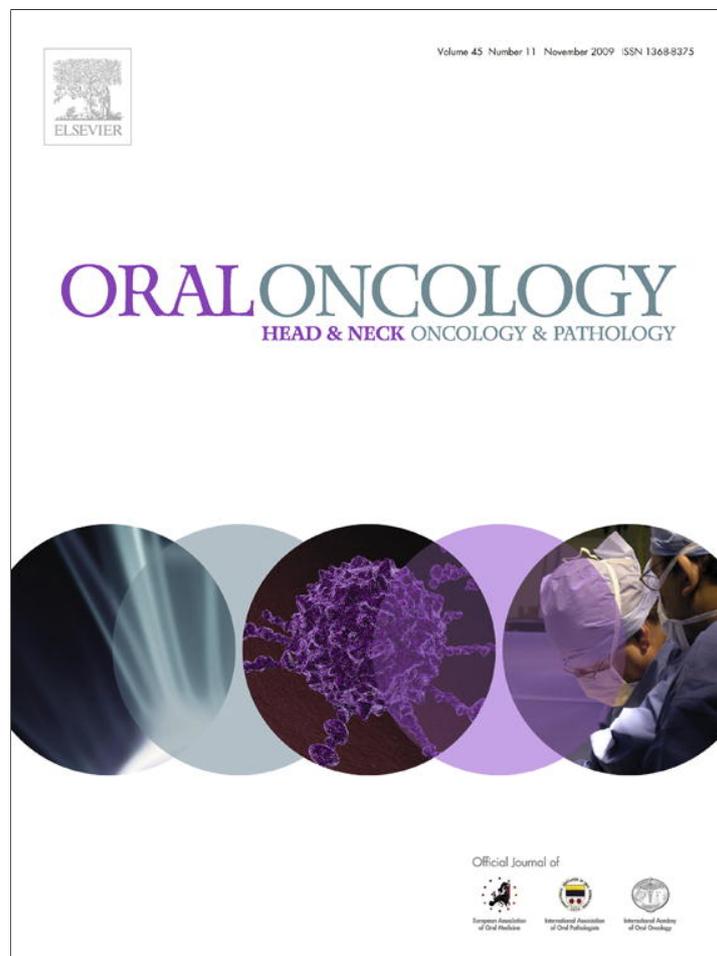


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## Isolation and characterization of normal hamster buccal pouch stem/stromal cells – A potential oral cancer stem/stem-like cell model

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## SUMMARY

The hamster buccal pouch (HBP) is an appropriate experimental model for buccal squamous cell carcinoma (SCC). Our objective was to isolate and characterize the stem/stromal cells from normal HBP. HBP stem/stromal cells were successfully derived from three of five normal pouch tissues, which differentiated into adipogenic, chondrogenic, and osteogenic lineages, and also expressed stem cell and differentiation markers, indicating their stem cell origin and differentiation capability. These cells showed high expression of CD29, CD90, and CD105, markers specific for bone marrow stem cells, and exhibited very low expression of CD14, CD34, and CD45, markers specific for hematopoietic cells. Of the HBP stem/stromal cells isolated, 90% stained positively for cytoplasmic keratin, whereas 10% stained positively for vimentin. In conclusion, normal HBP stem/stromal cells provide a potential avenue for future experimental trials of cancer stem/stem-like cells for treatment of buccal SCC. In vitro, we may detect the sequential changes of normal HBP stem/stromal cells during multistep oral carcinogenesis or the alternations of these cells upon irradiation treatment and/or chemotherapy.

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## Introduction

Oral squamous cell carcinoma (SCC) is an important health issue, as reflected by its high occurrence in many regions of the world.<sup>1</sup> Oral SCC ranked the 12th most prevalent global cancer<sup>2</sup> and the 8th most prevalent for males.<sup>3</sup> For those countries with a high prevalence of chewing betel-quinid and/or tobacco, SCC of the buccal mucosa is the most common oral cancer.<sup>4–8</sup> Thus, the buccal mucosa appears to be the site at greatest risk of contracting malignancy among habitual betel-quinid chewers who expose the buccal mucosa to high doses of mutagens and carcinogens.<sup>9</sup>

An animal model would be helpful to explore the molecular pathogenesis of SCC of the buccal mucosa. The hamster buccal pouch is an appropriate experimental model for examination of

buccal SCC.<sup>10</sup> Despite anatomic and histologic variations between hamster pouch buccal tissue and human buccal tissue, experimental carcinogenesis protocols for hamsters induce premalignant changes and carcinomas that resemble those that occur during analogous carcinogenic progression in human buccal tissue.<sup>11</sup> While cancer stem/stem-like cell studies are reported on human oral SCCs,<sup>12,13</sup> to our knowledge, no similar studies are reported on the hamster buccal pouch model. However, before applying the hamster buccal pouch model for the study of oral cancer stem/stem-like cells, the existence of normal stem/stromal cells in hamster buccal pouch tissue should first be identified and characterized. Therefore, the objective of the current study was to isolate and characterize stem/stromal cells from normal hamster buccal pouch tissues.

## Materials and methods

## Animals and treatments

Outbred, young (6-week-old), male Syrian golden hamsters (*Mesocricetus auratus*; five animals, purchased from the National

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Science Council Animal Breeding Center, Taipei, ROC), weighing approximately 100 g at the beginning of the experiment were housed under constant conditions (22 °C, 12-h light-dark cycle) and fed with tap water and standard Purina laboratory chow *ad libitum*. Appropriate animal care and experimental protocol ensured humane treatment, and all procedures were done with reference to the NIH *Guide for the Care and Use of Animals*.

After allowing all hamsters 1-week of adaptation to their new surroundings, the five animals were simultaneously killed by inhalation of a lethal dose of diethyl ether. Both pouches were dissected and then excised.

The right buccal pouch was immediately digested in collagenase type I (3 mg/ml, Invitrogen, Carlsbad, CA, USA) and dispase (4 mg/ml, Invitrogen) for 1 h at 37 °C.<sup>14,15</sup> The resulting cell suspensions were filtered through a 40- $\mu$ m cell strainer (Falcon, BD, Franklin Lakes, NJ), and the single cell suspensions were then cultured in a medium containing  $\alpha$ -modified Eagle's medium (Hyclone, Logan, UT, USA), 20% fetal bovine serum (FBS; Hyclone), 100  $\mu$ M of L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA), 100 U/ml of penicillin (Invitrogen), and 100  $\mu$ g/ml of streptomycin (Invitrogen). The cells derived were then cultured at 37 °C in 5% CO<sub>2</sub> incubator; the medium was refreshed every 48 h and 80% confluence was obtained for optimal cell harvesting.

The left buccal pouch was fixed in 10% neutral-buffered formalin solution for about 24 h, dehydrated in a series of ascending concentrations of alcohol solutions, cleared in xylene, and embedded in paraffin for routine histologic studies.

#### Proliferation rate

The proliferation rates the hamster buccal pouch stem/stem-cell like cells (HBPSCs) were determined by seeding 25,000 cells/well (at passage 3) into six-well plates; each pouch sample had three replicates and was subcultured and counted every 3 days. Cells were counted prior to the next passage and 25,000 cells/well were reseeded into a new six-well plate, for a total of four passages. The proliferation rate was then calculated by dividing the total cell number after every 3 days of culture by the initial 25,000 cells cultured.

#### Colony-forming unit (CFU) assay

The colony forming efficiency of the HBPSCs (at passage 3) was assessed. HBPSCs were seeded into a six-well plate at 40 cells/well, with three replicates. After 14 days in culture, the cells were fixed in 10% buffered formalin for 10 min and stained with 3% (v/v) crystal violet (Sigma) for 5 min; they were then washed twice with distilled water and the number of colonies counted. Only colonies greater than 2 mm in diameter were counted, as those any smaller could have broken off a larger colony or could have been satellite cells that were growing instead of the cell originally seeded. The percentage colony-forming efficiency was expressed as the total number of colonies divided by the initial number of cells that were seeded, multiplied by 100.<sup>14,15</sup>

#### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Five sets of oligoprimers were designed to detect stem cell markers (Oct-4, Rex-1 and Nanog; Table 1) and differentiation markers (Nestin and Osteonectin; Table 1). The specific oligoprimers sets<sup>14,15</sup> were designed in accordance with the published cDNA sequences in GenBank (Bethesda, MD, USA). All oligoprimers were intended to cross the junction between two exons, with the forward primer located at the 3' region of the leading exon; whereas, the reverse primer was located at the 5' region of the fol-

lowing exon to minimize the possibility of a false positive or contamination by genomic DNA.

Total RNA of HBPSCs (at passage 3) was obtained using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The RNA extracts were qualitatively checked by electrophoresis on 1.0% agarose gel and stained with ethidium bromide. The RNA concentrations were determined from the optical density at a wavelength of 260 nm (using an OD260 unit equivalent to 40  $\mu$ g/ml of RNA). Isolated total RNA (1  $\mu$ g) was reverse-transcribed to cDNA according to the SuperScript III protocol (Invitrogen). The obtained cDNA was then used for PCR amplification using a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as the positive control (Table 1) and H<sub>2</sub>O as the negative control. The amplification cycle consisted of 3 min at 94 °C, 25 s at 94 °C, 30 s at 62 °C, and 50 s at 72 °C, repeated 40 times, followed by 3 min at 72 °C. Amplification products were analyzed by electrophoresis in 2% agarose gels along with DNA molecular weight markers (Boehringer, Mannheim, Germany) containing ethidium bromide. The PCR products were visualized as bands using an ultraviolet transilluminator.

#### Immunohistochemistry

Immunohistochemical staining for keratin and vimentin was performed using a standard avidin-biotin peroxidase complex method.<sup>16</sup> Mouse monoclonal antibodies against pan cytokeratin (Abchem Corporation, Cambridge, UK; Cat. No. ab6401) and vimentin (Abchem Corporation; Cat. No. ab28028) were used.

Prior to immunohistochemical staining, endogenous peroxidase activity of the HBPSCs (at passage 3) was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 60 min. Then, a 10% solution of normal goat serum was applied for 60 min in order to inhibit non-specific staining. These sections were subsequently incubated respectively with antibodies against keratin (1:100) and vimentin (1:100) overnight at 4 °C. Following subsequent rinsing with Tris-buffered saline (TBS; three times, 10 min each), all tissue sections were incubated for 60 min at room temperature with biotin-conjugated goat anti-mouse IgG (Vector, Burlingame, CA, USA; 1:100). Following this, all sections were again washed with TBS (three times, 10 min each) and then incubated with an avidin-biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for another 60 min. After washing with TBS (three times, 10 min each), peroxidase binding was visualized as brown reaction products via a benzidine reaction. The sections were then counterstained with Mayer's hematoxylin. Each set of experiments included a specimen known to express keratin and vimentin, which served as a positive control and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

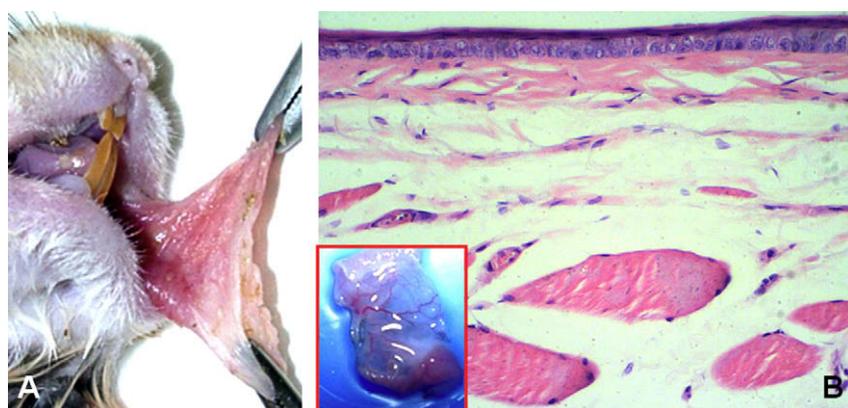
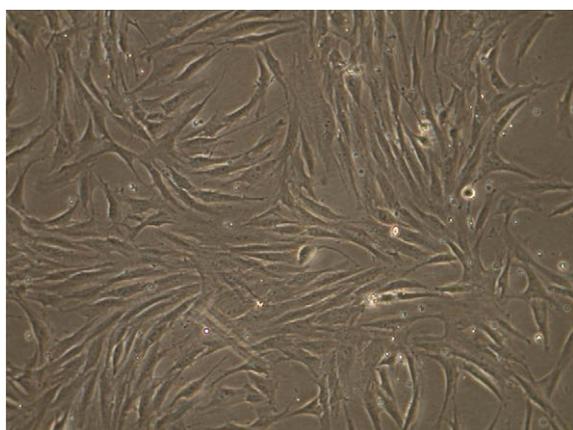
#### Adipogenic differentiation

The adipogenic differentiation capacity of HBPSCs (at passage 3) was assessed. HBPSCs were seeded at 400 cells/cm<sup>2</sup> in a 35-mm tissue culture dish and cultured for 11 days in HBPSC medium until confluence was attained. The HBPSC medium was then supplemented with 5  $\mu$ g/ml of insulin (Sigma), 50  $\mu$ M of indomethacin (Sigma), 1  $\mu$ M of dexamethasone (Sigma), and 0.5  $\mu$ M of isobutyl-1-methyl xanthine (Sigma) and the cultures were incubated at 37 °C and in a 5% CO<sub>2</sub> incubator for 21 days, with medium changes three times per week. As a control, HBPSC medium only was used. At the end of the cultivation period, the cells were fixed in 10% buffered formalin (10 min) and stained with Oil-Red-O (Sigma) for visualization of fat vacuoles.<sup>14,15</sup>

**Table 1**

Oligoprimers targeting stem cell markers and differentiation markers, as well as GAPDH, which was used as the control.

Oligoprimers	Sequences	PCR products
Oct-4	Forward: 5'-TGG AGA CTT TGC AGC CTG AG-3' Reverse: 5'-TGA AGT GGG GGC TTC CAT AG-3'	717-bp
Rex-1	Forward: 5'-GGC CAG TCC AGA ATA CCA GA-3' Reverse: 5'-GAA CTC GCT TCC AGA ACC TG-3'	232-bp
Nanog	Forward: 5'-AGG GTC TGC TAC TGA GAT GCT CTG-3' Reverse: 5'-CAA CCA CTG GTT TTT CTG CCA CCG-3'	364-p
Nestin	Forward: 5'-AGG AGT CGT TTC AGA TGT GGG AG-3' Reverse: 5'-CTC GTG CGC CAC GCG TAG AGC CT-3'	416-bp
Osteonectin	Forward: 5'-ATC TTC CTC CTT TGC CTG G -3' Reverse: 5'-GCT CAC CTC TCA AAC TCG CC-3'	323-bp
GAPDH	Forward: 5'-GAC TTC AAC AGC AAC TCC CAC-3' Reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3'	135-bp

**Figure 1** Representative sample of the hamster buccal pouch tissues revealed normal gross (A, inset) and histologic (B, hematoxylin and eosin stain,  $\times 200$ ) features.**Figure 2** Representative sample of the stem/stromal cells obtained from normal hamster buccal pouch tissue were spindle-shaped in morphology ( $\times 200$ ).

RT-PCR of PPAR $\gamma$  mRNA indicating adipogenic lineage of the HBSPCs (passage 3) was performed. PCR conditions were: 60.5 °C annealing with forward oligoprimers (5'-AGA CAA CAG ACA AAT CAC CAT-3') and reverse oligoprimers (5'-AAG TTT GAG TTT GCT GTG AAG-3') for 35 cycles, generating a PCR product of 401-bp. These primers detected both PPAR $\gamma$ -1 and -2.<sup>17</sup> GAPDH was used as the positive control and H<sub>2</sub>O as the negative control (Table 1).

#### Chondrogenic differentiation

The chondrogenic differentiation capacity of HBSPCs (at passage 3) was assessed. An aliquot of  $2.5 \times 10^5$  HBSPCs was centrifuged in

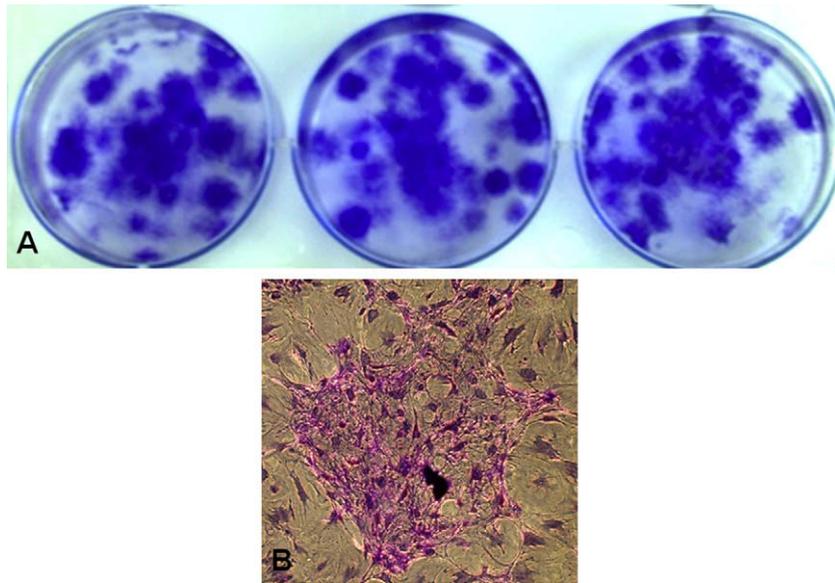
a 15-ml conical tube at 1000 rpm for 5 min. The pellet was maintained in HBSPC medium supplemented with 10  $\mu$ g/ml of ITS-X (Invitrogen), 5.35  $\mu$ g/ml of linoleic acid, 1.25  $\mu$ g/ml of bovine serum albumin, 1.0  $\mu$ g/ml of dexamethasone, and 10 ng/ml of TGF- $\beta_3$  (Chemicon, Billerica, MA) at 37 °C in a 5% CO<sub>2</sub> incubator. The medium was refreshed every 3–4 days for 4 weeks. As a control, HBSPC medium only was used. The chondroid pellets were then fixed in 4% paraformaldehyde (PFA) overnight, and paraffin-embedded sections (4  $\mu$ m) were stained with Alcian blue for visualization of the chondroid tissues.<sup>18</sup>

#### Osteogenic differentiation

The osteogenic differentiation capacity of HBSPCs (at passage 3) was assessed. HBSPCs were prepared as previously detailed, with the exception that 1 nM of dexamethasone (Sigma), 20 mM of  $\beta$ -glycerolphosphate (Sigma), and 50 ng/ml of L-thyroxine sodium pentahydrate (Sigma) were supplemented into the HBSPC medium on day 11. The cultures were incubated at 37 °C in a 5% CO<sub>2</sub> incubator for another 21 days, with medium changes three times per week. As a control, only HBSPC medium was used. At the end of the cultivation period, the cell cultures of HBSPCs were fixed in 10% buffered formalin for 10 min and stained with alizarin red S (Wako, Osaka, Japan) for 15 min at room temperature for visualization of bone mineral matrix.<sup>14,15</sup>

#### Flow cytometry

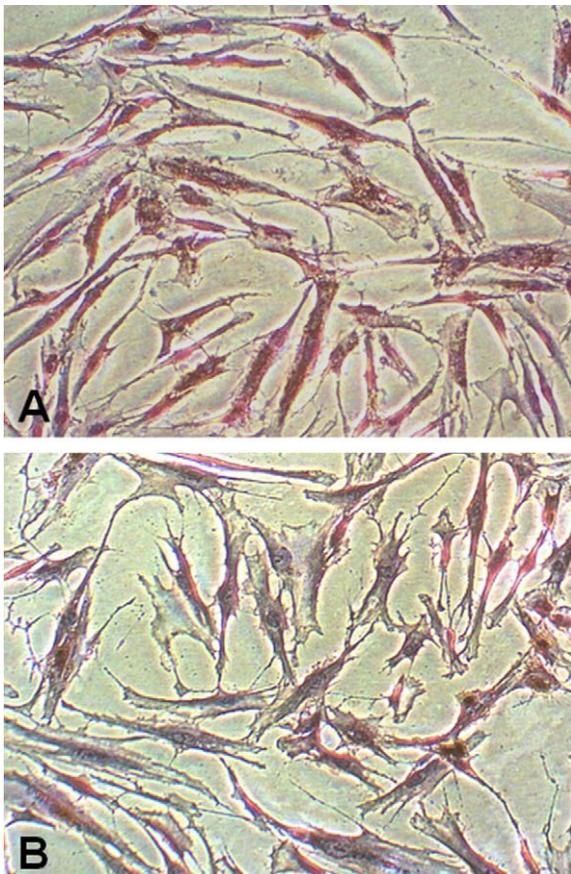
Flow cytometric analyses for stem cell markers and differentiation markers were performed. HBSPCs (at passage 3) were divided into six fluorescence-activated-cell sorting (FACS) round-bottom tubes (Becton Dickinson Falcon, Sunnyvale, CA, USA) at a concen-



**Figure 3** Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues were able to form colonies, stained with crystal violet (A; B,  $\times 100$ ).

tration of  $2 \times 10^5$  cells/tube and stained with IgG-fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated anti-CD14, -CD29, -CD34, -CD45, -CD90, -CD105 (Beckman Coulter, Villepinte, France, 20  $\mu$ l each). After a 20 min incubation at ambient temper-

ature in the dark, cells were washed twice with 2 ml of FACS wash solution (phosphate buffered saline (PBS) containing 0.1% FBS and 0.1%  $\text{NaN}_3$ ) and centrifuged 5 min at  $230 \times g$ . The supernatant was discarded and the cells were fixed with 1% formaldehyde (in PBS). Respective immunoglobulin G (IgG) isotype-matched controls (Beckman Coulter) were used as negative controls.<sup>19</sup> The data obtained using a Coulter Epics XL (Beckman Coulter, France) were analyzed with EXPO32 ADC software (Beckman Coulter) and Windows Multiple Document Interface for Flow Cytometry, WinMDI, version 2.8; freeware developed by Joe Trotter, downloadable at [methods.info/software/flow/winmdi.html](http://methods.info/software/flow/winmdi.html)).

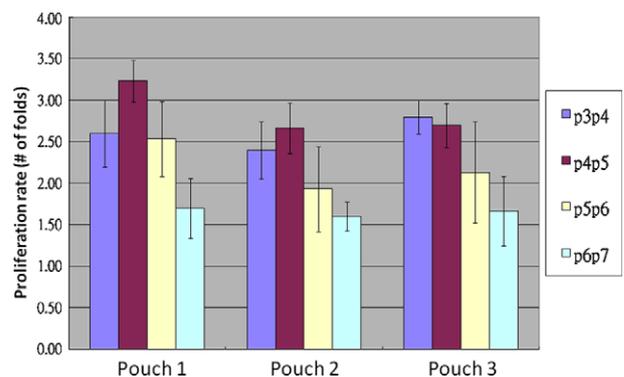


**Figure 4** Cytoplasmic keratin (A,  $\times 200$ ) and vimentin (B,  $\times 200$ ) stainings were noted for the representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues (avidin–biotin peroxidase complex stain).

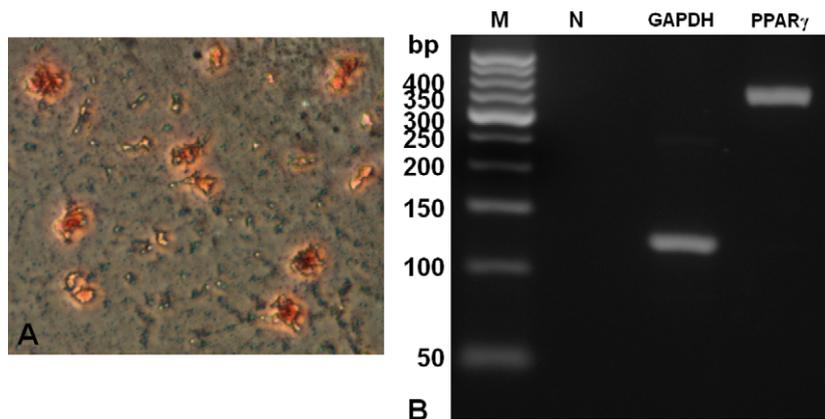
### Results

The left normal hamster buccal pouch tissues showed no obvious gross or histologic changes (Fig. 1A and B). During the early processing stage, cultures of two right pouch tissues were terminated due to contamination by microorganisms. HBPSCs for the other three right pouch samples were successfully processed, resulting in a culture processing success rate of 60%.

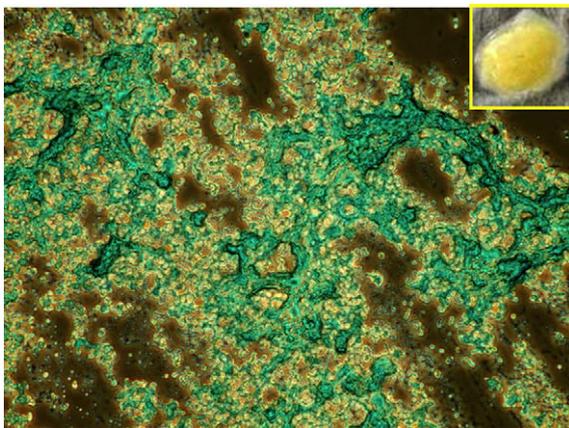
The HBPSCs proliferated with a spindle-shaped morphology (Fig. 2). After passage 3, homogenous populations of HBPSCs were obtained and maintained in culture based on their cell morphol-



**Figure 5** Proliferation rates for the stem/stromal cells obtained from the three normal hamster buccal pouch tissues (p: passage).

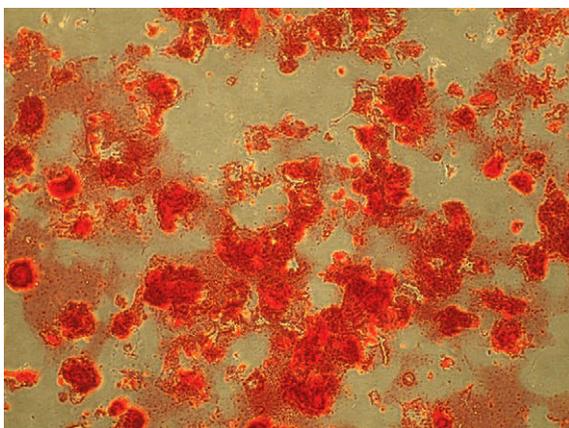


**Figure 6** (A) Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues were able to differentiate towards adipogenic lineage (Oil-Red-O,  $\times 200$ ). (B) Expression of PPAR $\gamma$  mRNA (401-bp) upon RT-PCR also indicates adipogenic lineage of HBPSCs obtained from normal hamster buccal pouch tissues; GAPDH (135-bp) was the positive control; H<sub>2</sub>O was the negative control (N); M: molecular weight marker.



**Figure 7** Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues were able to differentiate towards chondrogenic lineage (Alcian blue,  $\times 200$ ); inset: a yellowish chondroid pellet ( $\sim 3$  mm in diameter).

ogy. On average 80% of the HBPSCs were capable of forming colonies (Fig. 3). Positive cytoplasmic keratin staining was noted for about 90% of the HBPSCs (passage 3) (Fig. 4A). In contrast, only



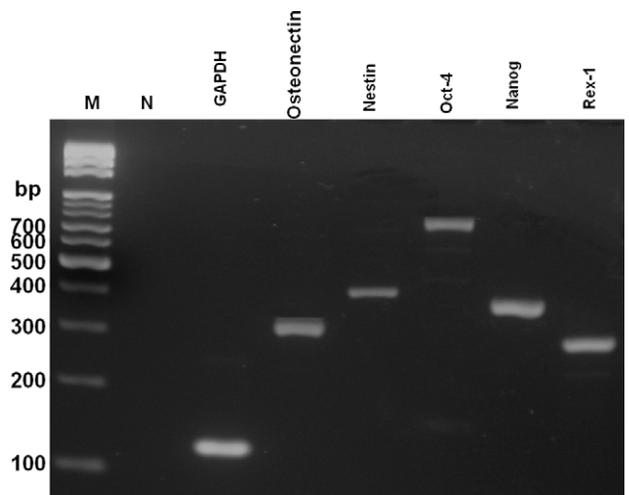
**Figure 8** Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues were able to differentiate towards osteogenic lineage (alizarin red S,  $\times 200$ ).

10% of the HBPSCs stained positive for vimentin (Fig. 4B). In addition, the HBPSCs had high proliferation rates during the early passages, but the rate decreased gradually as number of subcultures increased (Fig. 5).

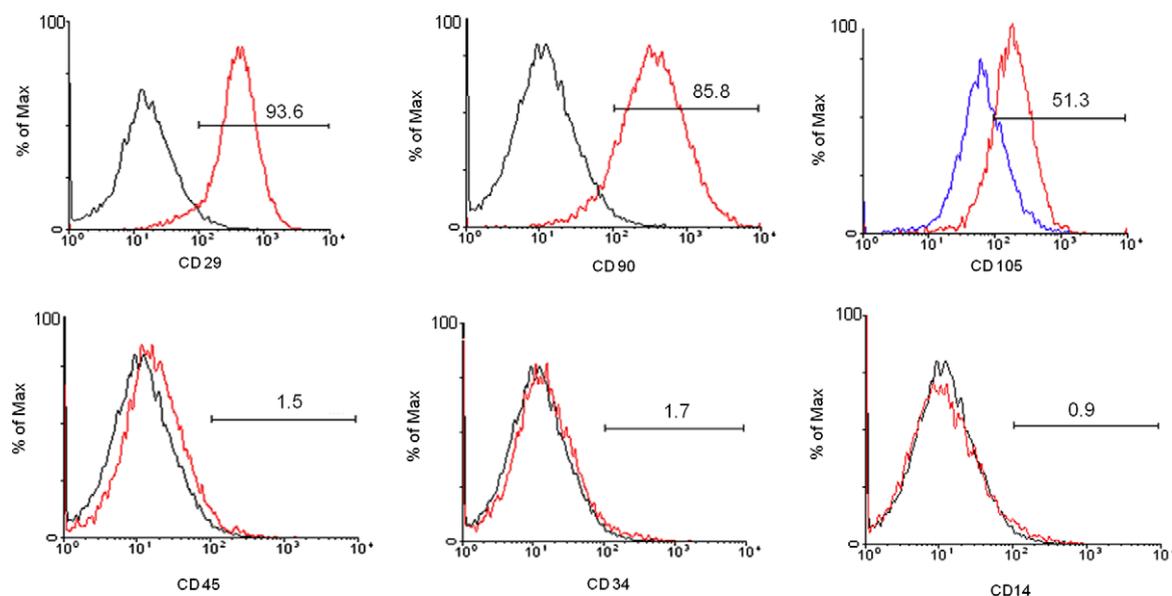
The HBPSCs differentiated into adipogenic (Fig. 6A and B), chondrogenic (Fig. 7), and osteogenic (Fig. 8) cell lineages. We also determined that the HBPSCs expressed stem cell markers and differentiation markers (Fig. 9). In addition, the HBPSCs highly expressed common bone marrow stem cell markers including CD29, CD90, and CD105 (Fig. 10). The HBPSCs expressed very low hematopoietic cell markers (CD14, CD34, and CD45) (Fig. 10).

**Discussion**

The classical model of cancer development indicates that tumors arise from a series of sequential mutations leading to genetic instability and/or environmental risk factors affecting normal cells.<sup>20</sup> Recently, the concept of cancer stem/stem-like cells (CSCs) has acquired much awareness. CSCs are tumorigenic, multipotential cells with poorly regulated self-renewal characteristics.<sup>21</sup> Upon asymmetric division, one daughter or ‘progenitor’ cell retains stem



**Figure 9** Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues expressed the differentiation markers (Osteonectin: 323-bp and Nestin: 416-bp) and stem cell markers (Nanog: 364-bp, Rex-1: 232-bp and Oct-4: 717-bp) upon RT-PCR. GAPDH (135-bp) was the positive control; H<sub>2</sub>O was the negative control (N); M: molecular weight marker.



**Figure 10** Representative sample of the stem/stromal cells obtained from the normal hamster buccal pouch tissues showed high expression for surface markers: CD29, CD90, and CD105 but very low expression for CD14, CD34, and CD45 (Black/blue line: isotype control, Red line: marker of interest; Max: maximum).

cell characteristics, while the other becomes committed to a lineage. CSCs are hypothesized to arise from developmentally arrested stem cells harboring mutations. The CSCs then replicate producing an exact copy of themselves as well as a continuous supply of heterogeneous tumor cells. In addition to self-renewal, CSCs have other stem cell features such as high proliferation abilities, high migration capacity, and drug resistance.<sup>22</sup> Furthermore, CSC theory suggests that only CSCs within a tumor can self-renew and proliferate extensively to form new tumors.<sup>21</sup> For several different types of cancers, a distinct subset of cells initiates tumors in vivo, whereas the remaining cells do not.<sup>23,24</sup> A minority population of CD44<sup>+</sup> cancer cells (<10% of the cells in a head and neck SCC), but not the CD44<sup>-</sup> cancer cells, generate new tumors in vivo.<sup>25,26</sup> Oral SCCs also possess stem cell markers such as Oct-4, Nanog, Nestin, CD117, and CD133.<sup>13</sup> Consequently, accumulated evidence shows CSCs in SCCs of head and neck, including oral cavity, function in initiation, maintenance, growth, and metastasis of tumors.

The rate of renewal for human normal oral epithelium is estimated to be approximately 14–24 days.<sup>27</sup> Therefore, most epithelial cells do not survive long enough to accrue the genetic alternations required for development of oral SCC.<sup>20</sup> In accordance with the genetic progression model, the formation of oral SCC requires months or years.<sup>28</sup> Thus, it is hypothesized that normal somatic stem cells, being long-term residents of the oral epithelium, are uniquely susceptible to accumulation of multiple oncogenic changes, giving rise to oral SCC. We successfully isolated HBPSCs from normal hamster buccal pouch tissue. Furthermore, we showed that the HBPSCs possessed adipogenic, chondrogenic, and osteogenic differentiation capacity, and they expressed stem cell markers.

The exact source(s) of CSCs in oral SCC remains to be completely elucidated. Progenitor cells within the basal layer of the oral epithelium are usually regarded as the putative source of CSCs in oral SCC. Nonetheless, non-epithelial sources in the oral epithelium including vessel wall-, muscle-, and adipose-derived stem cells are also hypothesized.<sup>21</sup> We isolated HBPSCs from the entire buccal pouch tissue consisting of both epithelial and connective tissue components.

The development of oral SCC has long been characterized as a multistep process consisting of the ‘initiation’, ‘promotion’, and

‘progression’ phases, which reflect accumulated genetic changes, inducing malignant transformation of normal oral mucosa.<sup>20</sup> It may not be straightforward to isolate the CSCs directly from hamster buccal pouch carcinomas due to ease of contamination with microorganisms during culture<sup>29</sup> and the very low number of CSCs.<sup>25,26</sup> With the isolation of normal HBPSCs, we may alternately follow in vitro sequential changes of normal HBPSCs during multi-step oral carcinogenesis or alternations of these cells upon irradiation treatment or chemotherapy or both. In conclusion, isolated normal HBPSCs, provide a potential avenue for the future study of CSC of buccal SCC.

#### Conflicts of Interest Statement

None declared.

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