STUDY ON THE EPIGENETIC REGULATION IN CANCER STEM CELLS DEVELOPMENT

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STUDY ON THE EPIGENETIC REGULATION IN CANCER STEM CELLS DEVELOPMENT

A dissertation submitted by **AUNG KO KO OO** in partial fulfilment of the requirements for the Doctor of Philosophy in Engineering in the Graduate School of Natural Science and Technology, Okayama University, Japan.

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CERTIFICATE

This is to certify that **Mr. Aung Ko Ko Oo** has worked on the dissertation entitled **"STUDY ON THE EPIGENETIC REGULATION IN CANCER STEM CELLS DEVELOPMENT"** under my supervision. This thesis is being submitted to the Graduate School of Natural Science and Technology, Okayama University for the partial fulfillment of the requirement for the degree of **Doctor of Philosophy in Medical Bioengineering**. It is an original record of the work conducted by the candidate and has not been submitted in full or part to any other university for the award of degree or diploma.

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SUMMARY

We have been investigating the new insight in the cancer stem cells (CSCs) by developing a CSC model that is derived from induced pluripotent stem cells (iPSCs). The evidence of CSCs was widely accepted as small percentage of cell population in tumor that have a self-renewal capability and are malignant. Microenvironment is crucial to regulate the proliferation, self-renewal ability and differentiation of normal stem cells. By extending this concept, microenvironment distorted by cancer cells could affect the diverse directions of stem cells and leading to the characteristics of CSCs. Even though CSC shared with normal stem cells in the characteristics of maintaining the stemness and differentiation potential, multiple genetic and epigenetic regulations are different to acquire the features of CSCs. Epigenetic mechanisms, such as DNA methylation, histone modification and non-coding RNA elements, are involved in stem-cell maintenance and in the regulation of differentiation of stem cells. On the other hand, the epigenetic alterations are relating to the tumorigenesis with the activation of oncogenes and silencing of tumor suppressor genes.

We have succeeded in converting mouse iPSCs (miPSCs) into CSC-Like cells (miPS-LLCcm) by treating the miPSCs with conditioned medium (CM) of Lewis Lung Carcinoma (LLC) cells. miPS-LLCcm cells developed highly angiogenic and malignant adenocarcinoma as well as lung metastasis when subcutaneously transplanted into nude mice. By the treatment, miPSCs obtained the ability of unlimited growth and the capacity to maintain their stemness, while they were allowed to differentiate without LIF. The subcutaneous transplantation of the survived cells into the mouse formed malignant tumors and metastasized into lung tissues. Thus, we concluded that miPSCs should be converted into CSCs without any intended genetic manipulation. Immunohistochemical

analysis revealed the heterogeneity of the tumors, in which miPS-LLCcm cells should on one hand maintain the undifferentiated population expressing GFP and differentiate on the other into adenocarcinoma phenotype expressing MUC1. The cells from tumors at primary site and metastatic nodules can be maintained *in vitro*.

In order to further confirm the acquisition of CSC-like phenotype, we characterized the miPS-LLCcm cells and its derived cells form tumor (Ptdc) and from lung metastatic nodules (LMN) with commonly known CSC markers. We evaluated the significantly higher expression of ALHD1 and CD44 in the Ptdc and LMN cells. Epithelial to mesenchymal transition (EMT) is believed to be the primary mechanisms in the transition of cellular stages during development, wound healing and cancer metastasis. The upregulated expression of EMT markers in Ptdc cells was suggesting the potential of partial and metastable epithelial-to-mesenchymal transition (EMT) phenotype in Ptdc cells.

Since this conversion is triggered only by the factor(s) contained in the conditioned medium, we have hypothesized that epigenetic alterations can induce CSCs from normal stem cells in the cancer microenvironment. Methylation and demethylation is generally considered to silence and activate gene expression, respectively. We tried to evaluate the epigenetic changes in early stages of cancer development in this research that has not yet been assessed. We traced the development of CSCs by the change of DNA methylation levels, which would provide the difference between the miPSCs and derived CSCs. Then, we compared the methylation in miPS-LLCcm, LMN and Ptdc cells with that in miPSC by three sets; (1) miPSCs vs miPS-LLCcm cells, (2) miPSCs vs Ptdc cells and (3) miPSCs vs LMN cells. All comparisons between the different cell populations were found to exhibit hypomethylation as compared to miPSCs and 926, 583 and 1105 differentially

methylated regions (DMRs) were identified respectively from the comparisons. DMRs-associated genes were further identified and segregated into hypo- and hyper-methylated genes categories. As the results, hypomethylation was found superior to hypermethylation in the CSCs.

The analysis of KEGG pathways relating to hypomethylated genes revealed the several notable pathways important in cancers. Validating certain pathways in the CSCs, that are corresponding to DMR-related genes showed the pathways relevant for carcinogenesis including Focal adhesion, PI3K-Akt signaling pathway, Calcium signaling pathway, Pathway in cancer and Transcriptional misregulation in cancer. Checking the upregulated expression of the genes included in the enriched pathways that are concordant with hypomethylation showed the trace of candidates relating to oncogenic potential of CSCs. The expression of hypomethylated genes relating to PI3K-Akt pathway was found significantly high among those of the other genes. We found Pik3r5, which is a regulatory subunit of Pik3cg enzyme, as a hypomethylated and highly up-regulated gene relating to PI3K-Akt pathway. In the recent reports, the PIK3CG and PIK3R5 were evaluated as a potential oncogene that are overexpressed in human cancers leading to oncogenic cellular transformation and malignancy. Overexpression of PI3K-Gamma candidates were relating to oncogenic potential of the CSC model. Together with the findings, the constitutive expression of Pik3r5 was detected by immunoblotting with anti-p101 antibody in miPS-LLCcm and its derivatives.

Activation of PI3K-Akt signaling pathway has been commonly reported as key driver of carcinogenesis. The upregulated expression of Pik3r5 should induce the activation of Akt resulting in the onset of tumorigenic and metastatic potential of miPS-LLCcm and Ptdc cells. We assessed the Akt activation with anti-phosphorylated Akt (p-

Akt) antibody and we found Akt was constitutively activated in miPS-LLCcm, Ptdc and LMN cells. Therefore, the hypomethylation of Pik3r5 gene was leading to the up-regulation and is closely related to the activation/phosphorylation of AKT that is the downstream target molecule.

Significant overall DNA hypomethylation during the conversion activated the certain proto-oncogene, with the activation of PI3K-Akt signaling pathway, which represent the malignant conversion even without mutations. In our study, we have successfully demonstrated the CSCs generated from iPSCs by the treatment with CM from cancer derived cells acquired the DNA hypomethylation that might be considered to be the new aspect in the early stage of CSCs.

CHAPTER (1)

GENERAL INTRODUCTION

1.1. Cancer Stem Cells

Cancer has been defined as an abnormal growth of cells with potential character of invasion or spreading into other parts of body [1]. These abnormal cells have the ability to proliferate rapidly, make the malignant tumors and invade the native tissue by producing enzymes. By contrast, benign tumors don't generally invade and usually push the normal tissue to the side [2,3]. There are over 100 types of cancer that can affect in human and their symptoms vary depending on the types of tissue, such as breast, skin, lung, colon, prostate, and lymphoma [1,4].

Research into cancers, nowadays, has been changed enormously with new and advance technologies. In the area of cancer research, there is still questioning how the research projects have been done and how the effectiveness of therapies come from the cancer research in the past. To get the fundamental progress in treating cancers, the cancer researchers are currently focusing into the advanced and more understanding of two areas; (a) the genetic underpinning of cancers and (b) the biology of cancers. Progressive research outcomes developed many types of cancer treatments which depend on the type of cancer and how advanced it is. Conventional therapy is widely accepted in cancer treatment and is different from alternative or complementary therapies [5,6]. Examples of conventional treatment for cancers include chemotherapy, radiation therapy, and surgery. With the new findings in the area of cancer research, the cancer treatment has also been improved dramatically, such as the adjuvant chemotherapy, hormonal therapy, immunotherapy, and target therapy.

Recent chemotherapeutic treatment can give the promising results in treating the different types of malignancies by combining with surgical removal or radiotherapy. After decades of making the drugs to kill proliferating tumor cells, the notable progresses in cancer treatment was resulted [7,8]. However, cancer researchers are facing the divergent nature of cancers such as, chemo-resistance, turning into more tumorigenic form, metastasis and cancer relapse. One of the reason is the heterogeneity of cancer where certain population of cancer cells have ability to survive against several cancer treatments. The chemotherapeutic agents can remove and kill the rapidly dividing cells of the bulk tumor but often miss the certain population of cells with distinct morphological and functional profile [9]. These issues could be explained by the presence of stem cell population in the tumor as subgroup of cancer cells. Previous findings strongly suggested that these stem cells are responsible for chemoresistance and caner relapse [10]. The remaining stem cells are able to comprise the whole tumor even in a few number left. These population was broadly named as cancer stem cells (CSCs) and thought to be involved in driving the cancer [11].

Cancer stem cell is normally identified as a cell within a tumor that possess the ability to self-renew and to cause the heterogenous lineages of tumor cells. There are many hypothesizes of CSC with their different points of understanding. The hypothesis has to be tested whether what evidence is consistence with it or not and cancer researchers refine the way of treating the cancers according their assessment.

The cancer research over several decades has been led by the mutation theory in that any cell in body could become cancer when it acquires a package of mutation [12,13]. Because of single and/or series of mutation, single gene or some group of genes, called oncogenes, are activated and some genes, called suppressor genes, are inactivated in cancer. In some cases, the combination of these two events was also involved in the carcinogenesis. Therefore, oncologists defined cancers as some kind of genetic mistake. Form this concern, all types of cells in the multicellular organisms could become cancer with certain reasons [14].

Another concern of cancers was evaluated with the evidence of cancer stem cells in the tumor. In this case, only a certain population of cells that have the stem cell properties are really prone to becoming malignant [8]. The hypothesis about the cancer stem cells is in an ongoing debate. There is still needed to answer whether CSCs represent a mature tissue stem cell which has undergone malignant change or whether differentiated cells dedifferentiated into stem cell program together with the malignancy [15]. Until now, cancer stem cells are purely defined only with their capacities of self-renewing and differentiation, considering independently from the concept of origin of cancers [7,16].

Cancers themselves organized through in very similar way to a normal organ, but the cells are proliferating abnormally [17]. Normal organ is organized in a hierarchy where at the apex of the hierarchy it is the stem cell and give rise to the other cells which form the bulk of an organ. In the

tumor, it is generally thought that cancer stem is the master cell that gives rise to heterogeneity of cancers [7].

Tumor initiating capacity of CSCs in tumor was shown by serial transplantation of limited tumor cells into immunocompromised animal models where undifferentiated cancer cells show more than the differentiated cancer cells [8,18]. Researchers had been trying to evaluate the markers to identify and isolate the population of stem cells from tumor cells. Even though the validity of CSC markers depends on different types of tissues, and/or modes of tumorigenesis, it is possible to address the population of CSC in the tumors with CSC markers detected in specific malignancies. Currently, CSCs were identified in human brain, breast, prostate, head and neck, pancreas, liver, ovary, and colon cancers, by using different markers, such as CD133, EpCAM, CD44, CD24, Lgr5 and ALDH1 [19]. With many questions about CSCs, the nature and characteristics of CSCs become interested and therapeutic CSC-targeting approaches popular in past decade of cancer research. Experimental limitation for the researchers is that CSCs are very small percentage of tumor cells, and the culturing and maintenance of these cells are still difficult [9]. Many researchers evaluated the properties and characteristics of CSCs by using the model of CSCs that are selected by the help of CSC-markers. The evaluations about CSCs are still depending on the validity of markers on these cancer types [19,20].

In conclusion, cancer stem cell is defined as a certain type oncogenic cell that have self-renewing and differentiation ability. Their self-renewing

properties drives the generation of more CSCs, and differentiation capacity generate the bulk of tumors. The metastasis and tumor relapse are also related with the properties of CSCs. Due to the properties of CSCs, it is very important to provide the therapy that eradicated CSCs completely.

1.2. iPSC-CSC

The tumor microenvironment (TME) is the cellular environment surrounding the tumor in which stromal fibroblasts, immune cells, macrophages, endothelial cells, leucocytes, and extracellular matrix exist and interact by releasing signaling molecules to promote tumor growth and metastasis. It has been reported the significant role of TME in disease progression like as cancer, but the clear-cut role has not been understood. A major concept of TME is that cancer cells interact closely with the surrounding cells, which together form the major construct of the TME leading into more complexity of cancer biology. In the case immune cells, the factors secreted in TME drive a chronic inflammatory, immunosuppressive, and pro-angiogenic environment. By getting adaptation in such environment, cancer cells are able to avoid the host immunosuppressive action and to get the new properties of metastasis and angiogenesis. The aberrant pathological process in tumor microenvironment can be related with divergent nature of cancers by changing the genetic or epigenetic regulation. Although it is a transient effect, it could activate the certain signaling pathways regulating cellular

proliferation and migration or in the case of stem cells it could be related with cell fate determination and differentiation.

Stem cells are found in certain population in multicellular organisms with their special characteristics different from others in the body. Stem cells are thought to be in blank state that can develop into cells with different specialized functions in different parts of the body during early life and growth. Stem cells have been termed as undifferentiated and self-renewing cells that can divide and make the unrestricted numbers of copies of themselves. Difference between stem cells and any other cells in the tissues is that when a stem cell divides, one remains by self-renewing as stem cell at exactly the same stage of differentiation and the other turns into a next stage of the differentiation down to a differentiated cell, such as a muscle cell or red blood cell, etc. [16,21]. Generally, type of stem cells can be divided into three types; (a) Embryonic stem cells (ESC) (b) Adult stem cells (c) Cancer stem cells. Embryonic stem cells are pluripotent, meaning they can develop into more than 200 cell types of the adult body and also have the ability to replicate indefinitely [22]. Adult stem cells found in adults can produce only a limited number of cell types. Cancer stem cells (CSCs) are tumor cells that have the principal properties of self-renewal, clonal tumor initiation capacity and clonal long-term repopulation potential [23].

Like the normal stem cells, CSCs are believed to reside in their own niches [24]. The niches for normal stem cells are crucial for proper differentiation of stem cells into certain progenitor cells and differentiated

cells. In contrast, the TME could affect on the differentiation of stem cells. Seno et.al explained the fate of stem cells distorted by TME using the conditioned medium of Lewis Lung Carcinoma (LLC) [24,25]. In their hypothesis, mouse induced pluripotent stem cells (miPSCs) should be induced to some kinds of progenitor cells, such as hematopoietic cells and neural stem cells, differentiating into various phenotypes, such as macrophage, monocytes, neural cells, cardiac cells and pancreatic b-cells, when they were exposed to the normal niche. On the other hand, they hypothesized that CSCs may also be derived from miPS cells only when exposure to a malignant niche [Figure 1.1].

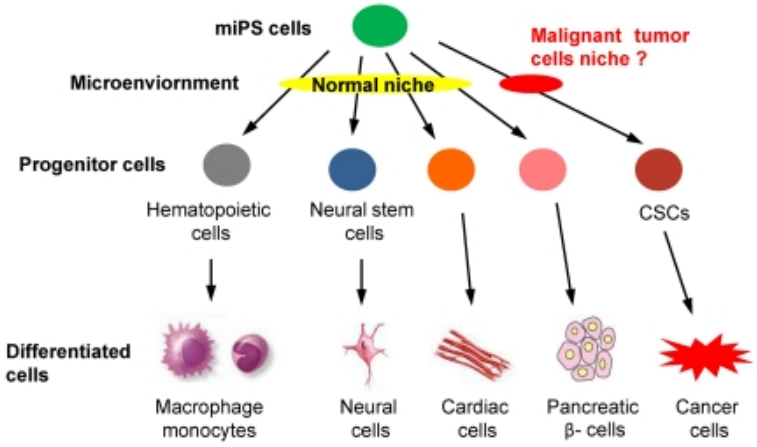


Figure 1.1 The hypothesis of miPSCs conversion into iPS-CSCs. CSCs are considered derived from normal stem cells affected by the microenvironment being influenced by cancer cells. [25]

They evaluated the new insight in the CSCs developed in cancer microenvironment, by extending the concept that the niche of the normal

cells is involved in the differentiation of stem cells into normal tissues. While the mouse induced pluripotent stem cells (miPSCs) were allowed to differentiate in the presence of cancer conditioned medium, they acquired the ability to maintain their stemness and differentiation potential, as well as formed malignant tumors with the features of adenocarcinoma and metastasized into lung tissues in vivo, considering the sign of CSCs. These models of cancer stem cell will provide the great advantages in cancer research and its applications in the future.

In iPS-CSC model, miPSCs were converted into CSCs without any intended genetic manipulation. The conversion could be considered through the transcriptional or translational changes determined by genetic or epigenetic alterations that are promoting tumorigenesis. Furthermore, they successfully generated various types CSC models converted from iPSCs with the aid of CM of various cancer cell lines such as human pancreatic carcinoma cell lines PK-8 cells and KLM-1 cells, human breast cancer cell lines T47D cells and BT549 cells, mouse carcinoma cell lines LLC cells, P19 cells, B16 cells and MC.E12 cells [14–16]. Anna et.al evaluated organ specific feature of xenografts tumors developed from iPS-CSC that were converted with conditioned medium of PDAC cells [26]. Furthermore, the story and origin of cancer associated fibroblast was explained with iPS-CSC model developed in the conditioned medium of breast cancer cells [27]. The iPS-CSC have differentiated into the fibroblast that support the tumorigenesis and metastasis of iPS-CSC. Interestingly, iPS-CSC model can also differentiate into the progenies of CSCs containing vascular endothelium [24,28].

In conclusion, the iPS-CSC is a model of CSCs that can explain biology and characteristics of CSC in nature. In addition, CSC generation from various kinds of cancer cells could be a source that provide a library of CSCs for customized cancer treatment. iPS-CSC model could be used in the evaluation of bona fide CSC markers and also the screening of chemotherapeutic drugs that are targeting the CSC population, to get better therapeutic approach of cancer.

1.3. CSC and Epigenetics

Carcinogenesis has been explained by the classical cancer initiation theory; evolutional accumulation of one or more mutations in a single or a few cells resulting in uncontrolled growth [29]. Genetic mutation was considered as the major causes of neoplasia [30]. However, it is now accepting the involvement epigenetic regulatory mechanisms in carcinogenesis. Genetic mutation is responsible for activation of tumor driver genes and silencing of tumor suppressors. In the second part, disruption of epigenetic regulation is leading to overexpression of oncogenes and downregulation of the tumor suppressor genes [31,32]. The genetic and epigenetic regulation were viewed as sole reason of abnormal gene expression.

The regulation performed by epigenetic mechanisms includes histone modification, chromatin remodeling factors, DNA methylation, microRNAs and post–translational modifications. The expression of

certain gene was determined by the status of DNA packaging at the regulation regions. These regions are normally the promoters and/or enhancers and insulators in chromatin. Some regulations were performed by the presence of transcription factors and chromatin modifying enzymes [33].

Without changing in DNA sequence, the ability to change the expression of genes is the primary role of epigenetic regulation. The epigenetic abnormalities involved in the tumorigenesis is not simple as the gain or loss of genes expression because the types of regulation will be different and complex depending on the stage and nature of carcinogenesis. During the tumor initiation and progression, DNA hypomethylation pattern was occurred in the cancer associated genes when comparing with normal tissues. Previously, the cancer-specific DNA methylation patterns was reported by comparing with associated tissues. The development of technologies in sequencing and microarray analysis are very supportive to examine and understand the aberrant epigenetic regulation specific to cancer.

During differentiation, ESCs start in a pluripotent state from which they sequentially develop into unique cell type with a narrower pluripotency. Histone methylation is short-term silencing mechanism by which some set of genes required for development were repressed at the stage of stem cell [34,35]. On the other hand, DNA methylation are long-term silencing mechanisms. Pluripotent stem cells express the set of transcription factors that are important for maintenance of stemness and

after development these genes are repressed by long-term silencing mechanisms. Therefore, in somatic cells the imprinted genes and pluripotency-associated genes were off by DNA hypermethylation [36,37]. The key developmental events with global epigenetic modifications and gene-expression patterns in mammalian cells were shown in **Figure 1.2.**

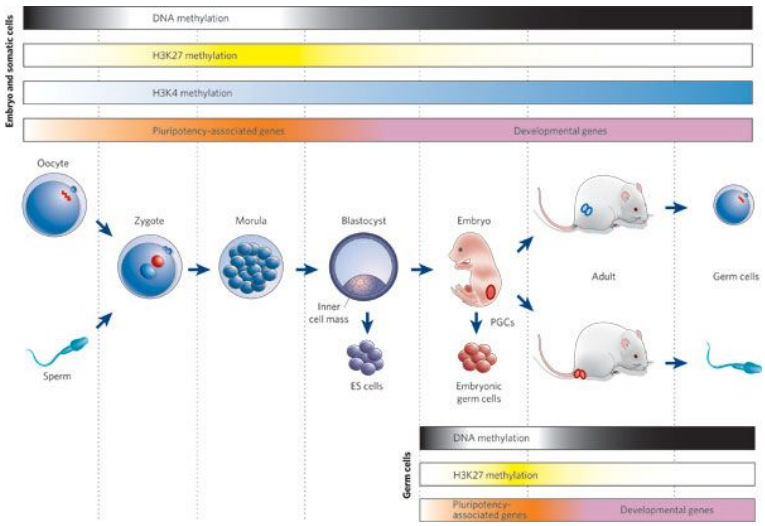


Figure 1.2 Key developmental events with global epigenetic modifications and gene-expression patterns mammalian cells. [37]

Some characteristics of CSC shared with normal stem cells including self-renewal and differentiation. Normal stem cells differentiated into the certain cell type in proper epigenetic regulation. Altered or aberrant epigenetic regulation was thought to be involved in CSC development required for the features of CSCs, highly proliferative, tumor formation, and metastatic ability to form the new tumor at distant site. The epigenetic regulation pattern for maintaining the stemness was occurred in CSC. The possibility of this evidence can be explained in two ways; one is dedifferentiation mechanisms gained in non-stem cancer cells

and another is the failure of DNA methylation mechanism in normal stem cell during differentiation.

Several key developmental or signaling pathways have been shown to play essential roles to get the CSCs functions, especially tumorigenesis, cancer initiation and maintenance of self-renewing. Epigenetic deregulation may contribute the alteration in such kinds of pathways. Generally, the Jak-STAT, Wnt, Notch, Hedgehog, PI3K, and NF-kB signaling were shown to be involved in mediating various stem cell properties, such as self-renewal, cell fate decisions, survival, proliferation, and differentiation [38–40]. Recent reports interestingly had reported that the regulation of these signaling pathways are imbalanced in cancers. One of the reasons for these evidences could be the abnormal epigenetic regulations. Recently, the distinct DNA hypomethylation of specific gene sets in MCF7-derived mammosheres caused the activation of Jak-STAT pathway that was considered for the maintenance of CSC properties to be involved in the regulation of stem cells [38,41]. In gastric cancers, activation of Wnt pathways in were more frequently affected by epigenetic alterations than by genetic alterations in the related genes [42]. Moreover, it was reported that the activation of Hedgehog was related with epigenetic changes in which, Shh promoter hypomethylation was suggested as a critical event in breast carcinogenesis [43]. The Notch signaling pathway play important roles in developmental process, and cell-cell communication for regulating the cell proliferation, differentiation and cell lineage progression but it is also dysregulated in many cancers [44]. One of the growing evidences in epigenetic dysregulation of notch signaling

pathway showed that overexpression of Notch ligand has been gained from enhanced histone acetylation at promoter region of this ligand in multiple myeloma.

In conclusion, although the experimental evidences of CSCs relating to their genetic and epigenetic regulation have been growing in number, the hypothesis of CSC still remain needed to understand how they develop, how different in characteristics depending on tissue, and dysregulation of key signaling pathways specific for their functions. It is very important to develop a CSC model that can sufficiently explain about the genetic or epigenetic profiles of CSC. The alteration mechanism in CSCs could be expected to be useful for development of specific therapy for cancer patients.

REFERENCES

1. GBD 2015 Mortality and Causes of Death Collaborators, G. B. D. 2015 M.; of Death, C. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet (London, England)* **2016**, *388*, 1459–1544, doi:10.1016/S0140-6736(16)31012-1.

2. National Cancer Institute NCI Dictionary of Cancer Terms: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/benign-tumor.

3. PubMed Health Benign Tumor: https://www.ncbi.nlm.nih.gov/pubmedhealth/PMHT0025285/.

4. Plummer, M.; de Martel, C.; Vignat, J.; Ferlay, J.; Bray, F.; Franceschi, S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob. Heal.* **2016**, *4*, e609–e616, doi:10.1016/S2214-109X(16)30143-7.

5. Tannock, I. F. Conventional cancer therapy: Promise broken or promise delayed? *Lancet* **1998**, *351*, doi:10.1016/S0140-6736(98)90327-0.

6. National Cancer Institute NCI Dictionary of Cancer Terms: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/conventional-treatment.

7. Magee, J. A.; Piskounova, E.; Morrison, S. J. Cancer Stem Cells: Impact, Heterogeneity, and Uncertainty. *Cancer Cell* 2012.

8. Reya, T.; Morrison, S. J.; Clarke, M. F.; Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **2001**, doi:10.1038/35102167.

9. Klonisch, T.; Wiechec, E.; Hombach-Klonisch, S.; Ande, S. R.; Wesselborg, S.; Schulze-Osthoff, K.; Los, M. Cancer stem cell markers in common cancers - therapeutic implications. *Trends Mol. Med.* 2008.

10. Dean, M.; Fojo, T.; Bates, S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer* 2005.

11. Dalerba, P.; Cho, R. W.; Clarke, M. F. Cancer Stem Cells: Models and Concepts. *Annu. Rev. Med.* **2007**, doi:10.1146/annurev.med.58.062105.204854.

12. Soto, A. M.; Sonnenschein, C. The somatic mutation theory of cancer: Growing problems with the paradigm? *BioEssays* 2004.

13. Blagosklonny, M. V. Molecular theory of Cancer. *Cancer Biol. Ther.* 2005.

14. Brücher, B. L. D. M.; Jamall, I. S. Somatic mutation theory - Why it's wrong for most cancers. *Cell. Physiol. Biochem.* 2016.

15. Hermann, P. C.; Huber, S. L.; Herrler, T.; Aicher, A.; Ellwart, J. W.; Guba, M.; Bruns, C. J.; Heeschen, C. Distinct Populations of Cancer

Stem Cells Determine Tumor Growth and Metastatic Activity in Human Pancreatic Cancer. *Cell Stem Cell* **2007**, *1*, 313–323, doi:10.1016/j.stem.2007.06.002.

16. Li, L.; Neaves, W. B. Normal stem cells and cancer stem cells: The niche matters. *Cancer Res.* 2006, *66*, 4553–4557.

17. Egeblad, M.; Nakasone, E. S.; Werb, Z. Tumors as organs: Complex tissues that interface with the entire organism. *Dev. Cell* 2010.

18. Al-Hajj, M.; Wicha, M. S.; Benito-Hernandez, A.; Morrison, S. J.; Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci.* **2003**, doi:10.1073/pnas.0530291100.

19. Karsten, U.; Goletz, S. What makes cancer stem cell markers different? *Springerplus* **2013**, doi:10.1186/2193-1801-2-301.

20. Lobo, N. A.; Shimono, Y.; Qian, D.; Clarke, M. F. The Biology of Cancer Stem Cells. *Annu. Rev. Cell Dev. Biol.* **2007**, doi:10.1146/annurev.cellbio.22.010305.104154.

21. He, S.; Nakada, D.; Morrison, S. J. Mechanisms of stem cell self-renewal. *Annu. Rev. Cell Dev. Biol.* **2009**, doi:10.1146/annurev.cellbio.042308.113248.

22. Keller, G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* **2005**, doi:10.1101/gad.1303605.

23. Plaks, V.; Kong, N.; Werb, Z. The cancer stem cell niche: How essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell* 2015.

24. Matsuda, S.; Yan, T.; Mizutani, A.; Sota, T.; Hiramoto, Y.; Prieto-Vila, M.; Chen, L.; Satoh, A.; Kudoh, T.; Kasai, T.; Murakami, H.; Fu, L.; Salomon, D. S.; Seno, M. Cancer stem cells maintain a hierarchy of differentiation by creating their niche. *Int. J. Cancer* **2014**, doi:10.1002/ijc.28648.

25. Chen, L.; Kasai, T.; Li, Y.; Sugii, Y.; Jin, G.; Okada, M.; Vaidyanath, A.; Mizutani, A.; Satoh, A.; Kudoh, T.; Hendrix, M. J. C.; Salomon, D. S.; Fu, L.; Seno, M. A model of cancer stem cells derived from mouse induced pluripotent stem cells. *PLoS One* **2012**, *7*, doi:10.1371/journal.pone.0033544.

26. Calle, A. S.; Nair, N.; Oo, A. K.; Prieto-Vila, M.; Koga, M.; Khayrani, A. C.; Hussein, M.; Hurley, L.; Vaidyanath, A.; Seno, A.; Iwasaki, Y.; Calle, M.; Kasai, T.; Seno, M. A new PDAC mouse model originated from iPSCs-converted pancreatic cancer stem cells (CSCcm). *Am. J. Cancer Res.* **2016**, *6*, 2799–2815.

27. Nair, N.; Calle, A. S.; Zahra, M. H.; Prieto-Vila, M.; Oo, A. K. K.; Hurley, L.; Vaidyanath, A.; Seno, A.; Masuda, J.; Iwasaki, Y.; Tanaka, H.; Kasai, T.; Seno, M. A cancer stem cell model as the point of origin of cancer-associated fibroblasts in tumor microenvironment. *Sci. Rep.* **2017**, *7*, 6838, doi:10.1038/s41598-017-07144-5.

28. Prieto-Vila, M.; Yan, T.; Calle, A. S.; Nair, N.; Hurley, L.; Kasai, T.; Kakuta, H.; Masuda, J.; Murakami, H.; Mizutani, A.; Seno, M. iPSC-derived cancer stem cells provide a model of tumor vasculature. *Am. J. Cancer Res.* **2016**, *6*, 1906–1921.

29. Rozhok, A. I.; Salstrom, J. L.; DeGregori, J. Stochastic modeling indicates that aging and somatic evolution in the hematopoietic system are driven by non-cell-autonomous processes. *Aging (Albany. NY).* **2014**, *6*, 1033–1048, doi:10.18632/aging.100707.

30. Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* 2011.

31. Baylin, S. B.; Herman, J. G. S.B. Baylin, J.G. Herman, DNA hypermethylation in tumorigenesis: epigenetics joins genetics, Trends Genet. 16 (2000) 168–174. - Google Search. *Trends Genet.* **2000**, *16*, 168–174.

32. Sandoval, J.; Esteller, M. Cancer epigenomics: Beyond genomics. *Curr. Opin. Genet. Dev.* 2012.

33. De Carvalho, D.; Sharma, S.; You, J. S.; Su, S. F.; Taberlay, P. C.; Kelly, T. K.; Yang, X.; Liang, G.; Jones, P. A. DNA Methylation Screening Identifies Driver Epigenetic Events of Cancer Cell Survival. *Cancer Cell* **2012**, doi:10.1016/j.ccr.2012.03.045.

34. Boyer, L. A.; Plath, K.; Zeitlinger, J.; Brambrink, T.; Medeiros, L. A.; Lee, T. I.; Levine, S. S.; Wernig, M.; Tajonar, A.; Ray, M. K.; Bell, G.

W.; Otte, A. P.; Vidal, M.; Gifford, D. K.; Young, R. A.; Jaenisch, R. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **2006**, doi:10.1038/nature04733.

35. Azuara, V.; Perry, P.; Sauer, S.; Spivakov, M.; Jørgensen, H. F.; John, R. M.; Gouti, M.; Casanova, M.; Warnes, G.; Merkenschlager, M.; Fisher, A. G. Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* **2006**, doi:10.1038/ncb1403.

36. Ohm, J. E.; McGarvey, K. M.; Yu, X.; Cheng, L.; Schuebel, K. E.; Cope, L.; Mohammad, H. P.; Chen, W.; Daniel, V. C.; Yu, W.; Berman, D. M.; Jenuwein, T.; Pruitt, K.; Sharkis, S. J.; Watkins, D. N.; Herman, J. G.; Baylin, S. B. A stem cell-like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing. *Nat. Genet.* **2007**, doi:10.1038/ng1972.

37. Reik, W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 2007.

38. Dreesen, O.; Brivanlou, A. H. Signaling pathways in cancer and embryonic stem cells. *Stem Cell Rev.* **2007**, doi:10.1007/s12015-007-0004-8.

39. Ghoshal, P.; Nganga, A. J.; Moran-Giuati, J.; Szafranek, A.; Johnson, T. R.; Bigelow, A. J.; Houde, C. M.; Avet-Loiseau, H.; Smiraglia, D. J.; Ersing, N.; Chanan-Khan, A. A.; Coignet, L. J. Loss of the SMRT/NCoR2 corepressor correlates with JAG2 overexpression in multiple myeloma. *Cancer Res.* **2009**, *69*, 4380–4387,

doi:10.1158/0008-5472.CAN-08-3467.

40. Toh, T. B.; Lim, J. J.; Chow, E. K.-H. Epigenetics in cancer stem cells. *Mol. Cancer* **2017**, *16*, 29, doi:10.1186/s12943-017-0596-9.

41. Onishi, K.; Zandstra, P. W. LIF signaling in stem cells and development. *Development* **2015**, doi:10.1242/dev.117598.

42. Yoda, Y.; Takeshima, H.; Niwa, T.; Kim, J. G.; Ando, T.; Kushima, R.; Sugiyama, T.; Katai, H.; Noshiro, H.; Ushijima, T. Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer. *Gastric Cancer* **2015**, doi:10.1007/s10120-014-0348-0.

43. Cui, W.; Wang, L.-H.; Wen, Y.-Y.; Song, M.; Li, B.-L.; Chen, X.-L.; Xu, M.; An, S.-X.; Zhao, J.; Lu, Y.-Y.; Mi, X.-Y.; Wang, E.-H. Expression and regulation mechanisms of Sonic Hedgehog in breast cancer. *Cancer Sci.* **2010**, doi:10.1111/j.1349-7006.2010.01495.x.

44. Bolós, V.; Grego-Bessa, J.; De La Pompa, J. L. Notch signaling in development and cancer. *Endocr. Rev.* 2007.

CHAPTER (2)

"iPSC DERIVED CSC MODEL WITH LUNG METASTASIS DEVELOPED IN THE MICROENVIRONMENT OF LUNG CARCINOMA"

ABSTRACT

Cancer stem cells (CSCs) are considered to be derived from normal stem cells affected by the tumor microenvironment through the genetic and epigenetic alterations that initiate malignant transformation. We have reported that the conditioned medium of Lewis lung carcinoma (LLC) cells can be used to convert mouse induced pluripotent stem cells (miPSCs) into cancer stem cell phenotype, which named miPS-LLCcm cells. miPS-LLCcm cells developed highly angiogenic and malignant adenocarcinoma after transplanted into nude mice. When miPS-LLCcm cells were subcutaneously injected into a nude mouse, malignant adenocarcinoma-like tumor was formed with lung metastasis. Immunohistochemical analysis with GFP antibody showed that the nodules formed in lung expressed GFP, of which expression was controlled by Nanog promoter, further proving that these cells were metastatic to lung. This should therefore be a model to study lung metastasis from a tumor formed by subcutaneous injection. In this study, we analyzed the expression of three types of candidate genes for stem cell, tumor driver genes and epithelial–mesenchymal transition (EMT) on four different stages of cells. The CSC-like cells developed from miPSC exhibited self-renewal activity and stem cell marker gene expression. The expression of EMT markers were analyzed in the primary culture derived from the tumor and nodules metastasized to lung. The primary cells from the tumor highly expressed the EMT markers; snail, slug, Twist and N-cadherin. The results demonstrated in this study indicate that primary cells from tumor are rich in CSCs with high metastatic potential.

2.1. INTRODUCTION

Tumors are composed of heterogeneous cancer cells with distinct morphological and functional profiles. This heterogeneity could be partially explained by the classical cancer initiation theory; evolutional accumulation of one or more mutations in a single or a few cells resulting in uncontrolled growth [1]. Another explanation is based on the presence of stem cells in tumors. Even in a few number the stem cells are considered to comprise the whole tumor in patient [2–4]. The heterogeneous population in cancer tissues are thought to be the progeny of stem cell population resulting from self-renewal and differentiation. Coupled with the malignant tumorigenic potential, the stem cells have been termed as cancer stem cells (CSCs) generating functionally hierarchical structure in a tumor.

There is a considerable evidence that many different cancers have a unique subpopulation of self-renewing cells that can generate the diverse tumor cells. CSCs were identified in brain, breast, prostate, head and neck, pancreas, liver, ovary, and colon cancers, by using different markers, such as CD133, EpCAM, CD44, CD24, Lgr5 and ALDH1. The question about the origin of CSC is still controversial. The leukemic stem cells were the first identified with the cell-surface markers, $CD34^{+}$ $CD38^{-}$ differentiating *in vivo* into leukemic blasts [2]. This particular approach led to emerge new studies which described tissue-specific markers for solid tumors. The phenotype associated with cancers including motility, invasion and chemo-/radio-resistance could be traced to CSCs. Metastasis through the activation of CXCR4 receptor was previously demonstrated by the migration of invasive CSCs defined with CD133 in

pancreatic cancer [5]. On the other hand, other studies attributed their metastatic potential to the epithelial-mesenchymal transition (EMT) events showing that differentiated cancer cells could turn into CSC-like mesenchymal cells [6].

CSCs is normally characterized as fraction of cancer cells that have ability for self-renewal, pluripotency and sustaining of the bulk of cancer. The best evidence for the existence of CSCs was demonstrated with cancer stem cells model that is derived from mouse induced pluripotent stem cells (miPSC). When miPSC are cultured in the presence of conditioned medium prepared from various cancer cell lines, it acquires the characters of CSCs with high tumorigenicity and stemness. We have evaluated miPS-LLCcm cells that were derived from miPSCs developed in the conditioned medium of Lewis lung carcinoma (LLC) [7]. This cancer stem cell model has the highly tumorigenic and angiogenic ability and also metastatic potential was observed when spheroid cells were injected into the mouse tail vein, multiple metastatic nodules were found in lung after one month.

Adult somatic cells have been successfully reprogrammed to pluripotent stem cells (iPSCs) with the transduction of four transcription factors [8]. The differentiation potential of iPSCs is largely expected to develop multiple potential avenues for the regenerative therapy. Immune rejection of embryonic stem cells can be avoided by replacing these with iPSCs. However, the risks of potential tumor development and other unpredictable biological changes during transplantation are still unresolved. The cellular interaction of transplanted cells in the microenvironment has been reported important to obtain successful results in regeneration therapy [9].

In this evidence, our hypothesis is that miPSCs are affected by the conditioned medium to become the miPS-CSCs through the post-transcriptional and translational or epigenetic regulations. It is a good model to study the behaviors of CSCs in primary tumor induction and metastasis.

2.2. RESULTS

2.2.1. Conversion of miPSCs into CSC-like cells with tumorigenic and metastatic potential

We have reported a model of CSC-like cells converted from miPSC by the exposure to the conditioned medium (CM) from various cancer cell lines [7,10,11]. The miPSCs used in the studies had GFP under the control of Nanog promoter wherein undifferentiated stem cells exhibit strong GFP expression and differentiated cells lose green fluorescence. miPSCs were found to be viable in the presence of conditioned medium even when the differentiation was allowed while they started differentiation and failed to survive beyond 10 days without CM. This scheme is briefly summarized in Figure 2.1A. After 4 weeks of treatment with CM, the survived and undifferentiated cells expressing GFP were named as miPS-LLCcm cells. The self-renewal of miPS-LLCcm cells, a specific character of undifferentiated stem cells, was also confirmed by sphere-forming assay as well as its differentiation potential was evident with highly adhesive fibroblast-like phenotype and loss of GFP expression (Figure 2.1B).

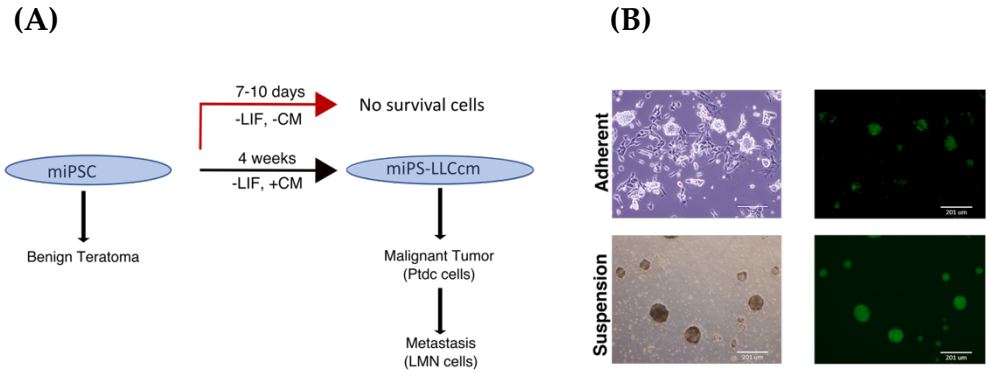


Figure 2.1. Conversion of miPSCs into miPS-LLCcm (A) Summarized scheme of conversion of miPSCs into miPS-LLCcm and its tumorigenic and metastatic activity. (B) Converted miPSCs in adherent culture (top) and in suspension culture (bottom).

The subcutaneous transplantation of miPS-LLCcm cells into immunocompromised Balb/c nude mice generated malignant tumor together with the metastasized nodule-like structures in the lung (Figure 2.2A and 2.2B). These tumors and lung nodules were subjected to primary cell culturing to isolate GFP expressing cells. The primary cultured cells from tumors was named as Ptdc cells and the cells from lung nodules was named as LMN cells. The Ptdc cells were subsequently transplanted to generate the secondary tumor again. In the subcutaneous injection of 10^6 cells, the growth of tumors was compared (Figure 2.2C). The Ptdc cells showed the most rapid growth when compared to miPS-LLCcm cells and miPSCs.

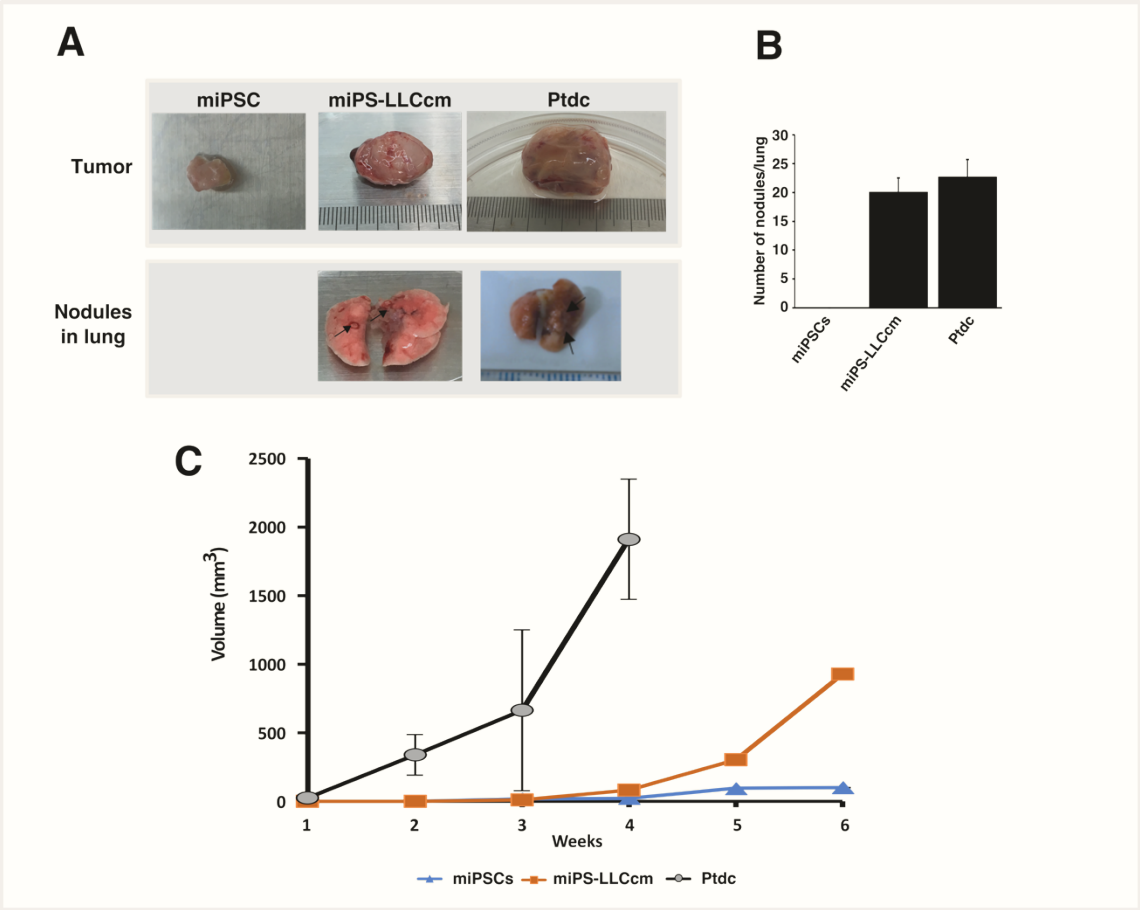


Figure 2.2. Tumorigenic and metastatic potential of miPS-LLCcm (A) Tumors and metastatic nodules in lungs generated by subcutaneously transplanted miPSCs, miPS-LLCcm cells and Ptdc cells into the Balb/c-nu mouse. Arrows in lung indicate the positions of nodules. (B) The histogram showed the average number of lung nodules for each of the three cells. (C) The sizes of tumors growing in 4-6 weeks.

Primary cultures derived from the tumors were positive for GFP signal, confirming they were originated from miPS-LLCcm cells and not from host cells, and displayed sphere-forming activity suggesting they preserve stem-like characteristics (Figure 2.3A and 2.3B). miPS-LLCcm cells, Ptdc cells and LMN cells maintained the expression of endogeneous stemness markers, such as

Nanog, Oct3/4, Sox2 and Klf4 without LIF similar with the untreated miPSCs cultured in the presence of LIF (Figure 2.4). Therefore, the stemness can be maintained in the presence of CM and this conversion supports the establishment of CSC-like features.

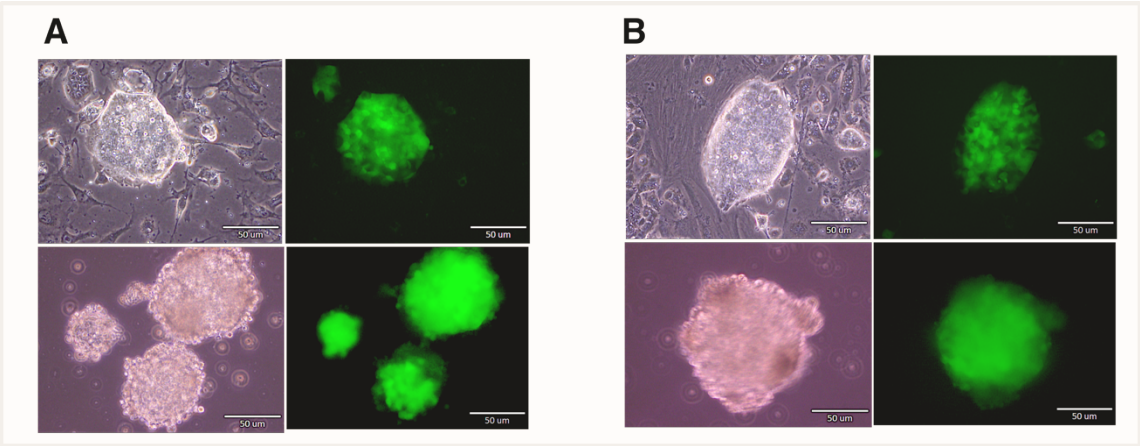


Figure 2.3. Primary cultured cells (A) Ptdc cells in adherent culture (top) and sphere formation in suspension culture (bottom) with the expression of GFP. (B) LMN cells in adherent culture (top) and sphere formation in suspension culture (bottom) with the expression of GFP.

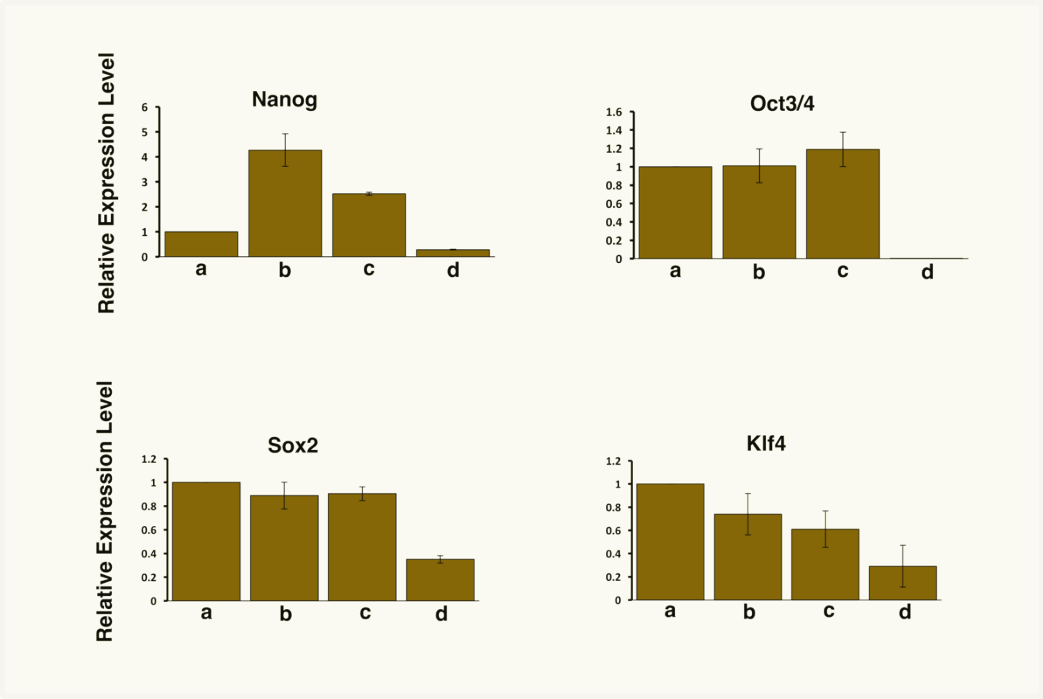


Figure 2.4. Comparison of the expression levels of stemness markers by rt-qPCR. a, miPSCs; b, miPS-LLCcm cells; c, Ptdc cells; d, LMN cells.

Histological analysis of the tumor showed poorly differentiated phenotype, high nuclear to cytoplasmic ratio, micrometastasis and some epithelial ductal-like structure, which are the signs of malignancy (Figure 2.5A). Metastatic node-like structures were observed in the lung of the mice (Figure 2.5B). Untreated miPSCs tumor displayed teratoma like phenotype with various germ layers (Figure 2.5C). These observations confirmed the self-renewing and tumorigenic potential of miPS-LLCcm cells.

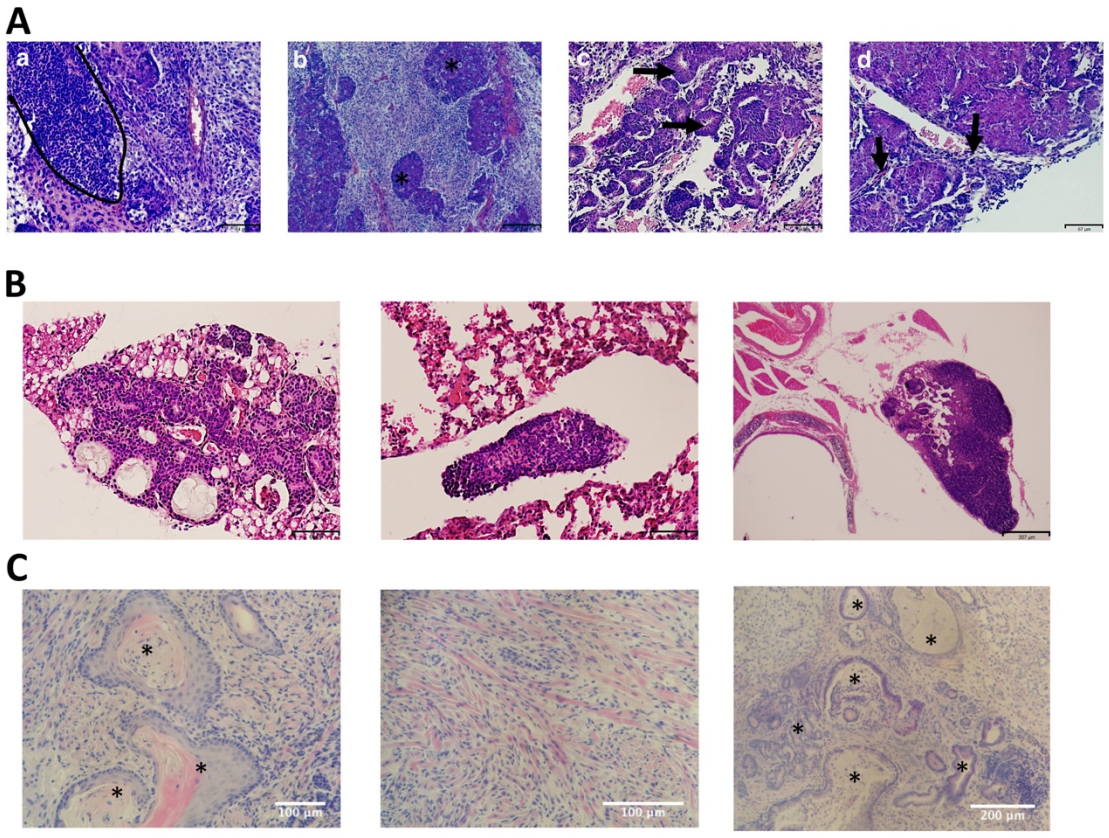
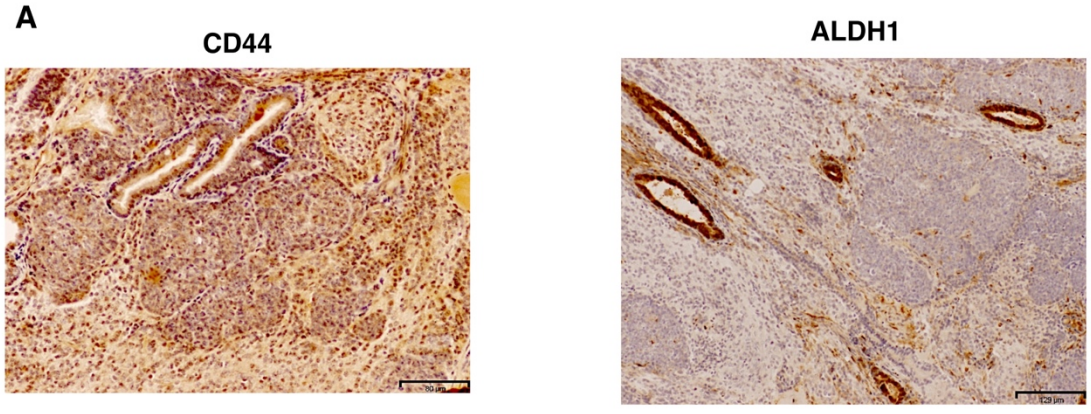


Figure 2.5. Histological analyses of allografts of miPS-LLCcm cells. (A) High nuclear to cytoplasmic ratio (a) and with the mass of undifferentiated cells (asterisks in (b)), granular epithelial structure (arrows in (c)) and micrometastasis (arrows in (d)). (B) Metastatic nodules-like tumor in the lung pulmonary tissue (left), in the lung tissue (middle), and in the chest (right). (C) Teratoma from untreated miPSCs showing the three germ layers: squamous epithelial tissue (asterisks) in ectoderm (left), muscle tissue in mesoderm (middle) and gland-like structures (asterisks) in endoderm (right). (A-C) H&E staining.

2.2.2. Characterization of miPS-LLCcm, Ptdc and LMN cells

In order to further confirm the acquisition of CSC-like phenotype, we assessed the expression of the commonly known CSC markers, CD44 and ALDH1 by rt-qPCR as well as immuno-histochemical analysis. (Figure 2.6A and 2.6B). The LMN and Ptdc cells showed the significantly higher expression of ALHD1 and CD44 than the miPSCs. The miPS-LLCcm derived tumor also showed the expression of these two markers.



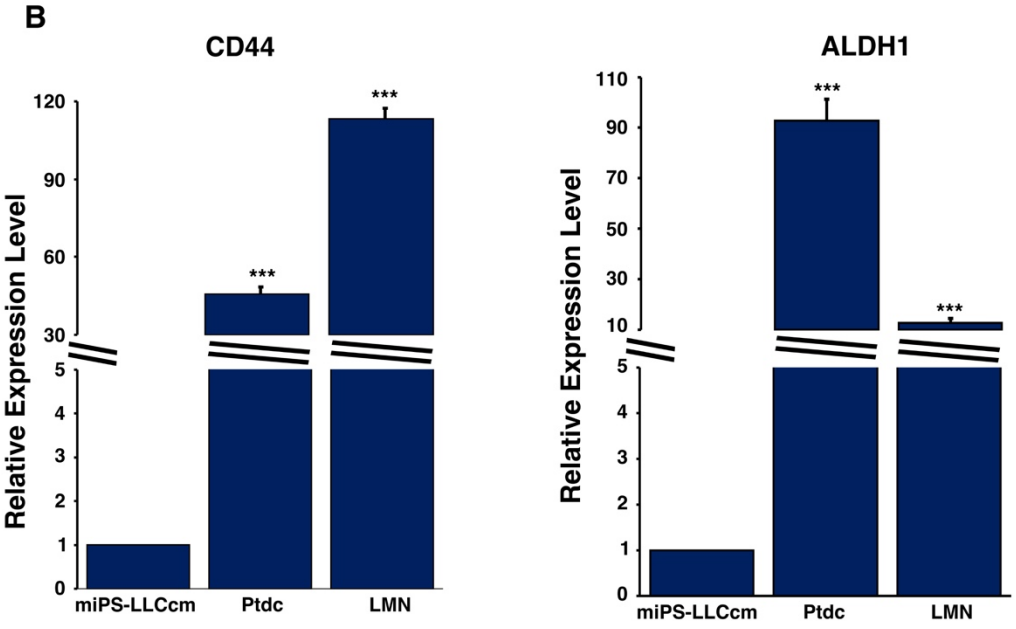


Figure 2.6. The localization and expression of CSC markers. (A) IHC analysis showing the expression of CD44 and ALDH1 in the tumor of the miPS-LLCcm cells. (B) The comparison of the expression of CD44 and ALDH1 the Ptdc and LMN cells with miPS-LLCcm by rt-qPCR analysis. ***P < .001, **P < .01, *P < .05.

2.2.3. *In vivo* tumorigenic differentiation of miPS-LLCcm

MUC1 plays a crucial role in cancer progression and is considered as a suitable marker for the adenocarcinoma tumor phenotype [12]. Immuno-histochemical analysis showed that tumor associated MUC1 expression was detected in tumor of miPS-LLCcm (Figure 2.7A). Mass of highly proliferating cells showed the expression of GFP as population of cells that are maintaining self-renewal ability.

Ptdc cells showed the upregulation of both E-cadherin, an epithelial marker, and N-cadherin, a mesenchymal marker, as compared to miPS-LLCcm and LMN cells (Figure 2.7B and 2.7C). The expression of Snail, Slug, Twist1 and Twist2 were also upregulated in Ptdc cells suggesting the potential of epithelial-to-mesenchymal transition (EMT) in Ptdc cells.

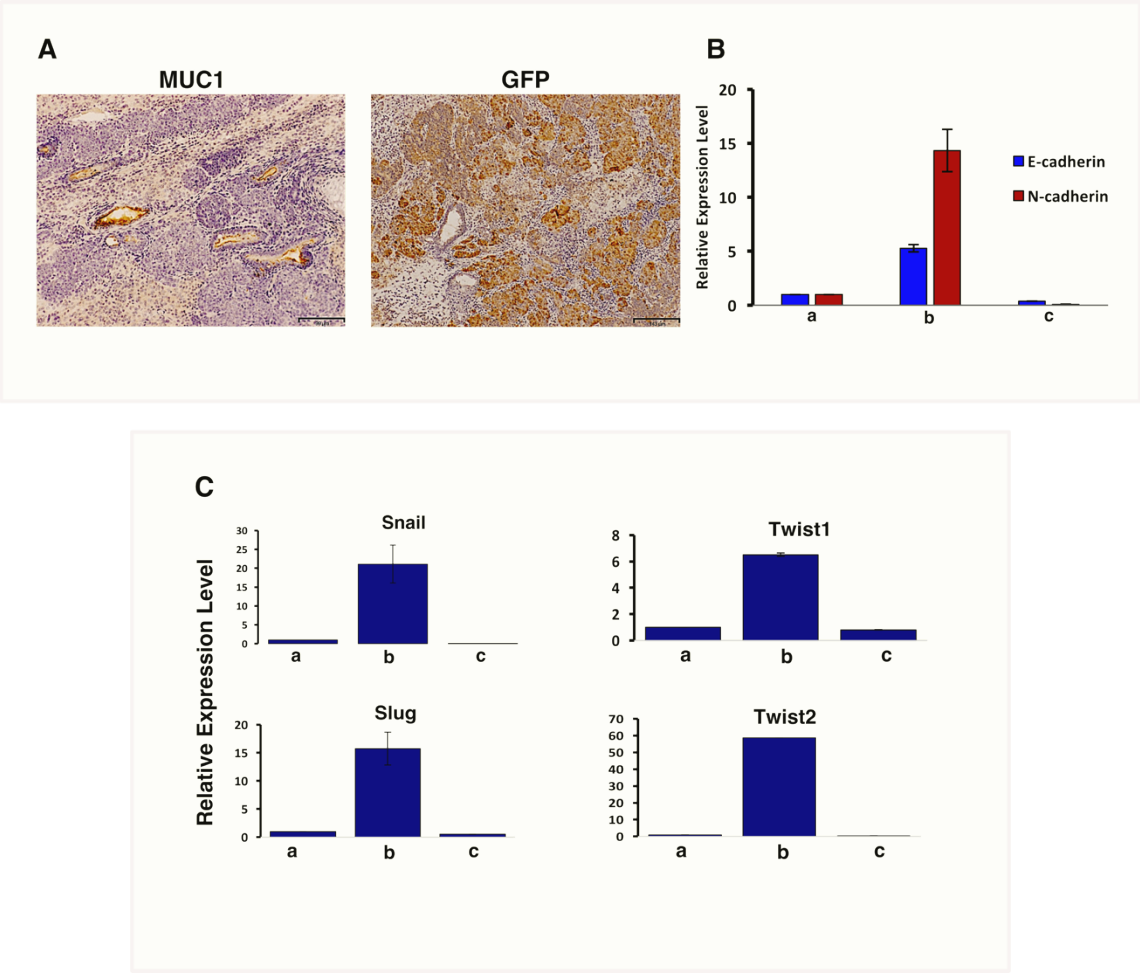


Figure 2.7. The localization and expression of EMT markers in miPS-LLCcm derived tumors. (A) Immuno-histochemical (IHC) analysis showed that ductal epithelial structure expressed MUC1 (left) and the undifferentiated mass of cells expressed the GFP (right). Comparison of the expression of (B) epithelial and mesenchymal markers (E-cadherin and N-cadherin) and (C) EMT markers in a, miPS-LLCcm cells; b, Ptdc cells and c, LMN cells.

2.3. MATERIALS AND METHODS

2.3.1. Cell Culture

Mouse Lewis Lung Carcinoma cell lines (LLC) were purchased from ATCC (USA) and maintained in DMEM (D5796 Sigma) medium containing 10% fetal bovine serum (FBS,Gibco,NY) and 100 U/ml penicillin/streptomycin (Wako,Japan). Mouse induced pluripotent stem cells (miPS, iPS-MEF-Ng-20D-17; Lot No.012, Fiken Cell Bank, Japan) were cultured in DMEM containing 15% FBS, 0.1 mM NEAA (100X NEAA, Gibco, NY), 2mM L-Glutamine (Nacalai Tesque, Japan), 50U/ml of penicillin/streptomycin (P/S), 0.1 mM 2-mercaptoethanol (Sigma) and 1000 U/ml of Leukemia Inhibitory Factor (LIF, Millipore, MA) on feeder layers of mitomycin treated mouse embryonic fibroblast (MEF) cells (Reprocell, Japan). In the case of feeder-less, the miPS cells were cultured on gelation (0.1%) coated dishes. The expression of GFP and cell morphology was observed and photographed using Olympus IX81 microscope equipped with a light fluorescence device (Olympus, Japan).

2.3.2. Conversion of miPSCs into the CSC-like cells

According to the methods reported by Chen L and Kasai T et al., we cultured the miPSCs in the presence of conditioned medium of LLC cells [7]. Nanog-GFP reporter expression was used in miPSC cells and the expression of GFP reflects the maintenance of stemness [13]. LLC cells were cultured and prior to collecting conditioned medium, the cells were changed into 5% serum medium at 70-80% confluency. After 48hrs incubation, the conditioned medium (CM) from LLC cells was collected and filtered through a 0.22 μm filter (Millipore, Ireland). The

miPSCs were treated with the CM for 4 weeks and miPSCs were cultured in the presence or absence of LIF as controls. The miPSCs cultured under feeder-less conditions were treated with the CM in 1:1 ratio of miPS medium and CM for four weeks.

2.3.3. Sphere Formation Assay

To generate the spheroids, serum free medium (DMEM 97.5%, NEAA 1%, L-Glutamine 1%, 100X Pen/Strep 0.5%, 0.1 mM 2-mercaptoethanol, and Insulin-transferrin-selenium-X 1/100 v/v (ITS-X, life technologies, CA) was used and single cells were plated on ultra-low attachment dishes (Corning incorporated, NY) at cell density of 1x104 cells/ml [14].

2.3.4. Animal experiments

The plan of animal experiments was reviewed and approved by the ethics committee for animal experiments of Okayama University under the IDs OKU-2013252, OKU-2014157, OKU-2014429 and OKU-2016078. All experiments were performed according to the Policy on the Care and Use of the Laboratory Animals, Okayama University. Nude mice (Balb/c-nu/nu, female, 4 weeks) were purchased CharlesRiver, Japan. Cells at 1x106 were suspended in 200 μl of HBSS (Hanks Balanced salt solution, Gibco, NY) and were subcutaneously transplanted into nude mice.

2.3.5. Preparation of primary cell culture

To prepare the primary culture from a mouse allograft, the tumor was excised and cut into small pieces (approximately 1 mm3) and washed in the

Hank's buffered salt solution (HBSS) for three times. These pieces were transferred into a 15-ml tube with 4 ml of dissociation buffer prepared in PBS containing 0.25% trypsin, 0.1% collagenase, 20% KnockOut™ Serum Replacement (Gibco, NY), 1 mM of CaCl2 and incubated at 37°C for 40 mins. To terminate the digestion, 5 ml of DMEM containing 10% FBS was then added. The cellular suspension transferred into the new tubes and centrifuged at 1000 rpm for 5 mins. The cell pellet was suspended in 5 ml of HBSS, and centrifuged at 1000 rpm for 5 min. The cell pellet was placed into an appropriate volume of miPS medium without LIF and the cell number was counted with hemocytometer. Then the cells at 5×105 were seeded per 60-mm dish. After a passage, the cells derived from mouse allografts were cultured in the presence of 1 μg/mL of puromycin for 24 hours to remove the host derived cells.

To prepare the primary culture from metastatic nodules in a lung, the lung tissue was excised and cut into small pieces (approximately 1 mm3) and washed in the HBSS for three times. And the same procedures with those for the cells from a tumor allograft were employed to prepare the cells. Finally, the expression of GFP and cell morphology was observed and photographed using Olympus IX81 microscope equipped with a light fluorescence device (Olympus, Japan).

2.3.6. RNA extraction, cDNA synthesis and qPCR mRNA expression analysis

Total RNA was extracted using RNAeasy Mini kit (QUIAGEN, Germany) according to the manufacturer's instructions and 1 μg of RNA was reverse transcribed using Superscript First strand kit (Invitrogen, CA). Quantitative real time PCR was performed with cycler 480 SYBR green I Master Mix (Roche,

Switzerland) according to manufacturer's instructions. Primers used for qPCR are listed in Table 2.1.

Table 2.1. List of Primers Used in the Experiments

No	Names	Forward Primer Sequence	Reverse Primer Sequence
1	Nanog	AGGGTCTGCTACTGAGATGCTCTG	AACCCAAGCACGTATCAGGG
2	Oct3/4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
3	Sox2	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA
4	Klf4	GGACTTACAAAATGCCAAGGGGTG	TCGCTTCCTCTTCCTCCGACACA
5	CD44	AGAAAAATGGCCGCTACAGTATC	TGCATGTTTCAAAACCCTTGC
6	ALDH1	AACACAGGTTGGCAAGTTAATCA	TGCGACACAACATTGGCCTT
7	E-cadherin	AACCCAAGCACGTATCAGGG	GGGGTCTGTGACAACAACGA
8	N-cadherin	CCTTGCTTCAGGCGTCTGTG	CTTGAAATCTGCTGGCTCGC
9	Snail	GGAGTTGACTACCGACCTTGC	TGGAAGGTGAACTCCACACAC
10	Slug	GCCCTTAAAGGCACTAACGAG	ATTCACGAAGGTGACGAGCC
11	Twist1	GCCGGAGACCTAGATGTCATTGT	TTAAAAGTGTGCCCCACGCC
12	Twist2	CTCACGAGCGTCTCAGCTAC	TTGTCCAGGTGCCGAAAGTC
13	GADPH	AACGGCACAGTCAAGGCCGA	ACCCTTTTGGCTCCACCCTT

2.3.7. Histological analysis and immunohistochemistry (IHC)

Paraffin embedded tumor sections (5μm) were stained with Hematoxylin (Sigma Aldrich,USA ;0.5%) and Eosin Y (Sigma Aldrich, USA) (HE) for histological analysis. Primary antibodies and dilutions used for IHC were used as follows; anti-GFP antibody 1:200 (#2956, Cell Signaling, USA), anti-MUC1

antibody 1:100 (Abcam/ab15481, UK), anti-ALDH1 antibody 1:200 (Abcam/ ab52492, UK) and Anti-CD44 antibody 1:200 (Abcam/ ab24504, UK).

2.3.8. Statistical analysis

The data were analyzed using two-tailed student's t-test and are presented as the mean ± standard deviation (SD) at least three-time determinations. A P-values less than 0.05 was considered to be statically significant, while less than 0.01 was highly significant.

2.4. DISCUSSION

The current study focused on the DNA methylation in the CSC model converted from iPSCs by the treatment with conditioned medium of cancer cells. By the treatment, miPSCs obtained the ability of unlimited growth and the capacity to maintain their stemness, while they were allowed to differentiate without LIF. The subcutaneous transplantation of the survived cells into the mouse formed malignant tumors and metastasized into lung tissues. Thus, we concluded that miPSCs should be converted into CSCs without any intended genetic manipulation. The immunohistochemical analysis revealed the heterogeneity of the tumors, in which miPS-LLCcm cells should on one hand maintain the undifferentiated population expressing GFP and differentiate on the other into adenocarcinoma phenotype expressing MUC1 while miPSCs developed benign teratoma showing the three germ-layers differentiation and the loss of undifferentiated phenotype. Furthermore, the cells from benign teratoma

cannot be maintained but the cells from tumors at primary site and metastatic nodules can be maintained in vitro.

The heterogeneous phenotypes characterized by the expression of E-cadherin and N-cadherin in the Ptdc cells should be implying the progression of cancer and metastatic potential of the tumor maintaining the plasticity of the transition between epithelial and mesenchymal states. Further study is required to confirm the cells are undergoing the EMT being involved in maintenance of stemness, invasiveness and metastasis of tumor cells.

Recently our group generated CSC models converted from iPSCs with the aid of CM of various cancer cell lines such as human pancreatic carcinoma cell lines PK-8 cells and KLM-1 cells, human breast cancer cell lines T47D cells and BT549 cells, mouse carcinoma cell lines LLC cells, P19 cells, B16 cells and MC.E12 cells [7,10,11]. In the conversion of pancreatic duct like adenocarcinoma (PDAC) like CSC model, there was no evidence relating to single point mutations even in Kras oncogene and its xenografts tumors showed the features of acinoductal metaplasia, pancreatic intraepithelial neoplasia and PDAC lesions[10]. We postulated that CSCs may be induced epigenetic changes without any known mutations.

Premature termination of reprogramming were reported to result in tumor development in various tissues with undifferentiated dysplastic cells exhibiting global changes in DNA methylation at H19 DMRs identifying IGF-2 expression up-regulated in the tumor initiating cells [31]. Since the changes in DNA methylation was considered responsible for the conversion of iPSCs into CSCs, the patterns of DNA methylation were compared between the converted cells

(miPS-LLCcm cells), tumor derived cells (Ptdc cells and LMN cells) and miPSCs. As the results of bisulfite sequencing, we evaluated the list of epigenetically affected genes regarding to the DMRs in the miPS-LLCcm cells and Ptdc cells and LMN cells. Hypo- and hypermethylated genes were identified and hypomethylation was found overall superior to hypermethylation in all CSCs when compared to the parental cell line miPSCs.

The analysis of KEGG pathways relating to hypomethylated genes revealed the several notable pathways important in cancers. Checking the expression of genes associated with these pathways, the expression of hypomethylated genes relating to PI3K-Akt pathway was found significantly high among those of the other genes. PI3K-Akt-mTOR signaling pathway has previously been reported as a key driver of carcinogenesis in several cancer types [32,33]. In this study, we found Pik3r5 (p101), which is a regulatory subunit of Pik3cg enzyme, as a hypomethylated and highly up-regulated gene relating to PI3K-Akt pathway. In the recent report, the evidence of PIK3CG as a potential oncogene were evaluated by analyzing the differential role each unit of PIK3CG, of which overexpression of the catalytic subunit PIK3CG (p110γ) or the regulatory subunit PIK3R5 (p101) leads to oncogenic cellular transformation and malignancy [34]. Therefore, the hypomethylation of Pik3r5 gene leading to the up-regulation is closely related to the activation/phosphorylation of AKT that is the downstream target molecule and Pik3cg should play a key role in carcinogenesis. In fact, the multiple myeloma cells derived from patients, the upregulation of PI3K components, in which PIK3CG has been proved to be a main regulator of cells adhesion and migration [35]. The PIK3CA gene has been reported to be hypomethylated in esophageal cancer cases when compared to the adjacent normal tissues [36]. On the other

hand, both Pik3r5 and Pik3cg were overexpressed resulting in the up-regulation of PI3K-gamma in the class IB PI3Ks, but not the PI3K-alpha in the class IA in our CSCs. Collectively, the activation of PI3K-Akt signaling pathway should significantly be relating with the conversion of miPSC into miPS-LLCcm cells resulting in the constitutive activation of Akt in Ptdc and LMN cells.

According to the recent reports, the tumor cells produced a variety of molecules such as growth factors, cytokines and chemokines, which exhibited various effects such as on tumor growth and angiogenesis, providing them with various microenvironments [37,38]. In our study, we have successfully demonstrated the CSCs generated from iPSCs by the treatment with CM from cancer derived cells acquired the DNA hypomethylation.

2.5. CONCLUSIONS

Significant overall DNA hypomethylation during the conversion should lead to the activation of certain proto-oncogene, which represent the malignant conversion even without mutations. In this context, the hypomethylation might be considered to contribute to the progression and metastasis of the cancer stem cells.

REFERENCES

1. Rozhok, A. I.; Salstrom, J. L.; DeGregori, J. Stochastic modeling indicates that aging and somatic evolution in the hematopoietic system are driven by non-cell-autonomous processes. *Aging (Albany. NY).* **2014**, *6*, 1033–1048, doi:10.18632/aging.100707.

2. Bonnet, D.; Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **1997**, *3*, 730–737, doi:10.1038/nm0797-730.

3. Reya, T.; Morrison, S. J.; Clarke, M. F.; Weissman, I. L. No Title. *Nature* **2001**, *414*, 105–111, doi:10.1038/35102167.

4. Clarke, M. F.; Dick, J. E.; Dirks, P. B.; Eaves, C. J.; Jamieson, C. H. M.; Jones, D. L.; Visvader, J.; Weissman, I. L.; Wahl, G. M. Cancer Stem Cells—Perspectives on Current Status and Future Directions: AACR Workshop on Cancer Stem Cells. *Cancer Res.* **2006**, *66*, 9339–9344, doi:10.1158/0008-5472.CAN-06-3126.

5. Hermann, P. C.; Huber, S. L.; Herrler, T.; Aicher, A.; Ellwart, J. W.; Guba, M.; Bruns, C. J.; Heeschen, C. Distinct Populations of Cancer Stem Cells Determine Tumor Growth and Metastatic Activity in Human Pancreatic Cancer. *Cell Stem Cell* **2007**, *1*, 313–323, doi:10.1016/j.stem.2007.06.002.

6. Sampieri, K.; Fodde, R. Cancer stem cells and metastasis. *Semin. Cancer Biol.* **2012**, *22*, 187–193, doi:10.1016/j.semcancer.2012.03.002.

7. Chen, L.; Kasai, T.; Li, Y.; Sugii, Y.; Jin, G.; Okada, M.; Vaidyanath, A.;

Mizutani, A.; Satoh, A.; Kudoh, T.; Hendrix, M. J. C.; Salomon, D. S.; Fu, L.; Seno, M. A model of cancer stem cells derived from mouse induced pluripotent stem cells. *PLoS One* **2012**, *7*, doi:10.1371/journal.pone.0033544.

8. Takahashi, K.; Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **2006**, *126*, 663–676, doi:10.1016/j.cell.2006.07.024.

9. Wan, P.-X. Importance of the stem cell microenvironment for ophthalmological cell-based therapy. *World J. Stem Cells* **2015**, *7*, 448, doi:10.4252/wjsc.v7.i2.448.

10. Calle, A. S.; Nair, N.; Oo, A. K.; Prieto-Vila, M.; Koga, M.; Khayrani, A. C.; Hussein, M.; Hurley, L.; Vaidyanath, A.; Seno, A.; Iwasaki, Y.; Calle, M.; Kasai, T.; Seno, M. A new PDAC mouse model originated from iPSCs-converted pancreatic cancer stem cells (CSCcm). *Am. J. Cancer Res.* **2016**, *6*, 2799–2815.

11. Nair, N.; Calle, A. S.; Zahra, M. H.; Prieto-Vila, M.; Oo, A. K. K.; Hurley, L.; Vaidyanath, A.; Seno, A.; Masuda, J.; Iwasaki, Y.; Tanaka, H.; Kasai, T.; Seno, M. A cancer stem cell model as the point of origin of cancer-associated fibroblasts in tumor microenvironment. *Sci. Rep.* **2017**, *7*, 6838, doi:10.1038/s41598-017-07144-5.

12. Nath, S.; Mukherjee, P. MUC1: A multifaceted oncoprotein with a key role in cancer progression. *Trends Mol. Med.* 2014, *20*, 332–342.

13. Okita, K.; Ichisaka, T.; Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **2007**, *448*, 313–317, doi:10.1038/nature05934.

14. Dontu, G.; Abdallah, W. M.; Foley, J. M.; Jackson, K. W.; Clarke, M. F.; Kawamura, M. J.; Wicha, M. S. In vitro propagation and transcriptional profiling of human mammary stem / progenitor cells. *Genes Dev.* **2003**, *17*, 1253–1270, doi:10.1101/gad.1061803.potential.

CHAPTER (3)

"Up-Regulation of PI 3-Kinases and the Activation of PI3K-Akt Signaling Pathway in Cancer Stem-Like Cells Through DNA Hypomethylation Mediated by the Cancer Microenvironment"

ABSTRACT

The tumors formed by subcutaneous injection of miPS-LLCcm cells that were converted from miPSCs, showed the structures with pathophysiological features consisting of undifferentiated and malignant phenotypes generally found in adenocarcinoma. Metastasis in the lung was also observed as nodule structures. Excising from the tumors, primary cultured cells from the tumor and the nodule showed self-renewal, differentiation potential as well as tumor forming ability, which are the essential characters of CSCs. Since this conversion is triggered only by the factor(s) contained in the conditioned medium, we have hypothesized that miPSCs are induced to cancer stem cells (CSCs) through epigenetic regulations without genetic modifications. We then characterized the epigenetic regulation occurring in the CSCs. By comparing the DNA methylation level of CG rich regions, the differentially methylated regions (DMRs) were evaluated in all stages of CSCs when compared with the parental iPSCs. In DMRs, hypomethylation was found superior to hypermethylation in the miPS-LLCcm cells and its derivatives. The hypo- and hypermethylated genes were used to nominate KEGG pathways related with CSC. As a result, several categories were defined in the KEGG pathways from which most related with cancers, significant and high expression of components was PI3K-AKT signaling pathway. Simultaneously, the AKT activation was also confirmed in the CSCs. The PI3K-Akt signaling pathway should be an important pathway for the CSCs established by the treatment with conditioned medium of LLC cells.

3.1. INTRODUCTION

Genetic mutation in tumor suppressors and oncogenic drivers have been widely described in the subsets of cancer patients. Mutation hypothesis is not only the sole reason of malignancy but also the epigenetic abnormalities have been involved in the tumorigenesis. Without changing in DNA sequence, the ability to change the expression of genes is the primary role of epigenetic regulation. Recent reports determined that epigenetic mechanisms, such as DNA methylation, can be involved in stem-cell maintenance and in the regulation of differentiation of stem cells [1,2]. Transcriptional silencing of tumor suppressor genes by promoter DNA hypermethylation is frequently found in human carcinogenesis [3]. However, hypomethylation was thought to be the epigenetic changes found in the early stages of carcinogenesis. Hepatocellular carcinoma lines that had been transformed with chemical carcinogens showed an aberrant hypomethylation of endogenous DNA during neoplastic transformation [4]. The global methylation difference between normal tissues and tumors showed that overall global hypomethylation is involved in oncogenesis or tumor progression [5].

It is well known that DNA methylation is one of the epigenetic modifications of DNA in all unicellular and multicellular organisms, associated with cell differentiation and proliferation [6]. Differentially methylated regions (DMRs) are stretches of DNA in the genome at which multiple adjacent CpG sites show differential methylation when comparing between two samples [7]. Growing evidences have shown that in many cancers, global DNA methylation changes contribute to alter gene expression programs, genomic instability, and

facilitate genetic mutations [8–10]. Most of researches in DNA methylation analyses have focused on aberrant methylation at the promoter, CpG rich and enhancer regions of related genes [11].

Recently, we demonstrated that the microenvironment of cancer cells can affect stem cells to convert iPSCs into CSC-like cells, with tumorigenic capacity as well as self-renewal and differentiation potential. In the present study, we tried to prove our hypothesis that epigenetic alterations can induce CSCs from normal stem cells in the tumor microenvironment.

3.2. RESULTS

3.2.1. Global DNA methylation analysis in Cancer Stem-like Cells

We analyzed the global DNA methylation status of miPS-LLCcm and its derivatives and used miPSC as control. After filtering low quality reads from raw RRBS sequencing data, we generated 4.9, 4.3, 3.9, 3.7 clean Gb of paired-end sequence data of miPSC, miPS-LLCcm, Ptdc, and LMN cells respectively **[Table S1]**. After filtering, clean reads were mapped to the reference sequence using BSMAP and the genome. From the clean reads 4.9, 4.3, 3.9, 3.7 Gb, uniquely mapping rate (77.15%, 74.82%, 75.9% and 77.79% respectively) were successfully aligned to the reference genome (mm10, GRC38 mouse), providing an average 12.02X, 11.7X, 8.9X, and 8.58X sequencing depth. The coverage of sequencing in all samples were 68.707%, 68.176%, and 663.969% and 62.886% of whole genome. The quality control items of sequencing data are corresponding to **Table S2**.

According to the sequence context of cytosine in the DNA sequence, it can be classified into three types, CG, CHG and CHH (H=A, G, or T). The decreasing

line in **Figure S1** reveled the range of effective sequencing depth of cytosine. These RRBS products cover the most number of promoters and CpG island of target regions, here we list the covered information include total number in genome, theoretical value in target region and the actual value which are covered by at least 5 CGs **[Table 3.1] [Figure S2].**

Table 3.1. Covered Promoter and CGI number of target regions

	PROMOTER	CGI
Whole Genome	24,445	16,023
Target Region	13,287	14,198
miPSC	9,368	11,719
miPSC_LLCcm	9,410	11,949
Ptdc cells	4,938	7,277
LMN Cells	5,181	7,687

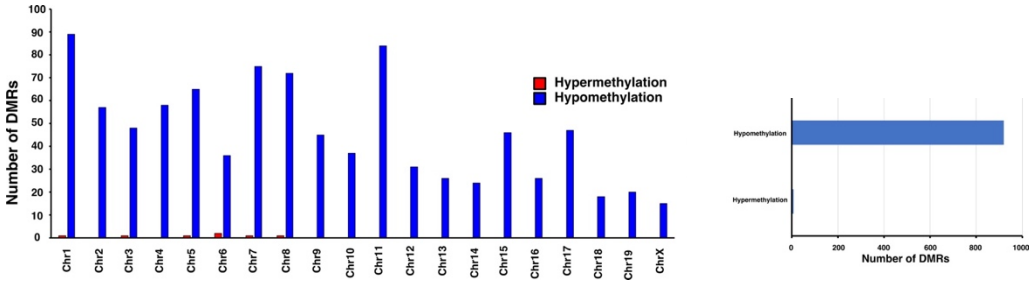
The coverage of methyl-cytosines were occurred in two featured regions; promoter and CpG island, the data for proportion of total methyl-cytosine was shown in **Table S3**. Among the methylated cytosine contexts, percentage of CG methylation pattern support the enough coverage. In the case of the proportions of methyl-cytosine pattern, mCG was the most common in both regions of all samples. Average Methylation level of each sample is shown in **Table S4** and determined by the reads which covered in cytosine, it was also equal the mC/C ratio at each reference cytosine. Methylation status of CG, CHG and CHH differ among species and even varies with different conditions concerning time, space and physiology within a single organism. The distribution of methyl-cytosine was analyzed according to methylation level, demonstrating that the different patterns were observed in four different stages of CSC model **[Figure S3].**

3.2.2. Analysis of the differentially methylated regions (DMRs) in the cancer stem cell model converted from iPSCs

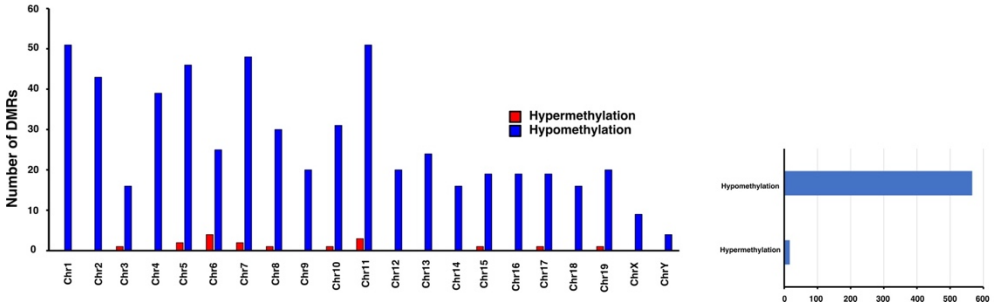
Epigenetic alterations have been attributed to play an important role in carcinogenesis. Methylation and demethylation is generally considered to silence and activate gene expression, respectively. However, the epigenetic changes in early stages of cancer development in this research has not yet been assessed. Since the 4-week treatment of iPSCs with LLC-CM appears to be the main source of conferring the potential of CSCs to generate miPS-LLCcm, we hypothesized that altered epigenetic regulation rather than gene mutations, may perform an essential role in this conversion process. Sliding-window approach identified differentially methylated regions (DMRs), which contains at least five CG sites. The methylation levels were significantly different between the samples when assessed by Fisher test ($P < 0.05$).

We compared the methylation in miPS-LLCcm, LMN and Ptdc cells with that in miPSC by three sets; (1) miPSCs vs. miPS-LLCcm cells, (2) miPSCs vs. Ptdc cells and (3) miPSCs vs. LMN cells. From these three comparisons, 926, 583 and 1105 DMRs were identified respectively **(Figure 3.1A-3.1C and Table 3.2)**. All DMRs between the different cell populations were found to exhibit hypomethylation as compared to miPSCs **(Figure 3.2)**.

(A)



(B)



(C)

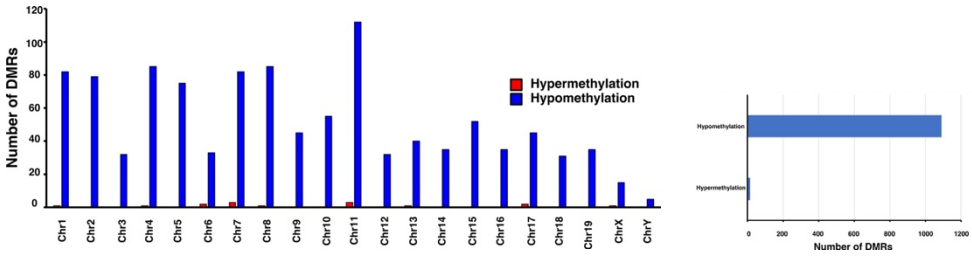


Figure 3.1. DMR in each chromosome and methylation pattern changes (Hyper and Hypomethylated DMRs) (A) miPSCs Vs miPS-LLCcm cells (B) miPSCs Vs Ptdc cells (C) miPSCs Vs LMN cells.

Table 3.2. Number and length of DMRs in each comparison with distribution in each chromosome

# Chr	miPSCs Vs miPS-LLCcm cells		miPSCs Vs Ptdc cells		miPSCs Vs LMN cells	
	DMR number	DMR length	DMR number	DMR length	DMR number	DMR length
chr1	90	22576	51	14197	83	23430
chr2	57	15754	43	12381	79	22178
chr3	49	12193	17	4575	32	8717
chr4	58	14374	39	10165	86	23431
chr5	66	17269	48	11344	75	21109
chr6	38	9749	29	8886	35	10530
chr7	76	18897	50	14132	85	23809
chr8	73	18255	31	8643	86	23922
chr9	45	11617	20	5377	45	12762
chr10	37	10686	32	7642	55	14107
chr11	84	22756	54	14689	115	33193
chr12	31	8340	20	4956	32	8975
chr13	26	7471	24	7108	41	11191
chr14	24	5650	16	4445	35	8621
chr15	46	11328	20	5208	52	13480
chr16	26	6278	19	4440	35	8311
chr17	47	11768	20	6246	47	14115
chr18	18	4935	16	4820	31	9282
chr19	20	5025	21	6078	35	10283
chrX	15	3763	9	2505	16	4616
chrY	0	0	4	734	5	1253
chrM	0	0	0	0	0	0
Total	926	238684	583	158571	1105	307315

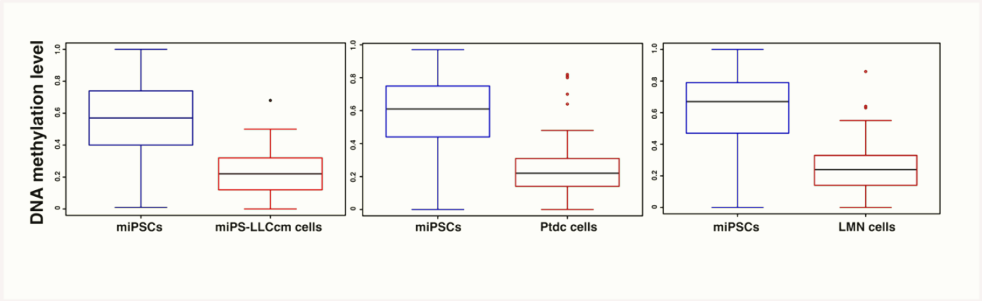


Figure 3.2. Relative DNA methylation levels of DMRs when miPS-LLCcm cells, Ptdc cells and LMN clells compared to miPSCs. miPSCs vs miPS-LLCcm cells (left), miPSCs vs Ptdc cells (middle) and miPSCs vs LMN cells (right).

DMRs-associated genes were further identified and segregated into hypo- and hyper-methylated genes categories (Dataset S2A,B, S3A,B, S4A,B). To validate whether the genes with DMRs were enriched for certain pathways, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for the hypomethylated genes in miPS-LLCcm, Ptdc and LMN cells. According the numbers of hypomethylated genes involved in the pathway, the top 15 KEGG pathways were nominated **(Figure 3.3A and 3.3C and Table S5-S7)** (Dataset S2C, S3C, S4C). We then selected the pathways most relevant for carcinogenesis including Focal adhesion, PI3K-Akt signaling pathway, Calcium signaling pathway, Pathway in cancer and Transcriptional misregulation in cancer.

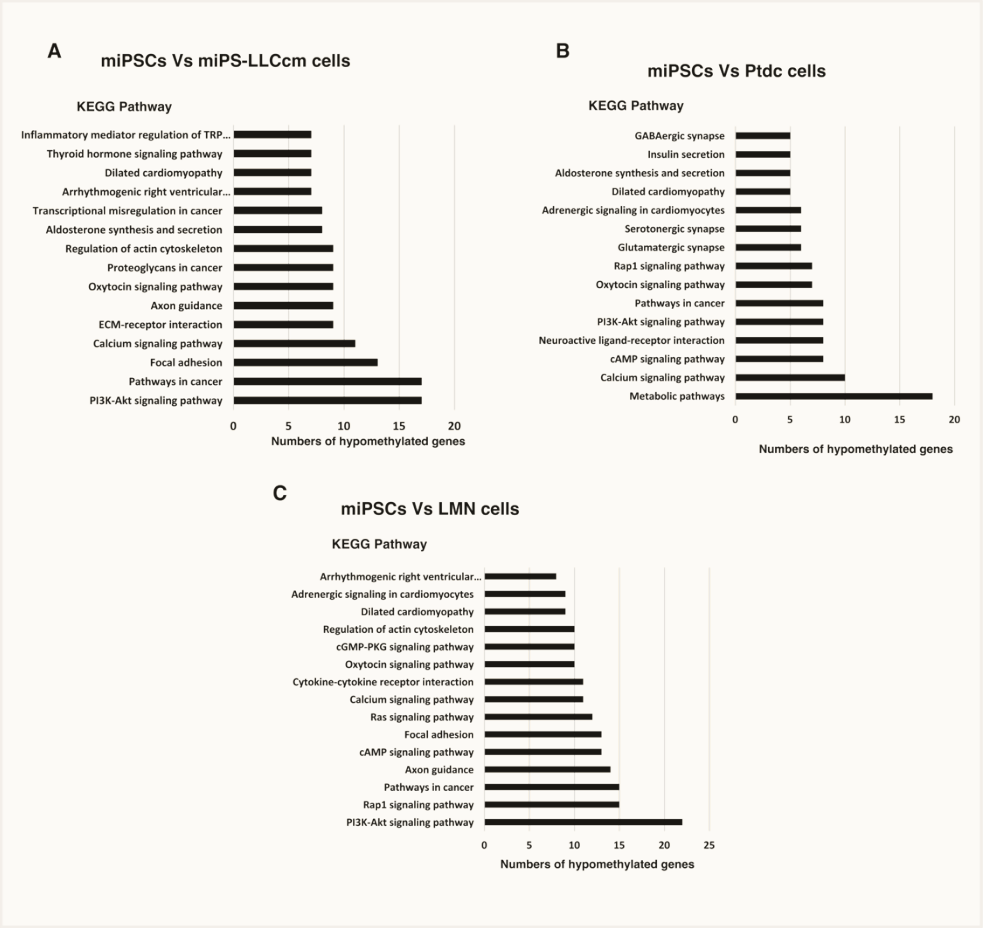


Figure 3.3. KEGG pathways nominated with the number of hypomethylated genes (A) miPSCs vs. miPS-LLCcm cells, (B) miPSCs vs. Ptdc cells (C) miPSCs vs. LMN cells.

3.2.3. Overexpression of PI3K-Gamma Candidates in the Model were Relating to Oncogenic Potential

To evaluate the up-regulated expression of the hypomethylated genes in the pathway selected above, we performed rt-qPCR analysis on the miPS-LLCcm cells together with the Ptdc and LMN cells. As the results, the expression of 11 genes were found up-regulated in miPS-LLCcm cells when compared to miPSCs **(Figure 3.4)**. Among the candidates, Pik3r5 (p101) showed significantly high expression in all the cells**.** Recent reports have suggested that the overexpression of the catalytic subunit Pik3cg (p110γ) or the regulatory subunit Pik3r5 leads to oncogenic cellular transformation and malignancy [12]. These two subunits are class IB PI3Ks and considered to make a heterodimer of PI3K-gamma. We assessed the expression levels of Pik3cg and Pik3ca, which were class IB and class IA PI3Ks, in the miPS-LLCcm, Ptdc and LMN cells. As the result, Pik3cg showed significantly higher expression ($P < 0.01$) than that of Pik3ca (Figure 3.5A).

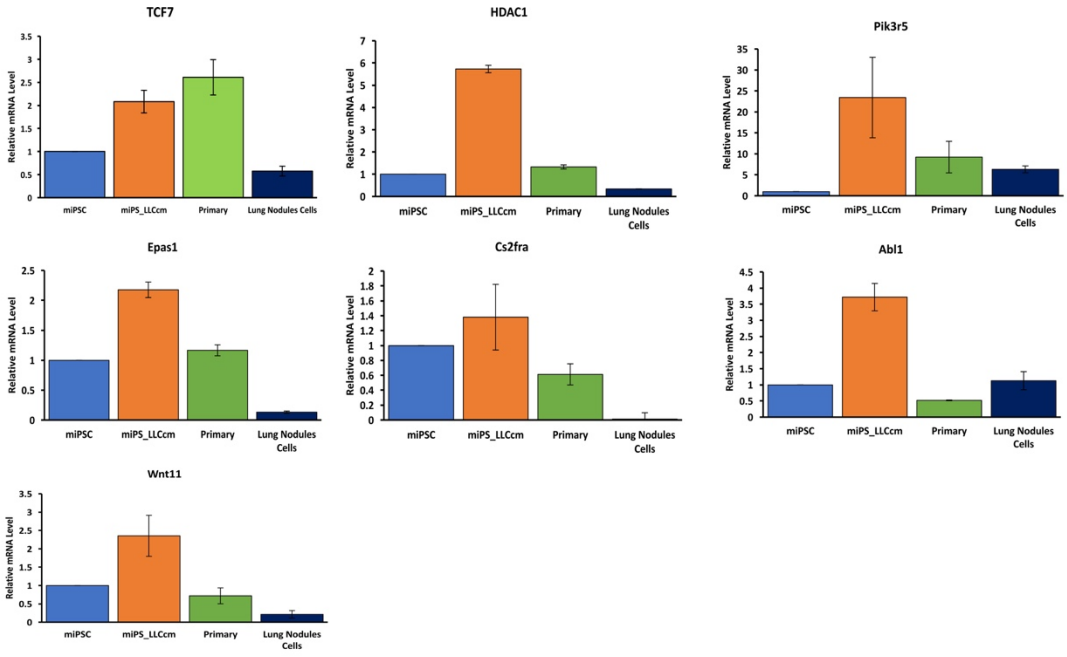


Figure 3.4. Expression of hypomethylated genes significant in KEGG-pathway related to cancer. rt-qPCR analyses of the candidate hypomethylated genes.

3.2.4. PI3K-Akt Activation Drives iPSC-CSCs Model

The up-regulated expression of Pik3r5 should induce the activation of Akt resulting in the onset of tumorigenicity and metastatic potential of miPS-LLCcm cells and Ptdc cells. Based on the evaluation in rt-qPCR, the constitutive expression of Pik3r5 was detected by immunoblotting with anti-p101 antibody in miPS-LLCcm and its derivatives **(Figure 3.5B)**. Akt activation was simultaneously assessed by Western blotting with anti-phosphorylated Akt (p-Akt) antibody. We found Akt was constitutively activated in miPS-LLCcm, Ptdc and LMN cells **(Figure 3.5B)**. In miPSCs, Akt was weakly activated in the presence of LIF but not in the absence of LIF. Taking these into consideration, the hypomethylation should lead to overexpress PI3K-gamma to enhance PI3K-Akt pathway in CSC-

like cells without mutations in the open reading frames of DNA **(Figure S4)**. These findings are consistent with the PI3K-Akt pathway as recognized one of the most frequent signaling pathway enhanced in human cancers [13].

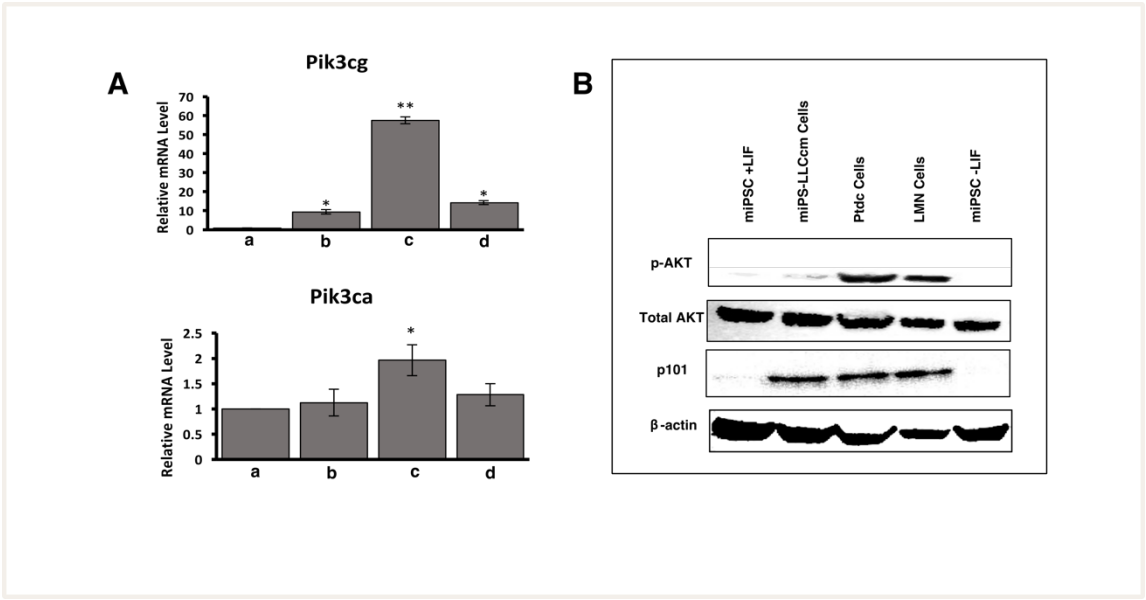


Figure 3.5. Evaluation of the candidate signaling pathway nominated by the KEGG analysis. (A) rt-qPCR analyses of Pik3cg (top) and Pik3ca (bottom); ***$P < .001$, **$P < .01$, *$P < .05$. (a, miPSCs; b, miPS-LLCcm cells; c, Ptdc cells; d, LMN cells). (B) Immunoblotting analysis of AKT activation and the expression of Pik3r5.

3.3. MATERIALS AND METHODS

3.3.1. RRBS DNA methylation analysis

The RRBS methylation analysis was performed by the BGI sequencing company. Extraction of DNA was carried out by QIAamp DNA Mini Kit

(QUIAGEN, Germany) according to manufacturing protocol. For RRBS library constructing, the DNA samples were digested with restriction enzyme after checking the sample quality test. This is followed by DNA-end repair, 3'-dA tailing, adapter ligation and size selection were carried out for fragments between 40 and 220 bps in length. Finally, bisulfite treatment was carried out with ZYMO EZ DNA Methylation-Gold kit (Zymo Research, USA) and converted fragments were amplified by PCR and the prepared RRBS library were further sequenced for the analysis.

RRBS libraries were sequenced on Illumina HiSeq 4000 using the PE100 sequencing strategy. Data filtering was performed to remove adaptor sequences, contamination and low-quality reads. The low-quality reads mean that the ratio of unknown base (N) is over 10 % and the ratio of quality less than 20 bases over 10%. After filtering the remaining reads are called clean reads and stored as FASTQ format. The clean reads were mapped to the reference sequence (mm10) using BSMAP in which the mapping rate and bisulfite conversion rate of each sample is calculated [14].

Methylation level (R_m) is determined by the reads which covered in cytosine, it was also equal the mC/C ratio of each reference cytosine [15,16]. The formula was as following;

$$R_m = \frac{N_m}{N_m + N_{nm}} * 100$$

(N_m represents the reads number of mC, while N_{nm} represents the reads number of non-methylation reads.)

Putative DMRs were identified by comparison of the sample1 and sample 2 methylomes using windows that contained at least 5 CpG (CHG or CHH) sites with a 2-fold change in methylation level and Fisher test pvalue<0.05. Two nearby DMRs would be considered interdependent and joined into one continuous DMR if the genomic region from the start of an upstream DMR to the end of a downstream DMR also had 2-fold methylation level differences between sample 1 and sample 2 with a pvalue<0.05. Otherwise, the two DMRs were viewed as independent. After iteratively merging interdependent DMRs, the final dataset of DMRs was made up of those that were independent from each other [17]. The DMR related genes was annotated with UCSC table browser tool [18,19].

3.3.2. KEGG Pathway Enrichment

Pathway-based analysis helps to further understand genes biological functions. DAVID bioinformatics resources is used to analyze the pathway enrichment of DMR -related genes [20–22]. This analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DMR -related genes comparing with the target regions background.

3.3.3. Western Blotting

The whole lysates of cells cultured in various conditions subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration in whole cell lysates was determined by BCA protein assay kit (Thermo Scientific;23235, USA). The protein was transferred from gel to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Germany) and probed with Antibodies against anit-Akt and anit-p-Akt (Ser473), anit-p110γ (D55D5), anit-p101 (D32A5) (Cell Signaling

Technology, USA) at a 1:1000 dilution followed by horseradish peroxidase-conjugated secondary antibodies (1: 2,000-1: 5,000, Cell signaling Technology). In -LIF experiment, miPSCs were cultured in miPSC medium without LIF for 6 hours before protein extraction. Immune-complex signals were developed with an ECL kit (PerkinElmer Inc., USA) and detected by Light Capture II (ATTO, Japan).

3.3.4. RNA extraction, cDNA synthesis and qPCR mRNA expression analysis

Total RNA was extracted using RNAeasy Mini kit (QUIAGEN, Germany) according to the manufacturer's instructions and 1 μg of RNA was reverse transcribed using Superscript First strand kit (Invitrogen, CA). Quantitative real time PCR was performed with cycler 480 SYBR green I Master Mix (Roche, Switzerland) according to manufacturer's instructions. Primers used for qPCR are listed in Table 3.3.

Table 3.3. List of Primers Used in the Experiments

No	Names	Forward Primer Sequence	Reverse Primer Sequence
1	TCF7	GCGCGGGATAACTACGGAAA	ACTGTCATCGGAAGGAACGG
2	Epas1	AGACACCCCAGGGAACACTA	TCACTGAAGTCCGTCTGGGT
3	HDAC1	CTGTGAACTACCCACTGCGA	TGGCGTGTCCTTTGATGGTC
4	Cs2fra	GTCCTCAACTCCACGGGTCA	CTCGAGCGCCTTCGTAGC
5	Wnt11	ACACTGTAAACAGCTGGAGGG	CGTGTACCTCTCTCCAGGTCAA
6	Abl1	ATCTCAGATGAGGTGGAGAAGG	GTGTCAGGCGCATCTTTCTG
7	CCND3	GATTCTGCACCGCCTGTCTC	GATACATCGCAAAGGTGTAATCTGT
8	Pik3ap1	GAAGGCCATTTCTGAAGATTCTGG	TCTCGTCCAGCTTGCATCTC
9	Thsb4	CCCAGCTGGACACTGACAAA	TCCTACCCCGTCATTGTTGC
10	Phllp1	GCCTGGAGCTGCTCAATAAC	CTCTCGGTTGTCACGGAAGT

11	Pik3r5	AAGTCCTTTGTCAGCAGTCCC	CTGGTAAACCTGCAGCAACAC
13	GADPH	AACGGCACAGTCAAGGCCGA	ACCCTTTTGGCTCCACCCTT

3.3.4. STATISTICAL ANALYSIS

The data were analyzed using two-tailed student's t-test and are presented as the mean ± standard deviation (SD) at least three-time repetition. A P-values less than 0.05 was considered to be statically significant, while less than 0.01 was highly significant.

3.4. DISCUSSION

The current study focused on the DNA methylation in the CSC model converted from iPSCs by the treatment with conditioned medium of cancer cells. In the conversion of pancreatic duct like adenocarcinoma (PDAC) like CSC model, there was no evidence relating to single point mutations even in Kras oncogene and its xenografts tumors showed the features of acinoductal metaplasia, pancreatic intraepithelial neoplasia and PDAC lesions[23]. We postulated that CSCs may be induced by epigenetic changes without any known mutations. Premature termination of reprogramming was reported to result in tumor development in various tissues with undifferentiated dysplastic cells exhibiting global changes in DNA methylation at H19 DMRs identifying IGF-2 expression up-regulated in the tumor initiating cells [24].

Since the changes in DNA methylation was considered responsible for the conversion of iPSCs into CSCs, the patterns of DNA methylation were compared

between the converted cells (miPS-LLCcm cells), tumor derived cells (Ptdc cells and LMN cells) and miPSCs. As the results of bisulfite sequencing, we evaluated the list of epigenetically affected genes regarding to the DMRs in the miPS-LLCcm cells and Ptdc cells and LMN cells. Hypo- and hypermethylated genes were identified and hypomethylation was found overall superior to hypermethylation in all CSCs when compared to the parental cell line miPSCs.

The analysis of KEGG pathways relating to hypomethylated genes revealed the several notable pathways important in cancers. Checking the expression of genes associated with these pathways, the expression of hypomethylated genes relating to PI3K-Akt pathway was found significantly high among those of the other genes. PI3K-Akt-mTOR signaling pathway has previously been reported as a key driver of carcinogenesis in several cancer types [25,26]. In this study, we found Pik3r5 (p101), which is a regulatory subunit of Pik3cg enzyme, as a hypomethylated and highly up-regulated gene relating to PI3K-Akt pathway. In the recent report, the evidence of PIK3CG as a potential oncogene were evaluated by analyzing the differential role each unit of PIK3CG, of which overexpression of the catalytic subunit PIK3CG (p110γ) or the regulatory subunit PIK3R5 (p101) leads to oncogenic cellular transformation and malignancy [27]. Therefore, the hypomethylation of Pik3r5 gene leading to the up-regulation is closely related to the activation/phosphorylation of AKT that is the downstream target molecule and Pik3cg should play a key role in carcinogenesis. In fact, the multiple myeloma cells derived from patients, the upregulation of PI3K components, in which PIK3CG has been proved to be a main regulator of cells adhesion and migration [28]. The PIK3CA gene has been reported to be hypomethylated in esophageal cancer cases when compared to the adjacent normal tissues [29]. On the other

hand, both Pik3r5 and Pik3cg were overexpressed resulting in the up-regulation of PI3K-gamma in the class IB PI3Ks, but not the PI3K-alpha in the class IA in our CSCs. Collectively, the activation of PI3K-Akt signaling pathway should significantly be relating with the conversion of miPSC into miPS-LLCcm cells resulting in the constitutive activation of Akt in Ptdc and LMN cells.

According to the recent reports, the tumor cells produced a variety of molecules such as growth factors, cytokines and chemokines, which exhibited various effects such as on tumor growth and angiogenesis, providing them with various microenvironments [30,31]. In our study, we have successfully demonstrated the CSCs generated from iPSCs by the treatment with CM from cancer derived cells acquired the DNA hypomethylation.

3.5. CONCLUSION

Significant overall DNA hypomethylation during the conversion should lead to the activation of certain proto-oncogene, which represent the malignant conversion even without mutations. In this context, the hypomethylation might be considered to contribute to the progression and metastasis of the cancer stem cells.

REFERENCES

1. Ortiz-sánchez, E. Overview : Epigenetic Regulation in Cancer Stem Cells by Methylation. **2014**, *1*, 3–5.

2. Toh, T. B.; Lim, J. J.; Chow, E. K.-H. Epigenetics in cancer stem cells. *Mol. Cancer* **2017**, *16*, 29, doi:10.1186/s12943-017-0596-9.

3. Baylin, S. B.; Herman, J. G. S.B. Baylin, J.G. Herman, DNA hypermethylation in tumorigenesis: epigenetics joins genetics, Trends Genet. 16 (2000) 168–174. - Google Search. *Trends Genet.* **2000**, *16*, 168–174.

4. Lapeyre, J. N.; Becker, F. F. 5-Methylcytosine content of nuclear DNA during chemical hepatocarcinogenesis and in carcinomas which result. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 698–705, doi:10.1016/0006-291X(79)92015-1.

5. Ehrlich, M.; Jiang, G.; Fiala, E.; Dome, J. S.; Yu, M. C.; Long, T. I.; Youn, B.; Sohn, O.-S.; Widschwendter, M.; Tomlinson, G. E.; Chintagumpala, M.; Champagne, M.; Parham, D.; Liang, G.; Malik, K.; Laird, P. W. Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene* **2002**, *21*, 6694–6702, doi:10.1038/sj.onc.1205890.

6. Reik, W.; Dean, W.; Walter, J. Epigenetic reprogramming in mammalian development. *Science* **2001**, doi:10.1126/science.1063443.

7. Rakyan, V. K.; Down, T. A.; Balding, D. J.; Beck, S. Epigenome-wide association studies for common human diseases. *Nat. Rev. Genet.* 2011.

8. Jones, P. A. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* 2012.

9. Pogribny, I. P.; Beland, F. a DNA hypomethylation in the origin and pathogenesis of human diseases. *Cell. Mol. Life Sci.* **2009**, doi:10.1007/s00018-009-0015-5.

10. You, J. S.; Jones, P. A. Cancer Genetics and Epigenetics: Two Sides of the Same Coin? *Cancer Cell* 2012.

11. Bell, R. E.; Golan, T.; Sheinboim, D.; Malcov, H.; Amar, D.; Salamon, A.; Liron, T.; Gelfman, S.; Gabet, Y.; Shamir, R.; Levy, C. Enhancer methylation dynamics contribute to cancer plasticity and patient mortality. *Genome Res.* **2016**, doi:10.1101/gr.197194.115.

12. Johnson, C.; Marriott, S. J.; Levy, L. S. Overexpression of p101 activates PI3Kgamma signaling in T cells and contributes to cell survival. *Oncogene* **2007**, *26*, 7049–7057, doi:10.1038/sj.onc.1210504.

13. Thorpe, L. M.; Yuzugullu, H.; Zhao, J. J. PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting. *Nat. Rev. Cancer* **2014**, *15*, 7–24, doi:10.1038/nrc3860.

14. Xi, Y.; Li, W. BSMAP: whole genome bisulfite sequence MAPping program. *BMC Bioinformatics* **2009**, *10*, 232, doi:10.1186/1471-2105-10-232.

15. Xiang, H.; Zhu, J.; Chen, Q.; Dai, F.; Li, X.; Li, M.; Zhang, H.; Zhang, G.; Li, D.; Dong, Y.; Zhao, L.; Lin, Y.; Cheng, D.; Yu, J.; Sun, J.; Zhou, X.; Ma, K.; He, Y.; Zhao, Y.; Guo, S.; Ye, M.; Guo, G.; Li, Y.; Li, R.; Zhang, X.; Ma, L.;

Kristiansen, K.; Guo, Q.; Jiang, J.; Beck, S.; Xia, Q.; Wang, W.; Wang, J. Single base–resolution methylome of the silkworm reveals a sparse epigenomic map. *Nat. Biotechnol.* **2010**, *28*, 516–520, doi:10.1038/nbt.1626.

16. Deng, J.; Shoemaker, R.; Xie, B.; Gore, A.; LeProust, E. M.; Antosiewicz-Bourget, J.; Egli, D.; Maherali, N.; Park, I.-H.; Yu, J.; Daley, G. Q.; Eggan, K.; Hochedlinger, K.; Thomson, J.; Wang, W.; Gao, Y.; Zhang, K. Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat. Biotechnol.* **2009**, *27*, 353–360, doi:10.1038/nbt.1530.

17. Cokus, S. J.; Feng, S.; Zhang, X.; Chen, Z.; Merriman, B.; Haudenschild, C. D.; Pradhan, S.; Nelson, S. F.; Pellegrini, M.; Jacobsen, S. E. Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature* **2008**, *452*, 215–219, doi:10.1038/nature06745.

18. Karolchik, D. The UCSC Table Browser data retrieval tool. *Nucleic Acids Res.* **2004**, *32*, 493D–496, doi:10.1093/nar/gkh103.

19. Zweig, A. S.; Karolchik, D.; Kuhn, R. M.; Haussler, D.; Kent, W. J. UCSC genome browser tutorial. *Genomics* 2008, *92*, 75–84.

20. Kanehisa, M.; Araki, M.; Goto, S.; Hattori, M.; Hirakawa, M.; Itoh, M.; Katayama, T.; Kawashima, S.; Okuda, S.; Tokimatsu, T.; Yamanishi, Y. KEGG for linking genomes to life and the environment. *Nucleic Acids Res.* **2008**, *36*, doi:10.1093/nar/gkm882.

21. Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Systematic and integrative

analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **2008**, *4*, 44–57, doi:10.1038/nprot.2008.211.

22. Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **2009**, *37*, 1–13, doi:10.1093/nar/gkn923.

23. Calle, A. S.; Nair, N.; Oo, A. K.; Prieto-Vila, M.; Koga, M.; Khayrani, A. C.; Hussein, M.; Hurley, L.; Vaidyanath, A.; Seno, A.; Iwasaki, Y.; Calle, M.; Kasai, T.; Seno, M. A new PDAC mouse model originated from iPSCs-converted pancreatic cancer stem cells (CSCcm). *Am. J. Cancer Res.* **2016**, *6*, 2799–2815.

24. Ohnishi, K.; Semi, K.; Yamamoto, T.; Shimizu, M.; Tanaka, A.; Mitsunaga, K.; Okita, K.; Osafune, K.; Arioka, Y.; Maeda, T.; Soejima, H.; Moriwaki, H.; Yamanaka, S.; Woltjen, K.; Yamada, Y. Premature termination of reprogramming in vivo leads to cancer development through altered epigenetic regulation. *Cell* **2014**, *156*, 663–677, doi:10.1016/j.cell.2014.01.005.

25. Chang, L.; Graham, P. H.; Ni, J.; Hao, J.; Bucci, J.; Cozzi, P. J.; Li, Y. Targeting PI3K/Akt/mTOR signaling pathway in the treatment of prostate cancer radioresistance. *Crit. Rev. Oncol. Hematol.* 2015, *96*, 507–517.

26. Papadimitrakopoulou, V. Development of PI3K/AKT/mTOR Pathway Inhibitors and Their Application in Personalized Therapy for Non-Small-Cell Lung Cancer. *J. Thorac. Oncol.* **2012**, *7*, 1315–26, doi:10.1097/JTO.0b013e31825493eb.

27. Brazzatti, J. A.; Klingler-Hoffmann, M.; Haylock-Jacobs, S.; Harata-Lee, Y.; Niu, M.; Higgins, M. D.; Kochetkova, M.; Hoffmann, P.; McColl, S. R. Differential roles for the p101 and p84 regulatory subunits of PI3Kγ in tumor growth and metastasis. *Oncogene* **2012**, *31*, 2350–2361, doi:10.1038/onc.2011.414.

28. Piddock, R. E.; Loughran, N.; Marlein, C. R.; Robinson, S. D.; Edwards, D. R.; Yu, S.; Pillinger, G. E.; Zhou, Z.; Zaitseva, L.; Auger, M. J.; Rushworth, S. A.; Bowles, K. M. PI3Kδ and PI3Kγ isoforms have distinct functions in regulating pro-tumoural signalling in the multiple myeloma microenvironment. *Blood Cancer J.* **2017**, *7*, e539, doi:10.1038/bcj.2017.16.

29. Wang, W.; Xie, Y.; Zhou, Z.; Qin, Z.; Wu, J.; He, J. PIK3CA hypomethylation plays a key role in activation of the PI3K/AKT pathway in esophageal cancer in Chinese patients. *Acta Pharmacol. Sin.* **2013**, *34*, 1560–1567, doi:10.1038/aps.2013.163.

30. Fukuyama, T.; Ichiki, Y.; Yamada, S.; Shigematsu, Y.; Baba, T.; Nagata, Y.; Mizukami, M.; Sugaya, M.; Takenoyama, M.; Hanagiri, T.; Sugio, K.; Yasumoto, K. Cytokine production of lung cancer cell lines: Correlation between their production and the inflammatory/immunological responses both in vivo and in vitro. *Cancer Sci.* **2007**, *98*, 1048–1054, doi:10.1111/j.1349-7006.2007.00507.x.

31. Yasmin, R.; Siraj, S.; Hassan, A.; Khan, A. R.; Abbasi, R.; Ahmad, N. Epigenetic regulation of inflammatory cytokines and associated genes in human malignancies. *Mediators Inflamm.* 2015, *2015*.

APPENDIX

Figure

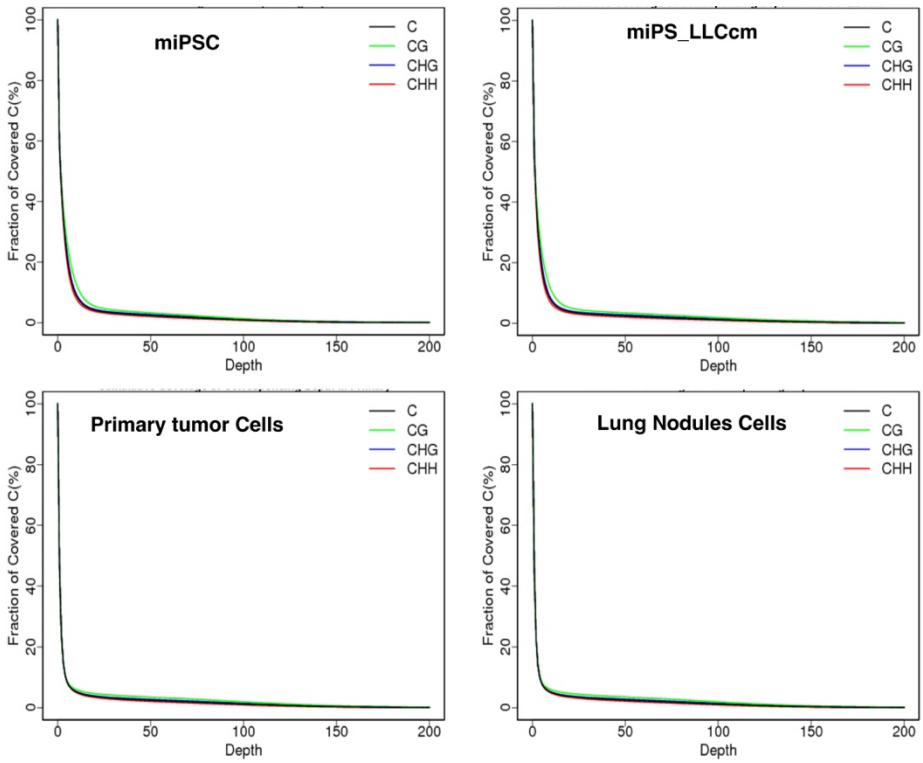


Figure S1. Cumulative distribution of effective sequencing depth of cytosine. X axis represents the effective sequencing depth of C base while Y axis represents the cumulative percentage of C base at a certain sequencing depth.

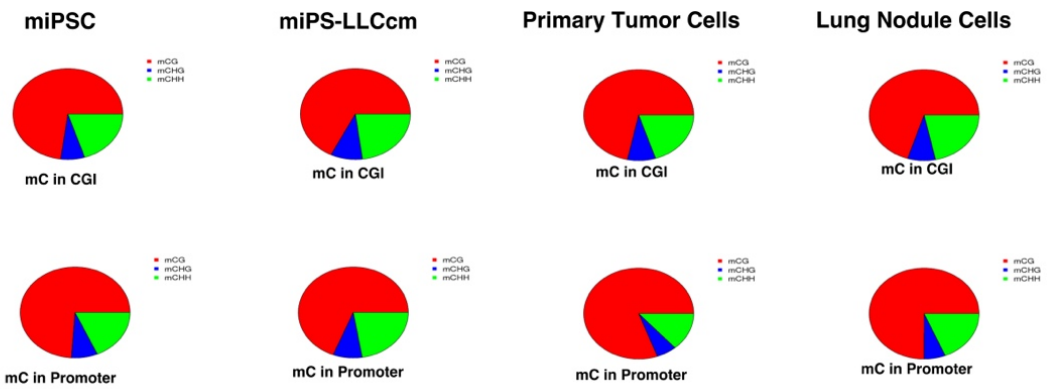


Figure S2. The proportion of mCG, mCHG, and mCHH. Proportion of different methyl-cytosine patterns. The pie graph has three colors, each color represents one methylation type (mCG, mCHG, mCHH), and the area represent the proportion of the mC.

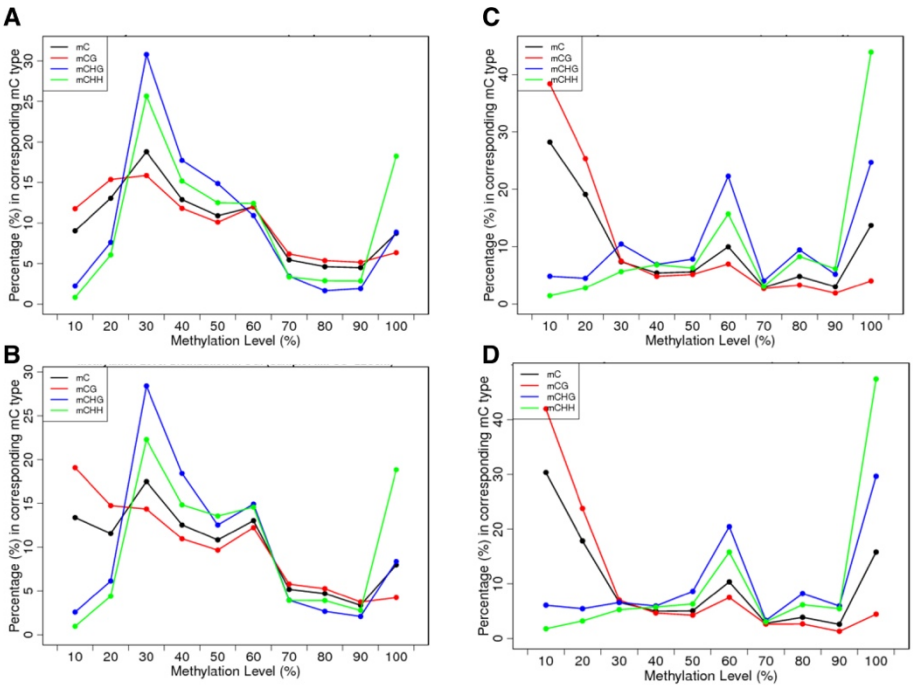


Figure S3. Methylation level distribution in CGI of four different stages of CSC cells, A. miPSC, B. miPS_LLCcm C. Primary Tumor Cells and D. Lung Nodules Cells.

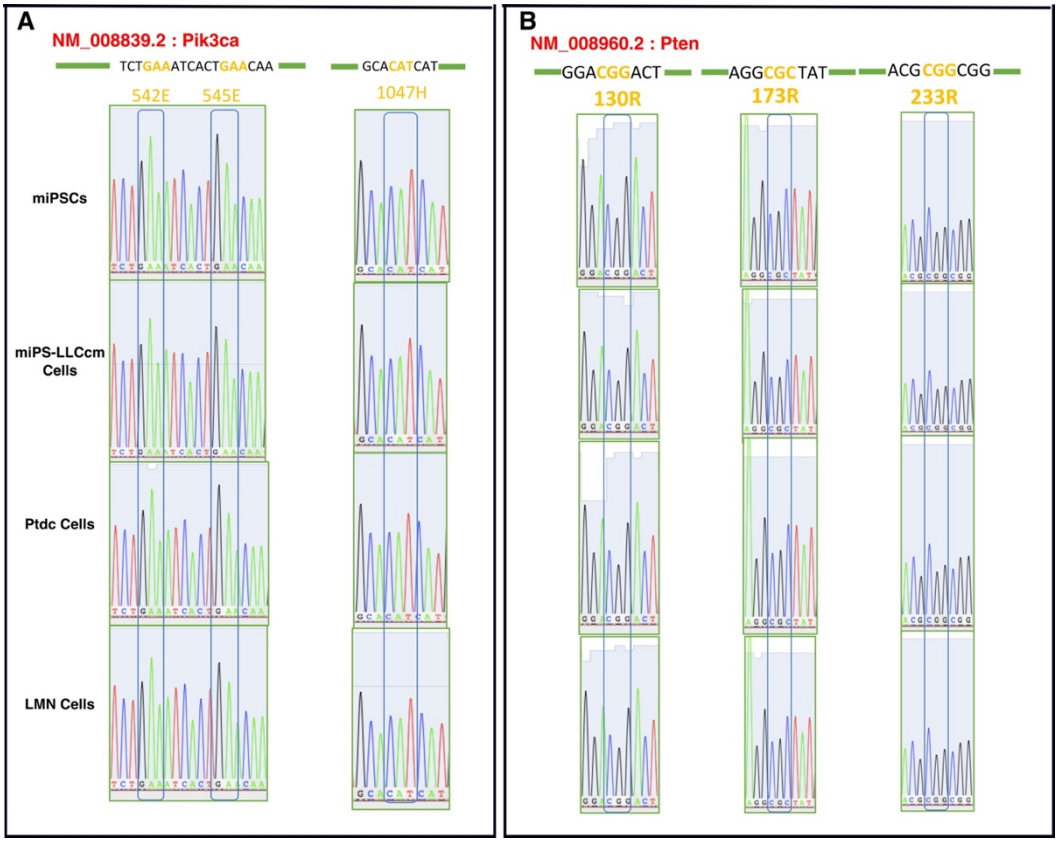


Figure S4. Trace data of codons in Pik3ca and Pten cDNA from miPSCs, miPS-LLCcm cells and Ptdc cells and LMN cells. The sequencing was carried out to cover the positions of codons that are frequently found mutated in human cancers, of the genes related to PI3K-Akt signaling pathway. (A) The sequencing of Pik3ca cDNA showed that there is no point mutation at the codons of 542E, 545E and 1047H in all cells. (B) The sequencing of Pten cDNA showed that there is no point mutation at the codons of 130R, 173R and 233R in all cells. (DNA sequencing and position of the codons are shown at the top. Names of the cells are indicated on the left.)

APPENDIX

TABLE

Table S1. Data Summary and QC

Samples	Fragment Length (bp)	Sequencing Strategy	Clean Reads No.	Clean Data Size (bp)	Clean Rate (%)
miPSCs	40~220	PE100	49108448	4910844800	68.01
miPSC_LLCcm	40~220	PE100	43937308	4393730800	52.25
Ptdc cells	40~220	PE100	39970510	3997051000	53.56
LMN cells	40~220	PE100	37446394	3744639400	56.22

Clean Rate (%) = Clean Data Size (bp)/Raw Data Size (bp)

Table S2. Alignment statistics with reference genome

Samples	Clean Reads	Mapped Reads	Mapping Rate (%)	Uniquely Mapped Reads	Uniquely Mapping Rate (%)	Enzyme Digestion Efficiency (%)
miPSC	49108448	44488548	90.59	37885255	77.15	33.783
miPSC_LLCcm	43937308	38450620	87.51	32873456	74.82	39.441
Primary	39970510	36058878	90.21	30337853	75.9	57.898
LMT	37446394	34785507	92.89	29131403	77.79	65.552

Table S3. Proportion of mCG, mCHG and mCHH

		in Promoter			in CGI		
		mCG	mCHG	mCHH	mCG	mCHG	mCHH
miPSC	mC number	9310	896	2556	21144	2199	5254
	Proportion (%)	72.932	7.026	20.042	73.938	7.69	18.373
miPS_LLCcm	mC number	6703	832	2173	14846	2059	5026
	Proportion (%)	69.046	8.57	22.384	67.694	9.389	22.917
Ptdc Cells	mC number	3713	304	805	7550	871	2108
	Proportion (%)	77.001	6.304	16.694	71.707	8.272	20.021
LMN Cells	mC number	3310	278	827	6800	793	2099
	Proportion (%)	74.972	6.297	18.732	70.161	8.182	21.657

Table.4 Average methylation level of mC

Elements	Sample	mC	mCG	mCHG	mCHH
promoter	miPSC	40.284	34.435	51	77.191
promoter	LMT	31.599	19.609	73.415	89.364
promoter	Primary	31.812	21.657	65.687	84.684
promoter	miPSC_LLCcm	36.447	27.234	60.194	85.299
CGI	miPSC	25.096	19.885	34.435	72.689
CGI	LMT	21.944	12.049	40.52	82.521
CGI	Primary	30.165	18.334	75.403	88.583
CGI	miPSC_LLCcm	20.431	14.563	28.361	58.732

Table S5. KEGG pathway analysis on hypomethylated DMR-associated genes (miPSCs Vs miPS-LLCcm cells)

Category	Term	Count	%	PValue	Genes
KEGG_PATHWAY	PI3K-Akt signaling pathway	17	4.009	0.00176	FGFR2, PHLPP1, COL4A3, COL4A2, COL4A1, PDGFA, ITGA11, ITGB4, NR4A1, EPHA2, VWF, CCND3, ITGB6, PIK3R5, PIK3AP1, NGFR, THBS4
KEGG_PATHWAY	Pathways in cancer	17	4.009	0.00592	FGFR2, COL4A3, TCF7, COL4A2, COL4A1, EPAS1, APC2, PDGFA, HDAC1, ADCY9, PLCG2, PTCH1, PIK3R5, WNT11, ABL1, CSF2RA, TRAF3
KEGG_PATHWAY	Focal adhesion	13	3.066	0.00090	VWF, COL4A3, COL4A2, COL4A1, CCND3, PDGFA, ITGB6, ITGB4, ITGA11, PIK3R5, VAV2, PXN, THBS4
KEGG_PATHWAY	Calcium signaling pathway	11	2.594	0.00328	GRM5, P2RX4, ADCY9, ERBB4, PDE1C, PLCG2, RYR1, PLCD3, CACNA1G, PLCD1, CAMK2B
KEGG_PATHWAY	ECM-receptor interaction	9	2.123	0.00036	VWF, COL4A3, COL4A2, COL4A1, ITGB6, ITGB4, ITGA11, SDC4, THBS4
KEGG_PATHWAY	Axon guidance	9	2.123	0.00432	ABLIM2, UNC5B, UNC5A, RGS3, NTNG1, ABL1, NTN1, EPHA2, EPHB2
KEGG_PATHWAY	Oxytocin signaling pathway	9	2.123	0.01404	KCNJ6, ADCY9, RYR1, NPR1, PIK3R5, CAMK2B, CACNA2D2, CAMKK2, CACNA2D4
KEGG_PATHWAY	Proteoglycans in cancer	9	2.123	0.05150	ANK1, ERBB4, PLCG2, PIK3R5, WNT11, PTCH1, CAMK2B, SDC4, PXN
KEGG_PATHWAY	Regulation of actin cytoskeleton	9	2.123	0.06603	FGFR2, APC2, PDGFA, ITGB6, ITGB4, ITGA11, PIK3R5, VAV2, PXN
KEGG_PATHWAY	Aldosterone synthesis and secretion	8	1.887	0.00164	DAGLA, ADCY9, HSD3B5, CACNA1G, NPR1, NR4A1, CAMK2B, PRKCE
KEGG_PATHWAY	Transcriptional misregulation in cancer	8	1.887	0.04940	ERG, CEBPE, HDAC1, PDGFA, SUPT3, NGFR, FCGR1, KLF3
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	7	1.651	0.00291	TCF7, DES, ITGB6, ITGB4, ITGA11, CACNA2D2, CACNA2D4
KEGG_PATHWAY	Dilated cardiomyopathy	7	1.651	0.00629	DES, ADCY9, ITGB6, ITGB4, ITGA11, CACNA2D2, CACNA2D4
KEGG_PATHWAY	Thyroid hormone signaling pathway	7	1.651	0.02589	HDAC1, PLCG2, ATP1A3, PLCD3, MED24, PLCD1, PIK3R5
KEGG_PATHWAY	Inflammatory mediator regulation of TRP channels	7	1.651	0.04095	ADCY9, P2RY2, ASIC4, PLCG2, PIK3R5, CAMK2B, PRKCE

Table S6. KEGG pathway analysis on hypomethylated DMR-associated genes (miPSCs Vs Ptdc cells)

Category	Term	Count	%	PValue	Genes
KEGG_PATHWAY	Metabolic pathways	18	5.901639344	0.403807798	PLD2, ME3, ALPPL2, KL, ALOX12E, HKDC1, CYP2J6, SGMS1, AGMAT, GGT5, GALM, SCLY, PYGL, PLCH2, MGAM, ATP6V0A1, FUK, ABO
KEGG_PATHWAY	Calcium signaling pathway	10	3.278688525	3.49E-04	P2RX4, ADCY4, ATP2A3, PDE1C, CACNA1G, GNAS, CAMK2B, CACNA1S, PTAFR, F2R
KEGG_PATHWAY	cAMP signaling pathway	8	2.62295082	0.010549721	PLD2, ADCY4, FFAR2, GNAS, CAMK2B, RAPGEF3, CACNA1S, F2R
KEGG_PATHWAY	Neuroactive ligand-receptor interaction	8	2.62295082	0.06103172	P2RX4, CHRM4, GRIK3, RXFP2, NPFFR1, PTAFR, CTSG, F2R
KEGG_PATHWAY	PI3K-Akt signaling pathway	8	2.62295082	0.140484875	VWF, COL4A3, YWHAG, PIK3AP1, NGFR, FGF1, F2R, ITGA2B
KEGG_PATHWAY	Pathways in cancer	8	2.62295082	0.215920682	COL4A3, ADCY4, PPARD, GNAS, FGF1, CSF2RA, F2R, ITGA2B
KEGG_PATHWAY	Oxytocin signaling pathway	7	2.295081967	0.013014035	ADCY4, KCNJ6, CACNB2, GNAS, CAMK2B, CACNA1S, MAP2K5
KEGG_PATHWAY	Rap1 signaling pathway	7	2.295081967	0.047927182	ADCY4, GNAS, RAPGEF3, NGFR, FGF1, F2R, ITGA2B
KEGG_PATHWAY	Glutamatergic synapse	6	1.967213115	0.013458719	PLD2, ADCY4, GRIK3, GNAS, SHANK1, SHANK2
KEGG_PATHWAY	Serotonergic synapse	6	1.967213115	0.023071277	KCNJ6, ALOX12E, CYP2J6, GNAS, RAPGEF3, CACNA1S
KEGG_PATHWAY	Adrenergic signaling in cardiomyocytes	6	1.967213115	0.037206634	ADCY4, CACNB2, GNAS, CAMK2B, RAPGEF3, CACNA1S
KEGG_PATHWAY	Dilated cardiomyopathy	5	1.639344262	0.018881129	ADCY4, CACNB2, GNAS, CACNA1S, ITGA2B
KEGG_PATHWAY	Aldosterone synthesis and secretion	5	1.639344262	0.021223303	ADCY4, CACNA1G, GNAS, CAMK2B, CACNA1S
KEGG_PATHWAY	Insulin secretion	5	1.639344262	0.021223303	ADCY4, FFAR1, GNAS, CAMK2B, CACNA1S
KEGG_PATHWAY	GABAergic synapse	5	1.639344262	0.022041932	PLCL1, ADCY4, KCNJ6, CACNA1S, NSF

Table S7. KEGG pathway analysis on hypomethylated DMR-associated genes (miPSCs Vs LMN cells)

Category	Term	Count	%	PValue	Genes
KEGG_PATHWAY	PI3K-Akt signaling pathway	22	3.716216216	6.25E-05	FGFR2, PPP2R5D, ITGB4, NR4A1, EPHA2, ITGA9, YWHAG, LAMB3, LAMB2, VEGFA, GYS1, PDGFRB, CREB3L1, PIK3AP1, PIK3R5, NGFR, COL1A1, THBS1, ANGPT2, ITGA2B, FN1, F2R
KEGG_PATHWAY	Rap1 signaling pathway	15	2.533783784	4.55E-04	FGFR2, GNAO1, GRIN2A, EPHA2, VEGFA, PDGFRB, GNAS, RAPGEF4, PIK3R5, RAPGEF3, NGFR, THBS1, ANGPT2, ITGA2B, F2R
KEGG_PATHWAY	Pathways in cancer	15	2.533783784	0.079528386	FGFR2, PPARD, LAMB3, LAMB2, VEGFA, WNT9B, MAPK9, PDGFRB, GNAS, PIK3R5, ABL1, CSF2RA, ITGA2B, FN1, F2R
KEGG_PATHWAY	Axon guidance	14	2.364864865	8.69E-06	NTNG2, SLIT1, EPHB1, EPHA2, SLIT3, EPHB2, NCK2, SEMA5B, UNC5B, EPHA8, NFATC4, ROBO3, ABL1, NFATC2
KEGG_PATHWAY	cAMP signaling pathway	13	2.195945946	0.002133177	PLD2, FFAR2, ATP1A3, GRIN2A, NPR1, GRIA1, CREB3L1, MAPK9, GNAS, RAPGEF4, PIK3R5, RAPGEF3, F2R
KEGG_PATHWAY	Focal adhesion	13	2.195945946	0.003211744	ITGA9, LAMB3, LAMB2, VEGFA, ITGB4, MAPK9, PDGFRB, MYLK2, PIK3R5, COL1A1, THBS1, ITGA2B, FN1
KEGG_PATHWAY	Ras signaling pathway	12	2.027027027	0.017974152	FGFR2, PLD2, VEGFA, GRIN2A, MAPK9, PDGFRB, PIK3R5, NGFR, ABL1, PLA2G3, ANGPT2, EPHA2
KEGG_PATHWAY	Calcium signaling pathway	11	1.858108108	0.009277251	P2RX4, TNNC2, PDE1C, RYR1, GRIN2A, PLCD3, CACNA1G, PDGFRB, MYLK2, GNAS, F2R
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	11	1.858108108	0.060185364	AMHR2, TNFRSF9, ACVR2B, LTBR, CXCR5, VEGFA, TNFRSF13B, PDGFRB, NGFR, CSF2RA, IL11
KEGG_PATHWAY	Oxytocin signaling pathway	10	1.689189189	0.011431376	GNAO1, RYR1, CACNB2, NPR1, MYLK2, GNAS, PIK3R5, NFATC4, NFATC2, CAMKK2
KEGG_PATHWAY	cGMP-PKG signaling pathway	10	1.689189189	0.018383555	GTF2I, GATA4, PDE5A, ATP1A3, NPR1, MYLK2, CREB3L1, PIK3R5, NFATC4, NFATC2
KEGG_PATHWAY	Regulation of actin cytoskeleton	10	1.689189189	0.062437352	FGFR2, ITGA9, CHRM4, ITGB4, PDGFRB, MYLK2, PIK3R5, F2R, ITGA2B, FN1
KEGG_PATHWAY	Dilated cardiomyopathy	9	1.52027027	6.64E-04	ITGA9, DES, MYBPC3, LMNA, ITGB4, CACNB2, GNAS, SGCA, ITGA2B
KEGG_PATHWAY	Adrenergic signaling in cardiomyocytes	9	1.52027027	0.023805439	PPP2R5D, ATP1A3, CACNB2, CREB3L1, GNAS, PIK3R5, RAPGEF4, RAPGEF3, SCN5A
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	8	1.351351351	0.00126012	ITGA9, DES, LMNA, ITGB4, CACNB2, DSP, SGCA, ITGA2B

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LISTS OF PUBLICATIONS

(1) Up-Regulation of PI 3-Kinases and the Activation of PI3K-Akt Signaling Pathway in Cancer Stem-Like Cells Through DNA Hypomethylation Mediated by the Cancer Microenvironment

Aung Ko Ko Oo, Anna Sanchez Calle, Neha Nair, Hafizah Mahmud, Arun Vaidyanath, Junya Yamauchi, Apriliana Cahya Khayrani, Juan Du, Md Jahangir Alam, Akimasa Seno, Akifumi Mizutani, Hiroshi Murakami, Yoshiaki Iwasaki, Ling Chen, Tomonari Kasai, and Masaharu Seno.

Translational Oncology (2018) Volume 11, No.3, pp 653–663.

https://doi.org/10.1016/j.tranon.2018.03.001

(2) Targeting Glioblastoma Cells Expressing CD44 with Liposomes Encapsulating Doxorubicin and Displaying Chlorotoxin-IgG Fc Fusion Protein

Hafizah Mahmud, Tomonari Kasai, Apriliana Cahya Khayrani, Mami Asakura, **Aung Ko Ko Oo**, Du Juan, Arun Vaidyanath, Samah El-Ghlban, Akifumi Mizutani, Akimasa Seno, Hiroshi Murakami, Junko Masuda and Masaharu Seno

International Journal of Molecular Sciences (2018), Volume 19, No3, pp659.

https://doi.org/10.3390/ijms19030659

(3) A Cancer Stem Cell Model as the Point of Origin of Cancer-Associated Fibroblasts in Tumor Microenvironment

Neha Nair, Anna Sanchez Calle, Maram Hussein Zahra, Marta Prieto-Vila, **Aung Ko Ko Oo**, Laura Hurley, Arun Vaidyanath, Akimasa Seno, Junko Masuda, Yoshiaki Iwasaki, Hiromi Tanaka, Tomonari Kasai & Masaharu Seno.

Scientific Reports (2017), Volume 7, Issue 1, pp6838.

https://doi.org/10.1038/s41598-017-07144-5

(4) Hyaluronic Acid Mediated Enrichment of CD44 Expressing Glioblastoma Stem Cells in U251MG Xenograft Mouse Model

Arun Vaidyanath, Hafizah Binti Mahmud, Apriliana Cahya Khayrani, **Aung Ko Ko Oo**, Akimasa Seno, Mami Asakura, Tomonari Kasai and Masaharu Seno.

Journal of Stem Cell Research & Therapy, Vol.7, No.384,

https://doi.org/10.4172/2157-7633.1000384

(5) A New PDAC Mouse Model Originated from IPSCs-Converted Pancreatic Cancer Stem Cells (CSCcm)

Anna Sanchez Calle, Neha Nair, **Aung Ko Ko Oo**, Marta Prieto-Vila, Megumi Koga, Apriliana Cahya Khayrani, Maram Hussein, Laura Hurley, Arun Vaidyanath, Akimasa Seno, Yoshiaki Iwasaki, Malu Calle, Tomonari Kasai, and Masaharu Seno

American Journal of Cancer Research (2016); Volume 6, No.12, pp2799–2815. PMID: 28042501

ORAL AND POSTER PRESENTATION

(1) iPSC derived CSC model with lung metastasis developed in the microenvironment of lung carcinoma (Poster presentation)

Aung Ko Ko Oo, Tomonari Kasai, Arun Vaidyanath, Hafizah Mahmud, Neha Nair, Anna Sanchez Calle, Masaharu Seno

The 2016 ASCB Annual Meeting, Mol. Biol. Cell 27, page1236, abstract P1946. (San Francisco, California, Dec 3-7, 2016)

(2) Analysis of the differential methylated regions in the cancer stem cell model converted from iPSCs (Poster presentation)

Aung Ko Ko Oo, Arun Vaidyanath, Anna Sanchez Calle, Hafizah Mahmud, Neha Nair, Apriliana Cahya Khayrani, Md Jahangir Alam, Tomonari Kasai, Masaharu Seno

Consortium of Biological Sciences 2017, The Molecular Society of Japan, No 40th Annual Meeting (Kobe, 2017-12-7).

(3) Conditioned Medium of Lewis Lung Carcinoma Cells Mediated the Oncogenic Conversion of iPSCs into Cancer Stem-like Cells Through DNA Hypomethylation with Lung Metastasis (Poster presentation)

Aung Ko Ko Oo, Aprilliana Cahya Khayrani, Juan Du, Md Jahangir Alam, Akimasa Seno, Yoshiaki Iwasaki, Ling Chen, Masaharu Seno

The 6th JCA-AACR Special Joint Conference (2018), The Latest Advances in Lung Cancer Research; From Basic Science to Therapeutics; July 10-12, 2018, Kyoto Tokyu Hotel, Kyoto, Japan.