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Cell recruitment by amnion chorion grafts promotes neovascularization

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Abstract

Background—Nonhealing wounds are a significant health burden. Stem and progenitor cells can accelerate wound repair and regeneration. Human amniotic membrane has demonstrated efficacy in promoting wound healing, though the underlying mechanisms remain unknown. A dehydrated human amnion chorion membrane (dHACM) was tested for its ability to recruit hematopoietic progenitor cells to a surgically implanted graft in a murine model of cutaneous ischemia.

Methods—dHACM was subcutaneously implanted under elevated skin (ischemic stimulus) in either wild-type mice or mice surgically parabiosed to green fluorescent protein (GFP) + reporter mice. A control acellular dermal matrix, elevated skin without an implant, and normal unwounded skin were used as controls. Wound tissue was harvested and processed for histology and flow cytometric analysis.

Results—Implanted dHACMs recruited significantly more progenitor cells compared with controls (**P* < 0.05) and displayed *in vivo* SDF-1 expression with incorporation of CD34 + progenitor cells within the matrix. Parabiosis modeling confirmed the circulatory origin of

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Disclosure

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Supplementary data

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recruited cells, which coexpressed progenitor cell markers and were localized to foci of neovascularization within implanted matrices.

Conclusions—In summary, dHACM effectively recruits circulating progenitor cells, likely because of stromal derived factor 1 (SDF-1) expression. The recruited cells express markers of "stemness" and localize to sites of neovascularization, providing a partial mechanism for the clinical efficacy of human amniotic membrane in the treatment of chronic wounds.

Keywords

Amniotic membrane; dHACM; Neovascularization; Chronic wounds; Progenitor cell recruitment; SDF-1; Hematopoietic progenitor cell

1. Background

Normal wound healing is a complex biological process that progresses through three overlapping phases as follows: inflammation, proliferation, and remodeling [1]. This process entails a well-coordinated interplay between numerous cell types regulated by extracellular matrix interactions and cytokine expression resulting in neovascularization, stromal deposition, and epithelialization [1]. Poor wound healing represents a significant health care burden, impacting patients' quality of life and costing the United States $25 billion annually [2]. Typically occurring in elderly and diabetic individuals [2], poor wound healing could be related to any of the previously mentioned processes, but is largely attributable to impaired new blood vessel formation (neovascularization) [1,3]. Effective therapies for these chronic wounds remain elusive [4], contributing to the large and growing incidence of 5–7 million cases per year in the United States alone [5].

Mesenchymal stromal cells (MSCs) [6–8], as well as hematopoietic progenitor cells (HPCs) [9–11], have been shown to play a critical role in wound healing, producing paracrine factors that stimulate cell migration and proliferation, and support angiogenesis, extracellular matrix production, and tissue regeneration. As such, these cell types have garnered significant attention for wound healing applications [12–15]. Two broad strategies for therapeutic application of stem cells in chronic wounds are (1) direct delivery of autologous or cryopreserved allogeneic stem cells to the wound and (2) recruiting endogenous stem cells to the wound and improving their wound healing potential. The experiments described here focus on the efficacy of a PURION processed dehydrated human amnion chorion membrane (dHACM) (EpiFix, MiMedx Group, Inc, Marietta, GA) for the recruitment of endogenous progenitor cells to ischemic skin and the promotion of neovascularization.

Human amniotic membranes have been successfully used to treat chronic cutaneous wounds [16–19], exhibiting low immunogenicity and reducing inflammation and pain while accelerating wound healing [20–24]. The exact molecular and cellular mechanisms underlying amniotic membrane's clinical benefit have yet to be fully elucidated. To gain a better understanding of the mechanism of action of amniotic membrane in healing chronic wounds, we previously investigated the intrinsic proteins contained within dHACM. We demonstrated that dHACM contains a variety of growth factors and cytokines, including

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platelet derived growth factor AA (PDGF-AA), transforming growth factor β1 (TGFβ1), vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF-2), and a number of inflammatory mediators, including interleukin (IL)-4, 6, 8, and 10, as well as tissue inhibitor of metalloproteinase 1 and 2 (TIMP 1 and 2) [25]. Moreover, dHACM was shown to promote fibroblast and endothelial cell proliferation, recruit MSCs, promote growth factor expression in native cells, and support perimatrix neovascularization [25–27]. In this study, we used two murine models of ischemia, including a parabiosis model, to investigate the recruitment of HPCs by dHACM, including the identification of a potential mechanism for recruitment, and the contribution of the recruited cells to neovascularization.

2. Materials and methods

2.1. Dehydrated human amnion and/or chorion membrane

dHACM (EpiFix, MiMedx Group), a dehydrated human allo-graft composed of laminated, placental amnion, and chorion membranes, PURION processed as previously described [28], was obtained from six healthy donors. Human placentas were donated under informed consent following cesarean sections, as regulated by the Food and Drug Administration's Good Tissue Practice and American Association of Tissue Banks.

2.2. Mice

Murine experiments were conducted in accordance with a protocol approved by the Stanford Administrative Panel on Laboratory Animal Care in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) accredited animal care facility. A 12-wk-old, FVB/NJ wild type (WT) and luciferase and/or green fluorescent protein (GFP) transgenic (FVB-Tg[CAG-luc,-GFP]L2G85Chco/J) female mice were obtained from Jackson Laboratories (Bar Harbor, ME). Only female mice were used to reduce sex-related variations in inflammatory response and wound healing [29]. Additionally, in our experience, female mice are more amenable to being paired with and parabiosed to a previously unknown mouse. The mice were housed in a room with a 12-h light–dark cycle, maintained at constant temperature, and given free access to food and water. Experiments were not commenced for at least 2 wk after arrival of the mice, allowing them to rest and adapt.

2.3. Surgical implantation

Three horizontal 6-mm incisions were created on the shaved dorsum of anesthetized mice. A subcutaneous pocket was bluntly dissected in the fascial plane underlying the panniculus carnosus and either a 5 mm × 5 mm square of dHACM or a 5 mm × 5 mm square of control acellular fetal bovine dermal matrix of nonplacental origin (PriMatrix; TEI Biosciences, Inc, Boston, MA) was inserted. The third pocket did not receive an implant and acted as the sham surgical control. After surgery, the mice were placed on warming pads and allowed to fully recover from anesthesia before being returned to the institutional animal facility in separate cages. All incisions were closed using interrupted 6-0 nylon sutures (Ethicon Inc, Somerville, NJ). At days 3, 7, 14, and 28 after operation, mice were euthanized (n = 3 mice) and the three surgical sites, including implant and overlying skin, along with uninjured skin as a further negative control, were harvested for either fixation in 4% paraformaldehyde (12

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h at 4°C) for histologic analysis or digested in 0.1% collagenase for flow cytometric analysis.

2.4. Murine parabiosis model

Luciferase and/or GFP "donor" and WT "recipient" mice were shaved and anesthetized. Parabiosis surgery was performed as previously described [30,31] with slight modification. Briefly, the corresponding flanks of mice were shaved and disinfected with Betadine solution and 70% ethanol three times. Matching skin incisions were made from the olecranon to the knee joint of each mouse. The skin edges were undermined to create about 1 cm of free skin. 6-0 nylon sutures were used to approximate the dorsal and ventral skin. The skin was oversewn to protect the suture line (Supplemental Figure 1A). Mice were allowed to recover as described previously. Buprenorphine was used for analgesia by subcutaneous injection every 8–12 h for 48 h after operation. After 2 wk, cross circulation was confirmed using fluorescent microscopy of the tail vein blood before surgical implantation in "recipient" mice as described previously (Supplemental Figure 1B).

2.5. Flow cytometric analysis of harvested tissue

After digestion, as described previously, samples were filtered, centrifuged, and incubated with fluorescently conjugated antibodies against CD45 (phycoerythrin; BD Biosciences, San Diego, CA), stem cell antigen-1 (Sca-1, fluorescein isothiocya-nate) (BD Biosciences), and lineage (Lin) markers (TER119, B220, CD4, CD8, CD11 b, Gr-1; [phycoerythrin-Cy5]) (eBio-sciences, San Diego, CA), for 30 min at 4°C in 2% fetal bovine serum (FBS) in phosphate-buffered saline as previously described [32]. Cells not stained with these antibodies were incubated with the appropriate isotype controls or left un-stained. Cells were then centrifuged and resuspended in propidium iodide for 1 min at 4°C. Samples were run on a Becton Dickinson-LSR Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). Data were analyzed using FlowJo digital fluorescence-activated cell sorting software by a single blinded evaluator (Tree Star Inc, Ashland, OR).

2.6. Histology and immunohistochemistry

Tissue was harvested and either embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc, Torrance, CA) from which 10-μm-thick frozen sections were serially cut or fixed, dehydrated, and embedded in paraffin blocks, from which 8-μm-thick sections were serially cut. Neovascularization was assessed using a polyclonal rabbit anti–mouse anti-CD31 primary antibody (1:100, Abcam, Cambridge, United Kingdom). Stromal derived factor 1α (SDF-1α) expression was assessed using a polyclonal rabbit anti–mouse anti-SDF-1α primary antibody (1:100, Abcam). CD34 expression was assessed using a polyclonal rabbit anti–mouse anti-CD34 primary antibody (1:100, Abcam). CD90 expression was assessed using a polyclonal rabbit anti-emouse anti-CD90 primary antibody (1:100, Abcam). Primary antibodies were incubated overnight at 4°C. Secondary staining was performed using either Alexa Fluor 594 Goat Anti-Rabbit IgG or Alexa Fluor 488 Goat Anti-Rabbit IgG at room temperature (1:400, Invitrogen, Carlsbad, CA). All samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were mounted with the VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and cover-slipped. A Zeiss Axioplan 2 fluorescence microscope was used to image the slides (Carl

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Zeiss, Inc, Thornwood, NY) by a blinded evaluator. A blinded evaluator then quantified fluorescence by analyzing at least three high-powered fields per wound using ImageJ software (NIH, Bethesda, MD).

2.7. Statistics

A power analysis using MATLAB R2010a (MathWorks Inc, Natick, MA) to determine the minimal sample size required to obtain significance levels of $\alpha = 0.05$ using a one-way analysis of variance across mouse and/or construct pairs determined that three measurements per group would be sufficient to power these assays, and as such we elected to use $n = 3$ murine pairs per group per time point to ensure adequate statistical precision while minimizing the potential distress that parabiosis creates for the animal subjects. Data are expressed as mean ± standard error of the mean. Statistical analyses were performed using a one-way analysis of variance or unpaired Student *t*-test. *P* values ≤0.05 were considered statistically significant.

3. Results

3.1. dHACM enhances recruitment of HPCs in WT mice

The dHACM and overlying skin contained significantly more HPCs (defined as Lin-/CD45+/c-Kit+/Sca-1+) (Fig.1A) than sham surgery and unwounded skin at days 7, 14, and 28 days after implantation ($P \leq 0.05$), with a ten-fold higher amount observed on day 14. At day 28, HPC numbers in the control acellular dermal matrix (ADM), sham surgery, and unwounded skin returned to baseline, but were persistently elevated in the dHACM ($P \leq 0.05$; Fig. 1B).

3.2. dHACM recruits and incorporates CD34 + progenitor cells

CD34 is a well-described marker for HPCs, predicting enhanced cellular function and improved therapeutic potential [33–35]. Immunohistochemistry demonstrated that dHACM stimulated a significant increase in CD34 + progenitor cells in the peri- implant space and surrounding skin compared with both sham surgical sites and healthy skin (4-fold, day 14; 3-fold, day 28; $P \leq 0.05$) and control ADM (2-fold, day 14; 3-fold, day 28; $P \leq 0.05$) (Fig. 2A and B). Furthermore, dHACM demonstrated significantly more incorporation of CD34 + progenitor cells within the implant compared with the control ADM at day 14, with persistence of these cells at day 28 ($P \leq 0.05$; Fig. 2C and D).

3.3. dHACM enhances recruitment of circulating bone marrow cells in a parabiosis model

GFP + circulating cells, derived from the bone marrow of “donor” mice, were elevated in the dHACM group compared with control ADM, sham, and unwounded groups on flow cytometric analysis of harvested tissue. This effect was significant on days 3, 7, and 14 compared with sham and unwounded samples, and on day 3 compared with control ADM ($P \leq 0.05$; Fig. 3A and B). Immunohistochemistry demonstrated that GFP + cells colocalized with SDF-1 expression within the dHACM (Fig. 4A), which was relatively increased compared with control ADM, sham, and unwounded skin (Fig. 4B), suggesting a possible mechanism for the enhanced recruitment.

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3.4. Recruited GFP + cells express progenitor cell markers and localize to foci of neovascularization

GFP + cells recruited by dHACM expressed CD90 (Fig. 5), a surface marker known to be expressed on multiple progenitor cells types and indicative of a more progenitor-like state [36–38], consistent with the recruitment of HPCs by the dHACM. Additionally, GFP + cells colocalized with areas of neovascularization (CD31 + staining) within the dHACM (Fig. 6A). The GFP/CD31 colocalization was more pronounced in dHACM compared with controls (Fig. 6B), suggesting a provascular effect of these recruited cells, in keeping with published literature on the angiogenic potential of CD90 + cells [39–42].

4. Discussion

Impaired neovascularization and poor wound healing exert a significant toll on both health care providers and patients [2,43–46]. There is a clear need for effective therapies that can address this clinical entity. Neovascularization, the growth of new blood vessels, and deposition of an extracellular matrix are essential for tissue repair and regeneration [3,8]. The ability of progenitor cells to support neovascularization and cell proliferation has gained them increased attention for tissue regeneration and wound healing [12–15].

Bioactive tissue matrices, such as dHACM, represent an attractive therapeutic modality for chronic wounds. Specifically, their cytokine and growth factor expression profile stimulates resident cells and recruits progenitor cells, whereas their structural component provides a scaffold for tissue regeneration [26]. A number of clinical trials have established the efficacy of human amniotic membrane in treating chronic, nonhealing wounds [16–19], and reducing pain and inflammation without immunological side effects [20–24]. In a recent clinical trial, dHACM healed 92% of diabetic foot ulcers after 6 wk compared with 8% in the standard care group [22].

In this study, using two murine models, dHACM effectively recruited HPCs and circulating progenitor cells to the site of implantation. It demonstrated enhanced recruitment and incorporation of progenitor cells as compared with control ADM, resulting in greater vascularization. *In vitro* experiments have shown that dHACM can directly cause MSC migration by releasing soluble factors [26]. The recruitment of progenitor cells by dHACM is likely due to the release of one or more soluble bioactive chemokines, with SDF-1 appearing to be a likely candidate. Alternatively, the cytokines or extracellular matrix components of dHACM may upregulate SDF-1 expression by endogenous cells localized near the implant. The increased expression of SDF-1 is particularly interesting, as it is decreased in the setting of diabetic wounds, which are subsequently impaired in their capacity to recruit progenitor cells [47]. Regardless of the specific mechanism, dHACM provides both a stimulus for recruitment of endogenous progenitor cells and a scaffold for their engraftment.

In addition to the angiogenic and trophic effects exerted by dHACM-recruited cells, modulation of the immune response may also play a role. Chronic inflammation in a wound stalls the healing process by preventing transition into the proliferative and remodeling phases, resulting in a nonhealing wound. CD90 + cells [48–50] and MSCs [51,52] are

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known for regulating immune and inflammatory responses during wound healing. These progenitor cells typically decrease secretion of the proinflammatory cytokines, tumor necrosis factor-α, and interferon-γ, while simultaneously increasing the production of anti-inflammatory cytokines IL 4 and IL 10 [53]. The recognized anti-inflammatory properties of amniotic membrane, and dHACM in particular, may result from synergistic effects of both the innate anti-inflammatory cytokines contained within the scaffold, as well as the secondary effects of recruited progenitors. The anti-inflammatory effect may also explain the decrease in GFP + cells in dHACM at day 28 (Fig. 3B) to baseline levels, allowing the wound healing process to progress. Furthermore, the effects of dHACM on HPCs were not assessed in this study, but after their engraftment, dHACM may potentially affect their functional capacity as a result of its immunomodulatory effects [26]. Further studies are needed to determine the effects of scaffold-cell interactions on recruited cell populations.

As mentioned previously, experimental and clinical evidence has demonstrated that progenitor cells promote repair and tissue regeneration. Both bone marrow-derived MSCs and adipose-derived stromal cells (ASCs) have been shown to promote wound healing [8,32,54–56], with ASCs in particular being the focus of significant research and clinical efforts due to their relative abundance and reproducible methods of procurement [57–59]. Unfortunately, ASC survival in the wound bed is limited, reducing the therapeutic window of applied ASCs [60]. Furthermore, autologous ASCs from diabetic individuals, who commonly suffer from chronic wounds, are dysfunctional [61], further limiting their therapeutic potential. Allogeneic MSCs, readily available from commercial sources, are subject to the same potential barriers as ASCs, including practical issues related to storage and delivery and functional issues, such as short-term engraftment in the wound with limited delivery of therapeutic factors.

dHACM, offers an alternative approach enhancing the recruitment and engraftment of endogenous progenitor cells capable of promoting neovascularization and wound repair. Moreover, it is stable at room temperature and does not require special storage or shipping. Our study indicates that the principal physiological activity of dHACM is its ability to recruit and incorporate endogenous progenitor cells, eliminating the need to harvest autologous cells from adipose tissue or bone marrow, or to commercially obtain cryopreserved allogeneic progenitor cells.

5. Conclusions

These experiments establish that dHACM is a biologically active scaffold that can recruit progenitor cells to a wound and thereby promote angiogenesis. Furthermore, dHACM may act synergistically with recruited cells to attenuate inflammation. The clinical benefits of dHACM may be partially explained by its ability to recruit circulating progenitor cells.

Supplementary Material

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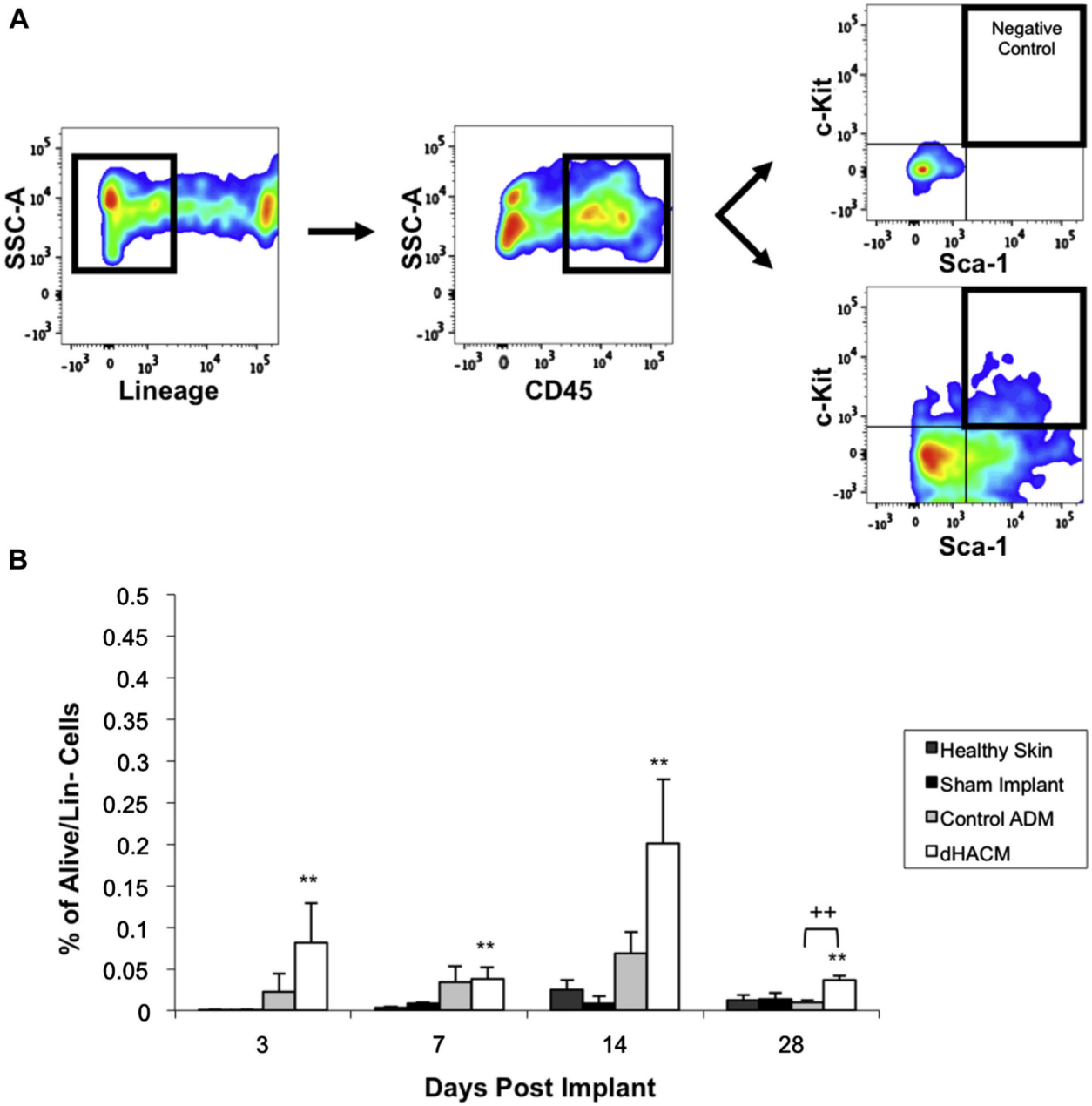

Fig. 1.

Fluorescence-activate cell sorting (FACS) analysis of HPCs in the implants and overlying skin. (A) A Lin-/CD45+/c-Kit+/Sca-1+ gating scheme was used to identify HPCs. (B) The relative number of HPCs in specimens of healthy skin, sham surgery sites, and control ADM are reduced compared with dHACM. ** indicates $P \leq 0.05$ for dHACM compared with healthy skin and sham implant. ++ indicates $P \leq 0.05$ for dHACM compared with control ADM. (Color version of the figure is available online.)

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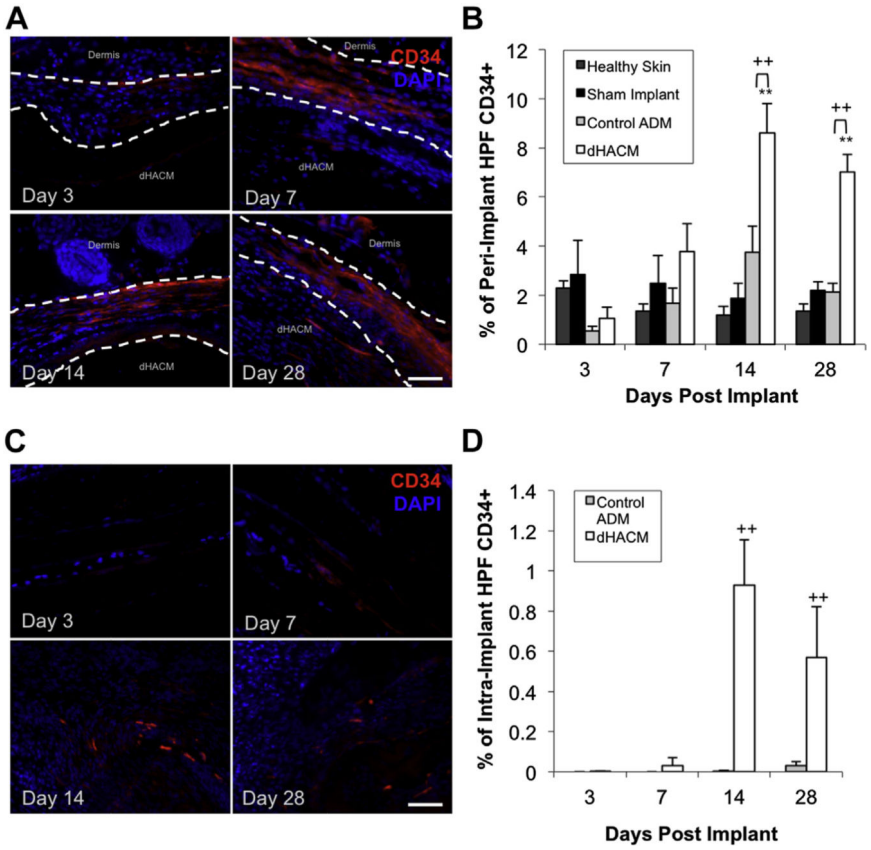

Fig. 2.

Immunohistochemistry for CD34 expression in dHACM and control groups. (A and B) Increased CD34 positive progenitor cells were visualized in the peri-implant space and skin overlying dHACM compared with controls. (C and D) Intraimplant CD34 + progenitor cell engraftment was increased in the dHACM group compared with control. ** indicates $P \leq 0.05$ for dHACM compared with healthy skin and sham implant. ++ indicates $P \leq 0.05$ for dHACM compared with control ADM. Scale bar: 50 μm. (Color version of the figure is available online.)

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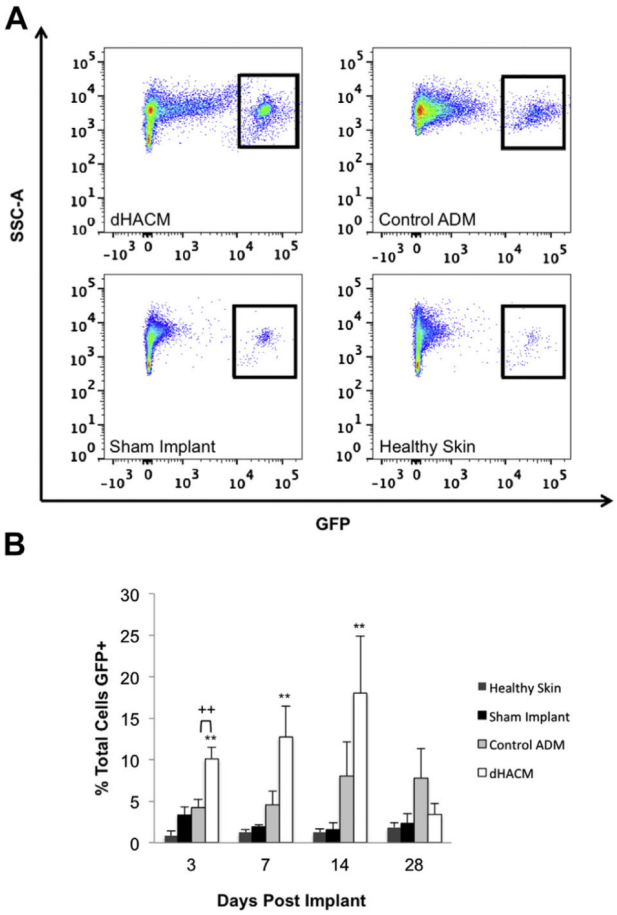

Fig. 3.

Flow cytometric analysis of GFP + cells in healthy skin, sham operated skin, control ADM, and dHACM after murine parabiosis. (A) Representative flow cytometry plots from day 7 samples identifying GFP + cells. (B) Flow cytometric quantification of GFP + cells showing increased recruitment by dHACM from day 3–day 14. ** indicates $P \leq 0.05$ for dHACM compared with healthy skin and sham implant. ++ indicates $P \leq 0.05$ for dHACM compared with control ADM. (Color version of the figure is available online.)

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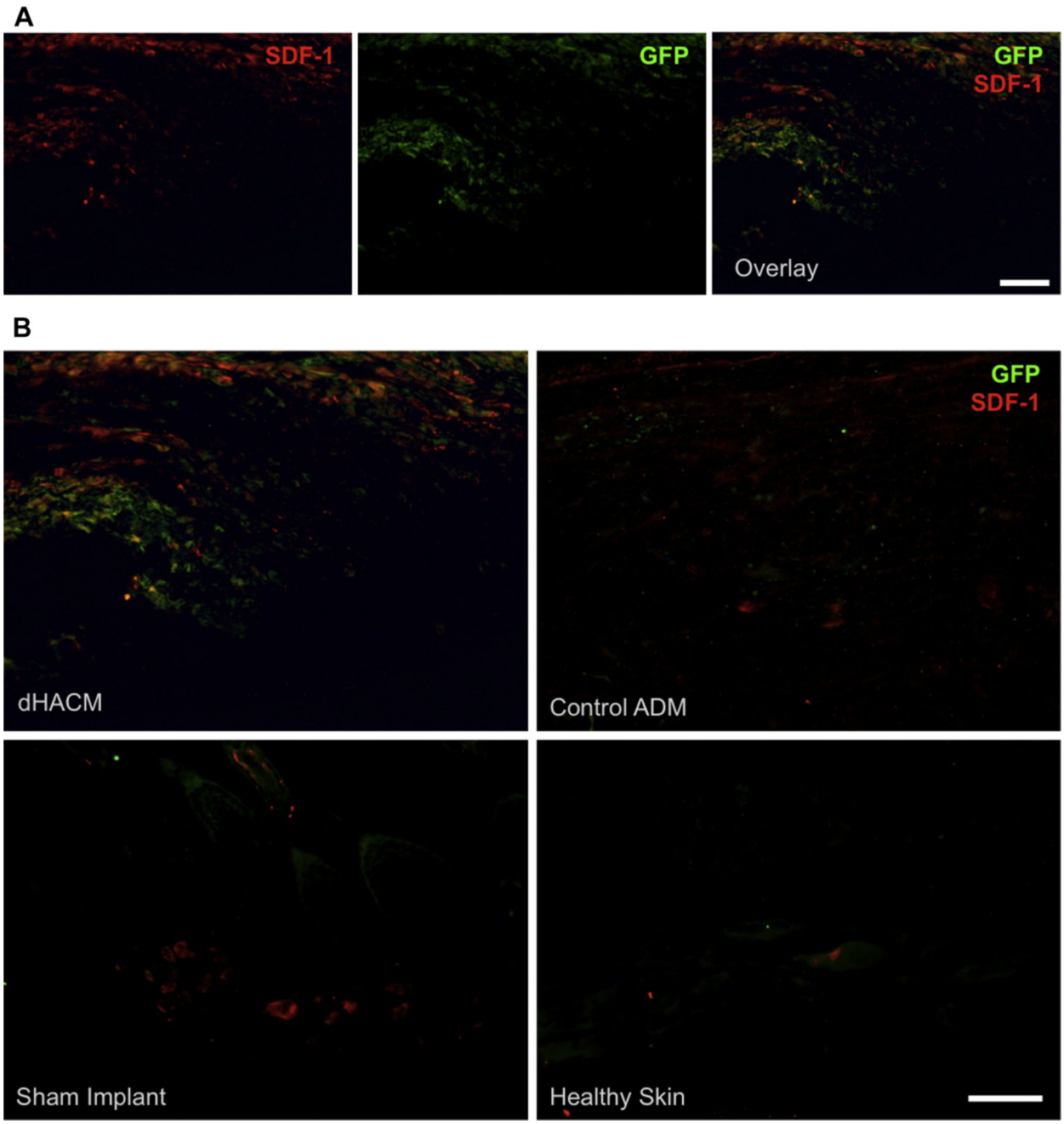

Fig. 4.

Immunohistochemistry of SDF-1 expression. (A) SDF-1 expression colocalizes with GFP expression in the dHACM group and (B) is relatively increased compared with controls. Scale bar: 50 μm. (Color version of the figure is available online.)

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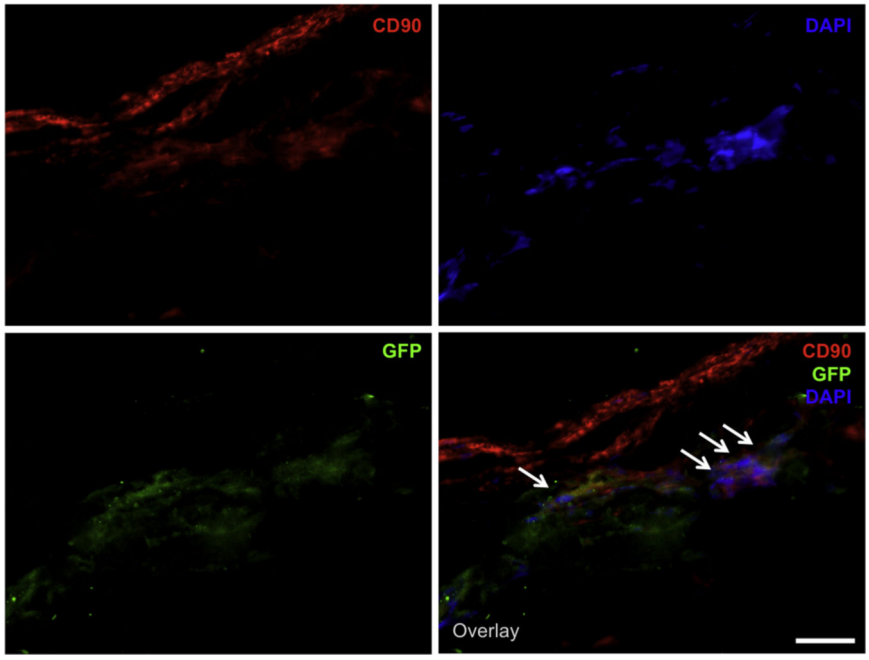

Fig. 5.

Immunohistochemistry of CD90 expression demonstrating coexpression with GFP and 4',6-diamidino-2-phenylindole (DAPI) nuclear stain within dHACM implants. White arrows demonstrate CD90+, GFP + recruited cells. Scale bar: 25 μm. (Color version of the figure is available online.)

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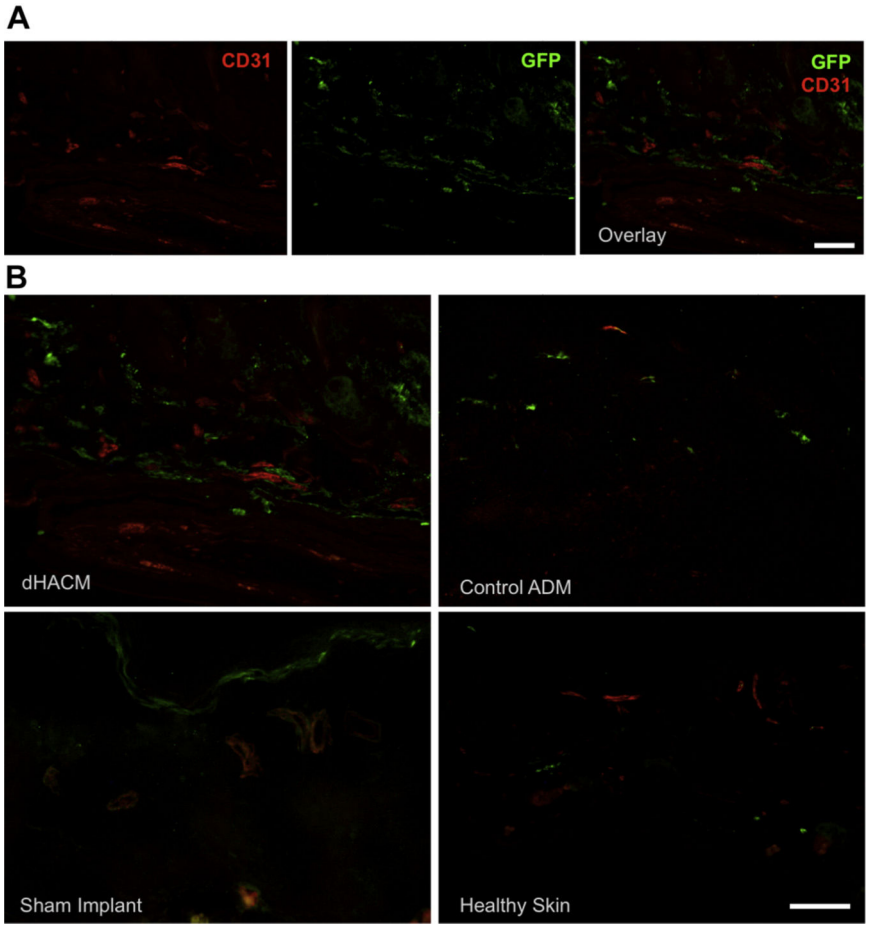

Fig. 6.

Immunohistochemical localization of recruited GFP + cells to sites of neovascularization. (A) GFP + cells are recruited to perivascular sites of CD31 expression within the dHACM; (B) a process that is relatively increased compared with control groups. Scale bar: 50 μm. (Color version of the figure is available online.)