Some pluripotency-related genes, including *Nanog*, *Oct3/4*, and *Lin28a*, were upregulated in the Dox-withdrawn kidney tumor cells as compared to normal kidney tissue, although the expression levels of both *Nanog* and endogenous *Oct3/4* were significantly lower than that of pluripotent stem cells (Figures 3C and S3C). Conversely, other pluripotency-related genes, such as *Esrrb*, were not upregulated in these tumors (Figures 3C).

To further characterize Dox-withdrawn tumor cells, we sought to identify tumor-cell-specific markers. We found that *Lgr5* is specifically upregulated in Dox-withdrawn kidney tumor cells, but not in adult kidney tissues or pluripotent stem cells (Figure 3D). Increased expression of *Lgr5* was similarly observed in the transplanted secondary tumors (Figure S3D). Therefore, we established iPSC lines from OSKM-inducible MEFs containing *Lgr5-EGFP* reporter allele in which *Lgr5* expression can be visualized by enhanced green fluorescent protein (EGFP) (Barker et al., 2007). The established *Lgr5*-reporter iPSCs do not express EGFP in ESC culture conditions (Figure S3E). OSKM-inducible *Lgr5* reporter chimeric mice at 4 weeks of age were treated with the 7+/7− Dox regimen. Again, these mice developed Dox-withdrawn kidney tumors consisting of dysplastic cells (Figure 3E). The scattered EGFP signals were observed in kidney tumors (Figure 3E), and immunohistochemical analysis revealed that *Lgr5* is specifically expressed in part of Dox-withdrawn kidney tumor cells (Figures 3E and S3E). These findings indicate that Dox-withdrawn kidney tumors contain *Lgr5*-positive cells and that the *Lgr5* reporter allele is available to specifically identify the Dox-withdrawn kidney tumor cells that are distinct from fully reprogrammed pluripotent stem cells. Of note, some of the *Lgr5*-expressing tumor cells also expressed Oct3/4 and Lin28b in immunohistochemical analysis (Figures 3F and S3F), thus suggesting that *Lgr5*-expressing tumor cells share some characteristics with pluripotent stem cells. The fact that Dox treatment for longer than 8 days followed by Dox withdrawal often results in teratoma formation supports the notion that partial reprogramming toward pluripotent stem cells is involved in the development of Dox-withdrawn tumors (data not shown). Altogether, our findings indicate that *Lgr5*-expressing tumor cells are distinct from pluripotent stem cells but contain partially reprogrammed cells.

Failed Repression of ESC-Polycomb Targets in Dox-Withdrawn Tumors

In contrast to ESC-like activation observed for both the ESC-Core and ESC-Myc modules, the ESC Polycomb repressive complex (PRC) module was differentially expressed between Dox-withdrawn tumors and ESCs (Figure 4A). We found that a number of ESC-PRC targeted genes are not repressed in both kidney tumors and transplanted secondary tumors (Figures 4A, S4A, and S4B), indicating that the failed repression of ESC-PRC targets is associated with the development of Dox-withdrawn tumors. Consistent with the notion, more than one-forth of the upregulated genes in tumor cells as compared to ESCs (greater than 3-fold upregulation) were targets of PRC in ESCs (Mikkelsen et al., 2007) (Table S1). We also found that Dox-withdrawn kidney tumors express kidney-precursor-expressing genes such as *Six2*, *Eya1*, and *Lgr5* (Barker et al., 2012; Kobayashi et al., 2008) (Figures 4B and S4C). In particular, *Six2* and *Lgr5*, which are also PRC targets in ESCs (Mikkelsen et al., 2007), are specifically upregulated in both Dox-withdrawn kidney tumors and secondary tumors when compared to both normal kidney tissues and pluripotent stem cells (Figures 3D, 4B, S3D, and S4D). Chromatin immunoprecipitation (ChIP)-qPCR experiments confirmed decreased H3K27me3 levels at both *Six2* and *Lgr5* promoter regions in Dox-withdrawn tumors when compared with those in normal kidney tissues (Figure S4E). Failed repression of the ESC-PRC module was also detectable in unsuccessfully reprogrammed kidney cells in vitro, which were established by the transient expression of reprogramming factors in isolated kidney tubule cells in vitro (Figure S4F).

We next examined the kinetics of transcriptional changes during the development of the Dox-withdrawn tumors. Immunohistochemical analysis revealed that early dysplastic cells at day 7 coincide with transgene-expressing cells (Figure S4G). Taking advantage of florescence-linked transgene expression in our mice, we fluorescence-activated cell sorted mCherry-positive kidney cells in OSKM mice given Dox for 7 days (D7), isolating early dysplastic cells for gene expression analysis. Fluorescence-activated cell-sorted D7 LacZ-mCherry-expressing kidney cells were used as a control (Figure S4H). Decreased expression of proximal tubule cell markers was observed in the D7 OSKM cells as compared to D7 LacZ cells, suggesting that the loss of kidney cell identity occurs in early dysplastic cells (Figure S4I). In contrast, increased expression of ectopic stem/progenitor cell markers was not evident in D7 OSKM cells (Figure S4I). These findings suggest that remodeling of global transcriptional profiles toward a stem/progenitor-like state is specifically associated with transgene-independent, late dysplastic cells.

To investigate cell-of-origin effects on failed reprogramming, we next performed a microarray analysis for Dox-withdrawn liver tumors and compared the data with that of kidney tumors. As observed in kidney tumors, the liver tumors displayed failed repression of the ESC-PRC module, accompanied by activation of both the ESC-Core and Myc modules (Figure S4J; Table S1). Although derepressed PRC module genes in kidney tumors and liver tumors often overlapped (Figure S5A; Table S1), we found differentially derepressed PRC genes between kidney and liver tumors. Notably, such differentially derepressed PRC genes were associated with kidney and liver development, respectively. These findings suggest that failed PRC repression in Dox-withdrawn tumors may be associated with the activation of a developmental transcription

(F) Dox-withdrawn tumors express pluripotency-related proteins. Double immunofluorescence for Lin28b and GFP (Lgr5) revealed that the GFP-positive tumor cells also expressed Lin28b. Double immunofluorescence for Oct3/4 and GFP (Lgr5) showed that a subset of GFP-positive tumor cells expressed Oct3/4 in the nucleus. Scale bars, 20 μm.
See also Figure S3.

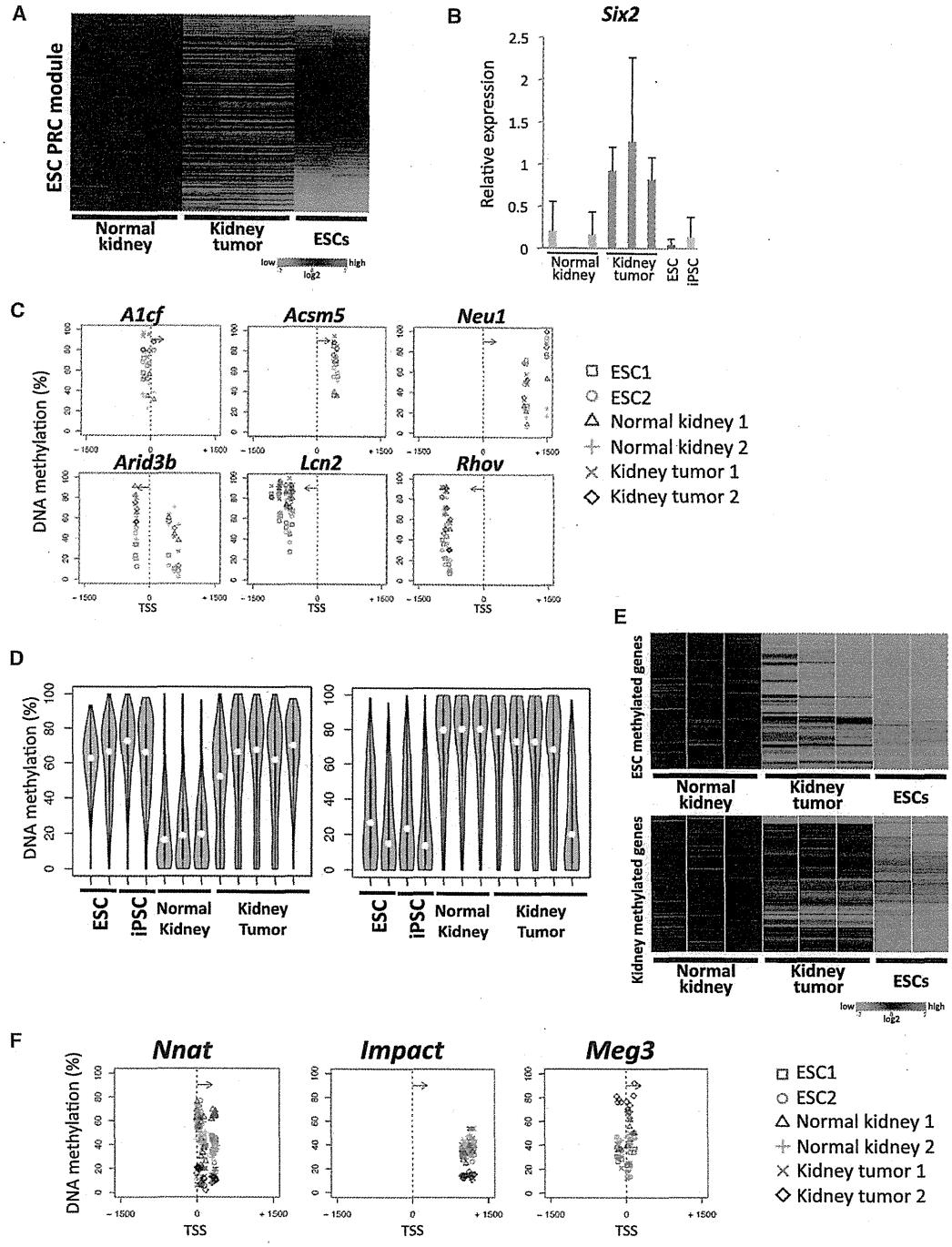

Figure 4. Altered Epigenetic Regulation in Dox-Withdrawn Tumors

(A) The microarray analyses revealed that ESC-PRC target genes were often activated in Dox-withdrawn kidney tumors compared to normal kidney tissues.

(B) *Six2* was highly expressed only in the Dox-withdrawn kidney tumors. Data are presented as mean ± SD. The mean level of kidney tumors was set to 1.

(C) Altered DNA methylation patterns in Dox-withdrawn tumors and the DNA methylation status of representative genes in the RRBS analyses.

(D) The global analyses for the DNA methylation levels. Genes that were differentially methylated between ESCs and normal kidney samples (more than 30% difference) were extracted and then analyzed for DNA methylation levels in Dox-withdrawn kidney tumors. Kidney tumors gain DNA methylation at ESC-methylated genes, whereas kidney-methylated genes often retain their methylation status in kidney tumors.

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program, which is affected in part by the cell of origin (Figure S5A).

Altered DNA Methylation in Dox-Withdrawn Kidney Tumor Cells

Somatic cell reprogramming is accompanied by global changes in DNA methylation patterns (Mikkelsen et al., 2008). The fact that failed reprogramming can cause tumor development suggests that altered epigenetic modifications play a role in tumorigenesis. To quantitatively profile DNA methylation in Dox-withdrawn tumors, we next performed reduced representation bisulfite sequencing (RRBS) (Meissner et al., 2005). We identified a number of genes with altered DNA methylation levels in Dox-withdrawn tumors as compared to normal kidney tissues. Dox-withdrawn tumors revealed frequent gains of DNA methylation at DNA-methylated genes in ESCs, whereas loss of methylation at DNA-methylated genes in kidney tissues was not evident (Figure 4C). To validate these findings, we next performed a global analysis. We first extracted genes differentially methylated between ESCs and normal kidney samples and then examined their DNA methylation in Dox-withdrawn tumors. The global analysis confirmed that Dox-withdrawn kidney tumors gained de novo methylation at ESC-methylated genes, whereas kidney-methylated genes often retain their methylation in Dox-withdrawn kidney tumors (Figure 4D). Consistent with these findings, ESC-methylated genes were frequently found to be repressed in Dox-withdrawn tumors, whereas kidney-methylated genes tended to remain silent in these tumors (Figure 4E). These results suggest that loss of somatic cell-specific DNA methylation is preceded by a gain of ESC-specific DNA methylation patterns during the reprogramming process.

Adult cancers generally exhibit two distinct patterns of alterations in DNA methylation: site-specific DNA hypermethylation and global DNA hypomethylation (Jones and Baylin, 2002; Yamada et al., 2005). We performed specified regional analyses for the DNA methylation in normal kidney tissues and Dox-withdrawn kidney tumors. DNA hypermethylation at promoter regions in Dox-withdrawn tumors was not detectable, regardless of the presence of CpG islands (Figure S5B). Additionally, decreased DNA methylation levels at intergenic regions were not obvious in Dox-withdrawn tumors (Figure S5B).

We found that Dox-withdrawn kidney tumors aberrantly express a number of imprinted genes and that altered expression levels are similar to those in ESCs (Figure S5C). When DNA methylation status at differentially methylated regions (DMRs) of imprinted genes were examined in Dox-withdrawn tumors using a MassARRAY platform (Ehrich et al., 2005), we found frequent alterations of DNA methylation status at DMRs in Dox-withdrawn tumors (Figure S5D). The aberrant genomic methylation levels at imprinted genes in Dox-withdrawn tumors were also confirmed by RRBS analysis (Figures 4F and S5E). Intriguingly, each Dox-withdrawn tumor revealed variable aberrations in DNA methylation at different imprinted genes. The aberrant methylation includes hypermethylation at the *Meg3* (*Gtl2*) DMR, which has been correlated with impaired differentiation properties of iPSCs (Stadtfeld et al., 2010a) (Figures 4F and S5E). Moreover, SNP analysis in the hybrid KH2 background revealed that the altered expression of some imprinted genes in Dox-withdrawn tumors arise from biallelic transcription, compared to monoallelic expression in the original OSKM-inducible ESCs (Figure S5F). Collectively, these results suggest that genomic imprinting is unstable in Dox-withdrawn tumors and provide additional evidence that altered gene expression underlying tumor development is associated with altered epigenetic signatures.

Dox-Withdrawn Kidney Tumors Resemble Wilms Tumors

Histological analysis revealed that Dox-withdrawn kidney tumors in reprogrammable mice resemble Wilms tumor, the most common pediatric kidney cancer (Figure 5A). A number of studies demonstrated that increased expression of *Igf2* with DNA hypermethylation at the *H19* DMR is one of the causative and most common alterations in Wilms tumors (Ogawa et al., 1993; Steenman et al., 1994). We confirmed that Dox-withdrawn tumors express a significantly higher level of *Igf2* than noninduced tissues (Figures 5B and S6A). Moreover, consistent with altered DNA methylation at other imprinted genes, the increased methylation at the *H19* DMR was detectable in some Dox-withdrawn kidney tumors (Figure 5C).

To additionally evaluate the similarity between Dox-withdrawn kidney tumors and Wilms tumors, we next compared global gene expression patterns. We first selected genes that are upregulated more than 5-fold in Dox-withdrawn kidney tumors in comparison with noninduced kidney tissues and then assessed expression of their human orthologs in human normal kidney tissues, Wilms tumors, and human ESCs (hESCs) using previously reported microarray data sets (Tchieu et al., 2010; Yusenko et al., 2009). We found that upregulated genes in Dox-withdrawn kidney tumors are frequently upregulated in both Wilms tumors and hESCs as compared to normal kidney samples (Figure 5D), whereas this upregulation is not evident in adult kidney cancers (renal cell carcinomas [RCCs]) (Figure S6B).

We also analyzed the expression of genes in ESC-Core, ESC-Myc, and ESC-PRC modules in Wilms tumors. Notably, ESC-upregulated genes in both ESC-Core and ESC-Myc modules are similarly activated in Wilms tumors (Figures 5D and S6C), although *NANOG* and *OCT3/4* are not expressed in Wilms tumors. In contrast, a fraction of ESC-PRC targeted genes expressed in kidney progenitors, such as *SIX2* and *LGR5*, are specifically upregulated in Wilms tumors as compared with

(E) DNA-methylation-associated gene regulation in Dox-withdrawn tumors. The vast majority of ESC-methylated genes were downregulated in Dox-withdrawn tumors, whereas a significant portion of kidney-methylated genes remained repressed in these tumors. ESC-methylated genes with decreased expression levels in ESCs and kidney-methylated genes with decreased expression levels in the kidney tissues were examined.

(F) Altered DNA methylation at the DMR of imprinting genes. Note that kidney tumor 2 showed aberrant methylation patterns at *Nnat*, *Impact*, and *Meg3*. In contrast, kidney tumor 1 showed an aberration only at *Nnat*.

See also Figures S4 and S5.

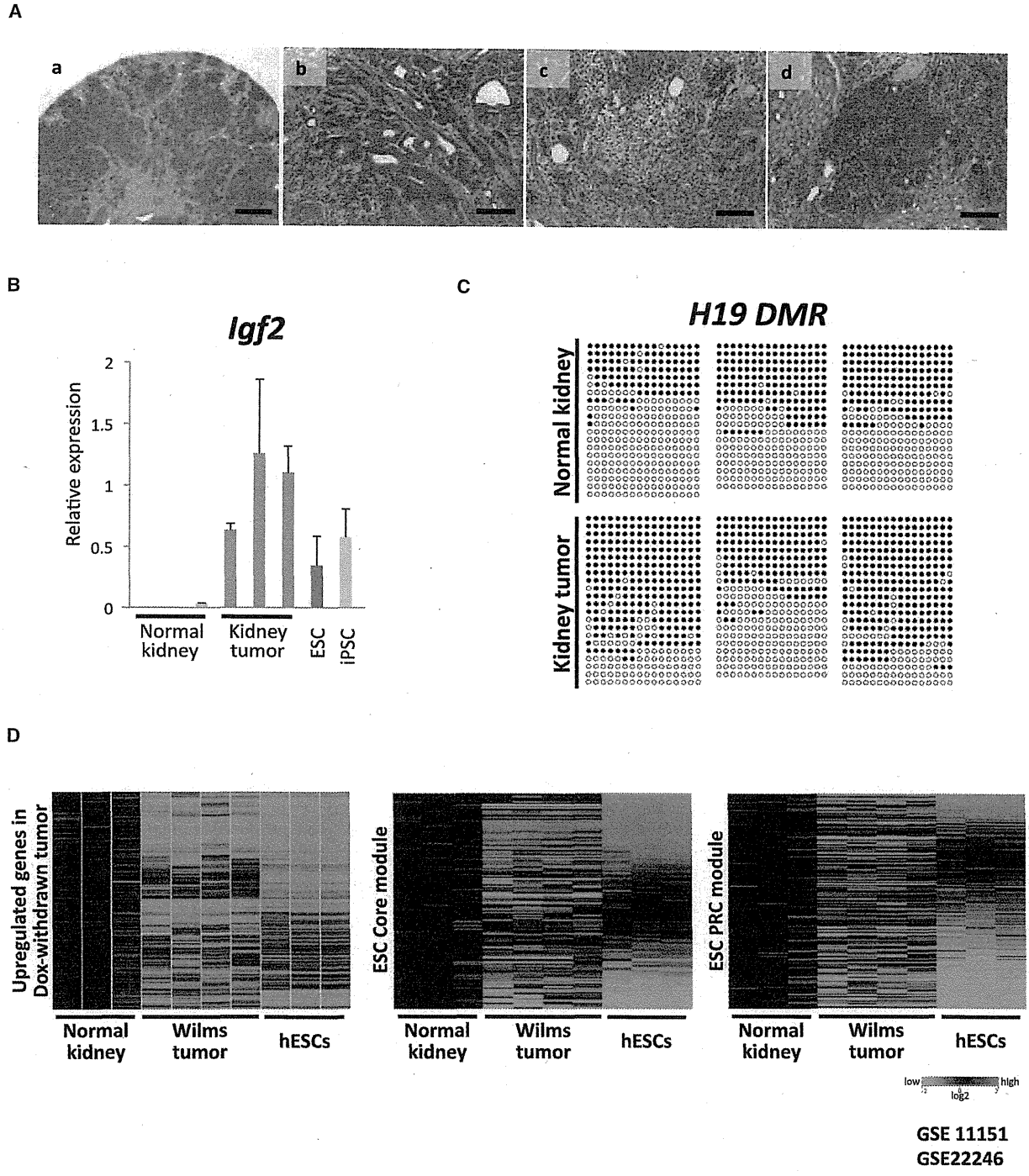

Figure 5. Dox-Withdrawn Kidney Tumors Resemble Wilms Tumors

(A) Representative histological findings of Dox-withdrawn kidney tumors (a–d). Tumors consisted of epithelial (b), stromal (c), and blastema-like (d) compartments, which are histological features of Wilms tumors. Scale bars, 500 μm (a) and 100 μm (b–d).

(B) The results of the qRT-PCR analysis for *Igf2*. *Igf2* was highly expressed in Dox-withdrawn kidney tumors. Data are presented as mean ± SD. The mean level of kidney tumors was set to 1.

(legend continued on next page)

those in normal kidney tissues, hESCs, and RCCs (Figures 5D and S6D) (Aiden et al., 2010). Collectively, kidney tumors induced by the transient expression of reprogramming factors display a number of shared characteristics with Wilms tumor. These findings also indicate that our mouse model may prove useful to uncover the pathogenesis of Wilms tumors.

iPSCs Derived from Dox-Withdrawn Kidney Tumors Contribute to Nonneoplastic Kidney Tissues in Chimeric Mice

We next tried to establish iPSCs from Dox-withdrawn kidney tumor cells. The tumor cell-specific *Lgr5-EGFP* reporter allele defined in this study was utilized to isolate tumor cells (Figures 3D, 3E, and S3E). *Lgr5*-expressing GFP-positive tumor cells were sorted and cultured in vitro with Dox to establish iPSCs from tumor cells (Figure 6A). During the culture of *Lgr5*-expressing tumor cells in vitro, *Nanog* expression at a level comparable to that in pluripotent stem cells was detected as early as 7 days after reprogramming factor induction (Figure 6B), a rate faster than the reprogramming process from normal kidney tubule cells in vitro (Figure S7A). After 2 weeks of culture with Dox exposure, more than 20 alkaline phosphatase (AP)-positive iPSC-like colonies were obtained from 100 *Lgr5*-expressing tumor cells (Figure S7B). We were able to establish Dox-independent iPSC lines from tumor cells at 3 weeks after transgene induction (Figure 6C), suggesting that the Dox-withdrawn tumor cells can be readily reprogrammed into pluripotent stem cells.

Cancers are believed to arise through the accumulation of multiple genetic abnormalities. We next investigated whether genetic abnormalities mandate the emergence of in Dox-withdrawn tumors. Exonic regions of 514 genes that include human-cancer-related genes in transplanted secondary kidney tumors were sequenced using a hybridization selection technique combined with next-generation sequencing (Table S3). Mutations in *Wt1*, *Wtx*, *Ctnnb1*, and *Trp53*, all of which have been identified in a subset of Wilms tumors, were not detected in three tumors examined. In addition, no cancer-related gene mutations were enriched in these tumors (data not shown). Array-based comparative genomic hybridization (CGH) revealed no prevalent chromosomal alteration in tumor samples (Figure S7C).

Finally, we injected the tumor-derived iPSCs into blastocysts to generate chimeric mice. Tumor-derived iPSCs contributed into adult chimeric mice (Figure 6D). Notably, the kidney-tumor-derived iPSCs differentiated into normal-looking kidney tissues (Figures 6E, 6F, and S7D). Moreover, these chimeric mice did not develop tumors even at 24 weeks of age (n = 8). To further demonstrate that tumorigenic cells can be reprogrammed into nonneoplastic cells, we also established iPSCs from the transplanted secondary tumors and confirmed their contribution to nonneoplastic kidney tissues (Figure S7E). These results substantiate that a genetic context of the Dox-withdrawn kidney tumor cells is not determinant of the cancer phenotype and support the conclusion that altered epigenetic regulations cause the abnormal growth in somatic cells, leading to the development of Dox-withdrawn tumors.

DISCUSSION

During somatic cell reprogramming, iPSCs gain the capacity for unlimited growth without particular genetic alterations. Using abbreviated reprogramming factor expression in vivo, we demonstrate that transient expression of reprogramming factors leads to tumor development. Such tumors display altered epigenetic modifications, indicating that epigenetic regulation characteristic of cellular reprogramming may also confer neoplastic growth properties to somatic cells. Intriguingly, Dox-withdrawn tumor cells are readily reprogrammed into pluripotent stem cells by additional 4F expression, indicating that the tumor cells represent a cellular state closer to iPSCs than the original somatic cells. Moreover, kidney tumor cell-derived iPSCs contribute to various somatic cell types and give rise to nonneoplastic kidney cells in mice. These data demonstrate that the abnormal growth of unsuccessfully reprogrammed cells depends predominantly on epigenetic regulations and raise the possibility that particular types of cancer may arise exclusively through altered epigenetic regulation.

Histological features of Dox-withdrawn tumors imply that unsuccessfully reprogrammed cells lack the ability to terminal differentiate along multiple lineages. It is noteworthy that Dox-withdrawn tumor cells fail to repress ESC-PRC targets yet share the activation of ESC core regulatory circuitry and Myc-related genes with pluripotent stem cells. It is conceivable that the repression of ESC-PRC targets would be exclusively associated with the acquisition of pluripotency, whereas activation of ESC core regulatory circuitry and Myc targets lead to self-renewing activity. This notion is also consistent with previous findings that PRC components are important for successful reprogramming in humans (Onder et al., 2012). Notably, the failed repression of the ESC-PRC module was detectable in previously reported partially reprogrammed cells in vitro (Polo et al., 2012), in which the activation of both ESC-Core and ESC-Myc modules had already occurred (Figure S7F). We also found that unsuccessfully reprogrammed kidney cells tend to retain DNA methylation at kidney-specific methylated genes. Considering that global epigenetic reorganization, including changes in both H3K27 methylation and DNA methylation, occurs during the later phase of iPSC generation (Polo et al., 2012), the expected repression of ESC-PRC targets and demethylation of somatic cell-specific genomic methylation might play a role in the final stages of successful somatic cell reprogramming.

Recently, Abad et al. reported that in vivo reprogramming allows the acquisition of totipotent features resulting in embryo-like cyst formation in reprogrammable mice (Abad et al., 2013). However, in the present study, we did not observe such cystic structures in Dox-treated reprogrammable mice.

(C) The bisulfite sequencing analysis revealed increased DNA methylation levels at the *H19* DMR containing two CTCF binding sites in Dox-withdrawn tumors.
(D) The results of the global expression analyses in Wilms tumors. The human orthologs of upregulated genes in Dox-withdrawn tumors and ESC module genes were assessed using previously reported microarray data sets (GSE11151 and GSE22246).
See also Figure S6.

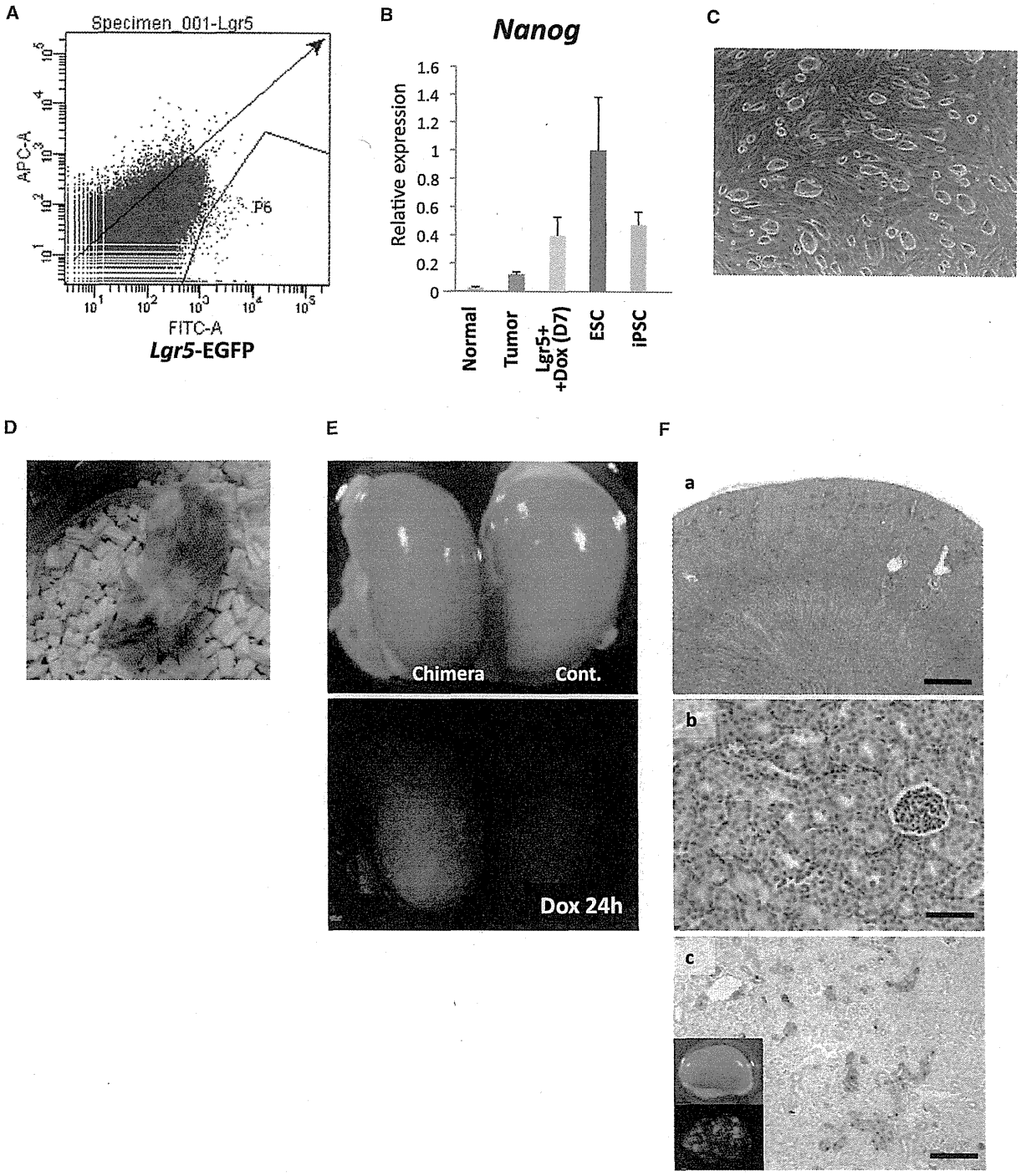

Figure 6. Generation of iPSCs from Dox-Withdrawn Tumors and Their Contribution to Normal-Looking Kidney Tissue

(A) The fluorescence-activated cell sorting analyses of Dox-withdrawn kidney tumor cells in a reprogrammable chimeric mouse with the *Lgr5*-EGFP reporter. GFP-positive *Lgr5*-expressing cells were sorted to exclusively isolate Dox-withdrawn tumor cells.

(B) Dox treatment of *Lgr5*-expressing tumor cells caused the rapid induction of *Nanog*. The *Nanog* levels were examined after seven days of treatment with Dox in vitro. Data are presented as mean ± SD. The level in ESCs was set to 1.

(C) An image of iPSCs derived from *Lgr5*-positive kidney tumor cells.

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Furthermore, teratoma-derived in vivo iPSCs in this study failed to differentiate into placental tissues despite robust fetal contribution upon injection into eight-cell-stage embryos (data not shown), suggesting that not all in vivo iPSCs are totipotent. Because the previous study was conducted using circulating iPSCs recovered in blood, the cell of origin for in vivo reprogramming might affect the acquisition of totipotent features. It should be also noted that Abad et al. utilized germline-transmitted transgenic mice that harbor lentivirus-mediated integration of inducible reprogramming factors (Carey et al., 2009) whereas we examined chimeric mice with transgenes at a targeted locus. The different levels of transgene induction caused by such distinct transgenic systems may underlie differences in the phenotypes observed between these two studies.

Here, we show that failed reprogramming-associated cancers resemble Wilms tumors in terms of histology and molecular characteristics, including aberrant expression of imprinted genes correlated with altered DNA methylation. It is well known that Wilms tumors have characteristics distinct from adult kidney cancers in many aspects. On the basis of our findings in Dox-withdrawn tumors, we discovered that Wilms tumors harbor an activated ESC core regulatory circuitry. This is in sharp contrast to previous findings that most adult cancers do not show activation of ESC core regulatory circuitry (Kim et al., 2010). We also found that many ESC-PRC targets are not repressed in Wilms tumors, despite common repression in many cancers (Ben-Porath et al., 2008; Kim et al., 2010). Gene Ontology analysis revealed that derepressed PRC genes in Wilms tumors include genes involved in kidney development, whereas they are not enriched in derepressed PRC genes in RCCs (data not shown), suggesting that activation of the embryonic kidney transcriptional network is associated with Wilms tumor development. Taken together, strongly active ESC-core regulatory circuitry and derepression of certain ESC-PRC targets may characterize Wilms tumors and may account for the characteristics distinctive of Wilms tumors and adult kidney cancers.

Although we revealed striking similarity between Dox-withdrawn kidney tumors and Wilms tumors, it remains unclear whether reprogramming processes play a role in the development of human Wilms tumors. It has been widely accepted that nephrogenic rests, abnormally persistent clusters of embryonal cells, are the precursors of Wilms tumors. Considering the artificial expression of reprogramming factors in our experimental system, the current study does not provide direct evidence that dedifferentiation is normally involved in the human Wilms tumor development. Yet, based on our findings, it is conceivable that a reprogramming process might cause cell-fate conversion into progenitor-like states, leading to the development of nephrogenic rests required for the early stages of Wilms tumorigenesis. Further detailed analyses using human samples are required to uncover the role of reprogramming in cancer development in humans.

In summary, we demonstrated that premature termination of in vivo reprogramming causes tumor development resembling Wilms tumor. Our findings suggest that altered epigenetic regulations relating to somatic cell reprogramming drive tumorigenesis, highlighting the importance of epigenetic regulation in cancer development.

EXPERIMENTAL PROCEDURES

Generation of OSMK-Inducible ESCs

A 7 kb fragment containing Oct3/4-P2A-Sox2-T2A-Klf4-E2A-c-Myc-ires-mCherry cDNA was generated (Carey et al., 2009) and ligated into the pBS31 vector (Beard et al., 2006). The resulting construct was electroporated into KH2 ESCs to obtain OSKM-inducible ESCs (Beard et al., 2006). OKS-, KMS-, O-, LacZ-inducible ESCs were also generated using the KH2 ESCs system.

Mice

Chimeric mice were generated using reprogramming factor-inducible ESCs by diploid blastocyst injection. *Lgr5-EGFP-ires-CreERT2* mice were obtained from The Jackson Laboratory and were crossed with OSKM-inducible mice to obtain embryos. The compound transgenic MEFs were treated with Dox to establish the OSKM-inducible iPSCs with the *Lgr5-EGFP* reporter allele. All animal experiments were approved by the CiRA Animal Experiment Committee, and the care of the animals was in accordance with institutional guidelines.

Doxycycline Treatment

Mice at 4 or 14 weeks of age were administered 2 mg/ml Dox in their drinking water supplemented with 10 mg/ml sucrose. For cell culture, Dox was used at a concentration of 2 μg/ml.

Secondary Tumor Development

Primary kidney tumors were minced and treated with collagenase (1 U/ml) followed by 0.25% trypsin digestion. The dissociated tumor cells were inoculated subcutaneously into BALB/cSlc-*nu/nu* mice or C.B-17/Icr-*scid*Jcl mice to form transplanted secondary tumors.

RNA Preparation, qRT-PCR and Microarray Analysis

Total RNA was isolated using the RNeasy Plus Mini kit (QIAGEN). The quantitative real-time PCR analysis was performed using the GoTaq qPCR Master Mix (Promega). The specific primer pairs used for amplification are shown in Table S2. The transcript levels were normalized to the *β-actin* level. The microarray analysis was performed using the Mouse Gene 1.0 ST Array (Affymetrix) in accordance with the manufacturer's instructions. All of the data analyses were performed using the GeneSpring GX software program (version 12; Agilent Technology).

DNA Methylation Analyses

The RRBS analysis was performed as described previously (Boyle et al., 2012). The samples were sequenced on an Illumina HiSeq 2000 machine. Three-kilobase regions flanking transcription start site (from −1,500 to +1,500) were analyzed to examine DNA methylation levels. The DNA methylation levels for each gene were determined based on the median of DNA methylation values at CpG sites within the region. The DNA methylation values at CpG sites

(D) Kidney tumor-derived iPSCs can contribute to adult chimeric mice.
(E) No tumor formation was observed in the kidneys of chimeric mice generated with kidney tumor-derived iPSCs. Note that Dox treatment for 24 hr confirmed the contribution of kidney-tumor-derived iPSCs to the normal-looking kidney.
(F) The histological analyses of the kidneys of chimeric mice demonstrated no detectable histological abnormalities (a and b). Kidney-tumor-derived iPSCs labeled with Venus could contribute to normal-looking kidney (c). Scale bars, 500 μm (a) and 100 μm (b, c).
See also Figure S7.

containing higher than 10× coverage in all comparative samples were used for the analysis.

Histological Analysis and Immunostaining

Normal and tumor tissue samples were fixed in 10% buffered formalin for 24 hr and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (H&E), and serial sections were used for the immunohistochemical analyses. The primary antibodies used were anti-Oct3/4 (1:100 dilution; BD Biosciences), anti-Ki-67 (1:100 dilution; Dako), anti-insulin (1:500 dilution; Dako), anti-BrdU (1:500 dilution; Abcam), anti-2A (1:250 dilution; Millipore), anti-Lin28b (1:100 dilution; Cell Signaling Technology), and anti-GFP (1:500 dilution; Invitrogen).

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray and RRBS data reported in this paper is GSE52304.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.01.005.

ACKNOWLEDGMENTS

We are grateful to T. Taya for CGH analysis and S. Sakurai and T. Sato for RRBS analysis. We also thank S. Masui, H. Sakurai, and members in Yamada laboratory for helpful discussions and T. Ukai, K. Osugi, and N. Nishimoto for assistance. The authors were supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT); the Ministry of Health, Labor, and Welfare of Japan; the JST; the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) of the Japanese Society for the Promotion of Science (JSPS); the Takeda Science Foundation; and the Naito Foundation. S.Y. is a member without salary of the scientific advisory boards of iPierian, iPS Academia Japan, Megakaryon Corporation, and HEALIOS K. K. Japan. The iCeMS is supported by World Premier International Research Center Initiative, MEXT, Japan.

Received: May 29, 2013
Revised: November 6, 2013
Accepted: January 3, 2014
Published: February 13, 2014

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Concise Review: Dedifferentiation Meets Cancer Development: Proof of Concept for Epigenetic Cancer

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Key Words. Induced pluripotent stem cells • Dedifferentiation • In vivo reprogramming • Epigenetic cancer • Wilms' tumor

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Received April 24, 2014; approved for publication June 26, 2014.

1066-5099/2014/$20.00/0

http://dx.doi.org/10.5966/sctm.2014-0090

ABSTRACT

The technology for generation of induced pluripotent stem cells (iPSCs) has made significant contributions to various scientific fields, and the field of cancer biology is no exception. Although cancer is generally believed to develop through accumulation of multiple genetic mutations, there is increasing evidence that cancer cells also acquire epigenetic abnormalities during development, maintenance, and progression. Because the epigenetic status of somatic cells changes dynamically through reprogramming, iPSC technology can be utilized to actively and globally alter the epigenetic status of differentiated cells. Using this technology, a recent study has revealed that some types of cancer can develop mainly through disruption of the epigenetic status triggered by dedifferentiation. In this paper, we outline the reprograming process and the epigenetic mechanism associated with the maintenance or conversion of cell identity. We then describe several observations suggesting that dedifferentiation can play an important role in cancer development. Finally, we introduce the system responsible for in vivo reprogramming to demonstrate the involvement of dedifferentiation-driven epigenetic disruption in cancer development, and propose that particular types of cancer can develop predominantly through epigenetic alterations. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1–6*

INTRODUCTION

For a long time it has been believed that once a cell differentiates into a particular cell type that has a distinctive function in the human body, it permanently loses the potential for diverse functions and stably maintains its identity. This unidirectional developmental process is frequently compared with a ball rolling down a hill into a landscape with peaks and valleys. The pluripotent stem cell is located at the top of the hill, and each differentiated cell state lies at the bottom of each distinct valley. Once development proceeds, cells are unable to climb back to the top of the hill and become stem cells again, and they cannot cross the ridges separating the individual valleys and transform into other differentiated cell types (Fig. 1). We now know that this dogma is not necessarily valid and that cells can be altered by artificial reprogramming technology. The pioneering studies that demonstrated this involved the nuclear transfer technique, which showed that a somatic nucleus can be reprogrammed to have pluripotency [1, 2]. It is also known that expression of a transcription factor, *MyoD*, induces myogenic differentiation of most cell types [3]. The decisive breakthrough came when Takahashi and Yamanaka succeeded in generating induced pluripotent stem cells (iPSCs), which are able to differentiate into any of the body's cell lineages [4, 5]. In the context of previous studies, they hypothesized that the factors playing important roles in the maintenance of embryonic stem cell (ESC) identity also play a critical role in the induction of pluripotency in somatic cells. As was expected, forced induction of four transcriptional factors—*Oct3/4*, *Sox2*, *Klf4*, and c-*Myc*, all of which are highly expressed and contribute to maintenance of ESC identity—resulted in generation of iPSCs. This iPSC-generation technique also opened the door to the direct reprogramming of specialized cells into other specialized types of cells [6–8]. As is the case in *MyoD*-induced transdifferentiation, direct reprogramming is basically achieved by inducing the transcription factors that govern the transcriptional network of the target cell lineage. Collectively, it is suggested that cell identity is controlled, at least in part, by a stable transcriptional network unique to the individual cell type and that, therefore, forced expression of transcription factors that activate the transcriptional network of the resulting cell type induce cell fate conversion (Fig. 1). A study demonstrating that expression of master transcription factors involving maintenance of cell identity interferes with reprogramming toward pluripotent stem cells supports this idea [9]. It is also important to note that the transcriptional network is closely linked with epigenetic modifications, suggesting that a stable transcriptional

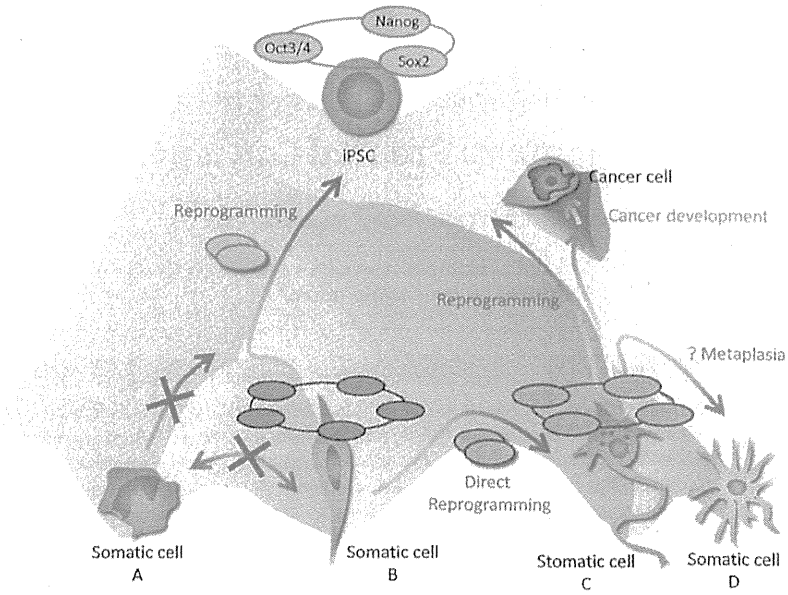

Figure 1. Dedifferentiation-driven cancer development in an epigenetic landscape. As a general rule, the developmental process is unidirectional and irreversible, like a ball rolling downhill (gray arrows). It is suggested that maintenance of cell identity is governed by cell type-specific transcriptional networks (halos over each cell type), accompanied by epigenetic regulation. Forced expression of the transcriptional factors (orange and violet ellipsoids) that activate the network of the target cells can induce lineage conversion, namely, reprogramming or direct reprogramming (blue arrows). External stimuli (e.g., infection, inflammation) and/or intrinsic plasticity might lead to dedifferentiation of mature cells as a natural phenomenon (red arrows). On the way toward pluripotency (by transcription factors or as a natural phenomenon), there may be a "hollow" near the path, leading to cancer development. Some partially reprogrammed cells that may already possess some cancer-like properties can fall to the hollow and expand as cancer cells (gold arrows). Abbreviation: iPSC, induced pluripotent stem cell.

network in a particular cell type is maintained by epigenetic regulation [10].

Epigenetic Regulation During Cell Identity Conversion

Epigenetics is defined as meiotically and mitotically inherited regulation of gene expression that is not accompanied by alteration of the DNA sequence. DNA methylation and histone modifications, both of which are major epigenetic modifications, also play critical roles in normal development. During the reprogramming process, dynamic alteration of epigenetic modifications can be observed, whereas the underlying DNA sequence remains unchanged. Consequently, epigenetic regulation during cell-fate conversion has been vigorously studied in the context of induced pluripotency. Although the road map has not been fully clarified, a number of detailed investigations have partly unraveled the processes operating during reprogramming [11–13]. Polo et al. have indicated that two epigenetic waves occur during reprogramming. In the first wave, which is driven by c-*Myc* or *Klf4*, an active mark, histone H3 lysine 4 (H3K4) trimethylation, becomes enriched at promoters for pluripotency-related genes. Gradual enrichment of H3K27 trimethylation then occurs at the same locus (bivalent domain, a characteristic mark of pluripotent cells) [12]. Finally, changes in DNA methylation take place after the second wave (*Oct4*-, *Sox2*-, or *Klf4*-driven wave), together with the altered histone modifications. Conversely, functional analyses have revealed that failure of epigenetic alteration can hamper the reprogramming process. It has been shown, for example, that failure to remove H3K9 methylation is one of the barriers preventing complete reprogramming into iPSCs [14].

As in the case of cell-fate conversion, evidence suggests that epigenetic regulation plays a key role in cancer initiation and progression. In the following section, we describe how the abnormality of epigenetic regulation occupies an important place in cancer development.

Epigenetic Abnormality During Cancer Initiation and Progression

Previous studies using the reverse genetics approach have revealed that cancer is caused by genetic mutations. Specifically, genetically modified rodents with mutations in oncogenes or tumor suppressor genes develop cancers, and this is a powerful tool for demonstrating that a mutation is functionally involved in cancer development in vivo. Mutation of the *APC* gene, for example, is known to be the first event in multistep carcinogenesis in the colon [15], and a causal relationship between *APC* gene mutation and colon cancer development has been established from the fact that *Apc*-mutant mice develop multiple intestinal tumors [16].

In contrast, accumulated evidence suggests that most cancer cells harbor alterations in epigenetic modifications, in addition to genetic mutations. Alteration of the DNA methylation pattern is the type of abnormality that has been analyzed most extensively. In particular, site-specific DNA hypermethylation and global DNA hypomethylation have been observed in the vast majority of cancers [17]. Although it remains unclear how genetic mutations affect epigenetic abnormalities, the functional involvement of epigenetic abnormalities in both cancer initiation and progression has also been highlighted by previous in vivo studies. Particularly noteworthy are detailed studies using *Apc*-mutant mice

that have revealed the role of abnormal DNA methylation in colon tumor development [18–21]. These studies suggest that forced reduction of DNA methylation causes chromosomal instability resulting in loss of *Apc* heterozygosity and promotes neoplastic transformation of colonic mucosa, whereas it suppresses the progression of early microadenomas into macroscopic tumors. In addition, de novo overexpression of DNA methyltransferase *Dnmt3b* accelerates the progression of colonic microadenoma to a macroscopic tumor, whereas deletion of *Dnmt3b* suppresses this progression.

Taken together, the available data suggest that cancer progresses through multistep processes involving both genetic mutations and epigenetic abnormalities; however, it still remains unclear how epigenetic abnormality occurs during cancer development. Previous studies have demonstrated that cancer-promoting inflammatory stimuli induce drastic changes in DNA methylation patterns [22]. These results suggest that external signals could be a cause of epigenetic abnormalities in cancer cells. In contrast, large-scale sequencing projects have identified a number of mutations of epigenetic regulator genes across a wide variety of cancer types [23]. These results clearly demonstrate that some of the epigenetic abnormalities observed in cancers are attributable to genetic mutations and highlight the primary role of genetic mutations, even against a background of epigenetic alterations.

Dedifferentiation in Cancer Initiation and Progression

Previous studies suggested that the concept of cancer stem cells is closely related to dedifferentiation of cancer cells. Because the role of dedifferentiation in cancer cell heterogeneity has been nicely described in other reviews [24, 25], we have not attempted comprehensive coverage of this topic in this review but rather focused on the possible role of dedifferentiation on cancer initiation and promotion.

Several studies suggested that cancer cells can arise from somatic stem cells [26, 27]. Baker et al. demonstrated that intestinal stem/progenitor cells are prone to transformation [26]. In contrast, other studies proposed that dedifferentiation of mature cells triggers cancer development [28–31]. Schwitalla et al. demonstrated that activated Wnt signaling together with elevated nuclear factor-κB (NF-kB) signaling can induce dedifferentiation of nonstem cells in the intestine, resulting in acquisition of tumor-initiating capacity with stem cell properties [28]. Using a conditional knockout system for *PAX5*, the master regulator of B-cell lineage, another group has shown that mature B cells can develop into aggressive lymphoma through dedifferentiation into the progenitor state [29]. Moreover, several studies that have explored the origin of tumor cells in glioma have suggested that this tumor can arise in differentiated lineages through dedifferentiation [30, 31].

In the field of surgical pathology, dedifferentiation (i.e.,morphological loss of lineage identity with tumor progression) is often recognized. Well-differentiated liposarcoma, for example, a common soft tissue sarcoma that shows characteristics of adipose tissue such as lipid droplets in cytoplasm, can progress to dedifferentiated liposarcoma, a high-grade malignancy lacking morphological features of fat tissue. Collectively, these findings suggest that dedifferentiation is involved in both initiation and promotion of cancer development; however, it is still unclear whether dedifferentiation is a driver of cancer development.

It is interesting to note that the reprogramming process, namely, dedifferentiation, has characteristics similar to those of cancer development. During reprogramming, somatic cells acquire unlimited proliferation properties and self-renewing activities, which are also well-accepted characteristics of cancer cells. It is also suggested that iPS cells and cancer cells share a similar metabolic status [32, 33]. Furthermore, poorly differentiated aggressive cancers have been shown to have an ESC-like transcriptional signature [34]. These similarities suggest that the processes of tumor development and reprogramming may be promoted by overlapping factors. Indeed, depletion of *p53*, a major tumor suppressor gene, significantly promotes the derivation of iPSCs, demonstrating that tumor suppression could be a roadblock on the path toward pluripotency [35, 36].

The fact that conversion of somatic cells to iPSCs is accompanied by dynamic changes in epigenetic status raises the possibility that epigenetic regulation of derivation of iPSCs might drive dedifferentiation-associated cancer development. Moreover, considering that iPSC derivation does not require any particular change in the underlying DNA sequence, it is possible that dedifferentiation-driven epigenetic disruption could be a primary driving force of a particular type of cancer development, independent of genetic mutations. Together, these findings provide a rationale for exploring the causal relationship between dedifferentiation and cancer development, using technology for iPSC derivation.

Dedifferentiation Can Induce Cancer Development: Indication of Epigenetics-Driven Cancer Development

We recently tried to elucidate the close association between dedifferentiation and cancer development using in vivo reprogrammable mice that have a transgenic system similar to that utilized in previous studies [37–40]. We used chimeric mice that were generated using ESCs in which reprogramming factors were inducible under the control of doxycycline. Continuous expression of reprogramming factors resulted in the development of multiple teratomas in various organs. It was noteworthy that in vitro culture of these teratoma cells resulted in derivation of iPSCs capable of chimeric contribution, confirming that somatic cells can be reprogrammed in vivo. Interestingly, when incomplete reprogramming was induced in these mice by withdrawal of doxycycline treatment before teratoma formation, the mice developed tumors consisting of undifferentiated dysplastic cells that were distinct from teratoma cells. These tumors showed invasion into the surrounding tissues, one of the hallmarks of cancer [41]. The doxycycline-withdrawn cancer cells arising in the kidney were considered to be intermediate between a differentiated (renal tubule cell) state and an iPSC state in terms of gene expression profile and DNA methylation pattern, consistent with the notion that these cancer cells are partially reprogrammed cells. Interestingly, these cancers resembled Wilms' tumor, a common pediatric kidney cancer, in many respects including histology, gene expression profile, and an abnormal expression pattern of *Ifg2*, a common imprinting gene, suggesting that this mouse could be a model for human Wilms' tumor. Importantly, no remarkable genetic mutations were observed in doxycycline-withdrawn cancer cells, and these cancer cells were readily reprogrammed into iPSCs with shorter latency and higher efficiency when compared with the process of reprogramming from normal kidney cells. Surprisingly, these kidney tumor-derived iPSCs contributed to

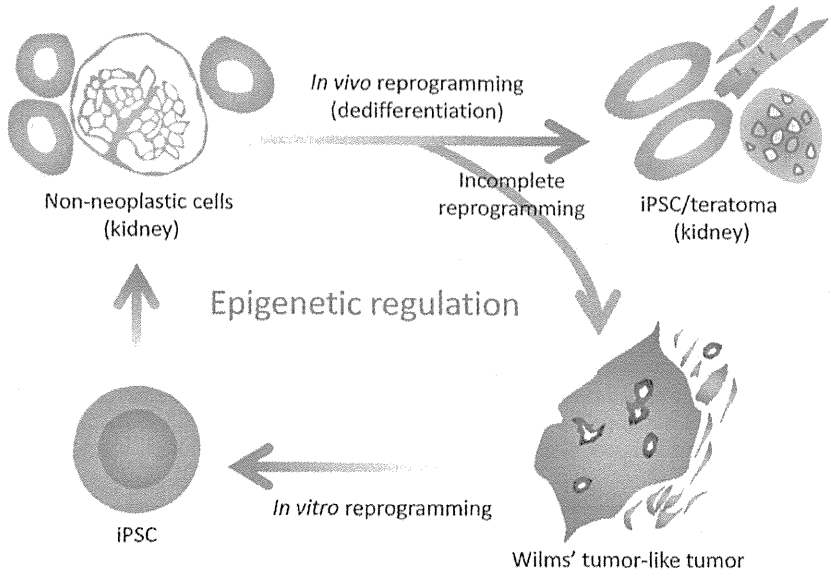

Figure 2. Proof of concept for epigenetic cancer. Non-neoplastic cells in the kidney can be reprogrammed into iPSCs in teratoma in vivo. However, incomplete reprogramming caused by transient expression of reprogramming factors results in the development of a cancer resembling Wilms' tumor, a common pediatric kidney cancer. The tumor cells are easily fully reprogrammed into iPSCs by additional expression of reprogramming factors in vitro. The kidney tumor-derived iPSCs differentiate into non-neoplastic kidney cells in chimeric mice, proving that kidney cancer cells in this model have not undergone irreversible genetic transformation. This result suggests that particular types of cancer can develop mainly through disruption of epigenetic regulation. Abbreviation: iPSC, induced pluripotent stem cell.

chimeric mice and differentiated into apparently normal non-neoplastic kidney cells. Considering that iPSC derivation and the differentiation process are not accompanied by changes in genomic sequence, these findings indicate that the kidney cancer genome in this model supports normal kidney development, resulting in terminal differentiation into non-neoplastic kidney cells. The results provide proof of concept for epigenetic cancer and suggest that particular types of cancer, such as Wilms' tumor, can arise mainly as a result of epigenetic disruption triggered by dedifferentiation (Fig. 2). The fact that human Wilms' tumors sometimes lack detectable genetic mutations at well-known genes for Wilms' tumor development supports this notion. Further analysis would be required to uncover molecular mechanisms for how somatic cells overcome the limited length of telomeres and the limited ability for cell proliferation with partial reprogramming [24, 41, 42], leading to the acquisition of neoplastic properties.

Given that epigenetic regulation can be modulated by chemical compounds, the epigenetic abnormality that drives cancer development could be a promising therapeutic target. It would be exciting to explore cancer types that depend mainly on epigenetic regulation for their development and maintenance. Childhood tumors, which appear not to accumulate genetic mutations in terms of patient age, may be candidates for epigenetically driven cancer. In fact, recent studies have demonstrated that childhood cancers, such as medulloblastoma, neuroblastoma, and rhabdoid tumor, have very few recurrent mutated genes [43–45]. Our experimental model described above utilizes artificial expression of reprogramming factors, and the present study does not provide direct evidence that dedifferentiation is actually involved in the development of human Wilms' tumor. Further detailed studies using human samples are needed to clarify the role of reprogramming in human cancer development. In addition, because it is unlikely that expression of Yamanaka factors is directly responsible for development of human cancers, it would be of great interest to identify a natural phenomenon that induces dedifferentiation.

The Possibility of Dedifferentiation as a Natural Phenomenon

Metaplasia is a reversible change in which one differentiated cell type is replaced by another mature cell type. It is a very common phenomenon and is regarded as an adaptive substitution of cells that are sensitive to stress by cell types better able to withstand the adverse environment. Importantly, metaplasia is of clinical significance because some metaplasias, such as Barrett esophagus and intestinal metaplasia of the stomach, are well known to have an increased risk of cancer development.

Nevertheless, the detailed mechanism of metaplasia still seems to be controversial [46, 47]. Although Wang et al. proposed that residual embryonic cells can be precursors of a Barrett-like metaplasia [46], Fujii et al. reported in a study of intestinal metaplasia of the stomach that chronic infection with *Helicobacter pylori* converts gastric epithelial cells to intestinal epithelial cells via tissue stem-like progenitor cells [48]: *H. pylori* infection induces aberrant expression of the intestine-specific caudal-related homeobox (CDX) transcription factors *CDX1* and *CDX2*. Ectopic *CDX1* activates the stemness-associated reprogrmming factors *SALL4* and *KLF5*, resulting in the reprogramming of gastric epithelial cells into tissue-stem like progenitors and leading to transdifferentiation into intestinal epithelial cells. This study supports the idea that external stimuli (i.e., a natural phenomenon) such as infection by a pathogenic organism (e.g., *H. pylori*) and subsequent inflammation can induce dedifferentiation of somatic cells. As mentioned above, it is noteworthy that inflammation-inducible

NF-kB signaling, one of the common cytokine signals, accelerates intestinal tumor formation initiated by dedifferentiation [28]. It is possible that the dedifferentiated cells, arising as a result of inflammation, may easily acquire cancer cell properties or already possess some aspects of them (Fig. 1). Given that accumulation of DNA methylation is observed at some loci depending on age [49], it is also possible that somatic cells in children may have more flexible plasticity than those of adults and that the characteristics of such cells may make them more prone to dedifferentiation, leading to tumor development (Fig. 1). The possibility of naturally occurring dedifferentiation is worth further exploration.

Recently, much attention has been given to "super-enhancers" for maintenance of cell identity. Super-enhancers are large clusters of transcription enhancers that have much stronger transcriptional activity than classic promoters and enhancers. Each cell type has unique super-enhancers, and these are involved in the proper expression of genes that define the distinct characteristics of both normal cells and cancer cells. Moreover, single nucleotide polymorphisms in super-enhancers are significantly correlated with lineage-associated disease [50, 51]. These findings indicate that loss of cell identity (i.e., dedifferentiation) plays a role in the pathogenesis of diverse diseases, including cancer.

Conclusion

Although the rationale for somatic dedifferentiation as a driver for general cancer development remains unclear, previous studies have suggested the involvement of dedifferentiation in cancer development. A recent study using iPSC technology that allows global changes in epigenetic status without affecting the underlying DNA sequence has provided stronger evidence for a causative and primary role of dedifferentiation-associated epigenetic regulation in a particular type of cancer development. Further studies aimed at identifying external stimuli that induce loss of cellular identity are warranted to explore the possibility of dedifferentiation as a natural causative phenomenon in human cancer development. The data obtained would be applicable for devising novel strategies for both prevention and treatment of specific types of cancer.

Acknowledgments

We thank members of the Yamada laboratory for helpful discussions. The authors were supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT); by the Ministry of Health, Labor, and Welfare of Japan; by the Japan Science and Technology Agency; by the Takeda Science Foundation; and by the Naito Foundation. The Institute for Integrated Cell-Material Sciences is supported by the World Premier International Research Center Initiative, MEXT, Japan.

Author Contributions

Yosuke Yamada, H.H., and Yasuhiro Yamada: manuscript writing; Yasuhiro Yamada: final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Pathology International

Pathology International 2014; **64**: 299–308

doi:10.1111/pin.12180

Review Article

Application of iPS cell technology to cancer epigenome study: Uncovering the mechanism of cell status conversion for drug resistance in tumor

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Recent studies imply that cancer cells possess the ability to reversibly change their properties between a drug sensitive state and a drug resistant state accompanied by epigenetic changes. This evidence indicates that better understanding of cancer epigenetics is important for efficient cancer therapies. Nevertheless, it had been difficult to deeply examine the epigenetic mechanisms because of lack of the tools to actively modify coordinated epigenetic events. In this stagnant situation, the reprogramming technology established by Yamanaka and coworkers have shed a new light. The novel reprogramming technology has made it possible for researchers to artificially introduce epigenetic remodeling into somatic cells. Accordingly, we might be able to use this technology as a tool to introduce the coordinated epigenetic reorganization.

In this review, we introduce the idea of cell state interconversion in cancer cells that is attributable to altered epigenetic regulations. We then depict the epigenetic modifications observed during the process of somatic cell reprogramming and give some examples of the difficulty in cancer cell reprogramming. Finally, we discuss how we can translate this reprogramming refractoriness of cancer cells into uncovering unique epigenetic regulations in cancer cells, which might be applicable eventually to the development of novel cancer therapeutics against drug resistant cancer cells.

Key words: *cancer cell reprogramming, cell status transition,* drug resistant cancer cells, epigenetic flexibility, epigenetics, iPS cell technology

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Disclosure Statement: Y.M. is employed by Chugai Pharmaceutical, Co., Ltd.

Received 12 April 2014. Accepted for publication 5 June 2014.

HYPOTHESIS WHICH EXPLAINS THE EMERGENCE OF CHEMOTHERAPY-REFRACTORY TUMORS

Discovery of the first oncogene, *Src* in the early 1970s has accelerated the speed of cancer research. During the 1980s and 1990s, a number of oncogenes and tumor suppressor genes were hunted by researchers and such achievements made the society rather optimistic about the cancer therapeutics in the future. It seemed that blocking the oncogene-dependent signaling pathway and/or blocking the pathway which is activated due to functional loss of tumor suppressor gene would induce growth retardation and apoptosis in cancer cells and eventually eliminate all the mass of the tumor lesion. However, soon after that, researchers had to be disappointed facing the reality that the eradication of cancer cells in tumor lesions is not as easy as initially expected, namely, disease is not always successfully cured simply by blocking the oncogene- and/or tumor suppressor gene-related pathways.

Up to now, many pharmaceutical companies have developed a variety of cytotoxic drugs and molecularly-targeted drugs, which specifically block a series of oncogenic signals. However, we are still facing difficulties curing cancers. First of all, tumors recur even after striking regression of their mass by the chemotherapy targeting oncogenic signals. Second, cancer lesions show heterogeneity so that it is not possible to sweep out all the malignant cells with a treatment targeting a single pathway. Third, cancer masses sometimes contain 'dormant' cancer cells, which escape from chemo-treatment and hide in patients' organs or bloodstreams. All these problems seem to be related to each other, and contribute to the refractoriness of cancer cells against chemotherapy.

The 'cancer stem cell hypothesis' might explain the difficulty in the eradication of cancer cells. This hypothesis claims the existence of 'cancer stem cells (CSCs)' in every tumor mass, defined as 'cells within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor'.[1] Previous

studies suggest that CSCs are resistant to chemotherapeutic drugs and harbor tissue-specific stem cell-like properties. Therefore, a single CSC can divide asymmetrically and eventually generate all types of the heterogeneous cancer cells within the tumor lesion. Given such properties of CSCs, it is now widely accepted that getting rid of CSCs is required to evade tumor recurrence.

In 1997, Bonnet and Dick reported isolation of cells which harbor a capacity of initiating human acute myeloid leukemia in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice). The isolated cell population provided the first conclusive evidence for human CSCs.[2] Following this study, CSCs in other types of tumors including breast cancer,[3] brain tumor,[4,5] prostate cancer,[6] and colon cancer[7] have been reported. Today, the list of isolated CSCs and molecular markers to sort CSCs are still expanding.

DYNAMIC CELL STATE INTERCONVERSION IN CANCER CELLS

To target CSCs as a novel cancer therapeutic, it is important to understand the mechanism of CSC emergence. The cell of origin for CSCs is one of the most actively discussed topics in the cancer research field recently. Since it is expected that CSCs possess biological characteristics similar to that of normal tissue specific stem cells in most instances (i.e., self-renewal property and capability to reconstitute the whole cancer cell types which exist in the original tumor lesion), tissue specific stem cells have long been considered to be a cell of origin of CSCs. However, it had been difficult to prove this in solid tumor models because of the lack of high-resolution lineage tracing methods.

In 2012, Clevers and coworkers succeeded in tracing a single clone of $Lgr5^{+}$ crypt tissue specific stem cell with *Apc* conditional knockout alleles in a mouse intestinal tumor model and proved that all cell types in the emerged tumor were offspring of a single crypt tissue specific stem cell harboring $Apc^{-/-}$ allele.[8] The fact that tissue specific stem cells with genomic mutation constitute whole tumor mass implied the existence of a 'unidirectional' hierarchy in the heterogenic tumor cell population. As symbolized in C. H. Waddington's 'Epigenetic landscape' described in 1957, it had long been believed to be impossible for normal somatic differentiated cells to go backwards into undifferentiated states. Considering the unidirectional differentiation of stem cells, it had also been anticipated that stem cell-derived cancer cells, which are presumably a similar entity of CSCs, also reside on the top of the unidirectional differentiation hierarchy of the cancer cell population and that the offspring of the CSCs never go back to CSC states (Fig. 1a).[9] However, recently the hierarchy of cancer cells ruled by CSCs has been discovered to be not as rigid as had been considered. Recent data suggest that CSCs and their offspring are 'bidirectionally' transiting from one state to the other. Therefore, it is reasonable to think that there might be a dynamic interconversion between CSC status and non-CSC status in a single cancer cell (Fig. 1b).

Evidence from a melanoma model supports this interconversion model. More than 25% of melanoma cells from patients have been shown to possess tumor initiating capacity in a xenotransplantation experiment. The fact that tumor initiating cells account for such a large proportion seems to contradict the rigid CSC hierarchy model.[10,11] $JARID1B^{high}/JARID1B^{low}$ transition model might give us a good explanation for this contradictory finding. Melanoma cells expressing JARID1B, a member of jumonji/ARID1 (JARID1) histone 3 lysine 4 (H3K4) demethylase family, are slow cycling and harbor CSC-like property. Herlyn and coworkers showed evidence of a bidirectional cell state transition between $JARID1B^{low}$ status and $JARID1B^{high}$ status in melanoma cells and their CSC property was also changed along with the expression level of JARID1B,[12] demonstrating the possibility that CSC and non-CSC are interconverting each other.

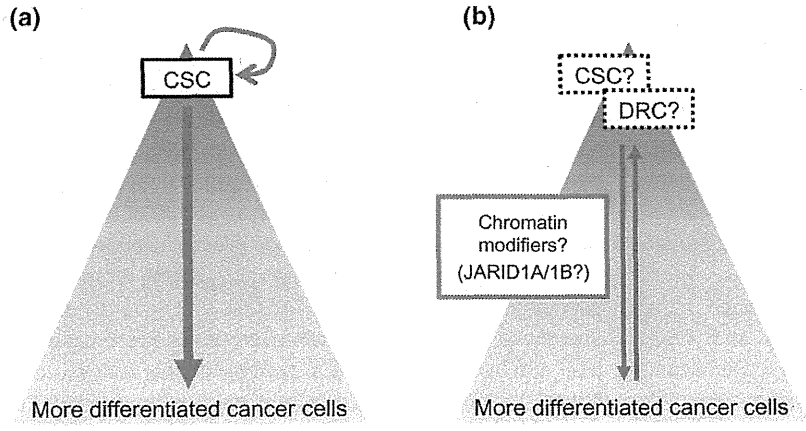

Figure 1 Cancer cells are reversibly converting their status in tumor lesions. (**a**) Hierarchy of cancer cell population in tumor lesion drawn based on classical cancer stem cell (CSC) hypothesis. CSC resides on the top of the hierarchy and all the rest of the cancer cells are CSC's offspring. Direction of the differentiation in the lesion is one-way and differentiated cancer cells never go back to the CSC state. (**b**) Recent data suggest that cancer cells are fluctuating in their status in tumor lesions. They are reversibly converting their characters e.g., from non-CSC state to CSC state and/or drug-sensitive cell state to drug-resistant cell (DRC) state. JARID1A and JARID1B have been reported to be involved in these conversions. Other chromatin modifiers might also play roles in similar conversions.

Bidirectional conversion of cancer cells from one state to another state is exemplified also in the process of acquisition/loss of drug resistance. Accumulating evidence suggests that CSCs are intrinsically more refractory to the effects of a variety of anti-cancer drugs, possibly via drug efflux machinery.[13] Indeed, the authors of the above-mentioned study have claimed that *in vitro* treatment of melanoma cells with cytotoxic reagent or BRAF V600E-targetted reagent, which selectively targets mutated *BRAF* oncogene in melanoma cells, uniformly resulted in enrichment for JARID1B-positive CSC-like cells.[12] Another member of the JARID1 H3K4 demethylase family, JARID1A, was also reported to play an important role in the acquisition of drug resistance in a series of cancer cell lines including lung cancer cell line and colorectal cancer cell line as well as melanoma cell line. These cells also show bidirectional conversion between drug sensitive and drug resistant states, which is implicated to be accompanied by global chromatin changes. In accordance with the expected relation between the drug resistant property and the stem-like property, JARID1A^{high} slow-cycling drug resistant cells express CSC markers such as CD133.[14] Since global demethylation of H3K4 by JARID1A and JARID1B is suggested to be a key event in the process of cancer cell senescence,[15] mechanistic background of slow-cycling CSC emergence might overlap with epigenetic regulations related to G0 state entry of cancer cells (Fig. 1b).

Methylation of histone H3K27 is another well-identified chromatin modification. While histone H3K4 methylation is known to be associated with transcriptional activation, H3K27 methylation is mainly associated with transcriptional repression. In a certain context such as key developmental genes in ES cells, the gene promoter region displays both H3K4me3 and H3K27me3 modifications. This specific modification pattern is termed 'bivalent domains' where gene transcription is silenced, yet kept to be poised for activation, namely the status in which cells can quickly alter their transcriptional profile in response to extracellular signals.[16] Interestingly, cancer cells might also utilize the bivalent domains to alter their characters. Weinberg and coworkers suggested that the conversion from non-CSC status to CSC status is mediated by the bivalent domains in the promoter region of *ZEB1*, a gene coding for Zinc finger E-box-binding homeobox 1 transcription factor. They have demonstrated that microenvironmental stimuli such as tumor growth factor β (TGFβ) signal alters the status of the *ZEB1* bivalent domain and increases the transcriptional activity, leading to the conversion from CD44^{low} non-CSC status into CD44^{high} CSC status in a breast cancer model.[17]

Altogether, this evidence suggests that cancer cells convert themselves between non-CSC status and CSC status possibly by altering the chromatin modifications. Although the involvement of chromatin modifications has not yet been demonstrated in animal models, evidence suggesting the conversion between non-CSC into CSC has been observed in a murine colon cancer model. Colon cancer is currently one of the most intensively-studied tumor types to understand the behavior of CSCs, not only because the differentiation hierarchy in normal colonic epithelium is well characterized, but also because the marker for colon tissue specific stem cells, Lgr5, is well established. Two models for the histopathogenesis of colon cancer progression have been proposed, namely the 'top-down' model and 'bottom-up' model. A 'top-down' model suggests that initially Lgr5$^-$ differentiated cells become dysplastic, spread laterally and then downward to replace normal crypts by neoplastic cells, while a 'bottom-up' model suggests that transformed Lgr5$^+$ crypt tissue stem cells expand upward during tumorigenesis.[18,19] The above-mentioned lineage tracing study of Lgr5$^+$ *Apc*$^{-/-}$ crypt cells by Clevers' group proposed a good example of the 'bottom-up' colon tumorigenesis model,[8] yet this does not exclude the possibility of the 'top-down' model. As for the 'top-down' model, Greten and coworkers have demonstrated by providing the genetic evidence that Lgr5$^-$ enterocytes have the potential to dedifferentiate and to reacquire stem cell properties with Lgr5 expression and tumor initiating capacity through NFkb-mediated activation of Wnt signaling.[20] This proposal of 'top-down' tumorigenesis further supports the notion that CSCs and non-CSCs interconvert each other within the tumor lesion.

The concept of status transition in cancer cells has also been suggested in tumor-derived tissues from actual patients. Patient-derived colon cancer lesions were xenoimplanted in the subcutaneous tissues of NOD/Shi-scid, IL-2Rγ(null) (NOG) mice and they could be serially transplanted for more than 10 passages in NOG mice. Number of Lgr5$^+$ CSC was increased during a series of *in vivo* passages and finally these Lgr5$^+$ cells were plated *in vitro* after dissection. Notably, treatment of the isolated Lgr5$^+$ cells with a cytotoxic drug, irinotecan, converted the cells into Lgr5 negative slow-cycling status and the following drug withdrawal re-converted the cells into Lgr5$^+$ cycling status,[21] demonstrating the dynamic cell state conversion between Lgr5-positive and negative colon cancer cells.

Given that the transition of cancer cell status are bidirectional, the underlying mechanism of the cell state interconversion is assumed not to be associated with genetic alterations, but to be epigenetic regulations. Furthermore, previous studies suggested that epigenetic machinery plays an important role in the emergence of CSCs. In a breast cancer model, rescue of the epigenetically silenced *SFRP1*, which is a tumor suppressor gene antagonizing Wnt signaling, decreases the number of CD44$^+$ cells and severely affect *in vivo* tumor onset as xenograft even under the activation of ERK signaling pathway. This exemplifies the endowment of the CSC property by an epigenetic mechanism.[22] Altogether, these studies imply that blockade of the epigenetic machinery

which converts non-CSCs to CSCs or drug-sensitive cancer cells to drug-resistant cancer cells is a promising strategy to target treatment-refractory cancer cells, that might eventually enable us to evade tumor recurrence and to achieve the complete cure of cancer disease through intervention of epigenetic regulations involving cell state interconversion.

iPSC TECHNOLOGY AS A TOOL TO INTRODUCE EPIGENETIC REORGANIZATION INTO SOMATIC CELLS

To develop the efficient therapeutics targeting CSCs, the intended study of epigenetic mechanisms behind CSC emergence is currently needed urgently based on the dynamic cell status transition in tumor cell mass. The concept of 'epigenetics' had been initially advocated in 1940s by a developmental biologist, Conrad Hal Waddington. He described epigenetics as a branch of biology which studies the causal interactions between genes and their products that bring the phenotype into being. In modern sense 'epigenetic regulation' is defined as all meiotically and mitotically inherited regulations of gene expression that are not accompanied by alteration of the DNA sequence. Such regulations of gene activity are achieved mainly through alterations of chromatin structure involving DNA methylation and post-translational modification of histone proteins.

While the role of genetic alterations in cancer development has been demonstrated in a number of studies using established experimental techniques including reverse genetic approaches, evidence indicating the functional significance of epigenetic abnormalities in cancer cells remains to be limited due to lack of the means to modify the epigenetic regulations. However, the ground-breaking studies of Yamanaka and coworkers reported in 2006 and 2007 raised the possibility of artificial control of the coordinated epigenetic modifications.[23,24]

Yamanaka and coworkers have demonstrated that overexpression of four transcription factors, *OCT3/4*, *SOX2*, *KLF4* and *MYC* (4Fs) reprograms differentiated cells into pluripotent stem cell state resulting in 'induced pluripotent stem cells (iPSCs)', both in mouse cells and in human cells. In the original study reporting the establishment of iPSCs, a retroviral system was used to introduce the exogenous 4F-coding genes. In general, retrovirally-introduced exogenous genes often become epigenetically silenced after some time, namely the exogenous 4F-coding genes are only transiently expressed in the process of the reprogramming. In the beginning, the researchers did not intend to induce the silencing of the exogenous genes during the reprogramming process. However, later studies have demonstrated that the epigenetic silencing of exogenous gene itself is required for the efficient accomplishment of cellular reprogramming.[25] Up to today, several other methods besides retroviral introduction have been developed to assure the 'transient' expression of exogenous 4Fs, including the methods that are not accompanied by genomic integrations, such as piggyBac transposon/transposase system,[26,27] Sendai virus[28] and episomal vecors.[29,30]

The important point here is that cellular reprogramming does not require alterations in genomic sequences. Exogenous expression of 4Fs only for a short period of time is enough to switch on the core transcriptional circuitry which is required for the induction and maintenance of the pluripotent state. Although the precise spatio-temporal epigenetic events undergoing during the reprogramming process are yet to be clarified, there certainly exist well-organized regulations of epigenome behind the acquisition of pluripotency. From this point of view, the novel technique for iPSC introduction is not merely a method to endow somatic cells pluripotency, but also a method to understand the coordinated epigenetic modifications underlying cell state transition.

EPIGENETIC MODIFICATIONS UNDERLYING CELLULAR REPROGRAMMING

It is noteworthy that cellular reprogramming is an epigenetic process, namely, it does not require alterations in genomic sequences. As a rough sketch, reprogramming steps are accompanied by erasure of the epigenetic modifications associated with the maintenance of cellular identity and also accompanied by induction of the epigenetic modifications similar to those found in early embryos.

One of the earliest events during the reprogramming process is genome-wide changes of the histone H3K4me2 modification at more than a thousand loci, including large subsets of pluripotency-related and developmentally-regulated gene promoters and enhancers. On the other hand, the pattern of the other histone modifications is also globally changed. Recent study using human cells suggests that the exogenous transcription factors form complexes and that majority of them bind to the distal enhancer elements of the target genes shortly after the 4F introduction.[31] Besides this 'distal enhancer binding', binding of the reprogramming factors to the megabase-scale swaths of the genomic region is also required to accomplish the reprogramming. During the latter step, histone H3K9me3 modification impedes the access of the 4Fs to the target regions. Thus, demethylation of H3K9 is a necessary step for the completion of the somatic cell reprogramming. Indeed, knocking-down of the genes coding for a series of H3K9 methyltransferases, *SUV39H1*, *SUV39H2* and *SETDB1*, enhances the efficiency of reprogramming either in mouse or human cells.[31–33] These findings suggest that changes of epigenetic modifications are the initial event during somatic cell reprogramming, and highlight the fundamental significance of epigenetic regulations in cell state transition.