Author's response to reviews

Title: Isolation and Characterization of Side Population Stem Cells in Articular Synovial Tissue

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Author's response to reviews: see over
Dear Dr Robin Cassady-Cain,

Assistant Editor, BMC-series journals

Re: Manuscript ID 1702346161772716 entitled "Isolation and Characterization of Side Population Stem Cells in Articular Synovial Tissue"

We are most grateful to you and the reviewers for your helpful comments regarding our manuscript. We have addressed all the comments by reviewers as indicated in the attached file, and we hope that the explanations and revisions of our work are satisfactory.

In the comment for original version, we received an advice that the manuscript must have been declared the approval of an appropriate ethics committee. However, all animal materials used in this study were by-products of industrial process in local slaughterhouse, and we think that the present work was exceptions of any experimental animal care guidelines.

Revised manuscript was reviewed and proofed by native English speakers.

I hope that the manuscript as revised addresses your concerns and will be approved for publication. I look forward to hearing further from you.
Yours sincerely.

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We are grateful to reviewer 1 for the critical comments and useful suggestions that have helped us to improve our manuscript considerably. As indicated in the response that follows, we have taken all the comments and suggestions into account in the revised version of our manuscript.

**Reviewer: Dr. Margarete K. Akens**

This manuscript describes a study investigating the side population of bovine synovial tissue cells. The authors illustrate a potential cell source for cartilaginous tissue engineering, where only a small amount of host tissue is needed to be expanded in cell culture.

However, the manuscript requires major compulsory revisions before a decision on publication can be reached.

**Background:** This section does not clearly outline the state-of-the-art knowledge and the novelty aspect of the presented study in comparison to already published papers.

A1: According your suggestion, we inserted the following sentence to obtain mutual interesting and understanding by readers “Stem cells from synovium membrane have been aspired as a fascinating source for use in regenerative medicine since it is possible to harvest from the joint without the damage on the healthy articular cartilage. Sekiya, et al. reported that synovial tissue is an excellent source since its-derived stem cells possessed high chondrogenic potential, however, the specific cell markers of these
have not yet been determined, and the way for efficient purification also
have not been developed.

Recent development of the stem cell biology demonstrated the
presence of side population (SP) cells. They are identified by their unique
fluorescence-activated cell sorting (FACS) profile, regardless of tissue
origin. When separated by a flow cytometer with a UV laser, SP cells are
distinct from cells that take up the Hoechst 33342 (Ho) dye. The SP
phenotype is determined by the BCRP1/ABCG2 gene that was expressed in
pluripotent ES cells and multipotent somatic stem cells. Isolation of SP stem
cells by the Ho-dye and FACS technique is reported in muscular tissue, liver,
lung, skin, uterus, testis and cornea by the present.

It was reported that SP stem cells of bone marrow origin displayed
strong hematopoietic reconstituting activity as measured by competitive
repopulation assays, and marrow SP cells also give rise to endothelial cells
and skeletal and cardiac muscles. Moreover, SP cells from the skeletal
muscle might be reconstitute both the skeletal muscle and the hematopoietic
system of recipients. From these, it is expected that the SP stem cells of
somatic tissues possess superior properties, and it’s easy to surmise that SP
cells in synovium tissues also possess valuable properties compared to other
type of somatic cells, and expected as more excellent source of regenerative
medicine.

SP stem cells of synovium tissue have been founded by Yamane et
al., and reported that it possess high chondrogenic properties; however, the precise characteristics such as stem cell marker gene expression profiles or multiple differentiation properties were not determined. Furthermore, the culture system for the SP stem cells have not been developed.

In the present study, we determined that the SP cells expressed important stem/progenitor cell markers CD34, Flk-1, c-Kit and Abcg-2, and it possessed multiple differentiation potentials to chondrocyte, osteocyte and myocyte. Furthermore, we also succeeded in expansion in vitro without losing their property. Our method suggested the way to amplify necessary stem cells from a little volume of tissue, it might be an effective way to prepare materials for the regenerative medicine”.

Methods: Harvesting synovial membrane; please verify the correctness of the joint used in the present study. How old were the animals?

A2: According to your suggestion, we added the sentence “Fibrous synovial membrane (wet weight 1.5–2.5g) was obtained aseptically from the metacarpophalangeal joint of freshly slaughtered calves about 10 months of age” in Materials and Methods.

Overall, the rationale for the methods used is not clearly described. The abbreviation PI is used without being introduced.

A3: In the revised version, we rewrote the Materials and Method section in more detail and introduced the abbreviation PI as “propidium iodide”.

Figure 4.
SEM should be replaced with SD. Student’s t-test will be inappropriate. S and N should be explained.

A4: According to your suggestion, we remade the figure using a mean value with S.D., and included it as Figure 3A. Observation of significant differences were performed by one-way ANOVA analysis followed by Tukey’s multiple comparison tests in the revised version. Furthermore, the experiments were replicated 6 times. This sentence was added to the Materials and Methods sections.

The abbreviation PI is used without being introduced.

A5: In the revised version, we introduce the abbreviation PI as “propidium iodide”.

PCR: In the text the genes of interest are not described. This reviewer is familiar with the gene expression determination based on Ct values, but it needs more explanation because it is not as widely known as the use of conventional PCR. How often were the experiments performed/repeated?

A6: According to your suggestion, we added a detailed explanation of real-time PCR based quantification as follows: “To quantify the relative expression of each gene, the Ct (threshold cycle) values were normalized for endogenous reference (delta Ct= Ct target- Ct β actin) and compared with a calibrator, using the “delta-delta Ct method” (delta-delta Ct= delta Ct sample – delta Ct calibrator). As a calibrator, we used the average Ct value of synovium
tissues obtained from three different animals. Using the Ct value, relative expression was calculated \( (2^{-\Delta \Delta \text{Ct}}) \). As the delta Ct method is only applicable when the amplification efficiencies of the target and the reference are essentially equal, we determined the efficiencies for 5 dilutions and the delta Ct values \( (\text{Ct}_{\text{target}} - \text{Ct}_{\beta \text{actin}}) \) were plotted against the dilution (log). The slope of the fitted line was then determined. A slope of less than 0.1 is then indicative of equal efficiencies (data not shown). The target genes in the present experiment were CD34, Flk-1, c-Kit, Abcg-2 and Mdr-1. All experiments included negative controls consisting of no cDNA for each primer pair. All samples were tested in duplicate, and the average values were used for quantification. Primers were designed to span exons to distinguish cDNA from genomic DNA products (table 1)” in the Materials and Methods section in the revised manuscript.

**Results:** Why do the authors show PCR products on a gel when the authors used the Applied Biosystems 7700 real-time PCR System? The Ct values could be expressed in graphs. The authors mentioned the use of statistical tests, but there are no values reported in the result section.

A7: In the experiment performed for determination of differentiation, we used the primers already constructed for RT-PCR in other project. So, not all of these were appropriate for using real-time PCR based assay about the melting temperature, amplification efficiency, and size of products. By the reasons, we performed the differentiation evaluation by RT-PCR using
ABI9700. In revised manuscript, we added the new sentence “The cDNA from each sample was diluted and used for an RT-PCR-based assay for Collagen II, Collagen I, CD-RAP, Aggrecan, Mvo-D, Myf-5 and Osteocalcin. PCR amplifications were performed at 95°C for 2 min followed by 35 cycles of 94°C, 20 s; 58°C, 20 s; and 72°C, 20 s using Platinum Taq PCRx DNA polymerase (Invitrogen) with appropriate primers (table 1) according to the manufacturer’s instructions. All primers for PCR were designed on two different putative exons so as to span one intron or designed to span a putative exon-exon junction. Amplicons were analyzed by agarose-gel electro-phoresis and ethidium bromide-staining.” in Materials and Methods

The statistical tests were performed again, and are annotated with “All values are mean ±S.D. of the 5 samples of SP (S) and NSP (N), and 6 samples of precultured SP (cSP) and precultured NSP (cN). Significant differences were found using ANOVA and Tukey’s multiple comparison tests. Asterisks on bars indicate significant difference (P<0.05).” in the figure legend of Figure 3A in the revised version.

Discussion: The authors write that the difference in their results compared with Yamane and Reddi are caused by different Hoechst 33342 concentrations. However, Yamane and Reddi harvested synovial membrane from a different joint (knee) compared to the present study. The present authors did not mention the age of their animals. This may
influence the amount of stem cells present in the tissue.

A8: In the revised version, we revised the discussion with the following sentence: “Recently, Yamane et al. reported that the SP fraction was only 0.6% in the synovium tissues at medial and lateral femoral condyles of 3-month-old calves. On the other hand, we observed SP fractions in about 2% of the total cells. Furthermore, the flow cytometric profiles of the two reports were totally different. This discrepancy might be due to differences of tissue origins, it was possible that the numbers of SP cells were different in the area of synovium tissues. Another factor might be based on the difference in the cell sorting condition. For instance, they used 10 µg/ml of Hoechst 33342 dye and we used it at 1.8 – 2.0 µg/ml. The experiment for determination of SP cells was dependent on the staining protocol which is subject to variations in timing, temperature, cell concentration, and Ho-dye concentration”.

What is the relevance for the increase of the stem cell markers Abcg-2 and Bmi-1 when cultured in StemPro-34SFM with 1% FBS?

A9: In the original version, we stated “When SP cells were cultured in StemPro-34SFM with 1% FBS, the expressions of two important stem cell markers Abcg-2 and Bmi-1 were dramatically increased (Figure 4). Abcg-2 is the molecular determinant for the SP phenotype and has been postulated as a universal stem cell marker [16]. And Bmi-1, a member of the polycomb gene family, is a critical determinant of the self-renewal capacity of stem
cells [17, 18]. These results also demonstrated that the stem cell proliferation was promoted by the preculture.”

We think that the elevation of the two markers Abcg-2 and Bmi-1 might be evidence for describing two types of conditions of cells, *i.e*

1. The cells proliferated (stem cell specific self-renewal)
2. The expression of Abcg-1 gene relating to Ho-efflux properties was maintained, which is reported as one of the stem cell specific characteristics.

From these, we suggested that the stem cells cultured in StemPro-34SFM with 1% FBS could proliferate while maintaining stem cell characteristics. The relevance of the relation of methods to results, StemPro-34SFM are originally developed for culture of CD34 positive hematopoietic stem cells. The medium also has been used for other types of stem cells such as germline stem cells (Kanatsu-Shinohara M et al. (2008) Biol Reprod (in press) Pluripotency of a Single Spermatogonial Stem Cell in Mice. Seandel M et al. (2007) Nature 449(7160):346-50. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors).

Therefore, it is acceptable that synovium stem cells selectively expanded in the medium. However, in the revised manuscript, we omitted the sentence and added more detailed experiments with more reliable evidence about self-renewal as *Figure 4.*

Revised manuscript was reviewed and proofed by native English speakers.
We are grateful to reviewer 2 for the critical comments and useful suggestions that have helped us to improve our manuscript considerably. As indicated in the response that follows, we have taken all the comments and suggestions into account in the revised version of our manuscript.

**Reviewer: Dr. Ichiro Sekiya**

The authors demonstrated the characteristics of SP cells in synovium-derived cells. The theme in this paper seems to be interesting.

The authors need to respond to the following comments and questions.

**Figure 1. (a) Are titles of X-axis and Y-axis correct?**

**How were R1 and R2 distinguished? (b) The titles of X-axis and Y-axis should be shown. How did the authors define SP cells in this paper?**

A1: In the original version, we showed FSC-SSC profiles that were obtained using settings of High-threshold and Low SSC. We received some important advice about the settings of the flow-cytometer. In the revised version, we uploaded the new figures about FACS based characteristics. We defined SP fractions using verapamil, which is an inhibitor for stem cell specific ABC transporter. The explanation of this was covered in the **Materials and Methods** as “The dye exclusion property of SP cells is partly due to the functional ABC transporters and verapamil effectively blocks the ABC membrane transporter from extruding the Hoechst dye. With the
addition of verapamil the presence of the SP fraction in region 3 was clearly
eliminated (See additional file 1), indicating that the cells in this region
were candidates for stem cells.”

Figure 2. What does tissue mean? What is the best concentration
of FBS to obtain highest rate of SP cells? The best concentration may be
less than 1%. The authors should discuss the optimal concentration of
FBS?

A2: In the revised version, we described the process of optimization of the
Hoechst33342 concentrations as “The rate of cell adhesion and growth was
significantly enhanced when serum was added at 1 to 5% concentrations to
the medium. In contrast, less than 1% serum addition could not support cell
adhesion and proliferation (see additional file 2), and over 10% serum
addition significantly diminished the expansion of SP cells (figure 2A). In
1% FBS containing StemPro-34SFM, small spindle-like cells were
expanded, and formations of small colonies were observed (figure 2B).” in
Results and as morphological data in additional file 2.

Figure 3. Can SP cells and non-SP cells be distinguished by
morphology? This should be discussed.

A3: In the revised version, we showed the phase-contrast images of the cells
in Figure 2B, and added a sentence as per your advice in the Discussion.

Figure 4.

SEM should be replaced with SD. Student’s t-test will be
inappropriate. â##Sâ## and â##Nâ## should be explained.

A4: According to your suggestion, we calculated the results by Tukey’s multiple comparison tests. In the text, we stated: “Significant difference was detected by one-way ANOVA analysis followed by Tukey’s multiple comparison tests. Comparisons between cultured SP cells and cultured MP cells in figure 4 were performed by Dunnet’s test and between SP cells at primary culture followed by student t-test. A P-value of less than 0.05 was considered significant.” in the Materials and Methods. About Figure 4 (Figure 3 in the revised version), the sentence “Significant differences were observed using ANOVA and Tukey’s multiple comparison tests. Asterisks on bars indicate significant differences (P<0.05).” were described in Legend.

The abbreviations were introduced in Figure Legend of Figure 3 in revised manuscript.

Figure 5.

The quality of cartilage appears to be poor.

Other pellets should be shown with a normal cartilage tissue as a positive control. Otherwise, histology of pellets should be deleted. Arrows in the pictures should be explained in the legends.

Cartilage-related gene expressions appear to be similar in â##SP Difâ## and â##NSP Difâ##. This should be discussed.

A4: In the revised version, higher-resolution images were used (shown in figure 5A) with normal cartilage tissue (shown in additional file 4). About
the chondrogenic potential of NSP cells, we stated as:” It was hypothesized that NSP fractions might include some kind of progenitor cells that could differentiate into chondrocyte or osteocyte”.

**Figure 6.** $(c$ and $d)$ is wrong. $(a$ and $b)$ is correct. Arrows in the pictures should be explained in the legends. The authors should discuss the difference of osteogenic potential between SP cells and NSP cells.

A5: According to your suggestion, we amended the abbreviation in legends, and referred to the differentiation potentials of SP and NSP in **Discussion** of the revised version: “In our study, NSP cells used as control also represented differentiation properties to chondrocyte and osteocyte. It was hypothesized that NSP fractions might include some kind of progenitor cells that could differentiate into chondrocyte or osteocyte”.

**Figure 7.** The authors should discuss the difference of myogenic potential between SP cells and NSP cells. Is ActinB similar to B-actin shown in Figure 5e?

A6: In the revised version, we addressed the myogenic potentials of SP cells in **Discussion** as follows: “On the other hand, myogenic properties were only observed in SP cells. As stimulating chemicals for myogenic differentiation from MSC 5’ azacytidine that is an analog of cytidine was used. The chemical functions by incorporation into DNA, and results in hypomethylation of the DNA. Although myocyte differentiation was
performed without any specific chemicals or cytokines in our study, about 1% of derivatives expressed Desmin, a muscle-specific intermediate filament protein. From these results, it was supposed that the SP cells possess high differentiation potentials”.

Abbreviation of β-actin was amended.

**Results**

Last passage of first paragraph, “The SP cells in region 2 were expressed stem cell makers such as CD34, Flk-1, c-Kit, Abcg-2 and Mdr-1. is duplicated. This sentence should be deleted here.

*Why is StemPro-34SFM superior to DMEM to obtain SP cells? This should be discussed.*

A7: In the revised version, we revised the last passage of the first paragraph per your suggestion. A sentence discussing about superior point of StemPro-34-SFM was added in **Discussion** of the revised version. As to the relevance of using StemPro-34SFM, the medium was originally developed for culture of CD34 positive hematopoietic stem cells. The medium also have been used for other types of stem cells such as germline stem cells (Kanatsu-Shinohara M et al (2008) Biol Reprod (in press) Pluripotency of a Single Spermatogonial Stem Cell in Mice, Seandel M et al (2007) Nature 449(7160):346-50. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors). Therefore, it is acceptable that synovium stem cells selectively expanded in the medium.
In revised manuscript, we added the following sentence “When ordinal culture medium DMEM was used as basic medium, any effects for expansion nor decreasing the rates of SP fractions dependent on serum concentrations were not observed. StemPro34-SFM is a medium specifically developed for CD34 expressing hematopoietic stem cell culture, and the effects for other type of stem cell such as spermatogonial stem cells were reported. Although the mechanism or molecules by which the expansion of SP cells were stimulated in the medium is not known, the factors necessary for the maintenance of these stem cells are the defined components of StemPro34-SFM, and may be common with hematopoietic stem cells.”

The authors state â##In 1% FBS containing StemPro-34SFM, small spindle-like cells were expanded, and formations of small colonies were also observed.â## The picture of colonies should be shown.

A8: According to your suggestion, we added figures in Figure 2B of the revised version.

The authors state â##Pellets from SP cells tend to have greater amounts of cartilage matrix than did pellets from non-SP cells, as shown by staining with toluidine blue and alcian blue (Figure 5a and 5b).â## If the authors insist the superiority of chondrogenic potential of SP cells, quantitative analysis is required.

A9: According to your suggestion, we added figures to Figure 2B of the revised version. About the quantitative analysis, we attempted to quantify
the matrix using a GAG quantification Kit (Primary Cells Co., Ltd.). But we failed in performing calculations of the control experiment using primary chondrocyte perhaps out of inexperience in quantification or because our differentiation system was not optimal. In the revised manuscript, we stated: “In chondrogenic culture, the cell pellets became spherical, and the pellets from SP cells tend to have greater amounts of cartilage matrix than did pellets from NSP cells”, and described that there is room for improvement in our differentiation systems.

We are planning to examine superior differentiation systems with serum-free, BMP supplemented systems (Sekiya I et al (2005) Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 320:269-76)

**Discussion**

Why did the authors used 1.8 \( \mu \)g/ml of Hoechst 33342 for analyzing, and used at 2.0 \( \mu \)g/ml for cell sorting instead of 10 \( \mu \)g/ml reported previously.

A10: The experiment for determination of SP cells was dependent on the staining protocol with regard to timing, temperature, cell concentration, and Ho-dye concentration. Furthermore, the FACS patterns were altered by “lot” of the dye. We first optimized our lot (so, we described the Lot No of Hoechst33342m verapamil and FBS in Materials and Methods in original version) and decided the range of 1.8 – 2.0\( \mu \)g/ml. The process of
optimization of the Hoechst33342 concentrations was shown as additional file 1 and Materials and Methods in the revised version. To explain the reason for the concentration difference between analyzing and sorting, we added the sentence “For analyzing the SP rates, cell suspensions were incubated in FACS buffer containing Ho-dye at 1.8 µg/ml for 90 minutes at 37°C. For sorting, cells were stained with 2.0 µg/ml under the same conditions to perform subsequent analysis closely” in Materials and Methods.

Did the high serum concentration repress the self-renewal of SP cells? Otherwise, did it differentiate SP cells into somatic cells? I think the authors can answer the question according to their results.

A11: In revised manuscript, we presented the figure shown the expansion of differentiated cells in additional file 2 and added the sentence “It is possible that the high serum concentration induced differentiation SP cells to differentiated somatic cells, promote expansion of differentiated cells and, as a result, the relative rate of SP fractions decreased” according to your suggestion.

Methods. Why were precultured and sorted cells cultured in the presence of bFGF, SCF and insulin-transferrin-serenium? Are these essential?

To confirm the differentiation potency, the authors used 7-times passaged cells. I am afraid the passage number may affect
differentiation potential. This should be discussed.

A12: About the questions, we performed a new experiment and presented them in **Results** and **Discussion** in the revised version. The results were described in **Figure 4**. Growth factor requirements were covered as follows:

“To achieve further enhancement of the cell number, we then examined the conditions for ex vivo expansion of sorted SP cells. For maintaining the SP cell proliferation while keeping their specific characters as stem cells, we found that the supplementation with three factors (bFGF, SCF and Fn) were effective. The FGF families are important factors for proliferation of variable stem cells such as MSC, neural stem cell, and embryonic stem cell. SCF plays an important role via a c-Kit receptor in the recruitment of adult hematopoietic stem cells, or formation of germ stem cells *in vivo*. Also *in vitro*, SCF is essential factor for the maintenance of hematopoietic stem cell.

Fn is a kind of extra-cellular matrix glycoprotein, which functions in promoting cell adhesion. In hematopoietic tissues, stem cells adhere to the Fn and endothelial vascular cell adhesion molecule (V-CAM) by integrin and cell surface glycosaminoglycans. And the molecule functions as the crucial factor for maintenance of *ex vivo* culture. The above culture condition enables the logarithmic expansion while maintaining some important original characteristics. After continued passaging and culturing, the expression of Abcg-2 mRNA was still maintained at a high-rate compared with SP cells *in vivo*. Importantly, this mRNA expression
reflected as the phenotype that the cultured SP cells also represented the Ho-efflux properties and Ver-sensitivity”.

To prepare RNA from cartilage pellets, how many pellets were used? How were they digested?

A13: According to your suggestion, we added a more detailed explanation in Materials and Methods as: “Sorted cells or cultured cells were treated with TRIzol reagent (Invitrogen) and mixed thoroughly with pipetting. To dissociate the pellets of chondrocyte obtained by differentiation assay, the pellet was disrupted using a 21-G needle and syringe in TRIzol reagent. The Trizol-lysates were then mixed with chloroform and centrifuged at 15,000 × g for 15 minutes. Following centrifugation, total RNA were obtained by isopropanol precipitation according to the manufacturer’s instructions”.

Revised manuscript was reviewed and proofed by native English speakers.
We are grateful to reviewer 3 for the critical comments and useful suggestions that have helped us to improve our manuscript considerably. As indicated in the response that follows, we have taken all the comments and suggestions into account in the revised version of our manuscript.

**Reviewer: Dr. Charles Archer**

The flow cytometry data is plausible, however it would have been enhanced by the presence of positive controls (bone marrow cells) as this method was designed with these cells in mind. Also the markers used are all haematopoietic stem cell markers I would like to have seen more mesenchymal stem cell markers. By culturing the cells in 1% serum an initial heterogeneous population appears to have been enriched for cells within a haematopoietic lineage. The multi-lineage differentiation studies produced good results for myogenesis, although I would like to see more images for osteogenesis and clearer images for chondrogenesis.

**General points**

1. **Why were different Hoechst concentrations used for analyzing and sorting?**

2. **Why was the concentration of Hoechst so low, can you include details of titration or optimization?**

   A1: In the original version, we showed FSC-SSC profiles that obtained by the settings of High-threshold and Low SSC. In revised experiment, we reexamined the settings of flow-cytometer according to your important advises. We uploaded the new figures about FACS based characteristics. Furthermore, the processes for optimization of the Hoechst33342
concentrations were showed as Additional file 1 and Materials and Methods in revised version.

**Figure 1A**

a. What are the cell types in the two regions?  
A2 for Q-a: In revision, we reexamined the Flow-cytometric analysis about mainly FSC/SSC profiles, live/dead cell evaluation and microscopic evaluation for the cells included in each population. The results of these experiments were stated as “In case of the bovine synovial cells, three distinct regions were observed after exclusion dead cells and debris (figure 1A). The cells included in population 1 (P1) and population 2 (R2) were smaller than that of population 3 (P3), no adherent cells were observed when they were seeded on culture dishes. On the other hand, population 3 were consisted with fibroblast-like cells and included in distinct side population (SP) (figure 1B).” in the Results section.

b. It may be useful to display a density dot plot, or reduce the percentage of data shown in the dot plot to give the reader a better indication of scatter.

c. The threshold on the machine was set very high (approx. 20,000) this does not allow us to see any of the debris/dead cells that are present in the sample and will be present in the side population plot.

d. The side scatter voltage should have been increased when running the sample to make it easier to analyze the sample.

A2 for Q-b to Q-d: These suggestions were very useful for the experiment in revision. We represented new figures as Figure 1. And in the new figures,
we represented the patterns obtained by the settings without any threshold. Furthermore, we changed the indication of the figures dot-plot to density-plot.

e. The dot plot seems to contain a lot of debris and doublets; this may have been helped by filtering immediately prior to running the sample and with the use of Propidium Iodide to exclude late apoptotic/dead cells.

A2 for Q-e: Also in the previous experiments, we had used filters to exclude debris and aggregates. In revised manuscript, we represented the figures certified exclusion of dead cells and debris in Figure 1.

Figure 1B

a. The plot size needs to increase to show clearer labeling.

b. The plots for test and control samples are the same for region1, the use of bone marrow cells as a positive control is required

A3 for Q-a and b: To our regret, we could not increase the plot size since synovium membranes used in our studies did not contain so enough number of cells. In the Figure 1 in revised version, we represented 30,000 counts (This scale was maximum in our experiment) per population 3. About the control experiment suggested above, our co-author S.K (Third author) already experienced to collect SP cells from mouse bone marrow in other project with some different groups. They all used same staining protocol and we don’t think that our system include serious problems.

Figure 2 - There is considerable variation of percentages for a small n-number. The SP decreases from 5% serum not over 10%. I would like to have seen more repeats (increase n-number) and side population dot plots for each concentration of
serum.

A4: We repeated the experiments according to your advice, and showed the values as N=6 in Figure 2. Side population dot plots for different concentration of serum were presented in the Additional file 3 in the revised manuscript.

a. I would like to see low power images to get a better idea of overall morphology.

b. C & D appear to have a number of rounded (apoptotic/dead) cells. Low power images would provide more information on their number in the sample.

A4: In the revised version, lower power images were represented. Furthermore, we prepared the new figure without floating cells. Lower power images of the cultures in various serum concentrations were represented as Additional file 2.

Figure 5E The annotation needs to be clearer, there is no difference between SP diff and NSP diff, are these the cultured cells?

Figure 6 The annotation needs to be clearer. The labelling in NSP and SP is very similar. What population does D relate to?

A5: According to your suggestion, we amended the abbreviation in Legends, and stated the differentiation potentials of SP and NSP in Discussion of the revised version. For differentiation experiment, we used cultured and passaged SP cells.

Figure 7 A&B I would like to see control images.

A6: In the present manuscript, we could not prepare any control figures about muscular tissues since we could not obtain appropriate tissues.
However, we already confirmed the specificity of the antibody in other experiment using mouse tissue.

Revised manuscript was reviewed and proofed by native English speakers.