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***REVIEW ARTICLE***

# Adipose Tissue-Derived Stem Cell Sheet Application for Tissue Healing *In Vivo*: A Systematic Review

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Adipose tissue-derived stem cells (ASCs) are known to be tissue-healing promoters due to their cellular plasticity and secretion of paracrine factors. Cultured ASC sheets provide a novel method of ASC application and can retain ASCs at the targeted tissue. The purpose of this systematic review is to evaluate preclinical studies using ASC sheet transplantation therapy for promoting tissue healing. First, we searched databases to identify studies of ASC sheet therapy in different experimental animal models, and then determined the quality score of studies using SYRCLE's risk bias tool. A total of 18 included studies examined the role of ASC sheets on tissue healing and function in models for myocardial infarction, dilated cardiomyopathy, full-thickness skin wounds, hind limb ischemia, esophageal strictures, and oral ulcers. ASC sheet application after myocardial infarction improved survival rate, cardiac function, and capillary density and reduced the extent of fibrosis. Application of ASC sheets to a full-thickness skin wound decreased the wound size and stimulated wound maturation. In the hind limb ischemia model, ASC sheet application improved limb perfusion and capillary density, and decreased the amount of ischemic tissue and inflammation. ASC sheet application to mucosal wounds of the digestive tract accelerated wound healing and decreased the degree of stricture and fibrosis. Taken together, transplanted ASC sheets had a positive effect on tissue healing and reconstruction in these preclinical studies. The reported favorable effects of ASC sheet therapy in various tissue healing applications may be implemented in future translational studies. It is suggested that future preclinical animal model studies of ASC sheet therapy should concern standardization of culture techniques and investigate the mechanisms of action. In addition, clearly indicated experimental setups according to the SYRCLE's guidelines should improve study quality and validity.

**Keywords:** adipose tissue-derived stem cells, cell sheet, *in vivo*, tissue healing, transplantation

## Introduction

Mesenchymal stem cells (MSCs) have been shown to be able to improve wound healing and regenerate tissue due to their paracrine effects and differentiation ability.[1,2] MSCs can be retrieved from various organs and connective tissues, including bone marrow,[3] adipose tissue,[4–6] dental pulp,[7–9] skin,[10,11] and umbilical cord.[12] Although these different stem cell populations are valuable, adipose tissue-derived stem cells (ASCs) are one of the most promising stem cell populations since human adipose tissue can be relatively easily obtained in large quantities with little donor site morbidity. Moreover, ASCs are immunoprivileged and can secrete bioactive factors that are important to tissue repair processes.[13]

ASCs have been injected as cell suspension or have been combined with biomaterials before delivering them to injured tissue[14–17] with variable degrees of success. Recently,

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cell sheet technology has been used to produce high-density cell sheet constructs that contain only cells and their secreted extracellular matrix. These cultured sheets are harvested as an intact monolayer or multilayer and can be transplanted to targeted host tissues without using biodegradable scaffolds and sutures. Cell sheets using two or more kinds of cell sources are also developed.[18,19]

ASC sheets consisting of ASCs and their secreted matrix have several advantages over ASC suspension or delivery of ASCs in a biomaterial since it immobilizes the delivered cells at the targeted site and requires no destruction of scaffold, suture, or glue when applied.[20,21]

To date, ASC sheets have been used in various tissues and pathologies, and different animal models. Therefore, the aim of this systematic review is to provide an overview of the effects of ASC sheets on the healing of different tissues. Results regarding the effects of ASC sheets on tissue healing and tissue reconstruction are discussed by relevant parameters in each disease model. ASC sheet features such as differentiation ability and secretion of paracrine factors are also discussed together with ASC sheet culture variables such as ASC source, seeding density, culture conditions and number of sheet layers. Finally, future directions for the use of ASC sheets in the field of regenerative medicine will be discussed.

## Materials and Methods

### *Inclusion and exclusion criteria*

Inclusion criteria were *in vivo* studies that examined the application of ASC sheets as local therapy to improve wound healing or tissue reconstruction. The study had to be written in English. The use of ASC sheets in combination with a scaffold, other cell types, and differentiated ASCs was excluded since these additions or modifications to ASC sheets can influence their behavior and consequently influence their effect on tissue healing. In addition, studies lacking a control group in which no ASC sheets were applied or in which ASCs were applied in any different way were also excluded. This last exclusion criterion was applied since a proper control is essential to evaluate the effect of ASC sheets in each different disease model.

### *Search strategy and data extraction*

The final systematic search was performed on February 9, 2017, in EMBASE, Medline (OvidSP), Cochrane, Web-of-science, Scopus, PubMed, and Google Scholar (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/teb). The systematic search was performed in collaboration with an information specialist from the Erasmus MC, University Medical Center library. The search strategy contained two main keywords: ASC and sheet. In the search, multiple synonyms were used for both topics. Conference abstracts were included in the primary search based on the keywords. After screening on title and abstract, full text was obtained from the relevant studies. The references of the included studies were screened for relevant studies.

Two independent reviewers (A.C. and P.S.) screened and included the studies separately and duplicates within the retrieved studies were removed. Disagreements were discussed. Studies were briefly screened by title and abstract based on whether ASC sheets were applied in an animal model followed by reviewing the full text based on inclusion and exclusion criteria.

### *Study characteristics and quality assessment*

Different characteristics were obtained from each study, including the type of transplantation, number of included animals, ASC sheet culture technology, time point of ASC sheet application, number of ASC sheet layers, and follow-up period after transplantation. *In vivo* measurements and (immuno-) histological outcomes were extracted to define wound healing and tissue reconstruction after sheet application.

For quality assessment of the studies, a modified version of SYRCLE's risk of bias tool[22] was used. Ten questions regarding different types of bias were answered by two independent reviewers (AC and PS, Supplementary Fig. S2). When the question was answered with ''Yes,'' that aspect was scored as bias free. When the question was answered with ''No,'' that aspect was scored as suspected bias. When the information was not sufficient to answer a question, the score was indicated as ''Unclear.''

## Results and Discussion

### *Study characteristics*

The search resulted in 2068 studies. After removal of duplicates, 910 studies were screened by abstract and title. Studies that did not examine the *in vivo* application of self-assembled ASC sheets were excluded. The full text was reviewed from 41 studies. Eighteen studies met all the inclusion criteria, contained no exclusion criteria, and thus were included in this systematic review (Fig. 1).

The outcomes of ASC sheet transplantations were compared to sham surgery, injection of phosphate-buffered saline (PBS), ASC suspensions injection, non-ASC sheets, or the application of modulated ASC sheets. Non-ASC sheets were made of dermal fibroblasts (DFB), ASC-derived cardiomyoblast-like cells (CLC), Sca-1-positive clonally expanded cardiac progenitor cells (CPC), and skeletal myoblast cells (SMBs). Modulated ASC sheets refer to ASC sheets that were modulated during culture before implantation using different methods, by inducing ASCs to overexpress vascular endothelial growth factor (VEGF) using baculovirus transduction[23,24] or by coculturing the ASC sheets with SMBs.[25] In some studies, ASC sheets with different numbers of cell layers were included and compared (Fig. 2).

Others also applied ASC sheets *in vivo*, such as in cardiac infraction in primates,[26] flexor tendon repair,[27] or pancreatic fistula treatment.[28] However, these studies do not fit in our inclusion criteria since these studies combined ASCs with other cell types or a collagen gel when preparing the cell sheet for transplantation without comparing this to an ASC sheet group only.

### *Study quality assessment*

The risk bias assessment according to a modified version of SYRCLE's guidelines[22] is summarized in Table 1. Quality assessment was difficult in most cases due to the lack of information about possible bias risks. Four studies in this were 5 or more[25,29–31] and are therefore considered a relatively low risk bias study in comparison to twelve included.

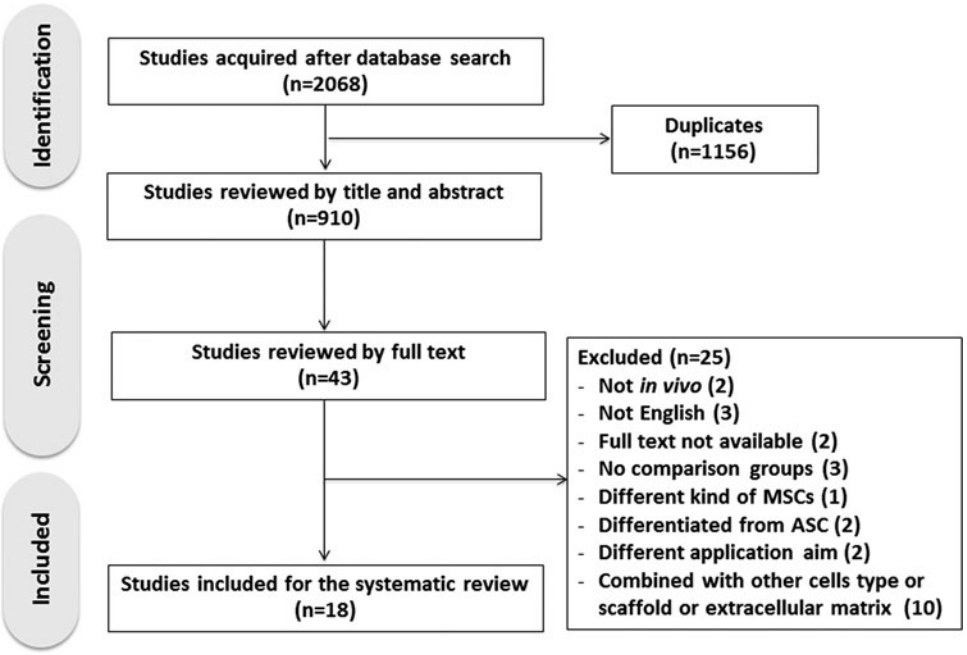


**FIG. 1.** Flow diagram of process for studies included in this systematic review. The number of studies in each phase is indicated between *brackets*.



*ASC sheets in cardiac injury*

Ten studies investigated the application of an ASC sheet in myocardial infarctions[23,25,29,32–39] and one study in dilated cardiomyopathy.[38] Nine studies used murine models.[25,29,33–35,37–39] Swine[32] and rabbit[23] were used in the other two studies. Xenogeneic transplantation of human ASC sheets into immunodeficient rats was performed in two studies[25,36] and mice ASC sheets were transplanted into rats given immunosuppressive drugs in one study.[39] Three out of

| Cardiac models (11) | Skin wound models (4) | Limb ischemia model (1) | Digestive tract models (2) |
|---|---|---|---|
| **Myocardial infarction (10)**<br>1. Sham surgery<br>2. PBS injection<br>3. ASC injection<br>4. Non-ASC sheet<br>- Cell free collagen sheet<br>- CPC sheet<br>- DFB sheet<br>- CLC sheet<br>- SMB sheet<br>5. Modulated ASC sheet<br>- VEGF overexpress ASC sheet<br>- ASC+ SMB sheet<br>- ASC+ CPC sheet<br>**Dilated cardiomyopathy (1)**<br>1. Sham surgery | 1. Sham surgery<br>2. ASC injection<br>3. layered ASC sheet | 1. Sham surgery<br>2. ASC injection<br>3. Modulated ASC sheet<br>- VEGF overexpress ASC sheet | **Esophageal wound (1)**<br>1. Sham surgery with supporting membrane<br>**Oral Ulcer (1)**<br>1. Sham surgery |

**FIG. 2.** Overview of different disease models in which ASC sheets were transplanted. The application of an ASC sheet was compared to many conditions. Sham surgery animals = untreated after disease is induced. ASC, adipose tissue-derived cell; CLC sheet, ASC-derived cardiomyoblast-like cell sheet; CPC sheet, cardiac progenitor cell sheet; DFB sheet, dermal fibroblast sheet; PBS, phosphate-buffered saline; SMB, skeletal myoblast cells; VEGF, vascular endothelial growth factor.

Table 1. Quality Assessment by Modified SYRCLE's Risk of Bias Tool

| | *1. Adequate sequence allocation* | *2. Similar groups at baseline* | *3. Concealed allocation to different groups* | *4. Animals randomly housed* | *5. Investigators blinded for intervention* | *6. Animals selected at random for outcome assessment* | *7. Outcome assessor blinded* | *8. Incomplete outcome data adequately addressed* | *9. Free of selective outcome reporting* | *10. Free of other problems* | *Total bias free score (0–10)* | *Suspected bias score (0–10)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cardiac models | | | | | | | | | | | | |
| Hamdi *et al.*[29] | **Yes** | **Yes** | **Un** | **Un** | **Un** | **Un** | **Yes** | **Yes** | **Yes** | **Yes** | **6** | **—** |
| Ishida *et al.*[32] | Un | Yes | Un | Un | Un | Un | Un | No | Yes | No | 2 | 2 |
| Ishii *et al.*[33] | Yes | No | Un | Un | Un | Un | Un | Yes | Yes | Yes | 4 | 1 |
| Kim *et al.*[39] | Un | Un | Un | Un | Un | Un | Yes | No | Yes | Yes | 2 | 1 |
| Matsuura *et al.*[34] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | Yes | 4 | — |
| Miyahara *et al.*[35] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | No | 3 | 1 |
| Okura *et al.*[36] | Un | Yes | Un | Un | Un | Un | Un | No | Yes | Yes | 3 | 1 |
| Otsuki *et al.*[37] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | Un | 3 | — |
| Shudo *et al.*[25] | **Yes** | **Yes** | **Un** | **Un** | **Yes** | **Un** | **Yes** | **Un** | **Yes** | **No** | **5** | **1** |
| Yeh *et al.*[23] | Yes | Yes | Un | Un | Un | Un | Un | Yes | Yes | Un | 4 | — |
| Hamdi *et al.*[38] | Un | No | Un | Un | Un | Un | Un | Un | Yes | Yes | 2 | 1 |
| Skin wound models | | | | | | | | | | | | |
| Cerqueira *et al.*[30] | **Yes** | **Yes** | **Un** | **Un** | **Un** | **Un** | **Un** | **Yes** | **Yes** | **Yes** | **5** | **—** |
| Kato *et al.*[42] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | No | 3 | 1 |
| Lin *et al.*[43] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | Un | 3 | — |
| Yu *et al.*[44] | Un | Un | Un | Un | Un | Un | Un | Un | Yes | Un | 1 | — |
| Limb ischemic model | | | | | | | | | | | | |
| Makarevich *et al.*[24] | Un | Un | Un | Un | Un | Un | Yes | Un | Yes | Yes | 4 | — |
| Digestive tract models | | | | | | | | | | | | |
| Lee *et al.*[45] | Yes | Yes | Un | Un | Un | Un | Un | Un | Yes | No | 3 | 1 |
| Perrod *et al.*[31] | **Yes** | **Yes** | **Un** | **Un** | **Un** | **Un** | **Un** | **Yes** | **Yes** | **Yes** | **5** | — |

Un, unclear, due to insufficient information. Studies with the least risk of bias are depicted in bold.

11 studies used an allogeneic source[23,38,40] and 5 out of 10 studies used an autologous source of ASCs[29,32,33,35,37] for transplantation into immunocompetent animals. The time between disease induction and sheet application differed from immediately to 4 weeks after injury. Follow-up time also varied from 3 to 16 weeks.

### Survival Rate

The survival rate of animals receiving an ASC sheet was reported in four different studies of the myocardial infarction model (Table 2). Survival rates differed between 70% and 100%.[29,33,35,36] The application of an ASC sheet improved survival rate in the myocardial infarction model when compared to sham surgery,[35,36] a PBS injection,[29] ASC injection,[29] or cell-free collagen sheets.[33] However, survival rates after application of CLC sheets were even higher than after application of ASC sheets[36] (Fig. 3A).

### Cardiac Function

All included studies evaluated the effect of ASC sheets on cardiac function (Table 2 and Fig. 3B) by examining echocardiographic measurements such as left ventricle ejection fraction,[25,29,32,36,37,39] left ventricle fractional shortening,[25,33–35,37–39] attenuation of maximum and minimum rate of change in left ventricular pressure (dP/dt),[25,34,35,37] left ventricle diastolic dimension,[34–36] left ventricle end diastolic pressure,[34,35] or left ventricle cavity.[23,25,35] Besides echocardiographic measurements to evaluate cardiac function, the biomarker for congestive heart failure, plasma atrial natriuretic peptide (ANP), was measured in three studies.[35–37] Compared with sham surgery and PBS or ASC injection, ASC sheet application improved cardiac function in nine studies after 3-, 4-, 8-, and 16-week follow-up.[25,29,32,33,35,37–39] In four studies, the application of an ASC sheet seemed to accelerate the improvement of cardiac function compared to sham surgery[23,36,40] or ASC injection,[39] eventually leading to the same cardiac function at the endpoint of the study.

Application of ASC sheets was also compared to non-ASC sheet application in six studies.[25,33–37] Restoration of cardiac function 4 weeks after ASC sheet application was found to be superior to a cell-free collagen or DFB sheet.[33,35] Cardiac function was similar after the application of SMB sheet.[25,37] However, superior cardiac function was observed after application of a CPC sheet (after 2 weeks) and a CLC sheet (after 4–9 weeks) when compared to ASC sheet application.[34,36]

Two studies evaluated whether modulated ASC sheets enhanced cardiac function.[23,25] ASC sheets overexpressing VEGF enhanced cardiac function compared to sham-operated animals, but not compared to sheets containing only ASC.[23] A composite ASC+SMB sheet enhanced cardiac function when compared to a sham surgery group or group treated with sheets containing only ASCs or myoblasts.[25]

ANP as biomarker for cardiovascular injury was measured in the plasma of animals in three studies. Lower plasma ANP levels were measured in all studies in rats receiving the ASC sheet than in rats receiving a DFB sheet or sham surgery.[35–37] However, after 8 weeks of application, rats receiving ASC sheets had higher plasma levels of ANP than rats receiving CLC sheets.[36]

### Infarct Size

Infarct size was measured by magnetic resonance imaging or by the weight of the infarcted cardiac tissue in two studies. Miyahara *et al.*[35] found no difference in infarct size between animals receiving an ASC sheet, DFB sheet, or sham surgery 4 weeks after implantation. On the other hand, at 4 weeks, Yeh *et al.*[23] found that ASC sheets and VEGF overexpressing ASC sheets decreased infarct size significantly compared to animals receiving sham surgery only.

### Vascularization

Improvement in cardiac muscle capillary density after ASC sheet application in comparison to sham surgery was found in six studies[23,25,32,33,37,39] and in comparison to ASC injection in one study.[39] No difference in capillary density after ASC sheet application was found in two studies.[29,34,38] One study found a significantly higher capillary density 1 week after ASC sheet application, which was no longer present at 5 weeks posttransplantation[34] (Fig. 3C).

When compared to cell-free collagen sheets, capillary density was improved 4 weeks after application of an ASC sheet.[33] When compared to SMB sheets or ASC injection, however, no difference in capillary density was found after 4 and 8 weeks.[25,29,37] Although ASC sheets initially improved cardiac muscle capillary density when compared to CPC sheets, at the end of the 4-week study period, capillary density in the ASC sheet group was less than the CPC group.[34] Modulated ASC sheets increased capillary density compared to animals either undergoing sham surgery or receiving non-modulated sheet or SMB sheet during a follow-up period of 8 weeks maximum.[23,25]

### Fibrosis

Eight studies measured the effect of ASC sheets on cardiac fibrosis after infarction.[23,25,29,33,34,37–39] ASC sheet transplantation decreased cardiac tissue fibrosis after cardiac injury when compared to sham surgery or cell-free collagen in six out of eight studies during 3–8 weeks of follow-up.[23,25,33,37–39] Only one study reported no effect of ASC sheet application on the extent of cardiac tissue fibrosis 4 weeks after infarction[34] (Fig. 3D). When compared to ASC injection and SMB sheet application, the fibrotic area after ASC sheet application was similar.[25,29,39] However, when compared to CPC sheets, the size of the fibrotic area after ASC sheet application was larger[34] (Fig. 3D). ASC sheets overexpressing VEGF and composite ASC+SMB sheets decreased cardiac tissue fibrosis even more than unmodulated ASC sheets.[23,25]

In summary, ASC sheet application after myocardial infarction improved the survival rate, cardiac function, capillary density, and the extent of fibrosis in 10 out of 11 studies, including 2 studies with the least risk of bias.[25,29] Both myocardial infarction and dilated cardiomyopathy seem to have better cardiac function recovery with ASC sheet application than after sham surgery, PBS injection, or ASC injection. In some studies,[36,40] the positive effects of ASC sheets were more obvious at early time points than at later follow-up time points. At early time points after injury and transplantation of the sheet, the damaged area can be ischemic and inflamed. Hypoxia and inflammatory cytokines are

## Table 2. Summary of Cardiac Study Characteristics

| *Author (year)*[ref.] | *ASC graft/ species of animal model* | *Number of animals in the study (animal in ASC sheet group)* | *Time between disease induction and sheet application (weeks)* | *Sheet technology ($x10^5$ cells/$cm^2$) and culture dish* | *ASC sheet modulated with* | *Sheet preimplantation cultured time (days)* | *Sheet layers (number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Modified ASC sheet compared to* | *Survival* | *Cardiac function* | *Infarct size* | *Capillary density* | *Fibrosis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Myocardial infarction* | | | | | | | | | | | | | | | |
| Hamdi *et al.* (2011)[29] | Autologous (Wistar rats) | 82 (21) | 4 | 3.6, thermoresponsive dish | | 1 | 3 | 8 | PBS injections | | ↑ | ↑ | | ↔ | ↔ |
| | | | | | | | | | ASC injections | | ↑ | ↑ | | ↔ | ↔ |
| Ishida *et al.* (2015)[32] | Autologous (Japanese pigs) | 14 (7) | 4 | 1.76, thermoresponsive dish | | Until confluence | 3 | 4 | Sham surgery | | | ↑ | | ↑ | |
| Ishii *et al.* (2014)[33] | Autologous (C57BL/6J mice) | 105 (25) | Immediately | 2.6, MCL on ultralow attachment plate | | 1 | Multi | 4 | Cell-free collagen gel sheet | | ↑ | ↑ | | ↑ | ↓ |
| Kim *et al.* (2017)[39] | Xenogenic (mice ASCs into rats) | 42 (16) | Immediately | 6.51, thermoresponsive dish | | 1 | 1 | 4 | Sham surgery | | | ↑ | | ↑ | ↓ |
| | | | | | | | | | ASC injection | | | ↔ | | ↑ | ↔ |
| Matsuura *et al.* (2009)[34] | Allogeneic (from GFP transgenic mice into C57BL/6J type mice) | 75 (25) | Immediately | 1.14, thermoresponsive dish | | 5 | 1 | 4 | Sham surgery | | | ↔ | | ↑&↔ | ↔ |
| | | | | | | | | | CPC sheet | | | ↓ | | ↑&↓ | ↑ |
| Miyahara *et al.* (2006)[35] | Autologous (Sprague-Dawley rats) | 46 (12) | 4 | 0.23, thermoresponsive dish | | 3 | 1 | 4 | Sham surgery | | ↑ | ↑ | ↔ | | |
| | | | | | | | | | DFB sheet | | | ↑ | ↔ | | |
| Okura *et al.* (2010)[36] | Xenogeneic (Human ASCs into nude SCID rats) | 85 (20) | 4 | 1.14, thermoresponsive dish | | 4 | 1 | 16 | Sham surgery | | ↑ | ↑&↔ | | | |
| | | | | | | | | | CLC sheet | | ↓ | ↓ | | | |
| Otsuki *et al.* (2015)[37] | Autologous (syngeneic Lewis rats) | 18 (11) | 4 | 2, thermoresponsive dish | | 1 | 1 | 4 | Sham surgery | | | ↑ | | ↑ | ↓ |
| | | | | | | | | | Myoblast sheet | | | ↔ | | ↔ | ↔ |

(*continued*)

Table 2. (Continued)

| *Author (year)*[ref.] | *ASC graft/ species of animal model* | *Number of animals in the study (animal in ASC sheet group)* | *Time between disease induction and sheet application (weeks)* | *Sheet technology ($x10^5$ cells/$cm^2$) and culture dish* | *ASC sheet modulated with* | *Sheet preimplantation cultured time (days)* | *Sheet layers (number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Modified ASC sheet compared to* | *Survival* | *Cardiac function* | *Infarct size* | *Capillary density* | *Fibrosis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Shudo *et al.* (2014)[25] | Xenogeneic (human ASCs into athymic nude rats) | 40 (10) | 2 | 0.85, thermoresponsive dish | Rat primary SMB cells | 4 | 3 | 8 | Sham surgery | | | ↑ | | ↑ | ↓ |
| | | | | | | | | | Modulated sheet | | | ↓ | | ↓ | ↑ |
| | | | | | | | | | Myoblast sheet | | | ↔ | | ↔ | ↔ |
| | | | | | | | | | | Sham surgery | | ↑ | | ↑ | ↓ |
| | | | | | | | | | | Myoblast sheet | | ↑ | | ↑ | ↓ |
| Yeh *et al.* (2014)[23] | Allogenic (New Zealand white rabbits) | 20 (5) | Immediately | 0.53, polystyrene plate | virus for VEGF overexpression | 3 | 1 | 8 | Sham surgery | | | ↔ | ↓ | ↑ | ↓ |
| | | | | | | | | | Modulated sheet | | | ↔ | ↑ | ↓ | ↑ |
| | | | | | | | | | | Sham surgery | | ↑ | ↓ | ↑ | ↓ |
| *Dilated cardiomyopathy* | | | | | | | | | | | | | | | |
| Hamdi *et al.* (2013)[38] | Allogeneic (from C57BL/6J into invalidated SRF mice) | 39 (20) | **3** | 3.4, thermoresponsive dish | | **2** | **1** | **3** | Sham surgery | | | ↑ | | ↔ | ↓ |

Summary of cardiac study characteristics.

Sham surgery animals are untreated after disease is induced. ↑ represents increase, ↓ represents decrease, ↔ represents not significantly different.

ASC, adipose tissue-derived stem cell; CLC sheet, ASC-derived cardiomyoblast-like cell sheet; CPC sheet, cardiac progenitor cell sheet; DFB sheet, dermal fibroblast sheet; MCL, magnetic cationic liposomes; PBS, phosphate-buffered saline; SMB, skeletal myoblast cells; VEGF, vascular endothelial growth factor.

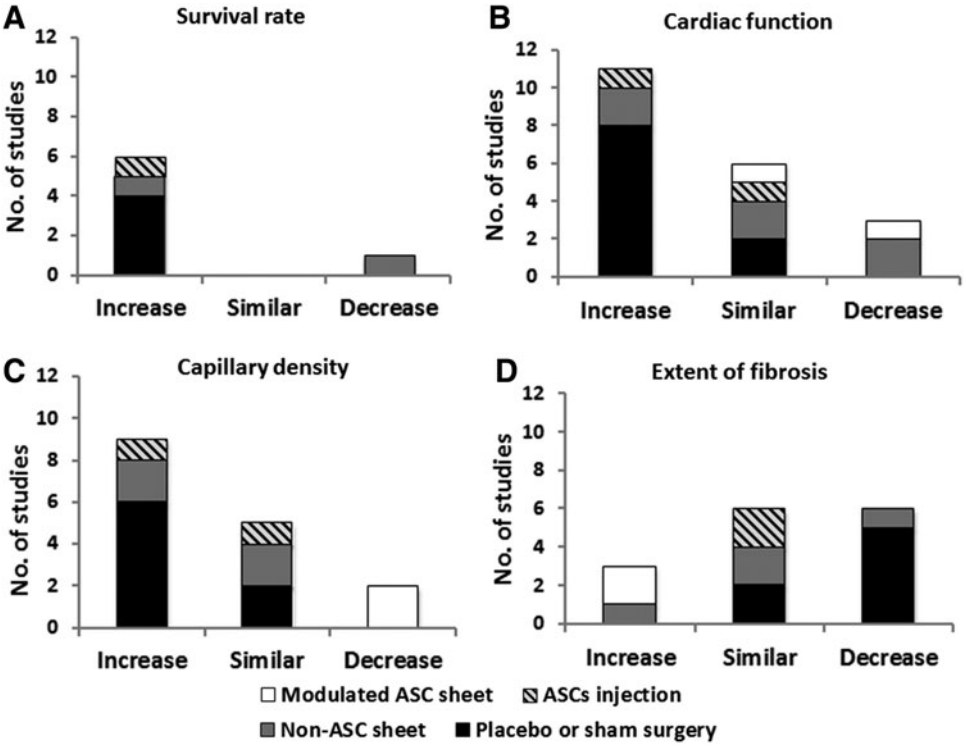


**FIG. 3.** Effects of ASC sheet application on the different read-out parameters after cardiac injury. **(A)** Survival rate; **(B)** cardiac function; **(C)** capillary density; **(D)** extent of fibrosis. Outcomes after application of ASC sheets were compared with different groups, each group represented by a different color; PBS injection (*black bars*) or sham surgery (disease without any treatment, *black bars*), application of a sheet containing collagen or other type of cells (non-ASC sheet, *dark gray bars*), ASC suspension injection (*light gray striped bars*) or modulated ASC sheet (*white bars*). Each bar represents the number of studies, of which characteristics can be found in Table 2.

known to enhance the paracrine ability of ASCs,[33,37,41] possibly contributing to the acceleration of cardiac repair in the short term, while not influencing regeneration at later time points when inflammation is diminished. Decreasing viability of ASC sheets at later time points after transplantation might also contribute to this finding.

### *ASC sheets in full-thickness skin wounds*

Four studies evaluated the effect of ASC sheets on the healing of full-thickness skin wounds.[30,42–44] In all studies, ASC sheets were applied immediately after wounding, in a diabetic rat model,[42] delayed wound healing models using mitomycin C,[44] weekly injections of depomedrol,[30] or splints.[43,44] One study used allogeneic ASC sheets for transplantation[42] and 3 studies used xenogenic human ASC sheets for transplantation in immunodeficient[43,44] or immunocompetent mice[30] (Table 3).

#### Wound Size and Wound Closure Time

Wound size and closure time were decreased in animals treated with an ASC sheet compared to animals receiving sham surgery and PBS injection in three studies.[42–44] In the study of Yu *et al.*,[44] a smaller wound size in the ASC sheet group was reported only at day 11 postwounding. Lin *et al.*[43] reported a smaller wound size at days 7, 10, and 14, but not at day 21 in animals receiving ASC sheets and a similar wound closure time to animals that received sham surgery only. In addition, mice treated with three-layer ASC sheets had significantly smaller wound sizes than animals receiving one-layer ASC sheets.[43] Cerqueira *et al.*[30] stated no difference in wound size or wound closure time between untreated animals and animals receiving ASC sheets after wounding (Fig. 4A, B).

#### Vascularization

Four studies evaluated capillary density between animals treated with an ASC sheet, sham surgery,[30,42,43] or ASC injection.[44] A higher capillary density 14 days postwounding was found in two studies after application of an ASC sheet than with sham surgery[42] or ASC injection[44] (Fig. 4C). Two studies found no difference in capillary density,[30,43] but found an increase in vessel diameter after the application of single or three-layered ASC sheets compared to sham surgery.[30]

#### Wound Maturation

At 14 days postwounding, an increase in connective tissue [42] and collagen,[43] or a higher number of epidermal cells[44] was reported after application of an ASC sheet to the dermal wound compared to the sham surgery group. Similarly, at 21 days postwounding, Cerqueira *et al.*[30] showed fully re-epithelialized wounds with hair follicles in the ASC sheet-treated group, whereas the wounds in the untreated group were only covered by a thin epithelial layer without hair follicles, suggesting a higher degree of epidermal maturation after application of an ASC sheet (Fig. 4D).

Taken together, beneficial effects of ASC sheets were seen in all four studies on regeneration of full-thickness skin wounds, both in models for (delayed) wound healing and diabetic skin wounds. A faster decrease in wound size and an increase in wound maturation were noted. These four studies also included a study with the least risk of bias. Based on this, it seems that ASC sheet application is also beneficial for skin healing. However, the small number of studies and lack of information to score the risk of bias make it difficult to draw firm conclusions for further clinical translation.

Table 3. Summary of Full-Thickness Skin Wound Study Characteristics

| *Author (year)*[ref.] | *ASC graft* | *Number of animals in the study (animal in ASC sheet group)* | *Impaired wound healing (yes or no)* | *Sheet technology (cells/cm$^2$) and culture dish* | *Sheet preimplantation culture time (days)* | *Sheet layers (number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Wound size* | *Wound closure time* | *Capillary density* | *Wound maturation* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cerqueira *et al.* (2013)[30] | Xenogeneic (human ASCs into Balb/c mice | 36 (24) | Yes | $3\times10^5$, thermoresponsive dish | 5 | 3 | 3 | Sham surgery | ↔ | ↔ | ↔ | ↑ |
| | | | | | | | | ASC sheet on polystyrene | ↔ | ↔ | ↔ | ↑ |
| | | | | $3\times10^5$, Polystyrene culture dish | 5 | 3 | 3 | Sham surgery | ↔ | ↔ | ↔ | ↑ |
| Kato *et al.* (2015)[42] | Allogeneic (ASCs from Lewis rats into Zucker diabetic fatty rats) | 48 (24) | Yes | $1.7\times10^4$, thermoresponsive dish | 7–8 | 1 | 6 | Sham surgery | ↓ | ↓ | ↑ | ↑ |
| Lin *et al.* (2013)[43] | Xenogeneic (human ASCs into athymic nude mice) | 18 (12) | No | Density not indicated, but $1\times10^6$ per cell sheet (unknown dish size) thermoresponsive dish | Until confluence | 1 | 3 | Sham surgery | ↓ | | ↔ | ↑ |
| | | | | | | | | Three layers | ↑ | | ↔ | ↓ |
| | | | | | | 3 | 3 | Sham surgery | ↓ | | ↔ | ↑ |
| Yu *et al.* (2014)[44] | Xenogenous (human ASCs into nude mice) | 15 (5) | Yes | $9.09\times10^3$, polystyrene culture dish | 7 | 1 | 2 | PBS injections | ↓ | ↓ | | ↑ |
| | | | | | | | | ASC injections | ↔ | ↔ | ↑ | |

Summary of full-thickness skin wound study characteristics. Sham surgery animals are untreated after disease is induced. ↑ represents increase, ↓represents decrease, ↔ represents not significantly different.

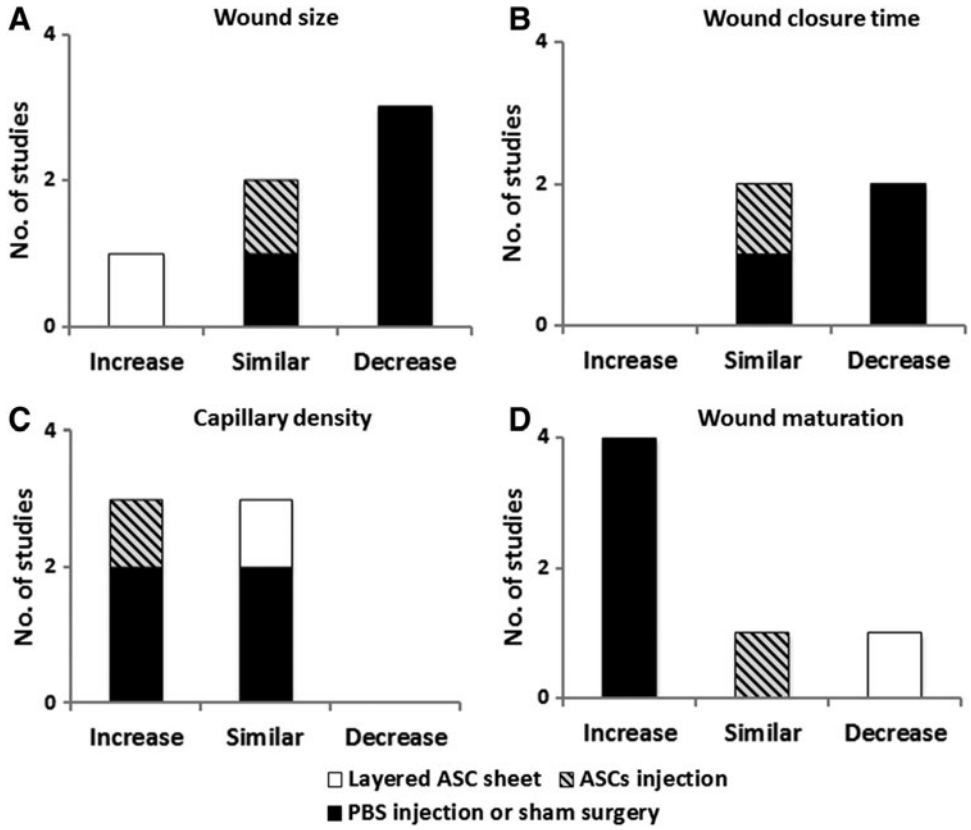


**FIG. 4.** Effects of ASC sheet application on different regenerative processes in full-thickness skin wounds. **(A)** Wound size; **(B)** wound closure; **(C)** capillary density; **(D)** wound maturation. Outcomes after application after application of ASC sheets were compared to different groups; PBS injection or sham surgery (*black bars*), injection of ASC suspension (*gray striped bars*), or an ASC sheet consisting of three layers (*white bars*). Each bar represents the number of studies, of which characteristics can be found in Table 3.



### *ASC sheet in hind limb ischemia*

One study[24] evaluated the effect of allogeneic ASC sheet application in a mouse model with induced hind limb ischemia. ASC sheets or ASC sheets overexpressing VEGF, ASC injection, or sham surgery were applied to the ischemic muscle after inducing hind limb ischemia in mice. Application of ASC sheets as well as VEGF overexpressing ASC sheets resulted in a higher capillary density and increased limb perfusion compared to ASC injection or sham surgery (Table 4). The VEGF overexpressing ASC sheets induced a significantly higher limb perfusion than ASC sheets 14 days after application. In addition, ASC sheets and especially VEGF overexpressing ASC sheets significantly reduced muscle necrosis and inflammation.

### *ASC sheets in digestive tract injury*

One study[31] evaluated the effect of allogeneic ASC sheet application in a porcine model after esophageal submucosal dissection. Two layers of ASC sheets supported by a membrane were endoscopically transplanted and compared with a control group that only received the membrane. Allogeneic ASC sheet application resulted in a lower incidence of severe strictures 3 days after surgery and less strictures at 2 and 4 weeks after surgery than the control group. ASC sheet application also caused a reduction in fibrosis and dysphagia, which resulted in more weight gain.

One other study[45] evaluated the effect of allogenic ASC sheet application on chemically induced oral mucosal ulcers in rabbits. ASC sheet application resulted in accelerated full-thickness healing of the anterior gingiva and buccal mucosa compared to sham operation (Table 5).

When applied to an esophageal wound, ASC sheets decreased the degree of stricture formation. When applied to oral mucosal ulcers, ASC sheets accelerated full-thickness healing. Even though these results are promising and one of these two studies is a relatively low risk bias study, more studies are needed to confirm the positive effect of ASC sheet therapy for these applications.

### *ASC sheet preparation techniques*

Even though in general ASC sheets were beneficial for tissue regeneration, not all parameters improved in all studies. This might be due to the fact that preparation technique, cell number, and cell density varied in the ASC sheets between the different studies and the measured parameters, methods, or time between disease induction and sheet application were different. To create ASC sheets, different protocols were used. ASC seeding density (seeding in high confluency versus seeding them in low density until confluence), culture time, type of culture dish (normal vs. thermoresponsive), and culture conditions used to obtain ASC sheets, as well as the number of ASC sheets stacked before transplantation (one sheet vs. three stacked sheets) varied between different studies as mentioned in Tables 2–5. As shown by articles included in this review, xenogeneic or allogeneic ASC sheet transplantation can be successfully used to promote tissue healing in both immunocompromised[25,36,43,44] and immunocompetent[23,24,30,31,38,42] recipients. These studies also included the least and low risk bias studies. Most of the studies in this review (12 out of 16) seeded ASCs at a density higher than $1 \times 10^5$ cells/mm$^2$ and culture time varied from 1 to 8 days or until confluence.

TABLE 4. CHARACTERISTICS OF HIND LIMB ISCHEMIA STUDY

| *Author (year)*[ref.] | *ASC graft* | *Number of animals in the study (animal in ASC sheet group)* | *Time between disease induction and sheet application (weeks)* | *Sheet seeding density (x $10^5$ cells/$cm^2$) and culture dish* | *ASC sheet modulated with* | *Sheet preimplantation culture time (days)* | *Sheet layers (Number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Modified ASC sheet compared to* | *Limb perfusion* | *Capillary density* | *Viable tissue (%)* | *Inflammation and necrosis (%)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Makarevich *et al.* (2015)[24] | Allogenic (C57/B6 mice) | 30 (14) | Immediately | 2.63, 12-well polystyrene culture dish | virus for VEGF overexpression | 2 | 1 | 2 | Sham surgery | | ↑ | ↑ | ↑ | ↑ |
| | | | | | | | | | Modulated sheet | | ↓ | ↓ | ↓ | ↓ |
| | | | | | | | | | ASC injection | | ↑ | ↑ | ↑ | ↑ |
| | | | | | | | | | | Sham surgery | ↑ | ↑ | ↑ | ↑ |
| | | | | | | | | | | ASC injection | ↑ | ↑ | ↑ | ↑ |

Characteristics of limb ischemia study. Sham surgery animals are untreated after disease is induced. ↑ represents increase, ↓ represents decrease, ↔ represents not significantly different.

Table 5. Characteristics of Digestive Tract Injury

*Esophageal submucosal dissection*

| *Author (year)*[ref.] | *ASC graft* | *Number of animals in the study (animal in ASC sheet group)* | *Time between disease induction and sheet application (days)* | *Sheet seeding density (cells/cm$^2$) and culture dish* | *Sheet preimplantation culture time (days)* | *Sheet layers (number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Dysphagia* | *Stricture (degree of stenosis)* | *Epithelium thickness* | *Fibrosis* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Perrod *et al.* (2016)[31] | Allogenic (porcine) | 11 (6) | Immediately | $4.3\times10^4$, thermoresponsive dish | 0.5 | 2 | 4 | Sham surgery with support membrane | ↓ | ↓ | ↑ | ↓ |

*Oral ulcer*

| *Author (year)*[ref.] | *ASC graft* | *Number of animals in the study (animal in ASC sheet group)* | *Time between disease induction and sheet application (days)* | *Sheet seeding density (cells/cm$^2$) and culture dish* | *Sheet preimplantation culture time (days)* | *Sheet layers (Number)* | *Follow-up period after sheet implantation (weeks)* | *ASC sheet group compared to* | *Ulcer size* | *Epithelium thickness* |
|---|---|---|---|---|---|---|---|---|---|---|
| Lee *et al.* (2016)[45] | Allogenic (rabbit) | 12 (6) | 2 | $2\times10^6$ per dish (unknown dish size), thermoresponsive dish | Unknown | 1 | 1 | Sham surgery | ↓ | ↑ |

Characteristics of Gastrointestinal injury study. Sham surgery animals are untreated after disease is induced. ↑ represents increase, ↓represents decrease, ↔ represents not significantly different.

Despite the fact that some *in vitro* studies report how different seeding densities influenced ASC paracrine ability,[17,46–48] the effect of ASC seeding density *in vivo* was not indicated in any of these studies. Whether seeding density affected the ability of ASC sheets to promote tissue healing is unknown.

Application of multilayered sheets instead of single-layered sheets on full-thickness skin wounds resulted in a smaller wound size, but not in a reduction of wound closure time. Wound capillary density was also lower after application of multilayered sheets compared to sham surgery.

Temperature-responsive culture plates were mainly used to produce the ASC sheets in the studies used for this review (12 out of 16). Alternative methods for creation of ASC sheets are the use of classical polystyrene culture plates or the use of ultralow attachment plates coated with magnetic cationic liposomes.[26] Two studies investigated whether a temperature-responsive plate altered the behavior of ASC sheets when compared to classical polystyrene plates. Hamdi *et al.* report similar gene expression patterns of ASC sheets after being cultured in classical polystyrene culture plates or in temperature-responsive culture plates.[29] On the other hand, Cerqueira *et al.* reported that ASC sheets from temperature-responsive plates could be more easily stacked to form an ASC construct than polystyrene-cultured and mechanically detached ASC sheets, and showed more significant improvement in full-thickness skin healing.[30] However, based on the included articles, it is difficult to identify one best method for producing ASC sheet for *in vivo* application since many factors contribute to the beneficial effect of ASC sheets.

Three studies with low risk of bias report beneficial effects of modulated ASC sheets using ASCs overexpressing VEGF[23,24] or making a composite sheet of ASCs with other cell types such as SMB.[25] Overexpression of VEGF by ASCs improved the therapeutic efficacy,[24] probably by promoting cell survival in the hypoxic environment (i.e., ischemic myocardium) after application.[23] The fact that ASC-SMB combined sheets resulted in an even greater improvement of cardiac function when compared to ASC sheets might be caused by a synergistic effect in their paracrine ability.[25] These results indicate possibilities to further enhance the beneficial effects of ASC sheets on tissue healing.

### *Hypothesized mechanism*

To investigate the mechanism behind the effect of ASC sheets on tissue healing, seven studies, including two studies that had the least risk of bias, detected endothelial cells that were derived from implanted ASCs. One study also claimed that ASCs differentiated toward pericytes.[33] Two of the five studies that investigated differentiation toward cardiomyocytes reported this actual differentiation, of which one had a low risk of bias.[29] Conflicting evidence exists on differentiation toward the epidermal lineage; one study with a low risk of bias reports that epidermal cells were derived from the transplanted ASC sheets[30] and one with a higher risk of bias indicated no dermal differentiation.[33] For some cardiac muscle healing parameters, CPC sheets and CLC sheets performed even better than ASC sheets in tissue regeneration. One explanation for these findings might be that these sheets contained already differentiated cells closely resembling the recipient tissue, but still with precursor characteristics, including the ability to secrete paracrine factors that promote tissue healing. In comparison to CLCs and CPCs, however, ASCs are more readily available, can be harvested less invasively, and require less complex culture protocols.[49]

Six studies showed an increase in gene expression and secretion of tissue healing factors after transplantation of ASC sheets compared to sham surgery. These factors included VEGF,[25,33,34,37] hepatocyte growth factor,[25,37] basic fibroblast growth factor (bFGF),[33,37,39] insulin-like growth

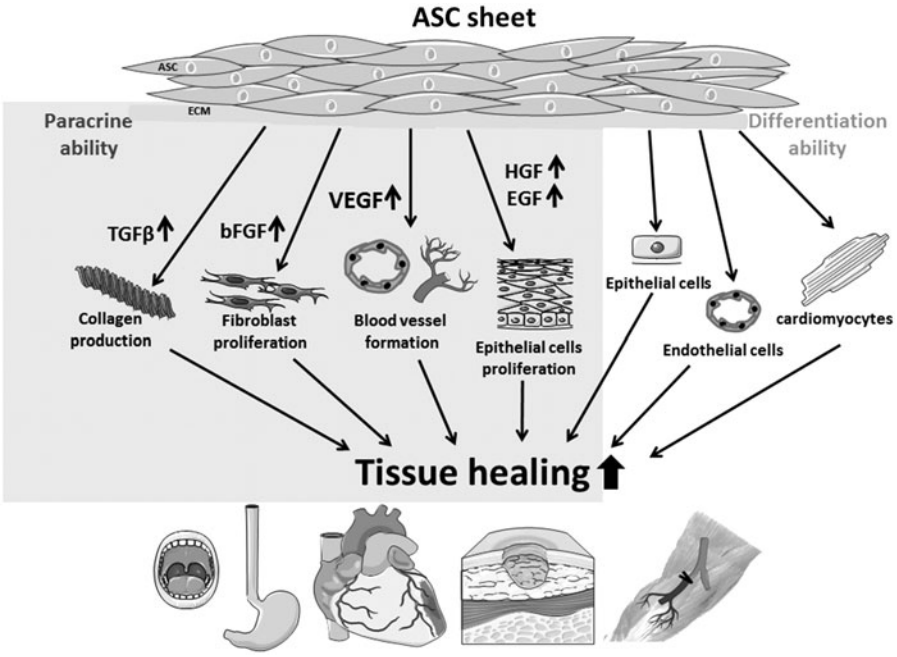


**FIG. 5.** The hypothesized working mechanism behind the beneficial effects of ASC sheets on tissue healing in different disease models. *Left panel* indicates the paracrine ability of ASC sheet. Each factor was investigated after ASC sheets were transplanted *in vivo*. *Right panel* indicates the differentiation ability of ASCs in the sheet toward different cell types that were investigated in the studies included in this review. bFGF, basic fibroblast growth factor; ECM, extracellular matrix; EGF, epithelial growth factor; HGF, hepatocyte growth factor; TGFβ, transforming growth factor beta.[50]

factor 1 and 2 (IGF1 and IGF2), transforming growth factor β1 (TGF-β1), and epidermal growth factor (EGF).[39] Four of these studies also showed the high production or expression of VEGF, bFGF, HFG, TGF-β1, EGF, IGF1 and 2, collagen I, interleukin 1 receptor antagonist, adiponectin, and fibronectin by the ASC sheets before transplantation.[25,33,39,40] In addition, some studies have shown that ASCs cultured in a high-density sheet have higher expression of several factors such as VEGF and FGF2 and lower expression of the proinflammatory cytokine such as tumor necrosis factor alpha than ASCs cultured in low density,[39,47] supporting the earlier mentioned results. Myocardial stress-induced biomarkers such as ANP and bone morphogenic protein 4 were decreased after the application of an ASC sheet.[38] Taken together, ASC sheet paracrine signaling and ASC differentiation into other cell types might have contributed to the beneficial effects of ASC sheets on tissue healing (Fig. 5).[50] Further investigation, however, needs to be done to confirm these results.

### *ASC sheets in different disease models*

A variety of disease models were used in the included studies in which the ASC sheets were applied, which have different effects on the created wounds and their healing. The methods of creating the wound or the wound environment (ischemic or normoxemic) most likely will alter the therapeutic effect of the ASC sheet. ASC sheet ability to increase capillary density seems obvious in a disease model with ischemia or hypoxia of the tissue such as cardiac infarction,[23,25,32,33,39] diabetic wounds,[42] or hind limb ischemia,[24] whereas this effect is less expected in dilated cardiomyopathy.[38] This might be due to the fact that ASC sheets in a hypoxic environment secrete or express factors beneficial for angiogenesis, such as VEGF and FGF2, at a higher level than with normoxia.[33,37]

## Conclusion

An array of preclinical studies has investigated the effect of ASC sheet transplantation on the healing of different tissues. Eighteen studies investigated the effect of ASC sheet application in animal models for the regeneration of five different tissues, namely cardiac tissue, skin, ischemic muscle, esophagus, and oral mucosa. In general, 17 out of these 18 studies found a beneficial effect of ASC sheets on tissue regeneration. ASC sheets promote tissue healing in at least cardiac tissue and skin, with the most evidence existing for cardiac experimental models. ASC sheets promoted tissue healing by stimulating angiogenesis and positively influencing other parameters—survival, function, capillary density, and wound size. Based on this, we conclude that ASC sheets are indeed beneficial for healing of cardiac muscle and full-thickness skin wounds. A similar beneficial effect of ASC sheets on tissue healing in a hind limb ischemia model and esophageal and oral mucosal wounds has only been demonstrated once to our knowledge.[24,31,45] In general, the application of an ASC sheet resulted in better tissue recovery, regeneration, and function than sham surgery, PBS or ASC injection, and non-ASC sheets. ASC sheets modulated to overexpress VEGF or composite ASC sheets enhanced the beneficial effects of ASC sheets on tissue healing. These promising results warrant future investigation of the effect of ASC sheets on the healing of other types of tissues. In addition, to enhance the beneficial effects of ASC sheets, modulating ASC sheets seems promising. Further *in vitro* and *in vivo* studies should focus on standardization of culture techniques and investigation of the mechanisms of action. Moreover, experimental setup details should be clearly indicated according to the SYRCLE's guidelines to be useful for translation to future clinical application.

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## Disclosure Statement

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