The effect of B27 supplement on promoting *in vitro* propagation of Her2/neu-transformed mammary tumorspheres

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A variety of human solid tumors, including breast cancer, are considered to embrace a hierarchical organization in which only rare tumor-initiating cancer stem cells are truly responsible for tumor formation. Cultivation of tumorspheres in non-adherent conditions is widely employed for enriching these putative cancer stem cells *in vitro*. However, the absence of a defined culture medium has handicapped further characterization and isolation of this cell population. In this study, we used mouse mammary tumor virus MMTV-HER2/neu transgenic mice that mimic the HER2/Neu-positive subtype of human breast cancer as a model system, and cultured primary tumor cells and tumorspheres from these mice under non-adherent conditions. In addition to essential growth factors, we found that B27 supplement played an important role in promoting tumorsphere formation and maintaining cultures through passaging. A tumorsphere-formation assay and measurements of average tumorsphere size provided insight into the characteristics of the putative breast cancer stem cells. Tumorspheres were enriched for cancer stem cells through serial passaging, while sphere size was determined by the innate properties of cancer stem cells and independent of the culture condition. Our study identifies B27 as an essential component of culture medium necessary for sustained propagation and enrichment of breast cancer stem cells *in vitro*.

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Introduction

Solid tumors account for an enormous cancer burden on society and represent an urgent therapeutic challenge. Epithelial cancers that derive from tissues including breast, lung, colon, prostate, pancreas, brain and ovary constitute approximately 80% of all cancers [1]. Breast cancer is the predominant malignancy in women, resulting in more than 40,000 fatalities annually in the United States alone and 10 times that number worldwide [2, 3]. Breast cancer arises from breast tissue, most commonly from inner lining of lactiferous ducts (ductal carcinoma) or the lactating lobules (lobular carcinoma) [4]. Different subtypes of breast cancer have been identified, and survival rates vary greatly depending on factors such as genetic alterations, stage, metastastic ability and therapeutic resistance [5].

Approximately 25~30% of human breast cancers have an amplification of the *Her2/neu* gene. The *Her2/neu* proto-oncogene encodes a transmembrane tyrosine kinase receptor that is a member of the epidermal growth factor receptor (EGFR) family [5-7]. This receptor is normally activated by growth factors that cause cells proliferation and division, and switched "off" in the absence of such growth factors. Overproduction of Her2/neu protein, which acts a key activator of proliferative signaling networks, leads to uncontrolled cell proliferation associated with an aggressive form

of breast cancer [8]. Her2/neu-positive breast cancer tends to be particularly invasive and is often associated with tumor relapse, enhanced metastasis, induced chemotherapy resistance, poorer prognosis and reduced overall survival rates [5, 9]. Her2/neu-positive breast cancer is also resistant to hormonal therapy [8]. Monoclonal antibodies such as trastuzumab (Herceptin) have been used to target Her2/neu, usually in combination with chemotherapy, to improve survival rate for this patient population [7, 9]. However, this therapy is expensive and not tolerated among all patients. This demands prospective insights into the underlying mechanisms involved in Her2/neu-positive breast cancer, which will contribute to the development of improved therapies to eradicate this malignancy.

To accomplish this goal, it is necessary to address the question of the cellular origins of solid tumors. Two major models have emerged over decades of cancer research. The stochastic model predicts that transformation is a result of random mutation and subsequent clonal selection [10]. In this model, any cell can accumulate neoplastic mutations and transform into a cancer cell, and any cancer cell has a similar potential for unlimited proliferation and tumor generation [10-12]. In contrast, the cancer stem cell theory views tumor as an aberrant organ, to which the principles of stem cell biology can be applied. In a similar way to normal tissues that are derived from somatic stem cells, the tumor is organized in a hierarchy that is initiated by tumorigenic cancer cells, or termed as cancer stem cells. The cancer stem cells acquire the capacity for extensive proliferation through accumulated mutations, and have the ability to initiate a tumor in immune-compromised mice that recapitulates the original heterogeneity of the patient tumor [10, 13]. A few additional properties are also shared by normal stem cells and cancer stem cells, which include the ability to migrate, the activation of membrane transporter activity, and resistance to apoptosis and chemotherapy [13]. The growth of cancer stem cells requires alterations in vital metabolic and signaling pathways and cell cycle progression that lead to the emergence of tumorigenic phenotypes [3, 14, 15]. As suggested in this theory, normal stem cells are targets of mutations that convert normal self-renewal potential into neoplastic engines, and only these transformed stem cells are capable of launching tumor formation, and comprise a tiny fraction of the vast tumor mass [11, 15-18].

There is growing evidence revealing the existence of cancer stem cells in a variety of human cancers, including leukemia [19] and several solid tumors such as breast [2, 20], brain [21, 22], melanoma [23], prostate [24], colon [25, 26], pancreatic [27] and lung cancer [28]. A number of methods have been developed for identifying putative cancer stem cells. As far as is known, cancer stem cells resemble normal stem cells in their ability to grow through self-renewal as non-adherent spherical clusters, known as tumorspheres, when an adherent substratum is not provided in the culture system [21, 29]. Therefore, the most widely used method is to grow primary tumor cells in serum-free non-adherent conditions, in which only the small fraction of tumor-initiating stem/progenitor cells within the tumor mass can survive and form tumorspheres in suspension [13, 20, 29]. In many cases, the tumorsphere can only survive for a few passages and gradually dies [26, 29]. In this study, we cultured tumorspheres from MMTV-Her2/Neu transgenic mouse mammary tumors and found that addition of one supplement,

B27, to the culture medium, can dramatically increase sphere-forming efficiency and sustain the propagation of tumorspheres for more than 20 passages. This finding will help to relieve the limitation of expanding cancer stem cells *in vitro*.

Materials and Methods

Isolation of cancer cells from MMTV-Neu transgenic mouse mammary tumors

Spontaneous mammary tumors that developed from MMTV-Neu transgenic mice were harvested, physically separated from connective and fat tissues, and transferred into DMEM/F12 medium (Gibco). Tumor tissues were minced into small pieces with a sterilized scalpel, and digested in DMEM/F12 medium with 1.5 mg/ml collagenase (Worthington) and 20 μg/ml hyaluronidase (MP Biomedicals) with agitation at 200 rpm for 2 hours at 37℃. The pellet was collected by centrifugation at 1200 rpm for 5 min, and incubated with 0.64% NH_4Cl (Stem Cell Technologies) for 3 min to lyse red blood cells. The suspension was centrifuged at 1200 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in DMEM/F12 medium and passed through a 40 μm strainer (PALL Corporation) to collect single cells, which were in turn transferred and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal-bovine serum (FBS, SAFC Biosciences) and 10 μg/ml insulin (Sigma) as primary tumor cells, which were named as parental cells.

In vitro propagation of tumor-initiating mammospheres

Cells harvested from spontaneous tumors were counted and then plated in petri dishes at a constant density of 40,000 viable cells per ml. Cells were grown in serum-free DMEM/F12 medium (Gibco), which was supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), 0.4% bovine serum albumin (BSA, Sigma), and 5 μg/ml insulin (Sigma). 1×B27 supplement (Gibco) was added to the treatment group to investigate its effect on tumorsphere formation. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 6 days, and collected by gentle centrifugation. The pelleted cells were enzymatically dissociated with accutase (Innovative Cell Technologies) for 10 minutes at room temperature, and mechanically dispersed by gently pipetting through a 23-gauge sterile needle. Single-cell suspensions were plated at the same density and culture conditions as described above, to generate the second generation of tumorspheres, and so forth.

RNA purification and PCR amplification

Total RNA was extracted from parental cells and tumorspheres using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 1 μg of total RNA was used for the reverse transcription assay to generate cDNA using M-MLV reverse transcriptase (Invitrogen). 1 μl of cDNA was used for a single PCR reaction to determine the expression levels of cell differentiation markers and stem cell markers. The following genes of interest were selected and tested by regular PCR: Klf4, Oct4, Sox2, Nanog, keratin 8, keratin 14, and keratin 18.

Quantitative real-time PCR analysis

The quantitative RT-PCR was performed using the fluorescent dye SYBR Green Master Mix (Qiagen, CA) following standard protocols on an ABI PRISM 7300 sequence detection system (Applied Biosystems, CA). The data were first analyzed using the Sequence Detector Software SDS 2.0 (Applied Biosystems). Results were

calculated and normalized relative to the GAPDH control by using the Microsoft Excel program. The relative expression values were calculated relative to GAPDH by using the 2-ΔCT method [40]. The data shown here represent the average of three independent experiments.

Tumorsphere-forming efficiency assay and size characterization

Tumorspheres were dissociated to single cells as described above, and plated in ultra-low attachment 96-well plate (Costar). 1000, 500, 200, 100 and 50 cells were plated in 100µl of serum-free medium per well, respectively in triplicate. The number of spheres formed in each well was determined after 6 days. Tumorspheres from every passage were dissociated and assayed for tumorsphere-forming efficiency. Meanwhile, tumorsphere size, defined as number of cells per sphere, was assessed for tumorspheres at every passage.

Results

Generation of mammary tumorspheres in non-adherent conditions

Spontaneous tumors from MMTV-Neu transgenic mice were dissociated to single cells and transferred into tissue culture flasks in DMEM medium with serum for cultivating primary tumor cells. After subculturing the cells for a few passages with enzymatic treatment to simultaneously remove fibroblasts and other non-tumor cells, primary tumor cells (named in this study as parental cells), formed an adherent monolayer with uniform morphology covering the bottom of a tissue culture flask (Fig. 1A). Tumor-initiating cells, as far as is known, resemble stem cells in their ability to grow through self-renewal as non-adherent spherical clusters, known as tumorspheres, with a non-adherent substratum [21, 29]. To test the ability of MMTV-Neu tumor cells to form tumorspheres, parental cells were collected and plated in serum-free DMEM/F12 medium that contained EGF, bFGF, BSA, insulin and B27 supplement. After six days of cultutre, the majority of the cells died, however a small fraction of cells survived and formed spherical clusters in suspension (tumorspheres) (Fig. 1B), which implied the existence of putative breast cancer stem cells that possessed stem cell-like self-renewal properties.

Candidate stem cell marker genes were overexpressed in tumorspheres

We examined the expression levels of a few known molecular markers of cancer stem cells in tumorspheres in relative to parental cells. This showed that putative stem cell markers, such as Oct4, Sox2, Klf4, Stem Cell Antigen-1 (Sca1) and Aldehyde dehydrogenase 1 (Aldh1) were overexpressed in tumorspheres, and differentiation markers for luminal epithelial cells like keratin 18 and myoepithelial cells like smooth muscle actin (SMA) were underexpressed in tumorspheres, compared with parental cells (Fig. 2). The expression profile of these marker genes suggests the presence of cancer stem cell population within the tumorspheres at a molecular level. Note that cell differentiation markers were not always underexpressed in tumorspheres relative to parental cells. The reason for this possibly lies in the presence of cancer stem cell-derived progeny within the tumorspheres that entered and stayed at various stages of differentiation. Stem/progenitor cells only constitute a tiny fraction of the heterogeneous cellular composition of tumorspheres. Hence the majority of cells at more differentiated stages was sufficient to give a high expression level of cell differentiation markers which almost equaled that of parental cells.

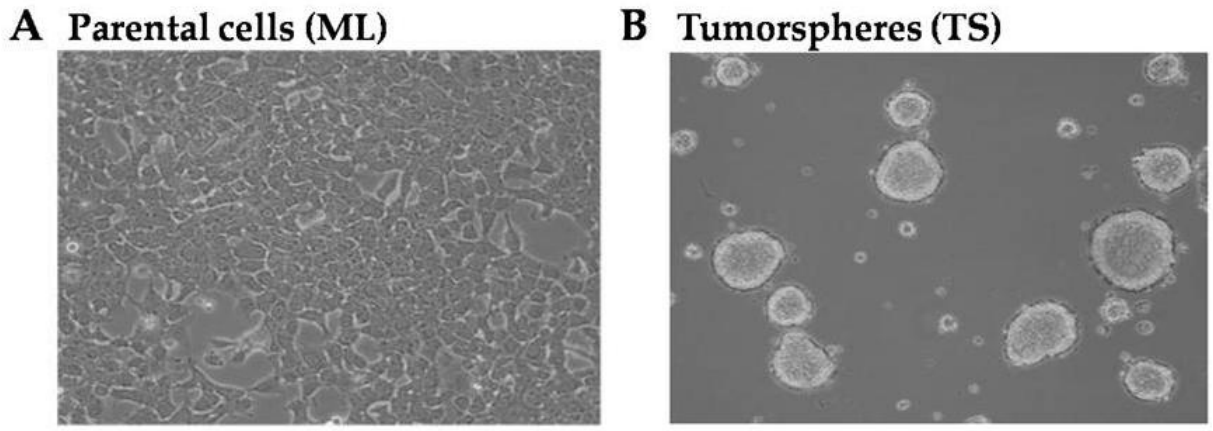

Figure 1. Culture of tumor cells isolated from spontaneous tumor tissues of MMTV-Her2/neu transgenic mice at different conditions. (A) Primary tumor cells were cultured in DMEM containing 10% FBS, exhibiting an epithelial-like morphology. **(B)** Mammary tumorspheres were grown in non-adherent suspension medium, presenting smooth-edged spherical phenotypes. Original magnification in A and B: ×100.

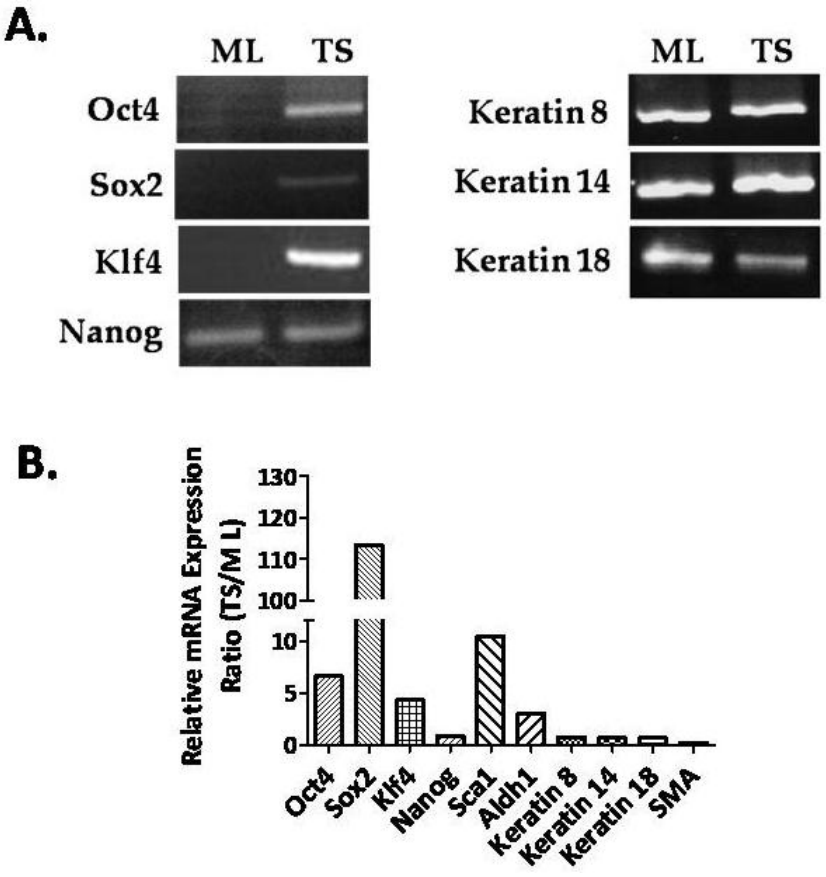

Figure 2. Gene profiling of stem cell markers and cell differentiation markers in monolayer (ML) cells and tumorspheres (TS). **(A)** RT-PCR analysis was performed to obtain the gene expression profiles in parental cells and tumorspheres. **(B)** Real-time PCR was performed to evaluate the relative expression levels of stem cell and differentiation marker genes in parental cells and tumorspheres. Putative stem cell markers, Oct4, Sox2, Klf4, Nanog, Stem Cell Antigen-1 (Sca1) and Aldehyde dehydrogenase 1 (Aldh1) were overexpressed in tumorspheres (TS), and cell differentiation markers, Keratin 8, Keratin 14, Keratin 18 and smooth muscle actin (SMA), were overexpressed in parental cells (ML).

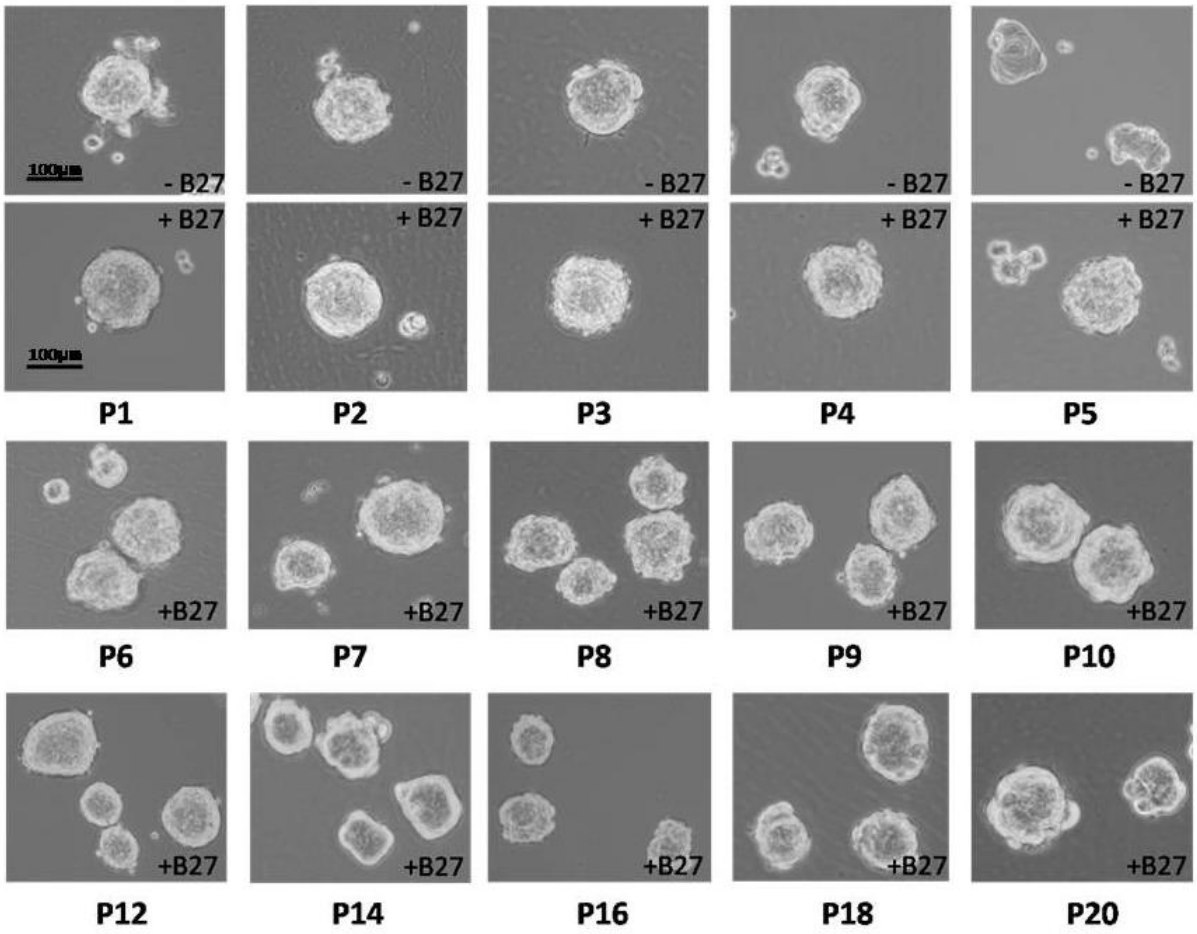

Figure 3. Optical microscopy for tumorspheres from serial cultivation in the suspension medium with or without the presence of B27 supplement. Morphological differences were visualized between tumorspheres maintained in two types of medium. As for +B27 medium, no significant morphological change was discerned over serial passaging. Original magnification: ×100.

B27 supplement is crucial for serially maintaining tumorspheres in vitro

Initially, we chose the simplest recipe of tumorsphere propagation medium containing only EGF and bFGF as essential growth factors and BSA and insulin as supplemental components [29, 30]. Although tumorspheres grew successfully in this medium for the first few passages, serial subcultures of tumorspheres eventually failed. Bubble-like hollow aggregates replaced normal tumorspheres after four or five passages, and repeated tests reproduced the same phenomenon. By comparing a large number of potential nutrients, we assumed that the addition of B27 supplement was essential and could replenish what was required for serial generation of stem cells (self-renewal) but missing in the previous medium [5, 20, 31-35]. Accordingly, tumorspheres were successfully maintained for more than 20 passages in B27-containing medium. In addition, the morphology of tumorspheres cultured in B27-free medium seemed to lose smoothness and gain roughness because of adherence of cells to the spherical edges during serial passaging (Fig. 3). The strong contrast between the two culture conditions implied that B27 is playing an important role in maintaining and promoting tumorsphere propagation *in vitro*. To test

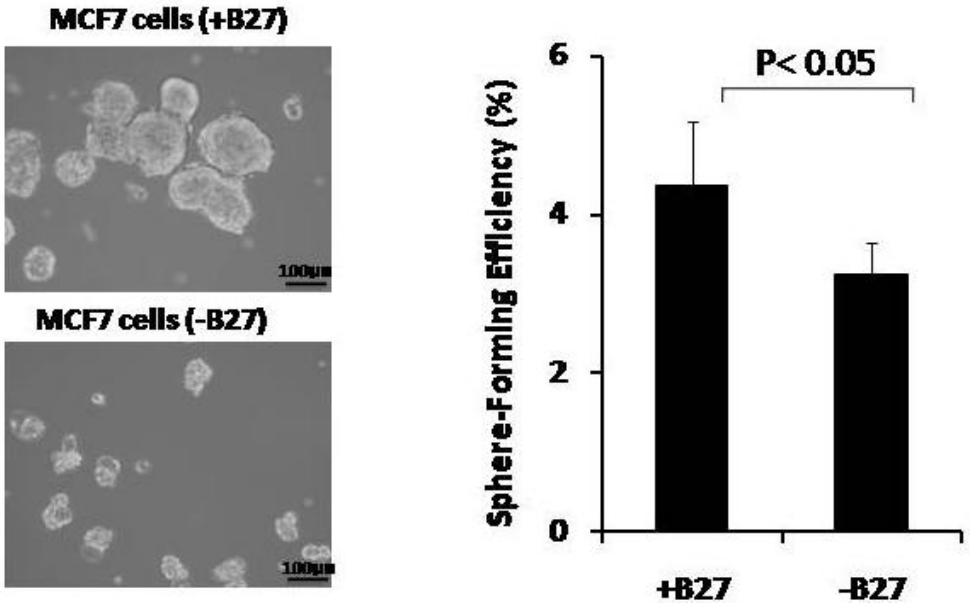

Figure 4. Tumorsphere culture of MCF7 breast cancer cells in the suspension medium with or without the presence of B27 supplement. (**A**) Morphological differences were visualized between tumorspheres maintained in two types of medium. As for +B27 medium, no significant morphological change was discerned over serial passaging. Original magnification: ×100. Scale bars represent 100μm. (**B**) Tumorsphere-forming efficiency assay. One hundred cells were seeded into ultra-low attachment 96-well plate. The sphere numbers are counted after 6 days. The Y axis shows the sphere-forming efficiency which represents the average number of tumorspheres per one hundred seeded cells.

whether that B27 has the similar effects on sphere formation of other cells, we cultured another human breast cancer cell line MCF7 in the presence and absence of B27 supplement. As expected, MCF7 cells in presence of B27 formed typical tumorsphere with higher efficiency (5.375% +B27 medium *vs.* 3.25% -B27 medium). In contrast, cells cultured in the B27-free medium formed atypical tumorspheres which were more like cell aggregates (Fig. 4).

Cancer stem cells were enriched through serial propagation

In order to passage tumorspheres, they were enzymatically and mechanically dissociated into a single cell suspension, and replated at a constant density of 40,000 viable cells per ml. Tumorsphere-forming efficiency was evaluated using an ultra-low attachment 96-well plate as described in materials and methods, and performed with tumorspheres from every passage in the presence and absence of B27 supplement. In this assay, tumorsphere-forming efficiency reflected the percentage of cells that were capable of forming tumorspheres, in other words, the proportion of cancer stem cells. This experiment clearly showed that the efficiency of tumorsphere-formation gradually increased in the presence of B27 (Fig. 5). This suggested that over time, an increasing number of cells had obtained the ability to self-renew and form tumorspheres, reflecting an enrichment of cancer stem cells. However, for tumorspheres maintained in B27-free medium, tumorsphere-forming efficiency rose slightly for the first three passages, resembling the trend observed in B27-containing medium, but dropped at the fourth generation when bubble-like aggregates began to replace typical spheres (Fig. 5), until tumorspheres were no longer observed after the fifth generation. This result supports our earlier observations that B27-free medium failed to maintain tumorsphere formation beyond four or five passages. Hence, B27 was

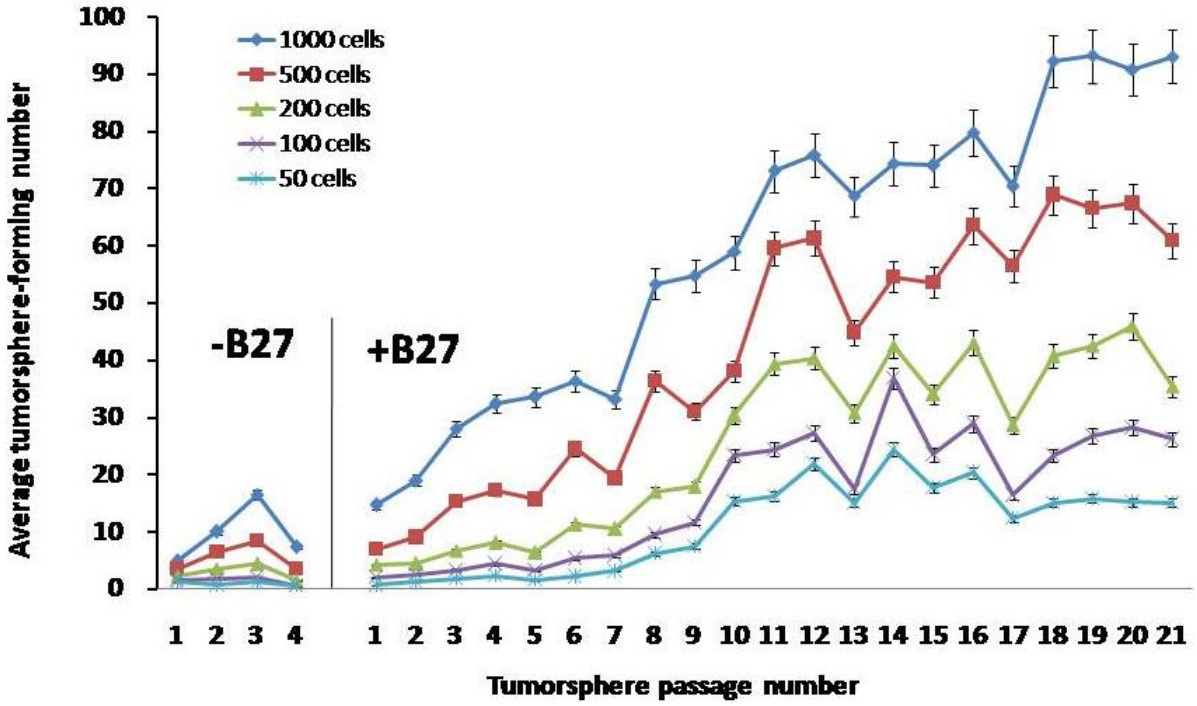

Figure 5. Tumorsphere-forming efficiency assay. Cells were seeded into ultra-low attachment 96-well plate. Five different cell densities were tested for +B27 and –B27 medium, respectively. Indicated numbers of the perpendicular axis showed average number of tumorspheres per well for a given starting cell density.

essential for promoting tumorsphere-forming efficiency *in vitro* as well as maintaining serial propagation through which cancer stem cells were enriched.

Tumorsphere size was dependent on the innate properties of cancer stem cells

Our data showed that B27-containing medium supported the serial culture of tumorspheres. We then grew tumorspheres for multiple passages under these conditions and measured the size of individual tumorspheres, by counting the number of cells per tumorsphere. Surprisingly, the average tumorsphere size did not exhibit a dramatic difference over serial passaging (Fig. 6). We therefore concluded that tumorsphere phenotypes such as morphology and size were determined by the innate properties of the cancer stem cells, rather than the number of cancer stem cells.

Discussions

The cancer stem cell hypothesis was proposed to resolve the question of cellular origins of malignancy, and to explain the observation that only a small proportion of cells within a tumor were tumorigenic [1, 10, 16]. Cancer stem cells, representing only a small fraction of cells within the bulk of tumor, have properties that render them capable of initiating a tumor, in contrast to the non-tumorigenic majority of cells [10, 15, 18]. Although cancer stem cells have not yet been isolated and fully characterized, there is a body of growing evidence revealing the existence of this population in a variety of human cancers [19-28]. The investigation and characterization of cancer stem cells has important implications for understanding cancer biology and in the development of efficient therapies [13, 20].

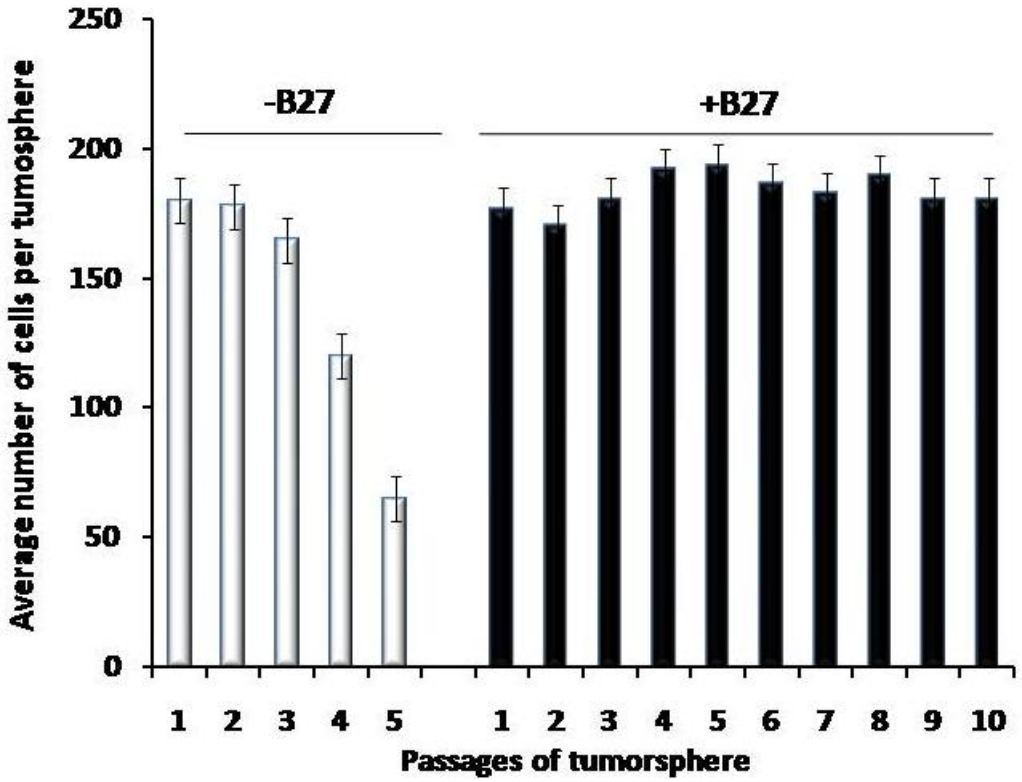

Figure 6. Characterization of average tumorsphere size. The tumorspheres cultured in +B27 and –B27 medium were collected and dissociated for size evaluation. The range of average size was 170~193 cells per sphere.

When primary cancer cells from the mouse mammary tumors in our study were cultivated on a solid substratum in the presence of serum-containing medium, cell underwent differentiation induced by environmental stimuli. A suitable system was urgently needed to maintain the cancer stem cells in an undifferentiated state, to allow serial passaging and propagation in culture. As cancer stem cells were speculated to resemble their non-mutated counterparts in the ability of forming spheres in non-adherent serum-free condition, a prospective method of propagating/enriching solid cancer stem cells *in vitro* was developed [5]. Major advances were achieved when an undifferentiated population of neural cells could be grown in suspension without losing multipotential differentiation capacity, and these cells formed non-adherent spherical clusters, termed as neurospheres, and contained 4~20% of stem cells [36, 37]. This method was instrumental in cancer stem cell research for various human malignancies, and was also employed in the study of breast cancer [2, 20, 29]. We adopted the same method, and have successfully grown tumorspheres from primary tumor cells in an anchorage-independent suspension in the presence of suitable growth factors (EGF and bFGF). At the molecular level, putative stem cell markers were overexpressed in these mammary tumorspheres, showing a molecular signature of their stem cell-like properties.

However, the absence of suitable defined systems to maintain cells in the undifferentiated state impeded further progress in the isolation and characterization of the

cancer stem cells, rendering this population elusive [13, 20, 38]. It is important to initially establish a reliable *in vitro* system of maintaining the scarce cancer stem cells. In our study of HER2/Neu-transformed breast cancer stem cells, we found the powerful effect of B27 supplement in promoting tumorsphere formation and maintaining serial propagation, compared with the B27-free medium. The advantage of B27 supplement was clearly apparent. Firstly, serial cultures of tumorspheres were achieved, allowing for a constant source of tumorspheres for the long-term study of breast cancer stem cells, owing to the role of B27 in increased survival of tumorspheres [20, 34, 39] and prevention of adherences [35]. Secondly, a tumorsphere-forming efficiency assay exhibited the role of B27 supplement in improving the tumorsphere-forming efficiency over serial passaging. Assessment of tumorsphere-forming efficiency revealed the enrichment of the cells capable of self-renewing and forming tumorspheres. The high productivity of "higher" passages of tumorspheres helps to save time and the investment in expensive tumorsphere cultivation. One of the potential pitfalls is that the tumorspheres are heterogeneous in nature. Despite the increase in sphere-forming efficiency, only a relatively small percentage of cells with each sphere hold the sphere-forming capacity. In addition, not all sphere-forming cells fulfill the criteria of being cancer stem cells. The heterogeneity of tumorsphere in cellular origin and composition makes it possible that the percentage of cancer stem cells may decrease after extended numbers of passages *in vitro*, e.g. the cancer stem cells may be outnumbered by cancer progenitor cells with capacity to form sphere. But the total cancer stem cells population will expand through self renewal with continuously passaging of tumorspheres. Lastly, B27 also seemed to be favorable in preserving adequate viability of frozen tumorspheres. Tumorspheres stored at -80ºC freezer showed a higher recovery rate in freezing medium that contained B27 supplement (data not shown). Despite the unknown effects of diverse components, B27 supplement, it is clearly an essential part of the culture medium necessary for sustained *in vitro* tumorsphere propagation and the study of cancer stem cells.

Conclusions

We grew primary tumor cells and tumorspheres out of mammary tumor tissue harvested from MMTV-Neu transgenic mice. In the process of characterization of mammary tumorspheres, we found that B27 supplement, in addition to essential growth factors, played an important role in promoting tumorsphere formation and sustaining serial cultures, compared with the counterparts maintained in B27-free medium. A tumorsphere-formation assay revealed the enrichment of breast cancer stem cells *in vitro* through serial passaging. The average tumorsphere size was, however, independent of the culture condition and inferred to be determined only by the innate properties of cancer stem cells. Yet the mechanism remains unknown, our study identifies that B27 supplement is necessary for sustained propagation and enrichment of breast cancer stem cells *in vitro*.

Acknowledgements

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