Review

Isolation of Stem Cells Using Spheroids from Fresh Surgical Specimen: An Analytic Mini-Review

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Abstract. *It is a commonly held belief that adult stem cells represent the "seeds" for normal cellular replenishment and also for carcinogenesis. The identification and characterization of stem cells for clinical therapeutic applications, however, is extremely challenging for a number of reasons. Recently, our group and others have attempted to isolate stem cells using spheroids from fresh surgical specimens and utilize them for in vitro and in vivo studies. This mini-review summarizes the major technical steps of these methods along with the primary findings. Besides, it critically analyzes the advantages and limitations of the concept and technical approaches. Finally, this mini-review presents our thoughts on the potential future directions of stem cell isolation and cancer stem cell-related research and clinical applications.*

In the adult human organs, only stem cells retain the potential for unlimited proliferation and multi-lineage differentiation (1-3). As the average life span of most cell types in most adult human organs is only about 100 days, stem cell-mediated normal cellular replenishment is vital for the maintenance of the structural and functional integrity of 1these organs in the systems; this is especially essential for the epithelial cells of the gastrointestinal tract, which turn over every 3-5 days (4-6). Stem cell-mediated normal cell growth is also essential for the repairing process after internal or external insults to the organism, such as burns, toxin, and injuries (7-10). Stem cell-mediated cell proliferation or differentiation constitutes the basis of Regenerative Medicine, which has been increasingly applied in the clinic to replace worn or lost body parts (11, 12). Aberrant alterations of the normal replenishment or repairing process has been linked to a wide variety of human diseases, such as atrophy, which is the result of reduced cell proliferation, or neoplasm, which is caused by increased cell proliferation (13-15). More importantly, reduction or loss of stem cell-mediated proliferation is the primary cause of aging and death of the organism. In addition, as cell proliferation is an absolute pre-requisite for the formation of a given tumor mass at a primary tissue site or a new tumor colony at a distant organ, stem cells are regarded as the "seeds" for benign and malignant tumors (16-18).

Identification or characterization of stem cells for therapeutic applications, however, is extremely challenging for a number of reasons: First, the absolute number of normal stem cells retained among people may differ significantly. It has been well-documented that the stem cell population progressively loses its volume and breadth of developmental potency during the aging process, which is significantly influenced by a great number of known and unknown factors (18-21).

Second, the ratio between normal stem and differentiated cells, or cancer stem *versus* tumor bulk cells, remains elusive. Although it is a common belief that cancer stem cells account for approximately 0.3-2% of the tumor bulk, a recent mathematical model shows that cancer stem cells "may

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comprise any possible proportion of the tumor, and that the higher the proportion the more aggressive the tumor is likely to be" (22-24).

Third, the histological origin of cancer stem cells remains elusive. Two exclusive theories of carcinogenesis, cancer stem cell and clonal evolution, were introduced in 1875 and 1976, respectively (25, 26). The cancer stem cell theory believes that cancer results from external or internal insults that cause genetic damages to multiple stem cells within a given tissue site, in which all damaged stem cells can progress and give rise to different cancer cell populations, in which each stem cell preserves its unique signatures (25). In contrast, the clonal evolution theory believes that carcinogenesis results from the accumulation of genetic abnormalities, and only the most dominant and aggressive clone can progress (26). Up to date, each of these theories appears to have experimental or clinical data to support.

Fourth, the normal pattern of stem cell replenishment has been a subject of debate. It has been proposed and demonstrated in some cases that normal stem cell self-renewal results from asymmetric-cell-division with non-random chromosomal cosegregation (ACD-NRCC), in which each chromosome in a stem cell contains one DNA strand that is conserved throughout asymmetric cell divisions (27-29). This hypothesis and supporting data, however, have been strongly objected by others (30-32).

It is a commonly held belief that both early normal morphogenesis and carcinogenesis are driven by stem cell-mediated cell proliferation, differentiation, and migration, which share the same mechanism and very similar processes. The comparison of the normal early morphogenesis with carcinogenesis, however, has been hampered by the lack of suitable *in vitro* or *in vivo* models. During the past, stem cell line- and xenograft-based approaches have been the primary, if not the sole, methods for studying morphogenesis and carcinogenesis. Stem cell line- and xenograft-based approaches, however, have three major drawbacks. First, most established stem cell lines tend to acquire artificial changes during the course of maintenance, which are not normally seen in the natural *in vivo* processes of cancer. Second, it is difficult, if not impossible, to accurately assess the interactions between cancer stem cells and their microenvironment, or interactions among different cell types. Third, results generated from cell lines or animal models often fail to reflect the intrinsic events in human due to the substantial structural, functional, and hormonal differences between them.

Recently, our group and others have attempted to isolate stem cells using spheroids from fresh surgical specimens and utilize them for *in vitro* and *in vivo* studies. This mini-review summarizes the major technical steps and primary findings, and also analyzes advantages and limitations of those approaches. In addition, this mini-review presents our thoughts on the potential future directions of stem cell isolation and cancer stem cell-related research.

Major Technical Steps and Primary Findings

Human hepatic cancer stem cells are characterized by common stemness traits and diverse oncogenic pathways (33) (Figure 1). Fresh surgical liver cancer specimens and liver cancer cell lines treated with zebularine generated a side-population with cancer stem cell properties demonstrated by self-renewal and superior tumor-initiating capacity in serial transplantations. These cancer stem cells are characterized by common stemness traits and diverse oncogenic pathways.

Establishment of human ultra-low passage colorectal cancer cell lines using spheroids from fresh surgical specimens suitable for vitro and in vivo studies (34) (Figure 2). Two ultra-low passage colorectal cancer cell lines were established using spheroids from fresh surgical specimens. Newly-established cell lines retain the morphological and immunohistochemical features of the original tumors, which are suitable for *in vitro* and *in vivo* studies of stem cells (see below).

Tumor initiating label-retaining cancer cells in human gastrointestinal cancers under asymmetric cell division (35) (Figure 3). A sub-populationof LRCC is actively dividing and exhibits stem cells and luripotency gene expression profiles, and also undergoes asymmetric cell division and non-random chromosomal cosegregation (ACD-NRCC). LRCCs have greater tumor-initiating capacity than non-LRCCs.

Identification of an adult stem/progenitor cell-like population in the human thyroid (36) (Figure 4). Spheroids with self-replicative potential were obtained from all thyroid specimens. The isolated population contained a subset of $CD34^{+}$ $CD45^{-}$ cells and it was able, in differentiation conditions, to generate follicles with thyroid hormonal production. When spheroids were co-cultured with a neuroblastoma cell line, they produced progeny expressing the neuronal marker beta-tubulin III. Spheroids were also able to undergo adipogenic differentiation when cultured in adipogenic medium.

Fresh surgical specimens yield breast stem/progenitor cells with oncogenic abnormalities (37) (Figure 5). The frequencies of stem/progenitor cells between benign and malignant tissues were similar. Stem cell-associated gene expression also was similar between benign and malignant stem cells. Genetic mutations in the PIK/AKT pathway were found in 73% of the tumors' stem cells, within two sub-populations. No mutations were found in stem/progenitor cell sub-populations from benign breast tissue.

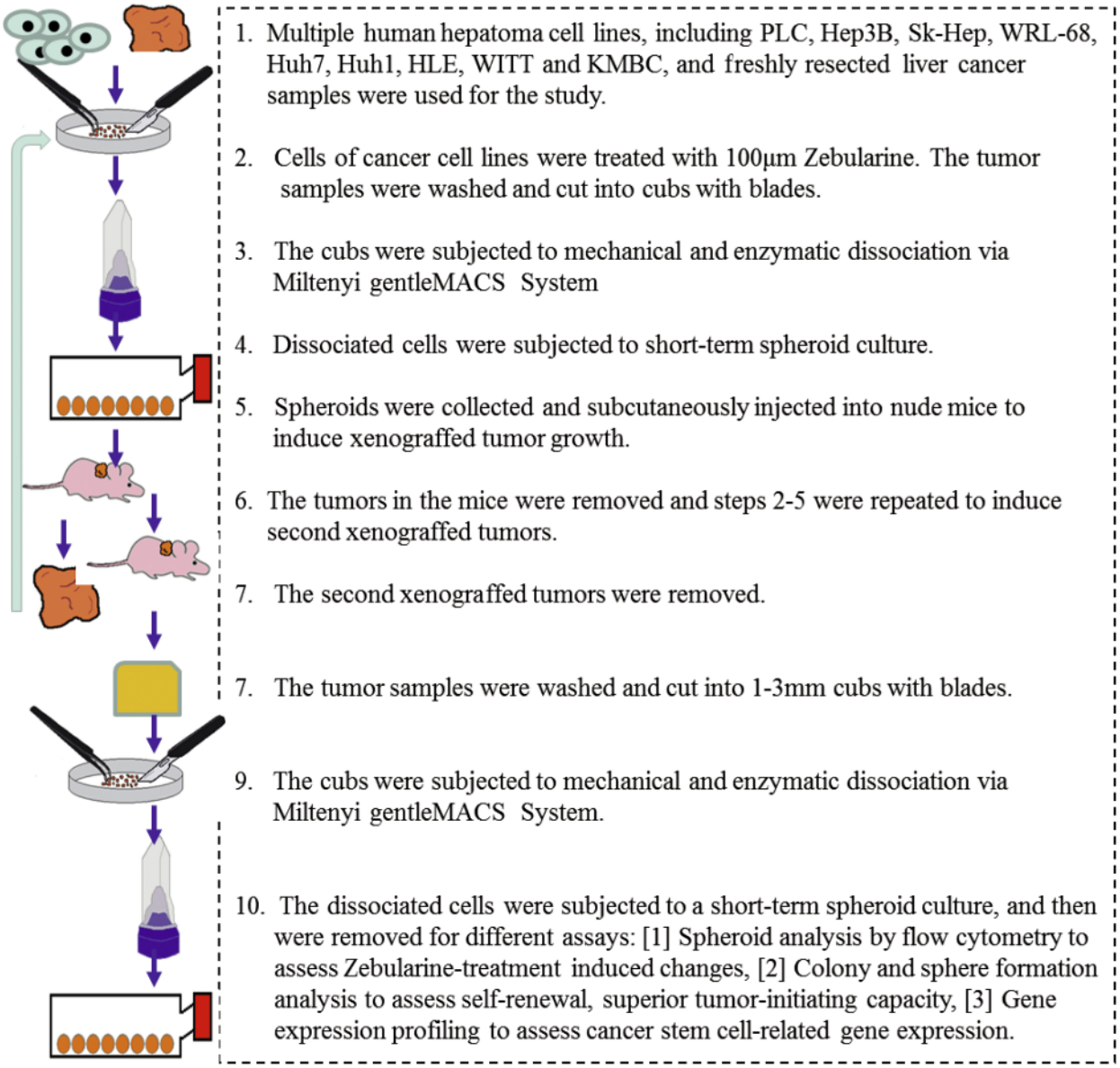

Figure 1. *Main steps for isolation of stem cells from human hepatoma cell lines and resected liver cancer samples.*

Strengths and Limitations of the Technical Approaches

Isolation of normal or cancer stem cells from fresh surgical specimens appears to have a number of advantages over the traditional stem cell line- or xenograft-based approaches. Firstly, surgery is a drastic stimulator, which could force stem cells to exit from quiescence, which consequently generates more stem cells for analyses. Secondly, the short-term spheroid culture could avoid any potential factors that may artificially alter the genetic or biological properties of the cultured cells. Thirdly, this approach could generate sufficient material to test for mutations, genetic signatures and molecular subtyping readily available for clinical therapeutic decision-making. Fourthly, the overall procedure is not complicated and the experimental circle is relatively short. With these advantages, isolation of normal or cancer stem cells from fresh surgical specimens appears to represent a more effective approach than traditional stem cell line- or xenograft-based method for stem cell research.

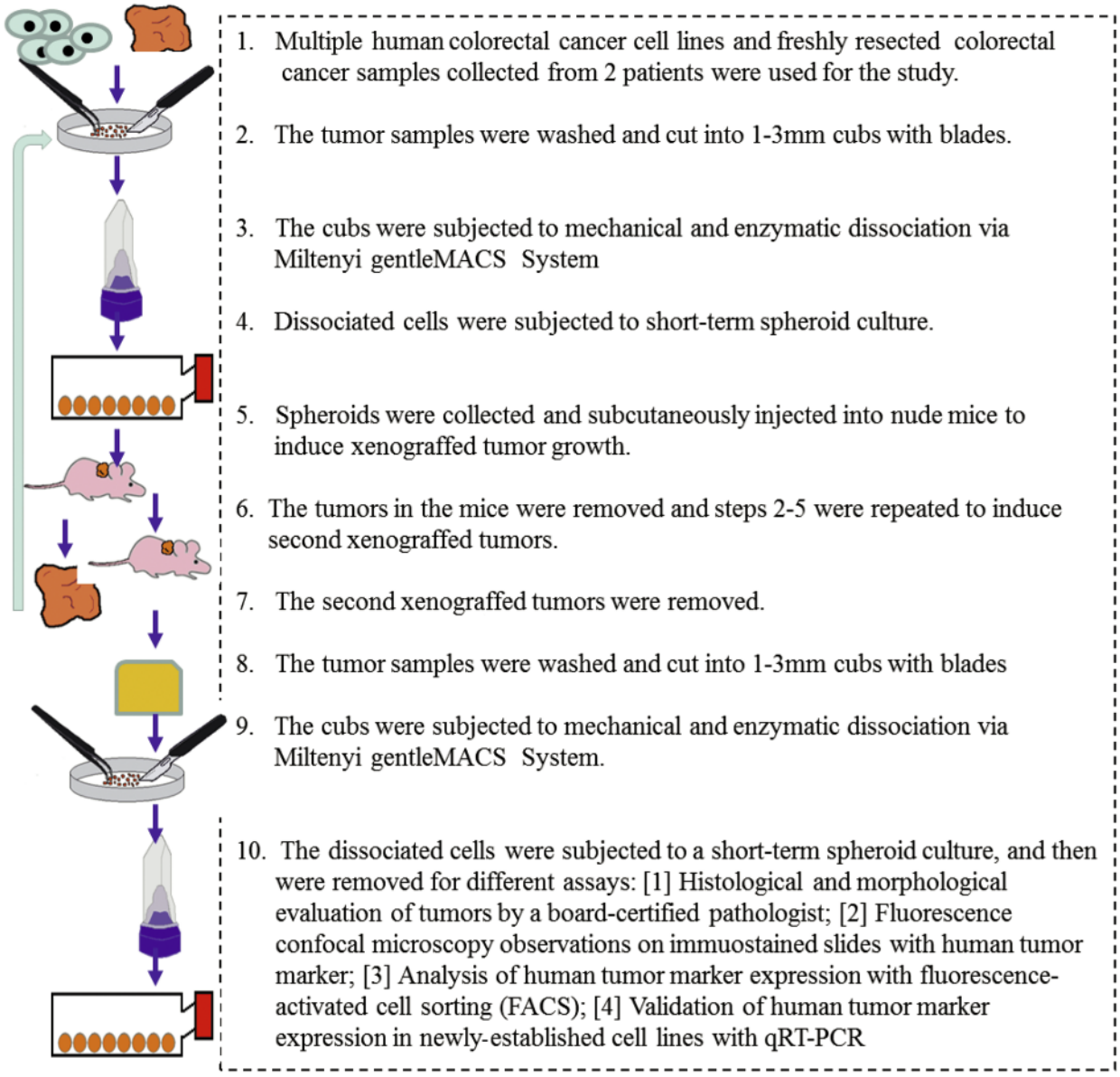

Figure 2. *Main steps for isolation of human ultra-low passage colorectal cancer cell lines using spheroids from fresh surgical specimens.*

Isolation of normal or cancer stem cells from fresh surgical specimens also appears to have a number of limitations. Firstly, surgery may represent a double-edged sword. On one hand, it may stimulate more stem cells to exit from quiescence that may give rise to a large number of stem cells for analyses. On the other hand, it could also stimulate the exit of partially-committed stem cells or committed progenitor cells, which may mask the genetic or biochemical profile of the "true" stem cells. Secondly, the short-term tissue culture may not be able to reliably determine whether the stemness of isolated stem cells is sustainable, or whether they are "true" stem cells. Thirdly, as surgically-removed tissue samples can undergo degenerative alterations very quickly and the size of the removed tissue samples varies substantially, it is technically challenging to select the adequate pieces for analysis. Fourthly, this approach may not be able to identify the true stem cell population that represent the "seeds" for tumor invasion and metastasis.

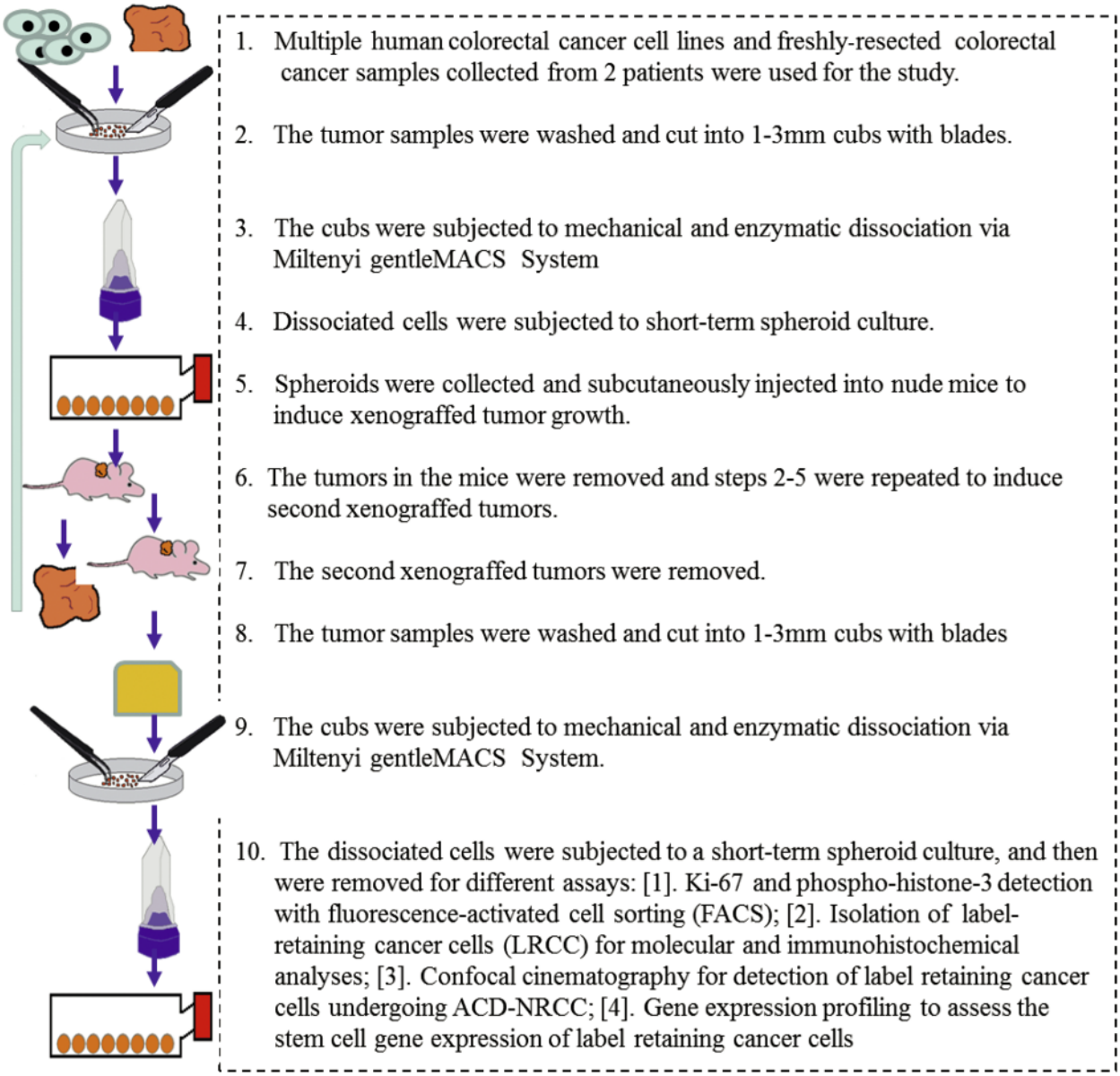

Figure 3. *Main steps for isolation of label-retaining cancer cells from human gastrointestinal cancer.*

Potential Future Directions of Stem Cell Isolation and Cancer Stem Cell-related Research

Our previous studies of human breast, prostate, cervical, lung, skin, and colorectal cancer have led to a novel hypothesis that carcinogenesis, cancer invasion, and cancer metastasis arise from the convergence of a tissue injury, the innate immune response to the injury, and the presence of cancer stem cells within the tumor capsules at the site of the injury (38-49). All epithelial cells and their derived pre-invasive cancer are completely surrounded by a dense fiber capsule, which consists mainly of collagen IV, fibronectin, and laminin. Focal capsule degeneration due to age or disease attracts lymphocyte infiltration that degrades the degenerating capsules resulting in focal disruptions in the capsule. If the focal disruption occurs in the capsule underlying progenitor and/or tumor stem cells, these cells are presented the opportunity to proliferate leading to the formation of proliferating cell "buds" overlying the disruption (38-49). Then, when lymphocytes infiltrate the

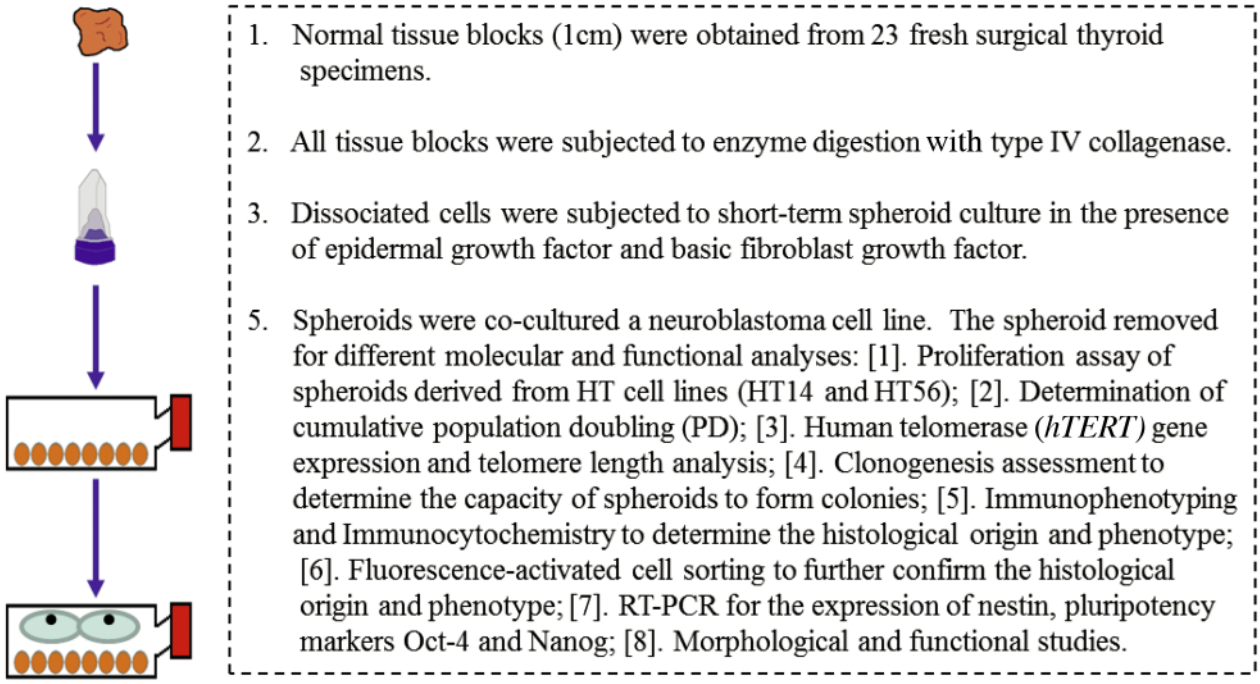

Figure 4. *Main steps for isolation of adult stem/progenitor cells from human thyroid.*

cell buds, they disrupt the inter-cellular junctions and surface adhesion molecules facilitating the dissociation of some proliferating stem cells from the primary site. During this process, lymphocytes and tumor cells can form tumor-lymphocyte chimeras (TLCs) through the fusion of cell membranes. Our confocal microscopic observations have shown that the membrane fusion does not trigger phagocytosis or tumor cell destruction (48, 49), suggesting that this fusion may be mediated by function-associated antigens expressed on the lymphocyte membrane and integrins or other surface molecules on the tumor cells (50-52). This perilous coupling allows lymphocytes to physically drag cancer stem cells to migrate, to breach cell barriers, to intravasate, circulate, and to travel to distant organs where cancer stem cell can form new colonies (48, 49). Our hypothesis has been recognized as more compatible with existing experimental evidence (53-55) than the traditional "proteolytic enzyme" theory (56). Our hypothesis is also applicable to all epithelium-derived cancers and can reasonably explain all major events of cancer invasion and metastasis. Finally, our hypothesis postulates, for the first time, a morphologically-defined precursor, the TLC, of metastatic cancer (48, 49).

Collectively, our hypothesis suggests that carcinogenesis, cancer invasion, and cancer metastasis could potentially occur at morphologically-benign or even normal-appearing epithelial structures as long as there is a convergence of a tissue injury, the innate immune response to the injury, and the presence of cancer stem cells within the tumor capsules at the site of the injury. Our hypothesis also suggests that cell clusters or "budding" cells overlying focally-disrupted epithelial capsule as that shown below are most likely to represent the direct precursor or "seeds" of invasive or metastatic cancer.

Consequently, surgically-removing benign or normal epithelial structures with and without focal capsule disruption, with and without overlying "budding" cells for spheroid or tissue culture appears to have a number of additional benefits for isolation of cancer stem cells. First, it could potentially pin-point the location of the potential cancer stem cells. Second, it may facilitate detection of potential cancer at the very early stage of disease. Third, as disruption of the tumor capsule is an absolute pre-requisite for invasion or metastasis of all epithelium-derived malignancies, elucidation of the genetic and biochemical profiles of cells overlying focally-disrupted capsules may lead to identification of the trigger factor(s) for invasion or metastasis of all cancers. Fourth, tissue culture of the entire epithelial structure with "budding" cells with suitable stimulating factors could quickly establish a stem cell line. In addition, as our recent studies of human breast, prostate, cervical, lung, skin, oral, and colorectal cancer have

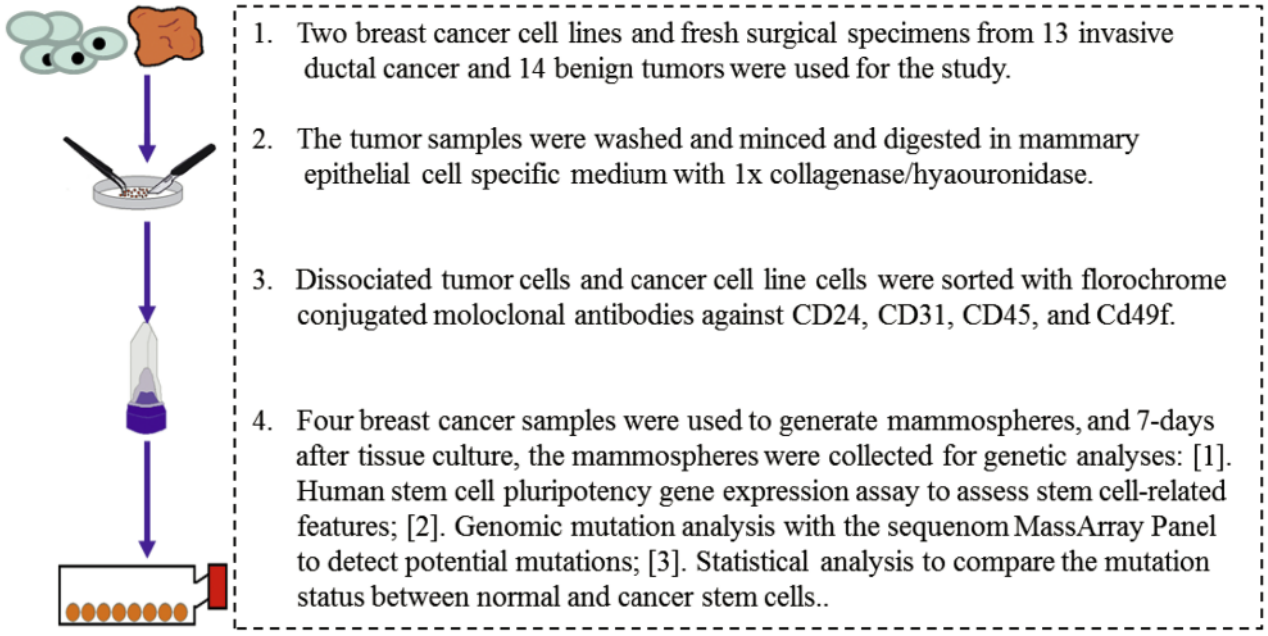

Figure 5. *Main steps for isolation of adult breast stem/progenitor cells from surgical specimens.*

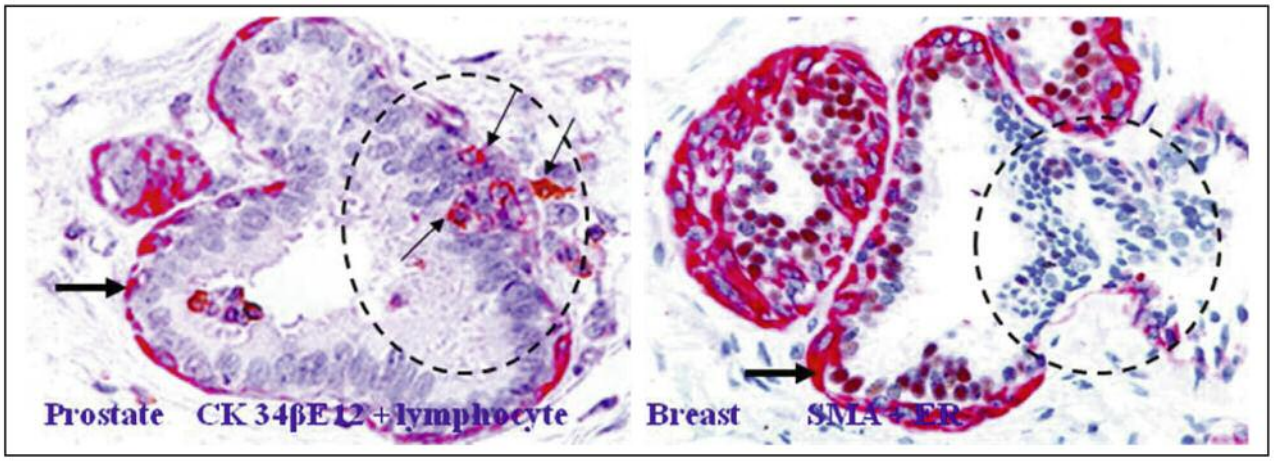

Figure 6. *Focal capsule disruptions in normal-appearing epithelial structures. Normal-appearing prostate and breast tissue sections were double-immunostained for capsule (thick arrows), lymphocytes (thin arrows), and estrogen receptor (brown). Circles identify focal capsule disruptions. Note that several lymphocytes infiltrate the epithelial cells overlying focal capsule disruption. Also note that epithelial cells overlying focal capsule disruptions lack ER expression. Original magnification, ×150.*

consistently shown that tumor-lymphocyte chimeras (TLCs) are detectable in benign or normal appearing epithelial structures with or without malignant lesions (48, 49), isolation of TLCs within the primary tumor site or regional lymph nodes, or blood for biochemical and molecular assays could potentially identify the specific molecules that are required for the formation of TLCs. The development of therapeutic agents to specifically target these molecules could potentially prevent cancer invasion and metastasis in the first place (57-61).

Disclosure

This manuscript has been read and approved by all Authors. This article is not under consideration by any other journal and has not been published elsewhere. The Authors of this article report no conflicts of interest. The Authors confirm that they have permission to reproduce any copyrighted material.

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