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Research Article

Adipose-Derived Stem Migration in the Vascular System after Transplantation and the Potential Colonization of Ectopic Sites in Swine

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Abstract

Background: Adipose-derived Stem Cells (ASC) are obtained from adipose tissue. They can be harvested by liposuction under local anesthesia, making these cells particularly desirable for use in tissue engineering or cell transplantation. However, little is known about the *in-vivo* characteristics of these cells post-transplantation.

Methods and Findings: Here we evaluate the potential of ASC to migrate systemically and the potential for accumulation and colonization in ectopic tissues in a pig model. ASC injected via ear vein can travel through the entire vasculature of the pig within 60 seconds and the cells continue to be present in the bloodstream for at least 1-hour post-transplantation. However, labeled cells were not present in the bloodstream at 2 or 4 weeks after ASC injection. The injected ASC appear to travel to areas of induced trauma and are not observed in filtering tissues of the body such as the spleen, liver, lung and liver.

Conclusion: These findings suggest that systemic administration of ASC could be a successful method of cell transplantation for tissue regeneration.

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Keywords

Regeneration; Porcine; Cell Transplantation; Adipose-Derived Stem Cells; Metastasis

Introduction

Achieving targeted trafficking and migration of stem cells is critical for successful tissue regeneration by inducing mobilization via vascular administration. Furthermore, the ability to manipulate stem cell homing could allow these cells to serve as vehicles for *in-vivo* delivery of therapeutic genes or drugs. Stem cells accomplish essential functions in the organization of embryonic tissues during development. In some cases, they are retained into adulthood, where they sustain homeostasis by continuously replacing senescent cells and restoring injured tissues. The self-renewal and trans-differentiation capabilities of stem cells can be considered insignificant unless their migration to target tissues can be coordinated appropriately [1-4]. Unfortunately, the molecular and cellular mechanisms underlying the migration and homing of Adipose-derived Stem Cells (ASC) are not entirely understood. However, there is evidence that adhesion molecules such as selectin, chemokine receptors and integrins play a role in migrating mesenchymal stem cells [2,5-7]. However, it is clear that MSC are capable of specific migration to a site of injury when delivered by intravenous injection [3,6,8-12]. Furthermore, the liberation of ASC from subcutaneous adipose tissue has been recently shown to regulate intramuscular adipocyte deposition in ectopic sites [13].

Although MSC transplants may offer solutions to many medical problems, there has been evidence of cell accumulation and colonization within untargeted tissues, leading to potential tumor formation [14-16]. For example, epithelial cancer formation (oral squamous cell carcinomas) occurred following allogeneic bone marrow transplantation [17]. Another study demonstrated tumor growth in nude mice in response to subcutaneous or intracranial transplantation of a combination of hASC and a tumor cell line [18]. MSC have been found to have a central role in the pathogenesis and progression of tumors [17-21]. The typical structure of solid tumors is composed of two separate compartments, which are dynamically intertwined. One part is parenchymal, which represents the neoplastic portion of the tumor. The other part is the stroma, which is a non-malignant supportive tissue. The stroma includes the extracellular matrix, blood vessels, immune and inflammatory cells, connective tissue and mesenchymal stem cells [22]. The stroma lies between malignant cells and normal host tissues, which is a crucial requirement for tumor growth. Studies demonstrate that MSC dynamically migrate to and proliferate in tumors and contribute to the tumor-associated stroma [21]. Evidence also shows that MSC localize in sites of inflammation within the stroma and are in close proximity or contact with tumor cells as a component of the remodeling process [19,20]. MSC that integrate into the tumor-associated stroma of epithelial tumors may encourage cancer cells to metastasize via MSC-to-tumor cell cross-talk, resulting in the spread of cancer cells [6,19-22].

The objective of the present study was to evaluate the migration and the potential colonization of ectopic sites, a prelude to tumor formation, associated with locally and systemically injecting ASC into the pig model. To establish the safety and the therapeutic benefits of a new technique such as stem cell transplantation, the use of animals as a model for preclinical trials is necessary. Using the Yorkshire pig as our biomedical model, we assessed the suitability of adipose tissue as a source of mesenchymal stem cells for tissue engineering applications. Here we evaluate the ability of ASC to migrate via the vasculature and the potential risk of cell colonization in multiple organs associated with transplanting ASC into the pig model.

Materials and Methods

Swine Model

This project used 15 Yorkshire pigs, weighing between 60-80 kg. Bilateral 10 mm surgical defects in the ramus of the mandible, followed by ASC transplant, were performed on each of the pigs. Surgeries were performed in IACUC approved facilities and all procedures were conducted under protocols (#07009) approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois. All pigs remained healthy up to 4 weeks post-ASC transplantation and at the time of sacrifice, all organs appeared to be normal and functional.

ASC Isolation, Culture, Cryopreservation and Passage

Cells were isolated, cultured, passaged and cryopreserved as previously described [23].

Labeling ASC with CFDA-SE and Preparing Reagent

Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (Vybrant™ CFDA-SE Cell Tracer Kit) was used to label the ASC before injection, making it possible to visualize the cells post sacrifice. The stock solution was Diluted in Phosphate-Buffered Saline (DPBS) to the desired working concentration (0.5-25 μ M). The stock solution was diluted in 45 mL of PBS for a 20 μ M working concentration.

Labeling Cells in Suspension

Cultured cells were trypsinized and centrifuged in a sterile 15 mL tube at 200 x g for 5 min to obtain a cell pellet. The supernatant was aspirated and the cells were gently resuspended in pre-warmed (37°C) DPBS containing the probe (CFDA-SE/DMSO mix). The tube was tightly sealed and incubated for 15 min at 37°C. Cells then were re-pelleted by centrifugation at 200 x g for 5 min and re-suspended in fresh pre-warmed DMEM. The cells were incubated for

another 30 min to ensure complete modification of the probe and finally washed again with DPBS.

Experiment 1: *In-vitro* Loading of Blood Samples with Labeled ASC Study

Blood Processing

Blood was collected from an average healthy male pig into six 10 ml EDTA-containing blood collection tubes and stored at room temperature for approximately 6 hours before processing. After the ASC were labeled and counted, the cells were separated into six 2 ml centrifuge tubes ($\approx 50,000$ cells in $25\mu\text{L}$ DMEM per tube). The labeled ASC were then fixed with 10% buffered formalin. One 2 mL tube of ASC was transferred to one tube of whole blood and the tube was gently mixed for approximately 30 sec to adequately disperse the cells throughout the blood sample. The whole blood was fractionated by centrifuging at $1500\text{--}2000 \times g$ for 10-15 min at room temperature. Small samples of each plasma, buffy coat and red cell layers were carefully collected and used to make cell smears on glass slides.

Bisbenzimidazole Staining

After the cell smears had completely dried, a small drop of 1 mg/ml bisbenzimidazole (Hoechst 33342, Calbiochem) was added and smeared over the slide with a clean glass slide. The bisbenzimidazole was allowed to completely dry on the slides before visualizing the slides with the microscope. Bisbenzimidazole is used as a fluorescent stain for DNA and has an excitation maximum of 358 nm and an emission maximum of 460 nm. The slides were all examined using a Leica DMI 4000 B inverted fluorescent microscope with FITC/DAPI filters and ImagePro Software to collect data from the slides.

Experiment 2: One Minute ASC Migration Study

After the ASC were labeled with CFDA-SE and counted, the cells were transferred to two 2 mL centrifuge tubes ($\approx 2.5 \times 10^6$ cells per 1.5 mL DMEM) and transported to the site of the sacrifice of the experimental pig. The labeled ASC were loaded into a 5 ml sterile syringe ($\approx 5 \times 10^6$ cells per 3 mL DMEM). The full complement of cells was injected directly into that ear vein of an average healthy male pig, weighing approximately 240 lbs. After 60 seconds, the pig was sacrificed by electrical stunning and exsanguination. Blood was collected immediately in six EDTA-containing blood tubes via jugular puncture. The blood tubes were stored at room temperature for approximately 1 hour before processing. Three blood smears were taken from the whole blood samples from each of the six tubes. Cell smears were stained with bisbenzimidazole and examined by fluorescent microscopy as described for Study 1.

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Experiment 3: Mandibular Defect Surgery with ASC Transplantation Flow Cytometry of Blood and Spleen Tissue Study

Surgery

Pigs were anesthetized with a sedative cocktail (TARK) consisting of Telazol® (tiletamine and zolazepam), atropine, xylazine and ketamine before surgery administered Intramuscularly (IM) and Intravenous (IV). The pig was monitored under anesthesia with Halothane®. Using an aseptic surgical technique, retromandibular and submandibular incisions were made through the skin and subcutaneous tissues to the muscular layer.

The pterygomasseteric sling was incised and the masseter muscle was elevated in a subperiosteal plane. The lateral cortex of the mandible was then exposed from the angle superiorly to the level of the condyle and anteriorly to the mental foramina. Bicortical surgical defects, 10 mm in diameter, were created in the posterior region (ramus) of the mandible using a bur technique with a right angle surgical drill and a 10 mm outer diameter trephine while supplying adequate irrigation.

Defect Injection (DI)

For the defect injection pigs, approximately 2.5 million CFDA-SE labeled adipose- derived stem cells, diluted in 0.5 ml of DMEM, were injected directly into each 10 mm defect. Before injecting the cells, a small pouch was sutured, with 3-0 Vicryl, in the periosteum to prevent the cells from spilling out of the defect.

Ear Injection (EVI)

For the ear vein injection pigs, approximately 5 million CFDA-SE labeled cells, diluted in 3 mL of DMEM, were injected into the ear vein via a catheter. Before injecting the cells, the incision was sutured completely. Before and after injecting the cells, the catheter was flushed with a Heparin-saline mixture, ensuring that the blood would not clot in the catheter and all cells would be entirely washed through the catheter.

Control

The same protocol was followed for the control pigs as the Ear Vein Injection group (EVI) except that no cells were administered. The incision was sutured entirely and instead of injecting the CFDA-SE labeled ASC, we injected 3 ml of DMEM without the cells. The catheter was flushed with the Heparin-Saline before and after DMEM injection.

Animal Sacrifice (One Hour Time Point)

For the 1-hour time point pigs, the normal surgery process was carried out until the suture procedure. For both experimental groups and control, the periosteum and fat layers were sutured as usual. However, the muscle and skin layers were "loosely" sutured. The incision was covered with ethanol pads to ensure sterile protocol. The pig remained on halothane at 5% for the one hour before sacrifice, with vitals every 15 min and constant monitoring. After one hour, the pig was removed from the anesthesia machine and rolled into the surgery prep room. At this point, the main arteries and veins of the throat area were severed with a knife allowing the pig to expire from exsanguination. The blood was then collected with 6 EDTA-containing blood tubes via the jugular. Ears, spleen, kidney, lung and liver were collected and put on ice for further analysis. The blood tubes were stored at room temperature for approximately 6 hours before processing.

Animal Sacrifice (2 and 4 Week Time Points)

The pig was sacrificed at the given time point (the 1-hour time point procedure differs slightly). The pig was electro-stunned, rendering it unconscious and euthanized by exsanguination. At that time, 6 EDTA-containing blood tubes were collected via jugular. Ears, spleen, kidney, lung and liver were collected and put on ice for further analysis.

The blood tubes were stored at room temperature for approximately 4 hours before processing.

Blood Processing

All blood samples were stored at room temperature and processed less than 24 hours after collection. Whole blood was fractionated by centrifuging at 1500-2000 x g for 10-15 min at room temperature. The plasma layer was aspirated off and discarded from all six tubes from each animal. The visible buffy coat material was carefully aspirated with a transfer pipette and added to a tube containing 1 ml of DMEM. The cells were gently pipetted up and down to rinse cells thoroughly, then centrifuged and the supernatant removed.

The pellet was resuspended in red blood cell lysing buffer to remove red blood cell contamination from the samples. After two min the samples were centrifuged and the supernatant was removed. The cells were then fixed in 10% buffered formalin, stored in DMEM and used for flow cytometry analysis.

Soft Tissue Processing

All tissue samples were processed within 3 hours after collection. Small sections of each tissue (spleen, liver, lung, liver) were cut into small pieces ($\approx 1 \text{ mm}^3$) and fixed in 10% buffered formalin. The ear vein was collected in the same manner. The ear vein was collected by moving

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the scalpel blade down both sides of the ear vein, completely removing the portion of the ear consisting of the vein from the rest of the ear and then cutting the vein horizontally into sections that were fixed in 10% buffered formalin. All collected pieces of tissue were used for histological analysis. The spleen tissue was treated slightly differently for flow cytometry purposes. The spleen's splenic artery and ventral regions were disaggregated using a glass microscope slide to tear apart the tissue and DMEM was used to collect the cells. The disaggregated tissue was then forced through a sterile mesh material, using a syringe plunger to move the cells through the mesh, flushing with DMEM simultaneously. The samples were then centrifuged, diluted and filtered. These spleen samples were then fixed in 10% formalin and stored in DMEM for use with the flow cytometry.

Microtome Sectioning and H and E Staining of Soft Tissue

The fixed tissues were dehydrated in a series of ethanol treatments and then cleared with xylene before processing paraffin embedding. Paraffin blocks were sectioned (6 μ m) on a rotary microtome with a disposable steel knife. The sections were floated on a 45°C water bath and collected with glass microscope slides. Slides were dried at 65°C for 60 min, allowed to cool and de-paraffinized in a series of xylene baths, followed by an ethanol bath to remove the xylene. Next, the slides were rehydrated in a descending series of ethanol baths and finally rinsed with tap water to remove residual ethanol for 5 min.

The slides were then put into a container filled with hematoxylin for 10 min and rinsed again with tap water to remove hematoxylin for ten more min. The slides were dipped into a jar containing 0.1% HCl 3 times and then into tap water 3 or 4 more times. The slides were immersed in the eosin stain for 3 min and then five times in acetic ethanol. The slides were then dipped in 100% ethanol five times and five more times in a fresh wash of 100% ethanol. The slides were then dipped five times in acetone and then five more times in a new acetone wash. Finally, the slides were immersed five times in xylene and five more times in a fresh xylene wash.

Fluorescent Microscopy

Once the sections were completely dried, they were visualized using a Leica DMI 4000 B inverted fluorescent microscope with the FITC filter to examine the possible existence of the CFDA-labeled stem cells that had been injected. The approximate excitation and emission peaks of CFDA-SE after hydrolysis are 492 nm and 517 nm. All sections were viewed at 40X to examine the sections for the presence of the CFDA-SE specific fluorescence. If specific fluorescence was detected, the questionable area was then observed at 200X.

Results

Experiment 1: *In-vitro* Loading of Blood Samples with Labeled ASC Study

Centrifugation of whole blood with labeled ASC demonstrated that the cells are only present in the white blood cell layer or buffy coat (Fig.1 and 2). By examining the blood smears stained with the bisbenzimidazole, all cells were stained blue and were visualized using the DAPI filter on the fluorescent microscope. The bisbenzimidazole stains the DNA in the nucleus of each cell, allowing us to distinguish between actual cells and debris. The results from the bisbenzimidazole staining revealed that the nuclei of the green fluorescing ASC were stained blue, confirming that the green fluorescing suspected ASC were in fact cells.

Figure 1: Fluorescent microscopy images of blood smears (FITC 200X). These images were taken from the blood smears of the fractionated blood that had been mixed with labeled ASC.

All images were taken with the same fluorescent filter, magnification and software settings. Panel A, is an image from the blood smear of the plasma layer. Panel B, is an image from the blood smear of the White Blood Cells (WBC) layer. Panel C, is an image from the blood smear of the Red Blood Cells (RBC) layer. Traces of labeled debris found in the other layers appeared to be broken or damaged parts of whole cells.

Figure 2: Fluorescent microscopy dual-stained blood cells from WBC blood smear. These images were captured using the FITC/DAPI filters at 20X. Panels a and b are images taken from the same blood smear. Panel a, labeled CFDA-SE ASC in WBC layer (FITC). Panel b, all nuclei is stained blue, including the nuclei of the CFDA-SE labeled ASC.

Experiment 2: One-Minute Migration Study

Labeled ASC are present in the blood smears collected from the jugular vein one minute after injection of the ASC via the ear vein, demonstrating that the labeled ASC migrated through the vasculature of the pig from the point of injection to the end of the collection in the jugular vein within 60 seconds (Fig. 3). The blood smears were stained with a nuclear stain (bisbenzimidazole) to confirm that the fluorescent ASC were intact cells and not debris (Fig. 3 and 4).

Figure 3: Fluorescent microscopy images of CFDA-SE labeled ASC found in blood smears from one-minute migration trial. Panel A, labeled ASC visualized in a blood smear using only the FITC filter. Panel B, all cells stained with bisbenzimidazole visualized in a blood smear using only the DAPI filter (Same view as panel A, but using a different fluorescent filter). The white arrow indicates the stained nucleus of the CFDA-SE labeled ASC. Panel C, dual filter image of panels a and b, showing the blue stained nucleus and the green fluorescing cytoplasm of the ASC.

Figure 4: Closer look at the fluorescence of the ASC found in a blood smear from the one-minute migration study. Panel a, dual filter image of ASC in a blood smear from the one-minute migration study. Panel b, this is an image of the same view from panel a, however the imaging software allows for local magnification. The white arrow indicates the area of local magnification. The image to the right in the smaller box is the actual local magnification. Panel c, a closer look at the local magnification in panel b. Here, you can see the blue located in the center of the cell (nucleus) and the green located on the outskirts of the cell (cytoplasm).

Experiment 3: Mandibular Defect Surgery with ASC Transplantation Flow Cytometry of Blood Study

Trauma was induced in the mandible of pigs by drilling a 10 mm diameter hole in the ramus of the jaw. Labeled ASC then were injected directly into the drilled hole in the mandible or systemically via ear vein. In the latter case, injected ASC were expected to migrate to the site of trauma in the mandible. Flow cytometry of blood collected 1 hour after Ear Vein Injection (EVI) of ASC, or Direct Injection (DI) into the defect site, indicated that labeled cells were still present systemically. The proportion of labeled cells was lower in the DI ($\leq 65\%$ gated cells) group than in the EVI ($\leq 1.34\%$ gated cells) group at 1-hour post-injection. However, labeled cells were not present in the bloodstream at 2 or 4 weeks after ASC injection (Fig. 5 and Fig. S1-S3).

Figure 5: Flow cytometry results from blood samples. Each panel represents the cells that were separated from the total cell population for each blood sample by gating. The small black box in the upper-right corner of each panel marks the area where the CFDA-SE labeled cells would appear on the graph, if present. Panel A is the 1- hour control. Panel B is the 1- hour DI using CELL1. ASC were present in this sample as seen in the small black box in the upper-right corner of the panel. Panel C is the 1-hour DI using CELL2. ASC were present in this sample as seen in the small black box in the upper-right corner of the panel. Panel D is the 1-hour EVI using CELL1. ASC were present in this sample as seen in the small black box in the upper-right corner of the panel. Panel E is the 1-hour EVI using CELL2. ASC were present in this sample as seen in the small black box in the upper- right corner of the panel. Panel F is the 2-week control. Panel G is the 2-week DI using CELL1. Panel H is the 2-week DI using CELL2. Panel I is the 2-week EVI using CELL1. Panel J is the 2-week EVI using CELL2. Panel K is the 4-week control. Panel L is the 4-week DI using CELL1. Panel M is the 4-week DI using CELL2. Panel N is the 4-week EVI using CELL1. Panel O is the 4-week EVI using CELL2.

Fluorescent Microscopy of Tissues

The results from the fluorescent microscopy of various tissues at the 1-hour time revealed that CFDA-SE specific fluorescence was apparent only in the ear vein of the right ear (Fig. 6). The right ear vein was the one used to inject the CFDA-SE labeled ASC for the surgery of that pig. The CFDA-SE specific fluorescence was not apparent in histological sections of any other

tissue at the 1-hour time. Similarly, results from the fluorescent microscopy in the 2-week time revealed that CFDA-SE specific fluorescence was apparent only in the ear vein of the right ear (Fig. 7). Again this was the ear vein used to inject the CFDA-SE labeled ASC for the surgery of that pig. CFDA-SE specific fluorescence was not observed in any other tissue at the 2-week time. No visible presence of CFDA-SE specific fluorescence was observed in any tissue at the 4-week time. Furthermore, the ear vein used to inject the CFDA-SE labeled ASC was evaluated extensively in the 4-week time pigs and there was no presence of the specific fluorescence.

Figure 6: Fluorescent microscopy and bright field images of the one-hour time point. Row (A) is composed of bright field images of the spleen (A1 @ 100X), liver (A2 @ 100X), lung (A3 @ 100X), kidney (A4 @ 100X), right ear (A5 @ 200X) and left ear (A6 @ 200X) from the control group in the 1-hour time point. Row (B) is composed of fluorescent micrographs of the spleen (B1 @ 100X), liver (B2 @ 100X), lung (B3 @ 100X), kidney (B4 @ 100X), right ear (B5 @ 200X) and left ear (B6 @ 200X) from the control group in the 1- hour time point. Row (C) is composed of bright field images of the spleen (C1 @ 100X), liver (C2 @ 100X), lung (C3 @ 100X), kidney (C4 @ 100X), right ear (C5 @ 200X) and left ear (C6 @ 200X) from the DI group in the 1-hour time point. Row (D) is composed of fluorescent micrographs of the spleen (D1 @ 100X), liver (D2 @ 100X), lung (D3 @ 100X), kidney (D4 @ 100X), right ear (D5 @ 200X) and left ear (D6 @ 200X) from the DI group in the 1-hour time point. Row E is composed of bright field images of the spleen (E1 @ 100X), liver (E2 @ 100X), lung (E3 @ 100X), kidney (E4 @ 100X), right ear (E5 @ 200X) and left ear (E6 @ 200X) from the EVI group in the 1-hour time point. Row (F) is composed of fluorescent micrographs of the spleen (F1 @ 100X), liver (F2 @ 100X), lung (F3 @ 100X), kidney (F4 @ 100X), right ear (F5 @ 200X) and left ear (F6 @ 200X) from the EVI group in the 1-hour time point. The white arrow in (F6) indicates CFDA-SE specific fluorescence in the ear vein of the left ear.

Figure 7: Fluorescent microscopy and bright field images of 2-week time point. Row (A) is composed of bright field images of the spleen (A1 @ 100X), liver (A2 @ 100X), lung (A3 @ 100X), kidney (A4 @ 100X), right ear (A5 @ 200X) and left ear (A6 @ 200X) from the control group in the 2-week time point. Row (B) is composed of fluorescent micrographs of the spleen (B1 @ 100X), liver (B2 @ 100X), lung (B3 @ 100X), kidney (B4 @ 100X), right ear (B5 @ 200X) and left ear (B6 @ 200X) from the control group in the 2-week time point. Row (C) is composed of bright field images of the spleen (C1 @ 100X), liver (C2 @ 100X), lung (C3 @ 100X), kidney (C4 @ 100X), right ear (C5 @ 200X) and left ear (C6 @ 200X) from the DI group in the 2-week time point. Row (D) is composed of fluorescent micrographs of the spleen (D1 @ 100X), liver (D2 @ 100X), lung (D3 @ 100X), kidney (D4 @ 100X), right ear (D5 @ 200X) and left ear (D6 @ 200X) from the DI group in the 2-week time point. Row (E) is composed of bright field images of the spleen (E1 @ 100X), liver (E2 @ 100X), lung (E3 @ 100X), kidney (E4 @ 100X), right ear (E5 @ 200X) and left ear (E6 @ 200X) from the EVI group in the 2-week time point. Row (F) is composed of fluorescent micrographs of the spleen (F1 @ 100X), liver (F2 @ 100X), lung (F3 @ 100X), kidney (F4 @ 100X), right ear (F5 @ 200X) and left ear (F6 @ 200X) from the EVI group in the 2-week time point. The white arrow in (F6) indicates CFDA-SE specific fluorescence in the ear vein of the left ear.

Flow Cytometry of Disaggregated Spleen Cells

Flow cytometry of disaggregated spleen tissue collected 1 hour after ASC injection revealed that CFDA-SE labeled ASC were present within spleen tissue in the EVI group (Fig. 8). The proportion of fluorescent cells present within the gated cells was low (<2% gated cells). This result was in contrast with those from the fluorescent microscopy. However, no CFDA-SE labeled ASC were found in any other treatment at 1 hour, nor were CFDA-SE labeled ASC

found in samples from spleen at 2 or 4 weeks post-injection by flow cytometry (Fig. 9 and Fig. S4-S6). These results were consistent with those of the fluorescent microscopy study.

Figure 8: Fluorescent microscopy and bright field images of the 4-week time point. Row (A) is composed of bright field images of the spleen (A1 @ 100X), liver (A2 @ 100X), lung (A3 @ 100X), kidney (A4 @ 100X), right ear (A5 @ 200X) and left ear (A6 @ 200X) from the control group in the 4-week time point. Row (B) is composed of fluorescent micrographs of the spleen (B1 @ 100X), liver (B2 @ 100X), lung (B3 @ 100X), kidney (B4 @ 100X), right ear (B5 @ 200X) and left ear (B6 @ 200X) from the control group in the 4-week time point. Row (C) is composed of bright field images of the spleen (C1 @ 100X), liver (C2 @ 100X), lung (C3 @ 100X), kidney (C4 @ 100X), right ear (C5 @ 200X) and left ear (C6 @ 200X) from the DI group in the 4-week time point. Row (D) is composed of fluorescent micrographs of the spleen (D1 @ 100X), liver (D2 @ 100X), lung (D3 @ 100X), kidney (D4 @ 100X), right ear (D5 @ 200X) and left ear (D6 @ 200X) from the DI group in the 4-week time point. Row E is composed of bright field images of the spleen (E1 @ 100X), liver (E2 @ 100X), lung (E3 @ 100X), kidney (E4 @ 100X), right ear (E5 @ 200X) and left ear (E6 @ 200X) from the EVI group in the 4-week time point. Row (F) is composed of fluorescent micrographs of the spleen (F1 @ 100X), liver (F2 @ 100X), lung (F3 @ 100X), kidney (F4 @ 100X), right ear (F5 @ 200X) and left ear (F6 @ 200X) from the EVI group in the 4-week time point.

Figure 9: Flow cytometry results from spleen samples. Each panel represents the cells that were separated from the total cell population for each blood sample by gating. The small black box in the upper-right corner of each panel marks the area where the CFDA-SE labeled cells would appear on the graph, if present. Panel A is the 1- hour control. Panel B is the 1- hour DI using CELL1. Panel C is the 1-hour DI using CELL2. Panel D is the 1-hour EVI using CELL1. ASC were present in this sample as seen in the small black box in the upper-right corner of the panel. Panel E is the 1-hour EVI using CELL2. ASC were present in this sample as seen in the small black box in the upper-right corner of the panel. Panel F is the 2- week control. Panel G is the 2-week DI using CELL1. Panel H is the 2-week DI using CELL2. Panel I is the 2-week EVI using CELL1. Panel J is the 2-week EVI using CELL2. Panel K is the 4-week control. Panel L is the 4-week DI using CELL1. Panel M is the 4-week DI using CELL2. Panel N is the 4-week EVI using CELL1. Panel O is the 4-week EVI using CELL2.

Gross Visualization of Soft Tissue Results

At the time of sacrifice for each time point, the spleen, liver, lung, kidney, left ear and right ear were collected from each animal involved in this experiment. The results from simple gross visualization of all of the spleens, livers, lungs, kidneys and ears collected from all experimental groups and control groups demonstrated that all organs looked normal and healthy. There were no physical signs of damage to any organ that was collected from any animal involved in this experiment. Additionally, all organs appeared to be of normal color, texture and structure for that specific organ.

H and E Staining Results

The purpose of staining some of the sections with H and E was to determine if all of the tissue was normal or not. We felt that fluorescent microscopy and bright field analysis of each section would not suffice in providing information about the normality of each tissue. Tissue sections of the spleen, liver, lung, kidney and both ears from the control group were stained with H and E to observe the structure of each tissue type. The same tissues from the experimental groups were then stained with H and E to observe and compare to the control sections. The results revealed that there were no structural differences between the control (Fig. 10) and experimental ear injection group (Fig. 11). At 10X and 200X, the experimental ear injection tissue samples appeared to be similar to those of the control samples.

Figure 10: Fluorescent microscopy and histology of control paraffin sections. All fluorescent micrographs were observed using a Leica DMI 4000 B inverted fluorescent microscope with the FITC filter for the FITC column and no filter for the Bright Field (BF) column. All Hematoxylin and Eosin (H and E) images were observed with a Nikon Diaphot Inverted Tissue Culture Microscope.

Figure 11: Fluorescent microscopy and histology of TRT2 paraffin sections. All fluorescent micrographs were observed using a Leica DMI 4000 B inverted fluorescent microscope with the FITC filter for the FITC column and no filter for the Bright Field (BF) column. All Hematoxylin and Eosin (H and E) images were observed with a nikon diaphot inverted tissue culture microscope.

Discussion

This study demonstrates the mobility of ASC when injected into the pig, either directly into a bone defect or via an ear vein. Cells migrate rapidly through the vascular system of the pig to sites of tissue trauma. Others have shown the potential migration of ASC by injecting cells into the muscle and then later identifying the cells in adjacent muscles after 50 days as well as ASC egress from subcutaneous adipose tissue, in mice fed high fat diets and incorporation into surrounding muscle [13,24]. Ferrari and collaborators injected bone marrow cells directly into degenerated muscle and demonstrated that injected cells were present in newly formed muscle fibers after 2 to 5 weeks [25]. The present study shows that these cells can travel from an ear vein to a jugular vein within 60 seconds in a swine model. These results demonstrate the capabilities of these cells to migrate systemically throughout the body to aid in the healing of damaged tissue potentially.

According to previous studies, cell accumulation and colonization in ectopic sites associated with intravenously injected stem cells are possible risks [16]. In rats, MSC that were infused into the jugular vein were mostly trapped in the lungs [26]. Intravenously injected ASC have been identified in the bone marrow, spleen and blood of mice [27,28]. In addition, ASC can migrate towards and engraft in blood and major hematopoietic organs [12,29]. In contrast, our results did not find labeled ASC in blood samples after 1 hour, nor were there signs of fluorescently labeled cells in the blood 2 or 4 weeks after cell injection.

The intrinsic physiological process of tissue regeneration *in-vivo* involves activating stem cells residing within the recipients' tissues or their recruitment from ectopic sites, particularly the bone marrow [30,31]. Signaling pathways and other molecular processes within the tissue environment of locally injected stem cell transplants may not be the most effective to achieve optimal tissue regeneration. Moreover, there could be a risk of the transplanted cells ascending to inappropriate lineages at the transplantation site. This study indicates that administering stem cells directly into the circulation may take advantage of naturally induced homing mechanisms to the site of trauma and provide an alternative to administration locally at the site of tissue trauma.

The capability of ASC to travel through the vasculature of the body also raises a potential risk of cell accumulation and colonization in untargeted tissues as the cells pass through the filtering organs. Our observations indicated that the ASC travel to areas of trauma, including the site of ear vein injection, but do not accumulate and remain in other tissues. Even the spleen tissue from the ear vein injected pigs had few CFDA-SE-labeled cells (2%) at 1-hour post-injection. We might expect labeled ASC to be present in the filtering organs after 1-hour post-transplantation. No labeled cells were observed in tissues at 2 or 4 weeks post-injection. Interestingly the exception was the continued presence of labeling at two weeks post-injection in the ear vein where the cells had been injected, suggesting the continued participation of those cells in the healing process of that site of trauma.

There have been reported instances of tumor formation after the injection of embryonic stem cells and some cases of induced tumor formation after MSC infusion [18,32,33]. Others have also shown that MSC treatment can decrease or inhibit tumor formation [34,35]. In the present study, we found no signs of tumor formation using the pig model, leading us to believe that most of the ASC injected migrated directly to the damaged mandible or were otherwise lost from the vasculature. The pig appears to be an excellent to examine the intricacies of ASC migration and possible colonization following infusion systemically.

Conclusion

ASC injected via ear vein can travel through the entire vasculature of the pig within 60 seconds and the cells continue to be present in the bloodstream for at least 1-hour post-transplantation. However, labeled cells were not present in the bloodstream at 2 or 4 weeks after ASC injection.

The injected ASC travel to areas of induced trauma and are not observed in filtering tissues of the body such as the spleen, liver, lung and liver.

These findings suggest that systemic administration of ASC could be a successful method of cell transplantation for tissue regeneration.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Smart N, Riley PR. The stem cell movement. *Circ Res*. 2008;102(10):1155-68.
2. Fu X, Liu G, Halim A, Ju Y, Luo Q, Song G. Mesenchymal stem cell migration and tissue repair. *Cells*. 2019;8(8):784.
3. Su P, Tian Y, Yang C, Ma X, Wang X, Pei J, et al. Mesenchymal stem cell migration during bone formation and bone diseases therapy. *Int J Mol Sci*. 2018;19(8):2343.
4. Lin W, Xu L, Zwingenberger S, Gibon E, Goodman SB, Li G. Mesenchymal stem cells homing to improve bone healing. *J Orthop Translat*. 2017;9:19-27.
5. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features and potential for homing. *Stem Cells*. 2007;25(11):2739-49.
6. Xiao Q, Wang SK, Tian H, Xin L, Zou ZG, Hu YL, et al. TNF- α increases bone marrow mesenchymal stem cell migration to ischemic tissues. *Cell Biochem Biophys*. 2012;62(3):409-14.
7. Li L, Jiang J. Regulatory factors of mesenchymal stem cell migration into injured tissues and their signal transduction mechanisms. *Front Med*. 2011;5(1):33-9.
8. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg*. 2002;73(6):1919-25.
9. Wang L, Li Y, Chen X, Chen J, Gautam SC, Xu Y, et al. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematology*. 2002;7(2):113-7.
10. Sohni A, Verfaillie CM. Mesenchymal stem cells migration homing and tracking. *Stem Cells International*. 2013;2013:130763.
11. de Lucas B, Pérez LM, Gálvez BG. Importance and regulation of adult stem cell migration. *J Cell Mol Med*. 2018;22(2):746-54.
12. Maeda A. Recruitment of mesenchymal stem cells to damaged sites by plant- derived components. *Frontiers in Cell and Developmental Biology*. 2020;8(437).

13. Girusse A, Gil-Ortega M, Bourlier V, Bergeaud C, Sastourné-Arrey Q, Moro C, et al. The release of adipose stromal cells from subcutaneous adipose tissue regulates ectopic intramuscular adipocyte deposition. *Cell Rep.* 2019;27(2):323-33.
14. Gianakos AL, Moya-Angeler J, Duggal S, Zambrana L, Fields KG, Mintz DN, et al. The efficacy of bisphosphonates with core decompression and mesenchymal stem cells compared with bisphosphonates alone in the treatment of osteonecrosis of the hip: a retrospective study. *Hss j.* 2016;12(2):137-44.
15. Houdek MT, Wyles CC, Collins MS, Howe BM, Terzic A, Behfar A, et al. Stem cells combined with platelet-rich plasma effectively treat corticosteroid-induced osteonecrosis of the hip: a prospective study. *Clin Orthop Relat Res.* 2018;476(2):388-97.
16. Pittenger MF, Discher DE, Péault BM, Phinney DG, Hare JM, Caplan AI. Mesenchymal stem cell perspective: cell biology to clinical progress. *NPJ Regen Med.* 2019;4:22.
17. Janin A, Murata H, Leboeuf C, Cayuela JM, Gluckman E, Legres L, et al. Donor-derived oral squamous cell carcinoma after allogeneic bone marrow transplantation. *Blood.* 2009;113(8):1834-40.
18. Yu JM, Jun ES, Bae YC, Jung JS. Mesenchymal stem cells derived from human adipose tissues favor tumor cell growth in vivo. *Stem Cells Dev.* 2008;17(3):463-73.
19. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature.* 2007;449(7162):557-63.
20. Laird DJ, von Andrian UH, Wagers AJ. Stem cell trafficking in tissue development, growth and disease. *Cell.* 2008;132(4):612-30.
21. López-Lázaro M. The migration ability of stem cells can explain the existence of cancer of unknown primary site. *Rethinking metastasis. Oncoscience.* 2015;2(5):467-75.
22. Li H, Fan X, Houghton J. Tumor microenvironment: the role of the tumor stroma in cancer. *J Cell Biochem.* 2007;101(4):805-15.
23. Monaco E, Lima A, Bionaz M, Maki A, Wilson S, Hurley W, et al. Morphological and transcriptomic comparison of adipose and bone marrow derived porcine stem cells. *The Open Tissue Engineer and Regene Med J.* 2009;2:20-33.
24. Rodriguez A-M, Pisani D, Dechesne CA, Turc-Carel C, Kurzenne J-Y, Wdziekonski B, et al. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J Exp Med.* 2005;201(9):1397-405.
25. Ferrari G, Cusella G, Angelis D, Coletta M, Paolucci E, Stornaiuolo A, et al. muscle regeneration by bone marrow-derived myogenic progenitors. *Science.* 1998;279(5356):1528-30.
26. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev.* 2009;18(5):683-92.
27. Devine SM, Peter S, Martin BJ, Barry F, McIntosh KR. Mesenchymal stem cells: stealth and suppression. *Cancer J.* 2001;7 Suppl 2:S76-82.
28. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage and lung in irradiated mice. *Proc Natl Acad Sci USA.* 1995;92(11):4857-61.
29. Cousin B, Andre M, Arnaud E, Penicaud L, Casteilla L. Reconstitution of lethally irradiated mice by cells isolated from adipose tissue. *Biochem Biophys Res Commun.* 2003;301(4):1016-22.
30. Iwaki A, Jingushi S, Oda Y, Izumi T, Shida JI, Tsuneyoshi M, et al. Localization and quantification of proliferating cells during rat fracture repair: detection of proliferating cell nuclear antigen by immunohistochemistry. *J Bone Miner Res.* 1997;12(1):96-102.
31. Shapiro F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *Eur Cell Mater.* 2008;15:53-76.
32. Przyborski SA. Differentiation of human embryonic stem cells after transplantation in immune-deficient mice. *Stem Cells.* 2005;23(9):1242-50.
33. Lee H-Y, Hong I-S. Double-edged sword of mesenchymal stem cells: Cancer-promoting versus therapeutic potential. *Cancer Sci.* 2017;108(10):1939-46.

34. Cai C, Hou L, Zhang J, Zhao D, Wang Z, Hu H, et al. The inhibitory effect of mesenchymal stem cells with rad-nk4 on liver cancer. *Appl Biochem Biotechnol.* 2017;183(1):444-59.
35. Ai J, Ketabchi N, Verdi J, Gheibi N, Khadem Haghighian H, Kavianpour M. Mesenchymal stromal cells induce inhibitory effects on hepatocellular carcinoma through various signaling pathways. *Cancer Cell Int.* 2019;19:329.

Supplemental Materials

Figure S1: Flow Cytometry Results from One-Hour Time Point Blood Samples

Block (A) consists of information pertaining to the 1-hour control blood. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 1-hour TRT1 blood. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

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Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 1-hour TRT2 blood. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (D3 and E3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

Figure S2: Flow Cytometry Results from 2-Week Time Point Blood Samples Block (A) consists of information pertaining to the 2-week control blood. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 2-week TRT1 blood. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 2-week TRT2 blood. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels

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(D3 and E3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

Figure S3: Flow Cytometry Results from 4-Week Time Point Blood Samples Block (A) consists of information pertaining to the 4-week control blood. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 4-week TRT1 blood. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 4-week TRT2 blood. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (D3 and E3) are graphs displaying the individual cell count within the total population (M2)

of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

Figure S4: Flow Cytometry Results from One-Hour Time Point Spleen Cells Block (A) consists of information pertaining to the 1-hour control spleen sample. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 1-hour TRT1 spleen samples. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 1-hour TRT2 spleen samples. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (D3 and E3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

Figure S5: Flow Cytometry Results from 2-Week Time Point Spleen Cells Block (A) consists of information pertaining to the 2-week control spleen sample. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 2-week TRT1 spleen samples. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 2-week TRT2 spleen samples. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (D3 and E3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.

Figure S6: Flow Cytometry Results from 4-Week Time Point Spleen Cells Block (A) consists of information pertaining to the 4-week control spleen sample. Panel (A1) displays the gated cells (red box) within the total population. Panel (A2) displays the area where the CFDA-SE labeled ASC should appear (blue box). Panel (A3) is a graph displaying the individual cell counts within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (B[cell type 1] and C[cell type 2]) consists of information pertaining to the 4-week TRT1 spleen samples. Panels (B1 and C1) display the gated cells (red box) within the total population. Panels (B2 and C2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (B3 and C3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar. Blocks (D[cell type 1] and E[cell type 2]) consists of information pertaining to the 4-week TRT2 spleen samples. Panels (D1 and E1) display the gated cells (red box) within the total population. Panels (D2 and E2) display the CFDA-SE labeled ASC population in the sample (blue box). Panels (D3 and E3) are graphs displaying the individual cell count within the total population (M2) of the sample and also indicating the presence of the CFDA-SE labeled cells, which fall under the M1 bar.