

Grafting of mesenchymal stem cell-seeded small intestinal submucosa to repair the deep partial-thickness burns

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ABSTRACT

Purpose: Regenerative medicine provides many treatments for burn wounds, of which cell-seeded substitutes are encouraging for large and deep burns. To assess the feasibility of mesenchymal stem cell (MSC)-seeded small intestinal submucosa (SIS) to repair the deep partial-thickness burns, a rat study was performed. **Materials & Methods:** The burn model was created by contacting the dorsal surface directly with boiled water for 10 seconds. MSCs at passage 3 were seeded on the SIS before implantation. Three days after burn injury, the grafts were implanted onto the burn area. At 3, 7, 14 and 21 days post implantation, gross observation and histological assessments were performed. **Results:** SIS alone and MSC-seeded SIS were able to accelerate the burn wound closure by enhancing granulation tissue formation, increasing wound maturity, improving revascularization, and inducing the proliferation of neo-epidermal cells. Additionally, MSC-seeded SIS was much more effective than SIS alone for the repair of deep partial-thickness burns. **Conclusion:** Both SIS and MSC-seeded SIS were able to repair the large and deep burn wounds and the loaded MSCs possessed positive effects to accelerate the wound closure in a rat model.

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Introduction

Graft-assisted healing is an important therapy for skin wounds. Porcine small-intestinal submucosa (SIS), a collagen-rich extracellular matrix, has been shown to be effective in the healing of excisional wounds (1). These SIS-based regenerative approaches, either alone or seeded with stem cells/growth factors, were able to enhance the wound healing rate, to promote angiogenesis, and to suppress the local inflammation (1–5). The promising success should be attributable to growth factors released from SIS and its important component-glycosaminoglycans after treatment (6,7), which jointly regulated the tissue development and remodeling.

Nevertheless, these encouraging outcomes from the excisional wound studies cannot be easily extrapolated to the burn wounds which are more complicated in the pattern of damage. Compared with the excisional wound, burns are much more difficult to be healed because of the

extensive necrosis and the ischemic dermal environment (8); in addition, the burn wounds are dynamic and can be deepened over time to form hypertrophic scarring (9,10). It was reported that the deep partial-thickness burn wounds could lead to death within 7–10 days after injury (11). Thus, early and effective therapies for burn wounds are required. Definitely, animal investigations are needed to be done if extending the SIS-based therapy from the excisional wounds to the burn wounds.

Other approaches for wound healing involve implanting active stem/progenitor cells and the aim is to replace those lost during burn injuries after the implanted cells can survive, differentiate and produce matrix macromolecules. Mesenchymal stem cells (MSCs) are one of the encouraging candidate cells. It was demonstrated that MSCs implantation was able to improving the rate of wound closure, increase granulation tissue formation and to enhance revascularization (12–14). To increase the anchoring rate of implanted cells, biomaterials, such

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as SIS and artificial substitutes, have been extensively employed for cell delivering. Of recent years, it was found that MSC-seeded SIS could suppress inflammation of the excisional wounds and increase the expression of skin regeneration-related growth factors, leading to rapid recovery of the skin injury (5). But few researchers have investigated the feasibility of combining MSCs with SIS for burn wound repair and the potentially regenerative mechanisms are still far from well understood.

In this study, it was thus hypothesized that MSC-seeded SIS was able to repair the burn wounds effectively. To test this hypothesis, the rat deep partial-thickness burns model was created and the burn wounds were covered by SIS and MSC-seeded SIS after tangential excision, respectively. Gross observation, histological and immunochemical studies were then performed accordingly.

Materials and methods

Experimental animals

Forty-nine adult male Sprague–Dawley (SD) rats (2-month old, 200–250 g) were purchased from the Laboratory Animal Center of Southwest Medical University, one of which was used for MSC isolation and the others for wound healing assessment. All the animal procedures were approved by the Animal Care and Use Committees of Southwest Medical University, following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolation of mesenchymal stem cells (MSCs)

The bone marrow was harvested by flushing the marrow cavity of femurs and tibiae with phosphate-buffered saline (PBS) after the rat was sacrificed. The solution that rich in bone marrow cells was placed in a tube and rinsed twice with PBS by centrifugation at 1000 g for 5 min. The fat layer was discarded. The remaining cells were seeded at a density of 2×10^5 cells/cm² in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin and 100 mg/ml streptomycin (Beyotime, Shanghai, China) at 37 °C in 5% CO₂ and 95% air atmospheric condition. The medium was replaced every 3 days to remove non-adherent cells. After 10 days in culture, spindle-shaped cells were trypsinized. MSCs were well identified as described in our previous study (15,16). The cells at passage 3 (Supplementary Figure 1A) were used in this study.

Small intestinal submucosa (SIS) preparation

Porcine SIS was prepared according to the standard procedures described previously (17). In brief, a segment of proximal jejunum was obtained from pig cadaver specimens. After carefully washing in a saline solution, the intestine was everted. The tunica mucosa, the serosa and tunica muscularis were removed by longitudinal wiping motions with moistened gauze. The remaining intestinal submucosa tube was split longitudinally and each was cut in to approximately 6 cm in length.

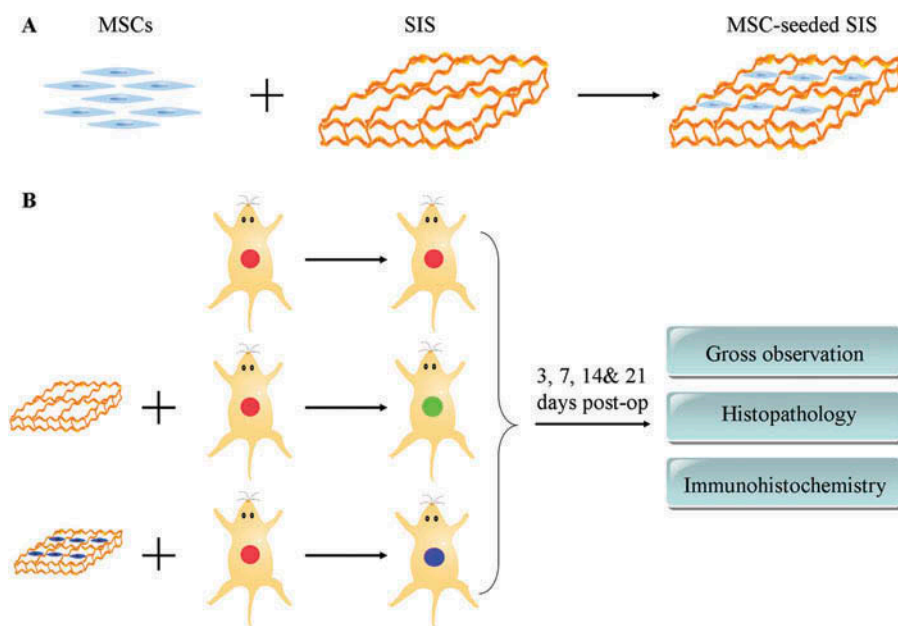


Figure 1. Schematic drawings illustrating the experimental designs of this study. (A) MSCs seeded into the SIS. (B) After implantation of SIS alone and MSC-seeded SIS in the rats, a series of tests was performed at 3, 7, 14 and 21 days.

After rinsed in 0.25% trypsin and vibrated in 0.1% sodium dodecyl sulfate (SDS) for 12 h, respectively, the resident cells were removed. The SIS sheets were sterilized with 0.1% peracetic acid, washed again with a saline solution and then freeze-dried at -55°C for 48 h. The resultant SIS was vacuum-sealed into hermetic packaging and terminally sterilized by ethylene oxide (Supplementary Figure 1B).

MSCs seeded into the SIS

The SIS sheet was taken out from the hermetic packaging and cut into fragments with a diameter of about 3 cm. The SIS fragments were rehydrated in six-well plates filled with culture medium. After 24 h culturing, the culture medium were removed and MSCs at passage 3 were seeded on the SIS patches (5×10^5 cells/cm²) according to our previous study (18). Patches were then cultured *in vitro* for 5–7 days before implantation. SIS alone was prepared following the same method. To observe the morphology of MSCs on SIS, after 5–7 days incubation, the patches were harvested and fixed in 10% paraformaldehyde for 20 minutes for H&E staining and scanning electron microscopy (SEM; Cambridge Sensotec S-250, Cambridge, UK) observation.

Animal burn model

A deep partial-thickness burn model was induced according to the previous study with minor modifications (19) and validated by pathologic examination (Supplementary Figure 2). Briefly, all animals were anesthetized by the intraperitoneal injection of 3% pentobarbital sodium solution at a dosage of 1.0 ml/kg. The back hair was removed with 8% Na₂S aqueous solution. The opening position of a glass pipe with a diameter of about 3 cm was placed on the dorsal skin. The duration of dorsal surface contacting directly with boiled water (100 °C) in the vitreous pipe was 10 seconds. Deep partial-thickness burn wounds with a diameter of 3 cm were then created for experiments.

Skin grafts implantation

The overview of the experiment design is described in Figure 1. Three days after burn modeling, 48 rats were randomly divided into three groups: MSC-seeded SIS group ($n = 16$), SIS alone group ($n = 16$) and the control group without any treatments ($n = 16$). All rats were anesthetized and the operating area was pre-sterilized with 0.5% iodophor. After removal of

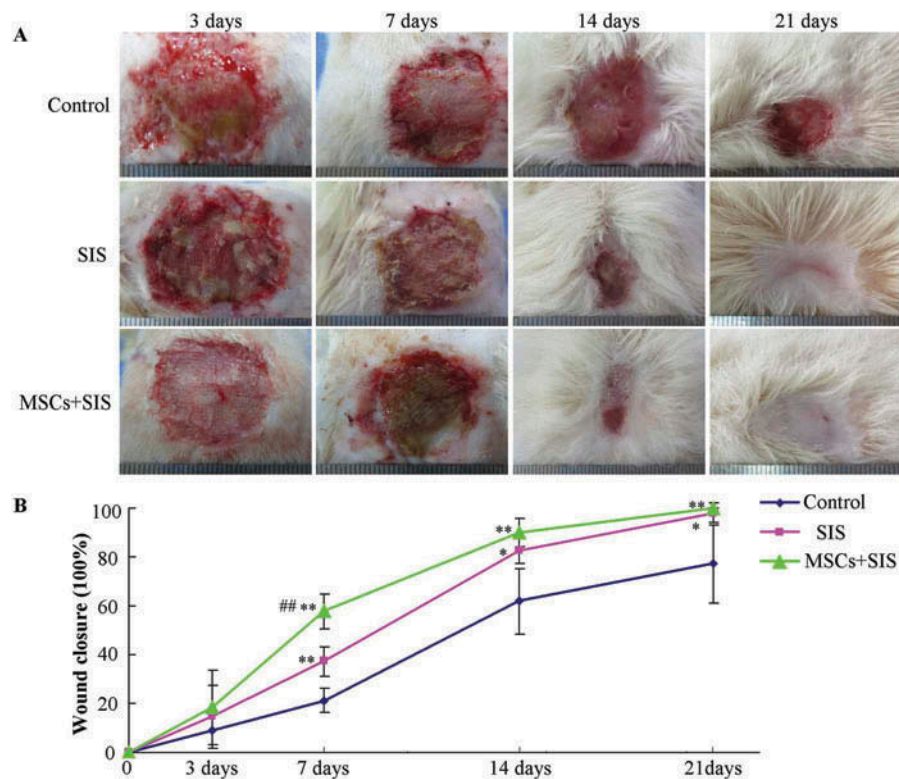


Figure 2. Both the SIS and MSC-seeded SIS enhanced the burn wounds repair. (A) Representative images illustrated the gross appearance of burned wounds treated with SIS alone, MSC-seeded SIS and the untreated group on the days 0, 3, 7, 14 and 21. Scale bar = 1 mm. (B) Statistical analysis of areas of burned wounds. ($n = 4$ per time point). * $P < 0.05$, vs. control group; ** $P < 0.01$, vs. control group; ## $P < 0.01$, vs. SIS group.

the necrotic crusts, MSC-seeded SIS and SIS alone were placed onto the burn area and sutured to the adjacent skin, respectively (Supplementary Figure 3). Then each rat received a pressure dressing (petrolatum gauze and elastoplast). The wounds that only covered with petrolatum gauze and elastoplast were served as control. Postoperatively, daily injections of ceftazidime and buprenex intraperitoneally were applied for 7 days to minimize discomfort experience and the opportunity of infection.

General observation and specimen harvest

The wounds were monitored and photographed every day. Wound area was calculated using the image analysis software (Image-Pro Plus 6.0). Wound closure rate was measured as the formula: (area of initial wound – area of actual wound)/area of initial wound $\times$ 100%. At the 3, 7, 14 and 21 days after implantation, four rats from each group were scarified under anesthesia and the full-thickness transverse biopsies of the wounds were harvested.

Histopathological examination

The tissue specimens were fixed in 10% buffered formalin for 24 h, dehydrated with a series of graded ethanol, embedded in paraffin, and then sectioned at a thickness of 5 μ m. Sections of 7 days after implantation were stained with H&E to measure the length of neoepithelium, and to assess the granulation tissue formation and maturity of the burn wound. Additionally, Masson's trichrome staining was performed on days 7 and 21 for the evaluation of collagen in the granulation tissue.

Assessment of granulation tissue formation and wound maturity

Granulation tissue formation was evaluated according to a scoring system described previously (20): score 1, no/minimal granulation tissue; score 2, low granulation tissue; score 3, moderate granulation tissue; score 4, extensive granulation tissue; score 5, very extensive granulation tissue. Besides, the scoring system for

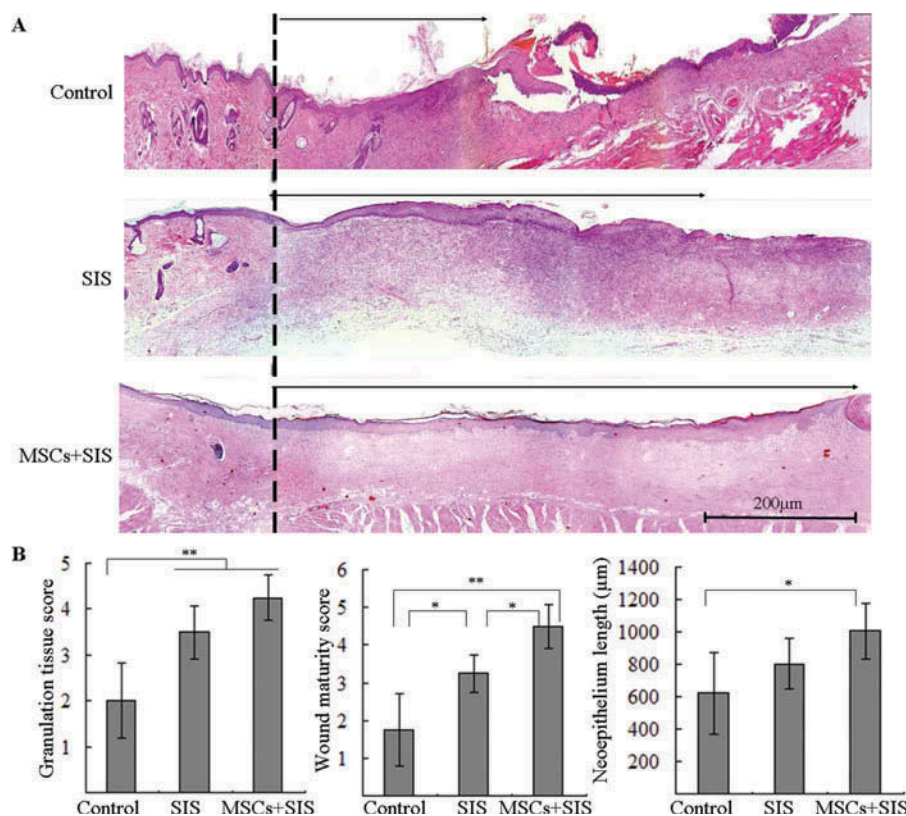


Figure 3. After SIS patches grafting, better epithelialization, granulation formation and wound maturity were achieved. (A) Histological images of the burned wounds in the control group, and after treatment with SIS alone and MSC-seeded SIS for 7 days. Black arrow indicated the neoepithelium. (B) MSC-seeded SIS accelerated the epithelialization when compared with control group on days 7. MSC-seeded SIS and SIS alone significantly accelerated the granulation formation (C) and wound maturity (D) at 7 days post implantation. ($n = 12$, 3 fields per section and 4 sections per group were selected randomly). ** $P < 0.01$, * $P < 0.05$.

wound maturity was also performed as follows (20): score 1, the number of cells appeared on the wound bed was limited or all the cells here were almost all inflammatory cells; score 2, predominantly inflammatory cells; score 3, equivalence between inflammatory cells and proliferative cells (fibroblasts, epidermal cells, and vascular endothelial cells); score 4, predominantly proliferative cells; score 5, almost all cells were proliferative cells. All the slides were evaluated separately by two pathologists in a blinded manner.

Immunohistochemistry

To assess the revascularization of tissue and the proliferation of epidermic cells, immunohistochemistry was performed for von Willebrand factor (vWF; Bioss, Beijing, China) and Ki-67 (Bioworld, Minneapolis, MN) antibodies. Briefly, the 5- μ m thick paraffin-embedded sections were deparaffinized, rehydrated, and blocked with 10% goat serum. Subsequently, the slides were incubated with the respective primary monoclonal antibody and secondary antibody (goat anti mouse or goat anti rabbit IgG label with HRP, ZSGB-BIO, Beijing, China) according to the manufacturer's instructions.

Blood vessels quantification and assessment of epidermic cells proliferation

Blood vessels were detected by the evaluation of immunohistochemical-stained sections of 7 days post implantation according to our previous study (18). The vWF positive blood vessels were identified and counted as brown lumens. Ten different fields limited to the wounded area were randomly selected, and the number of blood vessels was counted in each field under light microscopy (magnification $\times$ 400). The number of blood vessels in each field was averaged and blood vessel density was expressed as the number of blood vessels per unit area (0.2 mm²). The counting of Ki-67 positive epidermic cells in immunohistochemical-stained sections of 21 days after implantation was performed using the same method.

Statistical analysis

Quantitative data are expressed as mean $\pm$ SD. Comparisons of experimental data in different groups were performed with one-way ANOVA followed by LSD-test (SPSS 19.0 Inc., Chicago, IL). Significance was accepted at $P < 0.05$.

Results

SIS enhanced the burn wounds healing

MSCs maintained the fibroblast-like morphology after cultured in the SIS (Supplementary Figure 1D). After SIS and MSC-seeded SIS grafting, a serial of tests were performed (Figure 1). All the rats survived well without infection in the burn wounds. Compared to the untreated control, both the SIS and MSC-seeded SIS enhanced the burn wounds repair and the complete closure was seen at the last follow-up (Figure 2). In detail, no significant improvement of burn wound healing was found in the first 3 days; after implantation of SIS for 7 days, both SIS and MSC-seeded SIS significantly increased the burn wound healing and the closure rates were $37.12 \pm 6.05\%$ and $57.80 \pm 7.17\%$, respectively, while that in the control was $21.09 \pm 4.97\%$. Interestingly, the animals treated with MSC-seeded SIS showed higher wound closure rate than those treated with SIS alone on day 7 ($P < 0.01$). At 14 and 21 days post implantation, enhancement of wound closure by SIS and MSC-seeded SIS was recorded in comparison to that in the control group. As shown in Figure 2, the burn wound was almost closed after SIS treatment for 21 days, while that in the untreated group was not completed yet ($P < 0.05$).

To understand the mechanism of the repair potential of SIS, histological analysis was performed after grafting for 7 days. Based on the histological appearance of the skins (Figure 3), scoring results indicated that both the SIS with or without MSCs notably improved the granulation tissue formation, when compared with the untreated control ($P < 0.01$). Additionally, the burn wounds after the treatments with SIS and MSC-seeded SIS illustrated significantly higher maturity than that in the control group ($P < 0.05$). It was further found that the maturity score in the MSC-seeded SIS group was much higher than that in the SIS alone group ($P < 0.05$). Importantly, MSC-seeded SIS accelerating the epithelialization was determined; as shown in Figure 3, the neoepithelium length was $1006.62 \pm 170.74 \mu\text{m}$ after MSC-seeded SIS treatment, which was much higher than in the control group with the value of $620.44 \pm 253.74 \mu\text{m}$ ($P < 0.05$). This enhancement was not found in the SIS alone group.

Although the SIS patches with or without MSCs can be clearly noted on the surface of the burn wounds after treatment for 7 days, they completely integrated with dermal collagen fibers after 21 days (Figure 4). The SIS integration ensured the regular arrangement and extensive distribution of the regenerated collagen bundles, while loosely packed collagen fibers were seen in the control group.

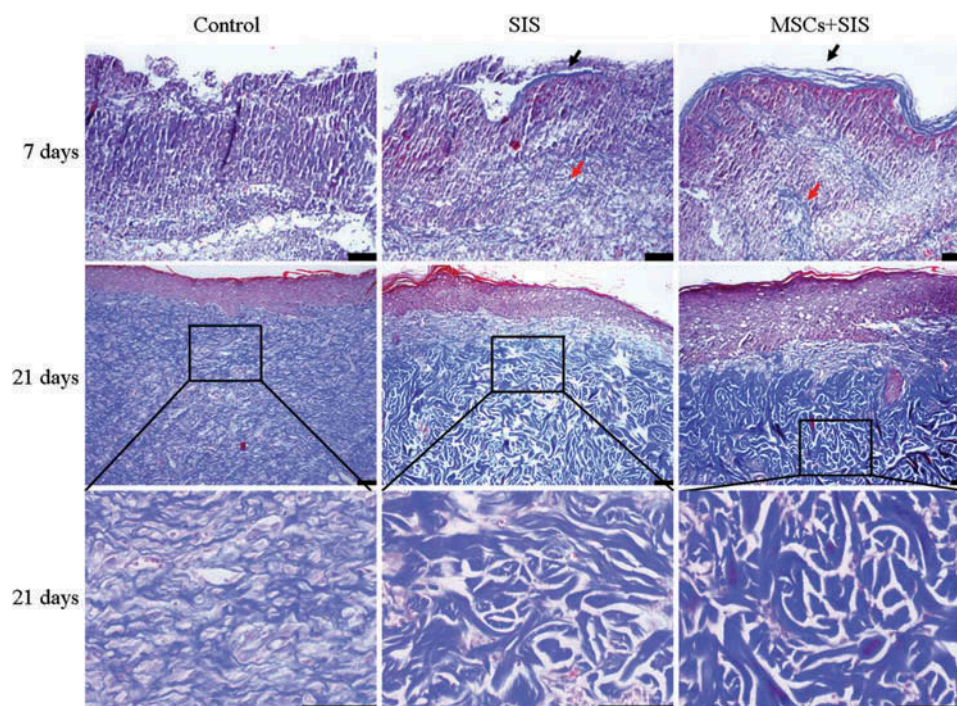


Figure 4. Masson's trichrome staining of the granulation tissue shows part of newly-formed collagen fibers distributed into the granulation tissue in SIS alone and MSC-seeded SIS group on the 7th day of wound healing. On day 21, the SIS patch completely integrated with the dermis; the regenerated collagen bundles of regular arrangement and extensive distribution were seen in SIS alone and MSC-seeded SIS group, while loosely packed collagen fibers with irregular arrangement were seen in the control group. The SIS patch on the epidermic surface was indicated by the black arrows and the newly formed collagen fibers in the dermal layer were indicated by the red triangles. Scale bar = 100 μ m.

MSC-seeded SIS improved the revascularization

After MSC-seeded SIS grafting for 7 days, revascularization of the tissue was assessed by blood vessels density. Obvious blood vessels formation, positive for vWF, was extensively identified in the dermis of the burn wounds (Figure 5A). The number of vWF-positive blood vessels was measured in the burn wound of the three groups (Figure 5B). Significantly increase of the blood vessels density was seen after the treatment of SIS alone and MSC-seeded SIS; and their values were 21.80 ± 7.00 and 27.50 ± 5.36 , respectively, while that of control was 15.40 ± 5.23 . Furthermore, compared with the SIS alone group, the mean numbers of blood vessels density were much higher in the burn wounds after treatment with MSC-seeded SIS ($P < 0.05$).

MSC-seeded SIS accelerated the proliferation of neo-epidermal cells

After SIS substitutes were implanted for 21 days, a large proportion of the burned area was re-epithelialized (Figure 3A). Immunohistochemistry for Ki-67 was performed and the proliferating epidermic cells could then be identified accordingly. As shown in

Figure 6A&B, MSC-seeded SIS treatment led to a higher number of Ki-67 positive cells (33.10 ± 6.52) in the epidermal layers of burn wounds, and the number was much more than that in the control group (24.10 ± 11.01) ($P < 0.05$). This enhancement was not found in the SIS alone group.

Discussion

As a completely decellularized matrix, SIS has been extensively applied for tissue regeneration; these applications include skin replacement, vascular repair and the other soft tissues reconstruction (1,21–23). In our previous study, SIS was used to repair the chronic myocardial infarction in rabbits and it was found that SIS was able to enhance myocardial regeneration (18). In this study, the feasibility of SIS to repair the deep partial-thickness burn wound in a rats was investigated. It determined that SIS with or without MSCs was able to accelerate the burn wound closure by enhancing the granulation tissue formation, increasing the wound maturity, and improving the revascularization. Similar findings were reported in other studies using the animal models with excisional wound models. For instance, SIS sponge has been reported to enhance the excisional

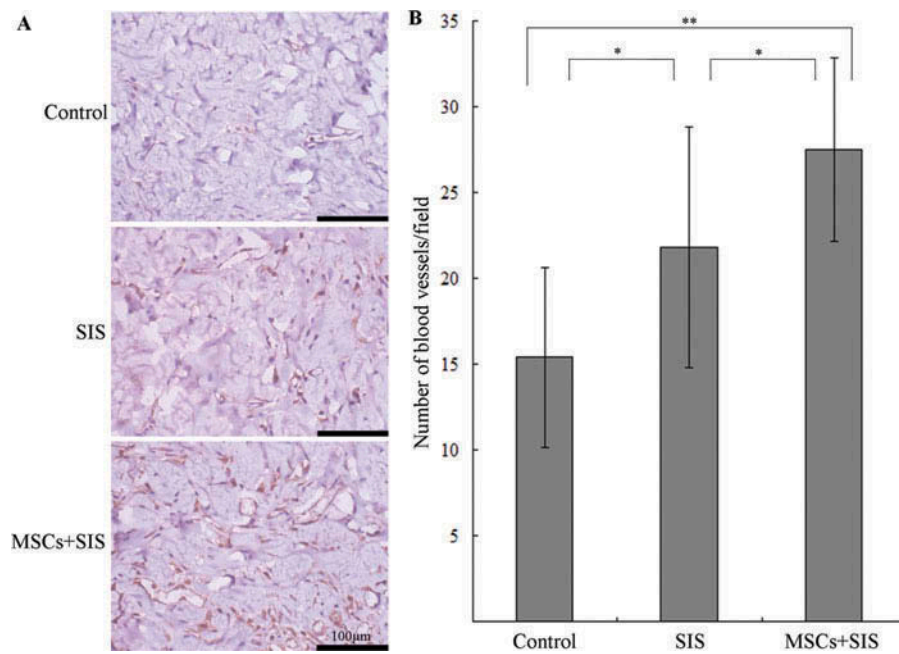


Figure 5. (A) Immunohistochemical staining images of burn wounds for vWF on 7 days post implantation. (B) Statistical analysis showed that compared with the control group, a higher blood vessel density was observed in wounds after treatment with MSC-seeded SIS and SIS alone; furthermore, the highest blood vessel density was observed in wounds treated with MSC-seeded SIS ($n = 10$). ** $P < 0.01$, * $P < 0.05$.

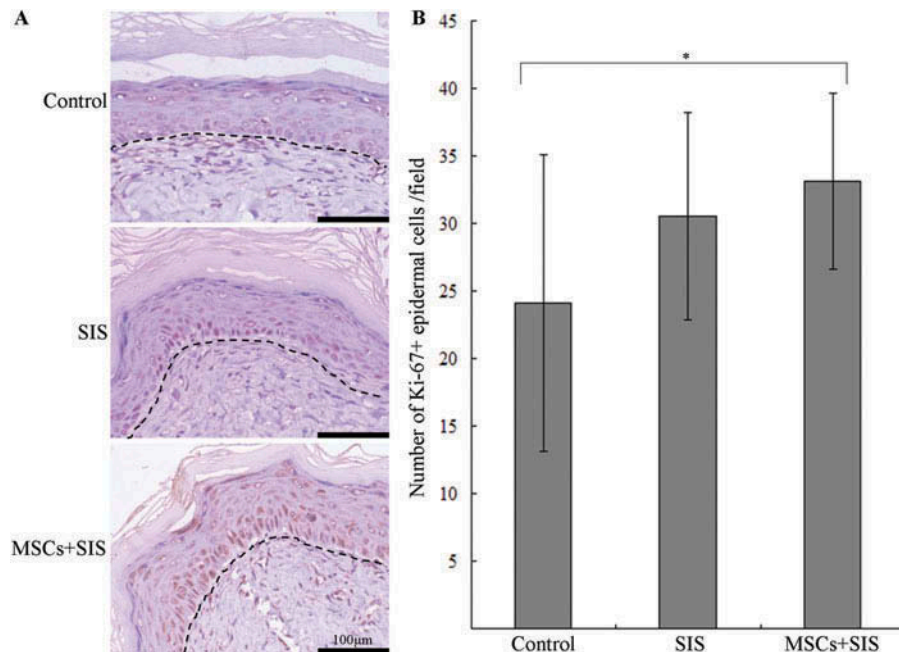


Figure 6. (A) Immunohistochemical staining images of burn wounds for Ki-67 in control, SIS alone and MSC-seeded SIS group on 7th day post implantation. The basement membrane is outlined using dashed lines. (B) Statistical analysis showed a significant increase in the density of Ki-67⁺ epidermal cells in the MSC-seeded SIS group compared with that of control group ($n = 10$). * $P < 0.05$.

wound healing by inducing the regeneration of dermal collagen and the progress of granulous tissue formation (4); wound healing enhancement was also found in a rabbit model with full-thickness square wounds (24). It is thus not surprising to observe the acceleration of

burn wound healing after SIS grafting, although the pathological appearance of burn wounds was much more complex than that of excisional wounds.

Beneficial effects were achieved after MSCs was seeded on the SIS for implantation. The highly porous structure

of SIS supports the nutrients diffusion and enables the adherence, growth and migration of seeded cells (25). MSC-seeded SIS has been developed for tissue regeneration (26,27). In this rat model, MSC-seeded SIS was developed to cover the burn wounds because it was able to promote angiogenesis, suppress the local inflammation, inhibit apoptosis and enhance the ECM deposition for repair of the excisional wounds (5,28,29). Nevertheless, this study was the first one to investigate the possibility of MSC-seeded SIS for burn wounds healing. The advantage was that the blood vessel density in the MSC-seeded SIS-treated burn wound was significantly increased. Engraftment of MSCs enhanced the revascularization of burn wounds, which was a crucial approach for wound healing to ensure the delivery of nutrient to the migrated cells in the epidermal and dermal layers. It was demonstrated the implanted MSCs may change the behaviors of the resident endothelial cells by secretion of growth factors and cytokines, thus induced the angiogenesis (16,30). The positive effect of SIS could not be ignored as it can release several kinds of angiogenic factors after degradation (17). Thus, the combination of MSCs with SIS could produce synergistic angiogenesis for promoting burn wounds repair.

The other advantage observed in this study was that the implanted MSCs could enhance the re-epithelialization as the density of Ki-67⁺ epidermal cells in the MSC-seed SIS was increased significantly. Epithelialization is essential for burn wound healing which provides a protective barrier to avoid infections and to maintain the fluid balance (8). In a human study, burn tissue treated with SIS presented good epithelialization which may be related with the expression of transforming growth factor- β_3 (1). In this study, the epithelialization was also found after SIS treatment but it was not as good as that after MSC-seeded SIS treatment. The enhancement of epithelialization was because MSCs was able to increase the proliferation of differentiated epidermal cells. However, other paracrine mechanisms could not be neglected as increasing evidences indicated that MSC-derived exosomes (MS-Ex) might stimulate the wound healing (31–33). Recently, in a rat burn wound model, it was found that MS-Ex not only promoted the epithelialization and cell proliferation through the activation of Wnt4/ β -catenin pathway, but also inhibited the apoptosis of skin cells through the activation of AKT pathway (34). Thus, further investigations are needed to fully understand of the underlying mechanisms regarding the positive role of MSCs in the SIS-based therapy for burn wounds repair.

To be honest, some limitations in this study should be acknowledged. First, the implanted MSCs were not tracked in the real-time pattern to monitor the distribution in the burn wounds over time. Second, the

MSCs seeded on the SIS patches were isolated from one donor rat, variable therapeutic effect of MSCs on the burn wound may be observed because of individual variation. Further experiments to investigate the protein and gene expression profiles in the burn wounds after MSC-seeded SIS treatments are underway.

In conclusion, SIS grafting was very effective for burn wounds repair. It could enhance the burn wound healing by increasing the granulation tissue formation, the wound maturity and the tissue revascularization. Importantly, MSCs seeding was able to enhance this positive effect, because MSCs were able to improve the re-epithelialization and the revascularization significantly. Therefore, the current findings could support the future treatment of large and deep burn wounds with SIS and MSC-seeded SIS.

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Declaration of interest

The authors declare no competing financial interests.

Notes on contributors

XG, BX, MYT and YCH contributed to the experimental design, data acquisition, analysis and interpretation. MYT, BX and YCH drafted the manuscript. XBL, ZJZ, ZL, WLL, ABX and LD provided technical support and assisted with the data analysis and interpretation. All the authors approved the final manuscript to be submitted.

References

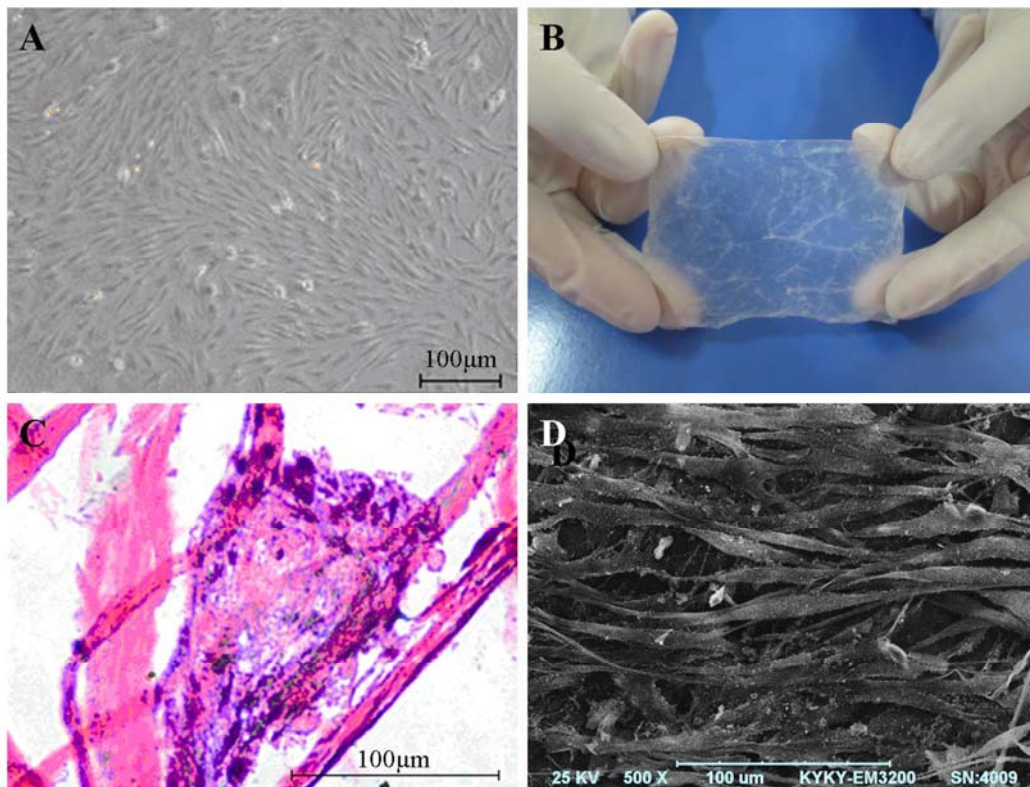
1. Salgado RM, Bravo L, Garcia M, Melchor JM, Krotzsch E. Histomorphometric analysis of early epithelialization and dermal changes in mid-partial-thickness burn wounds in humans treated with porcine small intestinal submucosa and silver-containing hydrofiber. *J Burn Care Res* 2014;35:e330–337.
2. Prevel CD, Eppley BL, Summerlin DJ, Sidner R, Jackson JR, McCarty M, Badyak SF. Small intestinal submucosa: utilization as a wound dressing in full-thickness rodent wounds. *Ann Plast Surg* 1995;35:381–8.
3. Bello YM, Falabella AF, Eaglstein WH. Tissue-engineered skin. Current status in wound healing. *Am J Clin Dermatol* 2001;2:305–13.
4. Kim MS, Hong KD, Shin HW, Kim SH, Lee MS, Jang WY, Khang G, Lee HB. Preparation of porcine small intestinal submucosa sponge and their application as a wound dressing in full-thickness skin defect of rat. *Int J Biol Macromol* 2005;36:54–60.

5. Yeum CE, Park EY, Lee SB, Chun HJ, Chae GT. Quantification of MSCs involved in wound healing: use of SIS to transfer MSCs to wound site and quantification of MSCs involved in skin wound healing. *J Tissue Eng Regen Med* 2013;7:279–91.
6. Hodde JP, Badylak SF, Brightman AO, Voytik-Harbin SL. Glycosaminoglycan content of small intestinal submucosa: a bioscaffold for tissue replacement. *Tissue Eng* 1996;2:209–17.
7. Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF. Identification of extractable growth factors from small intestinal submucosa. *J Cell Biochem* 1997;67:478–91.
8. Tiwari VK. Burn wound: How it differs from other wounds? *Indian J Plast Surg* 2012;45:364–73.
9. Neely AN, Brown RL, Clendening CE, Orloff MM, Gardner J, Greenhalgh DG. Proteolytic activity in human burn wounds. *Wound Repair Regen* 1997;5:302–9.
10. Chu CS, McManus AT, Mason AD, Jr., Okerberg CV, Pruitt BA, Jr. Multiple graft harvestings from deep partial-thickness scald wounds healed under the influence of weak direct current. *J Trauma* 1990;30:1044–9; discussion 1049–50.
11. Gravante G, Delogu D, Palmieri MB, Santeusano G, Montone A, Esposito G. Inverse relationship between the apoptotic rate and the time elapsed from thermal injuries in deep partial thickness burns. *Burns* 2008;34:228–33.
12. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med* 2012;1:142–9.
13. Clover AJ, Kumar AH, Isakson M, Whelan D, Stocca A, Gleeson BM, Caplice NM. Allogeneic mesenchymal stem cells, but not culture modified monocytes, improve burn wound healing. *Burns* 2014;41:548–57.
14. Leonardi D, Oberdoerfer D, Fernandes MC, Meurer RT, Pereira-Filho GA, Cruz P, Vargas M, Chem RC, Camassola M, Nardi NB. Mesenchymal stem cells combined with an artificial dermal substitute improve repair in full-thickness skin wounds. *Burns* 2012;38:1143–50.
15. Huang Y-C, Zhu H-M, Cai J-Q, Huang Y-Z, Xu J, Zhou Y, Chen X-H, Li X-Q, Yang Z-M, Deng L. Hypoxia inhibits the spontaneous calcification of bone marrow-derived mesenchymal stem cells. *Journal of Cellular Biochemistry* 2012;113:1407–15.
16. Xie HL, Zhang Y, Huang YZ, Li S, Wu CG, Jiao XF, Tan MY, Huang YC, Deng L. Regulation of high mobility group box 1 and hypoxia in the migration of mesenchymal stem cells. *Cell Biol Int* 2014;38:892–7.
17. Luo JC, Chen W, Chen XH, Qin TW, Huang YC, Xie HQ, Li XQ, Qian ZY, Yang ZM. A multi-step method for preparation of porcine small intestinal submucosa (SIS). *Biomaterials* 2011;32:706–13.
18. Tan MY, Zhi W, Wei RQ, Huang YC, Zhou KP, Tan B, Deng L, Luo JC, Li XQ, Xie HQ, Yang ZM. Repair of infarcted myocardium using mesenchymal stem cell seeded small intestinal submucosa in rabbits. *Biomaterials* 2009;30:3234–40.
19. Ren P, Guan DW, Zhao R, Ma WX, Zhang ST. Establishment of skin scald model in mice. *Fa Yi Xue Za Zhi* 2012;28:92–94, 99.
20. Gong C, Wu Q, Wang Y, Zhang D, Luo F, Zhao X, Wei Y, Qian Z. A biodegradable hydrogel system containing curcumin encapsulated in micelles for cutaneous wound healing. *Biomaterials* 2013;34:6377–87.
21. Sanchez-Palencia DM, Navarro J, Araque JC, Umana JB, Guerrero AF, Quijano LM, Lopez RD, Sandoval NF, Briceno JC. Effects of fabrication on early patency and regeneration of small intestinal submucosa vascular grafts. *ASAIO J* 2015;5:596–604.
22. Choi JS, Lee S, Kim da Y, Kim YM, Kim MS, Lim JY. Functional remodeling after vocal fold injury by small intestinal submucosa gel containing hepatocyte growth factor. *Biomaterials* 2015;40:98–106.
23. Mondalek FG, Fung KM, Yang Q, Wu W, Lu W, Palmer BW, Frimberger DC, Greenwood-Van Meerveld B, Hurst RE, Kropp BP, Lin HK. Temporal expression of hyaluronic acid and hyaluronic acid receptors in a porcine small intestinal submucosa-augmented rat bladder regeneration model. *World J Urol* 2015;33:1119–28.
24. Anilkumar TV, Vineetha VP, Revi D, Muhamed J, Rajan A. Biomaterial properties of cholecyst-derived scaffold recovered by a non-detergent/enzymatic method. *J Biomed Mater Res B Appl Biomater* 2014;102:1506–16.
25. Shi L, Ronfard V. Biochemical and biomechanical characterization of porcine small intestinal submucosa (SIS): a mini review. *Int J Burns Trauma* 2013;3:173–9.
26. Li Y, F Liu, Z Zhang, M Zhang, S Cao, Y Li, L Zhang, X Huang and Y Xu. Bone marrow mesenchymal stem cells could acquire the phenotypes of epithelial cells and accelerate vaginal reconstruction combined with small intestinal submucosa. *Cell Biol Int* 2015;39:1225–1233.
27. Hodonsky C, Mundada L, Wang S, Witt R, Raff G, Kaushal S, Si MS. Effects of scaffold material used in cardiovascular surgery on mesenchymal stem cells and cardiac progenitor cells. *Ann Thorac Surg* 2015;99:605–11.
28. Hocking AM, Gibran NS. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp Cell Res* 2010;316:2213–2219.
29. Choi JW, Park JK, Chang JW, Kim da Y, Kim MS, Shin YS, Kim CH. Small intestine submucosa and mesenchymal stem cells composite gel for scarless vocal fold regeneration. *Biomaterials* 2014;35:4911–8.
30. Bronckaers A, Hilkens P, Martens W, Gervois P, Ratajczak J, Struys T, Lambrechts I. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol Ther* 2014;143:181–96.
31. Xin H, Li Y, Buller B, Katakowski M, Zhang Y, Wang X, Shang X, Zhang ZG, Chopp M. Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 2012;30:1556–64.
32. Charrier A, Chen R, Chen L, Kemper S, Hattori T, Takigawa M, Brigstock DR. Exosomes mediate intercellular transfer of pro-fibrogenic connective tissue

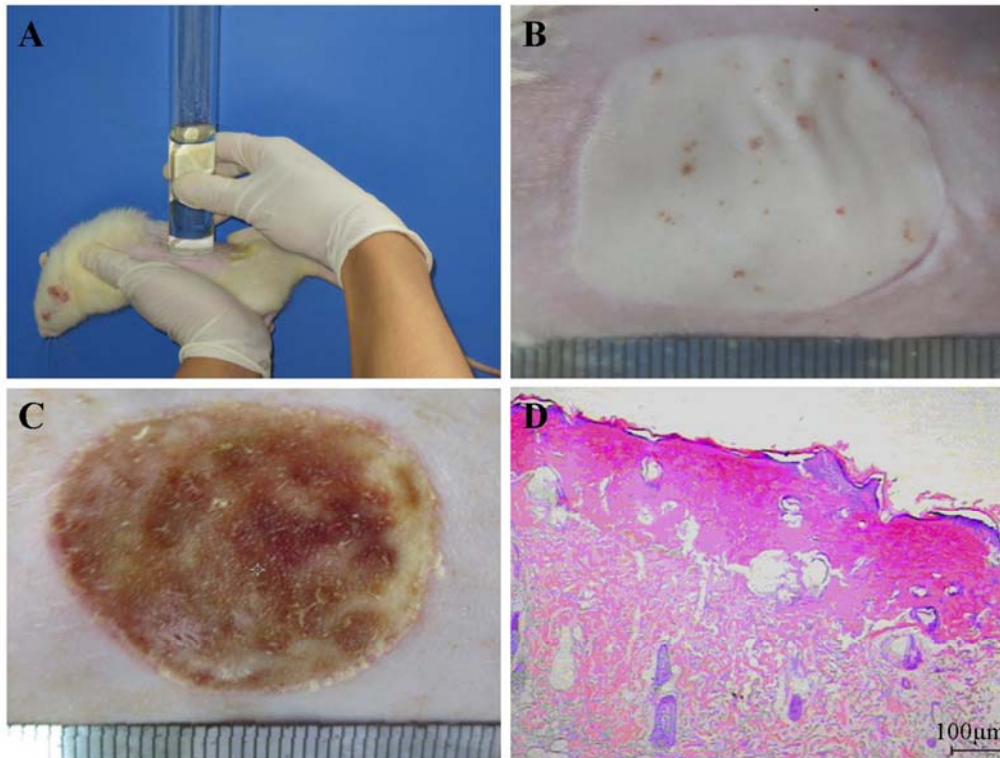
- growth factor (CCN2) between hepatic stellate cells, the principal fibrotic cells in the liver. *Surgery* 2014;156:548–55.
33. Zhou Y, Xu H, Xu W, Wang B, Wu H, Tao Y, Zhang B, Wang M, Mao F, Yan Y, Gao S, Gu H, Zhu W, Qian H. Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis in vivo and in vitro. *Stem Cell Res Ther* 2013;4:34.
34. Zhang B, Wang M, Gong A, Zhang X, Wu X, Zhu Y, Shi H, Wu L, Zhu W, Qian H, Xu W. HucMSC--exosome mediated--Wnt4 signaling is required for cutaneous wound healing. *Stem Cells* 2014;33: 2158–68.

Grafting of mesenchymal stem cell-seeded small intestinal submucosa to repair the deep partial-thickness burns

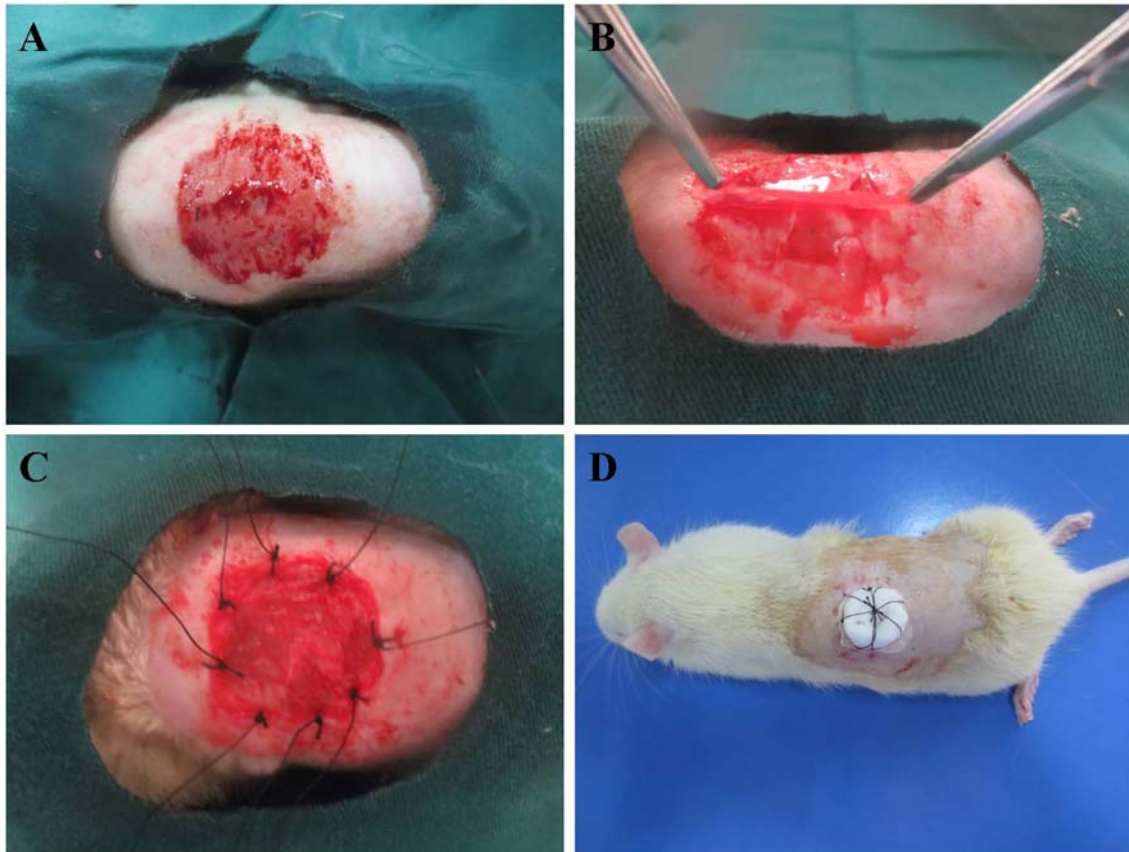
Supplemental Figures



Supplemental Figure 1 (A) Bone marrow MSCs at passage 3 possess the fibroblast-like morphology. (B) Gross observation of SIS. (C) H&E staining result showed that MSCs were observed in SIS. (D) SEM result showed that MSCs maintained the fibroblast-like morphology after seeding into SIS.



Supplemental Figure 2 (A) The deep partial thickness burns were created by contacting the rat dorsal surface directly with boiled water (100°C) for 10 seconds. (B) A circular heat-induced tissue coagulation area was formed on the dorsal surface after the dorsal surface was burned. (C) Three days after burning, the burned tissue completely turned into a scar; and (D) H&E staining result showed the epidermal and superficial dermal layers were filled with necrotic tissues. Scale bars in (B) and (C) represent 1 mm.



Supplemental Figure 3 After tangential excision, the burn wounds were covered by SIS or MSC-seeded SIS (A-C); after the skin grafts were sutured to the wound margins, each rat received a pressure dressing (D).