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Granulocyte-colony stimulating factor mediates neovascularization in acellular dermal matrix-transplanted areas by promoting endothelial progenitor cell homing

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Abstract. Acellular dermal matrix (ADM) is an ideal material for tissue engineering skin construction. Accelerating the vascularization of ADM is of great significance for improving the survival of skin transplantation. The purpose of this study is to investigate the function of granulocyte-colony stimulating factor (G-CSF) in endothelial progenitor cells (EPCs)-mediated neovascularization in ADM-transplanted skin area. Male Kunming mice were subcutaneous injected with 10 μg/kg G-CSF at 5 days before skin *in situ* replantation or porcine ADM transplantation. The surrounding tissues of implanted skin or venous blood was collected from the mice before the operation, and after the operation for 48 h, 72 h, 1 week, and 2 weeks, respectively. Cells co-expressing EPC markers, CD133, CD34, and Flk-1 were detected by flow cytometry. Immunohistochemistry of BrdU was performed to evaluate neovascularization in ADM-transplanted skin area. The results showed that G-CSF treatment increased the number of $CD133^+$-$CD34^+$ cells and $CD133^+$-$Flk\text{-}1^+$ cells in ADM-implanted area as well as the number of $CD34^+$-$Flk\text{-}1^+$ cells in peripheral blood. Likewise, G-CSF also increased the number of capillaries in ADM-transplanted areas. To sum up, G-CSF mobilizes EPC migration from bone marrow to peripheral blood and homing to wound sites, thus inducing neovascularization in ADM-transplanted areas.

Key words: Granulocyte-colony stimulating factor — Endothelial progenitor cells — Acellular dermal matrix — Neovascularization — Homing

Abbreviations: ADM, acellular dermal matrix; EPCs, endothelial progenitor cells; G-CSF, granulocyte-colony stimulating factor.

Introduction

The skin is the human body's biggest organ, and it functions as the barrier between the internal and external environment, protecting against ultraviolet radiation, pathogenic microbial agents, and mechanical disturbances (Lee et al. 2006). Even though the injured epidermis drives self-regeneration in the presence of stem cells, deep or large-area burns or wounds might result in a chronic wound that requires grafting (Herndon et al. 1989; Vig et al. 2017). Autografts and xenografts are used in traditional skin transplantation, however, autologous skin is limited when a large wound arises, and xenografts increase the chance of immunological rejection (Przekora 2020).

Traditional wound-healing procedures and tissue regeneration are being challenged by tissue-engineered skin

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substitutes. Due to its outstanding biocompatibility and tissue regeneration qualities, acellular dermal matrix (ADM) generated from human or animal skin has been recognized as a suitable material for tissue engineering and wound healing as tissue replacement, transplant, and wound dressing (Iorio et al. 2012; Guo et al. 2017). The collagen scaffold is kept while the cellular components are removed when making ADM from donor dermis; the resulting material can enable tissue regeneration and native cell ingrowth after transplantation (Daar et al. 2016), thus solving the issues brought by autografts and xenografts.

Vascularity deficiency leads to the impaired or delayed regeneration process during wound healing. Endothelial progenitor cells (EPCs), which are the precursors of endothelial cells derived from bone marrow, play a role in tissue revascularization and repair. The circulating EPCs migrate into the circulation upon various stimuli including hypoxia (Tepper et al. 2005), growth factors (Moore et al. 2001; Heeschen et al. 2003), or chemokines (Yamaguchi et al. 2003), and nest to the damaged locations, thereby participating in vascular homeostasis *via* differentiating into mature endothelial cells (Asahara et al. 1997; Kalka et al. 2000). Moreover, EPCs secrete a variety of angiogenic growth factors contributing to keratinocyte and fibroblast proliferation to stimulate endogenous angiogenesis during wound healing, resulting in collagen synthesis and re-epithelialization (Hur et al. 2004; Shantsila et al. 2007).

Chemokine administration, either systemic or local, is implicated in vascular healing. These chemokines facilitate EPC migration into the peripheral circulation, increasing the quantity of EPCs (Kaushik et al. 2019). Granulocyte-colony stimulating factor (G-CSF), a member of the growth factor family, is produced by multiple cells, including bone marrow stromal cells, fibroblasts, macrophages, and endothelial cells. It mainly exerts the positive effects on the development and function of neutrophils and the binding of G-CSF to its specific receptors (G-CSF-R) determines the proliferation and differentiation of progenitor neutrophilic cells (Rahi et al. 2021). G-CSF has been used for bone marrow transplantation in the clinical setting for many years. In a rat model of distraction osteogenesis, systemic administration of G-CSF improves bone regeneration and regulates progenitor cell mobilization (Roseren et al. 2021). Increasing evidence demonstrates the crucial role of G-CSF in the mobilization and homing of EPCs. The combination of G-CSF with AMD3100 promotes the complete healing of full-thickness wounds and neovascularization by improving the mobilization of endogenous EPCs (Lin et al. 2021). G-CSF induced effective mobilization of EPCs with high regenerative and proangiogenic potential in patients with acute lymphoblastic leukemia (Eljaszewicz et al. 2018). These findings reveal that G-CSF is an essential regulator in the mobilization of EPCs and neovascularization.

Based on the key role of G-CSF in the mobilization of EPCs, we speculated that G-CSF also promoted the wound healing progression after ADM transplantation *via* enhancing neovascularization. To confirm this hypothesis, in this research, we detected the effect of G-CSF administration on EPC amount and the number of the vessel in ADM-implanted skin tissues and EPC amount in peripheral blood. The findings in this research may provide an applicable technique for the rapid vascularization of clinical transplantation of ADM and other artificial scaffolds, and also provides new insight into the vascularization in skin tissue engineering.

Materials and Methods

ADM transplantation

ADM is a porous three-dimensional network scaffold with normal collagen and basement membrane that produced by removing epidermal cells and the cellular components of the dermal layer *via* physical and chemical methods, exhibiting low antigenicity, good histocompatibility, and stable physicochemical property in transplantation. Here, porcine ADM purchased from Unitrump Biomedical Technology (Jiangsu, Qidong, China) was used for transplantation. Male Kunming mice were obtained from the Laboratory Animal Center of Kunming Medical College. The mice were anesthetized *via* intraperitoneal injection with pentobarbital sodium (50 mg/kg). Then the skin located in the lateral chest wall of mice was resected in the area of 1.2 cm × 1.2 cm after disinfection to cause defects. The porcine ADM (1.0 cm × 1.0 cm) was implanted onto the wound surface and covered by the excised autologous dermis. Subsequently, the wound was closed using an absorbable suture (Covidien Medical Shanghai Co. Ltd.). This study is reported in accordance with ARRIVE guidelines. Animal experiments were approved by the Ethics Committee of First Affiliated Hospital of Jinan University in accordance with the National Institutes of Health's guidelines for the care and use of laboratory animals.

Animal grouping and treatment

A total of 120 mice were randomly divided into four groups (n = 30 in each): Control, G-CSF, ADM, and ADM+G-CSF. The mice in Control group were subcutaneous injected with physiological saline at 5 days before skin *in situ* replantation. The injection was performed daily and lasted for 7 days (5 days before the operation and 2 days after the operation). The mice in G-CSF group were treated as same as the mice in the Control group except that saline was replaced by 10 μg/kg granulocyte-colony stimulating factor (G-CSF; Qilu pharmaceutical, Jinan, China). The mice in ADM and

ADM+G-CSF groups underwent ADM implantation operation as described above, and those in ADM group were injected with saline while those in ADM+G-CSF group were injected with G-CSF. All mice were injected with 50 mg/kg 5-bromodeoxyuridine (BrdU) intraperitoneally daily to mark vessel formation. The mice were euthanized before the operation, and at 48 h, 72 h, 1 week, and 2 weeks after the operation, respectively ($n = 6$ each time point). Then the ADM-implanted skin with surrounding tissues was resected and fixed with paraformaldehyde, and the blood of mice was collected for the following analysis.

Flow cytometry

The co-expression of CD34, CD133, and Flk-1 (EPC markers) in implanted skin tissues or peripheral blood from mice was detected by flow cytometry. For isolation of single-cell suspensions, the skin tissues (1 cm × 0.5 cm) were grinded in pre-cooled phosphate buffer saline (PBS) and filtered through a nylon membrane (70 μm). After 5 min of centrifugation at 400 × *g*, the precipitated cells were washed with PBS for 3 times and then re-suspended in PBS containing 1% bovine serum albumin (BSA). Subsequently, the single-cell suspensions or blood samples were incubated with FITC-conjugated anti-CD133 (BD Biosciences), Phycoerythrin (PE)-conjugated anti-Flk-1 (BD Biosciences), and PE-Cy5-conjugated anti-CD34 (BD Biosciences) for 15 min in the dark. Red blood cells were removed using Red Blood Cell Lysis Buffer (Beyotime) for 5 min. Subsequently, the cells were washed, fixed with 2% paraformaldehyde, and kept in the dark at 4°C until fluorescence-activated cell sorter (FACS) analysis (BD Biosciences). EPC counts are expressed as a percentage of total cells in each sample. Flow cytometry was performed in a blinded fashion.

Immunohistochemistry

Immunohistochemical Strept avidin-biotin complex kit (Zhongshan Goldenbridge Biotechnology, Beijing, China) was used for BrdU staining. The skin tissues fixed with paraformaldehyde were embedded in paraffin and cut into 5-μm sections. After deparaffinization and hydration, the sections were incubated with 3% H_2O_2 and blocked with 5% BSA. Then the sections were incubated with the primary anti-BrdU (1:100, ab152095, Abcam) for 1 h at 37°C, followed by the incubation with biotinylated secondary antibody working solution and horseradish peroxidase-conjugated streptavidin working solution, respectively. Then these slices were exposed to diaminobenzidine before being observed under a microscope. Immunohistochemistry was performed in a blinded fashion.

Statistical analysis

The data in this paper were expressed as mean ± standard deviation. Two-way ANOVA followed by Sidak's or Tukey's

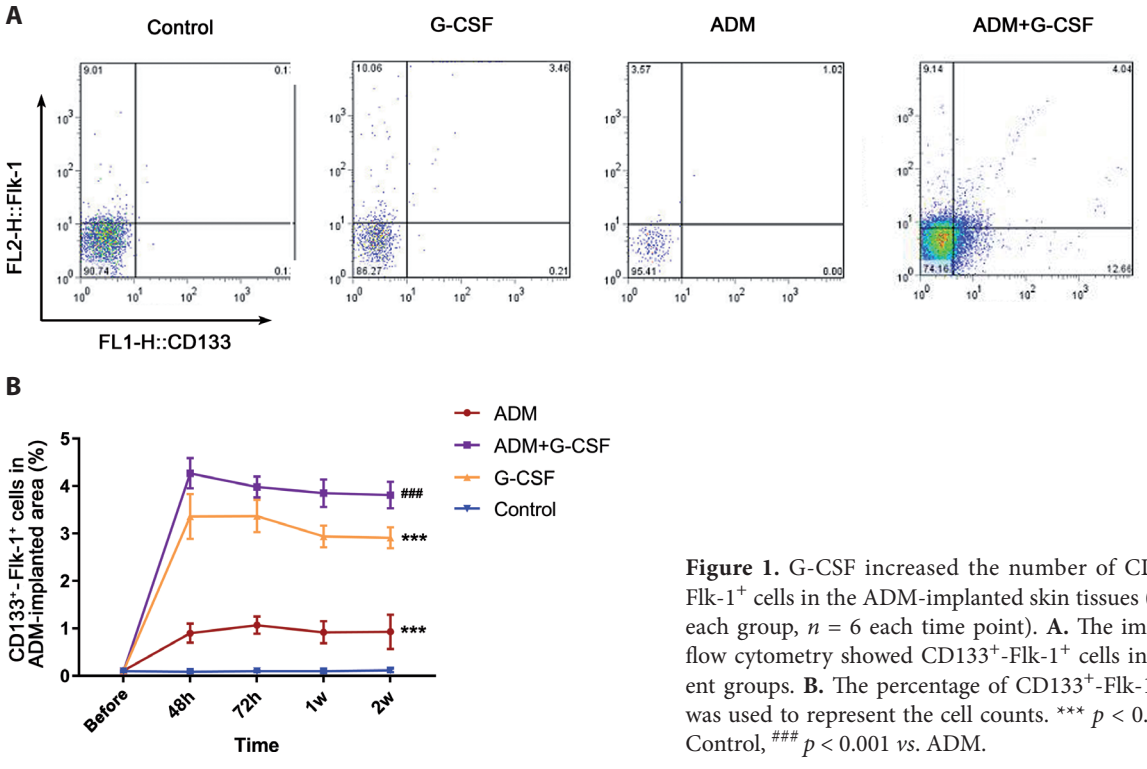

Figure 1. G-CSF increased the number of $CD133^+$-$Flk\text{-}1^+$ cells in the ADM-implanted skin tissues ($n = 30$ each group, $n = 6$ each time point). **A.** The images of flow cytometry showed $CD133^+$-$Flk\text{-}1^+$ cells in different groups. **B.** The percentage of $CD133^+$-$Flk\text{-}1^+$ cells was used to represent the cell counts. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* ADM.

post hoc test was used to compare the differences among these groups *via* GraphPad Prism 7 software. p value <0.05 was considered significant.

Results

G-CSF induces EPCs to nest to the ADM-transplanted area

Firstly, we detected EPCs in ADM-implanted skin tissues with flow cytometry. The percentages of cells co-expressing CD133 and Flk-1 were shown in Figure 1. Within 48 h after transplantation, the ratio of $CD133^+$-$Flk\text{-}1^+$ double-positive cells in all groups except Control group were rapidly elevated and came to a stable level almost unchanged in the following time points. Both the administration of G-CSF and ADM transplantation significantly increased the ratio of $CD133^+$-$Flk\text{-}1^+$ double-positive cells ($p < 0.001$ for G-CSF/ADM *vs.* Control). Besides, the percentage of $CD133^+$-$Flk\text{-}1^+$ double-positive cells in ADM+G-CSF group was higher than that in ADM group ($p < 0.001$ for ADM+G-CSF *vs.* ADM), which indicated that G-CSF mobilized the circulating EPCs to nest in the ADM-implanted area. Additionally, the number of $CD133^+$-$CD34^+$ cells was also examined in the operated area. As shown in Figure 2, the G-CSF exerted the same effects with $CD133^+$-$Flk\text{-}1^+$ on the percentage of $CD133^+$-$CD34^+$ cells ($p < 0.001$ for G-CSF/ADM *vs.* Control and ADM+G-CSF *vs.* ADM). These findings suggested that G-CSF promoted the migration of EPCs from bone marrow and induce its homing to the ADM-transplanted area. The detail statistical data of two-way ANOVA were shown in Table 1.

G-CSF promotes the migration of EPCs from bone marrow to peripheral blood

$CD34^+$-$Flk\text{-}1^+$ cells can be detected but $CD133^+$-$Flk\text{-}1^+$ cells are not detectable in the peripheral circulation. Therefore, we then detected the $CD34^+$-$Flk\text{-}1^+$ cells in the peripheral blood. The percentages of $CD34^+$-$Flk\text{-}1^+$ cells in Control group showed no alteration but those in the other three groups were rapidly elevated after transplantation. $CD34^+$-$Flk\text{-}1^+$ cell counts in peripheral blood of mice both in G-CSF and ADM groups were significantly higher than that in Control group ($p < 0.001$ for G-CSF/ADM *vs.* Control). G-CSF administration further increased the $CD34^+$-$Flk\text{-}1^+$ cells in mice receiving ADM transplantation ($p < 0.001$ for ADM+G-CSF *vs.* ADM), indicating that G-CSF promotes the migration of EPCs from bone marrow to peripheral blood (Fig. 3). The detail statistical data of two-way ANOVA were shown in Table 1.

G-CSF accelerates neovascularization in ADM-transplanted areas.

The neovascularization in ADM-transplanted area was assessed using immunohistochemical staining. The vessel

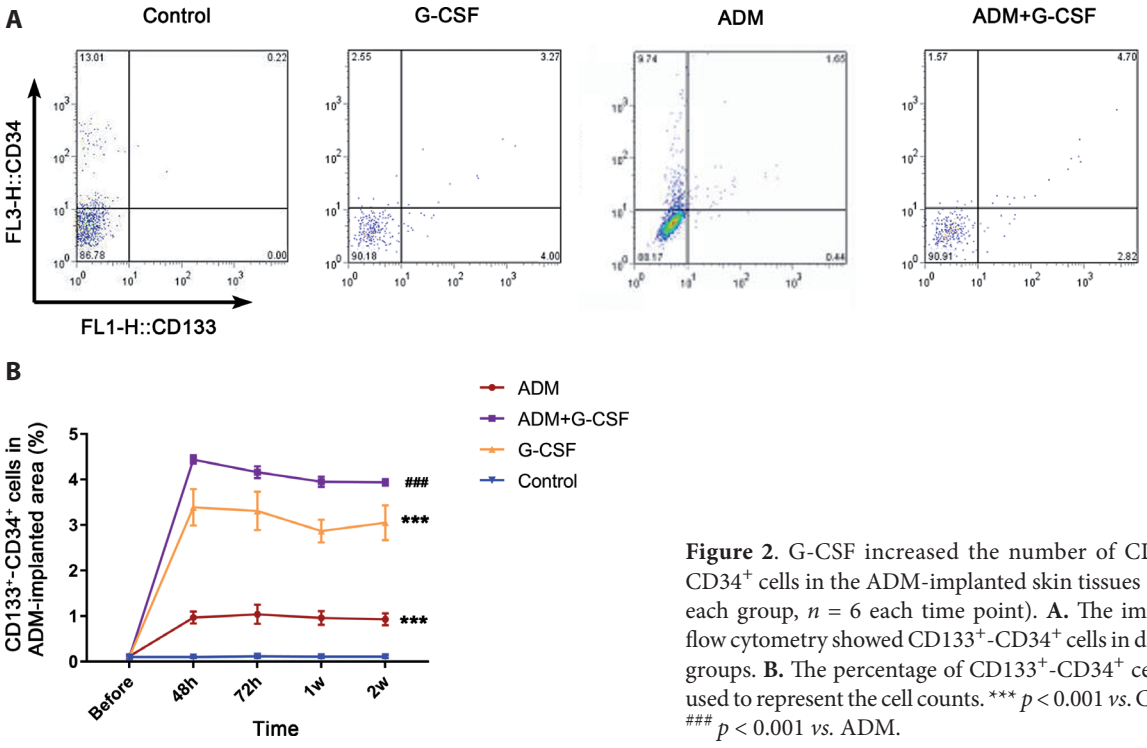

Figure 2. G-CSF increased the number of $CD133^+$-$CD34^+$ cells in the ADM-implanted skin tissues ($n = 30$ each group, $n = 6$ each time point). **A.** The images of flow cytometry showed $CD133^+$-$CD34^+$ cells in different groups. **B.** The percentage of $CD133^+$-$CD34^+$ cells was used to represent the cell counts. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* ADM.

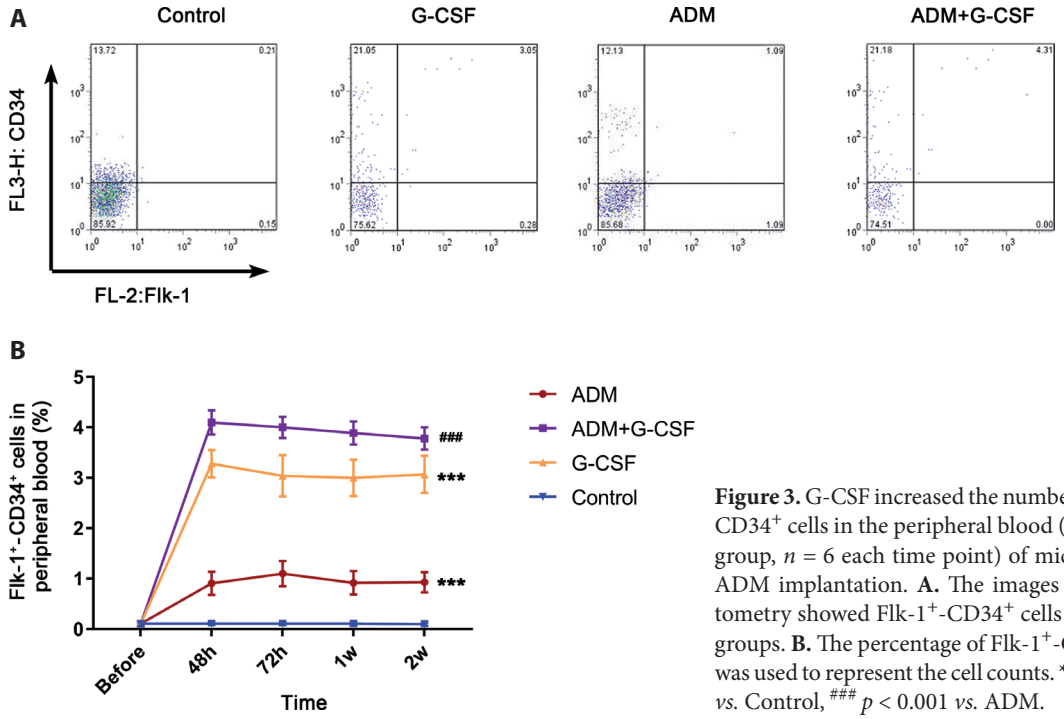

Figure 3. G-CSF increased the number of Flk-1$^+$-CD34$^+$ cells in the peripheral blood (n = 30 each group, n = 6 each time point) of mice receiving ADM implantation. **A.** The images of flow cytometry showed Flk-1$^+$-CD34$^+$ cells in different groups. **B.** The percentage of Flk-1$^+$-CD34$^+$ cells was used to represent the cell counts. *** $p < 0.001$ *vs.* Control, ### $p < 0.001$ *vs.* ADM.

density was represented as the number of capillaries. The results in Figure 4 showed that two weeks after ADM transplantation, the number of capillaries in transplanted areas was increased in ADM+G-CSF group compared with ADM group ($p < 0.01$ for ADM+G-CSF *vs.* ADM), indicating a positive effect of G-CSF on neovascularization in ADM-transplanted areas. The detail statistical data of two-way ANOVA were shown in Table 1.

Discussion

ADM has been widely used to improve wound healing and reconstruct damaged human tissue (Chen et al. 2013). In this study, we demonstrated that G-CSF increased the number of EPCs both in ADM-implanted skin and peripheral blood, as well as promoted the neovascularization in ADM-implanted skin, which suggested that G-CSF enhanced the mobilization of EPCs from bone marrow to peripheral circulation and then facilitated EPC homing to injured sites.

Inflammation, epithelialization, angiogenesis, remodeling, and scarring are all part of the cutaneous wound healing process, which is highly coordinated and complex (Takeo et al. 2015; Sorg et al. 2017). ADMs are known as soft tissue grafts generated by decellularizing tissue while maintaining the extracellular matrix. The cells of transplanted objects can use the matrix as a scaffold to develop and revascularize the graft. Wainwright first applied ADM to the treatment of a full-thickness burn injury and revealed

Table 1. The detailed statistical data of two-way ANOVA for studied parameters

	DF	F (DFn, DFd)	p value
CD133$^+$-Flk-1$^+$ cells			
Interaction	12	F (12, 80) = 105.5	< 0.001
Time	4	F (4, 80) = 476.6	< 0.001
Grouping	3	F (3, 20) = 832.3	< 0.001
ADM *vs.* Control[a]			< 0.001
G-CSF *vs.* Control[a]			< 0.001
ADM+G-CSF *vs.* ADM[a]			< 0.001
CD133$^+$-CD34$^+$ cells			
Interaction	12	F (12, 80) = 144.2	< 0.001
Time	4	F (4, 80) = 648.1	< 0.001
Grouping	3	F (3, 20) = 1211	< 0.001
ADM *vs.* Control[a]			< 0.001
G-CSF *vs.* Control[a]			< 0.001
ADM+G-CSF *vs.* ADM[a]			< 0.001
Flk-1$^+$-CD34$^+$ cells			
Interaction	12	F (12, 80) = 84.64	< 0.001
Time	4	F (4, 80) = 386.6	< 0.001
Grouping	3	F (3, 20) = 1274	< 0.001
ADM *vs.* Control[a]			< 0.001
G-CSF *vs.* Control[a]			< 0.001
ADM+G-CSF *vs.* ADM[a]			< 0.001
Number of capillaries			
Interaction	3	F (3, 40) = 3.636	= 0.02
Time	3	F (3, 40) = 22.8	< 0.001
Grouping	1	F (1, 40) = 8.399	= 0.006
ADM+G-CSF *vs.* ADM[b]			= 0.002

[a] Tukey's *post hoc* test; [b] Sidak's *post hoc* test.

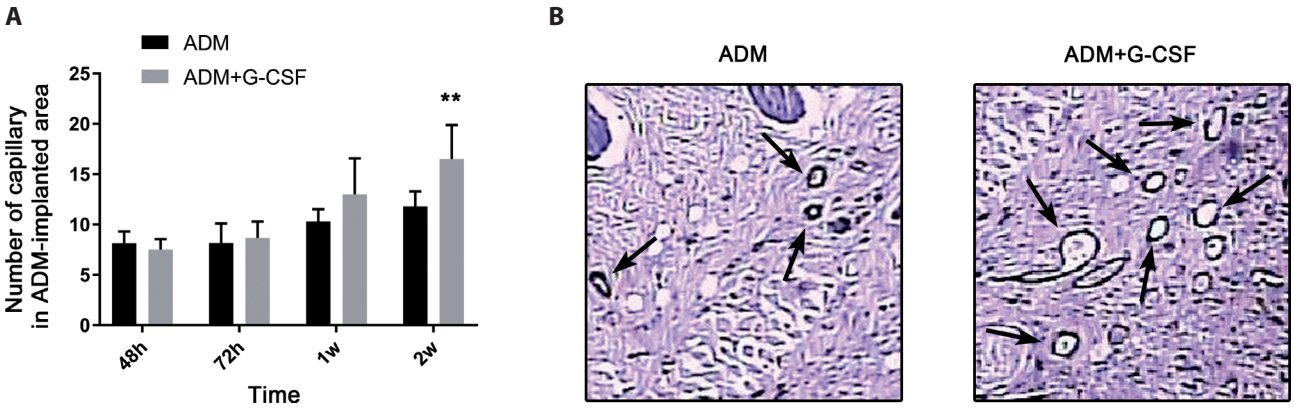

Figure 4. G-CSF promotes neovascularization in ADM-transplanted areas (n = 24 each group, n = 6 each time point). **A.** The number of BrdU-positive vessels was counted in the immunohistochemical staining. ** $p < 0.01$ *vs.* ADM. **B.** Representative images of immunohistochemical staining showed for BrdU-positive vessels in ADM-transplanted skin tissues two weeks after transplantation. The arrows represented capillaries. Magnification: ×40.

that ADM encouraged fibroblast infiltration, neovascularization, and epithelialization (Wainwright 1995). ADM has been commonly used for skin repair after injuries since then. Research also verifies that ADM has a promotional effect on wound healing (Chen X et al. 2020). In this study, by comparing the percentage of $CD133^+$-$Flk\text{-}1^+$ cells in the ADM-implanted area, we found that ADM promoted the mobilization of EPCs from bone marrow to the damaged locations.

EPCs account for roughly 0.001% of the overall stem cell population within the bone marrow. Although EPCs migrating from bone marrow constitute a tiny population of circulating cells in peripheral blood, they are capable of boosting vascular healing under ischemic or hypoxic conditions. EPCs adhere and mobilize in areas with ischemic damage. In response to ischemia insult, EPCs proliferate and differentiate into mature endothelial cells after nesting, forming new blood vessels and maintaining vascular homeostasis *via* direct intercellular connections, autocrine, and paracrine pathways (Fadini et al. 2012; Chopra et al. 2018). There is mounting evidence that bone marrow-derived EPCs can efficiently enhance neovascularization during tissue repair and endothelialization of vascular grafts. EPC implantation into dermal skin wounds improves new blood vessel formation by recruiting other inflammatory cells to the wound site, such as macrophages/monocytes (Suh et al. 2005), and accelerates the healing process. Moreover, exosomes from EPCs have been shown to stimulate cardiac fibroblast differentiation into endothelial cells by upregulating the expression of genes involved in the mesenchymal-endothelial transition (Ke et al. 2017). Various combinations of markers, such as $CD133^+$-$Flk\text{-}1^+$ (Peichev et al. 2000) and $CD34^+$-$Flk\text{-}1^+$ (Friedrich et al. 2006), have been used to identify the EPCs. Because mature endothelial cells do not express CD133, coexpression of Flk-1 and CD133 on $CD34^+$ cells phenotypically distinguishes a unique population of EPCs.

G-CSF is a cytokine that controls the proliferation, differentiation, and survival of neutrophils (Cox et al. 2014). G-CSF promotes the proliferation of all granulocyte lineages from myeloblasts (hematopoietic stem cells) to myeloid cells. It also can drive neutrophil differentiation, resulting in a rapid and sustained elevation in the number of circulating neutrophils (Basu et al. 2002; Chen W et al. 2020). Meanwhile, the function of G-CSF has been revealed in myocardial infarction. G-CSF recruits multipotent progenitor cells from the bone marrow into the peripheral bloodstream and mediated their differentiation into cardiomyocytes, vascular endothelial cells, or myofibroblasts, thus expediting healing processes (Orlic et al. 2001; Kawada et al. 2004; Harada et al. 2005). Besides, G-CSF administration, which triggers arteriogenesis in humans, markedly increases peripheral monocyte counts, alters arteriogenesis-related gene expression, and increased the number of EPCs (Meier et al. 2013). These findings implied the critical role of G-CSF in angiogenesis. As the results showed in this study, the percentages of $CD133^+$-$FLK\text{-}1^+$ and $CD133^+$-$CD34^+$ cells in the ADM-transplanted tissues were both increased after injection of G-CSF, which indicated that G-CSF induced more EPCs to home to the wound sites. $CD133^+$-$CD34^+$ cells are found to be mobilized to peripheral blood with G-CSF in large numbers (Camacho Villa et al. 2012), which is consistent with our findings. Furthermore, one study has shown that $CD34^+$-$Flk\text{-}1^+$ cells can be detected but $CD133^+$-$FLK\text{-}1^+$ cells are not detectable in the peripheral circulation (Asahara et al. 1997; Lanuti et al. 2016). Therefore, we then detected the $CD34^+$-$Flk\text{-}1^+$ cells and found that the percentage of $CD34^+$-$Flk\text{-}1^+$ cells

in the peripheral circulation was also increased due to G-CSF. In addition to the EPCs, the neovascularization in the ADM-transplanted area was also assessed. The increased number of capillaries after G-CSF treatment indicated that G-CSF induced neovascularization in ADM-transplanted skin. Similarly, in another research, G-CSF treatment is also demonstrated to increase the number of small arterials as well as microcapillary, thus inducing angiogenesis in myocardial infarction (Sato et al. 2008).

G-CSF was demonstrated to exert a positive role in the neovascularization of ADM-transplanted skin, which was mediated by its promotional effect on the mobilization and nesting of EPCs to the wound sites. However, the underlying pathway of G-CSF function remains unclear. G-CSF protects the human brain from vascular endothelial cell injury through MAPK and Akt signaling (Su et al. 2015). We wonder whether G-CSF regulates the neovascularization of ADM-transplanted skin by the same pathway and plan to confirm it in future research. In addition, the clinical value of G-CSF in the transplantation of ADM in human still needs to be confirmed.

In conclusion, G-CSF induces the mobilization and homing of EPCs to ADM-implanted skin to accelerate neovascularization, which may provide new insight into the rapid vascularization of clinical transplantation of ADM.

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Ethics approval. This study is reported in accordance with ARRIVE guidelines. Animal experiments were approved by the Ethics Committee of First Affiliated Hospital of Jinan University in accordance with the National Institutes of Health's guidelines for the care and use of laboratory animals.

Competing interests. The authors declare that they have no competing interests.

Availability of data and materials. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions. JW conceived and designed the present study. XL and XJ performed the experiments, analyzed the data, and drafted the article. HWL revised the article critically for important intellectual content. JW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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