Although some previous studies have reported postoperative complications after hepatectomy for HCC, there are no reports comparing postoperative complications between patients with FFP transfusion and those without FFP transfusion. Martin *et al*[25] reported the use of FFP after hepatic resection and suggested criteria for FFP transfusion to deal with postoperative complications after treatment of liver metastasis from colorectal cancer, but not for HCC with liver cirrhosis. Accordingly, their criteria should not be necessarily generalized to the use of FFP in hepatectomy for HCC. Therefore, this study is the first report in which the incidence of postoperative complications in HCC patients was compared between patients who received FFP and those who did not receive FFP transfusion.

To date, FFP has been traditionally used at hepatectomy for the purpose of hemostatic effect by correction of deficiency of coagulation factors and maintenance of circulating blood volume by supplementation of albumin, which is mainly responsible for the colloid osmotic pressure of plasma; in addition to the aforementioned purpose of prevention of hepatic failure[6-8]. Firstly, with regard to the hemostatic effect, recent improvements in surgical techniques allow hepatectomy to be performed with minimal bleeding[19,20]. Moreover, coagulopathy requiring FFP transfusion is generally reported to occur at a PT value of more than 2.0 times the control, whereas the mean PT level of patients of Group B1≥ 2000 mL and Group B2≥ 2000 mL in the present study did not drop to the applicable level, even though it was measured after hepatectomy[26-28]. Furthermore, the incidence of postoperative bleeding was low and was not different in the two groups. Taking these results into consideration, routine administration of FFP is not necessary in terms of the hemostatic effect. Secondly, the maintenance of appropriate circulating blood volume is important in order to prevent certain complications such as pulmonary edema and prerenal type of renal dysfunction. However, albumin products, which can be administered safely compared to FFP, can be substitutes for FFP in terms of maintenance of circulating blood volume. In fact, albumin products were administered perioperatively instead of FFP in this study, especially in the non-transfused group, and the incidence of these complications was not different between Group B1≥ 2000 mL and Group B2≥ 2000 mL. In this context, routine FFP administration is also suggested not to be necessary in terms of maintenance of the circulating blood volume. Thus, we suggest that the routine administration of FFP for the purpose of prevention of hepatic failure, hemostatic effect, and maintenance of circulating blood volume is not necessary.

Many adverse effects related to FFP transfusion have been identified, such as infection, allergic reactions, hemolysis, anaphylaxis, and TRALI[13-15]. In particular, TRALI, which is a rare and serious complication characterized by sudden onset of respiratory distress due to noncardiogenic pulmonary edema during or following transfusion, can be life-threatening. Fortunately, none of these transfusion-related complications occurred in our patients. However, since some of the reported adverse events can be life-threatening, one should refrain from inappropriate use of FFP.

Since an initial report by Foster *et al*[29] about survival advantages in patients undergoing colectomy for colon cancer, several other reports have shown that perioperative homologous blood transfusion to be an independent prognostic factor in many kinds of cancers[16,17,30-33]. However, a few suggested that homologous blood transfusion has no significant effect on the prognosis of cancer patients[34,35]. Thus, the association between transfusion and postoperative prognosis is still under debate. In the present study, postoperative prognosis did not correlate with FFP administration, but rather with tumor-related factors. Although the result was not powerful evidence to resolve the controversy, we can at least confirm that FFP administration does not improve prognosis of patients undergoing hepatectomy for HCC.

In fact, the guidelines of the Japanese Ministry of Health, Labour and Welfare state that administration of FFP should be limited only to supplement coagulation factors in those patients with a PT of more than 2.0 times normal or coagulation factor activity of ≤ 30%, and that the use of FFP for supplementation of circulation blood volume is inappropriate[18]. The guidelines do not mention administration of FFP for the prevention of hepatic failure. Thus, our suggestion is to obey the guidelines. Recently, Kaibori *et al*[36] reported the clinical value of FFP in surgery for HCC. They suggested that FFP transfusion was useful and recommended on the grounds of the results obtained from their analysis that the incidence of postoperative complications in patients with FFP transfusions was lower than that of patients with FFP and RCC transfusions, and was equal to that of non-transfused patients; long-term survival in patients with FFP transfusions was almost equal to that in non-transfused patients. However, their suggestion is perceived as groundless for the following reasons. To begin with, although there were some significant differences in many factors such as liver function and tumor progression among the groups in their study, they simply suggested that the difference in postoperative complications and long-term outcome resulted from the RCC and FFP transfusions. Secondly, since details of postoperative complications were not shown, especially for hepatic failure, postoperative bleeding, pulmonary edema and renal dysfunction, the examination of correlations between complications and FFP transfusions was insufficient. In addition, their suggestion completely ignored the recent guidelines of Japan.

The present analysis did not include HCC patients who underwent liver transplantation for treatment of liver cirrhosis. Therefore, the result of this study is not applicable to liver transplantation surgery. Considering that transfusion is performed for concomitant liver dysfunction at almost all liver transplantation surgery, it seems to be still too early to discuss the necessity of transfusion in such surgery.

In summary, FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis. Considering the previously reported FFP transfusion-related adverse effects in addition to the results of the present study, we suggest that FFP transfusion be abandoned in patients who undergo hepatectomy for HCC.

COMMENTS

Background

Fresh frozen plasma (FFP) has been frequently administered in the surgical treatment for hepatocellular carcinoma (HCC). Today, appropriate use of FFP is needed in terms of application and FFP transfusion-related potential adverse events. However, to our knowledge, there have been few reports investigating whether FFP transfusion affects outcomes following hepatic resection for HCC or any discussion of the need for FFP in surgery for HCC.

Research frontiers

The incidence of mortality and morbidity, postoperative liver function, and postoperative cancer prognosis were comparable between patients with intraoperative blood loss ≥ 2000 mL who had FFP transfusion and who did not have FFP transfusion.

Innovations and breakthroughs

This study showed that FFP transfusion did not affect outcomes following hepatic resection for HCC in terms of liver function, postoperative complications and cancer prognosis.

Applications

Considering the results of the present study, there is a suggestion that FFP transfusion should be abandoned in patients who undergo hepatectomy for HCC.

Peer review

The manuscript is a well-written paper that is adequately discussed with a reasonable number of literature references. Moreover, the topic is a current and popular one. Conclusions are well supplied by the results and literature.

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Cell Stem Cell

Reprogramming of Mouse and Human Cells to Pluripotency Using Mature MicroRNAs

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SUMMARY

Induced pluripotent stem cells (iPSCs) can be generated from differentiated human and mouse somatic cells using transcription factors such as Oct4, Sox2, Klf4, and c-Myc. It is possible to augment the reprogramming process with chemical compounds, but issues related to low reprogramming efficiencies and, with a number of protocols, residual vector sequences, remain to be resolved. We show here that it is possible to reprogram mouse and human cells to pluripotency by direct transfection of mature double-stranded microRNAs (miRNAs). Our approaches use a combination of mir-200c plus mir-302 s and mir-369 s family miRNAs. Because this reprogramming method does not require vector-based gene transfer, it holds significant potential for biomedical research and regenerative medicine.

Induced pluripotent stem cells (iPSCs) can be directly generated from fibroblast cultures by expression of only a few defined factors, such as Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka, 2006). Since the initial description of this approach, numerous studies have described modifications of the original protocol using additions or alternatives to these factors, including small molecules, with the aim of improving the efficiency and/or moving it toward potential clinical application (for example, Huangfu et al., 2008a, 2008b; Yoshida et al., 2009; Judson et al., 2009; Esteban et al., 2010). Genomic modification also remains a concern, because viral vector-mediated transduction of reprogramming genes involves random insertions of exogenous sequences into the genome. Some recent studies have indicated that iPSCs can be obtained with virus-free, removable PiggyBac transposons or episomal systems (Okita et al., 2008; Kaji et al., 2009; Woltjen et al., 2009; Jia et al., 2010), but these approaches still use DNA constructs, so the possibility of genomic integration of introduced sequences remains. Sendai virus has been used as an alternative (for example, in Seki et al., 2010), because it has an RNA genome, but it is still a virus. Reprogramming using just protein or mRNA has also been reported, but the protocols involved are technically challenging (Kim et al., 2009; Zhou et al., 2009; Warren et al., 2010).

MicroRNAs (miRNAs) are well-characterized regulators of development and differentiation (Lee et al., 1993; Ruvkun, 2001). Recent reports have demonstrated that specific miRNAs are highly expressed in embryonic stem cells (ESCs) and play a critical role in the control of pluripotency-related genes (Houbaviy et al., 2003; Judson et al., 2009; Suh et al., 2004). To identify candidate miRNAs to test for reprogramming activity, we analyzed miRNA expression in mouse ESCs, mouse iPSCs, and adult mouse adipose stromal cells (mASCs). miRNAs that were expressed >2-fold more strongly in mouse iPSCs and ESCs relative to mASCs (Figure S1A available online) were used for subsequent transfection assays (Figure S1D). The transfection efficiency was assessed by fluorescence microscopy using Fluorophore-labeled miRNAs, and we determined that 75.1% ± 0.1% of cells were fluorescent positive (data not shown). We introduced the miRNAs into mASCs obtained from *Nanog* promoter-driven green fluorescent protein (GFP) reporter mice (Okita et al., 2007). Real-time RT-PCR analysis indicated that transfected miRNA levels decreased markedly 72 hr post-transfection, so the transduction method was optimized to include four transfections at 48 hr intervals (Figures S1B and S1C; Table S1A available online). Eight days after initial transfection, the cells were passaged and grown in ESC-maintaining medium. We were able to detect GFP expression on day 14 after the transfection of mir-200c, mir-302 s, and mir-369 s family miRNAs, and by day 15 we observed approximately five GFP-positive colonies from 5×10^4 cells (Figures 1A–1D), giving an apparent efficiency that is comparable to that seen with the original report of retrovirus-mediated transcription factor introduction (Takahashi and Yamanaka, 2006). These colonies were not seen in mock transfectants in which negative control miRNAs were introduced (data not shown). Our analysis showed that introduction of all three of these miRNA candidates into mASCs from *Nanog* reporter mice successfully generated GFP-positive colonies, whereas the transduction of just one or two factors in

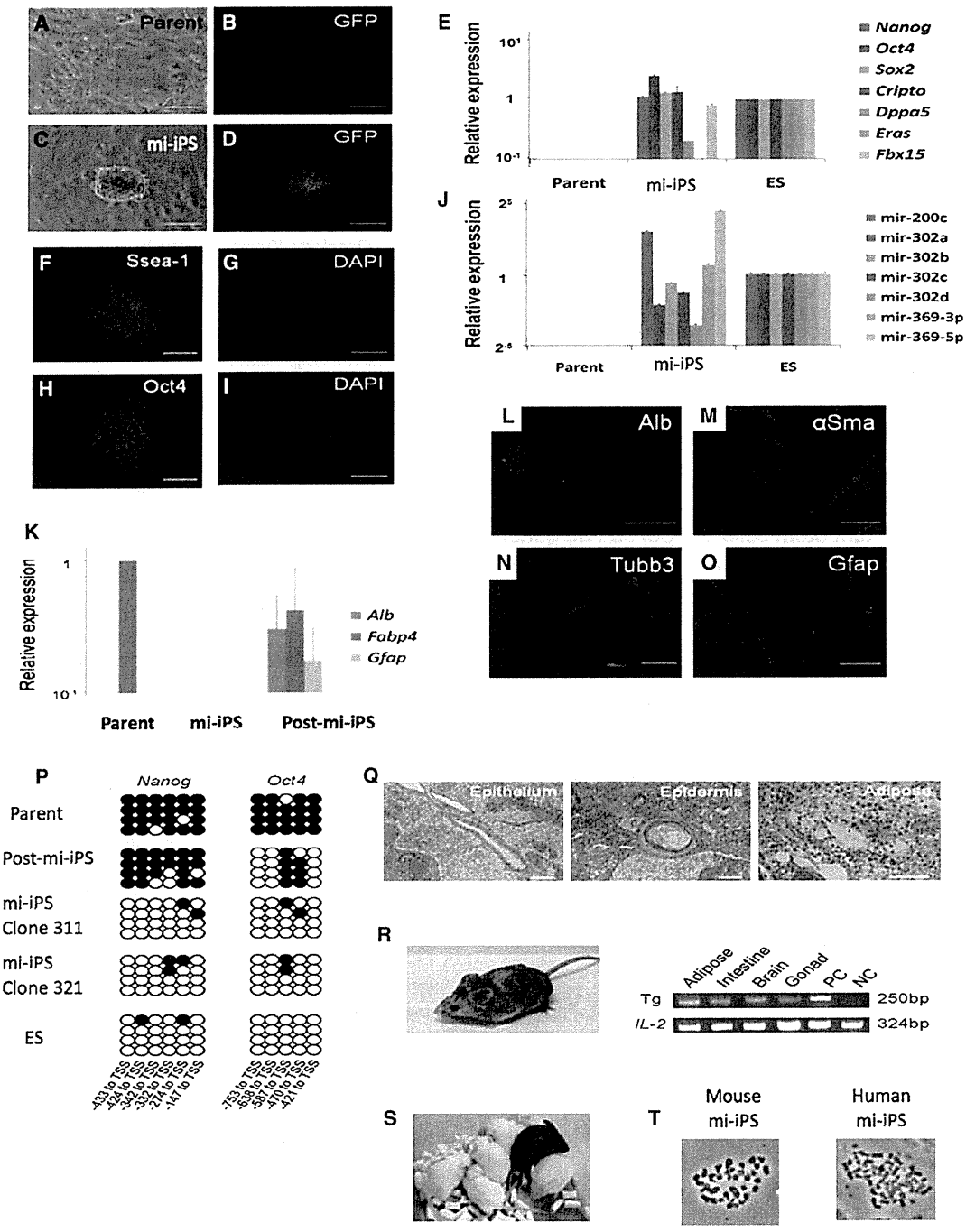

Figure 1. Induction of ES-like Cells from mASCs Using miRNAs

(A–D) Morphology of transfected mASCs. At day 15, colonies with defined margins and round shapes (C and D) that were distinct from parental cells (A and B) were observed. Brightfield (left) and fluorescence (right) images of parental and transfected cells are shown.

(E) The mi-iPSCs (clone 311) expressed undifferentiated ESC-marker genes. The expression of mRNA copies was normalized against *Gapdh* mRNA expression (mean ± SEM; n = 3).

(F–I) Immunocytochemistry revealed mi-iPSCs expressing Ssea-1 (F and G) and Oct4 (H and I).

(J) The expression of miRNA copies was normalized against U6 expression (mean ± SEM; n = 3).

(K–O) The differentiation of mi-iPSCs was induced by EB-like formation. The morphology of EB-like formation is shown in Figure S2D. (K) Real-time RT-PCR analysis verified the expression of differentiation markers, such as *Alb*, *Fabp4*, and *Gfap*. The expression of mRNA copies was normalized against *Gapdh* mRNA

any combination did not (experiments summarized in Figure S1D). We thus used all three candidates, mir-200c, mir-302 s, and mir-369 s, in subsequent studies. We refer to the reprogrammed cells generated using this approach as miRNA-induced pluripotent stem cells (mi-iPSCs).

Real-time RT-PCR (Table S1B) data revealed that on post-transfection day 30, mi-iPSCs expressed several genes characteristic of undifferentiated ESCs, such as *Nanog*, *Oct4*, *Sox2*, *Cripto*, *Dppa5*, and *Fbx15* (Figure 1E). The mi-iPSCs also expressed ESC-specific markers, such as Ssea-1 and Oct4 (International Stem Cell Initiative et al., 2007; Takahashi and Yamanaka, 2006) (Figures 1F–1I). In addition, greater mir-200c, mir-302 s, and mir-369 s expression was observed in mi-iPSCs than in the parental cells (Figure 1J). Expression profiling revealed the greater similarity of mi-iPSCs to mouse iPSCs (Takahashi and Yamanaka, 2006) and ESCs than mASCs (Figure S2E; accession number GSE28586). Karyotype analysis showed that 66.7% of the cells had a normal chromosome complement (40 mouse chromosomes in 8/12 cells) (Figure 1T).

To evaluate the differentiation capacity of mi-iPSCs, we examined the formation of embryoid bodies (EBs) in floating culture (Zhou et al., 2009) (Figure S2D). In suspension culture, mi-iPSCs formed ball-shaped EB-like structures. We transferred them to primary culture conditions (PCCs) consisting of gelatin-coated plates maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Attached cells, which we termed post-mi-iPSCs, were analyzed as described below and were compared with mi-iPS and parental cells. Twenty days after transfer to PCCs, the post-mi-iPSCs exhibited various morphological changes and expressed several genes characteristic of differentiation into all three germ layers. Real-time RT-PCR analysis confirmed *Alb* (endoderm), *Fabp4* (mesoderm), and *Gfap* (ectoderm) expression in post-mi-iPSCs (Figure 1K). The expression level of ES-like genes that were highly expressed in ESCs and mi-iPSCs was significantly reduced in post-mi-iPSCs (Figure S2A). Immunocytochemistry detected post-mi-iPSCs that were positive for Alb, αSma (mesoderm), Tubb3 (ectoderm), and Gfap (Figures 1L–1O and S2B). mir-200c and mir-302 s miRNA expression was lower in post-mi-iPSCs than in mi-iPSCs and ESCs (Figure S2C).

We next performed bisulfite genomic sequencing analyses to study the methylation status of cytosine guanine (CpG) dinucleotides in the promoter regions of pluripotency-associated genes, such as *Nanog* and *Oct4* (Table S1C; Zhou et al., 2009). The results revealed that the CpG dinucleotides of these gene promoters were less frequently methylated in mi-iPSCs and ESCs than in post-mi-iPSCs and parental cells (Figure 1P). These findings are consistent with reactivation of the promoter regions of immature status-related genes in mi-iPSCs.

To examine their in vivo differentiation properties, mouse mi-iPSCs were subcutaneously transplanted into the dorsal flanks of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Teratoma formation was observed in mice 12 weeks postinjection (Figure 1Q). Histological analysis revealed that the tumors contained various tissues found in epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) tissues. Furthermore, the injection of blastocysts with mi-iPSCs resulted in the generation of chimeric mice (Figure 1R and Table S1D). Genotyping (Table S1E) of the chimeric mice demonstrated the contribution of mi-iPSCs in intestinal, brain, adipose, and gonadal tissues, i.e., across all three germ layers. Crossing the chimeric mice resulted in the generation of progeny mice with the same coat color distribution as the original mi-iPSC parental strain, although the frequency of this occurrence was low (Figure 1S). Nevertheless, overall we have shown clearly that we can generate mouse mi-iPSCs that have acquired pluripotency via the introduction of synthesized miRNA from mASCs (Figure 1). We have also been successful in generating mi-iPSCs from MEFs, albeit with lower efficiency (one to five colonies from 5×10^5 cells) (data not shown).

To evaluate whether we can also reprogram human cells using this approach, we transfected mir-200c, mir-302 s, and mir-369 s into human ASCs (hASCs) and human dermal fibroblasts (HDFs) (results summarized in Table S2). Cells seeded at densities of 1×10^4 to 5×10^4 cells/well in 6-well culture plates were transfected with miRNAs through the same four-cycle protocol as for mouse cells at 48 hr intervals, and on day 8 the cells were harvested by trypsinization and transferred to ESC culture conditions according to methods described previously (Miyoshi et al., 2010). Twenty days after initial transfection, some colonies with sharp and defined margins and a different morphology from parental cells appeared (Figure 2A), at a frequency of two colonies from 1×10^5 adult human cells. We detected undifferentiated ESC marker gene expression in these colonies by immunocytochemistry (Figures 2B and 2C). Real-time RT-PCR revealed that these human mi-iPSCs expressed ES-like genes, including *NANOG*, *OCT4*, *SOX2*, and *LIN28* (Figure 2D). Using RT-PCR, we also found that hASC- and HDF-derived mi-iPSCs express high levels of mir-200c, mir-302 s, and mir-369 s (Figure 2E). The karyotype was predominantly normal (46 human chromosomes were observed in 10/12 cells [83.3%]; Figure 1T). To examine their differentiation

expression (mean ± SEM; n = 3). (L–O) Two weeks later, attached post-mi-iPSCs exhibited various morphologies, and immunocytochemistry confirmed the expression of Alb (L), αSma (M), Tubb3 (N), and Gfap (O) in these cells.

(P) *Nanog* and *Oct4* were not appreciably methylated in mi-iPSCs (clones 311 and 321), whereas the CpG dinucleotides of the regions were methylated in parental and post-mi-iPSCs (open and closed circles indicate unmethylated and methylated, respectively).

(Q) mi-iPSCs were subcutaneously transplanted into the dorsal flanks of NOD/SCID mice, and tumor formation was exhibited in various tissues as epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) layers.

(R) Chimeric mice were gauged by coat color. Genotyping of the chimeric mice demonstrated that mi-iPSCs contributed to adipose, intestinal, brain, and gonadal tissues. Il-2 was used as an internal control. Tail tips of a *Nanog* reporter mouse and parental mouse were used as positive and negative control templates, respectively. Tg, transgene characteristic of *Nanog* reporter mice; PC, positive control; NC, negative control.

(S) Germline transmission. The crossing of chimeric mice (>70% BL6 hair color contribution, as shown in R) with Bulb/c mice resulted in the generation of black mice at a frequency identical to that observed in the original murine mi-iPSC strain (the frequency was less than 1/20).

(T) The karyotype of human and mouse mi-iPSCs. Forty-six chromosomes in human mi-iPSCs and forty chromosomes in mouse mi-iPSCs are shown.

See also Figures S1 and S2 and Tables S1 and S2. Bar = 100 μm; original magnification, × 200.

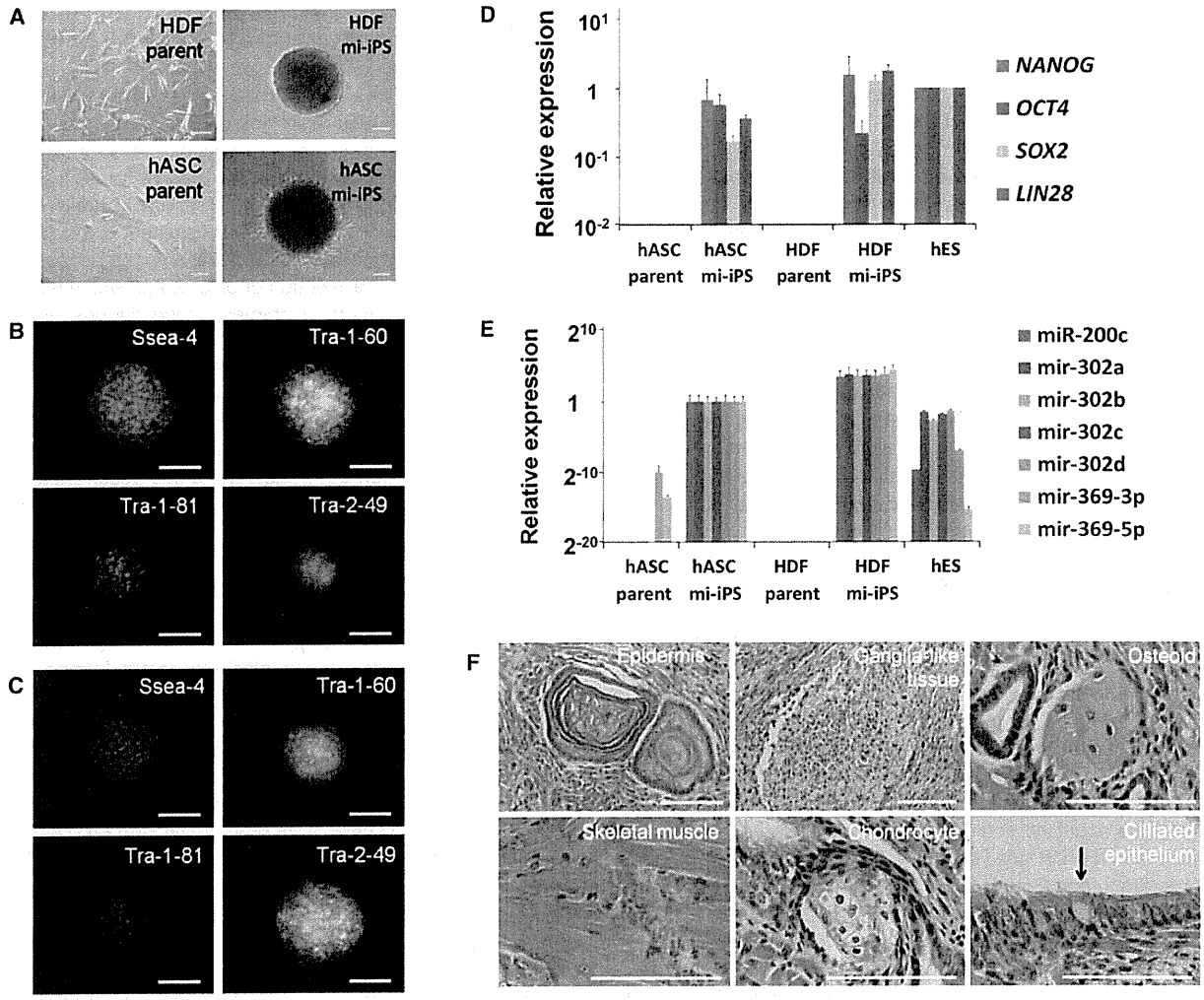

Figure 2. Pluripotency-Associated Gene Expression in mi-iPSCs from Human Somatic Cells

(A) At day 20, some colonies with sharp and defined margins appeared that were morphologically different from the parental hASCs and HDFs.

(B and C) Immunocytochemistry revealed mi-iPSCs from hASCs (B) and HDFs (C) expressing Ssea-4, Tra-1-60, Tra-1-81, and Tra-2-49.

(D) The mi-iPSCs from hASCs and HDFs highly expressed undifferentiated ESC-marker genes compared to the observed expression in their respective parental cells. The expression of mRNA copies was normalized against *GAPDH* mRNA expression (mean ± SEM; n = 3).

(E) Real-time RT-PCR analysis of miRNAs in parental mi-iPSCs. The expression of miRNA copies was normalized against *RNU48* expression, and the mean expression of hASC mi-iPSC was set to 1 for each gene (mean ± SEM; n = 3).

(F) mi-iPSCs (clone 3621A1) were transplanted subcutaneously into the dorsal flanks of NOD/SCID mice, and teratomas formed in various tissues and cells as epidermal, ganglia-like, osteoid, skeletal muscle, chondrocyte, and ciliated epithelial tissue (arrow, goblet cell).

See also Figure S2 and Tables S1 and S2. Bar = 100 μm; original magnification, × 200.

capacity, human mi-iPSCs were subcutaneously transplanted into the dorsal flanks of NOD/SCID mice. Teratoma formation was observed in mice 12 weeks postinjection (Figures 2F and S2F). Histological findings indicated that the tumors contained various tissues found in the epithelial (endoderm), epidermal (ectoderm), and adipose (mesoderm) tissues, representing all three germ layers. Overall, these findings suggest that direct transfection of mature miRNAs can also induce pluripotency in human somatic cells, and that these miRNA properties are conserved across species.

Our findings are consistent with previous analyses of miRNA function in pluripotent cells. Microarray analysis indicated that the promoter region of mir-302 s was conserved across species and driven by the transcription factor Oct4 (Marson et al., 2008). Members of the mir-302 s family were investigated as important key factors in the maintenance of ESC renewal and pluripotency as zygotic inhibitors of premature cell differentiation during early embryonic development (Rosa and Brivanlou, 2011). TargetScan (http://www.targetscan.org/) prediction for the mir-302 s family indicated that they inhibit the amine oxidase domain 1 (*Aof1*)

gene, which is associated with DNA methylation (Ciccone et al., 2009; Gregory et al., 2008). We found that *Aof1* was specifically repressed in mi-iPSCs, but not in parental cells (data not shown). During the course of reprogramming by defined factors, the morphology of somatic fibroblasts changed after cell-to-cell interactions, and iPSCs begin expressing E-cadherin, an epithelial cell marker highly expressed in ESCs (Gregory et al., 2008). These reports support our experimental results with mir-200c, which represses the epithelial-mesenchymal transition by inhibiting TGFβ signaling and is highly expressed in epithelial cells (Gregory et al., 2008). TargetScan prediction for mir-369 s also indicates that it inhibits *ZEB2*-related TGFβ signaling (Grimson et al., 2007) (data not shown). In our study, we could not generate GFP-positive colonies in mASCs from *Nanog* reporter mice using transfection of only one or two of these miRNAs. We concluded that all three of these factors are essential to generate PSCs from somatic cells. Other potential combinations of miRNAs that may also be able to generate pluripotent cells from somatic cells remain to be assessed.

Most of the reprogramming methods reported so far involve introduction of genetic materials and thus run the risk that exogenous vector sequences can be integrated into the host genome (Okita et al., 2008; Kaji et al., 2009; Woltjen et al., 2009; Jia et al., 2010). Very recently, highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency was reported (Anokye-Danso et al., 2011), but using integrating viral vectors and not direct transfection of mature miRNAs. Previous studies have also indicated that mir-302 s has reprogramming activity in human cells (Lin et al., 2011). Our study uses a different methodological approach, direct transfection of mature miRNAs, that has benefits from the perspective of potential clinical translation, although it does operate at considerably lower efficiency. We have successfully generated mi-iPSCs using this protocol more than six times. The resulting mi-iPSCs are subject to a reduced risk of mutations and tumorigenesis relative to most other protocols because mature miRNAs function without vectors or genomic integration. We also note that miRNA-based approaches are already under direct clinical investigation in a number of other therapeutic contexts. For example, a recent report demonstrated the utility and safety of therapy with miRNA in the treatment of hepatitis C (Lanford et al., 2010). We hope that mi-iPSC generation will eventually prove to be of significant benefit for both biochemical research and clinical regenerative medicine.

ACCESSION NUMBERS

The GenBank accession number for the expression profiling data reported in Figure S2E is GSE28586.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2011.05.001.

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REVIEW

Cancer stem cell theory in gastrointestinal malignancies: recent progress and upcoming challenges

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Abstract A growing body of evidence supports the notion that malignant tumors are heterogeneous and contain diverse subpopulations of cells with unique characteristics including the ability to initiate a tumor and metastasize. This phenomenon might be explained by the so-called cancer stem cell (CSC) theory. Recent technological developments have allowed a deeper understanding and characterization of CSCs. Even though the application of this theory to hematopoietic malignancies and solid tumors holds promise for new ways to treat cancer, it also brings some skepticism. Efficacious therapeutic approaches targeting the CSC population should be explored to overcome therapeutic failure and improve patient outcomes. This review will focus on the intrinsic and extrinsic regulation of CSCs, as well as the development of therapeutic approaches against CSCs, predominantly focusing on gastrointestinal malignancies.

Keywords Gastrointestinal cancer · Cancer stem cell · Epigenetic · Microenvironment

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Introduction

Cancer has been regarded as a heterogeneous population of cells having distinct characteristics in terms of capacity for tumor formation, metastasis, and drug resistance [1]. The heterogeneity of tumor cells might be explained by the concept of clonal evolution and cancer stem cell (CSC) models, as previously reviewed by Adams and Strasser [2]. The clonal evolution model suggests that most tumor cells have the capacity for self-renewal and tumor growth maintenance [2]. The CSC model defines a unique cancer cell population at the apex of the hierarchy, with indefinite proliferative potential that drives the formation of tumors and fuels their growth [3]. The term CSC does not imply the cell of origin but merely indicates functional properties [4].

Since the study of acute myeloid leukemia (AML) reported by Bonnet and Dick, evidence for the existence of tumor cells with stem cell-like properties has shed light on the field of cancer research [5]. This concept has been applied not only to hematopoietic malignancies but also to solid tumors. The study of breast cancer performed by Al-Hajj et al. demonstrated that breast cancer cells contained a $CD44^+/CD24^-$ subpopulation possessing CSC characteristics [6]. This CSC subpopulation is unique because it has distinctive phenotypic characteristics such as superior tumor-initiating and metastatic capabilities, chemoresistance, and radioresistance [7–9].

Given the contribution of CSCs to disease progression, the characterization of CSCs as targets for cancer therapy is necessary. The discovery of novel techniques to isolate and prospectively identify and characterize CSC populations will be a valuable step forward in this field of research, along with the investigation of the molecular signaling pathways, epigenetic mechanisms, and microenvironmental factors that govern them.

Identification of CSC populations

Several methods, including the side population (SP) assay or methods based on the utilization of a variety of surface markers have been widely used to facilitate the enrichment of CSC populations. However, some drawbacks still need to be considered.

SP assay

The identification of CSCs using the SP assay is based on the assumption that CSCs possess one of the characteristics of stem cells: the efflux of Hoechst dye by an ATP binding cassette (ABC) transporter protein expressed in the cell membrane [10]. SP cells were first identified in the mouse bone marrow as long-term repopulating cells [11]. This assay has been reported to successfully enrich stem cells or tissue progenitors, and also the liver [12], colorectal [13], and gastric [14] CSC populations. The SP assay has the advantage of detecting rare events in heterogeneous tumors, due to its high sensitivity. Even though the SP assay might enrich cancer cells with stem cell properties in several human malignancies, caution needs to be exercised in interpreting its results, due to the lack of specificity, because differentiated cells in adult tissues such as the liver [15] and the small intestine [16] also exhibit the SP phenotype. Other caveats resulting in misinterpretation of the SP assay include ploidy variation in tumor cells, which leads to a heterogeneous Hoechst profile, and its high sensitivity to slight discrepancies in staining conditions. Therefore, a combination of the SP assay and surface markers might provide a better way to further enrich CSC populations [17].

Surface markers

The utilization of unique surface markers that are expressed exclusively in CSC populations allows the prospective isolation of CSCs from specimens, either from cell lines or patients with primary tumors, and is therefore an important step in the investigation of CSCs. The discovery of consistent markers, either single or in combination that can identify CSC populations and reflect their malignant behavior, would provide a tremendous breakthrough.

CSC markers that have been found to date have been frequently identified in normal stem cells in previous studies and are expressed in an overlapping manner in cancers of various organs [18, 19]. Most of them are cell surface markers that allow the isolation of CSC populations using flow cytometry or magnetic cell sorting. The first CSC marker identified in solid tumors was CD44, a class I transmembrane glycoprotein, which has been reported to enrich breast CSCs [6]. Another example is CD133, which was previously defined as a marker in normal primitive hematopoietic cells [19] and neural stem cells [20], and is now widely used to enrich CSCs in colon cancer [21, 22] and hepatocellular carcinoma (HCC) [23]. However, intriguing evidence has shown that CD133 is not only expressed in CSCs but that it is also expressed in differentiated epithelial cells in various organs; furthermore, $CD133^-$ cancer cells can also initiate tumors [24]. This indicates the need for further validation of CSC markers. Here we describe several CSC markers that have been investigated so far in gastrointestinal (GI) malignancies. These are summarized in Table 1.

Esophageal cancer

Unlike the evidence in other GI malignancies, little evidence has supported the identification of CSC populations in esophageal cancer. Enrichment of SP cells from an esophageal cancer cell line has been performed, and SP cells showed 50 times higher tumorigenicity than non-SP cells, and higher expression of the stem cell–related genes OCT-4, SOX-2, BMI-1, and ZFX than non-SP cells. ATP-binding cassette (ABC) transporter genes (ABCG2 and ABCA5) and some genes related to the Wnt and Notch signaling pathways were also upregulated in SP cells [25]. Another marker that has been suggested as a CSC marker in esophageal cancer is podoplanin, a mucin-type transmembrane glycoprotein [26]. In esophageal squamous cell carcinoma, podoplanin is expressed at the outer edges of tumor cell nests, and podoplanin overexpression resulted in the enhancement of tumorigenicity, chemotherapy resistance, and invasiveness [26]. Zhang et al. [27] reported that a radioresistant population of esophageal cancer, which was established by continuous fractionated irradiation, displayed CSC-like characteristics, such as higher telomerase activity; enrichment of SP cells; and higher expression of β-catenin, Oct3/4, and β-integrin.

Gastric cancer

A study using gastric cancer cell lines has suggested that the $CD44^+$ fraction contains gastric CSCs; these CSCs show stem cell characteristics and exhibit increased resistance to chemotherapy and radiation-induced cell death [28]. However, the authors noted that gastric CSCs may comprise around 3% of the total $CD44^+$ fraction; therefore, CD44 is not highly specific for gastric CSCs because it is also expressed in non-tumorigenic gastric cancer cells [28]. Another report showed that diffuse-type gastric carcinoma cells contained SP cells that comprised between 1 and 4% of the total cells, and indicated that ABCG2 expression may serve as a marker for diffuse-type gastric CSCs. This SP fraction was diminished by treatment with transforming

Table 1 Identification of cancer stem cell markers in gastrointestinal malignancies

Organ	Marker	Frequency (%)	Minimum number of cells to generate tumor	Source	References
Esophagus	SP	0.3–1.4	n.d.	Cell line	[13]
Gastric	SP	1–4	5×10^4	Cell line	[15]
	$CD44^+$	5–94	2×10^4–3×10^4	Cell line	[25]
Colon	SP	0.3–0.7	n.d.	Cell line	[13]
	$CD133^+$	1.8–24.5	1×10^2	Clinical specimen	[21]
	$CD133^+$	0.7–6.1	3×10^3	Clinical specimen	[22]
	$CD44^+/CD166^+$	1.2–16	1×10^3	Clinical specimen	[31]
	$CD44^+/EpCAM^{high}$	0.03–38	2×10^2	Clinical specimen	[31]
	$ALDH1^+$	3.5	1×10^2	Clinical specimen	[119]
Liver	SP	0.25–0.80	1×10^3	Cell line	[12]
	$CD90^+$	0.04–2.34	1×10^3	Cell line	[32]
	$CD90^+/CD45^-$	0.74–6.2	5×10^3	Clinical specimen	[33]
	$CD90^+/CD44^+$	0.02–2.53	5×10^2	Cell line	[32]
	$CD90^+/CD44^+$	0.01–5.7	n.d	Clinical specimen	[32]
	$EpCAM^+$	24.3	2×10^2	Cell line	[34]
		1.4–5.2	1×10^4	Clinical specimen	
	$CD133^+$, $ALDH^+$	0.94–55.71	5×10^2	Cell line	[35]
	$CD133^+$	48–65	5×10^4	Cell line	[36]
	CD133	1–3	5×10^4	Clinical specimen	
	$CD133^+$, $CD44^+$	0.09–1.88	1×10^2	Cell line	[120]
Pancreas	$CD44^+$, $CD24^+$, ESA^+	0.2–0.8	1×10^2	Clinical specimen	[40]
	$CD133^+$	0.68–3.21	5×10^2	Clinical specimen	[41]

n.d not determined

growth factor (TGF)-β, thereby attenuating the tumor-forming ability of the population [14].

Colon cancer

Prominin-1, otherwise known as CD133, is among the best-characterized of CSC markers in colon cancer [21, 22, 29]. This marker has been used to successfully enrich tumorigenic and non-tumorigenic cells in brain tumors [30]. The work by O'Brien et al. [21] suggested that CD133 might be useful for separating tumorigenic and non-tumorigenic fractions of colorectal cancer cells, with the frequency of colon CSCs estimated at 1 in 262 $CD133^+$ cells. Although these authors found that CD133 was useful for significantly enriching the CSC population in colorectal cancer, not all $CD133^+$ cells were colon CSCs, thereby highlighting the need for additional markers [21]. Similar findings were revealed by Ricci-Vitiani et al. [22], who reported that $CD133^+$ cells accounted for 2.5% of the cells in clinical samples; these cells were considered to exist as a rare subpopulation within the bulk of tumor cells. CD133 was reported to be highly expressed in spheroids derived from colon cancer, and cells expressing it had the potential to differentiate in vitro and in vivo [2]. A work by Dalerba et al. [31] reported that CD166 could be used as a co-marker to identify CSCs in colon cancer in combination with CD44.

HCC

The presence of CSCs within HCC has been detected using several markers, such as CD90 [32, 33], EpCAM [34], ALDH1 [35], CD133 [36], and CD13 [37]. Both single markers and combinations of markers have been reported to enrich liver CSCs. CD90, a 25- to 37-kDa glycosyl-phosphatidylinositol-anchored glycoprotein [38], is expressed in liver stem/progenitor cells during hepatic development [39], and it has been investigated as a CSC marker in HCC [32]. The combination of positivity for CD90 and CD44 was associated with a more aggressive subpopulation of CSCs in HCC that showed metastatic potential compared with their $CD90^+CD44^-$ counterparts [32]. The $CD90^+CD44^-$cells were detected in 90% of blood samples from HCC patients, indicating the presence of circulating CSCs in HCC [33].

EpCAM, in combination with alpha-fetoprotein (AFP), has also been used for liver CSC enrichment. Besides their highly invasive and tumorigenic properties, the $EpCAM^+$ population had a gene expression pattern similar to that of

hepatocyte stem cells, and also showed activation of Wnt/β-catenin signaling [34]. A combination of CD133 and ALDH has also been reported to more specifically characterize the liver CSC population, because $CD133^+ALDH^+$ cells possess higher tumorigenicity than $CD133^+/ALDH^-$ cells [35]. The contribution of liver CSCs to chemoresistance has also been reported by Ma et al. [36]; they reported that $CD133^+$ HCC cells showed preferential activation of Akt/PKB and Bcl-2 cell survival responses. Another study has found that CD13, also known as aminopeptidase N, is a marker for the SP of HCC. $CD13^+$ cells show a low level of reactive oxygen species (ROS), which contributes to their chemoresistant and radioresistant properties [37].

Pancreatic cancer

Pancreatic CSCs have been enriched in the subpopulation characterized by the $CD44^+CD24^+ESA^+$phenotype, which accounted for 0.2–0.8% of pancreatic cancer cells. This population had a 100-fold increased tumorigenic potential compared with their non-tumorigenic counterparts, and also showed increased expression of sonic hedgehog (SHh), which plays an important role in the developmental signaling network [40]. Another marker of the CSC population in pancreatic cancer is CD133 [41]. As few as 500 $CD133^+$ cells from freshly isolated patient-derived tumor could give rise to a tumor; in contrast, even 10^6 $CD133^-$ cells could not generate a tumor. Moreover, a subpopulation of CSCs characterized by the expression of the CXCR4 receptor was found to have metastatic potential, indicating the existence of migrating CSCs in pancreatic cancer [41].

Functional assay for CSC properties

The functional characteristics that define CSCs are their ability for self-renewal and their capacity for recapitulation of the cellular heterogeneity present in the primary tumor. Until now, the gold standard for the assessment of the tumorigenic potential of a defined CSC population has come from the serial transplantation assay performed in immunosuppressed mice [42].

Concerns such as the choice of the recipient animal have been issues in determining the capabilities of CSCs through animal transplantation. Immunological disparity in the recipient mice has revealed different tumorigenic capacities of the CSC population [43]. Despite the wide utilization of the non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mouse for transplantation assays in most CSC-related investigations, a study of melanoma has proposed that the frequency of CSCs had been underestimated; this is because the utilization of severe immunodeficient mice, such as NOD/SCID interleukin-2 receptor γ chain-null ($Il2rg^{-/-}$) mice, which are deficient in natural killer cell activity, enhanced the detection of tumorigenic melanoma cells [43]. However, a study of colorectal CSCs, which also used ($Il2rg^{-/-}$) mice for the tumorigenicity assay, gave the same result as the study using NOD/SCID mice, suggesting that the immune status of the host does not influence the ability of CSCs to generate tumors [44].

Cancer stem cells and cellular origin of tumors

The term CSC does not necessarily imply the cellular origin of tumors, as it refers more to a population that propagates tumors, whereas cellular origin refers to a cell that received the first oncogenic hit [45, 46]. Tumors have been proposed to originate from transformed normal stem cells [47, 48] or progenitor cells [5]. The unique properties of stem cells, such as prolonged survival and self-renewal, would favor the accumulation of oncogenic mutations [49]. A study of hematopoietic malignancies by Bonnet and Dick has indicated that normal primitive cells rather than committed progenitor cells are the target for leukemic transformation [5]. Two leading-edge investigations also support this notion in solid tumor initiation. The deletion of adenomatous polyposis coli (APC) in intestinal crypt stem cells, marked by Lgr5, led to their transformation into microadenoma, while the deletion of APC in short-lived transit-amplifying cells failed to do so [47]. Another study has shown that prominin-1 (CD133) is a marker of stem cells in the small intestine and that endogenous Wnt signaling activation in these cells results in the disruption of the normal tissue maintenance that begins in the crypt, leading to aberrant expansion of the $Prom1^+$ stem cell population, which leads to the neoplastic transformation of the small intestinal mucosa [48].

The presence of CSCs within the bulk of a tumor (with these cells having the capacity to maintain tumor growth) has raised the question of where this subpopulation comes from. To obtain direct evidence as to whether CSCs originate from mutated adult stem cells remains difficult because the identification of adult tissue stem cells is still obscure in some organs [46]. Several studies have provided evidence that cells with CSC properties can be generated from progenitor cells or differentiated somatic cells. The expression of MLL-AF9 fusion protein has facilitated the conversion of a committed granulocyte macrophage progenitor to a leukemic stem cell [50].

Moreover, a concept that a CSC phenotype could be induced has also been postulated. The forced expression of Snail and Twist transcription factors, which lead to epithelial–mesenchymal transition (EMT), has been reported to generate cancer cells with stem cell-like properties [51]. Moreover, Wong et al. [52] have demonstrated that the

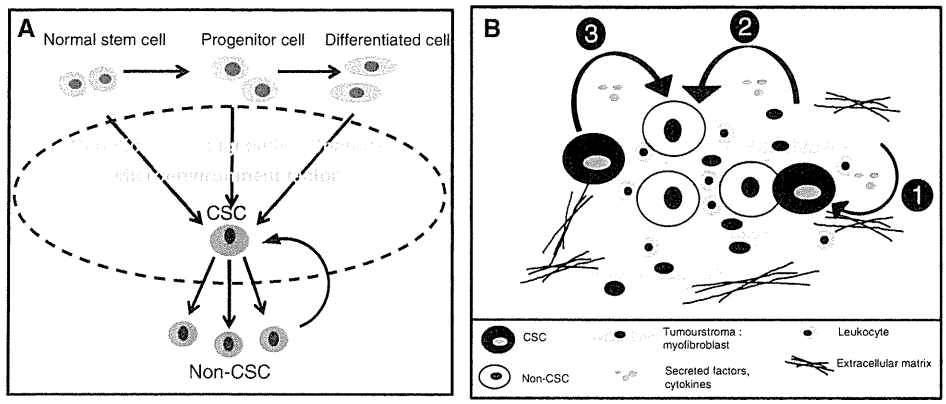

Fig. 1 **a** A model of cancer stem cell (*CSC*) formation. Primary as well as progressive aberrant genetic and epigenetic events in stem cells, progenitor cells, and probably more differentiated cells lead to uncontrolled self-renewal and formation of CSCs. Recent findings also highlight the possibility of the acquisition of stem cell properties in non-CSCs under permissive conditions. Some genetic and epigenetic events such as microRNA deregulation, DNA demethylation, and chromatin modification, as well as stimulation from the tumor microenvironment via specific cytokine secretion and hypoxia, might activate and deregulate stem cell-related pathways which lead to self-renewal activation that supports CSC survival and fuels malignant progression. **b** A model of the interaction between CSCs, non-CSCs, and the microenvironment. As occurs with stem cells, CSCs are also proposed to live in specific niches which support their growth and self-renewal activity (*1*). Specific stimuli from the niche, such as some cytokines and also low-oxygen conditions, might also promote transformation from non-CSCs into CSCs [44, 114] (*2*). Moreover, CSCs might also secrete some factors that stimulate the activation of the stem cell-related transcriptional network, and promote the growth of non-CSCs [110] (*3*)

embryonic stem cell gene expression module and the properties of CSCs in primary culture keratinocytes were inducible, and that c-Myc could be regarded as a key player in this process. Interestingly, a mouse fibroblast mutant for the Rb1 pathway was shown to undergo reprogramming toward the CSC phenotype as it formed spheres that were able to propagate in suspension cultures and generate SP cells with CSC characteristics. These SP cells were enriched for the high expression of ES-like genes (Nanog, Oct-4, Sox2, and c-Myc), the induction of Zeb1, the generation of a $CD44^{+}/CD24^{low}$ population, and the ability to form sarcoma in vivo [53].

Regulatory network in CSCs

Owing to the stem cell-like characteristics of CSCs, it could be hypothesized that they might be regulated by intrinsic and microenvironmental factors, similar to stem cells. Here we describe the stem cell signaling network, epigenetic regulation, and microenvironmental cues that might regulate CSC behavior (also described in Fig. 1).

Intrinsic factors

Stem cell-related signaling pathway

Overlapping molecular signaling has been found to regulate not only stem cells but also malignant progression [54]. An embryonic stem cell-like overexpression signature has been shown in poorly differentiated aggressive tumors [55], suggesting that the stem cell regulatory network has a pivotal role in some tumors. Here we discuss the role of several stem cell related signaling pathways in the modulation of CSC behavior.

Notch signaling

Notch signaling is an important player in GI organ development and oncogenesis [56–58]. The binding of the ligands Jagged1,2, Delta-like (Dll1-4), and Serrate to the transmembrane Notch receptors initiates the activation of Notch signaling. This event leads to the cleavage of Notch receptors and the release of the Notch intracellular domain (NICD), followed by the translocation of NICD to the nucleus. In the nucleus, the binding of NICD to the DNA-binding protein CSL facilitates the recruitment of the transcriptional coactivator Mastermind/Lag-3, which subsequently recruits the MED8 mediator transcription activation complex, and finally activates the downstream target genes [59].

Evidence for the involvement of Notch signaling in organ development comes from a study where its activation was found to impair intestinal cell differentiation into goblet and enteroendocrine cells [60]. Notch signaling is also vital for pancreatic cell type specification in the developing pancreas [61]. The differentiation of biliary epithelial cells has also been reported to be regulated by Notch signaling [62].

Aberrant Notch signaling also leads to malignant transformation and progression. A deficiency in Notch2,

one of the components of Notch signaling, leads to a phenotypic switch toward anaplastic pancreatic cancer with characteristics of EMT due to Myc modulation [63]. Recently, the inhibition of Notch signaling by amino-terminal enhancer of split (Aes) has been found to suppress colon cancer metastasis [64]. Notch inhibition also impairs esophageal squamous cell differentiation, resulting in basal cell hyperplasia and dysplasia [65]. Several studies have observed that Notch and Wnt signaling act synchronously. Increased Notch signaling is detected in benign adenomas, which may contribute to the APC-driven initiation of colorectal cancer [57]. The activation of Notch before the loss of the wild-type APC allele could lead to the localized expansion of early progenitor cells, thus increasing the chances of secondary mutations that drive tumor formation [57].

A study of colon cancer has shown that Notch signaling was required for the formation of CSCs and their self-renewal capacity. The upregulation of HES1 (the canonical Notch target gene), JAG1 (a pathway component), and Notch1 was also shown in colon CSCs compared with normal colon epithelium. The inhibition of Notch signaling by γ-secretase inhibitor promotes the differentiation of colon CSCs into goblet cells and also induces their apoptosis [66].

Wnt signaling

Wnt signaling pathways have been shown to play a major role in intestinal morphogenesis and homeostasis [67]. The activation of canonical Wnt signaling enables the binding of Wnt ligands to the receptor complex, the Frizzled family, and Lrp5/6, resulting in the uncoupling of β-catenin from the degradation complex, which elicits its translocation to the nucleus. In the nucleus, the β-catenin binds to Lef/Tcf transcription factors and activates target genes [67].

The importance of the Wnt pathway in controlling proliferation versus differentiation of normal and malignant intestinal epithelial cells has been shown through the disruption of β-catenin/TCF-4 activity that reduces c-MYC expression and releases $p21^{CIP1/WAF1}$ transcription. These molecular events subsequently mediate G1 arrest and differentiation [68]. Evidence supporting a regulatory role for Wnt signaling in colon CSCs comes from the study conducted by Vermeulen et al. [44], which suggests that Wnt signaling activity might serve as a functional marker for tumor cell clonogenicity and cancer stemness. The Wnt activity level was also correlated with the expression of previously described colon CSC surface markers such as CD133 and CD166 [44]. Recent findings have also revealed Oct-3/4 as a regulator in the Wnt pathway, because downregulation of Oct-3/4 increased β-catenin levels, which activated Wnt signaling and initiated EMT [69].

Hh signaling

The proliferation and differentiation of various embryonic tissues is commonly known to be dependent on the Hh signaling pathway. Because its deregulation is a frequent event in various human tumors, especially in GI malignancies, the maintenance of Hh signaling is important for the abrogation of aberrant proliferation and tumorigenesis [70].

The Hh–Gli1 pathway has been implicated in the colon CSC molecular regulatory circuit through regulation of the self-renewal activity and metastatic behavior of CSCs, which suggests that targeting Hh–GLI1 may provide a promising approach for the eradication of colon CSCs and metastases [71]. In the pancreas, increased expression of SHh has been found to promote the progression of precursor lesions of pancreatic cancer (PanIN lesions) to pancreatic adenocarcinoma [72]. The importance of the relation between SHh and cancer stemness is supported by Li et al.'s study, which showed a higher expression of SHh in a subpopulation of CSCs that were of the $CD44^+$/$CD24^+$/ESA^+ phenotype [40].

TGF-β signaling

The involvement of TGF-β in the development of various organs and in the maintenance of the pluripotent state of embryonic stem cells has been widely studied [73]. TGF-β is known to be involved in tumor suppression and promotion as well as tumor microenvironment interaction; hence, it is likely to play a role in the complex regulation of malignancies [74]. Loss of the Smad4 gene in TGF-β family signaling pathways has been shown to be involved in the progression from benign intestinal adenomas to highly invasive adenocarcinomas [75]. An important finding has highlighted the significance of TGF-β signaling in the acquisition of stem cell-like properties in cancer cells. Through EMT induction, TGF-β maintains stem cell-like properties and tumorigenic activity in breast cancer cells [51]. A study demonstrated that SP cells from pancreatic cancer cell lines underwent EMT in response to TGF-β treatment, and that the TGF-β responsiveness was greater in SP cells than that in the main population cells [76].

Although evidence exists suggesting that TGF-β stimulation promotes the generation of cancer cells with stem cell-like properties, other intriguing results have been obtained. Tang et al. [77] reported that TGF-β stimulation in transformed breast epithelial cells resulted in the loss of stem cell-like properties and the ability to form mammospheres.

Epigenetic factors

Epigenetic regulation refers to a mechanism that controls gene expression without alteration in the coding sequence. Several epigenetic regulators such as microRNAs, DNA methylation, and histone modification have been proven to widely affect and critically regulate many biological processes, including the development of malignancies [78, 79]. Evidence of a robust epigenetic switch comes from the cellular reprogramming phenomenon. The phenomenon that differentiated cells can be reprogrammed into pluripotent state, implies that epigenetic regulation is a powerful driving force that allows a change in cell fate and characteristics [80]. Thus, it is likely that the combined expression of specific stem cell-associated factors and oncogenes might also induce an undifferentiated state of cancer [55]. The contribution of epigenetic mechanisms to the regulation of CSCs has not yet been fully defined and deserves further investigation.

Histone and chromatin modification

Histone modifications are known to regulate gene expression because they serve as signals for the binding of chromatin-remodeling complexes, which modify chromatin into repressive or permissive states [81]. Embryonic stem cells (ESCs) have a bivalent histone mark, where the active mark H3K4me2 and the repressive mark H3K27me3 coexist in key developmental genes, thus allowing the tight regulation of gene expression [82]. In human malignancies, silenced genes showed histone H3 lysine 9 hypermethylation and histone H3 lysine 4 hypomethylation, and a defined heterochromatic structure [83]. The loss of H4 monoacetylation and trimethylation appears early and increases during oncogenic transformation [84]. The loss of H4 Lys 16 monoacetylation and H4 Lys20 trimethylation is a hallmark of human cancer due to its association with the hypomethylation of repetitive DNA sequences [85]. The role of histone modification in the CSC regulatory mechanism has not been widely studied. A higher enrichment of H3K4me2 and a significant reduction of H3K27me3 enrichment have been shown in CSCs compared with the bulk of a tumor. Therefore, this finding suggests that CSCs might be in a transcriptionally active state [86]. Moreover, specific changes in histone modifications have been reported to contribute to the molecular heterogeneity in prostate cancer and to underlie the differences in the clinical phenotypes of cancer patients [86]. As there are few studies of the epigenetic regulation of CSCs by histone modifications, this field remains open for further investigation.

DNA methylation

Although DNA methylation has been well described and studied in several human malignancies, to date very few studies have been performed to directly address the role of DNA methylation in the regulation of CSCs. A population of CSCs has been found to have a lower level of DNA methylation in several tumor suppressor genes than that in the bulk of tumor [85]. A recent study of HCC indicated that TGF-β1 stimulation inhibited global DNA methyltransferase (DNMT) activity and decreased DNMT1 and DNMT3β expression in a non-CSC population from a liver cancer cell line that was negative for CD133. This phenomenon was due to demethylation of the CD133 promoter-1 in $CD133^-$ cells following the TGF-β1 treatment [87].

Characterization of DNA promoter methylation in breast CSCs revealed the epigenetic deregulation of the Jak-STAT pathway, which is implicated in the maintenance of the features of breast CSCs. The level of CpG methylation was also reported to be significantly reduced in mammospheres compared with parental cells [88].

MicroRNAs

MicroRNAs are endogenous RNAs of approximately 22 nucleotides and are known to be potent regulatory molecules, because a single microRNA can target and regulate several genes [89]. Being important transcription regulators, microRNAs have been shown to contribute widely to oncogenic transformation, tumor suppressor activity, and the maintenance of pluripotency as well as differentiation [89–91]. Evidence for the key role of microRNAs in several biological processes comes from studies of the disruption of specific enzymes required in the microRNA biogenesis pathway. Global loss of microRNAs, as studied using Dicer knockout ESCs, compromises both self-renewal and the differentiation ability of ESCs, thus supporting the prominent contribution of microRNAs to the stem cell regulatory network [92, 93].

The importance of the role of microRNAs in the development of digestive organs has been investigated. Global loss of microRNAs, demonstrated in Dicer conditional knockout in a specific lineage of the intestine, showed disorganization in intestinal epithelial cells, a decrease in goblet cells, a dramatic increase in apoptosis in crypts of both jejunum and colon, and accelerated jejunal cell migration [94]. In the liver, the loss of Dicer compromised hepatocyte survival and enhanced proliferation as well as apoptosis [94]. Nevertheless, the loss of Dicer also promoted hepatocarcinogenesis [95].

The finding that human ESCs express a unique set of microRNAs [96] has given an insight into the contribution

of microRNAs in the regulation of the stem cell transcriptional network, which has also been shown to be involved in cancer. Global loss of microRNAs has been found to induce cellular transformation and tumorigenesis [97]. Global changes in the expression of microRNAs were associated with differentiation, and poorly differentiated tumors showed lower global expression levels of microRNAs [98].

Evidence supporting the role of microRNAs as a driving force and as epigenetic regulators of CSCs has been established from studies in several human malignancies. miR-181 has been found to be highly expressed in AFP^+EpCAM^+ CSCs in HCC and also upregulated in embryonic liver tissues and pluripotent hepatic stem/progenitor cells in human livers [99]. The role of miR-181 in maintaining an undifferentiated state in hepatic progenitor cells has been explained through the inhibition of its targets, GATA6 or CDX2, which are known as hepatocyte differentiation regulators, and also NLK, a Wnt/β-catenin signaling inhibitor [99]. miR-181 has also been found to regulate sphere formation in breast cancer cell lines through the inhibition of ATM, a serine/threonine kinase, which functions as a tumor suppressor gene [100]. TGF-β has been shown to induce the expression of miR-181b through a Smad4-dependent mechanism in hepatic cells [101]. miR-181b expression enhanced the tumorigenic potential and the resistance to doxorubicin of HCC cells [101]; therefore, evaluation of the TGF-β/miR-181 pathway as a target for overcoming drug resistance and eliminating CSCs awaits further investigation.

In HCC, other compelling evidence for the role of microRNAs in regulating CSCs was reported in a study that indicated that miR-130b regulated the self-renewal, tumorigenicity, and chemoresistance of $CD133^+$ CSCs [102]. The forced expression of miR-130b in $CD133^-$ CSCs rescued the phenotype of $CD133^+$ CSCs, as evidenced by the recovery of CSC characteristics such as self-renewal capacity, tumorigenicity, and chemoresistance [102].

Another study revealed the role of microRNAs in the regulation of CSCs in prostate cancer; miR-34a was found to inhibit prostate CSCs and metastasis by directly repressing CD44 [103]. In breast cancer, inhibition of miR-200b increased CSC formation through the direct repression of Suz12, a subunit of a polycomb repressor complex (PRC2), resulting in the reduction of Suz12 binding, H3-K27 trimethylation, and polycomb-mediated repression of the E-cadherin gene [104]. miR-200b has a protective effect because it negatively regulates CSC formation, as determined by reduced mammosphere formation and suppressed tumor growth in a mouse xenograft, in combination with chemotherapy [104]. The involvement of microRNAs in CSC regulation is summarized in Table 2.

Microenvironmental factors

The microenvironment has long been acknowledged as supporting the growth of stem cells as well as tumors [105–107]. Recently, several studies have provided remarkable evidence on the regulation of CSCs by the microenvironment. CSC properties have been shown to be influenced and induced by specific cues in their surrounding microenvironment. The β-catenin paradox, observed in colon cancer, dictates that not all cells within an APC-induced tumor have equal Wnt pathway activities [108], which raises questions on the mechanisms that regulate Wnt signaling activation. A recent study by Vermeulen et al. [44] indicates that Wnt signaling activity marks colon CSCs and is regulated by the microenvironment. A hepatocyte growth factor (HGF), which is secreted by myofibroblasts, is an important signal for Wnt signaling activation. This study also highlights the important concept that cancer stemness is a flexible feature and can be modulated by a specific microenvironment [44]. A study of brain tumors suggests that perivascular niches maintain brain CSC pool, whereas its disruption of the pool, compromising the self-renewal activity of brain tumors, leads to subsequent growth arrest [109].

The dynamic interaction between CSCs, non-CSCs, and the microenvironment has also been elucidated recently. A study of breast cancer revealed the maintenance of a dynamic equilibrium between CSCs and non-CSCs by specific secreted cytokines. Interleukin (IL)-6 secretion by CSCs facilitates the conversion of non-CSCs to CSCs. CSCs show self-renewal, continuously generating non-CSCs through differentiation and converting some non-CSCs back into CSCs by secreting IL-6 within the tumor [110]. Further studies are needed to confirm whether a similar mechanism exists in GI CSCs.

The investigation of extrinsic cues that modulate CSC behavior is very important. A recent study has shown that hypoxia is an important factor affecting niche biology. A specific pathway associated with stem cell transcription factor is activated by hypoxia. The availability of oxygen has a direct role in the regulation of ESC and adult neural stem cells through hypoxia-inducible factor (HIF)-1α-mediated Wnt/β-catenin signaling, in which HIF-1α enhances β-catenin activation and expression of the downstream effectors LEF-1 and TCF-1 [111].

Similar to ESCs, CSCs have also been reported to take advantage of hypoxia. Colorectal cell line-derived CSCs maintain their stem cell-like phenotype and prevent the differentiation of enterocytes and goblet cells under hypoxic conditions, and this effect is mediated by HIF1α [112]. In glioma, the CSC population retained the undifferentiated phenotype, showing enhanced self-renewal and proliferation in hypoxia. The response of CSCs to hypoxia involves

Table 2 MicroRNA signatures in cancer stem cells

CSC enrichment	Organ	MicroRNA	Expression in CSCs	Target gene	Function when overexpressed	References
$EpCAM^+$	Liver	miR-181s	Upregulated	CDX2, GATA6, NLK	Enrichment of CSC ($EpCAM^+$) population	[99]
Mammosphere	Breast	miR-181a/b	Upregulated	ATM	Induce sphere formation	[100]
$CD133^+$	Liver	miR-130b	Upregulated	TP53INP1	Increased ability to proliferate, self-renew, form tumors, and resist standard chemotherapy	[102]
$PROCR^+/ESA^+$	Breast	miR-495	Upregulated	E-cadherin REDD1	Promotes colony formation, tumorigenicity, and hypoxia resistance	[121]
$CD44^+$	Prostate	miR-34a	Downregulated	CD44,	Inhibits tumor development,	[103]
CD44high/CD24low	Breast	miR-200-b	Downregulated	Suz12	Inhibits CSC growth and formation	[104]
$CD44^+$	Liver	miR 199a-3p	Downregulated	CD44	Reduced in vitro invasion and sensitized the cells to doxorubicin	[122]
Chemotherapy-resistant mammosphere	Breast	Let7 family	Downregulated	HMGA2, HRAS	Inhibition of tumor formation, weakened self-renewal	[123]
$CD44^+CD24^{-/low}lin^-$	Breast	miR-200c	Downregulated	Bmi1	Inhibits clonal expansion, suppresses tumor formation	[124]
Chemotherapy-resistant mammosphere	Breast	miR-30 family	Downregulated	Ubc9, ITGB3	Inhibition of self-renewal, tumorigenesis, metastasis	[125]

CSC cancer stem cell

the activation of HIF-1 to enhance the self-renewal activity of $CD133^+$ cells and to inhibit CSC differentiation [113]. Moreover, hypoxia also induces a phenotypic shift from non-stem cell-like to stem-like phenotype in glioma [114].

Taken together, these studies indicate that CSCs that carry different genetic or epigenetic alterations may respond differently to the same cues in the cancer niche. Identification of factors in the tumor microenvironment that potentially regulate CSCs, as well as elucidation of the mechanism by which CSCs modify the tumor microenvironment and regulate their neighbor cells, are very critical areas of investigation, and these areas remain an open field for further study.

Therapeutic strategy against CSCs

CSCs are thought to be responsible for therapeutic failures in human tumors, and targeting this subpopulation is becoming an important concern. Understanding and modulating the signaling pathway that regulates CSC behavior might be a valuable therapeutic approach. CSCs might share molecular networks with normal stem cells, and therefore attention should be paid when targeting CSCs through stem cell regulatory pathways, as the resulting abrogation may not only disturb the CSC population but may also jeopardize the normal stem cell compartment. Hence, investigation of specific CSC regulatory pathways is necessary to overcome this challenge. Such a strategy could also be implemented with special attention focused on microenvironmental factors that promote the growth of CSCs.

Small compounds discovered through chemical screening have been found to successfully eradicate the CSC population in specific malignancies. In breast cancer, salinomycin, a potassium ionophore, has been found to reduce the proportion of CSCs by more than 100-fold relative to paclitaxel, to inhibit mammary tumor growth in vivo, and to induce epithelial differentiation [115]. The potent effect of salinomycin has also been observed for the eradication of colon cancer CSCs, as evidenced by the finding that it selectively targeted $CD133^+$ cell subpopulations and decreased their invasion and migration by promoting MET [116].

The complete elimination of the tumorigenic capacity of CSCs requires the combination of a specific stem cell pathway inhibitor and standard chemotherapy. The combined inhibition of the SHh pathway by cyclopamine and the mTOR pathway by rapamycin did not significantly reduce tumor progression. However, the addition of gemcitabine led to a complete reduction of tumorigenicity, suggesting that combination therapy might have an effect on the loss of cancer stemness characteristics, rendering the cells more susceptible to the cytotoxic effect of gemcitabine [117].

Immunological therapy has become another breakthrough in the search for specific targeted therapies against CSCs. In colorectal cancer, the eradication of $CD133^+$ cells with anti-IL4 antibody resulted in apoptosis through the reduction of anti-apoptotic protein. Combinations of anti-IL4 antibody with oxaliplatin or 5-fluorouracil have been shown to enhance the efficacy of chemotherapy [26].

Blocking the interaction between CSCs and their niche could provide another therapeutic strategy against these cells. The finding that CSCs are regulated by Wnt signaling activation, which is modulated by the microenvironment, suggests a promising strategy for targeting CSCs by blocking the interactions of these cells with the tumor microenvironment. Such a strategy might be achieved through the inhibition of c-Met, a Wnt signaling co-stimulator, resulting in inhibition of the Wnt pathway, thereby compromising CSC self-renewal activity [44]. Moreover, lessons learned from brain tumor research indicate that disruption of the perivascular niche compromises the self-renewal activity of CSCs; thus, the use of antiangiogenic drugs provides a therapeutic benefit in eradicating CSCs [109].

The selective eradication of CSCs has also been achieved in a study of AML leukemia stem cells (LSCs), where the CD44 inhibitor H90, which mediates the ligation of CD44 LSCs in vivo, blocked the interaction between LSCs and supportive microenvironments, and also altered the stem cell fate. The application of this finding to eradicate CSCs in solid tumors through interruption of their interaction with the niche should reward further investigation [118].

Concluding remarks

The CSC theory has significant implications in cancer research. It is a breakthrough not only in our understanding of the behavior of malignancies but also in our ability to develop new therapeutic approaches. The identification of reliable markers that specifically mark the CSC populations in a particular malignancy and reflect their phenotypic characteristics, which can be used in the clinical setting and could have the ability to predict patient outcomes, will be challenging. Although several CSC-targeted therapies have demonstrated considerable promise in eradicating CSC populations by various methods, confirmation of their efficacy in clinical settings is yet to be established. Moreover, elucidating the regulation of the CSC molecular network through various epigenetic mechanisms, which is now in progress, will certainly become the future trend of research and provide a valuable contribution in the investigation of CSCs.

Conflict of interest The authors declare that they have no conflict of interest.

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