Study of “Cancer Stem Cells and Tumor Microenvironment” using iPS cells

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Doctoral Course

OKAYAMA UNIVERSITY

Dedicated to all cancer fighters and those who've lost the fight

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CERTIFICATE

This is to certify that **Ms. Neha Nair** has worked on the dissertation entitled "**Study of "Cancer Stem Cells and Tumor microenvironment" using iPS cells"** 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. 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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PREFACE

Cancer is one of the leading causes of morbidity and deaths worldwide. In 2015, 8.8 million cancer-related deaths have been recorded and the number of new cases is expected to rise up to 70% in the next two decades. This has a significant and unprecedented effect on the economic growth of a country. Therefore, each year billions of funds are allocated towards the research and development of new therapeutic strategies to combat this growing menace. With the advent of new scientific technologies and advancement in molecular cancer research, the future does seems promising. Nevertheless, considerable measures need to be taken in order to solve key issues revolving around this disease such as late presentation of symptoms, development of resistance to available treatments and lack of awareness.

Targeted therapies such as kinase inhibitors and monoclonal antibodies have indeed altered cancer care in recent decades. They have improved the disease outcomes in many cancer types but still the most serious challenge often encountered in cancer therapy is the development of drug resistance. It is believed that this acquisition of resistance is typically caused by small population of cells in tumor with pre-existing alterations, which can drive the resistance. Since the concept of Cancer stem cells (CSC) has been introduced in late 90s, its importance in cancer research and development of novel anti-cancer therapies is widely accepted. CSC which are also known as 'tumor initiating cells' represent very small population of tumor sharing common properties with normal stem cells. They are unique cells with self-renewing and tumorigenic potential. Current radio and chemotherapies are able to eliminate the bulk of cancer cells but not cancer stem cells since they are endowed

with specific resistance mechanisms. They give rise to new tumors and metastases leading to relapse of the disease. The recurring tumors are more malignant, prone to faster metastasis and exhibits enhanced drug resistance. Overall, this leads to therapy failure reducing the survival rate of cancer patients.

Previous studies by our group have pioneered a cutting edge method to develop cancer stem cells from mouse and human induced pluripotent stem cells (iPSCs). The novelty of this method was the use of conditioned medium from cancer cell lines to direct the differentiation of iPS towards cancer stem like cells without undertaking any supporting genetic modifications. It was hypothesized that conditioned medium from cell lines should be enriched with factors released from cancer cells thereby mimicking tumor microenvironment. These factors mediated the conversion of iPSCs to cancer stem cells. First successful attempt was reported by Chen et al in 2012 wherein the conversion of mouse iPScs into cancer stem like cells using Lewis lung carcinoma (LLC) cell line conditioned medium was demonstrated. miPS cells used in this study was harboring GFP gene in the 5' untranslated region of an important pluripotent gene, *Nanog*. The resulting cells stably expressed GFP in undifferentiated state corresponding with the *Nanog* expression but lost its expression once differentiated. Intriguingly, the miPSCs survived the treatment with conditioned medium for four weeks and the resulting cells (termed as miPS-LLCcm) expressed key markers of stemness and self-renewal such as *Nanog, Eras, Rex1, Cripto* respectively. Furthermore, these cells fulfilled the primary criteria of cancer stem cells by exhibiting sphere forming ability in suspension cultures and tumorigenicity in balb/c nude mice (1).

Another study by Yan et al in continuation with the previous study demonstrated that tumor derived extracellular vesicles (tEVs) isolated from

conditioned medium of LLC cells alone facilitated the conversion of miPScs to CSC-like cells. The resulting cells (termed as miPS-LLCev) were observed to be similar in morphology and stemness when compared with miPS-LLCcm. These cells generated malignant liposarcoma and extensive angiogenesis when subcutaneously transplanted into balb/c nude mice. The results indicated that tEVs and/or other soluble factors are instrumental in supporting the differentiation of miPS-LLCcm. This study supported our hypothesis that CSC like cells or miPS-LLCcm and their differentiated progenies establishes a niche regulating their self renewal and thereby supporting its own sustenance in presence of tumor niche derived factors (2). Matsuda et al further reinforced this concept by showing that GFP^+ miPS-LLCcm could differentiate into GFP^- vascular endothelial lineage that together with other differentiated cells supported the maintenance and differentiation of miPS-LLCcm (3).

A pioneering work by Sanchez et al has provided interesting insights at the transcriptomic level of pancreatic CSCs generated from miPSCs following treatment with conditioned medium derived from human pancreatic cancer cell lines. In this study the in vitro converted cancer stem-like cells were enriched via subcutaneous transplantation in nude mice just like previous studies but subsequent transplantations were conducted directly into pancreas. Intriguingly, this orthotropic transplantation lead to the enrichment of pancreatic cancer stem cells which in turn generated tumors imitating pancreatic ductal adenocarcinoma phenotype (PDAC) with liver metastasis. The RNA sequencing analysis of the cancer stem cell line established indicated members from the transcriptional pancreatic progenitor network *Pdx1, Hes1, Foxa2, Hnf1a, Hnf4a, Pax6, Nr5a2, Rbpj, Rbpgl, MafA and MafB* were found to be expressed together with PDAC related hallmarks such as *Kras, Krt19, Col8a1, Col1a1, Cxcr4, Muc1, Muc5aC, Mmp2 or Malat1* as well as the most representative pancreatic CSC

markers *CD133, CD24a, EpCAM and CD44*. This study clearly indicated the crucial role of tumor microenvironment in deciding the fate of cancer stem cells and its lineage (4).

The present work discussed in this thesis is based on the inference drawn from the previous findings that in vitro generated CSC from iPSCs is a cutting edge model to understand role of tumor microenvironment in the maintenance of CSCs and their differentiated progenitor cells. In this study, the CSCcm or cancer stem cell lines generated from miPSCs on treatment with breast cancer cell line conditioned medium have been utilized to trace the functional roles of CSCs in subcutaneous and orthotropic tumor models respectively. This thesis is divided in to three *chapters* and each chapter is further subdivided into three sections: *Abstract, Introduction, Observation & Results and Discussion.*

In chapter 1, the establishment of CSC-like cell line from miPSCs is discussed. In this, human breast cancer cell lines, T47D and BT549 each representing different hormonal subtypes have been utilized to derive the respective conditioned medium. The miPSCs were exposed to the respective conditioned medium and the CSC-like cells were enriched by serial subcutaneous transplantations in nude mice. The resulting CSC-like cells had been characterized by morphological, histophathological and molecular techniques.

Chapter 2 deals with the experiments using iPSC-derived CSC model, which demonstrates that the cancer-associated fibroblast, a key component of stromal microenvironment, originates from cancer stem cells. These findings are the first to reported in literature and provide insights in to the ability of CSC to generate a self sustaining niche in the presence of tumor derived soluble and/or paracrine factors.

In chapter 3, generation of a potential breast cancer stem cell model using miPSC-derived CSC is discussed. The in vitro converted CSC-like cells were orthotropically transplanted in to mammary fat pad of female nude mice and the resulting primary cells were analyzed for the expression of key breast cancer stem cell markers. This study attempts to develop a useful murine breast cancer stem cell model that should support in designing novel anti-cancer therapeutics and personalized therapy.

CONTENTS

CHAPTER 1

iPSC DERIVED CANCER STEM CELL MODEL

ABSTRACT

In this chapter, the conversion of mouse induced pluripotent stem (miPS) cells into Cancer stem like cells is analyzed. For this, the miPS cells were treated with two human breast cancer cell lines, BT-549 and T47D derived conditioned medium respectively. Since miPS cells used in this study retained green fluorescent protein (GFP) gene under the control of Nanog gene promoter; therefore, self-renewing undifferentiated CSCs can be distinguished from differentiated CSCs by the presence and absence of GFP expression. The resulting CSC-like cells expressing key markers for stemnesss and pluripotency were enriched by serial subcutaneous transplantation. This study is based on the previously established protocol to generate CSC-like cells from miPS by Chen et al using conditioned culture medium from mouse cancer cell lines, suggesting that the medium contains a rich source of factors that are secreted from mouse cancer cells and have potential to mimic the tumor microenvironment.

INTRODUCTION

Concept of Cancer stem cells

In the past two decades, cancer research has witnessed several key milestones to control the growing menace of cancer. These have significantly improved our understanding of the molecular and cellular mechanisms underlying cancer progression but how tumor evades therapies is still elusive. The recent emergence of the concept of Cancer stem cells (CSC) has provided with interesting insights in the development of effective therapeutic strategies to deal with drug resistance and metastasis in tumor cells.

Normal stem cells and CSC both exhibit self-renewal capacity but this capacity is typically deregulated in CSC. They represent a distinct population in tumor with self-renewal and tumorigenic potential promoting metastasis and disease progression. There are several theories revolving around the development of cancer. **Clonal evolution theory or stochastic model** proposes that tumors are biologically homogenous and cells acquire heterogeneity by random accumulation of mutations and genetic alterations occurring either stochastically or randomly neoplastic cells. This leads to generation of benign or highly aggressive tumors depending on the type of genetic alteration acquired by the cells in the tumor. In contrast, the **CSC model or hierarchical model** argues that the cellular heterogeneity emanates from CSC. The CSC is biologically distinct from the rest of the cells and occupies the apex position in the hierarchy. Several reports supporting this model have demonstrated that in a given tumor non-differentiated tumorigenic cells gives rise to more differentiated cells. The tumor-initiating cells can be identified and purified from the bulk nontumorigenic population based on intrinsic characteristics.These differentiated cells were unable to

form tumors when compared with the their non-differentiated counterpart. Although, stochastic model can accommodate the existence of functional CSC, but the striking difference from the hierarchy model is that every cell is believed to possess the CSC potential (5).

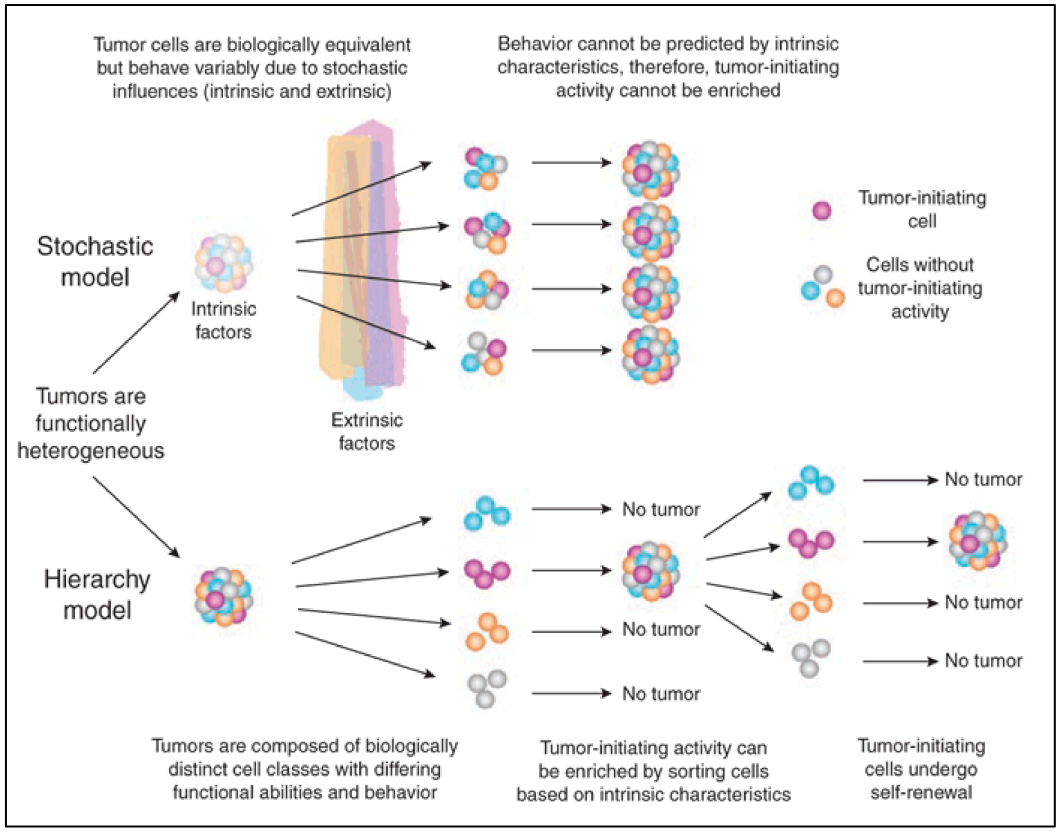


Fig. 1 Models of tumor heterogeneity. *Courtesy: J.E Dick. Looking ahead in cancer stem cells research. Nature Biotechnology, 27:44-46. 2009*

Both the models seem to be mutually exclusive but recent evidences are suggesting that both clonal evolution and hierarchical model should be considered simultaneously in defining the tumor heterogeneity. For example, Anderson and co-workers first directly proved the existence of genetic diversity of cancer propagating cells within individual ETV6-RUNX1-positive acute lymphoblastic leukemia (ALL) patients (6). Several other studies have also indicated that cancer stem cells exist but

might evolve over time. An ancestral clone gives rise to at least two clonal lineages that evolve independently, with each clone acquiring diverse genetic aberrations; one clone emerges as the dominant diagnostic clone, while the other clone gives rise to the predominant clone containing additional mutations at relapse (7).

Functional traits of cancer stem cells

a) Self-renewal potential

CSC represents a distinct population in a tumor with long term clonal and self-renewal potential. In vivo transplantation of tumor cells in immunocompromised mice is considered a standard assay to determine the presence of CSC. But in many cases it fails to completely recapitulate the in vivo condition of tumor often observed in human patients. Therefore, in addition to it, in vivo lineage tracing methods have been developed used in syngenic tumor mouse models and human xenograft models to assess the fate of cancer stem cells in tumors. Owing to their self-renewal potential it is considered that CSC gives rise to the non-CSC progeny forming the bulk tumor population.

b) Maintenance in quiescent, slow cycling or active states

Cancer stem cells have been reported to behave like adult stem cells by shuttling between quiescent and active states. For example, Trumpp et al have demonstrated the existence of quiescent leukemic stem cells (LSCs) in mouse model of acute myeloid leukemia (AML). In addition to it, a report has shown correlation between slow cycling CSC in breast cancer cells and the frequency of CSC, measured by the retention of a membrane dye PKH26 in mammosphere cultures, in tumorigenic assays. This feature of CSC is considered to be important in rendering it resistance to

most of the therapeutic drugs that majorly affects active cells. Moreover, it has been widely hypothesized that the cancer relapse could be attributed to the reactivation of the quiescent CSCs population after several years of treatment.

c) Resistance to conventional therapies

Recent studies have suggested CSC as a main player for chemoresistance against a variety of drugs including cisplatin and/or paclitaxel, temozolomide, etoposide, and doxorubicin or methotrexate in many types of cancer including gliomas and glioblastoma, breast, colorectal, AML, hepatic, lung, bone, pancreatic, ovarian and prostate cancers. Thus, the cellular and

molecular mechanisms underlying the CSC's chemoresistance have drawn accelerating attention for cancer research. (8). Several important features of CSCs render it chemoresistant to known therapeutic drugs. Firstly, they have increased drug efflux capacity mediated by expression of multi drug resistance (MDR) transporters. Second, many reports have suggested that CSC express higher levels of the enzyme Aldehyde dehydrogenase (ALDH1) compared with other cellular counterparts. For example, in a study highly metastatic breast cancer cells expressed elevated levels of ALDH1 and made them resistant to cyclophosphamide. Third, CSC exhibited increased resistance to radiation therapy due to increased DNA damage checkpoint reponse and DNA repair capacity as evident in CD133 expressing glioblastoma CSC-enriched population compared with $CD133^-$ populations. Lastly, CSC are considered capable to counterbalance the harmful effects of radiation induced reactive oxidation species (ROS) by expressing free oxygen radicals as observed in breast cancer models. Therefore, CSCs are endowed with the ability to counteract the inhibitory effects of therapeutic agents (5).

Potential biomarkers for identification of CSC

Breast	Colon	Glioma	Liver	Lung	Melanoma	Ovarian	Pancreatic	Prostate
ALDH1	ABCB5	CD15	CD13	ABCG2	ABCB5	CD24	ABCG2	ALDH1
CD24	ALDH1	CD90	CD24	ALDH1	ALDH1	CD44	ALDH1	CD44
CD44	β-catenin	CD133	CD44	CD90	CD20	CD117	CD24	CD133
CD90	activity	α_6-integrin	CD90	CD117	CD133	CD133	CD44	CD166
CD133	CD24	nestin	CD133	CD133	CD271		CD133	$\alpha_2\beta_1$-integrin
Hedgehog-Gli activity	CD26						c-Met	α_6-integrin
α_6-integrin	CD29						CXCR4	Trop2
	CD44						Nestin	
	CD133						Nodal-Activin	
	CD166							
	LGR5							

Table 1 Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Courtesy; J.L. Visvader, J.E., & Geoffrey. Nature reviews cancer,* ***8****: 755-768. 2008*

Table 1 shows the extensive list of popular markers used for the identification and isolation of CSC in solid tumors. However, these markers are not always considered reliable and stringent measures must be taken to ensure the purity of the isolated populations of CSC. Moreover, recent studies highlight the importance of using multiple markers for CSC identification and different approaches for its isolation. It is primarily due to the insufficient specificity of the markers. For example, CD44 that marks the breast cancer stem cell (BCSC) population has many splice variants and which of these variants actually identifies BCSC is highly uncertain. Further, widespread intra- and inter- tumor heterogeneity limits the specificity of the CSC marker expression. Such inter-tumour heterogeneity is observed for CD133, which is a relatively good FACS marker for CSCs, but is also frequently inactivated due to CpG island methylation Nevertheless, recent studies have successfully characterized and isolated CSC from solid tumors and analyzed its functional relevance for designing novel therapeutic drugs (9).

Relevance of CSC niche in cancer

The tumor microenvironment or niche is composed of diverse cells each contributing in the maintenance and homeostasis of the neoplastic cells in tumor. Just as the normal stem cells depend on its microenvironment for survival, self-renewal and differentiation CSCs are not autonomous. They require constant input from its surrounding microenvironment or niche. In glioma mouse xenograft model, endothelial cells maintain the stemness and tumorigenicity of the glioma CSC through notch and other diffusible factors. Some of the supportive endothelial cells were actually tumor derived and therefore, it was proposed that glioma CSC could generate its own niche. Activin and Nodal are not only produced by pancreatic CSCs, but also are secreted by stellate cells stimulating CSCs in a paracrine fashion. In breast cancer, mesenchymal cells are thought to support CSCs through a signalling loop dependent on the cytokines interleukin 6 (IL-6) and chemokine C-X-C motif ligand 7 (CXCL7), whereas in colon cancer myofibroblasts reportedly support CSCs through hepatocyte growth factor (HGF). Therefore, it can be postulated that the microenvironment plays a crucial role in the maintenance of CSC. Intriguingly, Kerso et al has postulated the existence of mutually exclusive model to determine tumor heterogeneity wherein the three fields of biology – cancer genetics, epigenetics and tumor microenvironment should be studied together to get better understanding of the processes that determine stemness and for designing cutting edge therapeutics (Fig 2). These three factors are thought to be working simultaneously to control the stemness but at the same time can act independently to so the same (10).

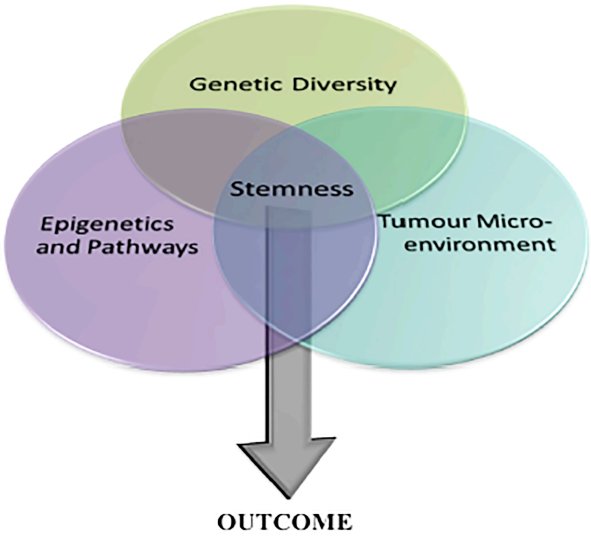


Fig 2. ***Evolution of the cancer stem cell model***
Courtesy; Kreso, A., & John E. D. Cell stem cell ***14****: 275-291. 2014*

Key signalling pathways related with CSC

Several pathways including PI3K/Akt, PTEN, JAK/STAT, Wnt/β-catenin, hedgehog, Notch, NF-κB, Bcl-2 plays important role in enabling CSC maintain their stem cell properties. In PI3K/Akt pathway, activation of Akt is reported in cellular transformation and tumorigenesis. Silencing or defective mutations of PTEN have been observed in various cancers, including T-cell acute lymphoblastic leukemia, prostate cancer, melanoma, glioblastoma and endometrial carcinoma.

The JAK/STAT signaling pathway is also involved in tumor initiation. Aberrations in the JAK/STAT pathway have been recognized in many cancers, especially leukemia. v-Abl is a strong non–receptor tyrosine kinase that can induce the malignant transformation of pre-B cells by affecting the JAK/STAT pathway.

Nuclear factor kappa B (NF-κB), a transcription factor that regulates the expression of multiple genes, participates in numerous cellular responses to stimuli such as cytokines, microbial antigens, free radicals and ultraviolet irradiation. An

increasing body of evidence has shown that NF-κB affects the expression of several apoptosis–related proteins, such as Bcl-xL, Bcl-2, survivin, cellular inhibitors of apoptosis (cIAPs), TRAF and cell cycle regulatory components. Aberrant NF-κB activation causes cancer development and progression, chemoresistance, chronic inflammation and autoimmune diseases.

The well-known Notch, Hedgehog and Wnt signaling pathways play fundamental roles in maintaining CSC populations. Notch signaling affects self-renewal and lineage-specific differentiation of normal human breast stem cells, Moreover, Notch4 activity is elevated in breast CSCs, and inhibiting Notch4 activity can reduce the breast CSC population, thereby suppressing tumor initiation.

Aberrant regulation of the Hedgehog pathway is associated with numerous human malignancies and is a critical factor affecting the outcome of treating the disease. Several agents targeting the Hedgehog pathway have shown promising pre-clinical results and are currently under investigation in phase I and II clinical trials.

The maintenance of CSC stem cell properties has been shown to be associated with the Wnt signaling pathway. Abnormal Wnt/β-catenin signaling has been identified in various malignancies, such as leukemia, colon, epidermal, breast and cutaneous carcinoma. Human colon carcinoma, one of the best studied tumors, is caused by defective mutations in the adenomatous polyposis coli (APC) gene. Defective mutations in APC result in the inappropriate stabilization of β-catenin, thus activating the Wnt cascade and inducing epithelial cell transformation (11-16).

Therapeutic targets in CSC

Current system of treatment for cancer fails to completely eliminate CSC leading to metastasis and cancer recurrence. Therefore, scientists are designing novel strategies designed to specifically wipe out the CSC population and by altering the niche supporting these cells. Fig. 3 gives a summary of the new promising CSC-targeting methods (17).

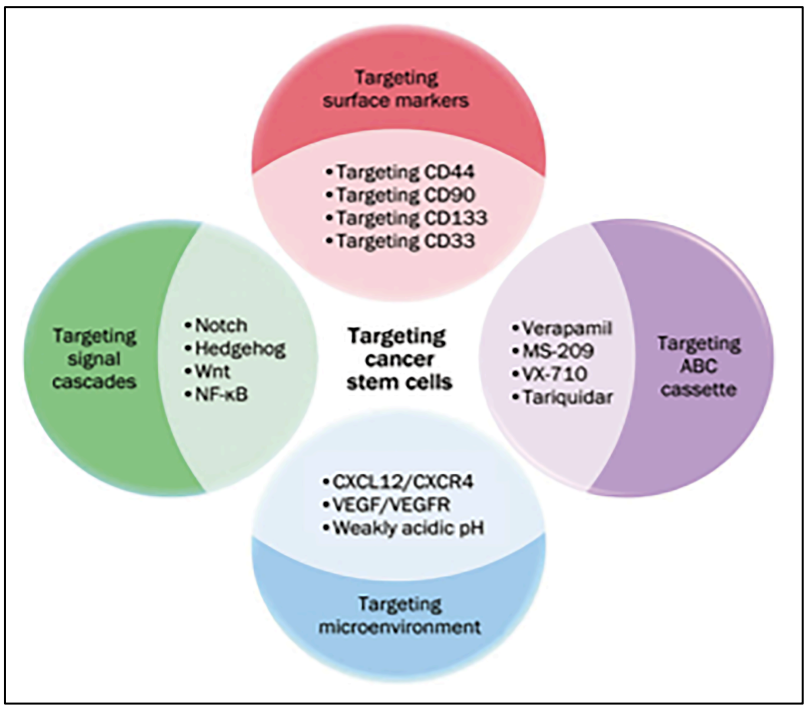


Fig. 3 Therapies targetting cancer stem cells.
*Courtesy; Understanding and targeting cancer stem cells: therapeutic implications and challenges. Chen, K., Huang, Y. H., & Chen, J. L. Acta Pharmacologica Sinica, **34**: 732-740. 2013*

RESULTS

miPS cells survived and proliferated in the presence of cancer cell line conditioned medium

Two-breast cancer cell lines, T47D and BT549 were cultured for deriving the conditioned medium. Respective culture medium was collected in 5% serum condition according to the previously established protocol. Subsequently, the miPS cells were treated with this conditioned medium for a period of four weeks by changing the medium every second day (Fig. 1a). As control experiments, miPS cells were treated with the Normal stem cell medium. In all the parallel experiments, LIF (Leukemia Inhibitory Factor) was withdrawn from the culture medium after miPS colonies established in feeder-less condition. It was observed that the untreated miPS cells failed to survive beyond one week in the presence of normal stem cell medium (Fig. 1b). On the contrary the cells rapidly proliferated in the presence of cancer cell line conditioned medium. Therefore, this treatment was continued for four weeks. The respective converted cells are termed as CM-T47D and CM-BT549. The conditioned medium-treated miPS cells proliferated heterogeneously along with a significant number of GFP-positive subpopulations.

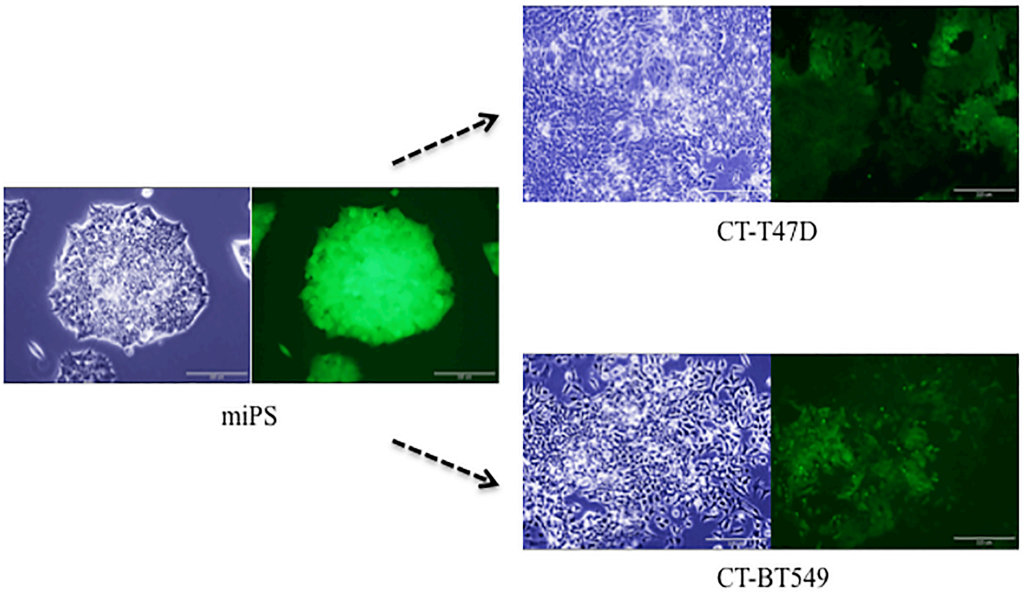


Fig. 1a Representative images of miPS cells and the conditioned medium treated cells, CT-T47D and CT-BT549 cells respectively. Scale bars represent 200µm.

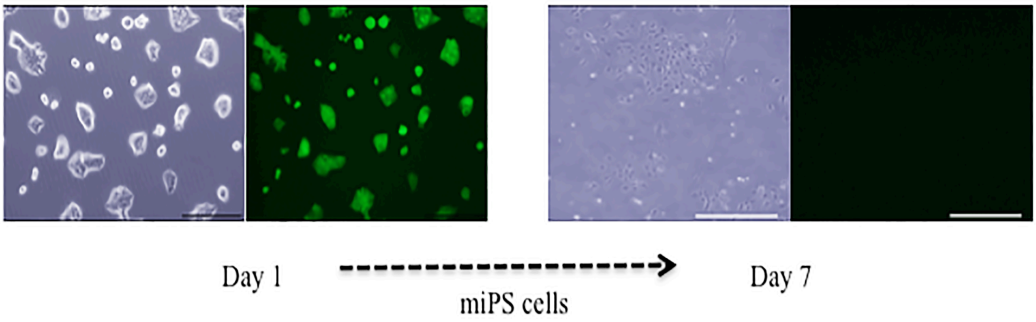


Fig. 1b Representative images of miPS cells in feeder-less condition. Cells could not be maintained without LIF for more than a week. Cells expire with concomitant loss of GFP expression. Scale bars represent 200µm (day 0) and 400µm (day7).

Converted cells exhibited features of CSC-like cells

To test whether the conditioned medium-treated miPS cells still express pluripotency markers, the morphologically heterogeneous cells (with both GFP positive and negative) for the expression of CSC and pluripotency markers by reverse

transcription quantitative PCR (RT-qPCR) were analyzed. It was observed that the conditioned medium-treated miPS cells (CM-T47D and CM-BT549) expressed Oct3/4 and Sox2, while they did not express Klf4 and c-Myc transgenes, suggesting that CM-T47D and CM-BT549 cells could be controlled primarily by other undetermined endogenous gene expression (Fig. 1c). Notably, the expression of prominent CSC makers such as CD133 and breast epithelial cancer cell marker EpCam were expressed 3- to 10-fold higher in both CM-T47D and CM-BT549 compared to the untreated miPS cells. Sustained expression of GFP also supports evidence that CM-T47D and CM-BT549 cells were converted into CSC-like cells.

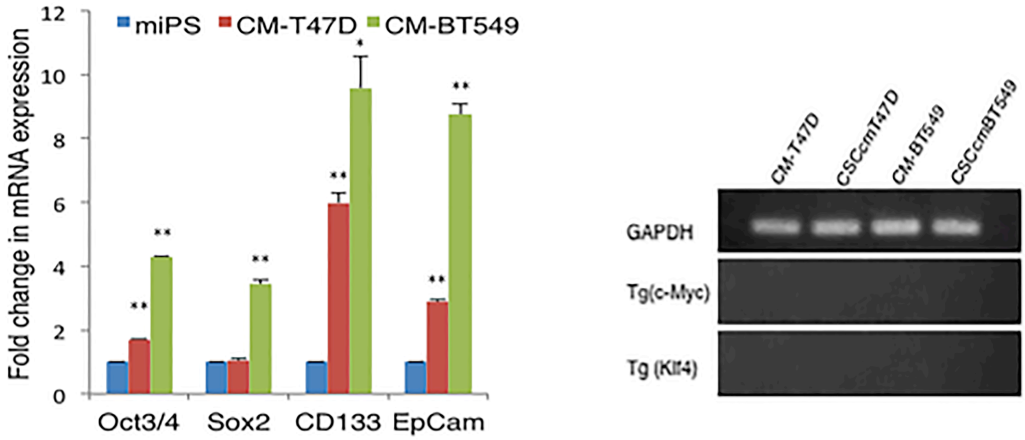


Fig. 1c RT-qPCR analysis of the CSC markers CD133 and EpCam, as well as pluripotency markers, Oct3/4 and Sox2 in the cells (CM-T47D or CM-BT549) after four weeks of the CM treatment. Expression values were normalized to GAPDH and compared with untreated miPS cells. RT-qPCR analysis of transgenes, Klf4 and c-Myc in the CM-treated miPS cells (CM-T47D or CM-BT549) after four weeks of the CM treatment and the primary cells (CSCcmBT549 or CSCcmT47D) generated from subcutaneous tumors. Agarose gel image shows no amplification products of Klf4 and c-Myc. Data are presented as mean ± standard deviation and analyzed using unpaired two-tailed student's *t*-test. The level of significance was set as $^{**}P<0.01$, $^{*}P<0.05$.

Generation of subcutaneous tumor in immunodeficient mice

At the end of four weeks, 1x 10^6 cells in Hanks balanced salt solution (HBSS) were injected in subcutaneously into balb/c (female) nude mice. All the transplanted mice presented rapidly growing tumor reaching up to a volume of 1000mm^3 within one

month. The primary cells were harvested and maintained in the 1:1 mixture of stem cell medium and respective conditioned medium. The primary cells thereafter are termed as CSCcmT47D and CSCcmBT549 based on the treatment of miPS cells with the respective conditioned medium Further, serial subcutaneous transplantations of the primary cells until tertiary level gave rise to tumor with the same vigor thereby indicating the enrichment of CSC population and maintenance of tumorigenicity (Fig. 1d).

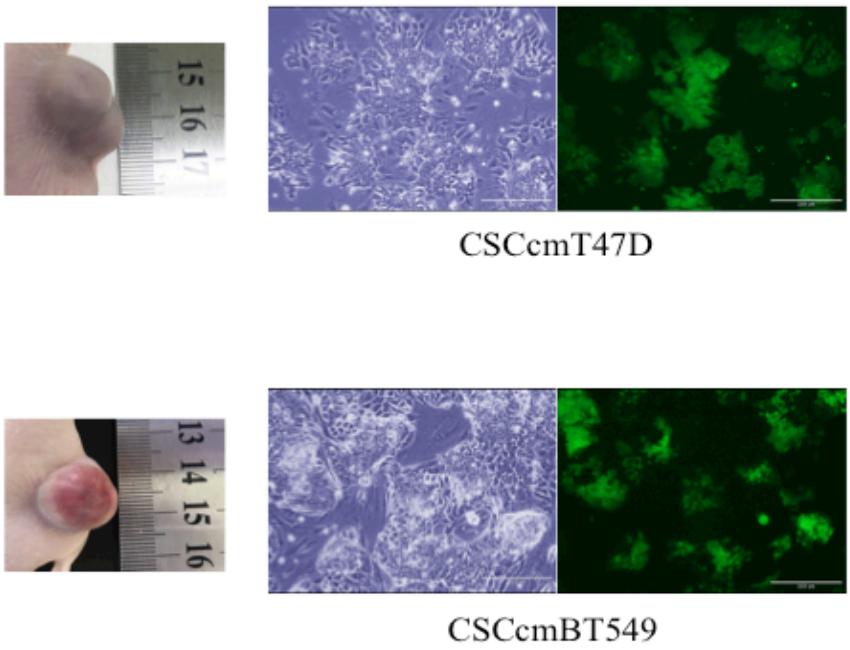
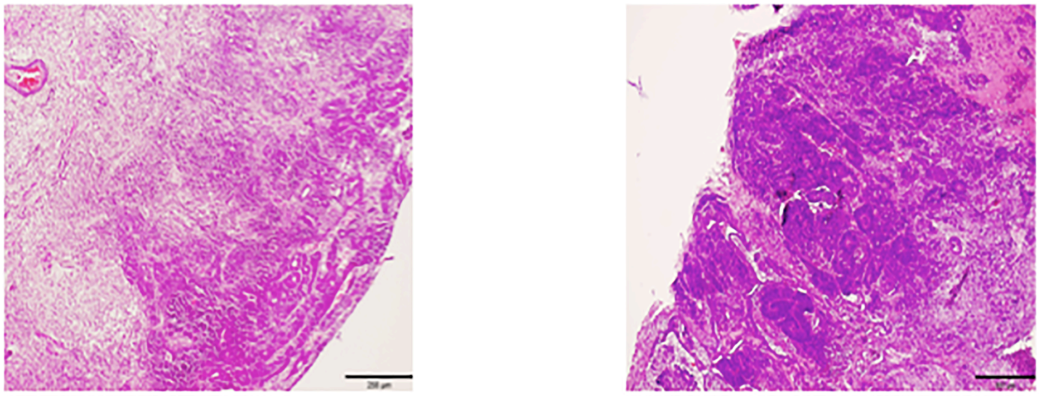


Fig. 1d Representative images of the subcutaneous tumors formed after transplantation of converted cells and the corresponding micrographs of the primary cells cultured from excised tumors. Scale bars represent 200μm.

Tumor histopathological analysis

Hematoxylin and eosin staining of tumor tissue sections revealed high nuclear to cytoplasmic ratio and poorly-differentiated glandular structures representative of adenocarcinoma.



CSCcmT47D CSCcmBT549

Fig. 1e Histopathological features of primary subcutaneous tumors CSCcmT47D and CSCcmBT549 analyzed by H&E staining. Scale bars represent 300µm.

Flow cytometer analysis for cancer stem cell markers

Both the conditioned medium treated miPS cells (CT-T47D and CT-BT549) and primary cells cultured from excised subcutaneous tumors (CSCcmT47D and CSCcmBT549) were analyzed for the expression of the prominent CSC surface markers, CD44 and CD24 respectively using flow cytometer. Marked enrichment of $CD44^{+}/CD24^{low}$ was observed in primary cells compared with converted cells indicating enrichment of the CSC population in vivo (Fig. 1f).

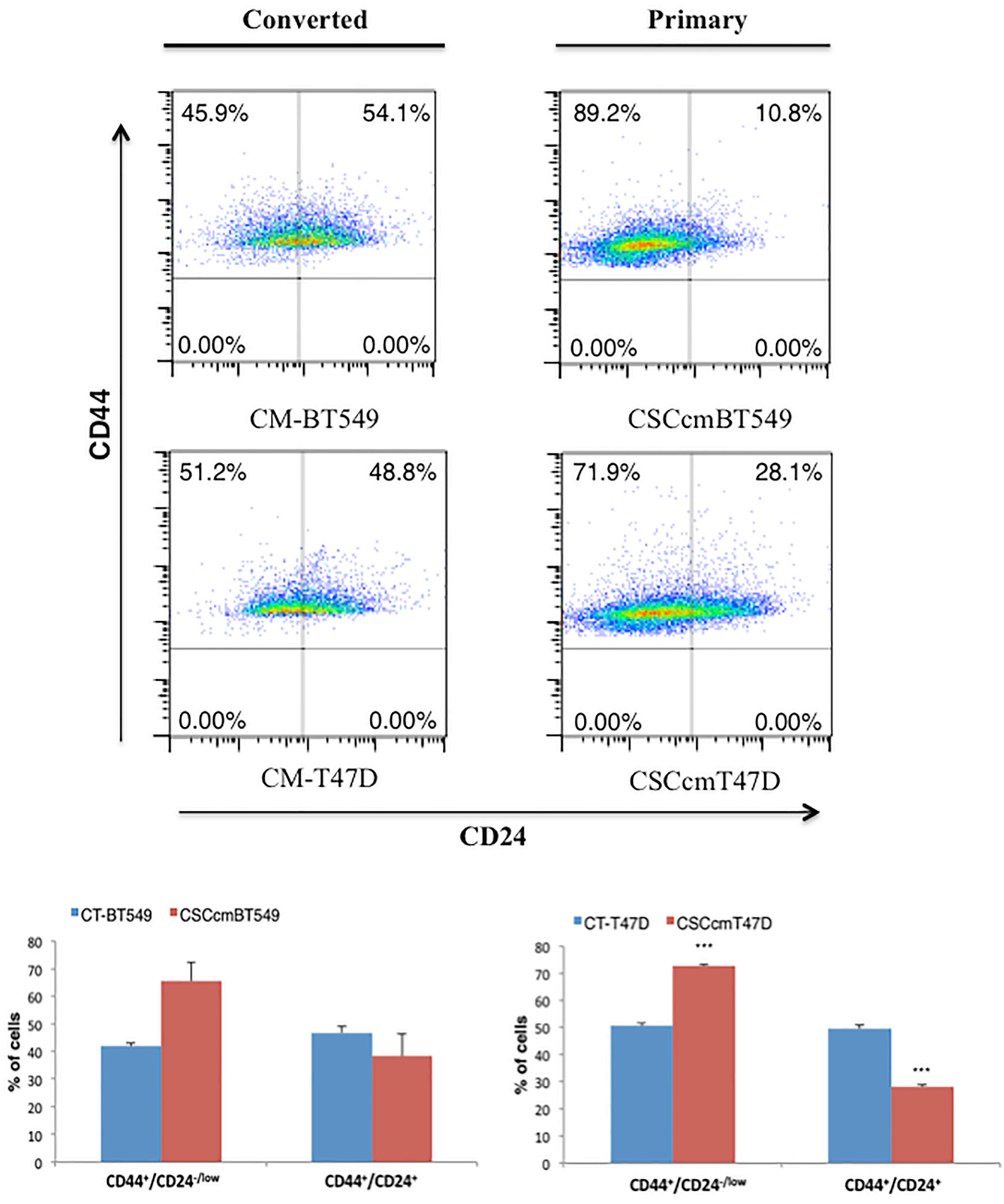


Fig 1f Representative plots and corresponding graphs of flow cytometer analysis demonstrating percentage of $CD24^{-/low}$ and $CD24^{+}$ in $CD44^{+}$ cells in 1) CT-BT549, 2) CSCcmBT549, 3) CT-T47D and 4) CSCcmT47D. Flow cytometer plots are representative of three independent experiments.

DISCUSSION

The cancer stem-like cells were generated from miPS cells by treating them with conditioned medium from two prominent human breast cancer cell lines, BT549 (claudin low) and T47D (luminal A). These cell lines were originally established from invasive ductal breast carcinoma and represent two major hormonal subtypes of breast cancer i.e. $ER^{+}/PR^{+}/Her2^{-}$ and $ER^{-}/PR^{-}/Her2^{-}$. This criterion of choosing the conditioned medium from these two cell lines was to facilitate the understanding of the effect of diverse tumor niche-derived factors. The conditioned medium treatment resulted in the establishment of GFP-expressing cells with enhanced expression of CSC markers, CD133 and EpCam indicative of conversion to CSC-like cells. The results corroborated with the previous findings wherein pancreatic cancer cell lines derived conditioned medium was used to treat the miPS cells. Further, the converted cells were morphologically heterogeneous containing GFP+ and GFP- cell population. Earlier reports suggest that *In vivo* transplantation is an important platform to identify the tumorigenic potential of CSC and the microenvironment provides the right conducive milieu to enrich this population. Therefore, subcutaneous transplantation into immunocompromised mice was considered necessary to enrich the CSC-like population. Transplanted cells were able to form tumor subcutaneously with high nuclear to cytoplasmic ratio and glandular structures representing adenocarcinoma. Moreover, subsequent serial transplantations gave rise to tumors with the same potential. Of note, no transgene expression of cMyc or Klf4 was observed which suggested that CM-T47D and CM-BT549 cells could be controlled primarily by other undetermined endogenous gene expression. Above all, flow cytometer analysis of the four weeks conditioned medium treated cells and primary cells revealed enrichment of highly tumorigenic $CD44^{+}/CD24^{-/low}$ cancer stem cell

population indicative of the fact that conditioned medium exerts the differentiation of miPS cell towards cancer stem cell like phenotype and in vivo microenvironment further enriches this population. Therefore, this model should be a useful tool for studying the hierarchy of CSCs and its progenitors *in vitro* with further enrichment in immunocompromised mice, indirectly supporting to overcome the drawbacks associated with isolation and study of CSC from cancer cell lines.

CHAPTER 2

CANCER STEM CELLS AS THE NOVEL POINT OF ORIGIN OF CANCER-ASSOCIATED FIBROBLASTS IN TUMOR MICROENVIRONMENT

ABSTRACT

In the present chapter, the potential of the CSC to generate CAF-like cells (CAFLc) in cancerous niche is tested. For this, primary cells from subcutaneous tumors (CSCcmT47D and CSCcmBT54) were cultured in serum free condition to enrich CSC spheres. These CSC spheres differentiated into various cell types including myofibroblast-like cells. The further characterization revealed that the myofibroblast-like cells corresponded phenotypically with CAFLc. The present findings support our new concept that CSC should be one of the major contributors of CAFs in tumor niche.

INTRODUCTION

Tumor microenvironment and Cancer-associated fibroblasts

The importance of stroma in tumor microenvironment is now considered more than beyond being just a physical support for the epithelial cells. They are crucial drivers of cancer progression mediating transformation, tumorigenicity, metastasis, inflammation and angiogenesis. A typical stroma consists of a variety of cells such as endothelial cells, immune cells, adipocytes, mesenchymal stem cells, and many more; each playing a distinct yet complimentary role forming a supportive structure for the cells residing in tumor. In a normal, non-cancerous TME stromal components play a very conducive role; fibroblasts secrete ECM supporting the tissue remodeling, adipose cells serves as energy reserves, endothelial cells organize themselves into blood vessels transporting oxygen and nutrients, immune cells mediating resistance against infections and stem cells like mesenchymal stem cells serving as progenitor cells constantly replacing these functional components of stroma. In cancer an imbalance in the functional roles of the stromal components is encountered in response with the changes in the mutated epithelial cells. This results in transformation of the stromal cells into tumor- associated stroma supporting the tumor malignancy.

Cancer-associated fibroblasts (CAFs) are the most abundant cell type in the tumor- associated stroma, which in many cases control the disease outcome. Normal fibroblast maintains the tissue homeostasis, supports wound healing, senescence and inhibit tumor cell growth. However, CAFs reside within the tumor margins and often infiltrate the tumor facilitating transformation and tumor progression. In breast and pancreatic cancer, they constitute more than 80% of the stromal compartment often

responsible for poor disease outcome. In recent years there is a quantum jump in the number of publications supporting the role of CAFs in tumor microenvironment is evident. Therefore, there is an emerging concept of CAF as architects of "cancer pathogenesis" (18).

Biomarkers for identifying CAF

It is a well-known fact that the heterogeneity of fibroblasts in stromal compartment is widespread. Similarly, multiple subpopulations of CAFs are present in the tumor milieu In addition to it, CAF also shares several markers with other components of the stroma, such as myofibroblasts, myoepithelial cells, mesenchymal stem cells and endothelia making it again difficult to identify and isolate them. The most widely used and reference marker for acquisition of a CAF phenotype is α-smooth muscle actin (α-SMA). Additional markers expressed in CAFs include fibroblast specific protein -1 (FSP1), vimentin, platelet derived growth factor- α (PDGF-α), PDGF-β, Neuron Glial Antigen-2 (NG2), Col1α1, Fibronectin (FN1), Podoplanin (PDPN), Prolyl-4-hydroxylase, Metalloproteinases (MMPs), Fibroblast activation protein (FAP). These markers are usually upregulated in CAFs. Among the downregulated ones are caveolin-1 and laminin (19). However, most of these markers are not CAF specific. Recently, markers restricted for CAF identification has been reported such as collagen-11 α-1 (COL11A1), Asporin, microfibrillar-associated protein 5 (MFAP5) which may become the potential biomarkers for reliable identification of CAFs (18).

Cellular origin of Cancer-associated Fibroblasts

The exact cellular origin of CAFs is a widely debated and controversial issue due to its apparent heterogeneity. Many origins of CAFs have been proposed in the literature and all seem to be relevant in understanding its functional role. One of the main sources is considered to be resident normal fibroblasts. The fibroblasts adjacent to the tumor acquire CAF phenotype mediated by several factors released by tumor cells activating protumorigenic pathways in them such as upregualtion of TGFβ, bone morphogentic protein (BMP), Wnt, Sonic hedgehog (Shh), platelet derived growth factor (PDGF) and integrin mediated signaling. Recent reports also propose Mesenchymal stem cells (MSCs) as possible sources of CAF. Prolonged exposure of human bone marrow-derived MSCs to conditioned medium from MDA-MB-231 breast cancer cells resulted in myofibroblast differentiation characterized by expression of CAF markers like α-SMA, vimentin, fibroblast surface protein (FSP) and CXCL12/SDF-1 (21). In several mouse models this has been confirmed by using labeled bone marrow cells injected into tumor bearing mice. CAF-like cells from bone marrow cell origin are present in the vicinity of the tumors. Epithelial cells are another plausible sources of CAFs in tumor milieu. This transformation is facilitated by epithelial-mesenchymal transition. The resulting mesenchymal like cells exhibit CAF-like morphology by expressing CAF markers alongwith residual expression of keratin confirming their epithelial origin. Another emerging source for CAFs is considered to be endothelial cells. Zeisberg et al has reported that the endothelial cells treated with TGFβ exhibit CAF morphology with elevated FSP1 expression with concomitant downregulation of endothelial marker CD31 (22). In addition to these, adipose derived stem cells have been reported to differentiate into CAF-like cells on exposure to conditioned medium from breast cancer cell lines (23). Overall, the

possibility for many sources of origin could be the underlying reason for the heterogeneity and multifunctional nature of CAFs in TME.

How to define CAF- a cell or state?

The aforementioned inconsistency in the knowledge of a specific marker to isolate CAF followed by its many possible sources of cellular origin, recent reports have suggested new definition for CAF. Intriguingly, CAFs in most of the cases have been found to be genetically stable. Madar et al defines CAF as a 'state' of fibroblast-like cells found in the vicinity of the tumor that promotes its progression (19). The maintenance of 'CAF state' is attributed to its continued exposure to factors released particularly by cancer cells in tumor niche. Many factors such as genetic mutations, epigenetic alterations, and persistent environmental effects have been considered the underlying reason for the acquisition of this state. Genetic mutations including loss of heterozygosity (LOH) has been reported in microdissected breast stromal tissues. Also, studies have shown mutations in P53 and PTEN in the stromal compartment of breast cancer patients. Nevertheless, several studies have also demonstrated that unlike epithelial cancer cells, genetic alterations, LOH are extremely rare in CAFs (4-5%) derived from human ovarian and breast tissues. In contrast, epigenetic alterations account for many early gene expression changes observed upon acquisition of CAF state. For example, TGF-β type II receptor (TGFBR2) is epigenetically silenced in 70% of prostate cancer patients and in host mouse prostate fibroblasts in murine xenograft model due to secretion of IL-6. This epigenetic alteration coincides with elevated DNA methyltransferase I (DNMT1) activity and histone H3 Lys9 trimethylation (H3K9me3). Another way for maintaining the 'CAF state' are the signals released from tumor cells, which includes release of interleukin 1 (IL-1),

tumor necrosis factor (TNF-α). Moreover, Sukowati et al have demonstrated the presence of multipotent primary CAF cells in hepatocellular carcinoma-affected liver tissue (24). Therefore, all these evidences provide concrete evidence in defining CAF as a 'state' rather than a cell type.

Role of CAFs in disease progression

Stromal cells play a significant role in tumor-associated malignancies. Normally, fibroblasts favor maintenance of epithelial morphology and constrain early dissemination, tumor growth and metastasis. However, CAF plays completely opposite roles. CAFs secrete various factors exemplified in figure 4 that mediate tumor-promoting effects thereby supporting cancer progression. CXCL12/ SDF1 is a chemokine that can induce angiogenesis and enhance proliferative capacity of cancer cells. This CXCL12 secreted from CAF act via TGF-β-regulated CXCR4 in adjacent epithelial cells, which in turn activate the AKT pathway eliciting tumorigenesis. Furthermore, CAFs secrete ECM modulators such as MMPs like MMP1, which has been reported to augment the migration and invasion of cancer cells. CAF secreted TGF-β1 activates TGF-β/Smad signaling pathway in breast cancer cells leading to acquisition of metastatic potential by cancer cells (18,19).

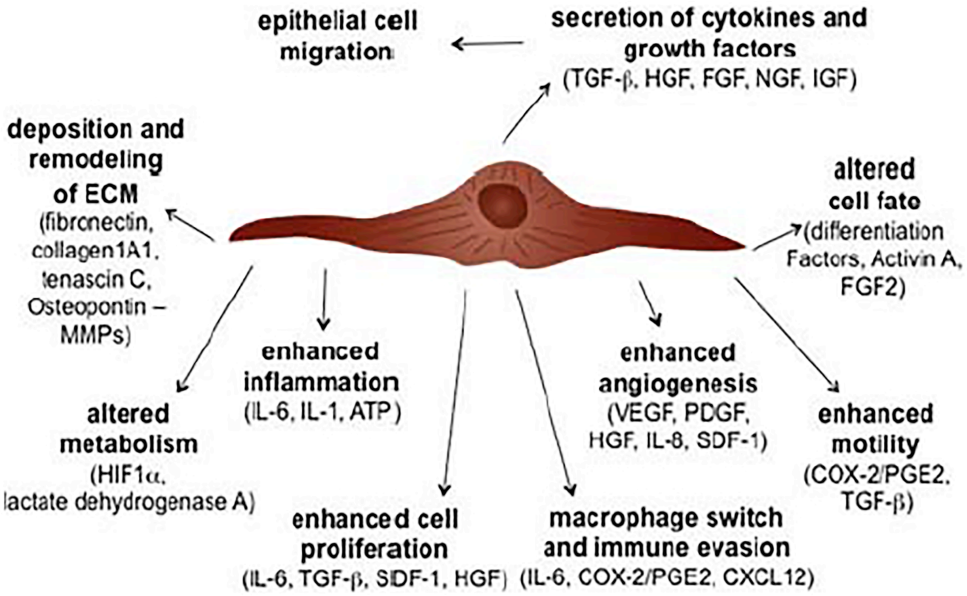


Fig. 4 **Multiple secreted factors and resultant phenotypes stimulated by CAFs.** *Courtesy ; Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. Gascard, P. Genes & development,* ***30****:1002-1019. 2016*

Recent studies have documented the contribution of CAFs in mediating the acquisition of premalignant phenotypes. One such example is the CAFs promoting the conversion of ductal carcinoma in situ (DCIS) to invasive breast carcinoma (IBC) via paracrine IL-6 signalling. Similarly, recent interesting studies have revealed the contribution of CAFs towards drug resistance by diverse mechanisms. They do so by 1) altering cell-matrix interaction that control epithelial cell sensitivity towards apoptosis, 2) secrete proteins that control epithelial cell survival and proliferation, 3) contribute to direct cell-cell interactions where cells can exchange membrane fragments and communicate, 4) create physical barrier that facilitate acquisition of drug resistance, 5) activate epigenetic plasticity in neighboring cells. Overall, CAF plays a multidimensional role in mediating cancer progression (18).

Potential therapeutic targets on CAFs

The current understanding of the effect of CAF on neoplastic progression is unprecedented. Therefore, exciting new clinical studies are being designed based on targeting CAFs in addition to targeting cancer cells in order to inhibit malignant phenotype. Drugs designed fall into two major categories; drugs aimed at 1) reverting the CAF phenotype to a normal fibroblast by targeting the pathways such as hedgehog, PDGF, or IL-6 CXCR4 signalling. 2) Eliminating CAFs by delivering proapoptic molecules using carriers recognizing CAF specific molecules such as FAP-specific antibodies or PDGF BH3 mimetics. Ongoing clinical trials are assessing the therapeutic value of human $CD8^+$ T cells engineered to target fibroblast activation protein (FAP) expressing cells (25). To summarize, simultaneous targeting of tumor stroma and epithelial cancer cells with combination therapies may be a successful therapeutic approach for treating various cancers and have the added benefit of preventing drug resistance.

RESULTS

Enrichment of primary cancer stem cell population by sphere formation

The primary cells cultured from excised tumor as discussed in previous chapter were treated with puromycin to deplete nude mice-originated cells completely. After the puromycin selection, the primary culture cells showed a high number of GFP-expressing colonies surrounded by myofibroblast-like cells. Sphere formation assay was set up with CSCcmT47D and CSCcmBT549 cells. GFP expressing spheres were observed in 4-5 days. The spheres, which were dissociated into single cells, also gave rise to new spheres during serial passage (Fig. 2a).

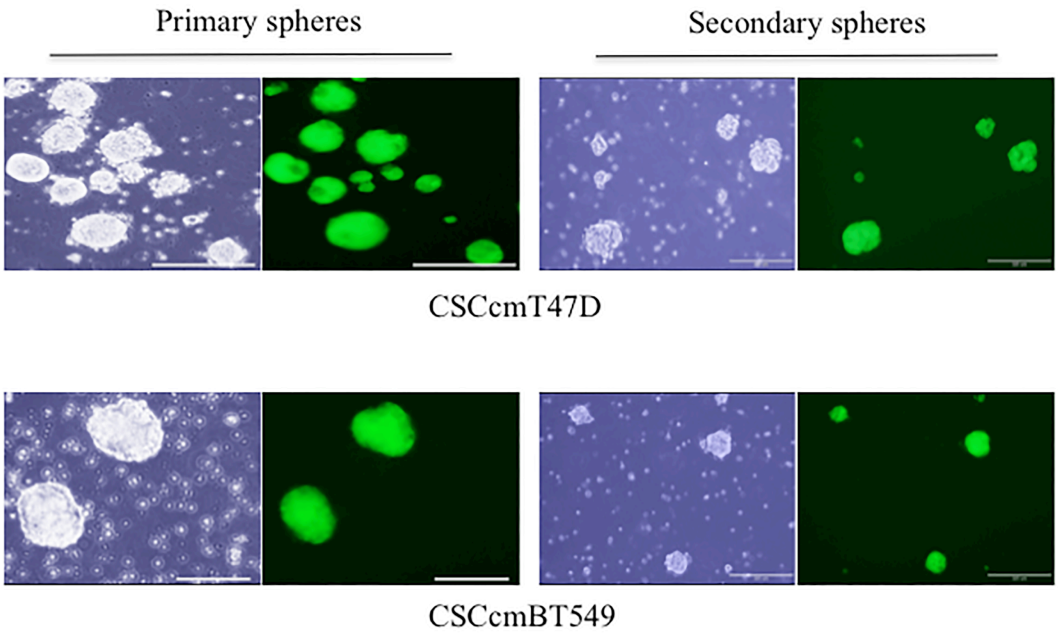


Fig. 2a Representative images of primary and secondary sphere-forming cells from tumor-derived primary cells (CSCmT47D and CSCcmBT549) cultured in serum-free condition. Scale bars represent 200 μm.

Tumor spheres exhibited key features of CSC

RT-qPCR analysis revealed that CSC marker CD133 were expressed higher in the cell population from spheres (Sphere-T and Sphere-B in Fig. 2b) compared with the primary cells (CSCcmT47D and CSCcmBT549), suggesting the enrichment of CSC-like cells. Also, the tumorigenicity of the sphere-forming cells was confirmed by the subsequent subcutaneous transplantation in to balb/c nude mice. The resulting tumors presented highly malignant phenotype, high nuclear to cytoplasmic ratio and enrichment of stromal population (Fig. 2c).

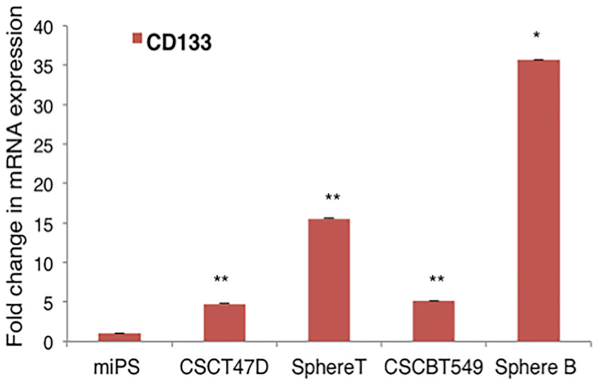
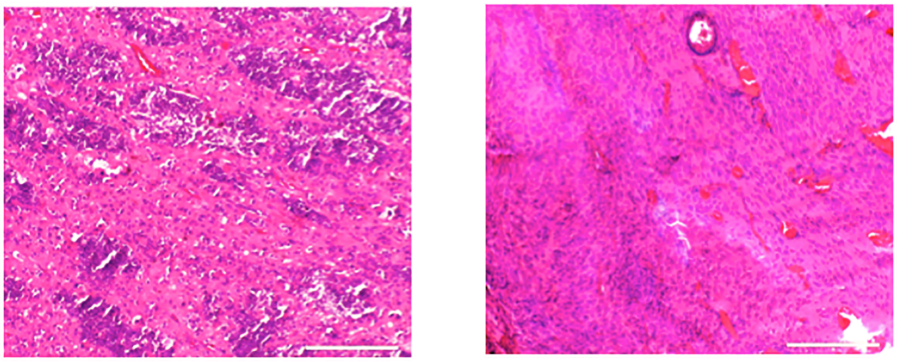


Fig. 2(b) RT-qPCR analysis of CD133 gene expression. The primary cells (CSCcmT47D and CSCcmBT549) and their corresponding spheroids (sphereT and sphereB) were used for the qPCR. Expression levels were compared with untreated miPS cells and normalized to GAPDH. Data are presented as mean ± standard deviation and analyzed using unpaired two-tailed student's *t*-test. The level of significance was set as $^{**}P<0.01$, $^{*}P<0.05$.



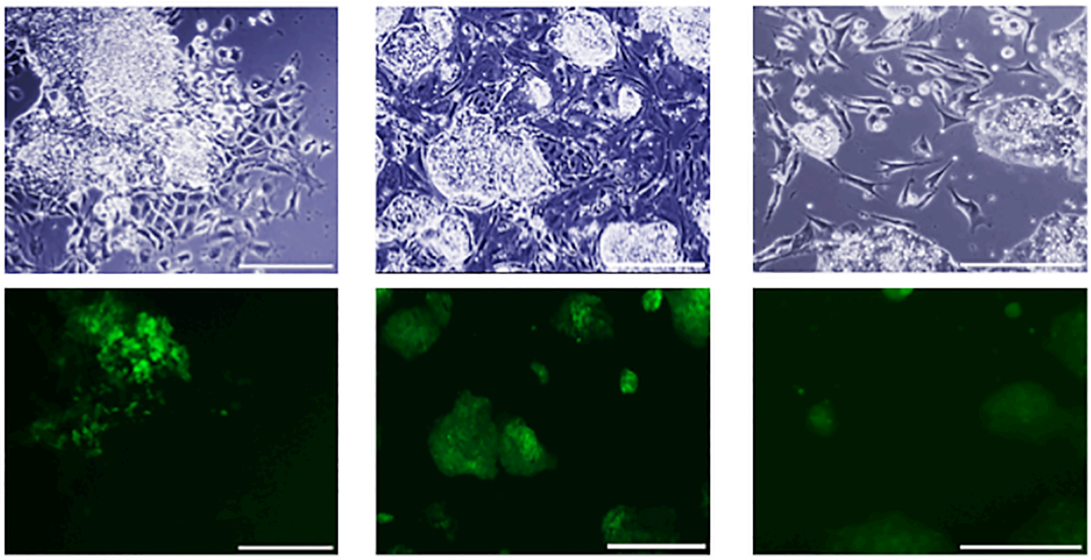
CSCcmT47D CSCcmBT549

Fig.2(c) Histopathology of the primary subcutaneous tumor formed by the injection of CSCcmT47D and CSCcmBT549 spheres. Scale bars represent 300 μm

Sphere differentiation and separation of myofibroblast-like cells

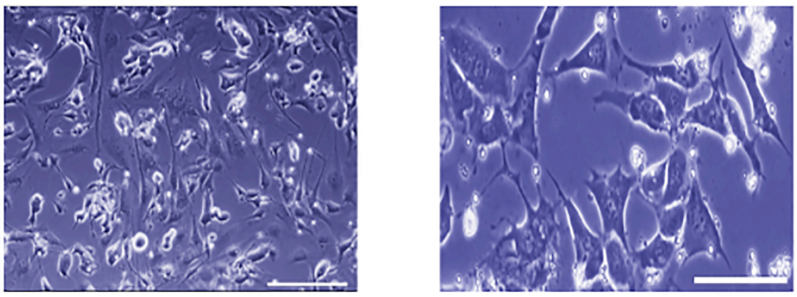
To determine the lineage of cells originating from the enriched CSCs, these spheres were then cultured and induced differentiation under anchorage-dependent conditions by adding respective primary cell-conditioned medium. The conditioned medium was collected from either CSCcmT47D or CSCcmBT549 cell culture in DMEM containing 5% knockout serum replacement (KSR). The differentiated cells resulted in the production of heterogeneous cell population, which are composed of sphere-like colonies expressing GFP and surrounding myofibroblast-like cells (Fig. 2d-e). Furthermore, subpopulation of myofibroblast-like cells was separated using mouse feeder-specific magnetic microbeads or Magnetic Activated Cell Sorting (MACS). This method enabled to separate the differentiated myofibroblast-like cells from the sphere-derived CSC population (Fig. 2f). As control, original miPS cells were differentiated to generate fibroblast-like cells according to previously reported

protocols. After differentiation, the cells were also separated using magnetic beads in order to isolate pure fibroblast cells termed as miPS-fibroblast (Fig. 2g).



d) e)

Fig. 2(d) Morphology of primary cells generated from CSCcmBT549 spheroids. The morphology of adherent CSCcmT47D spheroids was similar to that of CSCcmBT549 spheroids (data not shown). Scale bar represents 200µm. **Fig. 2(e)** Representative morphology of cells differentiated from CSCcmT47D and CSCcmBT549 spheres after two passages. Scale bars represent 200 µm and 400 µm.



CSCcmT47D CSCcmBT549

Fig. 2(f) Morphology of differentiated cells isolated from primary CSCcmT47D and CSCcmBT549 spheroids. The cells exhibited prominent nucleoli with long spindles. Scale bar represents 200 µm (*lef)9* and 100 µm (*right*).

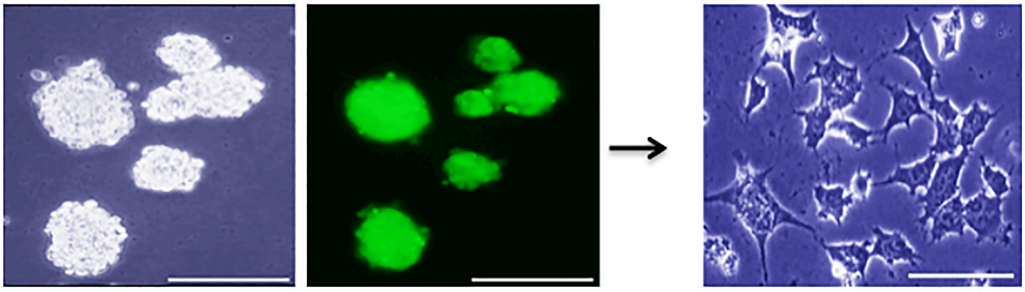


Fig. 2(g) Morphology of fibroblasts derived from miPS embryoid bodies. Scale bars represent 200 μm.

Separated myofibroblast-like cells displayed correspondence with cancer-associated fibroblast like cells

Quantitative gene expression analysis was performed by targeting the following genes: CAF markers such as Fibroblast specific protein (FSP1), α-smooth muscle actin (α-SMA), stromal derived factor-1 also known as CXCL12, transforming growth factor (TGFβ1) and platelet derived growth factor (PDGFα), alpha-1 type collagen (Col1α1) well as fibroblast-specific marker, vimentin, which were upregulated in the myofibroblast-like cells compared to miPS-fibroblast (Fig. 2h-i). Thus, we named these cells either CSCcmT47D-CAFLc or CSCcmBT549-CAFLc. In comparison to miPS-fibroblast, CSCcmT47D-CAFLc had increased expression of FSP1, CXCL12, TGFβ1, vimentin and PDGF-α, while α-SMA expression was undetectable (Fig. 2h and data not shown). On the other hand, CSCcmBT549-CAFLc was activated with considerable expression of the CAF secretory factors along with α-SMA, vimentin and Col1α1 expression (Fig. 2i).

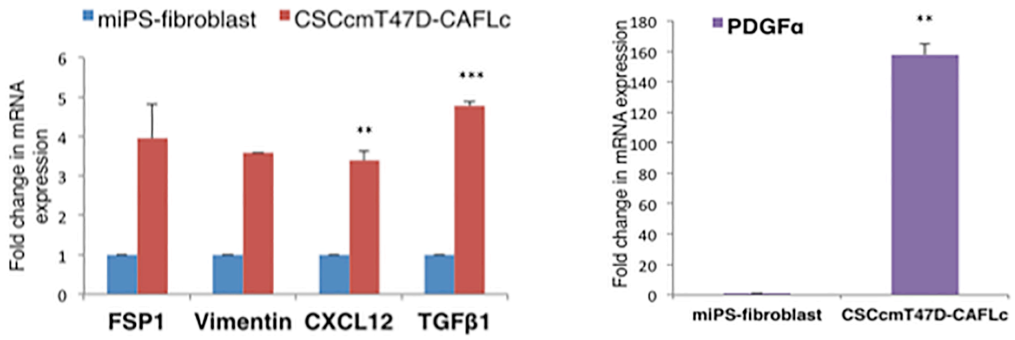


Fig. 2(h) RT-qPCR analysis of CAF markers, FSP1, Vimentin, TGFβ1, CXCL12, Col1α1, and PDGFα in CSCcmT47D-CAFLc. Data is presented as mean ± standard deviation and analyzed using unpaired two-tailed student's *t*-test. The level of significance was set as ***P<0.001,**P<0.01

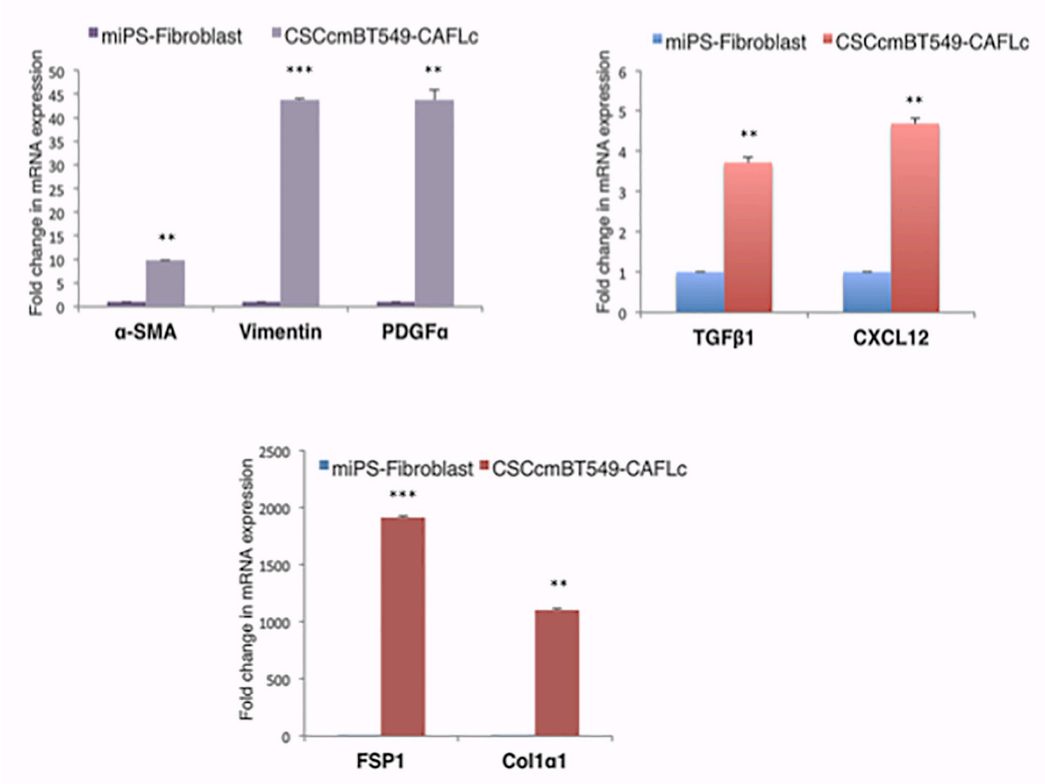


Fig. 2(i) RT-qPCR analysis of CAF markers, FSP1, Vimentin, TGFβ1, CXCL12, and PDGFα in CSCcmBT549-CAFLc. Data is presented as mean ± standard deviation and analyzed using unpaired two-tailed student's *t*-test. The level of significance was set as ***P<0.001, **P<0.01, *P<0.05

CSC-differentiated CAFs exhibited expression of prominent CAF proteins

Immunofluorescent staining in the separated CAFs for FSP-1, FAP and α-SMA was performed. The CSCcmBT549-CAFLc populations showed positive staining of all the three markers. In contrast, the CSCcmT47D-CAFLc subpopulations expressed primarily FSP-1 and FAP but not α-SMA (Fig. 2j).

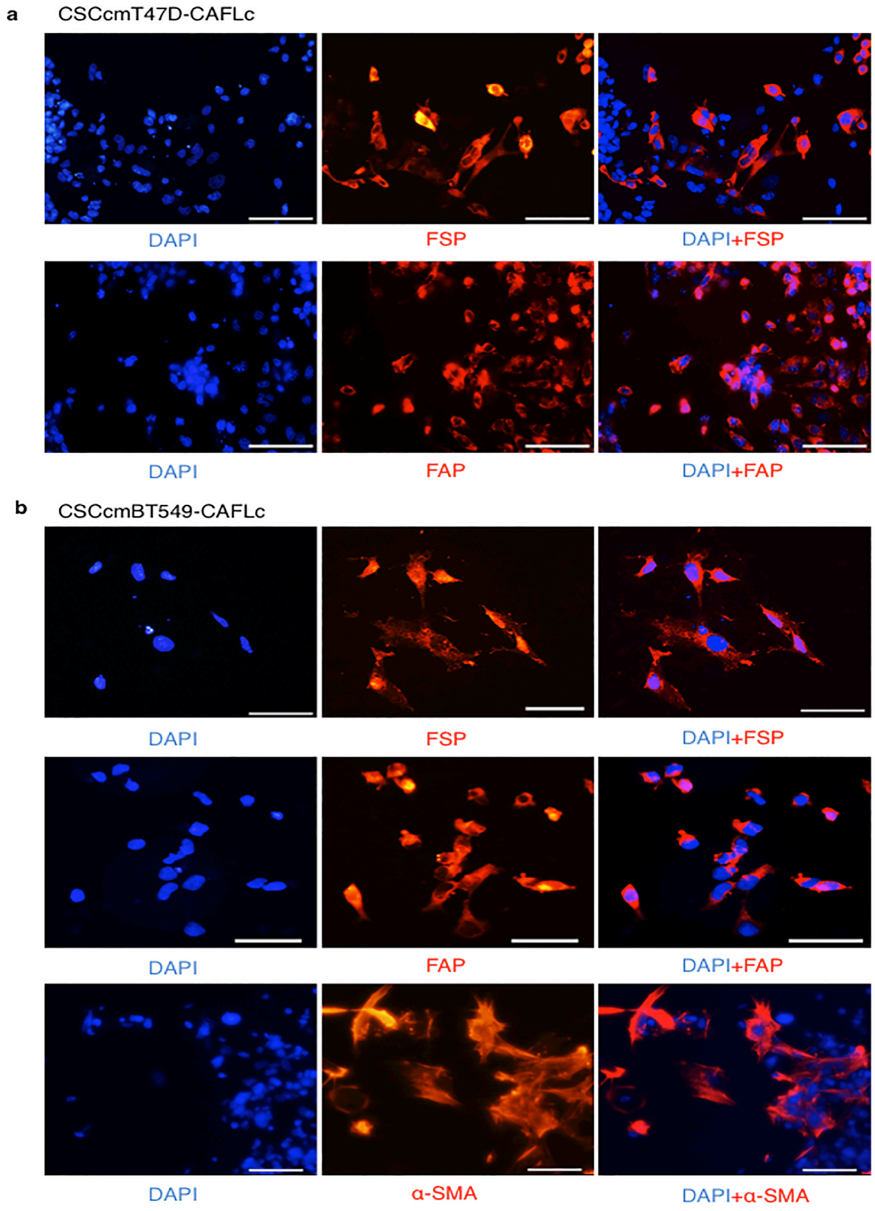


Fig. 2(j) Representative images of CSCcmT47D-CAFLc and CSCcmBT549-CAFLc stained separately with anti-rabbit FSP1, anti-rabbit α-SMA and anti-rabbit FAP antibodies. The nuclei were counterstained with DAPI (*blue*). Scale bars represent 50 μm.

CAF-like cells exhibited invasive potential

CAFs could lead in the tumor invasive front directing the migration of invading cancer cells as demonstrated by GFP-positive CAF cells. Therefore, matrigel invasion assay was performed and it confirmed that the generated CAFs were highly invasive as compared with miPS-generated fibroblasts (Fig. 2h).

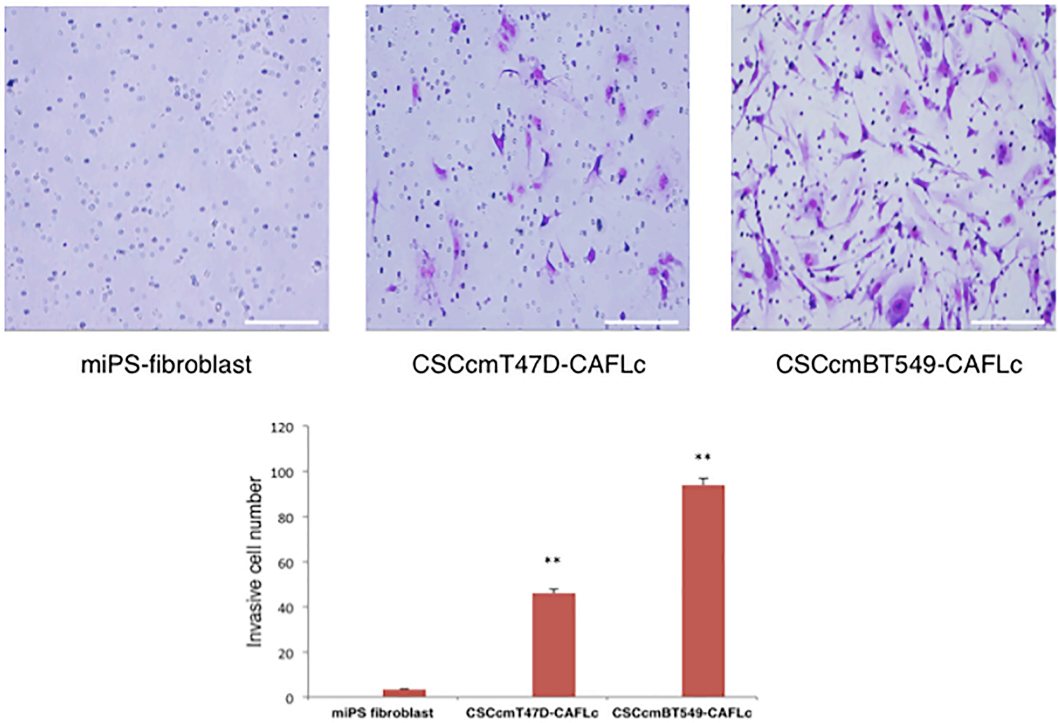


Fig. 2(h) CAFLc invasion ability was measured using Matrigel coated transverse inserts and the invaded cells were stained with giemsa and counted in several fields in triplicate. Scale bar represents 200 µm. The graph indicates quantitative results by counting invasive cells. CSCcmT47D-CAFLc and CSCcmBT549-CAFLc had higher number of cells invading than control miPS-fibroblasts. Data is presented as mean ± standard deviation and analyzed using unpaired two-tailed student's *t*-test. The level of significance was set as $^{**}P<0.01$.

Lineage tracing by GFP-RNA *in situ* hybridization in combination with immunofluorescence for FSP1

CSC cells are the source of origin of the differentiating CAFLc, they could be multipotent and thereby maintain plasticity in the phenotype. GFP-mRNA signal was significantly higher in the nucleus compared to the cytoplasm in addition to FSP-1 protein expression (Fig.2i). This observation was contrary to the attenuation of GFP florescence observed in the CAFLc in primary culture cells.

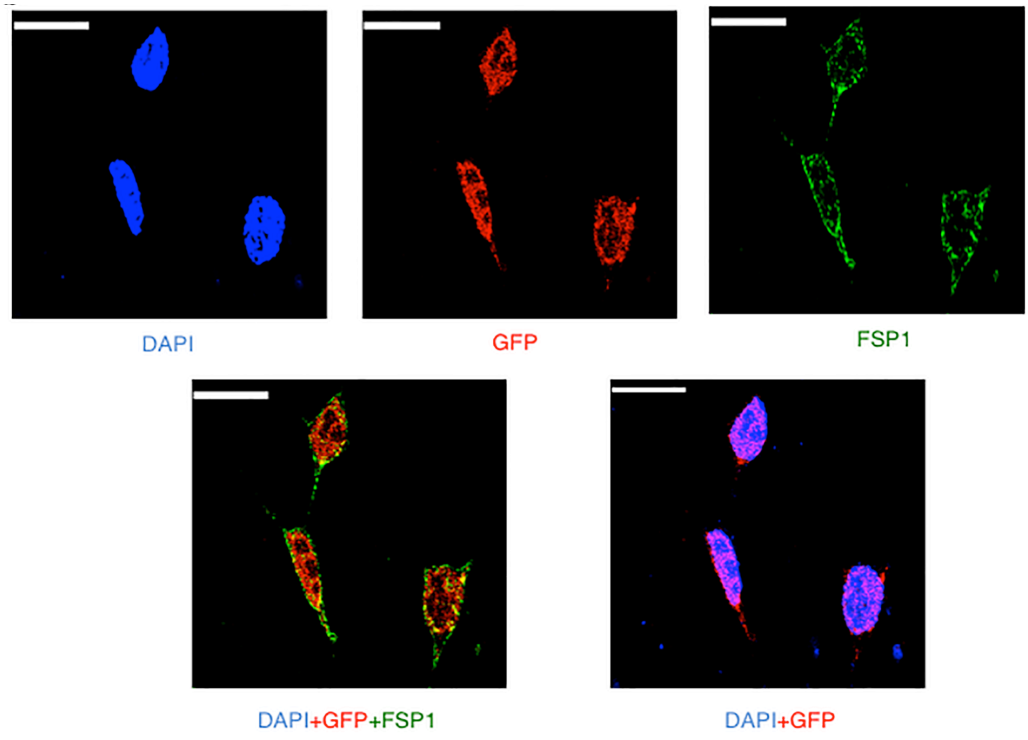


Fig. 2(i) *In Situ* Hybridization analysis with GFP-RNA probe for GFP-mRNA (*red*) combined with immunofluorescent analysis for FSP1 (*green*). DAPI staining is shown in *blue*. Strong GFP signal in the nucleus indicates plasticity of the CAF cells. Scale bar represents 10 µm.

DISCUSSION

The previous studies associated with the establishment of pancreatic CSC-like cells had demonstrated that the primary culture cells derived from subcutaneous tumors had highly enhanced expression of CSC markers and their associated pathways by the RNA sequencing analysis; therefore, it was decided that all the further experiments to be carried out using primary culture cells. Moreover, this observation was further corroborated with FACS analysis as discussed in the previous chapter.

The primary cells were enriched with puromycin treatment since the original miPS cells exhibit a puromycin resistance gene. This ensured depletion of the contaminating host cells. One of the striking observation notable was the establishment of myofibroblast-like cells encompassing the sphere-like cells even after repeating dissociation and passaging. Based on these observations, new concept is proposed that the close interaction between these subpopulations could be indispensable because the cell lineages maintain the defined organization under the so-called “self-sustaining” mechanism of CSCs

Several theories regarding the origin of CAF has been proposed in recent studies and in our present work we are proposing for the first time that CSC should be one of the important contributor of CAF in tumor microenvironment. To prove this hypothesis, we are demonstrating this phenomenon in the established CSC model. To begin with, the primary CSC-like cells were enriched from adherent cultures by sphere formation assay. Sphere-formation is an important property to determine the

functional phenotype of CSC population. Likewise, the established CSCs gave rise to a significant number of spheres in non-adherent serum-free condition. GFP expression ensured better selection of self-renewing spheres from aggregates. Sphere-forming CSCs are considered to be more tumorigenic as compared with the adherent counterpart. Accordingly, spheres on subcutaneous transplantation generated tumor with histopathological adenocarcinoma-like features, confirming their aggressive tumorigenic potential.

Since the expression of CD133 is considered to be vital in determining the enrichment of CSC population, the gene expression profile revealed 3- to 7-fold increase in its expression in the spheres when compared with the adherent primary cultures.

In adherent conditions, spheres have the distinctive ability to differentiate and proliferate, resulting in various cell types. One of the most distinguishing features observed during the CSC sphere differentiation was the proliferation of feeder-like myofibroblast cells surrounding undifferentiated population. This differentiation was carried out in the presence of primary cancer cell line, CSCcmBT549 or CSCcmT47D conditioned medium, collected by replacing FBS with low concentration of knockout serum replacement (KSR) medium in DMEM. Recently, numerous differentiation studies prefer to use this defined serum-free medium to avoid possible spontaneous and uncontrolled differentiation. Therefore, the serum-free primary CSC conditioned medium allowed for a slower and more controlled differentiation of spheres in adherent condition, enabling the understanding of the cell types that play an important role in maintenance of CSC population. The differentiated myofibroblast-like cells

were separated from the stem cell population using MACS. The separated cells had spindle-shape morphology with prominent nucleoli and the quantitative gene expression pattern for phenotypic markers like α-SMA, FSP1, and Vimentin. These results revealed that the differentiated myofibroblast-like cells resemble CAFs phenotypically.

In order to ascertain that the population does not constitute normal or activated fibroblasts, miPS cells were differentiated to fibroblasts using previously reported protocols. These fibroblasts were also enriched using the feeder-specific magnetic beads. Comparative gene expression analysis between the miPS-generated fibroblasts and CSC-derived fibroblasts provides evidence that the CSC-derived fibroblasts correspond to CAFs. Moreover, the expression of some of the well-known CAF secretory factors like CXCL12, TGF-β1, and PGDFα were up-regulated in CSC-derived fibroblast cells which promoted cancer cell invasion and progression. Concomitant results were obtained from immunocytochemistry of the separated cells for FSP, α-SMA and another prominent CAF marker FAP. The transcript level of CAF markers was notably up-regulated but did not reflect at the protein level. This contradiction could be due to the limitations in *in vitro* studies related with lack of paracrine signaling and inability to mimic a complete prototype of tumor niche. Further, the CAF cells had marked invasion ability through matrigel in comparison with the iPS generated fibroblasts corresponding with the previous study that CAFs isolated and cultured from patient samples were more invasive then tumor cells.

It was interesting to identify distinct subpopulation of CAFLc arising from the CSC cells. This study gives novel insight into the fact that heterogeneity between

different classes of CAFs could be attributed to their origin from CSCs modulated with epigenetic and microenvironmental changes. The populations of CAFs could not be maintained after their generation from CSCs for more than five passages. It is important to state that on separation, some CAFs subpopulations had the tendency to revert back to stem cell state and those fully differentiated did not survive. This phenomenon was not observed in case of normal miPS cells. In the primary cells CSCcmBT549 and CSCcmT47D, codependent GFP-positive stem cell colonies with differentiated fibroblast-like cells survived repeated passages were observed. Furthermore, it was observed that the CAF had the ability to switch between a stem cell state and a fully differentiated CAF-like state behaving as a supporting niche for the CSC sustenance.

Overall, by taking into account all the major observations and results regarding the conversion of CSC into CAF-Lc, we demonstrated that CSC could possibly be one of the important points of origin for CAF in the tumor niche and provides evidence regarding its heterogeneous identity.

CHAPTER 3

BREAST CANCER STEM CELL MODEL

ABSTRACT

The present chapter demonstrates preliminary studies leading to the development of a potential breast cancer stem cell model in immunocompromised nude mice. Converted miPS cells using cancer cell line conditioned medium as discussed in Chapter 1 has been used in the present study. These cells were transplanted orthotropically into mammary fat pad and the resulting tumors were analyzed for the enrichment of breast cancer stem cells.

INTRODUCTION

Breast cancer is the leading cause of death in women worldwide. Metastases, disease recurrence, resistant to conventional therapies are some significant hurdles in the complete evasion of this disease. Nevertheless, recent advancements in better screening methods and treatment of breast cancer are indeed hopeful but still there is still much to be done for achieving better disease survival rate. There has been a growing interest in understanding the role of cancer stem cells in breast cancer progression. Its study is providing with key insights in to its functional role in imparting drug resistance and metastasis.

Breast anatomy and physiology

Breast is a glandular organ with lobular organization. A normal human breast arises from the primary ectodermal growth in the second trimester of embryonic life. A typical breast lobe has a single lactiferous duct, which branches into several segmental ducts, ending into several terminal ducts and lobules, which represent the terminal ductal lobular units, **TDLU.** The ducts and ductules are lined by an inner layer of cuboidal to columnar epithelial cells and an outer layer of myoepithelial cells. The connective tissue within the lobule is composed of fibroblasts in a background of collagen and acid mucins, with histiocytes and occasional lymphocytes. The interlobular stroma is hypocellular and composed of fibroadipose tissue. The epithelium and lobular stroma are hormonally responsive.

The number of lobes and the basic morphology of the breast remain constant in a women's life but the size and form changes in response to the progressive and

regressive morphological processes occurring in response to age and hormonal status. During the reproductive period, the female breast undergoes a series of cyclical changes related to the menstrual cycle. Increased proliferation of the cells in ducts and lobules, which appears primarily in the luteul phase, leads to increased epithelial cell number and an increased number of acini per lobule; new bud formation from the terminal ducts increases the number of lobules. At the end of menstrual cycle, genetically programed cell death or apoptosiss appears in epithelial and myoepithelial cells and the process reverts to a status similar with the beginning of the menstrual cycle.

The mammary gland attains its full development in pregnancy with a substantial increase in the lobule number, size, and number of acini per lobule. After lactation, the mammary glands regress via collapse of the acini and small ducts, apoptosis of their epithelium and myoepithelium, and regeneration of the elements of interlobular stroma. During and after menopause, parenchyma undergoes involution resulting into diminished number of lobules, acini per lobules and terminal ducts in addition to stromal involution (26).

Deome et al first suggested the existence of self-renewing multipotent stem cells. It was hypothesizes that the entire mammary gland could be regenerated from the random fragments of breast epithelium (27). Furthermore, its also suggested that there is three distinct population of epithelial progeny cells in the breast: one capable of producing all epithelial cells and the other either give rise to secretory lobules or branching ducts. Therefore, the development and maintenance of breast lobes could be attributed to the complex processes involving several types of stem cells/ progenitor cells (28).

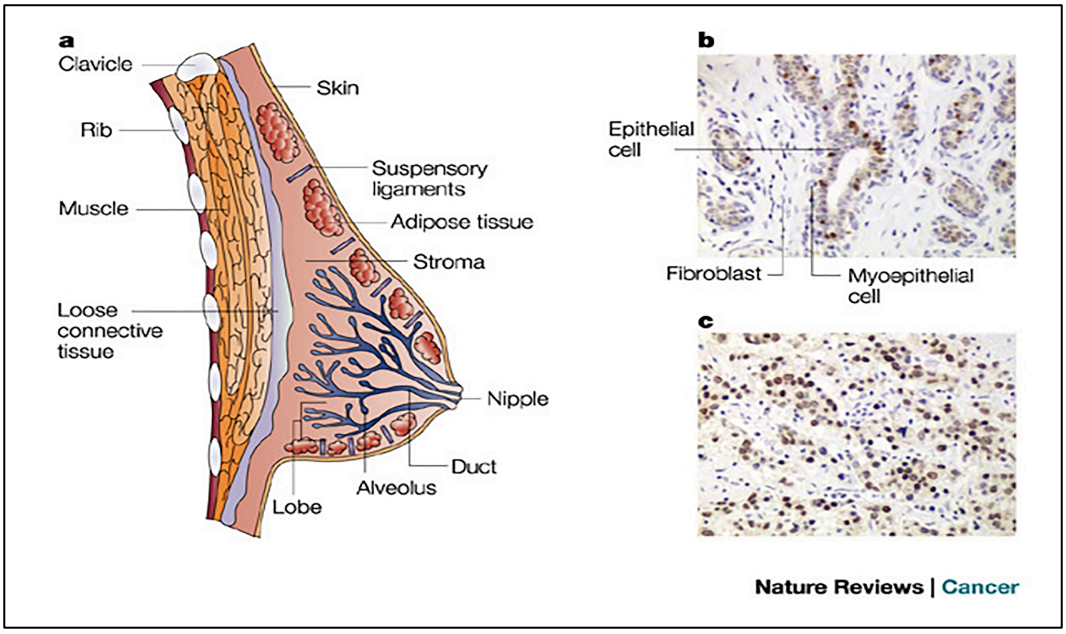


Fig. 1 a) Anatomy of the human mammary gland. b) Each duct is lined with a layer of epithelial cells and surrounded by an outer layer of myoepithelial cells. The glandular ducts are embedded in fibroblast stroma c) this structure breaks down in breast cancer, resulting in an epithelial cell mass. b) and c) are immunostained using antibodies to the estrogen receptor (ER; brown stained nucleus) showing that only a small portions of epithelial cells are ER^{+} in the normal breast. Courtesy: Ali et al; *Nature Reviews Cancer,* **2**:101-112. 2002

Breast cancer and its classification

Cancer that affects the breast tissue is named as breast cancer. Since it is a complex heterogeneous tissue therefore in the breast cancer classification, profiling is performed on the basis of various criteria. The major categories are based on the stage of the tumor, grade of the tumor, expression of specific hormone receptors, genes or proteins and on the basis of histopathology types. Most studies divide breast cancer into four major subtypes:

1. Luminal A – The cancer starts in the inner lining (luminal cells) of the mammary ducts. They tend to be positive for hormone receptors such as Estrogen-positive (ER^+), Progesterone-postive (PR^+) and herceptin receptor 2 negative ($Her2^-$). Of all the subtypes this has the best prognosis and highest survival rate.
2. Luminal B – They are usually ER^- and $Her2^{-/+}$. This subtype has comparatively lower survival rate depending on tumor size, tumor grade and number of lymph nodes.
3. Triple negative breast cancer – They are ER^-/PR^- and Her^- and considered as the most aggressive with poorest prognosis type of breast cancer. Most of the triple negative breast cancers are basal-like.
4. Her2 type – Most of this subtype are $Her2^+$ and some can be $Her2^-$ but they are ER-/PR-/Her2-. They correspond with poor tumor grade and lymph node positive.

Breast cancer stem cells

Neoplastic changes in cells are considered to be the result of a series of changes pertaining to disturburance in the cellular homeostasis and errors during cell cycle. A malignant tumor is composed of a heterogeneous population of mutant cells whose genotype and phenoytpe differs from the normal cell. But recent studies provides evidence that the tumors also consists of slow dividing cancer stem cells significantly controbuting towards the onset of tumorigenesis. Though they share similiarities with the normal stem cells but are not necessarily originated from stem cells. The main criteria to classify cells as cancer stem cells is based on its ability to;

1) form tumor in immunocompormised mice 2) self-renew by forming tumor in secondary mice and 3) differentiate into cells with non-stem cell characteistics.

Breast cancer is the first human carcinoma for which a putative cancer stem cell subpopulation has been isolated. Using cell surface markers, Al Hajj and colleagues isolated highly tumorigenic cell population expressing $CD44^{+}/CD24^{-/low}Lin^{-}$ from *in vitro*-separated tumorigenic cells from malignant human breast cancer derived pleural effusions. These cells had the ability to form tumors in immunocompromised NOD/SCID mice compared with $CD44^{+}/CD24^{+}Lin^{-}$. They shared the classical features of normal stem cells since it could be serially passaged (self renew) and formed heterogenous tumors (29). Another expression based CSC enrichment marker Aldehyde Dehydogenase (ALDH) is used to enrich breast CSCs population. The combination of measuring ALDH activity (ALDEFUOR assay) with the analysis of surface markers, $CD44^{high}/CD24^{-}$ is considered now as more refined methodology compared to either method alone. Daidone et al demonstrated the generation of non-adherant cells called mammospheres from single cell suspension obtained from primary breat tumors (2). These spheres showed positive enrichment of mammary cancer stem cells with majority of cells expressing $CD44^{high}/CD24^{-}$. Due to intra- and inter- tumor heterogeneity in breast cancer CSCs from different tumors have distinct expression profiles. Thus, isolation and chracterisation of CSC is cumbersome and requires a combination of various techniques to ensure isolation of pure CSCs (26,28).

Significance of breast cancer sem cells in cancer progression

Cancer stem cells are critical therapeutic targets due to its ability to support tumor aggressiveness. But breast cancer prognosis varies according to the subtype and

intriguingly this variation is also observed with CSC populations. For instance, in caludin-low, $Her2^{+}$ cancers, a gene set enrichment analysis showed that the proportion of cells expressing CSC markers was 3-4 folds times higher in undifferentiated tumors compared with well-differentiated tumors. Further, a comparative analysis for gene signature of surface marker, $CD44^{high}/CD24^{-}$between tumors cells and mammospheres confrimed that claudin-low tumors are enriched for CSCs and related with poor prognosis. Similiarly, Her2 expression has been linked to the expression of ALDH1 in human breast cancer with poor clinical outcome suggesting a direct link between CSC and tumor aggresiveness.

CSCs have been found to be associated with metstasis in recent studies. They play a pivotal role in dissemination and formation of metastatic lesions leading to cancer deaths. This is supported by various histopathological studies wherein a significant population of $CD44^{high}/CD24^{-}$ CSC populations was isolated from bone marrow lesions.

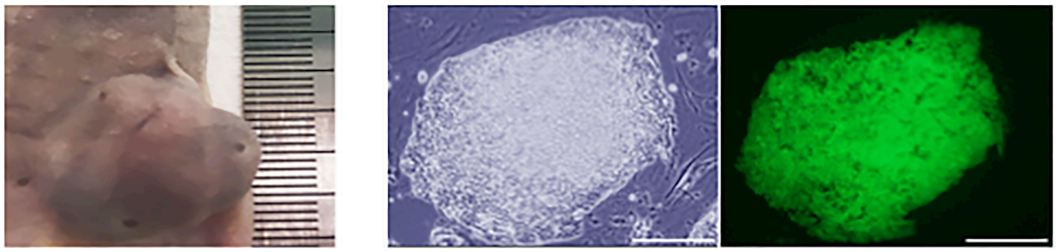
Breast cancer recurrence due to resistance to chemotherapy is another hurdle encountered in patients. CSCs are resistant to most of the known chemotherapeutics such as MCF-7 that is resistant to common drugs such as Adriamycin, 5-Fluorouracil, Methotrexate, cis-platinum. Interestingly, Gupta et al had demonstrated the enrichment of CSC-like cells on treatment of SUM159 and SUM149 with paclitaxel or 5-Fluorouracil respectively (30). Since the EMT mechanism and CSCs have a very close relationship it is often observed that cells undergoing EMT attain resistance by upregulating the expression of protein pumps associated with drug resistance. Furthermore, CSC helps in tumor maintenance since they differentiate to give rise to heterogeneous cell population and recapitulate malignant tumor. Thus, it is fairly

evident that breast cancer stem cells play an indispensible role in cancer disease progression and maintenance.

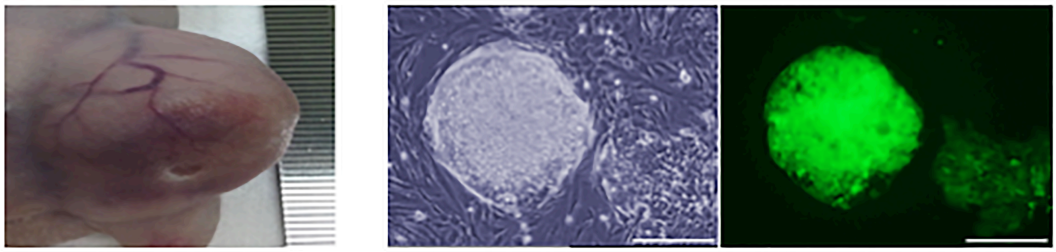
RESULTS

Orthotropic transplantation lead to enrichment of CSC-like cells

miPS cells were treated with conditioned medium derived from T47D and BT549 breast cancer cell lines following the similar protocol mentioned in Chapter 1. The primary cell line established from the subcutaneous tumors were injected into fourth mammary fat pad of female balb/c nude mice. Orthotropic tumors were observed within a short span of 25 days. Subsequent serial transplantation until tertiary level also generated tumors. The tumors were excised and harvested for primary cell culturing termed as T47DMaCSCcm and BT549MaCSCcm. Primary cells exhibited GFP^+ cancer stem cell like undifferentiated colonies surrounded by GFP^- cells (Fig. 3a). Metastatic nodes were observed on the lungs in at least 2/3 mice indicating metastasis. Lung tissue primary cultures also had GFP+ colonies but its number was less (Fig. 3b). This revealed that only a few cells acquired metastatic capability and migrated to the lung tissue forming metastatic nodes.



T47DMaCSCcm



BT549MaCSCcm

Fig. 3a Representative images of orthotropic tumor formed following mammary fat pad injection in nude mice and corresponding micrographs showing enrichment of GFP^+ CSC-like colonies in primary cultures. Scale bars represent 200µm.

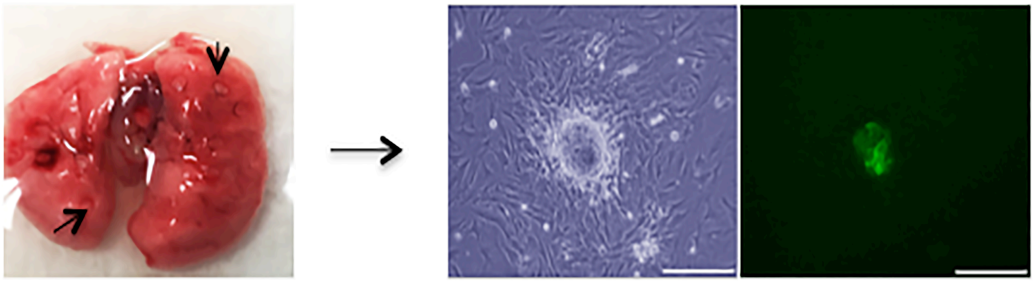


Fig. 3b Lung metastatic nodes observed in balb/c nude mice harboring orthotropic tumor formed by injecting T47D conditioned medium treated miPS cells and corresponding representative image of the primary lung culture showing CSC-like colony. Scale bar represents 200µm.

Characterization of primary cells revealed its correspondence with breast cancer stem-like cells

Quantitative gene expression analysis was performed for breast cancer stem cell markers: CD44, ALDHA1 and SOX2. Intriguingly, BT549MaCSCcm cell showed up regulation of CD44, ALDHA1 and SOX2 gene expression. Likewise, T47DMaCSCcm also revealed similar upregulation indicating positive enrichment of breast cancer stem cell population. On analyzing the expression level of Muc1, a breast adenocarcinoma marker, distinction was observed. BT549MaCSCcm cells had fairly significant expression of Muc1 but in T47DMaCSCcm it was undetectable (Fig. 3c). Furthermore, flow cytometer analysis confirmed enrichment of $CD44^{+}/CD24^{-/low}$ cell population (Fig. 3d).

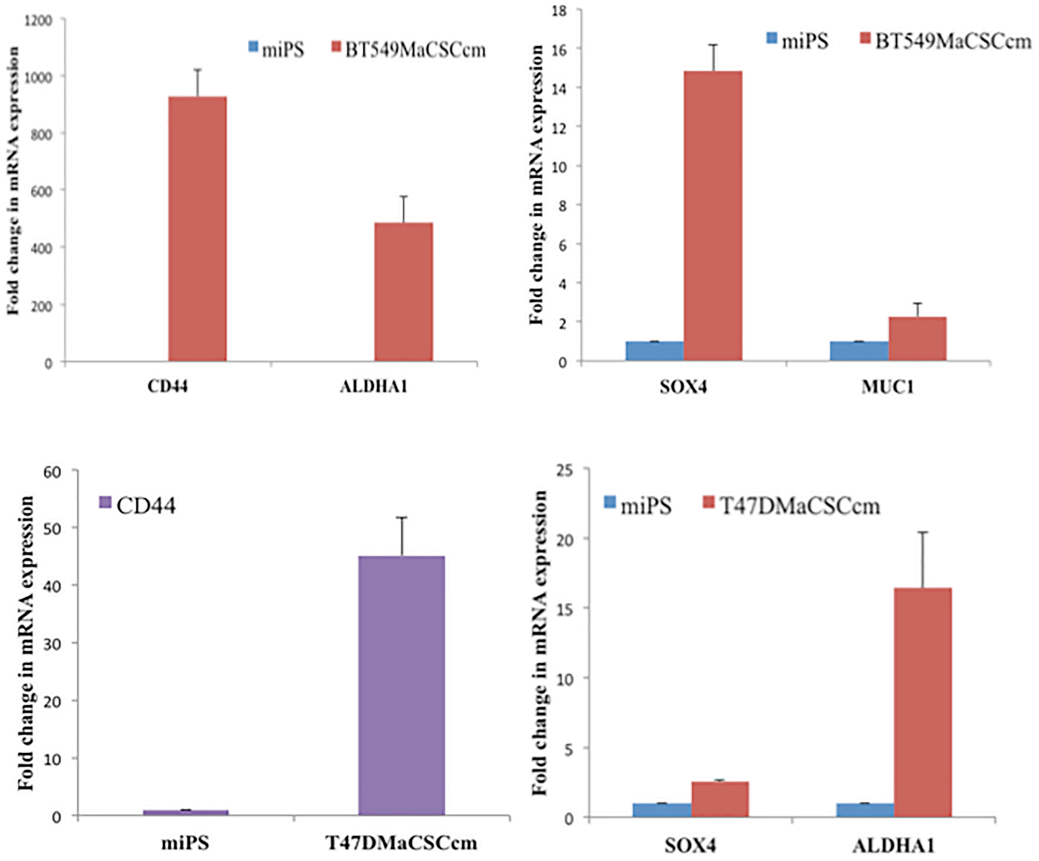


Fig. 3c qRT-PCR analysis showing enhanced expression of breast cancer stem cell markers, CD44 and ALDHA1 in primary cells cultured from tumors in comparison with miPS cells. Furthermore, the significant expression of MUC1, prognostic marker for metastatic progression and SOX4, master regulator of epithelial-mesenchymal transition is observed indicating a malignant breast cancer phenotype.

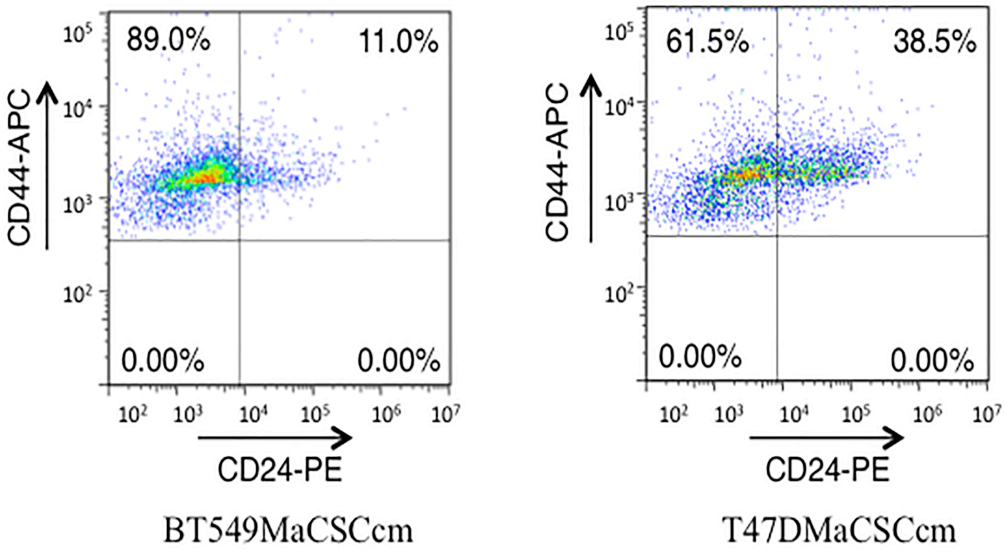
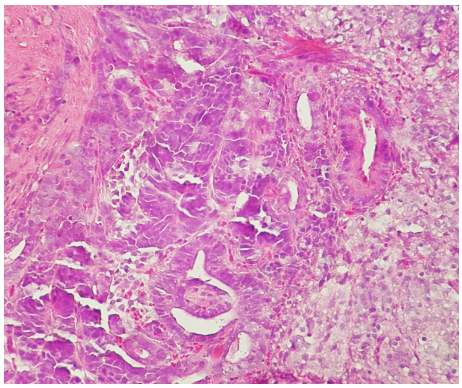


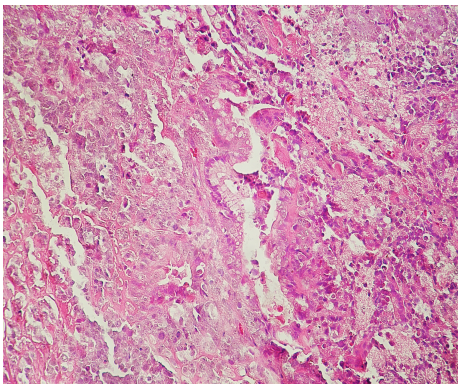
Fig. 3d Identification of breast cancer stem cell population expressing $CD24^{+}$ and $CD24^{-/low}$ in CD44 cells by flow cytometry. BT549MaCSCcm and T47DMaCSCcm primary cell lines shows enrichment of $CD44^{+}/CD24^{low}$ breast cancer stem population.

Immunohistochemical analysis of orthotropic tumor sections

The hematoxylin and eosin stained tumor sections confirmed the malignancy of the tumors characterized by high nuclear to cytoplasmic ratio and desmoplastic stroma (Fig. 3e). Her2/ ErbB2 receptor has recently reported being selectively expressed in breast cancer stem cell population in the triple negative subtype of human breast tumors. The tumor sections stained with Her2 receptor antibody revealed significant upregulation of this receptor in the ductal membranes (Fig. 3f). This indicated a enrichment of CSC population in vivo resulting in the generation of malignant tumors. Also, the respective tumor sections stained positive for Muc1, a common marker for identifying breast adenocarcinoma and epithelial tumors (Fig. 3g). Therefore, these results give preliminary evidence that these orthotropic tumors corresponded with breast tumors formed by CSC-like cells.

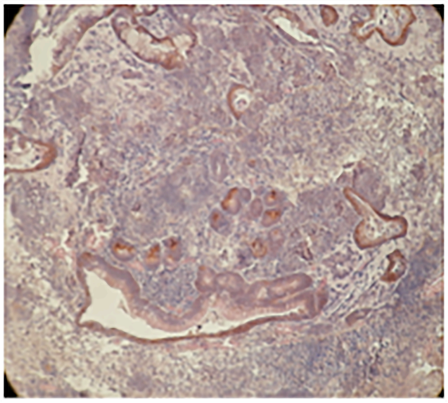


BT549MaCSCcm

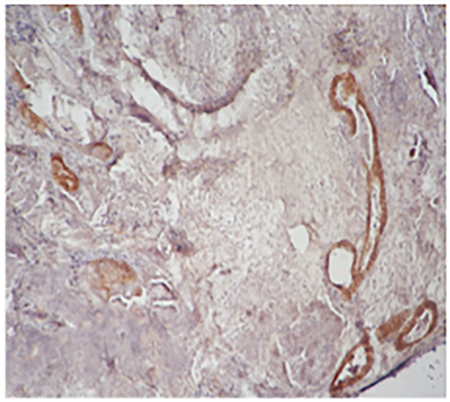


T47DMaCSCcm

Fig. 3e Representative images of Hematoxylin and Eosin (H&E) stained tissue section micrographs showing ductal structures in primary orthotropic tumors BT549MaCSCcm and T47DMaCSCcm.

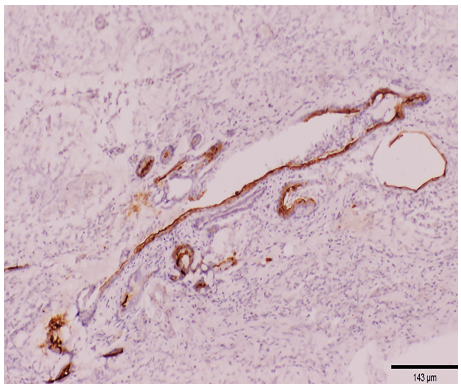


BT549MaCSCcm

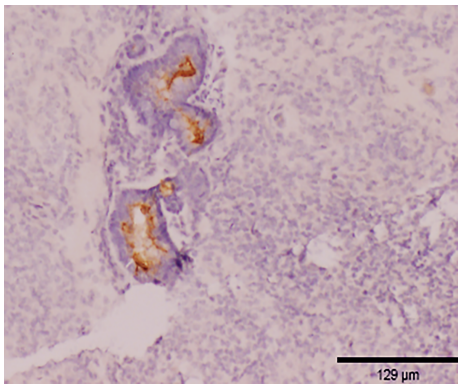


T47DMaCSCcm

Fig. 3f Immunographs of tissue sections stained with ErbB2/Her2 receptor demonstrated up regulation of Her2 receptor expression confirming enrichment of CSC-like cells.



BT549MaCSCcm



T47DMaCSCcm

Fig. 3g Immunograph of tissue section stained with Muc1 antibody confirming adenocarcinoma like phenotype of T47DMaCSCcm tumors.

DISCUSSION

Breast cancer is still one of the most pervasive diseases among women worldwide. Several therapeutic strategies have been devised but the problem of relapse and metastasis hinders its complete cure. The role of breast cancer stem cells is perceived as indispensable in the development and progression of this disease. The CSC niche maintains a heirarchy of heterogeneous cells, which facilitates the inception of the tumor until its invasion. Therefore, present study focuses on the development of an effective breast cancer stem cell model from mouse induced pluripotent stem cells (miPSCs). The miPSCs were treated with these conditioned media separately for a period of one month following the protocol previously discussed in chapter 1. The resulting survived cells were orthotopically transplanted into fourth gland of female Balb/c nude mice. Within a short span of 20-25 days, orthotopic tumors were developed. Therefore, these cells staisfied the key hallmark of tumorigencity usually exemplified by CSCs.

The primary cells cultured from excised tumors had numerous GFP+ colonies concomitant with the subcutaneous tumor cultures discussed in chapter 1. Intriuingly, the number and undifferentiated state of these colonies was observed to be more enhanced when compared with the subcutaneous tumors. This suggested that orthotropic microenvironment was more condusive in supporting the CSC-like population. This observation was further ascertained by quantitative gne expresssion analysis which showed high expression of breast CSC markers, CD44 and ALDHA1 respectively. SOX4, a master regulator of epithelial mesenchymel transition is considered to be accompanied by an enhanced number of cells with a $CD44^{+}/CD24^{-}$

phenotype and higher invasion and mobility of cancer cells *in vivo* and *in vitro*. Its expression was highly upregulated in both BT549MaCSCcm and T47DMaCSCcm primary cells respectively.

Furthermore, FACS analysis for CD44 and CD24 surface markers was performed to analyze the percentage of $CD24^{-/low}$ and $CD24^{+}$ in $CD44^{+}$ cells and the number of $CD44^{+}$ and $CD24^{-/low}$ expressing cells was observed to be upregulated. The combination of $CD44^{+}/CD24^{-/low}$ and/or ALDH1 is a widely used cancer stem cell marker in breast cancer. Therefore, orthotropic transplantation of conditioned medium treated miPS cells indeed lead to the generation and enrichment of breast CSC-like cells.

Muc1 is a gene encoding for mucin glycoproteins normally expressed in apical surface of mammary epithelial cells. But in many epithelial tumors particularly in breast adenocarcinomas its expression is upregulated on the cell surface. Its gene expression was observed being enhanced in BT549MaCSCcm but was undetectable in T47DMaCSCcm. Nevertheless, immunohistochemical analysis with antibody specific for Muc1 on T47DMaCSCcm tumor sections revealed its marked expression at protein level.

Recent studies have reported that CSC population selectively expresses Her2 receptor and this expression is regulated by the tumor microenvironment. Its blockade decreases the CSC population in breast cancer cell lines and mouse xenografts. Therefore, immunohistochemistry with Her2 receptor antibody was performed to analyze its expression in orthotropic tumor expression. Consistent with the previous reports BT549MaCSCcm and T47DMaCSCcm tumors displayed high

expression of Her2 receptor on the ductal membrane confirming marked enrichment of breast CSC-like cells by orthotropic transplantation.

The present study provides preliminary results related with the attempt to generate a mouse model that recapitulates breast cancer phenotype initiated by CSC-like cells. This model should contribute in the better understanding of the role of CSCs in development and progression of breast cancer.

MATERIALS AND METHODS

Cell culture

Two human breast cancer cell lines, T47D (ATCC HTB-133) and BT549 (ATCC HTB-122) were revived and cultured in RPMI-1640 (Sigma) medium containing 10% fetal bovine serum (FBS; Gibco, NY) and 100 U/ml penicillin/streptomycin (P/S; Wako, Japan). The conditioned medium (CM) was collected according to the previously established protocol by Chen et al. The cell lines were allowed to grow until it becomes 80% confluent in the same medium and then fresh medium containing 5% fetal bovine serum was added. The CM was collected and filtered using 0.22μm filter (Millipore, Ireland) and stored at -20°C until the time of use. miPS (iPS MEF-Ng-20D-17) were purchased from Riken Cell Bank, Japan and maintained in DMEM (D5796, Sigma) containing 15% FBS, 0.1 mM MEM non-essential amino acids (NEAA; Gibco, NY), 2 mM L-glutamine (Nacalai Tesque, Japan), 50 U/ml P/S, 0.1 mM 2-mercaptoethanol (Millipore, MA) and 1000 U/ml of Leukemia Inhibitory Factor (LIF; Millipore, MA) on feeder layer of mitomycin-treated mouse embryonic fibroblast (MEF) cells (Reprocell, Japan). The miPS cells express GFP controlled under Nanog gene promoter. For conditioned medium treatment, the miPS cells were cultured without LIF and MEF feeder cells in the presence of CM and miPS medium described above in 1:1 ratio with medium changed after every 24 hours for four weeks. The primary cancer stem cell line derived from mouse allografts was maintained in the same medium. For CM used for sphere differentiation, the primary cancer stem cells were treated with miPS medium, 10% FBS and P/S (50 U/ml) for until 80% cell confluence than the medium was changed to DMEM containing 5% knockout serum replacement (KSR) (Gibco, NY), 0.1mM

NEAA, 2 mM glutamine, 50 U/ml P/S which was collected after the cell confluence reached 100%. The CM was centrifuged at 1000 rpm for 5 minutes and filtered using 0.22 μm diameter pore filter (Millipore, Ireland) and stored at -20°C until the time of use.

Sphere formation and differentiation

5×10^4 cells were seeded on 6-cm ultra-low attachment dishes (Corning incorporated, NY) with the DMEM medium without FBS but with Insulin-transferrin-selenium-X (ITS-X; life technologies, CA), NEAA (0.1 mM), L-glutamine (2 mM) and P/S. After 3 – 4 days, the sphere-forming cells were collected by centrifuging at 300 rpm for 5 minutes and suspended in 1×phosphate buffer saline (PBS). After aspirating PBS, the spheres are dissociated by accutase (Sigma, USA) treatment and seeded at the density of 10^4 cells/ml in ultra-low attachment dishes with the above-described medium. After 4 days, spheres around 100 μm diameter were seeded in 0.1% gelatin (Sigma)-coated 6cm adherent dishes in presence of DMEM containing 5% KSR, P/S and conditioned medium collected from primary CSC cell line as described above in 1:1 ratio. Medium was changed every other day and the differentiated cells were dissociated and maintained for no longer than 2-3 passage.

Separation of myofibroblast-like cells

The myofibroblast-like cells differentiating cells from cancer stem cell populations were separated using feeder removal microbeads, mouse, LS column and Midi MACS separator (MACS, Miltenyi Biotec, Singapore). After separation, the cells were maintained in the same medium containing KSR up to five passages.

Animal experiments

a) Subcutaneous transplantation: Four-weeks-old female nude mice Balb/c-nu/nu (Charlesriver, Japan) were subcutaneously transplanted with 10^6 cells suspended in 200 µl of HBSS (Hanks Balanced salt solution, Gibco, NY).
b) Orthotropic transplantation: Five to six-weeks-old female nude mice Balb/c-nu/nu were injected unilaterally $5x10^5$ cells in 20 µl of HBSS in to the fourth abdominal mammary fat pad by subcutaneous injection at the base of the nipple.

Tumor growth was monitored using vernier caliper for one month and were harvested when they reached ~1000 mm^3. For assessing tumorigenic ability of tumorspheres, spheres from primary cancer stem cell lines were collected and resuspended in 200 µl of matrigel matrix (Corning Incorporated, NY) and injected subcutaneously into four weeks old nude mice. The plan of animal experiments was reviewed and approved by the Animal Care and Use Committee Okayama University under ID OKU2013252, OKU2014157, OKU2014429 and OKU2016078. All experiments were performed according to the Policy on the Care and Use of the Laboratory Animals, Okayama University.

Histological analysis

Tumors were excised from mice after four weeks, fixed in 4% paraformaldehye (Wako, Japan) and paraffin embedded. The paraffin tumor blocks were sectioned at 5µm thickness. Following deparaffinization, sections were stained with 0.5% Hematoxylin (Sigma Aldrich, USA) and Eosin Y (Sigma Aldrich, USA) for histological analysis.

Immunohistochemistry

Deparaffinization of the tumor sections was done by incubating in hot air oven at 60ºC for 30min and rehydrated using serial treatment with ethanol and xylene. Antigen retrieval was performed by treating the sections with 10mM citrate buffer (pH6.5) in a water bath at 100ºC for 30min. For quenching endogenous peroxidase activity, sections were blocked with 3% hydrogen peroxide (H_2O_2). Further, slides were blocked with PBS + 1.5% normal serum, incubated with primary antibodies diluted in 2.5% normal serum overnight at 4ºC for rabbit monoclonal Her2/ErbB2 (1:100, #CST 2165, Cell Signaling, MA), rabbit monoclonal GFP (1:200, #2956, Cell Signaling, MA), rabbit monoclonal MUC1 (1:100, #ab15481, Abcam, UK). Following incubation, the sections were washed with PBS and incubated with appropriate secondary antibody. Detection of the antibody was accomplished using Elite anti-rabbit ABC staining Vectastain kit (Vector, MI) and 3,30-diaminobenzidine tetrahydrochloride (DAB, Vector, MI) by following manufacturer's protocol. Finally, sections were counterstained with hematoxylin. Slides serving as negative controls were incubated with secondary antibody only.

Immunofluorescent analysis

10^5 cells/ml were seeded on 0.1% gelatin coated coverslips in 24-well culture plates. The cells were fixed with 4% paraformaldehye for 20 minutes at room temperature followed by permeabilisation with 0.2% Triton-X (Nacalai Tesque, Japan) in PBS. After 5 minutes, cells were washed and blocked with blocking buffer (PBS containing 5% BSA and FBS) for 1 hour. The cells were incubated with primary antibodies; (a) anti-FSP-1 (anti-rabbit, 1:300; MerckMillipore, Ireland) (b) anti-α-SMA (anti-rabbit,

1:200; Abcam) (c) anti-FAP alpha (anti-rabbit, 1:200; Abcam) for overnight at 4°C. Following washes with PBS, cells were incubated with Alexa Fluor 555 donkey anti-rabbit (1:300, Invitrogen, USA) for one hour. Nuclei were counterstained with 4', 6-diamino-3-phenylidole, dihydrochloride (Sigma, USA) and mounted on glass sides with Vectashield mounting medium (Vector Labs, USA). Images were acquired using Olympus microscope equipped with light fluorescence device (Olympus, Japan).

RNA extraction and Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from cells using RNAeasy Mini kit (QIAGEN, Germany) and treated with DNase I (Invitrogen, USA). 1 μg of RNA was reverse transcribed using Superscript First strand kit (Invitrogen, CA). Quantitative 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 Supplementary Table 1.

Cell Invasion assay

Matrigel Coated Transwell evaluated the invasion ability of the cells and Transwell inserts (Corning Matrigel Invasion chamber). 5×10^4 cells in 500 μl DMEM medium were added to upper chamber and medium containing 15% FBS was added into lower chamber. The cells were allowed to invade for 72 hours. The non-invading cells were removed by wiping and the invaded cells were first fixed with 4% paraformaldehye for 5 minutes followed by 20 minutes in methanol (Wako, Japan) and finally stained with Azure EMB geimsa (Merck, Ireland). The number of cells invaded was visualized under light microscope at 10x.

Combined Fluorescence *In Situ* Hybridization and Immunofluorescence

Combined FISH and immunofluorescence were performed according to the manufacture's protocol. Stellaris FISH probe, GFP labeled with Quasar 570 dye (VSMF-1018-5 Biosearch Technologies, Inc., Petaluma, CA) was used for FISH. Anti-FSP1 (1:300; Merck Millipore, Ireland) antibodies were used for immunofluorescence.

Flow cytometry

To isolate $CD44^{+}/CD24^{low}$ cells, primary cells derived from 3 independent tumor samples were separately stained with monoclonal anti-CD44 (Miltenyi Biotec), monoclonal anti-CD24 (CD24-PE: Miltenyi Biotec), and isotype controls (Rat IgG2b-APC and Rat IgG2b-PE) (Miltenyi Biotec) for 30 minutes on ice. The stained cells were analyzed using an Accuri™ flow cytometer (BD Biosciences) and FlowJo Software (Treestar Inc., San Carlos, CA).

Supplementary Table 1

List of mouse qPCR primers used in this study.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
CD133	CCTTGTGGTTCTTACGTTTGTTG	GTTGACGACATTCTCAAGCTG
EpCam	CTGGCGTCTAAATGCTTGGC	CCTTGTCGGTTCTTCGGACTC
Oct3/4	TCTTTCCACCAGGCCCCCGGCTC	TGCGGGCGGACATGGGGAGATCC
Sox2	TAGAGCTAGACTCCGGGCGATGA	TTGCCTTAAACAAGACCACGAAA
Tg Klf4	GCGAACTCACACAGGCGAGAAACC	TATCGTCGACCACTGTGCTGCTG
Tg c-Myc	CAGAGGAGGAACGAGCTGAAGCGC	TTATCGTCGACCACTGTGCTGCTG
FSP1	CAGGCAAAGAGGGTGACAAG	TGCAGGACAGGAAGACACAG
αSMA	GGAGAAGCCCAGCCAGTCGC	AGCCGGCCTTACAGAGCCCA
Vimentin	CGGCTGCGAGAGAAATTGC	CCACTTTCCGTTCAAGGTCAAG
Col1α1	GTCCTAGTCGATGGCTGCTC	CAATGTCCAGAGGTGCAATG
PDGFRα	ATGAGAGTGAGATCGAAGGCA	CGGCAAGGTATGATGGCAGAG
TGFβ1	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGAA
CXCL12	GAGCCAACGTCAAGCATCTG	CGGGTCAATGCACACTTGTC

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