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Abstract

Adult mammary stem cells have been identified in several species including the bovine. They are responsible for the development of the gland and for cyclic remodeling during estrous cycles and pregnancy. Epithelial cell subpopulations exist within the mammary gland. We and others showed previously that the Colony Forming Cell(CFC) assay can be used to detect lineage-restricted mammary progenitors. We carried out CFCs with bovine mammary cells and manually separated colonies with specific morphologies associated with either a luminal or a myoepithelial phenotype. Expression of specific markers was assessed by immunocytochemistry or by flow cytometry to confirm that the manual separation resulted in isolation of phenotipically different cells. When transplanted in recipient immunodeficient mice, we found that only myoepithelial-like colonies gave rise to outgrowths that resembled bovine mammary alveoli, thus proving that adult stem cells were maintained during culture and segregated with myoepithelial cells. After recovery of the cells from the transplanted mice and subsequent progenitor content analysis, we found a tendency to detect a higher progenitor frequency when myoepithelial-like colonies were transplanted. We here demonstrate that bovine adult mammary stem cells can be sustained in short-term culture and that they can be enriched by manually selecting for basal-like morphology.

Keywords: Bovine, Epithelial cells, Mammary gland, Stem cell, Xenotransplantation

1. Introduction

The mammary gland has a peculiar development, since it takes place mostly after birth [1]. Its parenchyma is a highly branched arborescent structure, in which alveoli constitute the functional units. The mammary epithelium is organized as a bilayer, where the inner luminal cells are responsible for milk production, while the outer myoepithelial cells have contractile capabilities [2]. The two different cell lineages can be identified also by the expression of specific markers. Luminal cells are cytokeratin (CK) 18^{+}/$CK14^{-}$, while myoepithelial cells are $CK14^{+}$/$CK18^{-}$. This tissue organization is well conserved among different mammalian species, including the human [3], mouse [4], goat [5] and bovine [2,6].

The mammary gland undergoes cyclic remodeling throughout the reproductive life of the organism. During each estrous cycle and more dramatically during pregnancy, epithelial cell number shows a marked increase and more

alveoli can be detected in the tissue [7]. Afterwards, involution takes place and the mammary gland revert to a quiescent state [8]. An adult stem cell population exists within the mammary tissue to sustain such a unique behavior. These stem cells have been identified and characterized by assessment of the expression of specific markers by flow cytometry or immunohistochemistry or through functional assays, such as transplantation in immunodeficient animals, to rigorously demonstrate their ability to self-renew and to differentiate. Species in which such characterization was carried out include the mouse [9], human [10] and more recently the bovine [11] and goat as well [5].

As we previously published, the bovine mammary gland is hierarchically organized: a small population of quiescent stem cells gives rise to different lineage restricted progenitors. These progenitors have the ability to extensively proliferate and to generate terminally differentiated cells [12]. They can be identified by their ability to form colonies in vitro over a short time. The cell morphology in these colonies can be used to infer the progenitor type that originated them in order to determine the progenitor content of the tissue of origin. An analysis based on surface markers of the different bovine mammary subpopulations has been previously carried out [13] but no in vivo functional assays were conducted on them.

In the present work we assessed whether the short-term in vitro culture system that is used to evaluate for bovine mammary progenitors can sustain the maintenance of more primitive stem cells. Moreover, we show that selection and collection of specific colony types can be used as a phenotype-based method to obtain a rough enrichment of bovine adult mammary stem cells.

2. Results

1. *CFC assays produce colonies with different morphologies*

When mammary epithelial cells are seeded at clonal density along with mitomycin C treated fibroblasts, different types of colony can be detected after 7-9 days as we previously reported [12].

Samples that were used for subsequent experiments were analyzed for progenitor content by measuring the frequency of colony forming cells. Moreover on randomly chosen dishes per each sample, cells were stained for CK14 and CK18 and for CK14 and p63 (a nuclear marker found in the mammary basal compartment) in order to confirm the luminal and myoepithelial nature of the colonies with different morphologies. More specifically,

colonies made by a high number of tightly packed cells with small cytoplasm had a $CK14^{+}/CK18^{-}$ phenotype and double positive $CK14^{+}/p63^{+}$ cells were evident (Fig. 1 i-ii) while colonies made by spindle shaped cells with an extended cytoplasm or cells with an irregular shape had a $CK14^{-}/CK18^{+}$ phenotype (Fig. 1 iii-iv).

Fig. 1 – **Immunofluorescence of representative colonies found in CFC assays**. CK14 (green) and CK18 (red) expression in myoepithelial like colonies (i) and luminal-like colonies (iii, iv). Luminal-like colonies can be found either with spindle-shaped cells and lower CK18 espression (iii) or with larger cells and higher CK18 expression (iv). CK14 (green) and p63 (red) expression in myoepithelial like colonies (ii). White arrows indicate areas where cells coexpress both basal markers. Nuclei were counterstained with DAPI (blue). Bars correspond to 500 µm (i and ii) or 250 µm (iii).

In luminal colonies the cells were also $p63^{-}$ (data not shown). In all samples some colonies with a different expression pattern were seen. The morphology of these colonies was similar to the myoepithelial ones, but only cells in the boundary area had a $CK14^{+}/CK18^{-}$ phenotype, while the cells in the inner region were weakly positive for both CK14 and CK18.

Progenitor frequency ranged from 1 out of 35 to 1 out of 7 with a similar distribution of luminal and myoepithelial progenitors. Frequency was calculated as number of colonies that formed in culture over total cell seeded.

2. *Cells from different colony types exhibit different phenotypes*

After a 9 days culture, cells seeded at clonogenic density formed colonies which were picked with a micropipette and split in different test tubes according to the morphology (luminal-like colonies and myoepithelial-like colonies). Colonies that showed CK14 positive cells and CK18 weakly positive cells were pooled with the myoepithelial-like colonies. After a brief enzymatic dissociation, the expression of CD49f (a myoepithelial marker which is associated with mammary epithelial stem cells) and ALDH1 (a marker of the luminal lineage which characterize also luminal unipotent progenitors) were assessed (Fig.2).

Fig.2 – Flow cytometry plots of cells collected from luminal and myoepithelial colonies. For each colony type, representative dot plots of ALDH1 expression vs side scatter (SSC), CD49f expression vs SSC and ALDH1 vs CD49f expression are shown. Each plot comprises 600 events. Events are live cells selected as DAPI negative events with forward scatter (FSC) and SSC parameters consistent with epithelial cells.

$CD49f^+$ cells were much more represented in the myoepithelial colonies as expected (90.1±2.8% in myoepithelial colonies vs 43.7±10.4% in luminal colonies, $p<0.01$), while overall expression of ALDH1 showed a modest variation according to colony lineage ($ALDH1^+$ cells are 22.9±10.1% in myoepithelial colonies vs 14.6±5.5% in luminal colonies, $p<0.01$). When the expression of both markers was examined at the same time, however, the percentage of $CD49f^-/ALDH1^-$ cells was much lower in the myoepithelial colonies (10.1±4.2% in myoepithelial colonies vs 56.4±9.9% in luminal colonies, $p<0.01$), while they had a higher proportion of $CD49f^+/ALDH1^-$ (63.9±0.2% in myoepithelial colonies vs 26.3±11.1% in luminal colonies, $p<0.01$). $CD49f^+/ALDH1^+$ cells were also detected with a higher frequency in myoepithelial colonies (25.0±4.9% vs 16.7±1.3% in luminal colonies, $p<0.01$). $CD49f^-/ALDH1^+$ cells were below 1% in every sample.

3. Xenografts of lineage restricted colonies

In a different experiment colonies were picked and split in different tubes according to morphology. Single cell suspensions were then made and implanted in recipient female mice. After 4 weeks gels were recovered and either dissociated to perform CFC assays or fixed and stained with mammary lineage markers.

No tubulo-alveolar structures could be detected in the gels where luminal-like colonies were transplanted. Only occasionally small pseudo-alveolar outgrowths with a distinct hollow lumen were detected (S1 Fig) in these gels. Several tubule-like structures were detectable in the gels seeded with myoepithelial-like colonies. CK14 expression could be detected while CK18 expression was undetectable in most outgrowths or very low in the remaining ones (Fig.3, (i)-(iv)).

Fig.3 – **Outcome of xenograft assays**. In panel (i)-(iv) a representative section of a collagen gel seeded with myoepithelial-like colonies after 4 weeks. CK14 is in green, CK18 in red and nuclei are counterstained with DAPI. Panel (iv) is an overlay of the fluorescent channels. Bars correspond to 250 µm. Images (v) and (vi) are representative 60 mm CFC dishes stained with crystal violet. Image (v) is a dish seeded with cells recovered from gels implanted with luminal-like colonies, while image (vi) is a dish seeded with cells recovered from gels where myoepithelial colonies were embedded.

When cells recovered from the gels were used to perform CFC assays, a difference was observed according to what colonies were originally implanted in the gels (luminal or myoepithelial). The average number of colonies found in the myoepithelial lineage was 180±23 while colonies generated from the gels containing luminal cells was 15±9. However the difference was not statistically significant (p=0.09) as calculated with Kolmogorov-Smirnov test.

3. Discussion

We previously showed that a population of adult stem cells reside in the mammary gland of ruminants (bovine [11] and goat[5]). These cells have the potential to regenerate fully functional alveoli which can produce and secrete milk when transplanted in a mouse model. However more committed progenitors can be found in the mammary tissue as well. Some of these progenitors have a more limited differentiation potential, since they are able to give rise only to luminal or myoepithelial cells.

In order to analyze and characterize better the different subsets of cells that compose the mammary hierarchy, it is necessary to find strategies to purify them. In other species this has been partially accomplished through flow cytometry and sorting based on the expression of different surface markers [9,10,14]. A similar approach was adopted by Rauner and Barash [13] in the bovine species. The authors described different populations according to the expression of surface markers CD49f and CD24 and analyzed their performance in several *in vitro* assays. However, no data have been produced about the regenerative abilities of these different subpopulations in an *in vivo* model. The CFC assay is widely used to assess the frequency and types of mammary progenitors in a sample, due to their consistent proliferation over a short time [15]. However, the data presented here demonstrate that adult stem cells

are maintained in culture at least for the duration of the CFC assay (tipically 7-9 days), since regenerated mammary structures can be detected when colonies are picked and transplanted *in vivo*. This is consistent with the concept that adult stem cells are a slowly replicating population. In physiological conditions they are almost quiescent in the mammary tissue, except for phases of the reproductive cycle when progesterone blood levels are high [16,17]. In the CFC context, colonies are generated by cells with a high proliferative index (and progenitors have this characteristic), while stem cells that might have been seeded along with them, do not proliferate extensively. Even though the assay condition should promote differentiation, it is possible that paracrine factors produced both by the epithelial colonies and the feeder layer might contribute to maintain a stem cell niche for the more primitive cells, thus preventing their loss through differentiation. A recent paper examined the proliferative properties of cultured bovine primitive cells [18]. The authors found that supplementing the culture medium with several growth factors increased proliferation as opposed to differentiation of these cells.

Based only on the morphology, colonies in the primary CFCs were divided into luminal-like and myoepithelial-like. As we have previously shown [11], morphology is closely associated with expression of markers of both mammary lineages. We further expand here such a characterization by assessing the expression of markers that are related to progenitor populations (CD49f and ALDH1). CD49f is part of the laminin-1 receptor and it is expressed in the mammary tissue by basal cells. A high CD49f expression has been associated with myoepithelial progenitors and mammary adult stem cells [10,14]. Recent papers expanded on the concept that mammary stem cells are closely associated with the basal layer by demonstrating that only cells of myoepithelial origin can differentiate in both mammary lineages [19] and that differentiated myoepithelial cells may spontaneously activate *in vitro* to expand the mammary stem cell compartment [20]. Hence, the data we present here are consistent with what was found in other species. High expression of ALDH1 has been associated with luminal progenitors which are not capable of regenerating a functional mammary epithelium [21]. When analyzing the differences in population frequencies, we found a higher proportions of CD49f positive cells (both in the $ALDH1^-$ and in the $ALDH1^+$ subfractions) in the basal fraction. Interestingly we found a double positive population both in the luminal and the myoepithelial colonies even though with slightly different frequencies. Further analysis of this population might allow us to ascertain the identity of these cells and to understand if they may be an early stage of bipotent progenitors or rather a transient intermediate for committed unipotent progenitors. As expected the $CD49f^+/ALDH1^-$ fraction was more represented

in the basal colonies and should account for myoepithelial progenitors and terminally differentiated basal cells. In the luminal colonies the frequency of double negative cells was higher as expected from terminally differentiated luminal cells.

It is then possible to use this approach as a method to roughly separate two different mammary subpopulations. Both the xenograft assay and the subsequent CFC assays we performed showed that mammary adult stem cells can be found only in the myoepithelial fraction and that the cells of the luminal lineage lack the repopulating ability of the more primitive cells. These data are consistent with reports of other authors who have found a correlation of adult mammary stem cells and the basal compartment in species such as the murine and the human ones [22-24].

The possibility to maintain in culture adult mammary stem cells, such as we demonstrated here, is appealing since it provides a useful tool to better understand their homeostasis in a much simpler in vitro environment. Moreover it allows scientists to explore techniques to manipulate them, such as expand them and influence their differentiation program either by exposing them to specific growth factors and hormones or by introducing exogenous genes. We also propose here an easy method to select and enrich stem cells based on the functional abilities of their progeny, namely the capacity to generate colonies with a specific morphology and marker expression pattern.

4. Experimental Section

1. *Collection of bovine mammary tissue*

Whole udders were collected from three different animals (11-17-96 months old animals) at a local abattoir within 2 hours of the time of slaughter. All samples were from Piedmontese cattle, a local beef breed. Sample collection was performed with the authorization and under the supervision of representatives of the Veterinary Services of the Italian National Health Service branch of the Ministry of Health.

Mammary tissue was then processed as previously described [11] in order to obtain a single cell suspension to be used for subsequent assays. Briefly tissue samples were dissociated overnight in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 Ham (DMEM/F12) 1:1 v/v mixture supplemented with 2% bovine serum albumin (BSA), 300U/ml collagenase type IV, 100 U/ml hyaluronidase type IV-S, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich)

Epithelial aggregates were then separated by differential centrifugation and enzymatically digested with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA followed by a digestion with 5 mg/ml dispase and 100 µg/ml DNAseI (all from Sigma-Aldrich)

2. *Colony forming cell (CFC) assay*

CFC assays were carried out as previously described [11]. Briefly, single cell suspensions of bovine mammary epithelial cells were seeded at very low density ($1x10^2$ to $1x10^3$ cells per 60mm dish) along with $2x10^5$ NIH 3T3 fibroblasts that were previously treated with mitomycin C (10 µg/ml for 2 hours).

Cells were cultured for 24 hours in human EpiCult B medium (STEMCELL Technologies) supplemented with 5 % fetal bovine serum (FBS) and 10^{-6}M hydrocortisone, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Sigma-Aldrich). Subsequently medium was replaced with fresh one without FBS and cells were cultured for 7-8 additional days.

Colonies were then examined under a Leica DM IL inverted contrast phase microscope (LEICA Microsystems) in order to assess their morphology. They were then picked from the dishes with a micropipette and transferred to 1,5 ml test tubes containing Hank's balanced salt solution (HBSS) supplemented with 2% FBS.

3. *Immunostaining*

Selected culture dishes were processed for immunostaining as described in [11]. Medium was removed from the dishes and cells were fixed with a 1:1 v/v mixture of acetone and methanol for 1 minute. Cells were then washed and blocked with Tris-HCl buffered saline (0.1 M Tris HCl, 0.14 M NaCl, pH 7.6) supplemented with 10% goat serum (all reagents from Sigma-Aldrich). Dishes were then incubated with primary antibodies for 1 hour at room temperature followed by another hour with secondary fluorochrome-conjugated antibodies. Nuclei were then counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at a concentration of 0,5 µg/ml.

The following antibodies were used in the protocol: anti-human cytokeratin 14 (CK14, 1:500 dilution, polyclonal AF-64, Covance), an anti-bovine CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich) and an anti-p63 (1:200 dilution, clone 4A4, Thermo Fisher Scientific) as well as secondary antibodies: AlexaFluor® 488-labelled goat anti-rabbit IgG and AlexaFluor® 594-labelled goat anti-mouse IgG (both from Life Technologies).

Negatively stained controls were performed for each antigen by replacing the primary antibody with a suitable isotype (normal mouse IgG or normal rabbit IgG from Santa Cruz Biotechnology Inc.) at the same concentration.

4. Flow Cytometry

After picking the colonies from the CFC assays, cells were digested with warm (37°C) trypsin (Sigma-Aldrich) for 2 minutes while pipetting. Trypsin was subsequently neutralized with cold HBSS supplemented with 2% FBS. The resulting single cell suspension was then stained for flow cytometry. Staining for aldehyde dehydrogenase I (ALDH1) was done with the ALDEFLUOR kit (STEMCELL Technologies) according to the instructions provided by the manufacturer. Cells were incubated for 30 minutes at 37°C with the ALDEFLUOR substrate and then stained with an R-PE conjugated anti-human CD49f (1: 25 dilution in 50 µl volume, clone GoH3, Santa Cruz Biotechnology Inc.). DAPI was then added at a concentration of 0.25 µg/ml in order to discriminate live from dead cells.
Cells were then run on an Attune® Cytometer (Life Technologies) equipped with a 405 nm and a 488 nm lasers.

5. Xenografts

Colonies were picked from the CFC assay, separated according to their morphology and single cell suspensions were prepared with the same protocol used for the flow cytometry analysis.
After dissociation, cells were mixed with 10T1/2 mouse fetal fibroblasts that were previously treated with mitomycin C at a concentration of 2 µg/ml for 16 hours. Cells were then embedded in rat tail collagen as previously described [11] and transferred under the kidney capsule of female NOD/SCID mice that received at the same time a silicone pellet (MED-4011, NuSil Technology) containing 2 mg of 17β-estradiol and 4 mg of progesterone (both from Sigma-Aldrich). The kidney capsule was chosen as the site for transplantation since it was previously described by other groups as able to support the growth of human mammary epithelial cells [10,27]. Moreover this site allowed for easier recovery of mammary cells at the end of the assay.
Each gel contained 2000 to 6000 epithelial cells and $1.6x10^5$ 10T1/2 fibroblasts. Six mice were transplanted: gels with luminal-like cells were placed in the left kidney, while gels prepared with myoepithelial-like cells were placed in the right kidney.

After 4 weeks the gels were recovered from the mice. Half of the gels were dissociated by incubation with collagenase (300U/ml, Sigma-Aldrich) for 5 hours at 37 °C in an incubator with 5% CO_2. Cell aggregates were then further digested with warm trypsin (Sigma-Aldrich) for 2 minutes and then used to perform CFC assays. Cells from a single gel were seeded in an individual dish.

The other gels were fixed in 10% formalin (Sigma-Aldrich) and processed for histological analysis.

6. Mice

Female NOD/SCID mice were bred and housed at the animal facility of the Department of Veterinary Science of the University of Turin according to the procedures and guidelines approved by the Italian Ministry of Health. Animal work described in this study has been reviewed and approved by the Italian Ministry of Health. Mice were used at 5 to 10 weeks of age for the transplants described. All surgical procedures were performed under anesthesia using an association of Xylazine and Zolazepam/Tiletamine. All efforts were made to minimize suffering, including administering Carprofen to provide post-surgery analgesia.

7. Statistics

Flow cytometry data were compared with either the univariate or multivariate Probability Binning algorithm described by Roederer et al. [25,26] using FlowJo vX.0.7 by FlowJo LLC.

Progenitor frequencies in CFC assays were compared using the Kolmogorov-Smirnov test.

Supplementary Material

Figure S1 – **Comparison of xenografts with luminal or myoepithelial colonies**. Top images ((i) through (iv)) show a representative section of a gel seeded with cells from myoepithelial (M) colonies. DAPI (i), CK 18 (ii), CK 14 (iii) staining are shown along with an overlay of all 3 stainings. Images (v) to (viii) show the same stainings on a representative section of a gel seeded with cells from luminal colonies. Bars correspond to 500 µm.

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Author contributions

EM conceived the study, carried out the flow cytometric analysis and drafted the manuscript. DC carried out the Colony Forming Cell assays. SM performed the transplantation in immunodeficient mice. PA carried out the immunofluorescence staining. MB participated in the study design and helped to draft the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Fig.1

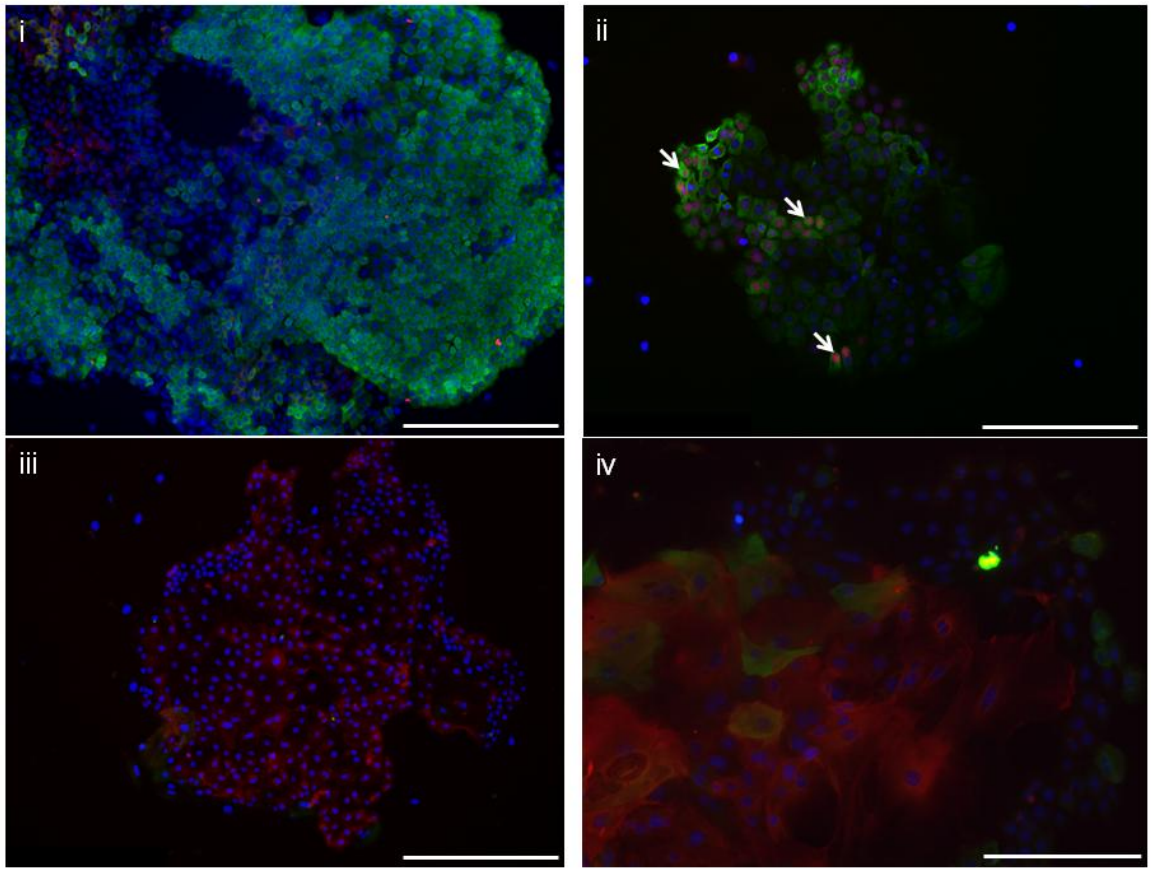
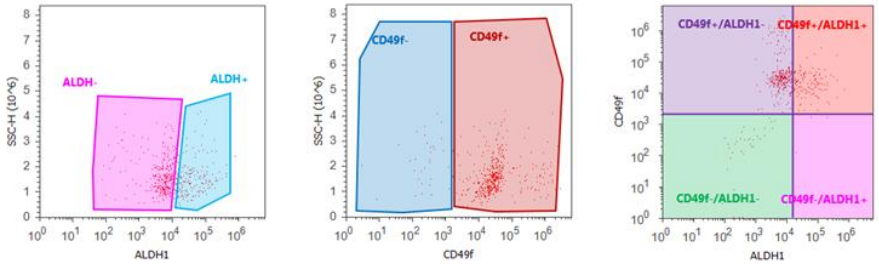


Fig.2

Myoepithelial



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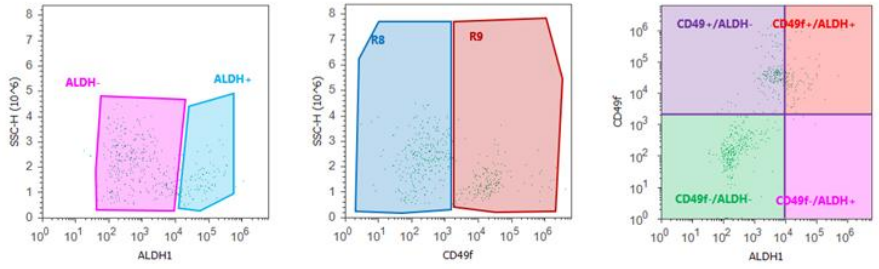
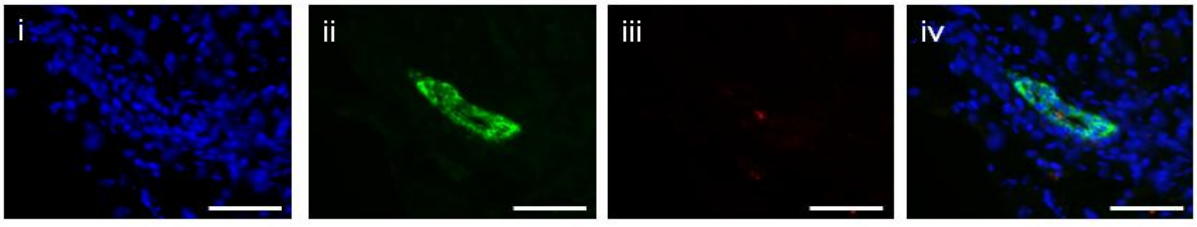
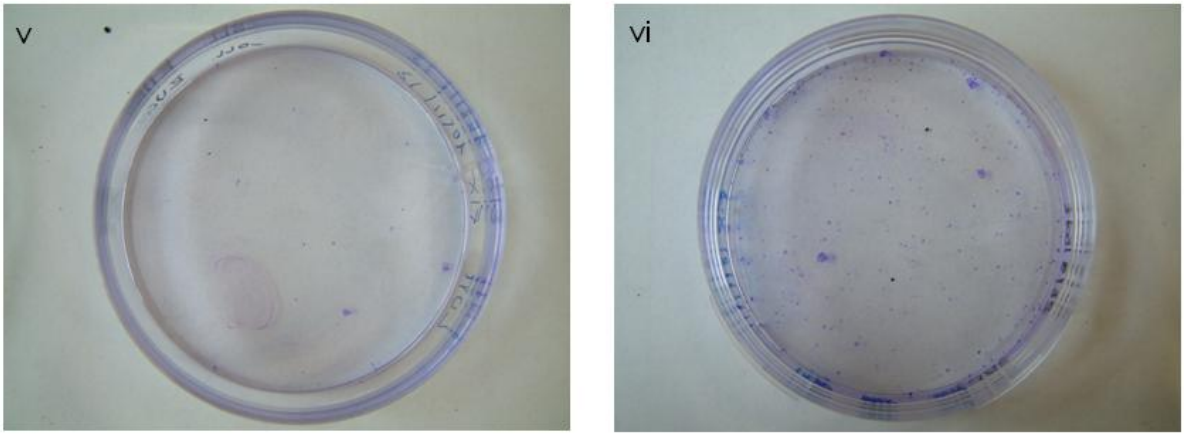


Fig.3





Supplementary Fig. 1