Transcriptomic study of dormant gastrointestinal cancer stem cells

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Abstract. We previously discovered the coexistence of dormant and proliferating cancer stem cells (CSCs) in gastrointestinal cancer, which leads to chemoradiation resistance. $CD13^-/CD90^+$ proliferating liver CSCs are sensitive to chemotherapy, and $CD13^+/CD90^-$ dormant CSCs have a limited proliferation ability, survive in hypoxic areas with reduced oxidative stress, and relapse and metastasize to other organs. In such $CD13^+$ dormant cells, non-homologous end-joining, an error-prone repair mechanism, is dominant after DNA damage, whereas high-fidelity homologous recombination is apparent in $CD13^-$ proliferating cells, suggesting the significance of dormancy as an essential protective mechanism of therapy resistance. However, this mechanism may also play a role in the generation and accumulation of heterogeneity during cancer progression, although the exact mechanism remains to be understood. Through transcriptomic study, we elucidated the underlying epigenetic mechanism for malignant behavior of dormant CSCs, i.e., simultaneous activation of several pathways including EZH2- and TP53-related proteins in response to microRNA101, suggesting that a pharmacogenomic approach would open an era to novel molecular targeting cancer therapy.

Introduction

Recent studies have revealed that cancer stem cells (CSCs) are a source of therapy resistance, disease recurrence, and metastasis to other organs (1-3). At least two types of CSCs, dormant (dCSC) and activated (aCSC), are involved in tumor homeostasis, which are in contrast to two types of stem cells, dormant and activated types in normal skin, intestine and the hematopoietic system (4). Our previous study indicated that $CD13^+CD90^-$ dCSCs of hepatocellular carcinoma survive in hypoxic areas in marginal regions in liver after therapy (5). In $CD13^+/CD90^-$ dCSCs, the occurrence of double-strand breaks (DSBs) in genomic DNA, a deleterious cellular event, and damage-induced repairs that are necessary for cellular survival (6), reduce after therapy presumably due to CD13/aminopeptidase N functioning as a scavenger of reactive oxygen species (ROS) (5) and partially due to error-prone repair such as non-homologous end-joining (NHEJ) (6,7). In a sharp contrast, $CD13^-/CD90^{+/-}$ dCSCs are sensitive to therapeutic insults from chemotherapeutic agents, which is associated with ROS-induced cell death after chemotherapy (8); however, damage is typically repaired though high-fidelity, error-free homologous recombination (HR) (6,7). Thus, dCSCs may be a cause of accumulation of deleterious mutations and should be targeted in therapy in terms of complete eradication of malignant cells, although hibernation therapy (the induction of dormancy) may be a viable option dependent on the patient's condition (7). Chemotherapy results in a shift from aCSCs to dCSCs and accumulation of dCSCs after treatment, and dormancy may function as a type of refuge for the survival of malignant cells. CD13 cells play a role in the inhibition of increase in ROS and the resultant suppression of cell death during the process of epithelial mesenchymal transition (EMT) of metastatic CSCs (9). The exposure to a CSC-specific inhibitor, ubenimex, resulted in considerable eradication of malignant cells *in vivo*, indicating an apparent benefit in the combination of conventional chemotherapy and a CSC-specific inhibitor.

Here, we performed transcriptome analysis for coding mRNAs and non-coding microRNAs (miRs) in $CD13^+/CD90^-$ dCSCs. This study allowed us to identify several pathways, which play a role in fundamental mechanisms in above-mentioned potentially malignant phenotype, and provided further clues for identification of molecular targets in therapeutic approaches for dCSCs.

Materials and methods

Cell cultures. Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan)

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Key words: transcriptome, cancer stem cells, dormancy, chemotherapy

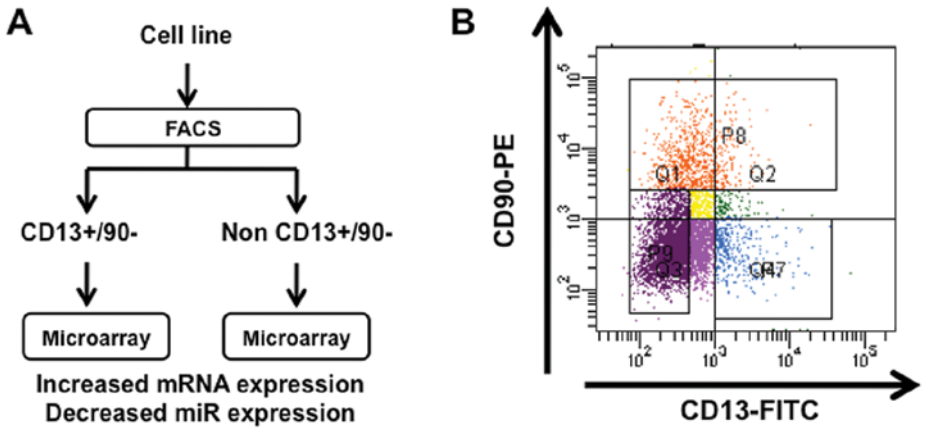

Figure 1. Isolation and analysis of liver dCSCs. (A) Cells were separated by FACS into dCSCs ($CD13^+/CD90^-$) and non-dCSCs ($CD13^+/CD90^+$, $CD13^-/CD90^+$, and $CD13^-/CD90^-$). Total-RNA was extracted and subjected to array screening for mRNA and miR. Here we focused on increased mRNA expression and decreased miR expression. (B) Representative data of FACS separation and sorting.

supplemented with 10% fetal bovine serum (FBS) at 37˚C in a 5% humidified CO_2 atmosphere.

Flow cytometric analysis and cell sorting. The antibodies used were purchased from BD Biosciences (Tokyo, Japan). In brief, cells were harvested with trypsin and EDTA. Doublet cells were eliminated using FSC-A/FSC-H and SSC-A/SSC-H. Dead and dying cells were eliminated with 7-AAD (BD Pharmingen, San Jose, CA, USA). Isotype controls (BD Biosciences) were used. FcR blocking was performed using an FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany).

RNA. Total-RNA was extracted using TRIzol reagent (Invitrogen/Life Technologies Japan, Tokyo, Japan). Reverse transcription was performed with SuperScript III reverse transcription kit (Invitrogen). qPCR was performed using the LightCycler TaqMan Master Kit (Roche Diagnostics, Tokyo, Japan) for cDNA amplification of target-specific genes. Purified cDNA from mouse ES cells was used as a positive control for target genes. The expression of mRNA copies was normalized to GAPDH (for mRNA) or RNU48 (for miR) expression, as indicated. The RNA samples were analyzed using SurePrint G3 Human GE 8x60K Microarray and the Human miRNA Microarray 8x15K Rel.12.0 (Takara, Kyoto, Japan).

Statistical analysis. For continuous variables, results are expressed as means ± SE. The relationship between the gene expression level and cell count was analyzed by chi-square and Wilcoxon rank tests. All data were analyzed using JMP software (SAS Institute, Cary, NC, USA). P-values of <0.05 were considered statistically significant.

Results and Discussion

A study of $CD13^+/CD90^-$ as dCSCs. $CD13^+$/aminopeptidase N is expressed in liver CSCs (5), where it is involved in the reduction of ROS through the glutathione reductase pathway. Considering that another independent study has shown $CD90^+$ as a candidate stem marker, critical to tumorigenicity in mice *in vivo* and clinical outcomes of patients (10), we indicated that $CD13^+/CD90^-$ cells exist in dormant phase of cell cycle, whereas $CD13^+/CD90^+$ cells are predominantly in the S phase and $CD13^-/CD90^+$ cells are in the G2/M phase (5). In previous studies, we have elucidated that following exposure to genotoxic insults, such as chemotherapy or radiation therapy, $CD13^-$ cells shift to the $CD13^+$ fraction in dormant phase of cell cycle. In dCSCs, double stranded breaks (DSBs) are repaired predominantly through the error-prone NHEJ mechanism (6,7). In sharp contrast, the high-fidelity HR-type repair proteins are increased in non-dormant CSCs compared with NHEJ proteins, of which cells are usually sensitized through chemoradiation therapy (6,7). Thus, after chemoradiation therapy, NHEJ supposedly contributes to the generation of misrepair after DSBs, which may cause chromosomal deletions, insertions, or translocations, and subsequent genomic instability (11). Such genomic alterations lead to the inactivation of tumor suppressor genes and activation of oncogenes, which become more apparent during tumor development of primary lesions, recurrence and metastasis (12). Nevertheless, there remains an important issue to be addressed, i.e., the identification of molecular mechanisms fundamental for initiation and development of tumor tissues composed of stem cell hierarchy, and moreover, the type of mechanism involved in CSC-based heterogeneous tumors. We began with transcriptome assessment, i.e., the expression of mRNAs and miRs and their association with $CD13^+/CD90^-$ cells in supporting or maintaining CSC survival in the absence of genotoxic stimuli, which may be beneficial in the study of the basal situation and may help understand the differences between therapy-resistant clones and *de novo* tumor-initiating cells.

As shown in Fig. 1, we separated liver cancer cells by FACS sorting into $CD13^+CD90^-$ dCSCs from other non-CSCs. Considering that miRs play a role in the regulation of mRNA in its stability and translation as an inhibitory regulation system, we focused on increased expression of mRNA clones and decreased expression of miR clones. The data of high-density array screening indicated 17 clones of increased expression in $CD13^+CD90^-$ populations compared with unsorted cells with more than 2-fold significant increase. The data were almost consistent in $CD13^+/CD90^-$ populations

Table I. mRNAs expressed highly in $CD13^+CD90^-$ PLC cells.

mRNA	$CD13^+CD90^-$/ unsorted	$CD13^+CD90^-$/ non-$CD13^+CD90^-$
Cadherin 6, type 2, K-cadherin (CDH6)	3.33	3.21
Interleukin 8 (IL8)	3.27	1.50
Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	3.26	2.61
Endothelin 1 (EDN1)	3.20	1.16
Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1)	3.16	2.06
Tumor necrosis factor, α-induced protein 6 (TNFAIP6)	3.13	1.83
Chemokine (C-X-C motif) ligand 1 (CXCL1)	3.08	1.25
Vascular cell adhesion molecule 1 (VCAM1)	2.94	2.49
L1 cell adhesion molecule (L1CAM)	2.72	2.40
Wingless-type MMTV integration site family member 2 (WNT2)	2.66	1.10
Carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3)	2.65	1.99
Chemokine (C-X-C motif) ligand 3 (CXCL3)	2.29	1.79
Chemokine (C-C motif) ligand 20 (CCL20)	2.24	2.44
Chemokine (C-X-C motif) ligand 12 (CXCL12)	2.11	1.53
Tumor necrosis factor (TNF superfamily, member 2) (TNF)	2.07	1.59
Chemokine (C-X-C motif) ligand 5 (CXCL5)	2.04	1.52
Wingless-type MMTV integration site family, member 7B (WNT7B)	2.02	1.16

The relative expressions in $CD13^+CD90^-$ PLC cells are shown as the ratio to unsorted, or to excluded populations (non $CD13^+CD90^-$).

Table II. miRs expressed highly in $CD13^+CD90^-$ PLC cells.

miR	$CD13^+CD90^-$/ unsorted	Non $CD13^+CD90^-$/ unsorted	Function
hsa-miR-374a	-8.24	0.19	Downregulated upon cisplatin exposure
hsa-miR-489	-7.17	0.41	miR-489 inhibited cell growth in all head and neck cancer cell lines
hsa-miR-223	-6.69	0.16	Reduced miR-223 expression in primary MEF leads to increased Fbw7 expression and decreased cyclin-E activity
hsa-miR-101	-6.68	-0.21	miR-101 could sensitize hepatoma cell lines to both serum starvation- and chemotherapeutic drug-induced apoptosis. Genomic loss of miR 101 leads to overexpression of histone methyltransferase EZH2 in cancer
hsa-miR-9	-6.29	0.70	Directly repress Lin28
hsa-miR-378	-6.21	-0.06	Novel target of the c-Myc oncoprotein that is able to cooperate with activated Ras or HER2 to promote cellular transformation
hsa-miR-182	-6.14	-0.68	Antagonizing miR-182 enhances BRCA1 protein levels and protects them from IR-induced cell death
hsa-miR-421	-5.69	0.20	Overexpression of miR-421 in pancreatic cancer cells promoted cell proliferation and colony formation
hsa-miR-125a-3p	-4.99	0.01	Hypoxia regulated microRNA

The relative expressions in $CD13^+CD90^-$ PLC cells are shown as the ratio to unsorted, or to excluded populations (non $CD13^+CD90^-$).

compared with non-$CD13^+/CD90^-$ cells (Table I). Next, we analyzed miR expression and successfully isolated nine miRs in $CD13^+/CD90^-$ population compared with unsorted cells with more than 4-fold significant decrease. The data were almost consistent in $CD13^+/CD90^-$ populations compared with non-$CD13^+/CD90^-$ cells (Table II).

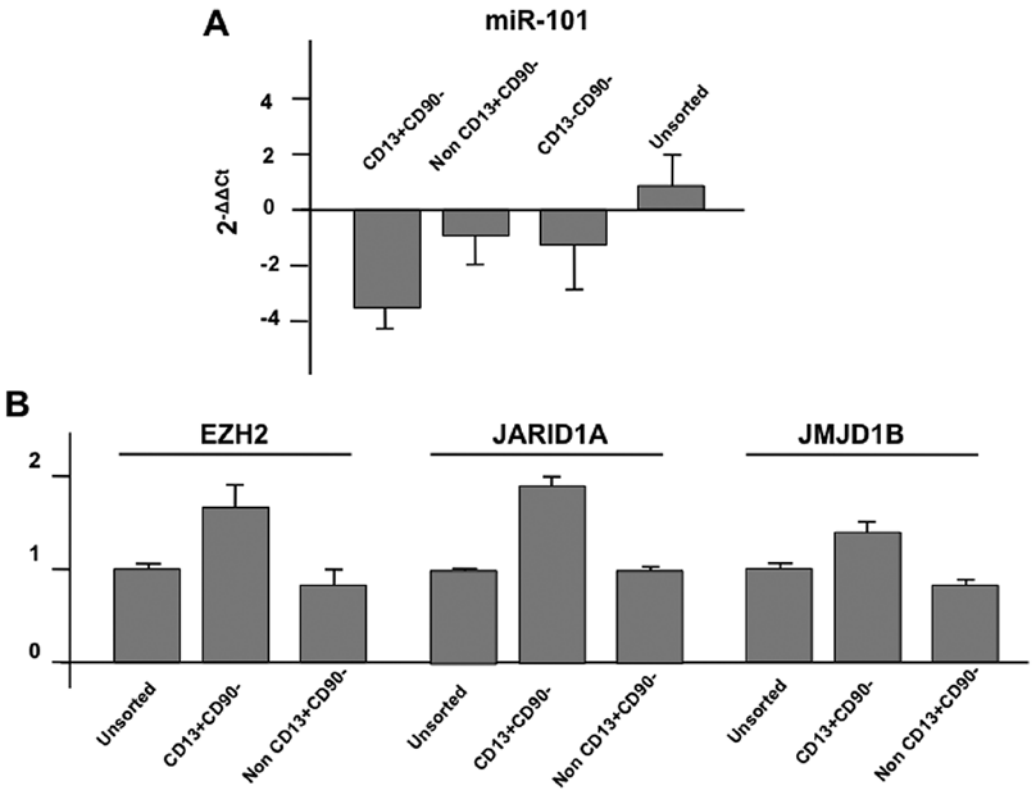

Figure 2. Quantitative analysis of isolated mRNA and miR transcripts by RT-PCR. RNAs from cells were extracted and subjected to qRT-PCR. Data of (A) miR study and (B) mRNA study are shown.

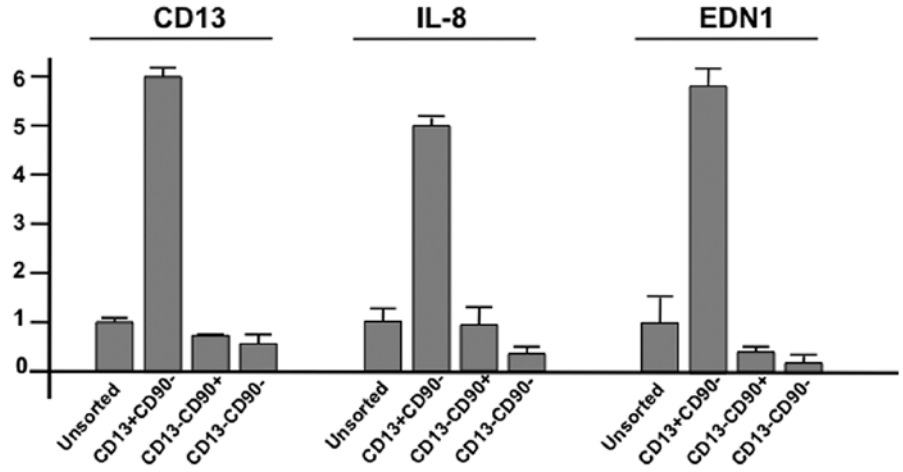

Figure 3. Quantitative analysis of isolated mRNA and miR transcripts by RT-PCR. RNAs from cells were extracted and subjected to qRT-PCR. Data of mRNA study are shown.

Identification of regulatory networks. By assessment of pairs of miRs and its putative target mRNAs using prediction software (http://www.targetscan.org/; http://www.microrna.org/microrna/home.do), we confirmed the data of the array by quantitative PCR. As shown in the representative data, the expression of miR-101 was downregulated in $CD13^+/CD90^-$ cells compared with non-$CD13^+/CD90^-$ cells or $CD13^-/CD90^-$ cells; in sharp contrast, the expression of putative targets, EZH2 (enhancer of zeste homolog 2; http://www.genecards.org/cgi-bin/carddisp.pl?gene=EZH2&search = EZH2), JARID1A; (http://www.genecards.org/cgi-bin/carddisp.pl?gene =KDM5A&search=JARID1A), and JMJD1B (http://www.genecards.org/cgi-bin/carddisp.pl?gene=KDM3B&search=JMJD1B) were increased (Fig. 2; summarized in Fig. 3). CD13 mRNA expression was increased in $CD13^+/CD90^-$ cells, but not in $CD13^-/CD90^+$, $CD13^-/CD90^-$, or unsorted cells. The expression of interleukin-8 (IL-8; http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL8&search=Interleukin+8) and endothelin 1 (EDN1; http://www.genecards.org/cgi-bin/carddisp.pl?gene=EDN1&search=Endothelin+1) was increased in

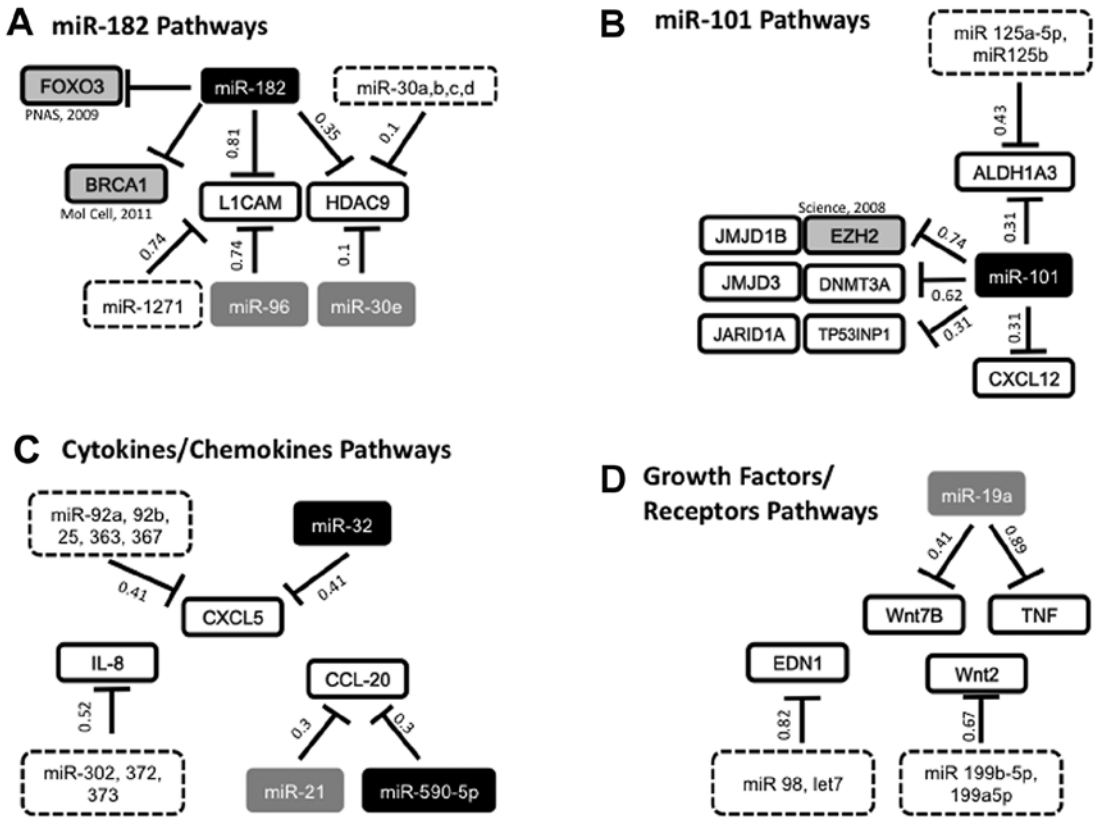

Figure 4. Summary of identified networks. The present study identified several networks, i.e., (A) miR-182 pathways, (B) miR-101 pathways, (C) cytokine/chemokine pathways and (D) growth factors and their receptor pathways. Closed characters in black (mir-101, mir-182, miR-32 and miR-590-5p) indicate identified and verified molecules in this study, whereas closed characters in gray (miR-96, miR-30e, miR21, miR019a) show molecules of which significance was already suggested in previous studies (3,25). Closed characters by dashed lines indicate molecules that were not verified in this study as linked to $CD13^+/CD90^-$ dormancy. As target mRNAs, closed characters in gray (FOXO3, BRCA1 and EZH2, and miR-182) indicate identified and verified molecules in this study and already reported as significant in previous publications, whereas open characters in bold (LICAM, HDAC9, JMJD1B, JMJD3, DNMT3A, JARID1A, TP32INP1, ALDH1A3, CXCL12, CXCL5, IL-8, CCL-20, Wnt7B, TNF, EDN1 and Wnt2) denote novel unpublished molecules identified and verified in this study.

$CD13^+/CD90^-$ cells, but not in others (Fig. 3; summarized in Fig. 3). Through this study, we were able to find at least three core regulatory networks for the maintenance of dormant $CD13^+/CD90^-$ cells, but not in others, i.e., miR-182 pathways, miR-101 pathways, cytokines/chemokines pathways (IL-8, CXCL5 [http://www.genecards.org/cgi-bin/carddisp.pl?gene=CXCL5&search=CXCL5], CCL-20 [http://www.genecards.org/cgi-bin/carddisp.pl?gene=CCL20& search=CCL-20]), growth factors and their receptor pathways [EDN1, Wnt2 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=WNT2&search=Wnt2), Wnt7B (http://www.genecards.org/cgi-bin/carddisp.pl?gene=WNT7B&search = Wnt7B), and TNF (http://www.genecards.org/cgi-bin/carddisp.pl?gene =TNF&search=TNF)]. As noted in this study, we did not detect any apparent involvement in DNA damage response machineries, except for an association underlined by TP53INP1 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=TP53INP1&search =TP53INP1) and BRCA1 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=BRCA1& search = BRCA1), suggesting that (1) the damage response in dCSCs is characteristic after exposure to genotoxic stimuli, whereas in the absence of damage insults, they are not apparent and (2) the DNA damage response was regulated mainly by the modification of proteins such as phosphorylation or ubiquitination, and the expression array technology was less sensitive to pathway detection and other networks may have been missed.

Significance of regulatory networks (Fig. 4). The EZH2 gene encodes a member of the Polycomb group (PcG) family, which forms multimeric protein complexes involved in maintaining a transcriptionally repressive state of genes over successive cellular generations (13). Reportedly, the genomic loss of miR-101, a putative tumor suppressor, leads to overexpression of histone methyltransferase EZH2 in cancer (14,15), hypoxia, and androgen-dependent conditions (16) as well as in gastric cancer (17), pancreatic cancer (18), lung cancer (19) glioblastoma (20) and invasive squamous cell carcinoma (21). Thus, PcG proteins are critical epigenetic mediators of stem cell pluripotency and CSC functions, which may be implicated in human cancer pathogenesis, probably indicating candidacy for novel pharmacological targets of cancer therapy. Recently, it was reported that the administration of diflourinated-curcumin (CDF), a novel analogue of the turmeric spice component curcumin, has antioxidant properties and inhibits tumor growth through reduced expression of EZH2, Notch-1, CD44, EpCAM, and Nanog and increased expression of let-7, miR-26a, and miR-101 (18). These findings indicated that CDF inhibited CSC growth by targeting an EZH2-miRNA regulatory circuit for epigenetically controlled gene expression. In the present study,

we identified various miR-101 targets, including JARID1A, JMJD1B, TP53INP1, and EZH2, suggesting that these target molecules act together to maintain CSC dormancy, and proposed the possible significance of the miR-101 pathway. We also identified the miR-182 pathway, in which miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to poly-ADP ribose polymerase (PARP) inhibitors (22). The overexpression of miR-182 was reported in high-grade ovarian papillary serous carcinoma (23). Reportedly, aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 (24). Taken together with our study, the miR-182 pathway may be involved in the maintenance of CSC function in a similar manner. As summarized in Fig. 4, we identified other cytokines, chemokines, growth factors, receptors and pathways. These findings may facilitate further studies on the regulatory mechanisms of the dormant phase of gastrointestinal CSCs.

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