iPSC generation. *Cell Stem Cell* 2008; **3**: 475–9.
59 Moon JH, Ishii H, Dewi DL *et al.* Gain-of-function oncogenic mutations in TP53 enhance defined factor-mediated cellular reprogramming. Nature Precedings, 2011.
60 Lin SL, Chang DC, Ying SY, Leu D, Wu DT. MicroRNA miR-302 inhibits the tumorigenecity of human pluripotent stem cells by coordinate suppression of the CDK2 and CDK4/6 cell cycle pathways. *Cancer Res* 2010; **70**: 9473–82.
61 Fareh M, Turchi L, Virolle V *et al.* The miR 302-367 cluster drastically affects self-renewal and infiltration properties of glioma-initiating cells through CXCR4 repression and consequent disruption of the SHH-GLI-NANOG network. *Cell Death Differ* 2011; DOI: 10.1038/cdd.2011.89. [Epub ahead of print].
62 Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. *Science* 2010; **327**: 542–5.
63 Arai F, Hirao A, Ohmura M *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004; **118**: 149–61.
64 Fuchs E, Segre JA. Stem cells: a new lease on life. *Cell* 2000; **100**: 143–55.
65 Haraguchi N, Ishii H, Mimori K *et al.* CD13 is a therapeutic target in human liver cancer stem cells. *J Clin Invest* 2010; **120**: 3326–39.
66 Ben-Porath I, Thomson MW, Carey VJ *et al.* An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008; **40**: 499–507.
67 Ratajczak MZ, Shin DM, Liu R *et al.* Epiblast/germ line hypothesis of cancer development revisited: lesson from the presence of Oct-4+ cells in adult tissues. *Stem Cell Rev* 2010; **6**: 307–16.
68 Kuroda Y, Kitada M, Wakao S *et al.* Unique multipotent cells in adult human mesenchymal cell populations. *Proc Natl Acad Sci USA* 2010; **107**: 8639–43.
69 Wakao S, Kitada M, Kuroda Y *et al.* Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. *Proc Natl Acad Sci USA* 2011; **108**: 9875–80.
70 Yamanaka S. Elite and stochastic models for induced pluripotent stem cell generation. *Nature* 2009; **460**: 49–52.
71 Yu J, Vodyanik MA, Smuga-Otto K *et al.* Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science* 2007; **318**: 1917–20.
72 Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science* 2008; **322**: 945–9.
73 Aoi T, Yae K, Nakagawa M *et al.* Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 2008; **321**: 699–702.
74 Kim JB, Zaehres H, Wu G *et al.* Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 2008; **454**: 646–50.
75 Huangfu D, Maehr R, Guo W *et al.* Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol* 2008; **26**: 795–7.
76 Wernig M, Lengner CJ, Hanna J *et al.* A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat Biotechnol* 2008; **26**: 916–24.
77 Sun N, Panetta NJ, Gupta DM *et al.* Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci USA* 2009; **106**: 15720–5.
78 Shi Y, Do JT, Desponts C, Hahm HS, Schöler HR, Ding S. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* 2008; **2**: 525–8.
79 Judson RL, Babiarz JE, Venere M, Blelloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009; **27**: 459–61.
80 Zhu S, Li W, Zhou H *et al.* Reprogramming of human primary somatic cells by OCT4 and chemical compounds. *Cell Stem Cell* 2010; **7**: 651–5.
81 Li Z, Yang CS, Nakashima K, Rana TM. Small RNA-mediated regulation of iPS cell generation. *EMBO J* 2011; **30**: 823–34.
82 Subramanyam D, Lamouille S, Judson RL *et al.* Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nat Biotechnol* 2011; **29**: 443–8.
83 Li L, Connelly MC, Wetmore C, Curran T, Morgan JI. Mouse embryos cloned from brain tumors. *Cancer Res* 2003; **63**: 2733–36.
84 Hochedlinger K, Blelloch R, Brennan C *et al.* Reprogramming of a melanoma genome by nuclear transplantation. *Genes Dev* 2004; **18**: 1875–85.
85 Blelloch RH, Hochedlinger K, Yamada Y *et al.* Nuclear cloning of embryonal carcinoma cells. *Proc Natl Acad Sci USA* 2004; **101**: 13985–90.
86 Postovit LM, Margaryan NV, Seftor EA *et al.* Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. *Proc Natl Acad Sci USA* 2008; **105**: 4329–34.
87 Utikal J, Maherali N, Kulalert W, Hochedlinger K. Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J Cell Sci* 2009; **122**: 3502–10.
88 Carette JE, Pruszak J, Varadarajan M, Blomen VA, Gokhale S. Generation of iPSCs from cultured human malignant cells. *Blood* 2010; **115**: 4039–42.
89 Mathieu J, Zhang Z, Zhou W, Wang AJ, Heddleston JM. HIF induces human embryonic stem cell markers in cancer cells. *Cancer Res* 2011; **71**: 4640–52.
90 Allegrucci C, Rushton MD, Dixon JE *et al.* Epigenetic reprogramming of breast cancer cells with oocyte extracts. *Mol Cancer* 2011; **10**: 7.

Ann Surg Oncol
DOI 10.1245/s10434-011-2040-5

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Increased CD13 Expression Reduces Reactive Oxygen Species, Promoting Survival of Liver Cancer Stem Cells via an Epithelial–Mesenchymal Transition-like Phenomenon

Ho Min Kim[1], Naotsugu Haraguchi[1], Hideshi Ishii[1,2], Masahisa Ohkuma[1,3], Miho Okano[1], Koshi Mimori[2], Hidetoshi Eguchi[1], Hirofumi Yamamoto[1], Hiroaki Nagano[1], Mitsugu Sekimoto[1], Yuichiro Doki[1], and Masaki Mori[1]

[1]Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Osaka, Japan; [2]Department of Molecular and Cellular Biology, Division of Molecular and Surgical Oncology, Kyushu University, Medical Institute of Bioregulation, Beppu, Ohita, Japan; [3]Department of Gastroenterological Surgery, Jikei Collage Graduate School of Medicine, Tokyo, Japan

ABSTRACT

Background. Recently, it has been reported that a small population of cancer stem cells (CSCs) play a role in resistance to chemotherapy and radiation therapy. We reported that $CD13^+$ liver CSCs survive in hypoxic lesions after chemotherapy, presumably through increased expression of CD13/Aminopeptidase N, which is a scavenger enzyme in the reactive oxygen species (ROS) metabolic pathway. On the other hand, the concept of epithelial–mesenchymal transition (EMT) was indicated by a recent study showing an increased plasticity linked to the cellular "stemness" of CSCs.

Methods. To study the relationship between CSCs and EMT, we examined biological characteristics of liver cancer cell lines with EMT by exposing transforming growth factor-β (TGF-β).

Results. We showed that a TGF-β-induced EMT-like phenomenon is associated with increased CD13 expression in liver cancer cells. This phenomenon prevents further increases in the ROS level as well as the induction of apoptosis, promoting the survival of $CD13^+$ CSCs, whereas inhibition of CD13 stimulates apoptosis. Immunohistochemical analysis also indicated that after chemotherapy, CD13 was coexpressed with N-cadherin in surviving cancer cells within fibrous capsules. We have demonstrated that CD13 expression plays a role in supporting the survival of CSCs and that there is an EMT-associated reduction in ROS elevation.

Conclusions. This novel and consistent linkage between functional CSC markers and the EMT phenomenon suggests a bona fide candidate for targeted therapy for EMT-mediated invasion and metastasis of liver cancer.

Cancer stem cells (CSCs) are believed to share unique characters with normal stem cells, self-renewal ability, and produce differentiated cells.[1,2] Additionally, CSCs play a key role in resistance to chemotherapy and radiotherapy. CSCs give rise to cancer recurrence even though most cancer cells have been disrupted after this therapy.[3] Since the identification of CSCs in leukemia,[4–6] CSCs have been reported in various solid tumors; brain, head and neck, breast, and colon.[7–12]

In studies of hepatocellular carcinoma, side population (SP) fraction, CD133, CD90, CD44, and epithelial cell adhesion molecules (EpCAMs) were reported as markers of CSCs or cancer-/tumor-initiating cells.[13–20] We have reported CD13 as a novel liver CSCs marker and as candidate therapeutic target with some sets of functional analyses.[21] We showed that CD13+ cells mainly existed in SP fraction, contained potentially dye-long retaining cell, and had high tumorigenic activity. Additionally, CD13 + CSCs showed strong chemoradiation resistance in vitro and in vivo, by protecting cells from DNA damage via regulation of the

Electronic supplementary material The online version of this article (doi:10.1245/s10434-011-2040-5) contains supplementary material, which is available to authorized users.

First Received: 14 March 2011

H. Ishii
e-mail: hishii@cfs.med.osaka-u.ac.jp

M. Mori
e-mail: mmori@gesurg.med.osaka-u.ac.jp

Published online: 31 August 2011

reactive oxygen species (ROS) level. Inhibition of CD13-induced tumor cell apoptosis resulted in tumor disruption via blocking self-renewal activity. CD13 also has been associated with tumor invasion, regulation of angiogenesis, and resistance to apoptosis.[22–24]

Epithelial–mesenchymal transition (EMT) plays crucial roles in developmental processes, such as neural crest and mesoderm formation during embryogenesis, and has been focused as a key process in tumor invasion, metastasis, and tumorigenicity.[25,26] EMT has been well characterized by numerous pathways of transcriptional factors, including Snail, Twist, and Zeb, which are generally promoted by transforming growth factor (TGF) β superfamily.[25] In the study of mammary epithelial cells, it was reported that EMT generates normal mammary epithelial cells with properties of stem cells.[27] In the study of breast cancer CSCs, it has reported that breast cancer cells increase malignant potential with increased CSCs phenotype by the treatment of EMT inducers.[27] Furthermore, ROS have been proposed recently to be involved in tumor metastasis, which is a complicated process, including EMT of cancer cells and the microenvironment inside or around the tumor lesion.[28,29]

In the present study, we identify a TGF-β-induced EMT-like phenomenon is associated with increased CD13 expression in liver cancer cells and plays a role in the reduction of the intracellular ROS level, promoting the survival of liver CSCs. The present data indicate a novel and consistent linkage between CD13, a functional CSC marker, and the EMT phenomenon.

MATERIALS AND METHODS

Cell Culture

Human hepatocellular carcinoma (HCC) cells, HuH7, and PLC/PRF/5, obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan) were cultured in an RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (FBS; GIBCO). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Induction and Reversal of EMT

Cells were seeded and incubated in a standard medium for 48 h and then incubated in a serum-free medium supplemented with 5 μg/ml insulin and 10 ng/ml endothelial growth factor at 37°C in an atmosphere containing 5% CO_2 for 48–72 hr with TGF-β1 (R&D) at concentrations of 0–5 ng/ml with daily replacement of the fresh culture medium.[30]

Gene Expression Study

Total RNA was prepared using TRIzol reagent (Invitrogen). Reverse transcription was performed with SuperScriptIII (Invitrogen). Quantitative real-time (qRT)-PCR was performed using a Light Cycler TaqMan Master kit (Roche Diagnostics). The expression of mRNA copies was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The PCR primers used for amplification were as follows: GAPDH, 5′-GTCAACGGATTTGGTC TGTATT-3′ (forward) and 5′-AGTCTTCTGGGTGGCAGT-GAT-3′ (reverse); N-cadherin, 5′-ACAGTGGCCACCTA CAAAGG-3′ (forward) and 5′-CCGAGATGGGGTTGA TAATG-3′ (reverse); fibronectin, 5′-CAGTGGGAGACCTC GAGAAG-3′ (forward) and 5′-TCCCTCGGAACATCA GAAAC-3′ (reverse); vimentin, 5′-GAGTCCACTGAGTAC CGGAGAC-3′ (forward) and 5′-TGTAGGTGGCAATCT CAATGTC-3′ (reverse); E-cadherin, 5′-ACACCATCCTCAG CCAAGA-3′ (forward) and 5′-CGTAGGGAAACTCTCT CGGT-3′ (reverse); Bmi-1, 5′-TGTAAAACGTGTATTGTT CGTTAC-3′ (forward) and 5′-CAATATCTTGGAGAGTT TTATCTGACC-3′ (reverse); Notch-1, 5′-CGCACAAG GTGTCTTCCAG-3′ (forward) and 5′-AGGATCAGTGG CGTCGTG-3′ (reverse); Gclm, 5′-GAAGAAGATATTTT TCCTGTCATTGAT-3′ (forward) and 5′-CCATTCATG TATTGAAGAGTGAATTT-3′ (reverse); Gss, 5′-CCTGCTA GTGGATGCTGTCA-3′ (forward) and 5′-TCATCCTGTTT GATGGTGCT-3′ (reverse); c-Maf, 5′-AATACGAGAAGC TGGTGAGCAA-3′ (forward) and 5′-CGGGAGAGGAAGG GTTGTC-3′ (reverse).

Flow Cytometric Analysis and Cell Sorting

The following antibodies were used to characterize cancer cells: allophycocyanin (APC)-conjugated anti-human CD133/1 (clone AC133, Miltenyi-Biotec), APC-conjugated anti-human CD90 (clone 5E10, BioLegend), APC-conjugated anti-human CD13 (clone WM-15, eBioscience), APC-conjugated anti-human CD324 (E-cadherin, clone 67A4, Bio Legend), phycoerythrin (PE)-conjugated anti-human CD90 (BioLegend), PE-conjugated anti-human CD325 (N-cadherin, clone 8C11, eBioscience), and fluorescein isothiocyanate-conjugated anti-human CD13 (eBioscience).

Dissociated cells were stained with anti-human CD13 with N-cadherin. The labeled cells were analyzed and sorted by the BD FACSAria II Cell Sorter System (Becton Dickinson), and the data were analyzed with Diva software (Becton Dickinson).

Colony Formation Assay

Cells were plated in soft agar at a density of 1×10^3–1×10^4 cells/3.5-cm dish. The base layer was 0.6% agar in the RPMI 1640 medium and the top was 0.2%. Cells were incubated for 30 days; colonies were stained with Diff-Quik kit (Sysmex).

Transplantation of Cancer Cells into Nonobese Diabetic/Severe Combined Immunodeficient (NOD/SCID) Mice

Isolated cells were resuspended in 50 μl of PBS after sorting, and cell aliquots were diluted 1:1 with Growth Factor Reduced Matrigel Matrix (BD Biosciences) and injected subcutaneously into the flank of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice at densities of 5×10^2, 2.5×10^3, and 1×10^4 cells under anesthesia. After 15 weeks, all tumors were removed and analyzed. All of the animal studies were approved by ethics board of animal study of Osaka University.

ROS Assay

The ROS assay was performed as described previously.[21] Cells were loaded with 10 μM of 2′,7′-dichlorofluorescein diacetate (DCF-DA) at 37°C for 30 min, and cells were pretreated with 100–500 μg/ml of ubenimex (Nihon Kayaku) at 37°C for 24 h and stained with DCF-DA. To evaluate the effect of ROS to induce EMT, 0 to 10 μM of *N*-acetyl cysteine (NAC) (Wako Pure Chemical Industries), which has been known as a general antioxidant agent and ROS inhibitor, was added with or without TGF-β1.

Immunohistochemistry

Ten, fresh, HCC, surgical tissue specimens were obtained from Osaka University, Japan, with the patients' informed consent and the approval of the Research Ethics Board of Osaka University. The VECTASTAIN Elite ABC Kit (Vector Laboratories) was used to detect signals from the antigen-antibody reactions. The primary anti-CD13 mouse polyclonal antibody (38C12; Neomarkers Laboratory Vision) and the primary anti-N-cadherin rabbit polyclonal antibody (YS; Immuno-Biological Laboratories) were used.

Statistical Analysis

Statistical analyses were performed using JMP 8.0.1 for Windows (SAS Institute). Possible differences between groups were analyzed using Student's t test, χ^2 test, or Wilcoxon test. A probability level of 0.05 was chosen to indicate statistical significance.

RESULTS

TGF-β-Induced EMT Phenotypes in Liver Cancer Cells

To determine whether liver cancer cells (HuH7, PLC/PRF/5) induced the EMT phenotype, we exposed liver cancer cells to TGF-β. Culturing the liver cancer cells with TGF-β changed their phenotype to spindle-shape morphology (Fig. 1a); this cell phenotype was detected at levels higher than 0.5 ng/ml for HuH7 and 1 ng/ml for PLC/PRF/5. An expression study using qRT-PCR indicated that the mesenchymal markers N-cadherin, fibronectin, and vimentin also were significantly elevated (Fig. 1b). FACS analysis showed that expression of the epithelial marker E-cadherin was decreased (Fig. 1c).

Induced EMT Associated with Increased CD13 Expression in Liver Cancer Cells

To assess whether TGF-β-induced EMT phenotype associate to liver cancer stem cell markers, HuH7 and PLC/PRF/5 cells were incubated with or without TGF-β. By the treatment of TGF-β, expression of CD133 and CD90 was not changed in both HuH7 and PLC/PRF/5.[15–19] In contrast, expression of CD13 was increased almost double in both HuH7 and PLC/PRF/5 (Fig. 2a).[21] Multicolor analysis of N-cadherin and CD13 revealed that N-cadherin$^+$ cells were localized in CD13$^+$ cell fraction (Fig. 2b). Because the CD13 fraction was <3% in HuH7 and >30% in PLC/PRF/5 cells under standard conditions, we used PLC cells in the subsequent studies.

Tumorigenicity of TGF-β-Induced CD13$^+$ Cells

To investigate colony formation ability, the soft agar assay was performed in CD13$^+$/N-cadherin$^-$, CD13$^+$/N-cadherin$^+$, and CD13$^-$/N-cadherin$^-$ cell fractions. The number of CD13$^-$/N-cadherin$^+$ cells was not sufficient for analysis. The soft agar assay showed that after TGF-β stimulation, CD13$^+$/N-cadherin$^-$, and CD13$^+$/N-cadherin$^+$ cells revealed significantly higher colony-forming ability compared with CD13$^-$/N-cadherin$^-$ cells ($P < 0.001$; Fig. 3a and b). There was no appreciable difference between N-cadherin$^+$ and N-cadherin$^-$ cells, which indicated that CD13$^+$ cells exhibited significantly higher colony-forming ability than CD13$^-$ cells. To investigate tumorigenic activity of CD13$^+$/N-cadherin$^-$, CD13$^+$/N-cadherin$^+$, and CD13$^-$/N-cadherin$^-$ cells, isolated 5×10^2, 2.5×10^3, and 1×10^4 cells of each fraction were inoculated into NOD-SCID mice. Serial transplantation experiment indicated that CD13$^+$/N-cadherin$^-$ cells possess higher tumorigenic activity compared with CD13$^-$/N-cadherin$^-$ cells. CD13$^+$/N-cadherin$^+$ cells formed tumor