

conducted by Ji et al. [54], who reported that plasma levels of miR-208, which is produced exclusively in the heart, increased in isoproterenol-induced myocardial injury. Plasma levels of miR-208 show good correlation with plasma levels of cardiac troponin I, a classic and gold-standard marker of myocardial injury [55]. In addition to miR-208, other skeletal muscle-enriched miRNAs, such as miR-1, miR-133a/b, and miR-499, have been evaluated.

As far as we know, only the study by Ji et al. [54] has demonstrated a relationship between circulating miRNA levels and drug-induced cardiovascular injury. Thus, circulating miRNAs as a biomarker of acute myocardial infarction (AMI) are also described in this section to explore their potential as biomarkers of drug-induced cardiovascular toxicity. Cheng et al. [56] reported that serum levels of circulating miR-1 were significantly increased in patients with AMI and were positive correlated with serum creatine kinase-MB levels. Moreover, they reported that the levels of circulating miR-1 in urine were significantly elevated in patients with AMI and showed a positive correlation with serum troponin I levels [57]. In addition, a time-course study using rats showed an obvious delay in the increase of miR-1 levels in urine when compared with that in blood, but the increase of miR-1 levels in urine was sustained longer than that in blood. Consistently, serum levels of miR-133, which belongs to the same cluster and is cotranscribed with miR-1, have been found to be elevated in humans after AMI [58–61]. Several additional studies have shown that circulating levels of the myosin-related miR-499 are elevated in patients after AMI [59, 60, 62]. Despite these encouraging results, the number of samples in the aforementioned studies is insufficient to provide clear proof of the diagnostic power of miRNA signatures and their value for clinical testing of AMI patients. Oerlemans et al. [63] then examined the expression of several miRNAs in the serum of 106 acute coronary syndrome (ACS) patients and 226 patients who had chest pain but were not diagnosed as having ACS. The expression levels of the combined three miRNAs (miR-1, -21, and -499) were significantly higher in the ACS patients than in the non-ACS patients. Interestingly, the combination of these three miRNAs resulted in a significantly greater area under the receiver operating characteristic curve (AUC) of 0.94 than that of high-sensitivity troponin T (0.89). This suggests that these three circulating miRNAs in blood are strong biomarkers for detecting myocardial injury. However, almost all of the miRNAs described in this section, especially in the second half, may not necessarily relate to cardiovascular injury induced by drugs, because few miRNA studies have investigated drug-induced cardiovascular toxicity. Therefore, it is necessary to investigate circulating miRNAs as reliable biomarkers of cardiovascular injury induced by drugs in humans.

4 A Remaining Question in Methodology: How to Normalize Circulating miRNAs

Detection of miRNAs by real-time quantitative polymerase chain reaction (qPCR) has the advantages of being robust, relatively inexpensive, and sensitive to even small amounts, because of signal amplification [64]. Yet many factors dictate the quality of real-time qPCR results, such as differences in the quality of the starting materials and RNA extraction or transcript efficiencies [65]. Thus, a suitable normalizer is required to eliminate as much variation as possible to increase the accuracy of expression measurements. Although small RNA molecules such as 5S and U6 are frequently used as reference genes, they may change widely depending on pathological conditions [66], thus suggesting that these small RNA molecules are not suitable as internal controls in all studies. Synthetic spike-in miRNAs, mainly *C. elegans* miRNAs without homology to mammalian miRNAs, are also used for normalization, but they cannot correct sample-to-sample variations and are unstable in crude plasma [67]; thus, no housekeeping miRNA/small RNA or universal normalizer has yet been established. In addition, evidence reported in previous studies suggests that the transcript levels of some housekeeping genes vary considerably in response to changes in experimental conditions and/or patient conditions [68, 69].

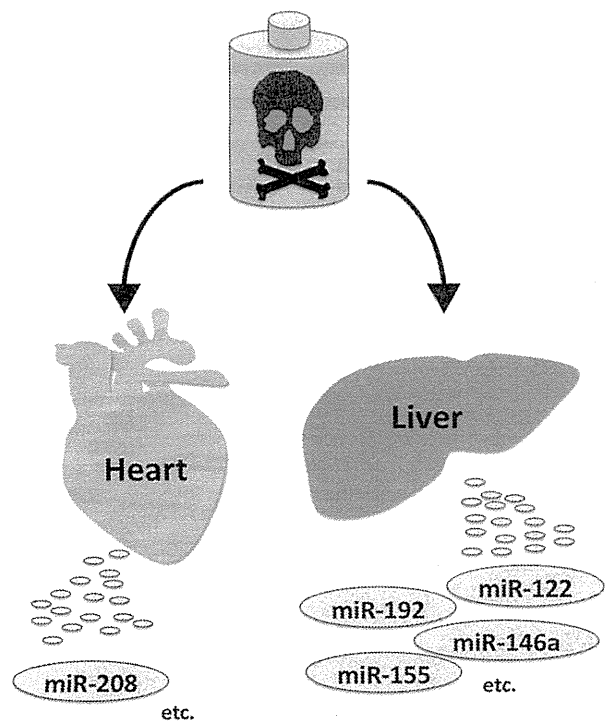


Fig. 1 Circulating microRNAs (miRNAs) as possible biomarkers of liver and heart injury induced by drugs

Wang et al. [70] reported that miR-103 was a suitable reference gene for plasma miRNA analysis in an acetaminophen-induced liver injury model in rats. This suggests that miR-103 is a suitable normalizer in miRNA analysis using plasma/serum, although it will be necessary to evaluate stability in various drug-induced injury models. According to this view, it may be necessary to perform a well-controlled analysis of circulating miRNAs in a large cohort of patients and healthy volunteers. These studies provide further evidence that miRNAs may be useful as serum biomarkers for clinical use.

5 Conclusion

In this article, we discuss recent findings regarding the possibility of circulating miRNA as a biomarker of liver and cardiovascular toxicity (Fig. 1). Although the field of miRNA-related toxicological studies is still in its infancy, novel, reliable, and sensitive miRNA biomarkers that can be used for assessment of tissue-specific toxicity will continue to be discovered in the future, and it is hoped that the disadvantages of circulating miRNAs as biomarkers will be conquered, especially for use in clinical applications. Circulating miRNAs in biological fluids have great potential to contribute to drug development and clinical therapy.

Acknowledgments and Disclosures This work was supported in part by a grant-in-aid for Research on Applying Health Technology from the Ministry of Health, Labour and Welfare, Japan. The authors have no financial relationships to disclose that are directly relevant to the content of this article.

References

- Bartel DP. MicroRNAs: genomics biogenesis, mechanism, and function. *Cell*. 2004;116:281–97.
- Stefani G, Slack FJ. Small noncoding RNAs in animal development. *Nat Rev Mol Cell Biol*. 2008;9:219–30.
- Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993;75:843–54.
- Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *C. elegans*. *Science*. 2001;294:858–62.
- <http://www.mirbase.org/>. Accessed Jun 2013.
- Bentwich I, Avniel A, Karov Y, et al. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet*. 2005;37:766–70.
- Lagos-Quintana M, Rauhut R, Meyer J, et al. New microRNAs from mouse and human. *RNA*. 2003;9:175–9.
- Shah AA, Leidinger P, Blin N, et al. miRNA: small molecules as potential novel biomarkers in cancer. *Curr Med Chem*. 2010;17:4427–32.
- Wang K, Zhang S, Marzolf B, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci USA*. 2009;106:4402–7.
- Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56:1733–41.
- Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;10:997–1006.
- Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*. 2010;101:2087–92.
- Kosaka N, Ochiya T. Unraveling the mystery of cancer by secretory microRNA: horizontal microRNA transfer between living cells. *Front Genet*. 2011;2:97.
- Turchinovich A, Weiz L, Langheinze A, et al. Characterization of extracellular circulating microRNA. *Nucleic Acid Res*. 2011;39:7223–33.
- Chim SS, Shing TK, Hung EC, et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem*. 2008;54:482–90.
- Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008;141:672–5.
- Vickers KC, Palmisano BT, Shoucri BM, et al. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13:423–33.
- Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci USA*. 2011;108:5003–8.
- Ghosh AK, Secreto CR, Knox TR, et al. Circulating microvesicles in B-cell chronic lymphocytic leukemia can stimulate marrow stromal cells: implications for disease progression. *Blood*. 2010;115:1755–64.
- Lima LG, Chammas R, Monteiro RQ, et al. Tumor-derived microvesicles modulate the establishment of metastatic melanoma in a phosphatidylserine-dependent manner. *Cancer Lett*. 2009;283:168–75.
- Skog J, Wurdinger T, Van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10:1470–6.
- Wolfers J, Lozier A, Raposo G, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med*. 2001;7:297–303.
- Holmgren L, Szeles A, Rajnavolgyi E, et al. Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood*. 1999;93:3956–63.
- Zernecke A, Bidzhekov K, Noels H, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal*. 2009;2:ra81.
- Gilad S, Meiri E, Yogev Y, et al. Serum microRNAs are promising novel biomarkers. *PLoS One*. 2008;3:e3148.
- Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105:10513–8.
- Brase JC, Wutting D, Kuner R, et al. Serum microRNAs as non-invasive biomarkers for cancer. *Mol Cancer*. 2010;9:306.
- Wang JF, Yu ML, Yu G, et al. Serum miR-146a and miR-223 as potential new biomarkers for sepsis. *Biochem Biophys Res Commun*. 2010;394:184–8.
- Moussay E, Wang K, Cho JH, et al. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2011;108:6573–8.
- Hu Z, Chen X, Zhao Y, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol*. 2010;28:1721–6.

31. Ng EK, Chong WW, Jin H, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut*. 2009;58:1375–81.
32. Madhavan D, Cuk K, Burwinkel B, et al. Cancer diagnosis and prognosis decoded by blood-based circulating microRNA signatures. *Front Genet*. 2013;4:116.
33. Chang CY, Schiano TD. Drug hepatotoxicity. *Aliment Pharmacol Ther*. 2007;25:1135–51.
34. Halegoua-De Marzio D, Navarro VJ. Drug-induced hepatotoxicity in humans. *Curr Opin Drug Discov Dev*. 2008;11:53–9.
35. Fannin RD, Russo M, O'Connell TM, et al. Acetaminophen dosing of humans results in blood transcriptome and metabolome changes consistent with impaired oxidative phosphorylation. *Hepatology*. 2010;51:227–36.
36. Shi Q, Hong H, Senior J, et al. Biomarkers for drug-induced liver injury. *Expert Rev Gastroenterol Hepatol*. 2010;4:225–34.
37. Kim WR, Flamm SL, Di Bisceglie AM, et al. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology*. 2008;47:1363–70.
38. Nathwani RA, Pais S, Reynolds TB, et al. Serum alanine aminotransferase in skeletal muscle diseases. *Hepatology*. 2005;41:380–2.
39. Antoine DJ, Williams DP, Jenkins AK, et al. High-mobility group box-1 protein and keratin-18, circulating serum proteins informative of acetaminophen-induced necrosis, and apoptosis in vivo. *Toxicol Sci*. 2009;112:521–31.
40. Boone L, Meyer D, Cusick P, et al. Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies. *Vet Clin Pathol*. 2005;34:182–8.
41. Zhang Y, Jia Y, Zheng R, et al. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin Chem*. 2010;56:1830–8.
42. Antoine DJ, Mercer AE, Williams DP, et al. Mechanism-based bioanalysis and biomarkers for hepatic chemical stress. *Xenobiotica*. 2009;39:565–77.
43. Laterza OF, Lim L, Garrett-Engele PW, et al. Plasma microRNAs as sensitive and specific biomarkers of tissue injury. *Clin Chem*. 2009;55:1977–83.
44. Starkey Lewis PJ, Dear J, Platt V, et al. Circulating microRNA as potential markers of human drug-induced liver injury. *Hepatology*. 2011;54:1767–76.
45. Xu J, Wu C, Che X, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog*. 2011;50:136–42.
46. Bihrer V, Friedrich-Rust M, Kronenberger B, et al. Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. *Am J Gastroenterol*. 2011;106:1663–9.
47. Cermelli S, Ruggieri A, Marrero J, et al. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS One*. 2011;6:e23937.
48. Yamamura Y, Nakajima M, Takagi S, et al. Plasma microRNA profiles in rat models of hepatocellular injury, cholestasis, and steatosis. *PLoS One*. 2012;7:e30250.
49. Bala S, Petrasek J, Mundkur S, et al. Circulating microRNAs in exosome indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology*. 2012;56:1946–57.
50. Yang X, Greenhaw J, Shi Q, et al. Identification of urinary microRNA profiles in rats that may diagnose hepatotoxicity. *Toxicol Sci*. 2012;125:335–44.
51. Fung M, Thornton A, Mybeck K, et al. Evaluation of the characteristics of safety withdrawal of prescription drugs from worldwide pharmaceutical markets: 1960 to 1999. *Drug Inf J*. 2001;35:293–317.
52. de Winter RJ, Koster RW, Sturk A, et al. Value of myoglobin, troponin T, and CK-MB mass in ruling out an acute myocardial infarction in the emergency room. *Circulation*. 1995;92:3401–7.
53. Condorelli G, Latronico MVG, Dorn GW. microRNA in heart disease: putative novel therapeutic targets? *Eur Heart J*. 2010;31:649–58.
54. Ji X, Takahashi R, Hiura Y, et al. Plasma miR-208 as a biomarker of myocardial injury. *Clin Chem*. 2009;55:1944–9.
55. Adams JE, Schechtman KB, Landt Y, et al. Comparable detection of acute myocardial infarction by creatine kinase MB isoenzyme and cardiac troponin I. *Clin Chem*. 1994;40:1291–5.
56. Cheng Y, Tan N, Yang J, et al. A translational study of circulating cell-free microRNA-1 in acute myocardial infarction. *Clin Sci*. 2010;119:87–95.
57. Chen Y, Wang X, Yang J, et al. A translational study of urine miRNA in acute myocardial infarction. *J Mol Cell Cardiol*. 2012;53:668–76.
58. Wang GK, Zhu JQ, Zhang JT, et al. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J*. 2010;31:659–66.
59. Corsten MF, Dennert R, Jochems S, et al. Circulating microRNA-208b and microRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet*. 2010;3:499–506.
60. D'Alessandra Y, Devanna P, Limana F, et al. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction. *Eur Heart J*. 2010;31:2765–73.
61. Kuwabara Y, Ono K, Horie T, et al. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate the existence of myocardial damage. *Circ Cardiovasc Genet*. 2011;4:446–54.
62. Adachi T, Nakanishi M, Otsuka Y, et al. Plasma microRNA 499 as a biomarker of acute myocardial infarction. *Clin Chem*. 2010;56:1183–5.
63. Oerlemans MIFJ, Mosterd A, Dekker MS, et al. Early assessment of acute coronary syndromes in the emergency department: the potential diagnostic value of circulating microRNAs. *EMBO Mol Med*. 2012;4:1176–85.
64. Bustin SA, Benes V, Nolan T, et al. Quantitative real-time RT-PCR: a perspective. *J Mol Endocrinol*. 2005;34:597–601.
65. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA*. 2008;14:844–52.
66. Iwamoto H, Kanda Y, Sejima T, et al. Serum miR-210 as a potential biomarker of early clear cell renal cell carcinoma. *Int J Oncol*. 2013 (in press).
67. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*. 2008;105:10513–8.
68. Guenin S, Mauriat M, Pelloux J, et al. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *J Exp Bot*. 2009;60:487–93.
69. Song J, Bai Z, Han W, et al. Identification of suitable reference genes for qPCR analysis of serum microRNA in gastric cancer patients. *Dig Dis Sci*. 2012;57:897–904.
70. Wang Y, Tang N, Hui T, et al. Identification of endogenous reference genes for RT-qPCR analysis of plasma microRNAs levels in rats with acetaminophen-induced hepatotoxicity. *J Appl Toxicol*. 2013 (in press).



The role of microRNAs in the regulation of cancer stem cells

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Cancer stem cells (CSCs) have been reported in many human tumors and are proposed to drive tumor initiation and progression. CSCs share a variety of biological properties with normal somatic stem cells such as the capacity for self-renewal, the propagation of differentiated progeny, and the expression of specific cell surface markers and stem cell genes. However, CSCs differ from normal stem cells in their chemoresistance and tumorigenic and metastatic activities. Despite their potential clinical importance, the regulation of CSCs at the molecular level is not well-understood. MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that play an important role in the regulation of several cellular, physiological, and developmental processes. Aberrant miRNA expression is associated with many human diseases including cancer. miRNAs have been implicated in the regulation of CSC properties; therefore, a better understanding of the modulation of CSC gene expression by miRNAs could aid the identification of promising biomarkers and therapeutic targets. In the present review, we summarize the major findings on the regulation of CSCs by miRNAs and discuss recent advances that have improved our understanding of the regulation of CSCs by miRNA networks and may lead to the development of miRNA therapeutics specifically targeting CSCs.

Keywords: microRNA, cancer stem cells (CSCs), tumor initiation, therapy resistance, metastasis

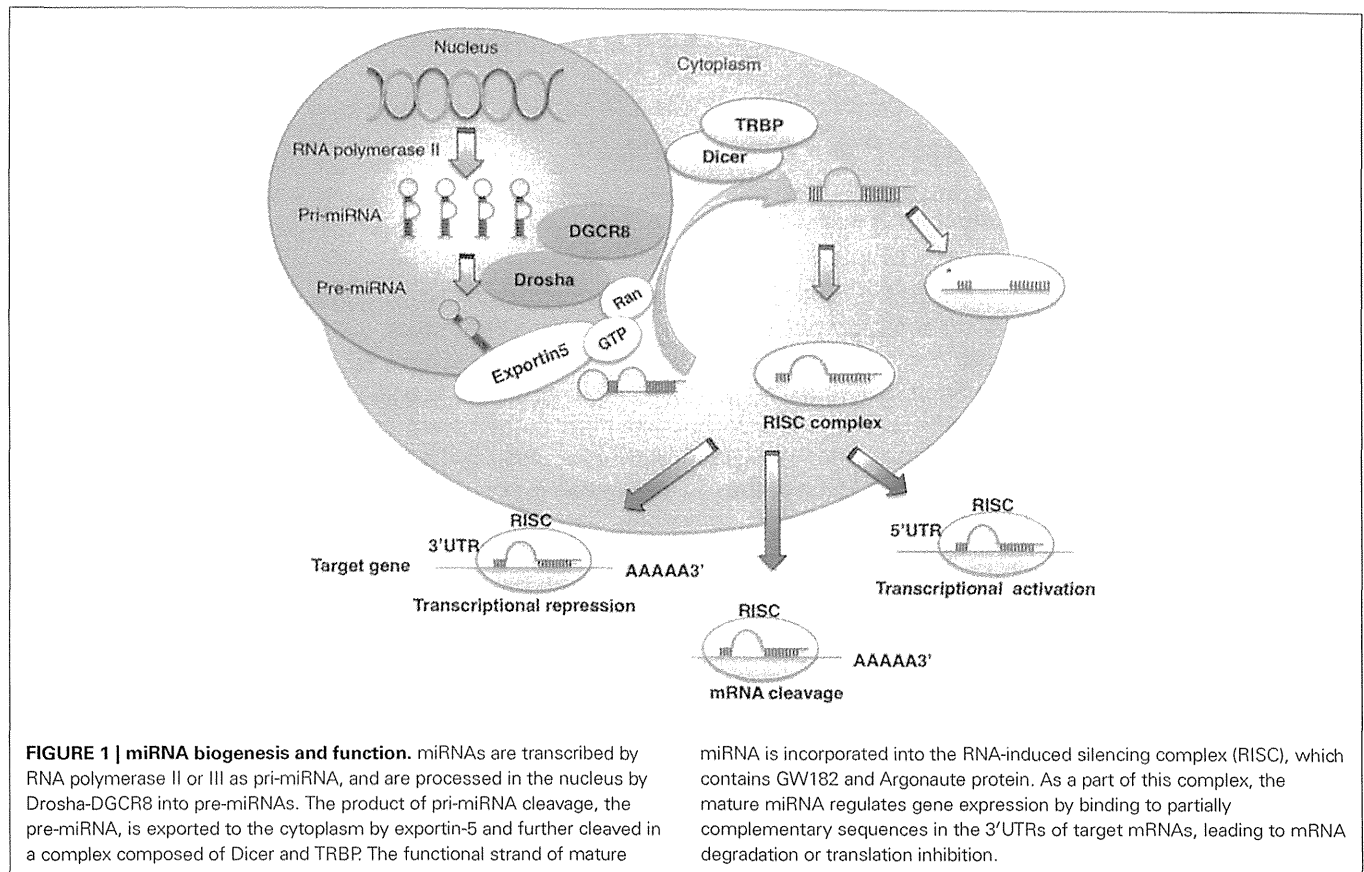
BACKGROUND

The CSC theory, which is based on the concept that cancer might arise from a rare population of cells with stem cell properties, was proposed approximately 150 years ago (Cohnheim, 1875; Wicha et al., 2006). Recent technological developments (flow cytometry analysis and cell sorting) and the establishment of new animal models have provided evidence supporting the CSC theory. Moreover, CSCs are resistant to conventional treatments and are therefore not only of academic interest, but may also be an important consideration in clinical practice. Therefore, a better understanding of the characteristics of CSCs and the identification of therapeutic agents capable of targeting the CSC population are critical issues. Cancer researchers have investigated protein-coding genes and products, including surface markers that are involved in the self-renewal and asymmetric cell division of CSCs. Recently, in addition to alterations in protein-coding genes, abnormalities in non-coding RNAs [miRNAs and long intergenic non-coding RNAs] have been observed in various types of cancers and have been shown to play important roles in the regulation of CSC properties such as asymmetric cell division, tumorigenicity, and drug resistance. In the present review, we discuss the general features of CSCs and the role of miRNAs in the regulation of CSC properties, and summarize the current therapeutic strategies targeting miRNAs for CSC therapy.

BIOGENESIS AND FUNCTIONS OF miRNAs

miRNAs are 21–25 nucleotides long, non-coding RNAs that regulate gene expression at the post-transcriptional level by

binding to the 3'-untranslated regions (3'UTRs) or the open reading frames of target genes, leading to the degradation of target mRNAs or repression of mRNA translation (Iorio and Croce, 2012). miRNAs are transcribed for the most part by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRNA), and are processed in the nucleus by RNase III Drosha into 70–100 nucleotide long precursor miRNAs (pre-miRNAs) in combination with cofactors such as DGCR8, an evolutionarily conserved protein that interacts with proline-rich peptides through its WW domain (Gregory et al., 2004; Lee et al., 2004) (Figure 1). DGCR8 is located on chromosome region 22q11.2, whose heterozygous deletion results in the most common human genetic deletion syndrome, known as DiGeorge syndrome. The clinical symptoms of the disease are highly variable and in approximately 75% of patients, congenital heart defects are observed (Shiohama et al., 2003; Yamagishi and Srivastava, 2003). The product of pri-miRNA cleavage, the pre-miRNA, is exported to the cytoplasm by exportin-5, a member of the Ran-dependent nuclear transport receptor family (Lee et al., 2004) and further cleaved in a complex composed of RNase III Dicer and the transactivating response RNA-binding protein (TRBP) into a miRNA:miRNA* complex. While one of the two strands is selected as a guide strand, the complementary strand (miRNA*) is usually degraded (Iorio and Croce, 2012). miRNA* was originally considered to have no function and to be degraded; however, recent evidence suggests that it can be used as a functional strand and may play significant biological roles (Uchino et al., 2013; Yang et al., 2013).



The mature miRNA is incorporated into a complex known as the RNA-induced silencing complex (RISC), which contains the GW182 and Argonaute proteins. As a part of this complex, the mature miRNA regulates gene expression by binding to partially complementary sequences in the 3'UTRs of target mRNAs, leading to mRNA degradation or translation inhibition (Iorio and Croce, 2012). Several studies have reported that miRNAs also bind to the 5'UTR or the open reading frame (Orom et al., 2008; Mandke et al., 2012) and can promote the translation of their target genes under growth arrest conditions (Vasudevan et al., 2007). Recently, Nishi et al. showed that TNRC6A, a human GW182 paralog, shuttles Ago2 into the nucleus and the colocalization of Ago2-TNRC6A with miRNAs mediates gene silencing (Nishi et al., 2013).

MICRORNAs REGULATE PLURIPOTENCY AND DIFFERENTIATION

The discovery of two miRNAs, *lin-4* and *let-7*, in *Caenorhabditis elegans* suggested that miRNAs are important regulators of embryonic development and stem cell functions in mammals (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). The function of miRNAs in mouse and human embryonic stem cells (ESCs) has been investigated using cells lacking Dicer1 and DGCR8, which are critical for miRNA biogenesis. Deletion of Dicer1 leads to embryonic lethality in mice (Bernstein et al., 2003) and DGCR8-deficient mouse ESCs show alterations in the regulation of the cell cycle and differentiation that are associated with

failure to silence stemness markers, such as *Oct4*, *Rex1*, *Sox2*, and *Nanog*, as well as delayed expression of differentiation markers (Wang et al., 2007).

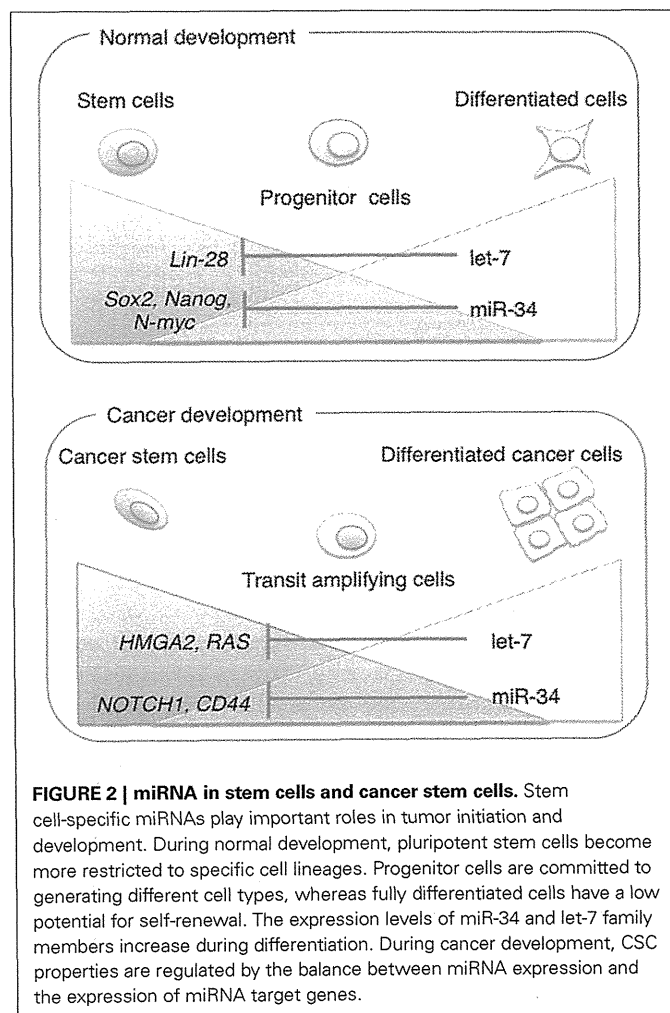
In a comparative transcriptome analysis, Dicer1-deficient mouse ESCs lacking miRNAs showed a significant increase in transcripts containing a GCACUU motif in the 3'UTR (Sinkkonen et al., 2008). This sequence is complementary to the AAGUGC seed sequence of the miR-290-295 cluster (miR-290, miR-291a, miR-292, miR-291b, miR-294, and miR-295) and the miR-302/367 cluster (miR-302a, miR-302b, miR-302c, miR-302d, and miR-367) in mouse ESCs. Using a similar approach, novel stem cell-specific miRNAs were initially identified in human ESCs. These miRNAs include two clusters: miR-302/367 and the miR-371 cluster (miR-372 and miR-373). The expression of the miR-371 cluster is downregulated before that of the miR-302/367 cluster, suggesting a temporal hierarchy in the duration of specific miRNA activity (Stadler et al., 2010; Kim et al., 2011). Members of the miR-302 family rescue the proliferation defects of DGCR8-mutant mouse ESCs (Wang et al., 2008) and reprogram human skin cancer cells into a pluripotent ESC-like state (Lin et al., 2008).

The *Let-7* family is another critical regulator of ESC differentiation. Mature *let-7* family members are essentially absent in ESCs and accumulate only upon ESC differentiation (Viswanathan et al., 2008). Melton et al. reported that whereas transfection of *let-7c* into wild-type cells had no effect on the expression of pluripotency genes, *let-7c* rescued the differentiation defect in DGCR8^{-/-} cells by downregulating *Oct4*, *Sox2*, and *Nanog*

(Melton et al., 2010). Lin-28, a marker of undifferentiated ESCs, is also used to induce pluripotent stem cells (Yu et al., 2007b). A negative feedback loop between Lin-28 and let-7 family members precisely controls the levels of these miRNAs. Although Lin-28 regulates the expression of let-7 miRNAs by binding to the precursors and blocking their maturation, the let-7 family is highly expressed and targets Lin-28 mRNA in mouse differentiated cells and embryonic carcinoma cells (Yu et al., 2007b) (Figure 2). Members of the miR-34 family of miRNAs are direct targets of p53 and function as tumor suppressors, inhibiting reprogramming through the repression of pluripotency genes such as *Nanog*, *Sox2*, and *N-myc* (Choi et al., 2011) (Figure 2). Since the cell cycle regulator p21 also represses reprogramming efficiency, these findings suggest that p53 represses pluripotency via two distinct mechanisms. Evidence that let-7 and miR-34 family members are tumor suppressor miRNAs (Takamizawa et al., 2004; Johnson et al., 2005; Tazawa et al., 2007) suggests that stem cell-specific miRNAs play important roles in tumor initiation and development.

miRNA REGULATION IN CANCER

miRNAs play a crucial role in the progression of human cancer, and expression profiling in human malignancies has identified



signatures associated with cancer development, progression, and prognosis (Liu et al., 2012; Volinia and Croce, 2013). Chromosomal regions coding for oncogenic miRNAs that are involved in the negative regulation of a tumor suppressor gene can be amplified in association with cancer development. This amplification would result in the upregulation of oncogenic miRNAs and silencing of tumor suppressor genes (He et al., 2005). On the other hand, miRNAs targeting oncogenes are often located in fragile site, where deletions or mutations can occur, leading to the reduction or loss of miRNAs and the overexpression of their target oncogenes. Dysregulation of miRNA expression affects processes associated with cancer progression such as the induction of anti-apoptotic activity, drug resistance, tissue invasion, and metastasis (Cimmino et al., 2005; Tavazoie et al., 2008; To et al., 2008). Recent evidence suggests that miRNAs are involved in tumor initiation through the regulation of CSC properties such as self-renewal ability, tumorigenicity and drug-resistance (Yu et al., 2007a; Shimono et al., 2009; Song et al., 2013a,b).

CSCs

Accumulating lines of evidence suggest that CSCs share a variety of biological properties with normal somatic stem cells such as the capacity for self-renewal, the propagation of differentiated progenitors, and the expression of specific stem cell genes (Colmont et al., 2012). However, CSCs differ from normal stem cells in their chemoresistance and tumorigenic and metastatic activities (Colmont et al., 2012 and Table 1). In addition, recently glycosylation patterns are found to be different between normal stem cells and CSCs (Karsten and Goletz, 2013). The CSC theory is generally accepted in the field of cancer research, not only in basic research but also with regard to cancer drug discovery.

Normal stem cells and CSCs act via common signaling pathways that regulate self-renewal activity, including Wnt, Notch,

Table 1 | Representative cell surface markers for human CSCs.

Cancer type	CSC marker	References
AML	CD34 ⁺ /CD38 ⁻	Bonnet and Dick, 1997
Breast	CD44 ⁺ /CD24 ^{-/low}	Al-Hajj et al., 2003
	ALDH1	Ginestier et al., 2007
Glioma	CD133	Singh et al., 2003, 2004
Colon	CD133	O'Brien et al., 2007; Ricci-Vitiani et al., 2007
	CD44/EpCAM/CD166	Dalerba et al., 2007
Metastatic Colon	CD133 ⁺ /CD26 ⁺	Pang et al., 2010
Melanoma	CD20	Fang et al., 2005
	CD271	Boiko et al., 2010
Pancreatic	ESA/CD44/CD24	Hermann et al., 2007
Metastatic Pancreatic	CD133/CXCR4	Li et al., 2007a
Prostate	CD44/a2β1/CD133	Collins et al., 2005
Lung	CD133	Eramo et al., 2008
Hepatic	EpCAM/AFP	Yamashita et al., 2010
Gastric	CD44	Takaishi et al., 2009

AML, acute myelogenous leukemia; ALDH, aldehyde dehydrogenase; EpCAM, epithelial cell adhesion molecule; CXCR4, CXC chemokine receptor 4; AFP, alpha-fetoprotein.

and Sonic Hedgehog, and dysregulation of these pathways plays a role in tumor initiation and development (Reya et al., 2001). Jamieson et al. showed that aberrations in the Wnt/ β -catenin pathway enhance self-renewal activity during leukemia stem cell propagation (Jamieson et al., 2004). Korkaya et al. reported that the Wnt/ β -catenin pathway is involved in the regulation of normal and malignant mammary stem/progenitor cell populations (Korkaya et al., 2009). Several studies have shown that the Notch pathway is activated in breast, glioblastoma, and colon CSCs (Hoey et al., 2009; Taketo, 2011). Alterations in Hedgehog signaling have been reported in colon, breast, and glioblastoma CSCs (Liu et al., 2006; Varnat et al., 2009; Takezaki et al., 2011).

The development of fluorescent antibodies, flow cytometry, and cell sorting techniques enabled the identification of cell populations possessing CSC properties. Furthermore, the development of severely immunodeficient mouse strains facilitated the evaluation of tumor formation ability. These methods have enabled the identification and isolation of CSCs from various cancers (Bonnet and Dick, 1997; Al-Hajj et al., 2003; Collins et al., 2005; Fang et al., 2005; Ginestier et al., 2007; Hermann et al., 2007; Li et al., 2007a; Eramo et al., 2008; Takaishi et al., 2009; Boiko et al., 2010; Pang et al., 2010; Yamashita et al., 2010) (Table 1). In this review, we discuss the major findings of recent studies highlighting the roles of certain “CSC-specific” miRNAs in representative cancer types (Table 2). From these discussions, we present an emerging theme that several miRNAs may exert a functional role in the regulation of the key biological properties of CSCs.

LEUKEMIA STEM CELLS

Through an integrated approach that combined miRNA expression analysis and bioinformatic prediction of mRNA targets, distinct miRNA signatures were shown to fine-tune each step of hematopoiesis, including the reconstitution potential of hematopoietic stem cells (Arnold et al., 2011). The miR-17-92 cluster functions as an oncogenic miRNA by enhancing the formation of Myc-driven B-cell lymphomas in a mouse model (He et al., 2005). Single miRNAs function as oncogenes. The overexpression of miR-155 in early B-cells leads to polyclonal expansion of the pro-B-cell compartment (Costinean et al., 2006), and retroviral expression of miR-155 in immature mouse hematopoietic cells resulted in the expansion of granulocyte/monocyte populations displaying pathological features characteristic of myeloid neoplasia without progression to acute myeloid leukemia (AML) (O’connell et al., 2008). Recently, dysregulation of single miRNAs was shown to contribute to hematological malignancies, including AML and myelodysplastic syndrome (Han et al., 2010; Song et al., 2013a). Han et al. reported that miR-29a regulates early hematopoiesis and induces AML by converting myeloid progenitors into self-renewing leukemia stem cells via targeting several tumor suppressors and cell cycle regulators (Han et al., 2010). miR-22-induced inhibition of the ten-eleven-translocation gene 2 (*TET2*) tumor suppressor increased the methylation of *TET2* target genes, such as *Aim2*, *Hal*, *Igfbt2*, and *Sp140*, and resulted in positive effects on hematopoietic stem cell self-renewal and transformation. This has led to the suggestion that miR-22 is associated with myelodysplastic syndrome and hematological malignancies (Song et al., 2013a).

BREAST CSCs

The first solid tumor CSCs were identified in and isolated from breast tumors in 2003 (Al-Hajj et al., 2003). Al-Hajj et al. described a $CD44^+/CD24^{-/low}$ cell population that had a markedly high tumor-initiating capacity. In 2007, Yu et al. identified let-7 as a master regulator of breast CSC properties (Yu et al., 2007a). In breast CSCs, reduced let-7 expression controls self-renewal and differentiation through *RAS* and *HMGA2*, respectively (Figure 2). Since *HMGA2* plays a role in the control of differentiation and proliferation of both human and mouse ESCs (Li et al., 2007b), these findings also suggest that let-7 is involved in the growth and differentiation of ESCs beyond tumorigenesis.

Epithelial-to-mesenchymal transition (EMT) is an evolutionarily conserved process that occurs during embryonic development in many species of mammals (Liu et al., 2006). Since the EMT program is often activated during tumor invasion and metastasis, the genetic controls and biochemical mechanisms underlying the acquisition of invasiveness and the subsequent systemic spread of cancer cells have been areas of intensive research. The EMT phenotype is characterized by the downregulation of epithelial markers such as E-cadherin, the expression of mesenchymal markers such as N-cadherin and vimentin, the loss of cell-cell contact and cell polarity, and the acquisition of cell invasive capabilities. Mani et al. reported that EMT is also associated with the acquisition of CSC properties (Mani et al., 2008). A $CD44^+/CD24^{-/low}$ cell population purified from cancer tissues shows the features of an EMT phenotype, and human cancer cells induced to undergo EMT exhibit a $CD44^+/CD24^{-/low}$ antigen phenotype and high tumorigenicity.

Recently, two studies reported the clinical relevance of CSCs in breast cancer specimens (Giordano et al., 2013; Yu et al., 2013). In early breast cancer patients, the presence of $CD44^+/CD24^{-/low}$ cells in bone marrow was indicative of a poor prognosis (Giordano et al., 2013). Circulating tumor cells (CTCs) in breast cancer patients also showed the EMT phenotype (Yu et al., 2013). Progressive disease patients undergoing therapy had a higher number of mesenchymal marker positive CTCs than epithelial marker positive CTCs. These results suggest that the CSC phenotype is clinically important not only as a therapeutic target but also as a potential biomarker for the prognostic evaluation of patients undergoing cancer treatment.

A molecular link between EMT and the miR-200 family is provided by the zinc-finger E-box-binding homeobox protein encoding genes (*ZEB1/ZEB2*) (Gregory et al., 2008; Park et al., 2008). The miR-200 family consists of five members that are classified into two clusters: miR-200a, miR-200b, and miR-429 on human chromosome 1; and miR-200c and miR-141 on human chromosome 12 (Gregory et al., 2008). Expression of the miR-200 family strongly inhibits the EMT phenotype induced by TGF- β , and a reciprocal feedback loop between the miR-200 family and the ZEB family of transcription factors tightly regulates both EMT and mesenchymal-to-epithelial transition (Burk et al., 2008). MiR-200 family members are downregulated in normal human and mouse mammary stem cells and breast CSCs, and miR-200c inhibits the formation of mammary ducts from mammary stem cells and tumor formation from breast

Table 2 | The regulatory roles of miRNAs in CSCs.

Cancer Type	miRNA	Target gene	Role of miRNA in CSC properties	References
Leukemia (AML and MDS)	miR-22	<i>TET2</i>	Promotion of self-renewal	Song et al., 2013a
Breast	Let-7	<i>RAS</i> and <i>HMGA2</i>	Inhibition of self-renewal and de-differentiation	Yu et al., 2007a
	miR-200 family	<i>ZEB1/ZEB2</i>	Inhibition of EMT	Gregory et al., 2008
		<i>BMI-1</i>	Inhibition of self-renewal	Shimono et al., 2009
		<i>SUZ12</i>	Inhibition of mammosphere formation	Iliopoulos et al., 2010
miR-22	<i>TET</i> family (<i>TET1-3</i>)	Suppression of miR-200 family expression	Song et al., 2013b	
Brain	miR-9/9*, miR-17	<i>CAMTA1</i>	Promotion of CD133 ⁺ cell proliferation	Schraivogel et al., 2011
	miR-128	<i>BMI-1</i>	Inhibition of self-renewal	Godlewski et al., 2008
	miR-199b-5p	<i>HES1</i>	Reduction of the CD133 ⁺ cell fraction	Garzia et al., 2009
Colon	miR-193	<i>PLAU</i> and <i>K-RAS</i>	Inhibition of tumorigenicity and invasiveness	Iliopoulos et al., 2011
	miR-451	<i>MIF</i> and <i>COX-2</i>	Inhibition of self-renewal and tumorigenicity	Bitarte et al., 2011
	miR-34a	<i>NOTCH 1</i>	Suppression of asymmetric cell division	Bu et al., 2013
Prostate	miR-34a	<i>CD44</i>	Inhibition of self-renewal and metastasis	Liu et al., 2011
	miR-320	β -catenin	Inhibition of Wnt/ β -catenin pathway	Hsieh et al., 2013

AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.

CSCs (Shimono et al., 2009). Members of the miR-200 family also modulate the self-renewal ability of CSCs by targeting B-lymphoma Mo-MLV insertion region 1 homolog (*BMI-1*) and *SUZ12*, a subunit of a polycomb repressor complex (Iliopoulos et al., 2010). *BMI-1* regulates the self-renewal and differentiation of several types of stem cells, including hematopoietic, brain, and mammary stem cells (Molofsky et al., 2003; Park et al., 2003; Pietersen et al., 2008). Therefore, modulation of the activity of the miR-200 family using conventional therapy could be a promising approach to improve the effectiveness of breast cancer treatments.

Normal human and mouse mammary stem cells can be isolated and characterized on the basis of their aldehyde dehydrogenase (ALDH) activities (Ginestier et al., 2007). Using ALDH activity, Ibara et al. determined that miR-205 and miR-22 were highly expressed in mouse mammary progenitor cells (Ibarra et al., 2007). MiR-22 was recently shown to be an epigenetic modifier that promotes stemness and metastasis in breast cancer by directly targeting enzymes in the TET family, which regulate DNA demethylation (Song et al., 2013b). The TET family is involved in the demethylation of the miR-200 promoter, and miR-22 promotes CSC properties such as EMT and a metastatic phenotype through the suppression of the miR-200 family. This provides the first evidence that chromatin-remodeling systems with opposing effects on cell fate (self-renewal vs. differentiation) are regulated by opposing sets of miRNAs.

BRAIN CSCs

The pentaspan membrane glycoprotein CD133, also known as Prominin-1, was first identified as a marker of hematopoietic stem cells and progenitor cells, and was subsequently used to

detect malignancies (Miraglia et al., 1997; Yin et al., 1997). In solid cancers, CD133 was first used to identify CSCs in different types of human brain tumors including glioblastoma, medulloblastoma, and ependymomas (Singh et al., 2003, 2004; Yu et al., 2010). In these studies, patient tumor cells were separated based on the expression of CD133. The CD133⁺ cell population is highly tumorigenic *in vivo*, whereas CD133⁻ cells do not form tumors even at high numbers (Singh et al., 2003, 2004; Yu et al., 2010). CD133⁺ cells are also resistant to radiation and chemotherapy. These findings led to the hypothesis that glioblastomas are maintained by CSCs, and that this treatment-resistant subpopulation is a promising target for effective therapies. CD133 has been instrumental for the identification of CSCs in colorectal (Ricci-Vitiani et al., 2007) and pancreatic (Hermann et al., 2007) carcinomas. CD133 itself is a marker of normal neural stem cells in both humans (Uchida et al., 2000) and mice (Lee et al., 2005).

In cancer cells, the deacetylase HDAC6 directly interacts with and regulates the intracellular localization of CD133 (Mak et al., 2012). CD133 forms a stable protein complex with HDAC6 and β -catenin, which leads to the activation of β -catenin signaling targets in different types of cancer. CD133 is also associated with phosphoinositide 3-kinase (PI3K) 85 kDa regulatory subunit (p85) in glioma stem cells (GSCs) (Wei et al., 2013). The PI3K pathway is a key regulator of tumorigenesis in glioblastoma and other cancers (Godlewski et al., 2010). Therefore, activation of the PI3K/Akt pathway by the physical interaction between CD133 and p85 promotes tumorigenicity in GSCs. The function of CD133 in brain tumors should be fully characterized in the near future, which may shed light on the role of CD133 as a functional marker of GSCs.

Schraivogel et al. reported that miR-9, miR-9* (miR-9/9*), miR-17, and miR-106b are highly abundant in the CD133⁺ cell population in glioblastoma cell lines. Among the upregulated miRNAs in the CD133⁺ cell population, inhibition of miR-9/9* or miR-17 leads to reduced neurosphere formation and stimulates cell differentiation. Functional analysis of these miRNAs showed that miR-9/9* and miR-17 target calmodulin-binding transcription activator 1 (*CAMTA1*), a putative transcription factor of the anti-proliferative cardiac hormone natriuretic peptide A (*NPPA*). Clinical studies also demonstrated that *CAMTA1* and *NPPA* expression is correlated with patient survival. These findings could provide a basis for the design of novel treatment strategies for glioblastoma (Schraivogel et al., 2011).

MiR-124 and miR-128 are the most highly expressed miRNAs in the adult brain and are preferentially expressed in neurons (Smirnova et al., 2005). Patients with high-grade glioma show significant downregulation of miR-128 expression. Functional analyses showed that miR-128 expression inhibits glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo* (Godlewski et al., 2008). In addition, miR-128 specifically inhibits the self-renewal capacity of GSCs by directly targeting *BMI-1*, a polycomb family transcriptional repressor required for postnatal maintenance of neural stem cells in the peripheral and central nervous system (Molofsky et al., 2003). Since *BMI-1* maintains neural stem cells in an undifferentiated self-renewing state, the regulation of *BMI-1* by miR-128 may contribute to normal stem cell regulation.

Another study showed that miR-199b-5p downregulation was associated with metastatic spread in medulloblastoma. In medulloblastoma cells, miR-199b-5p directly targets *HES1*, a transcription factor of the Notch signaling pathway (Garzia et al., 2009). During brain development, Notch functions as a critical regulator of cell fate, by which gliogenesis can only occur when Notch signaling specifically represses the neuronal pathway in progenitor cells (Karamboulas and Ailles, 2013). MiR-199b-5p blocks Notch signaling, inhibiting the self-renewal capacity of medulloblastoma cells by reducing the CD133⁺ subpopulation (Garzia et al., 2009). Recently, miR-34a was shown to regulate Notch signaling by targeting *Notch-1* and *Notch-2* in medulloblastoma cells (Li et al., 2009). Therefore, miR-199b-5p and miR-34a are important for the self-renewal potential of GSCs via the Notch signaling pathway.

COLON CSCs

CD133 was initially used to identify and isolate colon CSCs (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), which was followed by the identification of CD44, epithelial surface antigen (EpCAM), and CD166 as alternative colon CSC markers (Dalerba et al., 2007). CD166 is a mesenchymal stem cell marker whose expression is correlated with poor prognosis in colon cancer patients (Weichert et al., 2004). Compared to CD44⁻/EpCAM^{low} cells, CD44⁺/EpCAM^{high} cells from primary tumors show high tumorigenic activity in NOD/SCID mice. Moreover, CD166⁺ cells in the CD44⁺/EpCAM^{high} cell fraction contribute to the tumorigenic activity of colon CSCs. In addition to CD133, CD44, EpCAM, and CD166, the expression of leucine-rich repeat-containing G-protein-coupled receptor 5 (*Lgr5*) varies among

colorectal cancer (CRC) cases and is significantly correlated with lymphatic and vascular invasion, lymph node metastasis, and drug resistance (Vermeulen et al., 2008; Merlos-Suarez et al., 2011; Kobayashi et al., 2012).

Iliopoulos et al. reported that the expression of miR-193a is inversely correlated with *K-RAS* and plasminogen activator urokinase (*PLAU*) expression in human colon adenocarcinomas, and that miR-193 expression inhibits tumorigenicity and invasiveness by directly targeting *K-RAS* and *PLAU*, respectively (Iliopoulos et al., 2011). MiR-451 is another regulator of CSC properties such as self-renewal, tumorigenicity, and drug resistance. In spheroid cell culture, downregulation of miR-451 induces the upregulation of macrophage migration inhibitory factor (MIF) and COX-2, resulting in the acquisition of self-renewal and tumorigenic properties (Bitarte et al., 2011). MIF and Cox-2 are involved in the activation of the Wnt pathway, which is functionally essential for the maintenance of colon CSCs (Vermeulen et al., 2010), suggesting that miR-451 could regulate the properties of colon CSCs by suppressing the Wnt pathway.

Notch signaling is frequently activated in CRCs, and is dysregulated directly by epigenetic and genetic changes and indirectly by synergistic interactions with the Wnt pathway, which is also activated in CRC (Taketo, 2011). Notch signaling promotes the self-renewal activity of intestine and colon stem cells (Taketo, 2011). Therefore, colon CSCs in CRC are thought to arise from, or at least share common properties with, normal colon stem cells (Clevers, 2011; O'Brien et al., 2012). Bu et al. reported that miR-34a determines whether colon CSCs undergo symmetric or asymmetric division, and that inhibition of asymmetric cell division suppresses tumorigenicity (Bu et al., 2013). MiR-34a inhibits Notch signaling by directly targeting Notch receptors (Li et al., 2009), suggesting that the upregulation of miR-34a weakens Notch signaling and promotes the generation of daughter cells (non-CSCs), whereas low miR-34a levels promote Notch signaling and lead to the maintenance of CSCs. This study also demonstrated that the expression level of miR-34a correlates more closely with the differentiation of daughter cells than the presence of Numb, which also suppresses Notch signaling by promoting the degradation of membrane-bound Notch and its intracellular domain (Bu et al., 2013).

PROSTATE CSCs

In prostate cancer (PCa), $\alpha_2\beta_1$ integrin, CD133, and CD44 were initially used to identify and isolate CSCs (Collins et al., 2005; Patrawala et al., 2006, 2007). Patrawala et al. reported that CD44⁺ PCa cells have higher proliferative, tumorigenic, and metastatic potentials than CD44⁻ PCa cells (Patrawala et al., 2006), and showed that androgen receptor (AR)-negative CD44⁺ PCa cells differentiate into AR-positive CD44⁻ PCa cells. Consistent with this report, prostate-specific antigen (PSA)-negative or -low PCa cells that are resistant to androgen ablation have a highly tumorigenic phenotype (Qin et al., 2012). In addition, PSA^{-/low} PCa cells generate PSA⁺ PCa cells through asymmetric cell division, and highly tumorigenic PSA^{-/low} PCa cells are characterized by an ALDH⁺/CD44⁺/ $\alpha_2\beta_1$ integrin⁺ phenotype (Qin et al., 2012).

Liu et al. reported that miR-34a is downregulated in CD44⁺ PCa cells purified from xenografts and primary tumors, and

that miR-34a directly regulates the expression of *CD44* at the post-transcriptional level by binding to its 3'UTR (Liu et al., 2011). Expression of miR-34a in CD44⁺ PCa cells inhibits tumor migration and metastasis in a xenograft model (Liu et al., 2011), and miR-34a inhibits Notch and AR signaling in PCa cells (Li et al., 2009; Kashat et al., 2012), suggesting that miR-34a suppresses the self-renewal activity of CSCs in PCa cells.

Another miRNA that regulates CSC properties is miR-320, which acts by directly targeting β -catenin in PCa cells (Hsieh et al., 2013). miR-320 and β -catenin expression is inversely correlated in CD44⁺ PCa cells. Furthermore, gene expression profiling of miR-320-overexpressing PCa cells showed a significant decrease in downstream target genes of the Wnt/ β -catenin pathway and CSC markers (Hsieh et al., 2013).

THERAPEUTIC APPROACHES TO TARGET CSCs

The development of therapies against CSCs has resulted in the establishment of a new generation of cancer therapeutics, which is particularly important in the treatment of intractable cancers. Since CSCs are molecularly distinct from non-CSCs and bulk tumor cells, a high-throughput screening approach was used to identify small compounds that eliminate or reduce levels of CSCs (Gupta et al., 2009; Sachlos et al., 2012). Gupta et al. identified salinomycin as a selective inhibitor of breast CSCs (Gupta et al., 2009) by screening a library of 16,000 natural and commercial chemical compounds in a search for small compounds capable of killing breast CSCs. Although the precise molecular mechanisms underlying the elimination of CSCs by salinomycin are not fully understood, several studies have improved our understanding of the mechanisms and pharmacological action of salinomycin in human CSCs (Fuchs et al., 2010; Lu et al., 2011; Tang et al., 2011). Systemic salinomycin therapy induces a marked regression of subcutaneous thoracic metastases of breast cancer, and combination therapy of salinomycin with erlotinib resulted in significant tumor regression in metastatic squamous cell carcinoma (Naujokat and Steinhart, 2012).

High-throughput screening using neoplastic and normal human pluripotent stem cells (hPSC) showed that among 590 compounds, only thioridazine significantly promoted differentiation of neoplastic hPSCs but not of normal hPSCs (Sachlos et al., 2012). Thioridazine acts through dopamine receptors (dopamine receptor1-5) (Seeman and Lee, 1975), indicating that its selective interference with human CSCs is mediated by dopamine receptor antagonism.

The development of therapies against CSCs is challenging because both bulk cancer cells and CSCs must be eliminated. As CSCs are molecularly distinct from bulk tumor cells, they can be targeted by exploiting their molecular differences as described above (Tables 1, 2). One of the most promising approaches is the cell based delivery of miRNAs or miRNA inhibitors. Several studies demonstrated that miRNAs are secreted through "exosomes," which are small endosome-derived vesicles (30–100 nm) secreted from different cell types, such as dendritic cells, hepatocyte, and tumor cells (Mittelbrunn et al., 2011; Luga et al., 2012; Ramakrishnaiah et al., 2013). The exosome secreted from mesenchymal stem cells (MSC) is selectively transferred to the glioblastoma multiforme (GBM) (Munoz et al., 2013).

Since miR-9 is involved in the upregulation of p-glycoprotein, Munoz et al. developed an MSC derived exosome containing anti-miR-9 that efficiently suppressed p-glycoprotein expression in the temozolomide-resistant GBM.

The glycosylation pattern of CSC markers on CSCs is different from normal stem cells (Karsten and Goletz, 2013). Some CSC markers such as CD44 and CD133 are also expressed in normal stem and progenitor cells (Karsten and Goletz, 2013), which might have negative implications for the development of CSC-targeted delivery. This problem could be addressed by the development of liposomes or nanoparticles conjugated to antibodies against CSC specific glycans that permit the selective delivery of CSC suppressive miRNAs or small molecules.

Recent studies have shown that several dietary compounds can directly or indirectly affect the properties of CSCs (Li et al., 2011). Therefore, natural dietary compounds have received increasing attention in cancer chemoprevention, and several natural compounds that induce the elimination or differentiation of breast CSCs have been identified (Kakarala et al., 2010; Li et al., 2010; Hagiwara et al., 2012). Resveratrol is a non-toxic natural product that is found in grapes, berries, peanuts and red wine (Aziz et al., 2003). Nowadays, resveratrol is widely consumed as a nutritional supplement (Prasad, 2012), and its multifaceted biological effects include anti-mutagenic and anti-cancer properties (Prasad, 2012; Patel et al., 2013). Hagiwara et al. found that resveratrol enhances miRNA functions through the upregulation of Ago2 expression, which leads to the suppression of CSC properties (Hagiwara et al., 2012). These results suggest that the identification of non-toxic natural compounds capable of suppressing the properties of CSCs through the regulation of miRNA expression is a promising approach to support conventional chemotherapy.

CONCLUSIONS

Accumulating lines of evidence have shown that the heterogeneity and plasticity of cancer cells is reflected in the transition from a non-CSC to a CSC phenotype. Therefore, clinical oncologists and cancer researchers need to determine which cancer cells have the potential to contribute to tumor initiation and progression, including therapeutic resistance and metastasis. Several studies reviewed here have shown that miRNAs can function as tumor suppressors or oncogenes and play important roles in various aspects of CSC properties. In this regard, miRNAs are considered to be functional markers of CSCs. Therefore, a more detailed understanding of the function of miRNAs in CSC biology may improve cancer treatments and possibly lead to the clinical application of miRNAs in cancer diagnosis, treatment, and prognosis.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control of Japan; a grant-in-aid for Scientific Research on Priority Areas Cancer from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

REFERENCES

- Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3983–3988. doi: 10.1073/pnas.0530291100
- Arnold, C. P., Tan, R., Zhou, B., Yue, S. B., Schaffert, S., Biggs, J. R., et al. (2011). MicroRNA programs in normal and aberrant stem and progenitor cells. *Genome Res.* 21, 798–810. doi: 10.1101/gr.111385.110
- Azim, M. H., Kumar, R., and Ahmad, N. (2003). Cancer chemoprevention by resveratrol: *in vitro* and *in vivo* studies and the underlying mechanisms (review). *Int. J. Oncol.* 23, 17–28.
- Bernstein, E., Kim, S. Y., Carmell, M. A., Murchison, E. P., Alcorn, H., Li, M. Z., et al. (2003). Dicer is essential for mouse development. *Nat. Genet.* 35, 215–217. doi: 10.1038/ng1253
- Bitarte, N., Bandres, E., Boni, V., Zarate, R., Rodriguez, J., Gonzalez-Huarriz, M., et al. (2011). MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. *Stem Cells* 29, 1661–1671. doi: 10.1002/stem.741
- Boiko, A. D., Razorenova, O. V., Van De Rijn, M., Swetter, S. M., Johnson, D. L., Ly, D. P., et al. (2010). Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. *Nature* 466, 133–137. doi: 10.1038/nature09161
- Bonnet, D., and Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* 3, 730–737. doi: 10.1038/nm0797-730
- Bu, P., Chen, K. Y., Chen, J. H., Wang, L., Walters, J., Shin, Y. J., et al. (2013). A microRNA miR-34a-regulated bimodal switch targets notch in colon cancer stem cells. *Cell Stem Cell* 12, 602–615. doi: 10.1016/j.stem.2013.03.002
- Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., et al. (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep.* 9, 582–589. doi: 10.1038/embor.2008.74
- Choi, Y. J., Lin, C. P., Ho, J. J., He, X., Okada, N., Bu, P., et al. (2011). miR-34 miRNAs provide a barrier for somatic cell reprogramming. *Nat. Cell Biol.* 13, 1353–1360. doi: 10.1038/ncb2366
- Cimmino, A., Calin, G. A., Fabbri, M., Iorio, M. V., Ferracin, M., Shimizu, M., et al. (2005). miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13944–13949. doi: 10.1073/pnas.0506654102
- Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nat. Med.* 17, 313–319. doi: 10.1038/nm.2304
- Cohnheim, J. (1875). Congenitales, quergestreiftes Muskelsarkom der Nieren. *Virchows Arch.* 65:64. doi: 10.1007/BF01978936
- Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., and Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* 65, 10946–10951. doi: 10.1158/0008-5472.CAN-05-2018
- Colmont, C. S., Harding, K. G., Piguet, V., and Patel, G. K. (2012). Human skin cancer stem cells: a tale of mice and men. *Exp. Dermatol.* 21, 576–580. doi: 10.1111/j.1600-0625.2012.01533.x
- Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N., et al. (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7024–7029. doi: 10.1073/pnas.0602266103
- Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W., et al. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10158–10163. doi: 10.1073/pnas.0703478104
- Eramo, A., Lotti, F., Sette, G., Pillozzi, E., Biffoni, M., Di Virgilio, A., et al. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ.* 15, 504–514. doi: 10.1038/sj.cdd.4402283
- Fang, D., Nguyen, T. K., Leishear, K., Finko, R., Kulp, A. N., Hotz, S., et al. (2005). A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res.* 65, 9328–9337. doi: 10.1158/0008-5472.CAN-05-1343
- Fuchs, D., Daniel, V., Sadeghi, M., Opelz, G., and Naujokat, C. (2010). Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. *Biochem. Biophys. Res. Commun.* 394, 1098–1104. doi: 10.1016/j.bbrc.2010.03.138
- Garzia, L., Andolfo, I., Cusanelli, E., Marino, N., Petrosino, G., De Martino, D., et al. (2009). MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. *PLoS ONE* 4:e4998. doi: 10.1371/journal.pone.0004998
- Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 1, 555–567. doi: 10.1016/j.stem.2007.08.014
- Giordano, A., Gao, H., Cohen, E. N., Anfossi, S., Khoury, J., Hess, K., et al. (2013). Clinical relevance of cancer stem cells in bone marrow of early breast cancer patients. *Ann. Oncol.* 24, 2515–2521. doi: 10.1093/annonc/mdt223
- Godlewski, J., Newton, H. B., Chiocca, E. A., and Lawler, S. E. (2010). MicroRNAs and glioblastoma; the stem cell connection. *Cell Death Differ.* 17, 221–228. doi: 10.1038/cdd.2009.71
- Godlewski, J., Nowicki, M. O., Bronisz, A., Williams, S., Otsuki, A., Nuovo, G., et al. (2008). Targeting of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. *Cancer Res.* 68, 9125–9130. doi: 10.1158/0008-5472.CAN-08-2629
- Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., et al. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* 432, 235–240. doi: 10.1038/nature03120
- Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., et al. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* 10, 593–601. doi: 10.1038/ncb1722
- Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A., et al. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* 138, 645–659. doi: 10.1016/j.cell.2009.06.034
- Hagiwara, K., Kosaka, N., Yoshioka, Y., Takahashi, R. U., Takeshita, F., and Ochiya, T. (2012). Stilbene derivatives promote Ago2-dependent tumour-suppressive microRNA activity. *Sci. Rep.* 2, 314. doi: 10.1038/srep00314
- Han, Y. C., Park, C. Y., Bhagat, G., Zhang, J., Wang, Y., Fan, J. B., et al. (2010). microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. *J. Exp. Med.* 207, 475–489. doi: 10.1084/jem.20090831
- He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., et al. (2005). A microRNA polycistron as a potential human oncogene. *Nature* 435, 828–833. doi: 10.1038/nature03552
- Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., et al. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1, 313–323. doi: 10.1016/j.stem.2007.06.002
- Hoey, T., Yen, W. C., Axelrod, F., Basi, J., Donigian, L., Dylla, S., et al. (2009). DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 5, 168–177. doi: 10.1016/j.stem.2009.05.019
- Hsieh, I. S., Chang, K. C., Tsai, Y. T., Ke, J. Y., Lu, P. J., Lee, K. H., et al. (2013). MicroRNA-320 suppresses the stem cell-like characteristics of prostate cancer cells by downregulating the Wnt/beta-catenin signaling pathway. *Carcinogenesis* 34, 530–538. doi: 10.1093/carcin/bgs371
- Ibarra, I., Erlich, Y., Muthuswamy, S. K., Sachidanandam, R., and Hannon, G. J. (2007). A role for microRNAs in maintenance of mouse mammary epithelial progenitor cells. *Genes Dev.* 21, 3238–3243. doi: 10.1101/gad.1616307
- Iliopoulos, D., Lindahl-Alten, M., Polytarouchou, C., Hirsch, H. A., Tschlis, P. N., and Struhl, K. (2010). Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. *Mol. Cell* 39, 761–772. doi: 10.1016/j.molcel.2010.08.013
- Iliopoulos, D., Rotem, A., and Struhl, K. (2011). Inhibition of miR-193a expression by Max and RXRalpha activates K-Ras and PLAU to mediate distinct aspects of cellular transformation. *Cancer Res.* 71, 5144–5153. doi: 10.1158/0008-5472.CAN-11-0425
- Iorio, M. V., and Croce, C. M. (2012). MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol. Med.* 4, 143–159. doi: 10.1002/emmm.201100209
- Jamieson, C. H., Ailles, L. E., Dylla, S. J., Muijtens, M., Jones, C., Zehnder, J. L., et al. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N. Engl. J. Med.* 351, 657–667. doi: 10.1056/NEJMoa040258
- Johnson, S. M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., et al. (2005). RAS is regulated by the let-7 microRNA family. *Cell* 120, 635–647. doi: 10.1016/j.cell.2005.01.014
- Kakarala, M., Brenner, D. E., Korkaya, H., Cheng, C., Tazi, K., Ginestier, C., et al. (2010). Targeting breast stem cells with the cancer preventive

- compounds curcumin and piperine. *Breast Cancer Res. Treat.* 122, 777–785. doi: 10.1007/s10549-009-0612-x
- Karamboulas, C., and Ailles, L. (2013). Developmental signaling pathways in cancer stem cells of solid tumors. *Biochim. Biophys. Acta* 1830, 2481–2495. doi: 10.1016/j.bbagen.2012.11.008
- Karsten, U., and Goletz, S. (2013). What makes cancer stem cell markers different? *Springerplus* 2, 301. doi: 10.1186/2193-1801-2-301
- Kashat, M., Azzouz, L., Sarkar, S. H., Kong, D., Li, Y., and Sarkar, F. H. (2012). Inactivation of AR and Notch-1 signaling by miR-34a attenuates prostate cancer aggressiveness. *Am. J. Transl. Res.* 4, 432–442.
- Kim, H., Lee, G., Ganat, Y., Papapetrou, E. P., Lipchina, I., Socci, N. D., et al. (2011). miR-371-3 expression predicts neural differentiation propensity in human pluripotent stem cells. *Cell Stem Cell* 8, 695–706. doi: 10.1016/j.stem.2011.04.002
- Kobayashi, S., Yamada-Okabe, H., Suzuki, M., Natori, O., Kato, A., Matsubara, K., et al. (2012). LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution. *Stem Cells* 30, 2631–2644. doi: 10.1002/stem.1257
- Korkaya, H., Paulson, A., Charafe-Jauffret, E., Ginestier, C., Brown, M., Dutcher, J., et al. (2009). Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol.* 7:e1000121. doi: 10.1371/journal.pbio.1000121
- Lee, A., Kessler, J. D., Read, T. A., Kaiser, C., Corbeil, D., Huttner, W. B., et al. (2005). Isolation of neural stem cells from the postnatal cerebellum. *Nat. Neurosci.* 8, 723–729. doi: 10.1038/nn1473
- Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75, 843–854. doi: 10.1016/0092-8674(93)90529-Y
- Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* 23, 4051–4060. doi: 10.1038/sj.emboj.7600385
- Li, C., Heidt, D. G., Dalerba, P., Burant, C. F., Zhang, L., Adsay, V., et al. (2007a). Identification of pancreatic cancer stem cells. *Cancer Res.* 67, 1030–1037. doi: 10.1158/0008-5472.CAN-06-2030
- Li, O., Li, J., and Droge, P. (2007b). DNA architectural factor and proto-oncogene HMGA2 regulates key developmental genes in pluripotent human embryonic stem cells. *FEBS Lett.* 581, 3533–3537. doi: 10.1016/j.febslet.2007.06.072
- Li, Y., Guessous, F., Zhang, Y., Dipierro, C., Kefas, B., Johnson, E., et al. (2009). MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. *Cancer Res.* 69, 7569–7576. doi: 10.1158/0008-5472.CAN-09-0529
- Li, Y., Wicha, M. S., Schwartz, S. J., and Sun, D. (2011). Implications of cancer stem cell theory for cancer chemoprevention by natural dietary compounds. *J. Nutr. Biochem.* 22, 799–806. doi: 10.1016/j.jnutbio.2010.11.001
- Li, Y., Zhang, T., Korkaya, H., Liu, S., Lee, H. F., Newman, B., et al. (2010). Sulforaphane, a dietary component of broccoli/broccoli sprouts, inhibits breast cancer stem cells. *Clin. Cancer Res.* 16, 2580–2590. doi: 10.1158/1078-0432.CCR-09-2937
- Lin, S. L., Chang, D. C., Chang-Lin, S., Lin, C. H., Wu, D. T., Chen, D. T., et al. (2008). Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* 14, 2115–2124. doi: 10.1261/rna.1162708
- Liu, C., Kelnar, K., Liu, B., Chen, X., Calhoun-Davis, T., Li, H., et al. (2011). The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat. Med.* 17, 211–215. doi: 10.1038/nm.2284
- Liu, N., Chen, N. Y., Cui, R. X., Li, W. F., Li, Y., Wei, R. R., et al. (2012). Prognostic value of a microRNA signature in nasopharyngeal carcinoma: a microRNA expression analysis. *Lancet Oncol.* 13, 633–641. doi: 10.1016/S1470-2045(12)70102-X
- Liu, S., Dontu, G., Mantle, I. D., Patel, S., Ahn, N. S., Jackson, K. W., et al. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res.* 66, 6063–6071. doi: 10.1158/0008-5472.CAN-06-0054
- Lu, D., Choi, M. Y., Yu, J., Castro, J. E., Kipps, T. J., and Carson, D. A. (2011). Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemia cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13253–13257. doi: 10.1073/pnas.1110431108
- Luga, V., Zhang, L., Vitoria-Petit, A. M., Ogunjimi, A. A., Inanlou, M. R., Chiu, E., et al. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151, 1542–1556. doi: 10.1016/j.cell.2012.11.024
- Mak, A. B., Nixon, A. M., Kittanakom, S., Stewart, J. M., Chen, G. I., Curak, J., et al. (2012). Regulation of CD133 by HDAC6 promotes beta-catenin signaling to suppress cancer cell differentiation. *Cell Rep.* 2, 951–963. doi: 10.1016/j.celrep.2012.09.016
- Mandke, P., Wyatt, N., Fraser, J., Bates, B., Berberich, S. J., and Markey, M. P. (2012). MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame. *PLoS ONE* 7:e42034. doi: 10.1371/journal.pone.0042034
- Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., et al. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133, 704–715. doi: 10.1016/j.cell.2008.03.027
- Melton, C., Judson, R. L., and Belloch, R. (2010). Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature* 463, 621–626. doi: 10.1038/nature08725
- Merlos-Suarez, A., Barriga, F. M., Jung, P., Iglesias, M., Cespedes, M. V., Rossell, D., et al. (2011). The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell Stem Cell* 8, 511–524. doi: 10.1016/j.stem.2011.02.020
- Miraglia, S., Godfrey, W., Yin, A. H., Atkins, K., Warnke, R., Holden, J. T., et al. (1997). A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 90, 5013–5021.
- Mittelbrunn, M., Gutierrez-Vazquez, C., Villarroya-Beltri, C., Gonzalez, S., Sanchez-Cabo, F., Gonzalez, M. A., et al. (2011). Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat. Commun.* 2, 282. doi: 10.1038/ncomms1285
- Molofsky, A. V., Pardal, R., Iwashita, T., Park, I. K., Clarke, M. F., and Morrison, S. J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature* 425, 962–967. doi: 10.1038/nature02060
- Munoz, J. L., Bliss, S. A., Greco, S. J., Ramkissoon, S. H., Ligon, K. L., and Rameshwar, P. (2013). Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. *Mol. Ther. Nucleic Acids* 2, e126. doi: 10.1038/mtna.2013.60
- Naujokat, C., and Steinhart, R. (2012). Salinomycin as a drug for targeting human cancer stem cells. *J. Biomed. Biotechnol.* 2012, 950658. doi: 10.1155/2012/950658
- Nishi, K., Nishi, A., Nagasawa, T., and Ui-Tei, K. (2013). Human TNRC6A is an Argonaute-navigator protein for microRNA-mediated gene silencing in the nucleus. *RNA* 19, 17–35. doi: 10.1261/rna.034769.112
- O'Brien, C. A., Kreso, A., Ryan, P., Hermans, K. G., Gibson, L., Wang, Y., et al. (2012). ID1 and ID3 regulate the self-renewal capacity of human colon cancer-initiating cells through p21. *Cancer Cell* 21, 777–792. doi: 10.1016/j.ccr.2012.04.036
- O'Brien, C. A., Pollett, A., Gallinger, S., and Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 445, 106–110. doi: 10.1038/nature05372
- O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., Boldin, M. P., Taganov, K. D., Nicoll, J., et al. (2008). Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* 205, 585–594. doi: 10.1084/jem.20072108
- Orom, U. A., Nielsen, F. C., and Lund, A. H. (2008). MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol. Cell* 30, 460–471. doi: 10.1016/j.molcel.2008.05.001
- Pang, R., Law, W. L., Chu, A. C., Poon, J. T., Lam, C. S., Chow, A. K., et al. (2010). A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. *Cell Stem Cell* 6, 603–615. doi: 10.1016/j.stem.2010.04.001
- Park, I. K., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., et al. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423, 302–305. doi: 10.1038/nature01587
- Park, S. M., Gaur, A. B., Lengyel, E., and Peter, M. E. (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* 22, 894–907. doi: 10.1101/gad.1640608
- Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408, 86–89. doi: 10.1038/35040556
- Patel, K. R., Andreadi, C., Britton, R. G., Horner-Glister, E., Karmokar, A., Sale, S., et al. (2013). Sulfate metabolites provide an intracellular pool for resveratrol generation and induce autophagy with senescence. *Sci. Transl. Med.* 5, 205ra133. doi: 10.1126/scitranslmed.3005870

- Patrawala, L., Calhoun-Davis, T., Schneider-Broussard, R., and Tang, D. G. (2007). Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. *Cancer Res.* 67, 6796–6805. doi: 10.1158/0008-5472.CAN-07-0490
- Patrawala, L., Calhoun, T., Schneider-Broussard, R., Li, H., Bhatia, B., Tang, S., et al. (2006). Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 25, 1696–1708. doi: 10.1038/sj.onc.1209327
- Pietersen, A. M., Evers, B., Prasad, A. A., Tanger, E., Cornelissen-Steijger, P., Jonkers, J., et al. (2008). Bmi1 regulates stem cells and proliferation and differentiation of committed cells in mammary epithelium. *Curr. Biol.* 18, 1094–1099. doi: 10.1016/j.cub.2008.06.070
- Prasad, K. (2012). Resveratrol, wine, and atherosclerosis. *Int. J. Angiol.* 21, 7–18. doi: 10.1055/s-0032-1306417
- Qin, J., Liu, X., Laffin, B., Chen, X., Choy, G., Jeter, C. R., et al. (2012). The PSA(-/lo) prostate cancer cell population harbors self-renewing long-term tumor-propagating cells that resist castration. *Cell Stem Cell* 10, 556–569. doi: 10.1016/j.stem.2012.03.009
- Ramakrishnaiah, V., Thumann, C., Fofana, I., Habersetzer, F., Pan, Q., De Ruiter, P. E., et al. (2013). Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, 13109–13113. doi: 10.1073/pnas.1221899110
- Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901–906. doi: 10.1038/35002607
- Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* 414, 105–111. doi: 10.1038/35102167
- Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., et al. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature* 445, 111–115. doi: 10.1038/nature05384
- Sachlos, E., Risueno, R. M., Laronde, S., Shapovalova, Z., Lee, J. H., Russell, J., et al. (2012). Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* 149, 1284–1297. doi: 10.1016/j.cell.2012.03.049
- Schraivogel, D., Weinmann, L., Beier, D., Tabatabai, G., Eichner, A., Zhu, J. Y., et al. (2011). CAMTA1 is a novel tumour suppressor regulated by miR-9/9* in glioblastoma stem cells. *EMBO J.* 30, 4309–4322. doi: 10.1038/emboj.2011.301
- Seeman, P., and Lee, T. (1975). Antipsychotic drugs: direct correlation between clinical potency and presynaptic action on dopamine neurons. *Science* 188, 1217–1219. doi: 10.1126/science.1145194
- Shiohama, A., Sasaki, T., Noda, S., Minoshima, S., and Shimizu, N. (2003). Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. *Biochem. Biophys. Res. Commun.* 304, 184–190. doi: 10.1016/S0006-291X(03)00554-0
- Shimono, Y., Zabala, M., Cho, R. W., Lobo, N., Dalerba, P., Qian, D., et al. (2009). Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. *Cell* 138, 592–603. doi: 10.1016/j.cell.2009.07.011
- Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., et al. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res.* 63, 5821–5828.
- Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., et al. (2004). Identification of human brain tumour initiating cells. *Nature* 432, 396–401. doi: 10.1038/nature03128
- Sinkkonen, L., Hugenschmidt, T., Berninger, P., Gaidatzis, D., Mohn, F., Artus-Revel, C. G., et al. (2008). MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* 15, 259–267. doi: 10.1038/nsmb.1391
- Smirnova, L., Grafe, A., Seiler, A., Schumacher, S., Nitsch, R., and Wulczyn, F. G. (2005). Regulation of miRNA expression during neural cell specification. *Eur. J. Neurosci.* 21, 1469–1477. doi: 10.1111/j.1460-9568.2005.03978.x
- Song, S. J., Ito, K., Ala, U., Kats, L., Webster, K., Sun, S. M., et al. (2013a). The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation. *Cell Stem Cell* 13, 87–101. doi: 10.1016/j.stem.2013.06.003
- Song, S. J., Poliseno, L., Song, M. S., Ala, U., Webster, K., Ng, C., et al. (2013b). MicroRNA-antagonism regulates breast cancer stemness and metastasis via TET-family-dependent chromatin remodeling. *Cell* 154, 311–324. doi: 10.1016/j.cell.2013.06.026
- Stadler, B., Ivanovska, I., Mehta, K., Song, S., Nelson, A., Tan, Y., et al. (2010). Characterization of microRNAs involved in embryonic stem cell states. *Stem Cells Dev.* 19, 935–950. doi: 10.1089/scd.2009.0426
- Takaishi, S., Okumura, T., Tu, S., Wang, S. S., Shibata, W., Vigneshwaran, R., et al. (2009). Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 27, 1006–1020. doi: 10.1002/stem.30
- Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., et al. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 64, 3753–3756. doi: 10.1158/0008-5472.CAN-04-0637
- Taketo, M. M. (2011). Reflections on the spread of metastasis to cancer prevention. *Cancer Prev. Res. (Phila.)* 4, 324–328. doi: 10.1158/1940-6207.CAPR-11-0046
- Takezaki, T., Hide, T., Takana, H., Nakamura, H., Kuratsu, J., and Kondo, T. (2011). Essential role of the Hedgehog signaling pathway in human glioma-initiating cells. *Cancer Sci.* 102, 1306–1312. doi: 10.1111/j.1349-7006.2011.01943.x
- Tang, Q. L., Zhao, Z. Q., Li, J. C., Liang, Y., Yin, J. Q., Zou, C. Y., et al. (2011). Salinomycin inhibits osteosarcoma by targeting its tumor stem cells. *Cancer Lett.* 311, 113–121. doi: 10.1016/j.canlet.2011.07.016
- Tavazoie, S. F., Alarcon, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P. D., et al. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* 451, 147–152. doi: 10.1038/nature06487
- Tazawa, H., Tsuchiya, N., Izumiya, M., and Nakagama, H. (2007). Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15472–15477. doi: 10.1073/pnas.0707351104
- To, K. K., Zhan, Z., Litman, T., and Bates, S. E. (2008). Regulation of ABCG2 expression at the 3' untranslated region of its mRNA through modulation of transcript stability and protein translation by a putative microRNA in the S1 colon cancer cell line. *Mol. Cell Biol.* 28, 5147–5161. doi: 10.1128/MCB.00331-08
- Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., et al. (2000). Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 97, 14720–14725. doi: 10.1073/pnas.97.26.14720
- Uchino, K., Takeshita, F., Takahashi, R. U., Kosaka, N., Fujiwara, K., Naruoka, H., et al. (2013). Therapeutic effects of microRNA-582-5p and -3p on the inhibition of bladder cancer progression. *Mol. Ther.* 21, 610–619. doi: 10.1038/mt.2012.269
- Varnat, F., Duquet, A., Malerba, M., Zbinden, M., Mas, C., Gervaz, P., et al. (2009). Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Mol. Med.* 1, 338–351. doi: 10.1002/emmm.200900039
- Vasudevan, S., Tong, Y., and Steitz, J. A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. *Science* 318, 1931–1934. doi: 10.1126/science.1149460
- Vermeulen, L., De Sousa, E. M. F., Van Der Heijden, M., Cameron, K., De Jong, J. H., Borovski, T., et al. (2010). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat. Cell Biol.* 12, 468–476. doi: 10.1038/ncb2048
- Vermeulen, L., Todaro, M., De Sousa Mello, F., Sprick, M. R., Kemper, K., Perez Alea, M., et al. (2008). Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13427–13432. doi: 10.1073/pnas.0805706105
- Viswanathan, S. R., Daley, G. Q., and Gregory, R. I. (2008). Selective blockade of microRNA processing by Lin28. *Science* 320, 97–100. doi: 10.1126/science.1154040
- Volinia, S., and Croce, C. M. (2013). Prognostic microRNA/mRNA signature from the integrated analysis of patients with invasive breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* 110, 7413–7417. doi: 10.1073/pnas.1304977110
- Wang, Y., Baskerville, S., Shenoy, A., Babiarz, J. E., Baehner, L., and Blelloch, R. (2008). Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nat. Genet.* 40, 1478–1483. doi: 10.1038/ng.250
- Wang, Y., Medvid, R., Melton, C., Jaenisch, R., and Blelloch, R. (2007). DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat. Genet.* 39, 380–385. doi: 10.1038/ng1969

- Wei, Y., Jiang, Y., Zou, F., Liu, Y., Wang, S., Xu, N., et al. (2013). Activation of PI3K/Akt pathway by CD133-p85 interaction promotes tumorigenic capacity of glioma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6829–6834. doi: 10.1073/pnas.1217002110
- Weichert, W., Knosel, T., Bellach, J., Dietel, M., and Kristiansen, G. (2004). ALCAM/CD166 is overexpressed in colorectal carcinoma and correlates with shortened patient survival. *J. Clin. Pathol.* 57, 1160–1164. doi: 10.1136/jcp.2004.016238
- Wicha, M. S., Liu, S., and Dontu, G. (2006). Cancer stem cells: an old idea—a paradigm shift. *Cancer Res.* 66, 1883–1890. discussion: 1895–1886. doi: 10.1158/0008-5472.CAN-05-3153
- Yamagishi, H., and Srivastava, D. (2003). Unraveling the genetic and developmental mysteries of 22q11 deletion syndrome. *Trends Mol. Med.* 9, 383–389. doi: 10.1016/S1471-4914(03)00141-2
- Yamashita, T., Honda, M., Nio, K., Nakamoto, Y., Yamashita, T., Takamura, H., et al. (2010). Oncostatin m renders epithelial cell adhesion molecule-positive liver cancer stem cells sensitive to 5-Fluorouracil by inducing hepatocytic differentiation. *Cancer Res.* 70, 4687–4697. doi: 10.1158/0008-5472.CAN-09-4210
- Yang, X., Du, W. W., Li, H., Liu, F., Khorshidi, A., Rutnam, Z. J., et al. (2013). Both mature miR-17-5p and passenger strand miR-17-3p target TIMP3 and induce prostate tumor growth and invasion. *Nucleic Acids Res.* 41, 9688–9704. doi: 10.1093/nar/gkt680
- Yin, A. H., Miraglia, S., Zanjani, E. D., Almeida-Porada, G., Ogawa, M., Leary, A. G., et al. (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 90, 5002–5012.
- Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., et al. (2007a). let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell* 131, 1109–1123. doi: 10.1016/j.cell.2007.10.054
- Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., et al. (2007b). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920. doi: 10.1126/science.1151526
- Yu, L., Baxter, P. A., Voicu, H., Gurusiddappa, S., Zhao, Y., Adesina, A., et al. (2010). A clinically relevant orthotopic xenograft model of ependymoma that maintains the genomic signature of the primary tumor and preserves cancer stem cells *in vivo*. *Neuro Oncol.* 12, 580–594. doi: 10.1093/neuonc/nop056
- Yu, M., Bardia, A., Wittner, B. S., Stott, S. L., Smas, M. E., Ting, D. T., et al. (2013). Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* 339, 580–584. doi: 10.1126/science.1228522

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 October 2013; accepted: 03 December 2013; published online: 03 January 2014.

Citation: Takahashi R-u, Miyazaki H and Ochiya T (2014) The role of microRNAs in the regulation of cancer stem cells. *Front. Genet.* 4:295. doi: 10.3389/fgene.2013.00295
This article was submitted to *Epigenomics and Epigenetics*, a section of the journal *Frontiers in Genetics*.

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Premature Termination of Reprogramming In Vivo Leads to Cancer Development through Altered Epigenetic Regulation

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<http://dx.doi.org/10.1016/j.cell.2014.01.005>

SUMMARY

Cancer is believed to arise primarily through accumulation of genetic mutations. Although induced pluripotent stem cell (iPSC) generation does not require changes in genomic sequence, iPSCs acquire unlimited growth potential, a characteristic shared with cancer cells. Here, we describe a murine system in which reprogramming factor expression in vivo can be controlled temporally with doxycycline (Dox). Notably, transient expression of reprogramming factors in vivo results in tumor development in various tissues consisting of undifferentiated dysplastic cells exhibiting global changes in DNA methylation patterns. The Dox-withdrawn tumors arising in the kidney share a number of characteristics with Wilms tumor, a common pediatric kidney cancer. We also demonstrate that iPSCs derived from Dox-withdrawn kidney tumor cells give rise to nonneoplastic kidney cells in mice, proving that they have not undergone irreversible genetic transformation. These findings suggest that epigenetic regulation associated with iPSC derivation may drive development of particular types of cancer.

INTRODUCTION

Induced pluripotent stem cells (iPSCs) can be established from differentiated somatic cells by the forced induction of four transcription factors: *Oct3/4*, *Klf4*, *Sox2*, and *c-Myc* (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007; Woltjen et al., 2009). To achieve somatic cell reprogramming, multiple cellular

processes act synergistically in a sequential manner (Brambrink et al., 2008; Polo et al., 2012; Samavarchi-Tehrani et al., 2010). Despite extensive studies, the precise mechanism of somatic cell reprogramming still remains unclear (Rais et al., 2013). It is known that non-iPSC-like colonies often appear at the intermediate stage of cellular reprogramming in vitro. In addition, there are several reports describing partial iPSCs that deviate successful reprogramming (Fussner et al., 2011; Mikkelsen et al., 2008; Sridharan et al., 2009). However, the characteristics of such failed reprogramming states are largely unknown, and no study has elucidated the failed reprogramming state from cell types other than fibroblasts.

The process of iPSC derivation shares many characteristics with cancer development. During reprogramming, somatic differentiated cells acquire the properties of self-renewal along with unlimited proliferation and exhibit global alterations of the transcriptional program, which are also critical events during carcinogenesis (Ben-Porath et al., 2008). The metabolic switch to glycolysis that occurs during somatic cell reprogramming is similarly observed in cancer development (Folmes et al., 2011). Such similarities suggest that reprogramming processes and cancer development may be partly promoted by overlapping mechanisms (Hong et al., 2009). Practically, the forced induction of the critical reprogramming factor *Oct3/4* in adult somatic cells results in dysplastic growth in epithelial tissues through the inhibition of cellular differentiation in a manner similar to that in embryonic cells (Hochedlinger et al., 2005). These studies provided a possible link between transcription-factor-mediated reprogramming and cancer development.

To elucidate the involvement of failed reprogramming in cancer development, in the present study, we generated an in vivo reprogramming mouse system using reprogramming factor-inducible alleles and examined the effects of reprogramming factor expression in somatic cells in vivo. We show that failed reprogramming-associated cells behave similarly to cancer cells



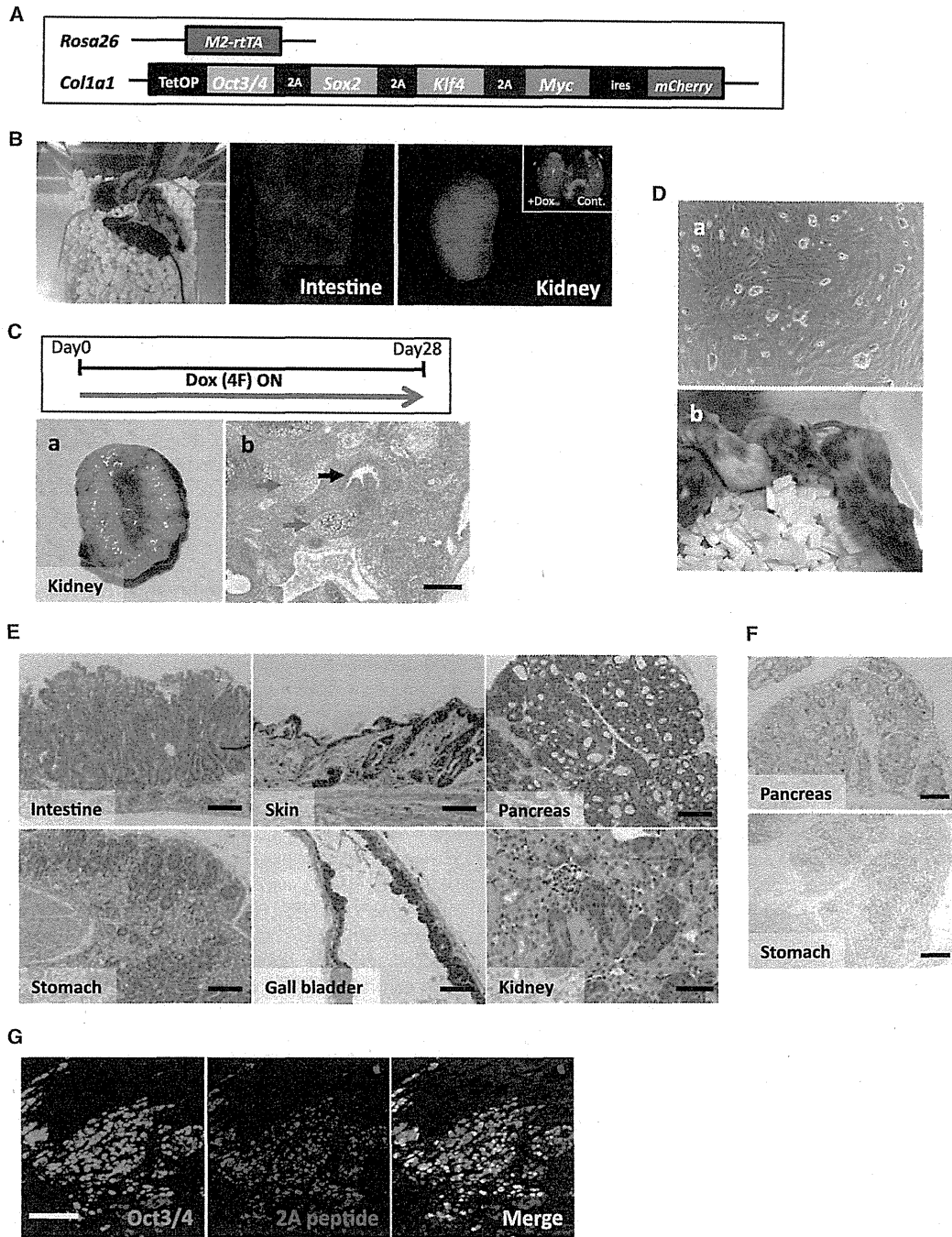


Figure 1. Reprogramming of Somatic Cells In Vivo

(A) Generation of four-factor-inducible ESCs. TetOP, tetracycline-dependent promoter.

(B) Generation of chimeric mice using OSKM-inducible ESCs. mCherry signals could be detected in various organs after Dox treatment for 3 days.

(C) Treatment of chimeric mice with Dox for 28 days resulted in the development of multiple tumors containing pluripotent stem cells. (a) A representative macroscopic image of the cut surface of the kidney tumor. (b) A histological section of the kidney tumor showing the differentiation of tumor cells into three germ layers, indicating teratoma formation. The blue, red, and black arrows represent neuronal, cartilage, and glandular epithelial components, respectively. Scale bar, 200 μ m.

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and cause neoplasia resembling Wilms tumor, a childhood blastoma in the kidney. Moreover, we demonstrate that altered epigenetic regulations cause the abnormal growth of such failed reprogramming-associated cancer cells.

RESULTS

In Vivo Reprogrammable Mouse

To establish the reprogrammable mouse system, we generated embryonic stem cells (ESCs) in which reprogramming factors can be induced under the control of doxycycline (Dox) (Figure 1A) (Carey et al., 2010; Stadtfeld et al., 2010b). We used KH2 ESCs with the optimized reverse tetracycline-dependent transactivator at the *ROSA 26* locus (Beard et al., 2006). A polycistronic cassette encoding four reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) (Carey et al., 2010), followed by *ires-mCherry*, was targeted into the *Col1a1* gene locus under the tetracycline-dependent promoter of KH2 ESCs (Figure 1A).

Next, we generated chimeric mice via blastocyst injection of four-factor (4F)-inducible ESCs. To confirm inducible expression of the reprogramming factors and mCherry in vivo, Dox-containing water was provided to chimeric mice starting at 4 weeks of age. On day 3 of Dox treatment, we could detect the mCherry signal in various organs, including stomach, intestine, liver, pancreas, kidney, gallbladder, and skin (Figure 1B). We also confirmed the expression of reprogramming factors in germline-transmitted mouse tissues by quantitative RT-PCR (qRT-PCR) (Figure S1A available online).

Mouse embryonic fibroblasts (MEFs) containing these reprogramming factor-inducible alleles could give rise to iPSCs after Dox treatment in vitro (Figure S1B). We next asked whether responding somatic cells could be reprogrammed in vivo. The chimeric and germline-transmitted mice given Dox-containing water (2 mg/ml) from 4 weeks of age became morbid within 7–10 days and a few days, respectively. A small proportion of chimeric mice could be treated with Dox for 4 weeks, presumably because of a lower contribution of ESCs in responding tissues. Notably, mice treated with Dox for 4 weeks developed multiple tumors in several organs, such as the kidney and pancreas (Figure 1Ca), whereas tumor formation was never observed in nontreated mice ($n = 7$, 7 months of age). Histological analysis revealed that these tumors differentiated into three different germ layers, indicating that they are teratomas (Figure 1Cb). When teratoma cells were cultured ex vivo in the absence of Dox (no additional 4F expressions), iPSC-like cells were established (Figure 1Da). Importantly, the teratoma-derived iPSC-like cells contributed to adult chimeric mice when they were injected into blastocysts (Figure 1Db). Therefore, we

conclude that somatic cells can be reprogrammed in vivo to pluripotency in our reprogrammable mouse system.

Forced Expression of Reprogramming Factors In Vivo Leads to Rapid Expansion of Dysplastic Cells

We next examined the early changes after expression of reprogramming factors in somatic cells in vivo. After treatment of 4-week-old mice with Dox for 3–9 days, all mice developed dysplastic lesions in epithelial tissues of various organs (Figure 1E), although there were variations in severity of the phenotype among chimeras. Dysplastic cells proliferated actively, as revealed by Ki67 staining (Figure 1F). Abnormal proliferation of somatic cells was observed as early as 3 days after Dox treatment (Figure S1C), and by day 7, such dysplastic cell growth was detected even for pancreatic and kidney cells, which typically do not divide actively under physiological conditions (Figures 1E and 1F). Immunofluorescent analysis of Oct3/4 and the 2A peptide (forming transgene connections) demonstrated that the dysplastic cells expressed reprogramming factors (Figure 1G). Collectively, the forced expression of reprogramming factors caused dysplastic cell expansion of epithelial tissues in vivo.

The Fate of Early Dysplastic Cells after Withdrawal of Dox

To examine whether subsequent expansion of such dysplastic cells depends on the continuous expression of reprogramming factors, we withdrew Dox for 7 days after an initial 4- to 7-day treatment (Figure 2A). Although Dox treatment for 4–7 days caused active cell proliferation in a variety of tissues of all mice, we did not observe any dysplastic cells in some mice after withdrawal of Dox (Figure 2A; Table 1). Of particular note, mice treated with Dox for periods less than 5 days before withdrawal often revealed a lack of dysplastic cells (Table 1). These data suggest that early dysplastic cell growth requires continuous expression of reprogramming factors. We next investigated the fate of eliminated dysplastic proliferating cells after the withdrawal of Dox. Bromodeoxyuridine (BrdU) was injected into mice during Dox treatment to label proliferating cells caused by reprogramming factor expression during the first 7 days (Hochedlinger et al., 2005), and then mice were sacrificed after the withdrawal of Dox for 7 days, on day 14. Notably, BrdU-labeled cells were often observed in normal-looking pancreatic and kidney tissues at day 14 (Figure 2B). Furthermore, BrdU-labeled cells in the pancreatic islets also expressed insulin (Figure 2B). This suggests that the expanded cells caused by the transient expression of reprogramming factors were, at least in part, integrated into normal-looking tissues after Dox withdrawal.

(D) Teratomas contain pluripotent stem cells. (a) Ex vivo teratoma culture gave rise to iPSC-like colonies without Dox exposure. (b) Teratoma-derived iPSCs contributed to adult chimeric mice.

(E) Dysplastic cell expansion by the forced expression of reprogramming factors in vivo. The histology of various organs of mice treated with Dox for 3 to 9 days. Scale bars, 200 μm (intestine, skin, pancreas, stomach, and gall bladder) and 100 μm (kidney).

(F) Ki67 immunostaining revealed active proliferation of the dysplastic cells in the pancreas and stomach. Scale bars, 200 μm .

(G) Immunofluorescent staining for Oct3/4 and 2A peptide in the intestine of an OSKM chimeric mouse treated with Dox for 7 days. The 2A antibody used here recognizes both Oct3/4-P2A and Sox2-T2A. Dysplastic cells showed positive staining for both Oct3/4 and 2A. Scale bar, 50 μm .

See also Figure S1.

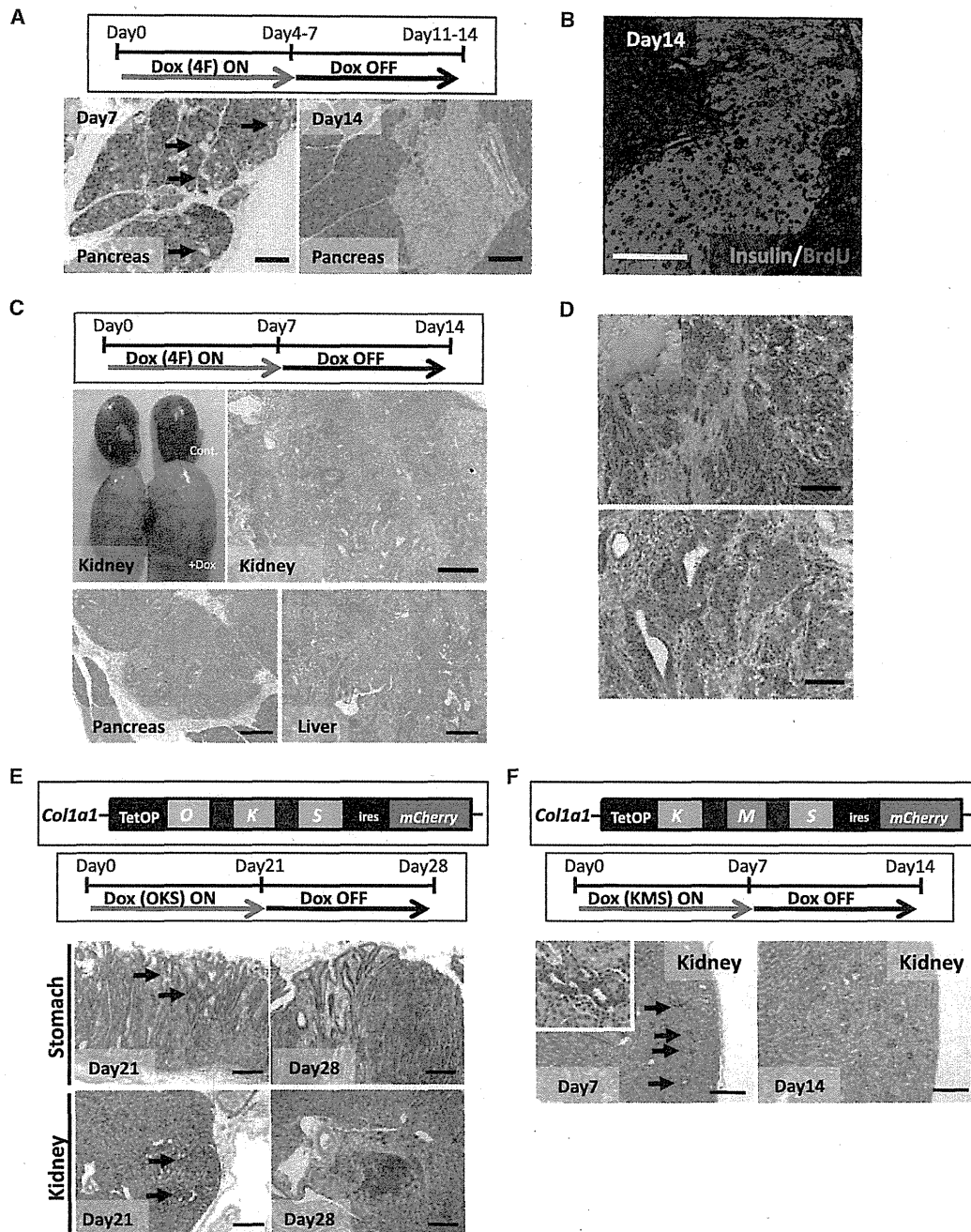


Figure 2. Transient Expression of Reprogramming Factors Causes Neoplasia

(A) A schematic drawing of the experiment and histological sections of the pancreas taken on days 7 and 14. Dysplastic cell growth was induced by treatment with Dox for 7 days (arrows on day 7). The pancreatic section taken on day 14 revealed normal histology. Scale bars, 200 μ m.

(B) Double immunofluorescence for insulin and BrdU in the pancreas on day 14. For the pulse and chase experiment, BrdU was injected intraperitoneally every day during Dox administration starting on day 2 (days 2–7), followed by withdrawal of Dox for 7 days. BrdU-positive cells were frequently observed in normal-looking pancreatic islet cells, which also expressed insulin. Scale bar, 100 μ m.

(C) Treatment of OSKM chimeric mice with Dox for 7 days, followed by the withdrawal of Dox for another 7 days. The macroscopic image shows the development of bilateral kidney tumors on day 14. Representative histological images are shown for Dox-withdrawn tumors in the kidney, pancreas, and liver. Scale bars, 200 μ m.

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Table 1. Transient Expression of Reprogramming Factors Causes Tumor Development

Dox Treatment	n	Kidney		Pancreas		Liver	
		No Phenotype	Dysplastic Growth	No Phenotype	Dysplastic Growth	No Phenotype	Dysplastic Growth
4 days ON → OFF	4	2	2	4	0	3	1
5 days ON → OFF	2	1	1	2	0	2	0
6 days ON → OFF	5	1	4	2	3	3	2
7 days ON → OFF	33	7	26	22	11	25	8

Prolonged Expression of Reprogramming Factors Leads to Transgene-Independent Tumor Formation in Somatic Cells

In contrast to the reversion of early dysplastic proliferating cells into normal-looking cells, mice that had been given Dox for 7 days often went on to develop tumors in multiple responding organs even after Dox withdrawal (Figure 2C; Table 1). The developed tumors consisted of histologically undifferentiated dysplastic cells, which were distinct from teratoma cells (Figures 2C and S2A). The dysplastic cells invaded the surrounding tissues, which is one of the hallmarks of cancer cell growth (Figure S2A). Dox-withdrawn tumor cells were negative for 2A staining, affirming that they grew independent of transgene expression (Figure S2B). Dox-withdrawn kidney tumors were similarly observed in elderly mice given Dox starting at 14 weeks of age (13 out of 19 mice). When Dox-withdrawn kidney tumor cells were transplanted into the subcutaneous tissues of immunocompromised mice, they formed secondary tumors within 3 weeks without Dox administration (Figures 2D and S2C), reflecting the neoplastic potential of Dox-withdrawn tumor cells.

Reprogramming factors in our transgenic system include *c-Myc*, a well-known oncogene. To investigate the contribution of *c-Myc* on the development of Dox-withdrawn tumors, we generated three-factor-inducible chimeric mice, which express *Oct3/4*, *Sox2*, and *Klf4* (OKS), but not *c-Myc*, by the targeted insertion of transgenes into the identical locus as 4F (OSKM)-inducible mice (Figure 2E). Similar to 4F-induced mice, OKS induction *in vivo* caused dysplastic cell growth in various organs yet required longer periods of treatment (Figure 2E). After 3 weeks of induction of OKS followed by withdrawal for 7 days, these mice developed the Dox-withdrawn tumors consisting of undifferentiated dysplastic cells in multiple organs (4 out of 8 mice; Figure 2E). Therefore, transgenic *c-Myc* is dispensable for the development of Dox-withdrawn tumors.

Oct3/4 plays a critical role in cellular reprogramming, and expression of three factors (*Klf4*, *c-Myc*, and *Sox2*) in the absence of *Oct3/4* is not sufficient for iPSC generation (Takahashi and Yamanaka, 2006). To further demonstrate a link between

cellular reprogramming and Dox-withdrawn tumor development, we generated chimeric mice in which *Klf4*, *c-Myc*, and *Sox2* (KMS), but not *Oct3/4*, can be induced upon Dox treatment (Figure 2F). Following Dox treatment for 7 days, we observed dysplastic cell growth in the kidney of KMS-inducible mice (three out of six mice; Figure 2F). However, in sharp contrast to OSKM/OKS-induced mice, the withdrawal of Dox eliminated the dysplastic cells in the kidney of KMS-induced mice ($n = 17$; Figure 2F). A previous study demonstrated that ectopic expression of *Oct3/4* alone can induce dysplastic growth whereas the transgene withdrawal leads to complete reversion of such dysplasia (Hochedlinger et al., 2005). Consistent with the previous observation, the *Oct3/4*-single induction under the same experimental condition failed to form Dox-withdrawn tumors ($n = 18$; Figure S2D). Taken together, we conclude that reprogramming pressure toward pluripotency driven by the combination of reprogramming factors is associated with the development of Dox-withdrawn tumors.

Loss of Cell Identity and Gain of ESC-Related Gene Expression in Dox-Withdrawn Tumors

To characterize Dox-withdrawn tumor cells, we examined gene expression in kidney tumors that arose in OSKM-inducible mice treated with the 7+7– Dox regimen. In the KH2 system, transgene expression in the kidney is induced exclusively in the tubule cells (Beard et al., 2006). We observed decreased expression of kidney tubule cell-specific genes in Dox-withdrawn kidney tumors, indicating loss of kidney cell identity (Figure 3A). A previous study dissected the gene expression signature of ESCs into three functional modules: core pluripotency factors, Polycomb complex factors, and Myc-related factors (Kim et al., 2010). Notably, microarray analysis revealed that the ESC-Core module is similarly activated in Dox-withdrawn kidney tumors and ESCs (Figure 3B) (Ohta et al., 2013). We also found that the Myc module displays similar activation between Dox-withdrawn tumors and ESCs (Figure S3A). The activation of ESC-Core and ESC-Myc modules was similarly confirmed in transplanted secondary tumors (Figure S3B).

(D) Minced Dox-withdrawn tumor cells were injected in the subcutaneous tissues of immunocompromised mice. A histological section of one of the tumors phenocopied the original Dox-withdrawn tumor. Scale bars, 200 μm (upper panel) and 100 μm (lower panel).

(E) A schematic drawing of the OKS transgene at the *Col1a1* locus. A histological section of the kidney on days 21 and 28. The expansion of dysplastic cells was observed in the stomach and kidneys on day 21 (arrows). The dysplastic cell growth could be detected even after the withdrawal of Dox in OKS-induced mice (day 28). Scale bars, 200 μm .

(F) A schematic drawing of the KMS transgene. A histological section of a kidney after the treatment with Dox for 7 days (day 7) and the withdrawal of Dox for another 7 days (day 14). KMS induction leads to dysplastic growth in the kidney tubule cells (arrows for day 7). The inset shows a higher-magnification image. No dysplastic cells were detectable in the kidneys of KMS-induced mice after the withdrawal of Dox (day 14). Scale bars, 200 μm .

See also Figure S2.

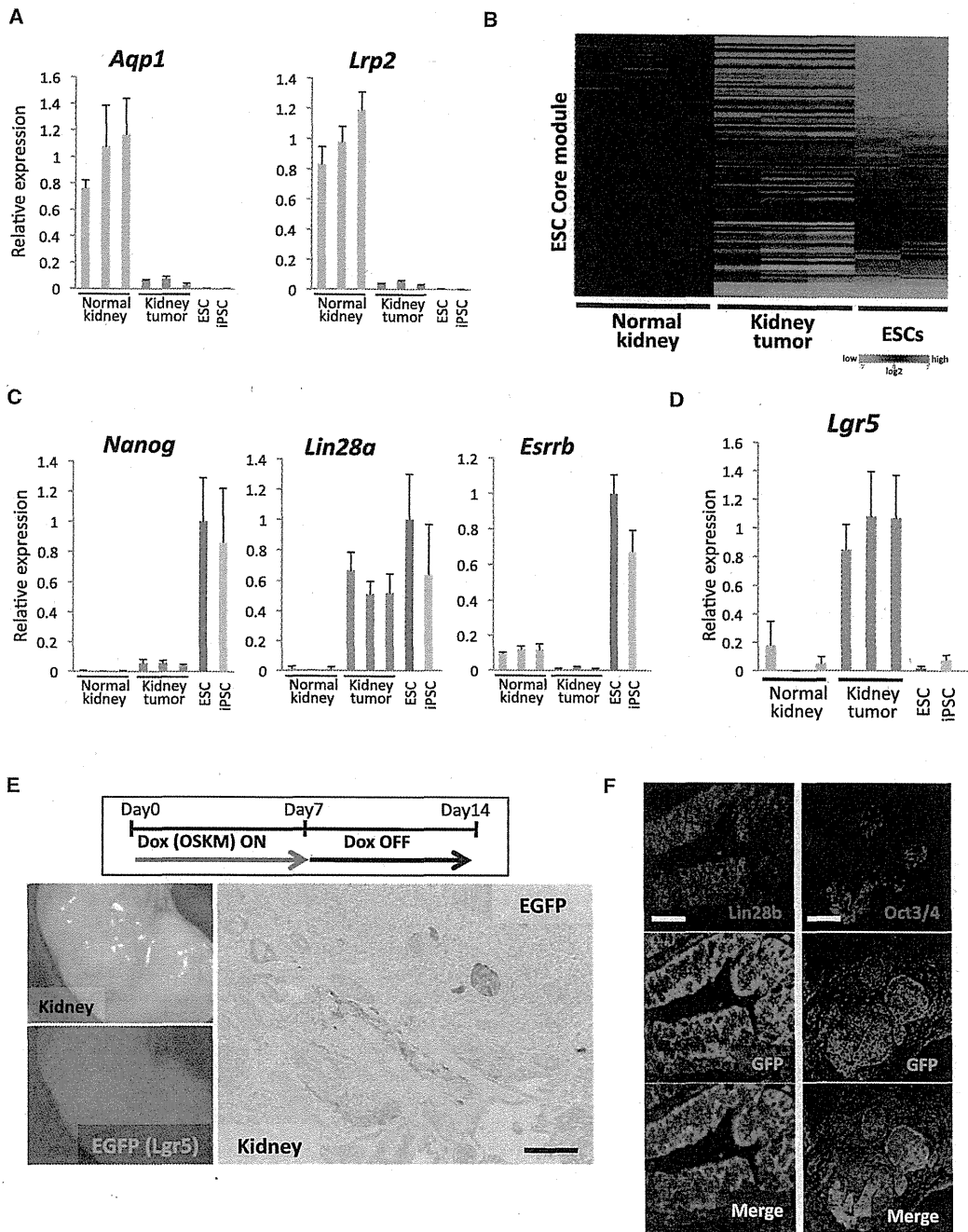


Figure 3. Loss of Cell Identity and Gain of ESC-Related Gene Expression in the Dox-Withdrawn Tumors

(A) The results of the qRT-PCR analyses of *Aqp1* and *Lrp2*. The expression levels of *Aqp1* and *Lrp2* were significantly downregulated in the Dox-withdrawn kidney tumors. Data are presented as mean \pm SD. The mean level of normal kidney samples was set to 1.

(B) The microarray analyses revealed the activation of the ESC Core module in Dox-withdrawn kidney tumors.

(C) The results of the qRT-PCR analyses of pluripotency-related genes. Data are presented as mean \pm SD. The transcript level in ESCs was set to 1.

(D) *Lgr5* as a candidate marker of Dox-withdrawn kidney tumor cells. *Lgr5* was specifically expressed in Dox-withdrawn kidney tumors. Data are presented as mean \pm SD. The mean level of kidney tumors was set to 1.

(E) A schematic drawing of the experimental protocol using chimeric mice with both reprogrammable alleles and the *Lgr5-EGFP* allele. Macroscopic images of the Dox-withdrawn kidney tumor with the *Lgr5-EGFP* allele showing scattered EGFP signals in the kidney tumor. GFP immunostaining of kidney tumor sections revealed that the GFP signals are detectable specifically in tumor cells. Scale bar, 100 μ m.

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