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INTRODUCTION

- Several forms of evidence suggest that heterologous transplantation of porcine adipose-derived stem cells (ADSC) enhances bone healing (*Wilson et al., 2010*).
- Freshly harvested ADSC are a heterogeneous population, which contain several types of cells other than stem cells.
- The isolation of highly purified ADSC can be of clinical importance.
- The CD34 protein is a known marker of hematopoietic stem cells and freshly harvested ADSC appear to have high abundance of CD34+ cells; thus, this marker might be specific for ADSC.
- The stemness, features, and clinical efficacy of ADSC CD34+ cells need to be evaluated.

OBJECTIVE

- Main objective was to compare *in vitro* growth and osteogenic capacity and *in vivo* bone healing potential of ADSC freshly harvested and ADSC separated using CD34 as a marker.

MATERIALS AND METHODS

In vitro

- ADSC were extracted from back fat of 4 male pigs of 6 months of age.
- Immediately after extraction aliquots of the ADSC were sorted using magnetic beads (Figure 1) into CD34 positive (CD34+) and negative (CD34-) cells.
- Aliquots of ADSC were analyzed by flow cytometry to evaluate efficacy of sorting by magnetic beads (Figure 1). Viability was assessed after sorting using Trypan Blue staining.
- For the *in vitro* experiment, the unsorted ADSC (ADSC), plus the CD34+, CD34– cells, and a 50:50 mixture of CD34+ and CD34- cells (MIX) were plated in 24 well plates, cultured to ~80% confluence, and differentiated into osteocytes for 18 days [d].
- At 3, 6, and 18 d after addition of osteogenic medium the number of bone nodules was counted in each well and ≥ 7 color pictures of representative nodules per each well was taken. Dimension and density of nodules was assessed by Image J software (only part of the data shown).
- Two wells of each cell type and from each pig were trypsinazed before cell plating, on several time points during expansion, at confluence, and at 6, 12, and 18 d of differentiation for cell counting and RNA extraction.
- Real-time RTPCR (qPCR) was performed for *CD34*, and two osteogenic marker *COL1A1* [collagenase A1] and *SPARC* [osteonectin] genes (*Monaco et al., 2009*). qPCR data were normalized using geometric mean of 4 (out of 6 tested) internal control genes (*BANF1*, *RPS15A*, *NSUN5*, and *NUBP1*) as previously reported (*Monaco et al., 2010*).
- At 18 d differentiation samples for each cell type and each pig were fixed in 10% formalin and stained with alizarin red (AR). Images of the whole well were taken at 100X magnitude by a robotic stage inverted microscope (Olympus IX71) and nodules number and area analyzed using Olympus MicroSuite™. After image analysis AR was extracted and quantify as previously reported (*Gregory et al., 2004*) using a 5 points standard curve.

In vivo

- Mandibular osteoectomies with 10 or 25 mm diameter defects were performed in male pigs (6 month old) and duplicates of ADSC, CD34+, and CD34- cells were injected directly in the defect before suturing (Table 1). Pig were allowed to heal for 8 wks before collection of mandibles after euthanization.
- Mandibles were scanned with Hologic QDR 4500A Dual Energy X-ray Absorptiometry and images analyzed with Subregion Hi-Res software (V. 11.2:3). Difference in bone mineral density (BMD) between the original bone (preserved in liquid N_2) and new-formed bone in the defect was measured.

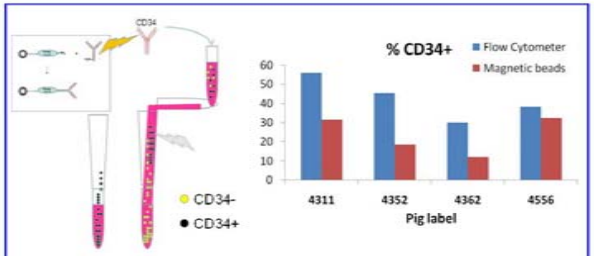

Figure 1. Magnetic sorting of CD34+ cells. ***Left panel*** shows schematic representation of magnetic beads sorting of freshly isolated ADSC CD34+ cells. ***Right panel*** illustrates efficacy of magnetic beads sorting where aliquots of freshly isolated ADSC were sorted by flow cytometer. Data indicate that magnetic sorting was able to isolate between 40 and 80% of CD34+ cells. Viability was similar between cell types (overall mean 82.3±18.0%).

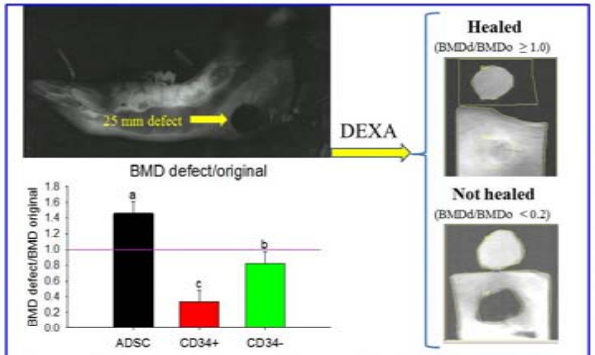

Figure 4. *In vivo* healing capacity. Aliquots of freshly isolated ADSC were sorted by flow cytometer in CD34+ and CD34- cells. The 3 cell types were transplanted in 10 or 25 mm mandible defects and let heal for 8 weeks. The mandibles were then analyzed by DEXA (see example in ***top and right panels***). The data (***bottom left panel***) clearly indicate a larger and complete healing by freshly isolated and unsorted ADSC compared to sorted cells. Among sorted cells CD34- cells had higher healing capacity compare to CD34+ cells.

DISCUSSION

In vitro

- The magnetic beads sorting had between 40 and 80% efficacy (Figure 1); thus, CD34- cells were not pure but contained a considerable amount of CD34+. Porcine ADSC had 42.3±11.0% CD34+ cells, which is >100 fold the usual concentration of CD34+ cells found in bone marrow (<0.1%).
- The ADSC reached confluence earlier compared to sorted cells (Figure 2). The pg RNA/cell decreased during the first 6 d after plating and increased in ADSC after initiation of differentiation. Surprisingly, expression of *CD34* was similar between cell types (Figure 2). As expected its expression decreased after plating and was not affected by differentiation. Among osteogenic genes only expression of *SPARC* increased significantly during differentiation (Figure 2). Those data indicate that ADSC were more active and prone to respond to the *in vitro* conditions compared to the sorting cells. This is partly explainable by the stress due to sorting method.
- ADSC had overall larger osteogenesis compared to sorted cells. The latter had no overall significant differences as shown in Figure 3.

In vivo

- Unsorted ADSC largely enhanced bone healing in both 10 and 25 mm defects (the 25 mm is considered a critical size defect). The ADSC had a significant larger healing compared to sorted cells (Figure 4). Among those CD34- cells appear to be more efficacious compared to CD34+ cells.

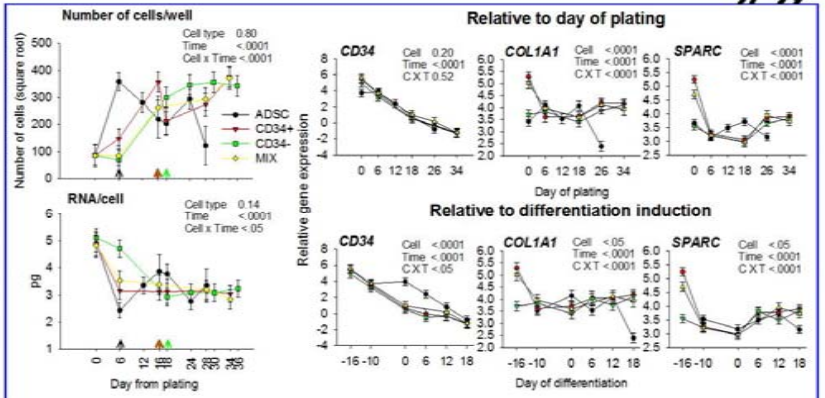

Figure 2. Cell count, RNA/cell, and qPCR. ***Left panels*** illustrate the number of cells counted and pg RNA/cell at several time points relative to plating in ADSC, CD34+, CD34-, and MIX cells. Vertical arrows denote the beginning of osteogenic differentiation (ca. 80% confluence) for ADSC (black), CD34+ (red), CD34- (green), and MIX (dark yellow) cells. ***Right panels*** illustrate temporal mRNA transcription relative to the day of plating (top panels; time 0 = prior to plating) or to the beginning of differentiation (bottom panels; time -16 = prior to plating) of the marker *CD34* and of the osteogenic genes *COL1A1*, and *SPARC*.

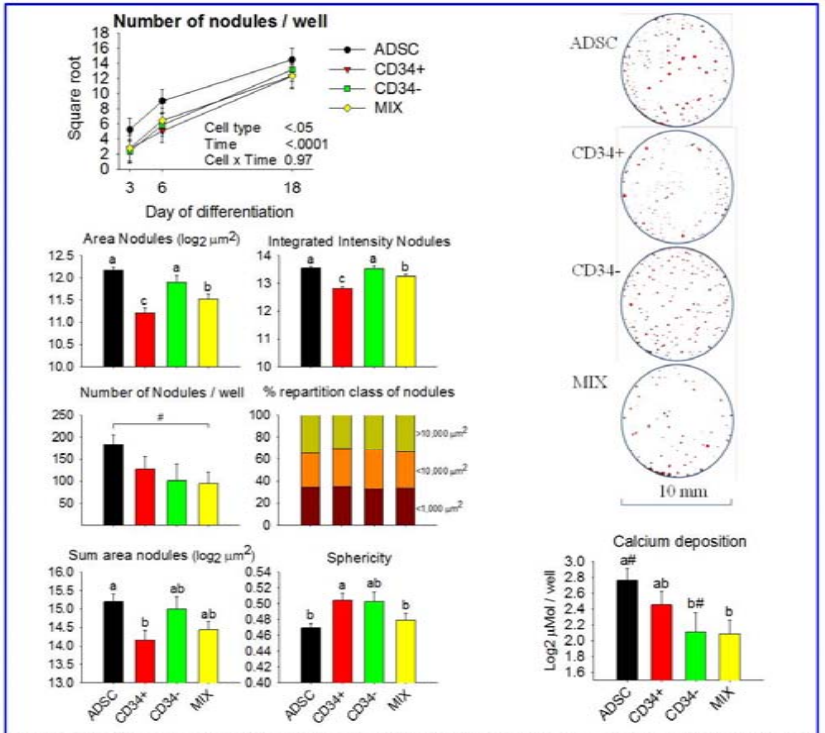

Figure 3. *In vitro* osteogenic differentiation. ***Top left panel*** illustrates the temporal increase in the number of nodules / well. ADSC had the greatest number of nodules in all points evaluated; no differences were observed between the sorted cells. ***Bottom left panels*** show several measurements of nodule formation at 18 d of differentiation. ***Right bottom panel*** illustrates calcium deposition / well through relative quantification of alizarin red (1 mol AR = 2 Mol calcium) at 18 d differentiation. ***Right top figures*** are representative examples for each cell type of nodule formation in the whole well (false red color). (a-b-c denote differences with p<.05 between cell types; # denote tendency [p<0.10]).

LITERATURE CITED

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