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Potential of salivary gland stem cells in regenerative medicine

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Maimets, M. (2016). *Potential of salivary gland stem cells in regenerative medicine*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

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CHAPTER 2

REGENERATION OF IRRADIATED SALIVARY GLANDS WITH STEM CELL MARKER EXPRESSING CELLS

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Radiother Oncol. 2011 Jun;99(3):367-72.

ABSTRACT

Background and Purpose: Stem cell therapy could be a potential way for reducing radiation-induced hyposalivation and improving the patient's quality of life. However, the identification and purification of salivary gland stem cells have not been accomplished. This study aims to better characterize the stem/progenitor cell population with regenerative potential residing in the mouse salivary gland.

Materials and Methods: Mouse submandibular gland tissue, isolated cells and cultured 3 day old salispheres were tested for their expression of stem cell markers c-Kit, CD133, CD49f, and CD24 using immunohistochemistry for tissue and flow cytometry for cells. Mice were locally irradiated with a single dose of 15 Gy and transplanted with cells expressing defined markers.

Results: Cells expressing known stem cell markers are localized in the larger ducts of the mouse salivary gland. Isolated cells and cells from day 3 salispheres also express these markers: c-Kit (0.058% vs. 0.65%), CD133 (6% vs. 5%), CD49f (78% vs. 51%), and CD24 (60% vs. 60%, respectively). Intraglandular transplantation of these cells into irradiated salivary glands of mice resulted in stem cell marker-specific recovery of salivary gland function.

Conclusions: Different stem cell-associated markers are expressed in mouse salivary gland cells, which upon transplantation are able to regenerate the irradiation-damaged salivary gland.

INTRODUCTION

Every year more than 500,000 new patients are diagnosed with head and neck cancer worldwide (Jemal et al., 2006). The treatment for these patients is usually radiotherapy either alone or in combination with surgery and chemotherapy. Radiation-induced damage to normal tissues may result in organ dysfunction, and/or cause a reduction in the patient's post-treatment quality of life (Jensen et al., 2010). Radiotherapy of head and neck cancer patients, which often involves co-irradiation of the salivary glands, can lead to hyposalivation. This mostly irreversible side effect can result in the development of xerostomia, a multi-faceted syndrome including symptoms, such as oral dryness and infections, dental caries, and difficulties with speech and food mastication (Vissink et al., 2003). Even with unilateral sparing (Jellema et al., 2007) and Intensity Modulated Radiation Therapy (IMRT) approximately 40% of these patients develop hyposalivation and consequential life-long complaints (Vergeer et al., 2009). To date, no satisfactory clinical management of xerostomia exists (Vissink et al., 2003). Transplantation of salivary gland stem cells may offer a potential treatment for radiation-induced hyposalivation (Coppes et al., 2009). As a model, radiation-induced salivary gland damage in rodents develops in a very similar way as in patients (Coppes et al., 2002). Salivary glands of rodents consist of several cell types, similar to humans: acinar cells which are responsible for water and protein secretion, myoepithelial cells surrounding the acini and ducts, and ductal cells which mainly modulate the composition of the saliva. The ductal system consists of intercalated, striated/granular convoluted tubules and excretory duct cells (Denny et al., 1999; Gresik, 1994). Regeneration of the salivary gland after ductal obstruction (leading to acinar cell atrophy) has been attributed to putative stem cells residing in the ductal compartment. Complete recovery of the gland is induced within a week of ductal obstruction (Burford-Mason et al., 1993; Takahashi et al., 2000; Takahashi et al., 2004). Increased proliferation of these stem/progenitor cells seems to underlie the protective effect of prophylactic pilocarpine (Burlage et al., 2009; Burlage et al., 2008) and KGF (Lombaert et al., 2008b) treatment on radiation-induced damage to the parotid gland. Interestingly, we recently showed that transplantation of duct derived c-Kit$^+$ stem/progenitor cells in mice rescued salivary glands from irradiation damage (Lombaert et al., 2008a). Furthermore, human salivary gland excretory ducts also contain c-Kit$^+$ cells similar to rodent excretory ducts (Feng et al., 2009). Other preclinical studies, however, suggest that also CD49f and CD29 might be used as stem cells markers for the salivary gland (Matsumoto et al., 2007; Sato et al., 2007). In other glandular tissues stem cells are identified by using the markers CD49f (Lawson et al., 2007), CD133 and CD117 (c-Kit) (Leong et al., 2008), and specifically CD29 and CD24 for mammary gland (Shackleton et al., 2006). Furthermore, since we have observed that also some of the cells in the c-Kit$^+$ population may have regenerative potential (Lombaert et al., 2008a) further characterization of the stem/progenitor pool is warranted.

Therefore, the aim of this study was to better characterize stem/progenitor populations with regenerative potential residing in the mouse salivary gland by using c-Kit, CD133, CD49f, and CD24/CD29 as potential stem cell markers. In mice, the presence of these markers was investigated in submandibular gland and cultured salispheres. Subsequently, cells expressing these markers were isolated by fluorescence activated cell sorting and transplanted into salivary glands of irradiated recipients after which the saliva secretion was assessed as a measure of gland function.

MATERIALS AND METHODS

Animals

8–12 week old female C57BL/6 mice were purchased from Harlan (The Netherlands). The mice were maintained under conventional conditions and fed *ad libitum* with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and water. All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

Immunohistochemical analysis of salivary gland

Mice were desanguinized under isofluorane anesthesia and the submandibular glands were carefully dissected. In order to perform immunohistochemical staining extirpated mouse submandibular glands were incubated for 30 h at room temperature in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin and sliced into 5 µm sections. The sections were de-waxed and labeled for the following markers: c-Kit (1:75) (R&D Systems, Minneapolis, MN; MAB1356), CD24 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA; SC-7034), CD133 (1:50) (Abcam, Cambridge, MA; ab19898), CD49f (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA; SC-6596). Visualization for bright field microscopy was accomplished by adding specific secondary biotin carrying antibodies (Dako, Carpinteria, CA), an avidin–biotin-horse radish peroxidase complex (ELITE ABC Kit, Vector Laboratories, Burlingame, CA) and the diaminobenzidine (DAB) chromogen. Nuclear counterstaining was performed with hematoxylin. Control sections without primary antibodies did not show positive immunostaining.

Isolation of salivary gland stem cells

Following submandibular gland dissection, salivary gland cells were isolated and cultured as published previously (Lombaert et al., 2008a; Lombaert et al., 2008b; Pringle et al., 2011). Cell suspensions were prepared by first mechanically disrupting the gland, followed by enzymatic digestion with collagenase type II and hyaluronidase enzymes and CaCl2. After filtering, the cell

suspension was plated in non-coated 12 wells plates at 400,000 cells per well. Per gland 2–4.6 x 10^6 cells could be isolated. The culture medium consisted of DMEM/F12 (Gibco, Carlsbad, CA), penicillin, streptomycin, glutamax, EGF (20 ng/mL), FGF-2 (20 ng/mL), N2 (1/100), insulin (10 μg/mL), and dexamethasone (1 μM). Fresh medium was added every 3 days. All growth factors were pur- chased from Sigma–Aldrich (St. Louis, MO), except for N2 (Gibco, Carlsbad, CA).

Flow cytometric analysis

Cultured cells growing in spheres were dissociated using 0.05% trypsin–EDTA (Gibco, Invitrogen). Pacific Blue anti-mouse CD117 (c-Kit) (Biolegend, San Diego, CA), PE anti-human/mouse CD49f (Biolegend, San Diego, CA), PE anti-mouse Prominin I (CD133) (eBioscience, San Diego, CA), FITC anti-mouse CD24 (BD Biosci- ences Pharmingen) and FITC anti-rat CD29 (BD Biosciences Pharmingen) antibody incubations were performed at 4 °C for 20 min, followed by a wash step in PBS/0.2% bovine serum albumin (BSA). Finally, Propidium Iodide (PI, 1 μg/mL) was added to the cells before analysis using a FACS LSR-II Flow Cytometer (BD). At least 50,000 events for each measurement were recorded. Data were analyzed with FlowJo software (Tree Star, Ashland, OR). Gates for viable c-Kit$^+$, CD24$^+$, CD29$^+$, CD133$^+$, and CD49f$^+$ were set by using isotype controls for Pacific Blue, PE and FITC. Due to a difference in the isotype controls for isolated and cultured c-Kit+ cells the gates were different (see Supplement Fig. 1).

Irradiation and saliva collection from mouse salivary glands

Salivary glands were locally irradiated with a single dose of 15Gy (Precision X-ray Inc. – X-rad 320, 200kV, 20mA, 1.843 Gy/min). This dose is known to induce hyposalivation without compromising the general health of the animals. At 120 days post-irradiation saliva flow rate was determined. Animals were placed in a restraining device after pilocarpine injection (2.5 mg/ kg, s.c.). Saliva was collected for 15 min, and the quantity was determined gravimetrically, assuming a density of 1mg/mL for saliva. Percentage flow rate of saliva of an animal is calculated by denoting the pre-irradiation saliva as 100%. The principle behind this functional assay is that in vivo regeneration is the most rigorous test for stem cell function.

Intra-glandular injection of cultured cells

Day 3 cultured spheres were collected and dissociated by 0.05% trypsin–EDTA for transplantation studies. Cell sorting from dissociated spheres for c-Kit$^+$, c-Kit$^-$, CD133$^+$ and CD49f$^+$, and CD24$^+$/CD29$^+$ cells was performed using MoFlo flow cytometer (Beckman Coulter, Carpinteria, CA). After sorting the cells were suspended in an equal volumes of a-MEM (Gibco, Invitrogen, Carlsbad, CA) containing 2% of fetal calf serum (Gibco, Invitrogen, Carlsbad, CA) and Indian Ink (1:200 dilution) solution to

visualize the injected fluid. Thirty days post-irradiation, under general anesthesia using a 28G needle and a Microliter Syringe (Hamilton, Reno, NV), both submandibular glands of irradiated mice were injected with 5 µL of cell suspension with 5000 CD133^{+} or 150 c-Kit^{+} and 67,096 CD49f^{+} or 5000 CD24^{+}/CD29^{+} cells.

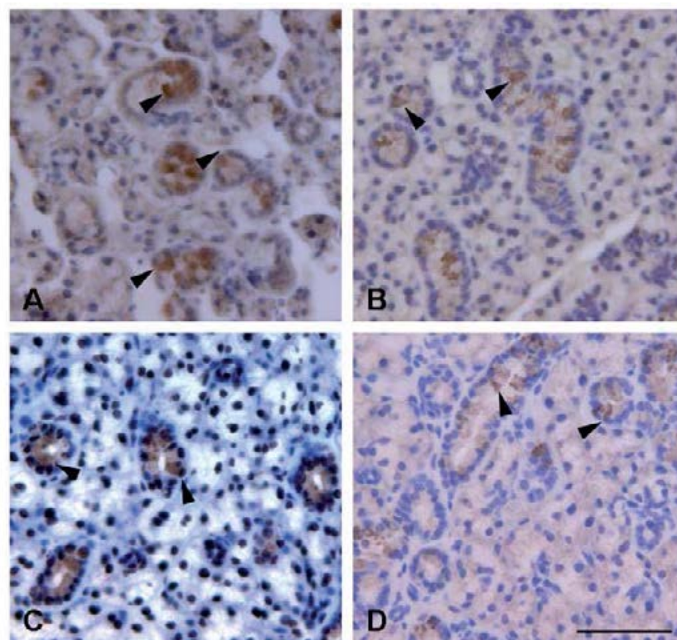

Figure 1. Stem cell-associated marker expression was observed in the ducts of intact mouse submandibular glands. Arrowheads represent DAB staining (brown) of c-Kit (**(A)** CD117), CD49f (**(B)** Integrin alpha-6), CD133 (**(C)** Prominin 1) and CD24 **(D)**. Scale bar = 50 µm. Nuclei are counterstained with hematoxylin (blue).

RESULTS

Expression of stem cell markers in murine submandibular gland – in tissue and in culture

To investigate whether mouse submandibular glands express stem cell markers, we performed immunohistochemical staining on mouse submandibular gland tissue for known stem cell markers c-Kit, CD133, CD24, and CD49f (Fig. 1). Positive staining for c-Kit, CD133, CD24, CD29 (not shown), and CD49f was observed solely in cells within the excretory duct of the submandibular gland. This indicates that, as expected, the major ducts of the mouse submandibular gland contain putative stem/progenitor cells expressing multiple stem cell markers.

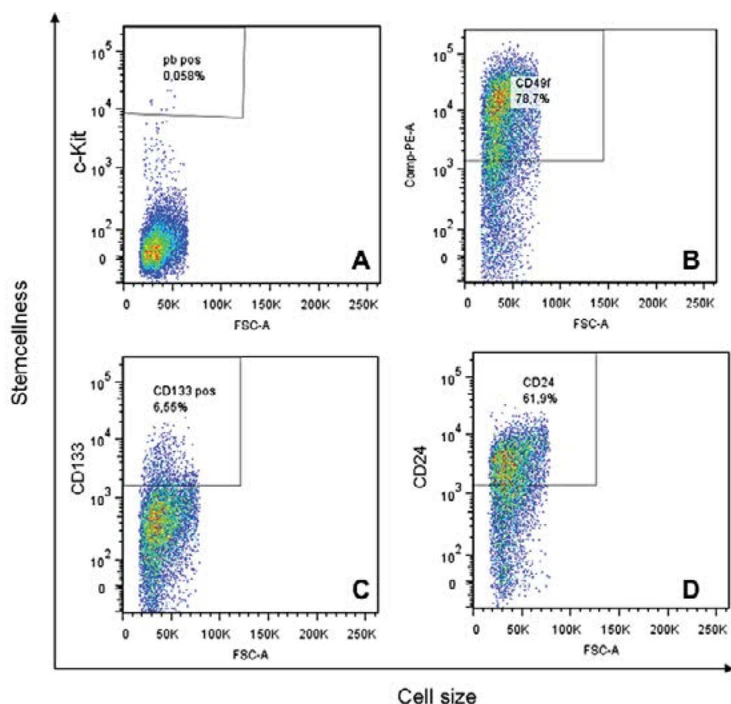

Figure 2. Stem cell associated marker expression was observed immediately after isolating murine salivary gland cells. Flow cytometric analysis of single cells obtained from submandibular glands revealed the presence of c-Kit (**(A)** CD117), CD49f (**(B)** Integrin alpha-6), CD133 (**(C)** Prominin 1) and CD24 **(D)** expressing cells. Positive populations are gated according to isotype controls. The numbers in the gates represent percentage of viable cells positive for certain stem cell-marker.

Recently, we showed that salispheres cultured from dispersed mouse submandibular gland express the stem cell marker c-Kit and are able to differentiate and self-renew in vitro (Feng et al., 2009; Lombaert et al., 2008a). Therefore, we quantified the number of cells expressing other stem cell markers. To investigate the presence and potential enrichment of these markers in our salisphere-culture, we fluorescently labeled cells from freshly dissociated mouse submandibular gland tissue (Fig. 2) and from 3 day old salispheres (Fig. 3).

Analysis of flow cytometry data shows that from freshly isolated cells from mouse submandibular gland, approximately 6% express CD133, 78% CD49f, 60% CD24, and only 0.058% c-Kit. Interestingly, in 3-day salisphere cultures we observed that the expression levels of CD133 (5.0 ± 3.8%), CD49f (51 ± 12%) and CD24 (60 ± 13%) remained relatively stable but c-Kit expression increased more than 10-fold to 0.65 ± 0.93% (all n = 3). Interestingly, no cells were found to express both CD133 and c-Kit (Supplement Fig. 1). These data indicate that culturing salivary gland cells as salispheres enriches the cultures for c-Kit positive cells.

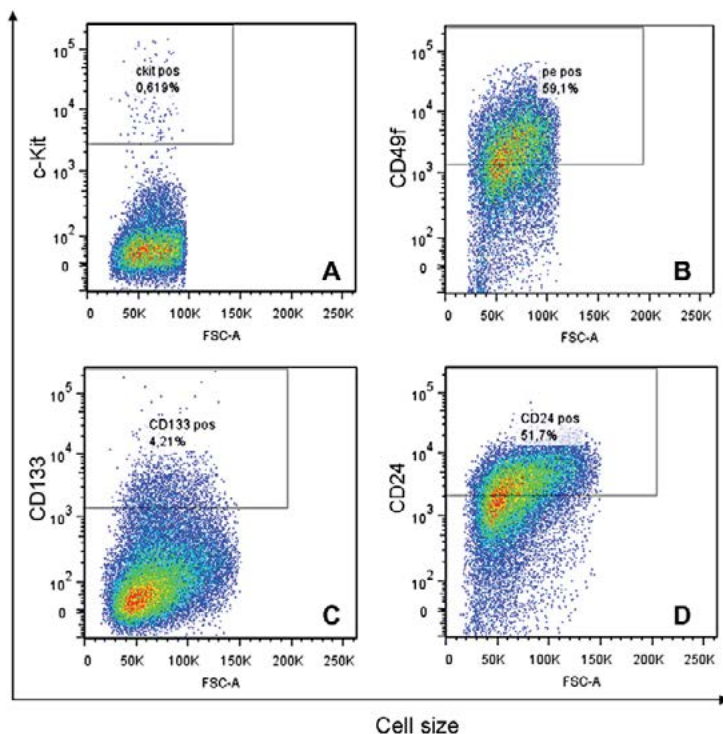

Figure 3. Stem cell-associated cell surface marker expression was observed in 3-day cultured salispheres. Flow cytometric analysis of single cells obtained from salispheres revealed the presence of c-Kit (**(A)** CD117), CD49f (**(B)** Integrin alpha-6), CD133 (**(C)** Prominin 1) and CD24 **(D)** expressing cells. Positive populations are gated according to isotype controls. The numbers in the gates represent percentage of viable cells positive for certain stem cell-associated marker.

Regenerative potential of stem cell markers expressing mouse submandibular gland cells

To investigate the regenerative potential of cells expressing these stem cell markers, we transplanted selected cells from day 3 salispheres into irradiated submandibular glands of mice, measured their saliva production and compared the results with irradiated, non-transplanted control animals (Fig. 4). The number of cells transplanted was dependent on the number that could be isolated from one donor gland, varies in a range of 4–5 million on day 0.

One hundred and twenty days post-irradiation a significant increase in saliva production was observed in mice transplanted with as few as 300 c-Kit$^+$ cells and in mice transplanted with 10,000 CD133+ cells. We also observed an increase in the saliva production of mice transplanted with 134,000 CD49f$^+$ cells, and 10,000 CD24$^+$/CD29$^+$ cells but less potently than the regeneration obtained with c-Kit$^+$ and CD133$^+$ cells. These results suggest that mouse submandibular cells expressing

multiple stem cell markers are capable of regenerating the submandibular gland after local transplantation in the irradiated gland.

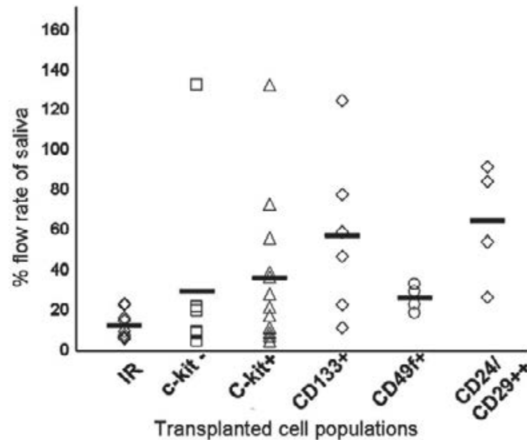

Figure 4. Functional recovery of irradiated salivary glands following transplantation with marker-expressing cells is dependent on cell phenotype. Percentage of control (non-irradiated) flow rate of saliva at 120 days post-irradiation, from 10 irradiated non-transplanted mice (IR) and from 12 mice transplanted with 300 c-Kit^{+}, 6 mice with 10,000 CD133^{+}, 4 mice with 134,000 CD49f^{+}, and 4 mice with 10,000 CD24^{+}/CD29^{+} cell populations. c-Kit data are partially taken from Lombaert et al. (Lombaert et al., 2008a).

DISCUSSION

Salivary glands of head and neck cancer patients undergoing radiotherapy may be damaged due to side effects of irradiation, resulting in impairment of saliva production. To these patients stem cell therapy is an attractive option for restoring the irradiation-damaged salivary gland. The aim of the present study was to further characterize putative stem/progenitor cells with regenerative potential present in the salivary gland, using a mouse model.

From previous studies (Lombaert et al., 2008a) we know that there might be a population of stem/progenitor cells, negative for c-Kit, but which can contribute to gland restoration. Interestingly, we found expression of multiple stem cell markers in mouse salivary gland, such as CD24, CD49f, and CD133. These immunohistochemical findings reveal that cells expressing these stem cell markers are located in the ducts of the salivary gland, where it has been suggested that tissue stem cells reside (Lombaert et al., 2008a).

Interestingly, we observed a variable intensity of immunohistochemical staining which indicates that some cells express these markers to a greater extent than others. This high expression might reflect their stem cell potential, and is supported by the fact that cells with greatest expression of stem cell markers are the most potent stem cell population in mammary gland (Shackleton et al., 2006).

By flow cytometry, we found that mouse submandibular cells on day 0 express high levels of CD24 and CD49f, but low levels of CD133 and c-Kit. The c-Kit marker and to a lesser extent CD133 are enriched during culture under high growth factor conditions, which may indicate positive selection for cells with stem cell characteristics.

Day 3 salispheres express high levels of CD49f, which were shown in another study to be co-expressed with Thy-1 in stem/progenitor cells of salivary glands of mouse (Hisatomi et al., 2004), rat (Matsumoto et al., 2007), and human (Sato et al., 2007). CD49f was also expressed in stem cells of other glands like mouse prostate gland (Lawson et al., 2007). CD24 expressed in day 3 spheres, could also be a potential stem cell marker in salivary gland, as it is reported that CD24 contributes to stem cell activity, when co-expressed with CD29 in mammary gland (Shackleton et al., 2006). However, the high frequency of CD24+ cells in salispheres makes it unlikely that CD24 can be used as a single stem cell marker, but CD24 may be more usefully employed in combination with other markers. We found that $CD24^+/CD29^+$ cells represent a subpopulation of cells, which contributed to regeneration in some mice transplanted with this population. Other combinations of marker expression with CD24 may also be worthwhile testing, in terms of defining the optimal cell phenotype for stem or progenitor cells in the mouse salivary gland.

Our in vivo transplantation results indicate that c-Kit^+ cells show more stem cell like characteristics than $CD133^+$, $CD49f^+$, and $CD24^+$, as they were able to rescue salivary gland dysfunction with as few as 300 cells. Our previous studies using serial transplantations with c-Kit^+ cells definitively established it as stem cell marker in the salivary gland, making further studies with combinations of other markers with c-Kit a high priority. Moreover, c-Kit cells were shown to be able to rescue the irradiated salivary gland in a serial transplantation experiment (Lombaert et al., 2008a), indicating self-renewal ability, an essential characteristic of stem cells. This potential still has to be determined for the other stem cell marker expressing cells.

The aim of our studies on salivary gland stem cells is to rescue irradiation damaged salivary gland in head and neck cancer patients. Therefore, it would be very desirable to be able to obtain maximum regeneration with the most efficient combination of stem and progenitor cells, especially when a low amount of donor tissue is available. In this respect, it is interesting to mention that we could not find c-Kit/CD133 double positive cells in the mouse salivary gland culture system, indicating that

salispheres may contain different subpopulations Therefore, it might be a better option to combine c-Kit$^+$ cells with CD133$^+$ (or CD49f$^+$) progenitor like cells, facilitating early and prolonged restoration of irradiation damaged salivary gland.

In conclusion, this study showed that different stem cell markers are expressed in mouse salivary gland. Combinations of these putative stem cell populations from mouse salivary gland may provide the best regeneration of the irradiation damaged salivary gland, since transplantation of a mixture of progenitor and stem cells may be clinically the most promising treatment.

Conflict of interest

The authors declare no conflicts of interests.

Acknowledgements

This work was supported financially by a grant from the Dutch Society for Cancer Research (RUG 2008-4022).

SUPPLEMENTARY FIGURES

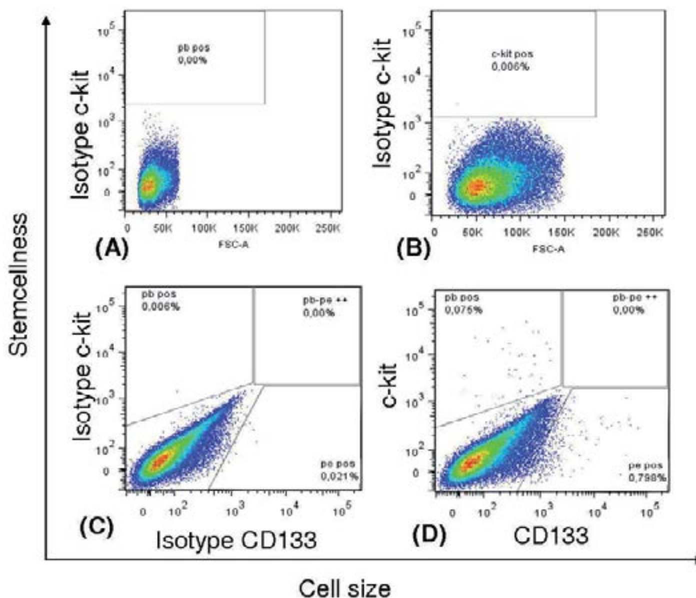

Figure S1. FACS plots of isotype controls and c-Kit/CD133. Isotype controls for c-Kit staining. (A) Immediately following isolation, (B) 3 days after isolation, and (C) for both c- Kit and CD133, 3 days after isolation. Flow cytometry for c-Kit and CD133 showed no co- expression of these markers (D).

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