Mini Review

# Collagen vitrigel membrane: a powerful tool for skin regeneration

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**Severe burn patients lose wide areas of their skin and are confronted with a high risk of death because they lose an indispensable barrier against the invader, which in turn promotes water evaporation. Cultured skin is expected to be a promising technology for extensive skin defect patients, while faultless cultured skin has not been established. A skin sheet, tough and durable enough in clinical use, is urgently needed because typical cell sheets available now are fragile and difficult to handle at the time of surgery. Collagen vitrigel membrane is a novel biomaterial consisting of high-density collagen fibrils equivalent to connective tissues *in vivo*. With this novel collagen material utilized as a scaffold, we established a novel cultured skin sheet composed of keratinocytes and mesenchymal cell types. This cultured skin showed a fully differentiated epidermal layer, and could be handled with a tweezers. Interestingly, transplantation of a skin sheet revealed that acceleration of healing or inhibition against scarring depended on mesenchymal cell types in the skin sheet. Our findings suggest that the cultured skin sheet utilizing collagen vitrigel membrane cultured with keratinocytes and mesenchymal cell types will be a powerful tool for the wide skin defect and injury.**

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## Introduction

The skin primarily serves as a barrier separating an organism's inner space from its outer space. This barrier function is afforded by the physical character of epidermis and dermis: the tightly packed epidermis reduces the gap in the barrier, and the collagen-producing dermal fibroblast supports the barrier's biomechanical strength and the skin's biological microenvironment.

Severe burn patients lose wide areas of their skin and are confronted with a high risk of death because they lose a "castle wall against the invader" and, as a result, water evaporation is promoted. The standard in treating severe skin loss even today is a conventional surgical treatment by skin grafting. The utilization of cultured skin to cover extensive burn wounds appeared very promising at first, but the technique has several limitations to overcome so as to become clinically applicable. It takes a long time to culture an area of skin, and the dermal layer cannot be made strong enough. To surmount this obstacle and establish a new strategy for skin regeneration, it is necessary to explore the type of cell source that serves as supportive cell for skin regeneration.

The aim of this review is to outline the utility of collagen vitrigel membrane and the mesenchymal cell support for parenchymal cell for cultured skin. Cultured skin sheet is expected to play a pivotal role in skin regeneration, especially in the case of extensive and intractable wounds. Progress in research into the skin regeneration technology will address these issues and, if successful, patients will benefit immensely with a better clinical outcome and improved quality of life.

## Collagen vitrigel and xerogel membrane

Collagen is a major component of connective tissues and is the most abundant compositional protein in mammals. Since commercial collagen products exist in various states such as powder, aqueous solution, sol, gel, membrane, sponge, etc., it was impossible to reproduce the mechanical property of collagen fibrils *in vivo*. Meanwhile, Takezawa et al. developed a novel collagen-based biomaterial consisting of high-density collagen fibrils equivalent to connective tissues *in vivo* and named it "collagen vitrigel" because its preparation required a vitrification process[1]. A collagen vitrigel membrane becomes a collagen xerogel membrane by drying-out and vice versa, the collagen xerogel membrane is easily converted into a collagen vitrigel by hydration (Fig.1)[2]. This novel collagen-based biomaterial may easily be handled with tweezers, and possesses excellent transparency and permeability to proteins with high molecular weights. Hence, its excellent utility has been demonstrated in various studies both *in vitro* and *in vivo*[1, 3-5]. With other colleagues of us, we recently succeeded in developing a collagen vitrigel membrane chamber useful for reconstructing culture models, such as "tissue sheets" composed solely of epithelial cells, mesenchymal cells, or endothelial cells, and "organoid plates" composed of more than two types of cells[6-8].

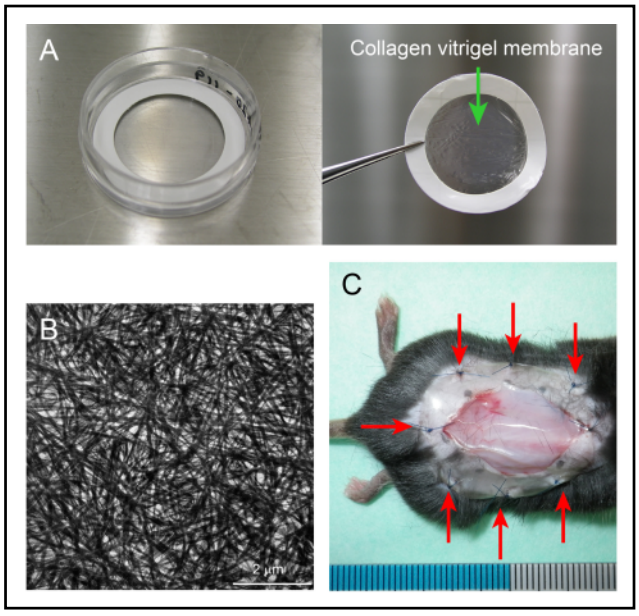


Fig.1 Collagen vitrigel membrane

(A) Appearance of collagen xerogel membrane in dry condition. Collagen xerogel is easily converted into collagen vitrigel by hydration. (B) Collagen vitrigel membrane consists of high-density collagen fibrils. (C) Thick collagen xerogel membrane allows direct suture (arrow) to a tissue defect in the skin.

## Mesenchymal cells support epidermal regeneration

Mesenchymal-epithelial interaction plays an essential role in organogenesis and tissue regeneration at both embryonic and adult stages[9, 10]. Green et al. demonstrated that fibroblastic feeder cell was able to promote the proliferation of keratinocytes *in vitro*, and brought on a revolutionary development of cultured skin and other organs[11, 12]. In general, dermal mesenchymal fibroblasts provide keratinocytes with a number of cytokines, such as keratinocyte growth

factor, stromal-derived factor-1 and interleukin-6, as critical crosstalk molecules[13, 14]. It is well known that decubitus ulcer patients suffer from poor wound healing because of their unhealthy granulation tissue. This unhealthy granulation tissue is believed to delay epidermal repair through mesenchymal cell dysfunction, especially inappropriate productions of cytokines[15, 16].

For the last decade, adult stem cells, such as bone marrow mesenchymal stem cells (BMSCs) and stem cells derived from adipose tissue have come to the front of tissue regeneration[17-19]. Bone marrow-derived and adipose-derived mesenchymal stromal cells contain multipotent stem cells that are capable of differentiating into numerous cell types, including fibroblast, bone, cartilage and muscle (skeletal and smooth) cells. These mesenchymal stem cells are not lineage-restricted and produce functional non-hematopoietic cell types when transplanted into foreign tissues. Together with other colleagues of ours, we demonstrated that these cell types participated in the skin regeneration[20-22].

Moreover, our previous study demonstrated that not only skin-localized mesenchymal cells but also non-skin-localized mesenchymal cells lacking the stem cell feature enabled keratinocytes to undergo normal proliferation and differentiation[21]. Mesenchymal cells derived from heart, spleen, lung, liver and kidney promoted growth and differentiation of keratinocytes. The vast majority of these mesenchymal cells were commonly positive for vimentin and the positive staining proportion was dependent upon the derived organ type. Notably, our previous study showed that not only normal cells but also cancer-associated fibroblasts (CAF) isolated from a poorly differentiated adenocarcinoma in a male gastric cancer patient in his seventies supported epidermal regeneration in a skin reconstruction model[22]. Although further research is needed, these findings suggest that CAFs themselves may be involved in the tissue regeneration of non-cancer cells. Focusing on this point, researchers must look into the cross-organ specificity of the biology of mesenchymal cells, especially for the sake of future regenerative medicine.

## Cultured skin sheet with the use of collagen vitrigel membrane

As mentioned above, collagen vitrigel membrane is suitable as a cell culture scaffold. In this experiment, cell components of skin sheet were derived from green rat (SD-Tg (CAG-EGFP)) to render the cell kinetics traceable. We prepared three types of vitrigel skin sheets: i) keratinocytes with dermal fibroblasts (vitrigel-KD); ii) keratinocytes with BMSCs (vitrigel-KB); and iii) keratinocytes alone (vitrigel-K). At culture day 7, both dermal fibroblasts and BMSCs clearly promoted the proliferation and stratification of keratinocytes, giving rise to the formation of basal, prickle, granular and horny cell layers. In contrast, keratinocytes cultured without mesenchymal cells showed only fragmented keratin material, and collagen vitrigel membrane disappeared in this condition (Fig.2).

The efficacy of the transplantation of skin sheets made as above was evaluated by the wound healing test: full-thickness dermal wounds induced in nude rat (F344/N-rmu) were treated with cultured skin sheet transplantation (Fig.3). In this experiment, five types of vitrigel skin sheets were prepared: i) vitrigel-K; ii) vitrigel-KD; iii) vitrigel-KB); iv) keratinocytes with splenic fibroblasts (vitrigel-KS); and v) vitrigel control without cell components (vitrigel-Cont). At day 21, the skin wound was closed in groups in which vitrigel-K and vitrigel-KD were transplanted. These two regenerated types of skin had a contracted scar formation. No treatment group and the group transplanted with vitrigel-

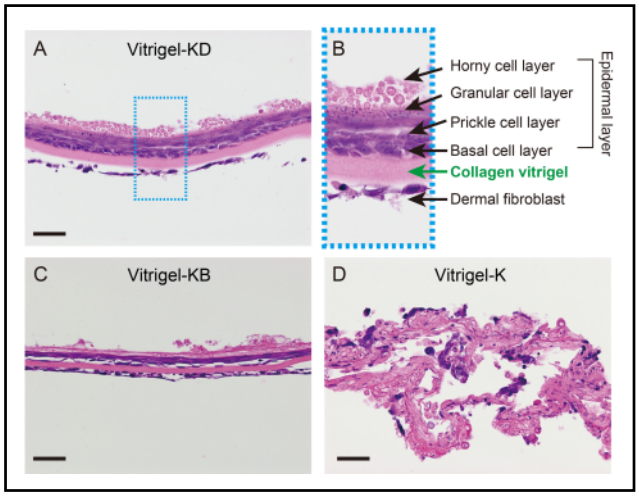


Fig.2 Structures of the skin sheet

(A) Cultured skin sheet composed of keratinocytes and dermal fibroblasts utilizing collagen vitrigel membrane (vitrigel-KD) shows differentiated epidermal layer. (B) Basal, prickle, granular and horny cell layers are seen in skin sheet (corresponding to inset of Fig.1A). (C) Cultured skin sheet of vitrigel-KB shows differentiated epidermal layer. (D) Cultured skin sheet composed of keratinocytes alone shows only fragmented keratin material, and collagen vitrigel membrane has disappeared. Bar, 50 $\mu$m.

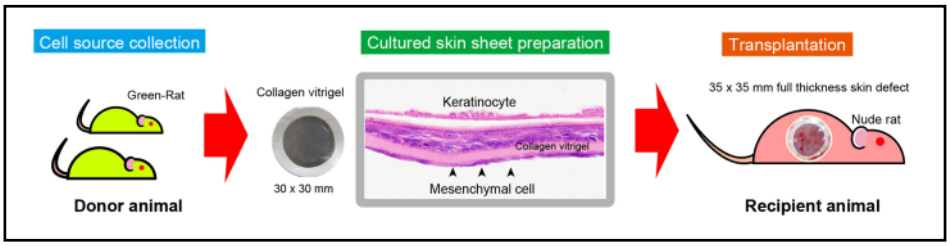


Fig.3 Scheme of transplantation of cultured skin sheet

Cell components of skin sheet derived from green rat. The skin sheets were transplanted onto nude rat having been treated with full thickness skin defect.

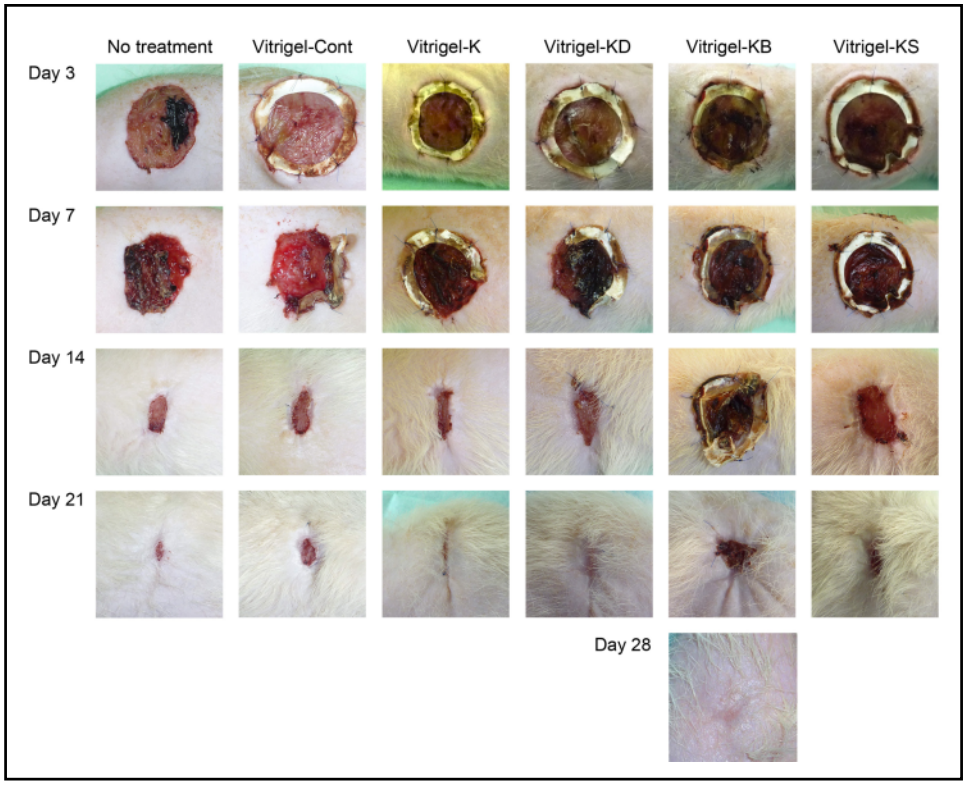


Fig.4 Macroscopic findings of the treatment course

In every group, wound area becomes contracted with time. At day 21, skin wound in the group transplanted with vitrigel-K or with vitrigel-KD is closed and contracted. No treatment group and vitrigel-Cont group each shows a small area of granulation tissue. Wound closure was delayed in group transplanted with vitrigel-KB or in group with vitrigel-KS. These wounds show wide granulation tissue with crust. Regeneration tissue treated with vitrigel-KB shows non-scarring and flat surface of regenerated skin at day 28.

Cont showed a small area of granulation tissue and did not finish epithelialization. The group transplanted with vitrigel-KB or with vitrigel-KS had poor wound healing progression and showed relatively wide granulation tissue with crust (Fig.4). Notably, the regeneration tissue treated with vitrigel-KB showed scar-free and flat regenerated skin at day 28.

As shown in Figure 5, the regenerative epidermis in the group transplanted with vitrigel-K or with vitrigel-KD showed a fully differentiated epidermal layer with a rete-ridge structure at day 21. In the vitrigel-K group, regenerative dermal tissue shows a disarrayed arrangement of mesenchymal cells. The vitrigel-KD group, on the other hand, has an arrangement of dermal mesenchymal cells in a horizontally ordered alignment. The wound tissue in vitrigel-KB or in vitrigel-KS showed inflammatory granulation tissue in the regenerative dermis with crust containing fibrin, inflammatory cells and necrotic tissue. No granulomatous inflammatory reaction or foreign-body type reaction was observed in skin lesions in groups treated with skin sheet. In every condition, we detected very few or no GFP-positive donor-derived cells in regenerative tissue. At day 28, the regenerated skin in the group treated with vitrigel-KB showed a fully differentiated epidermal layer with rete-ridge structure and horizontally ordered fibroblast in dermis like a vitrigel-KD group.

Contrary to our initial expectation, the healing effect of

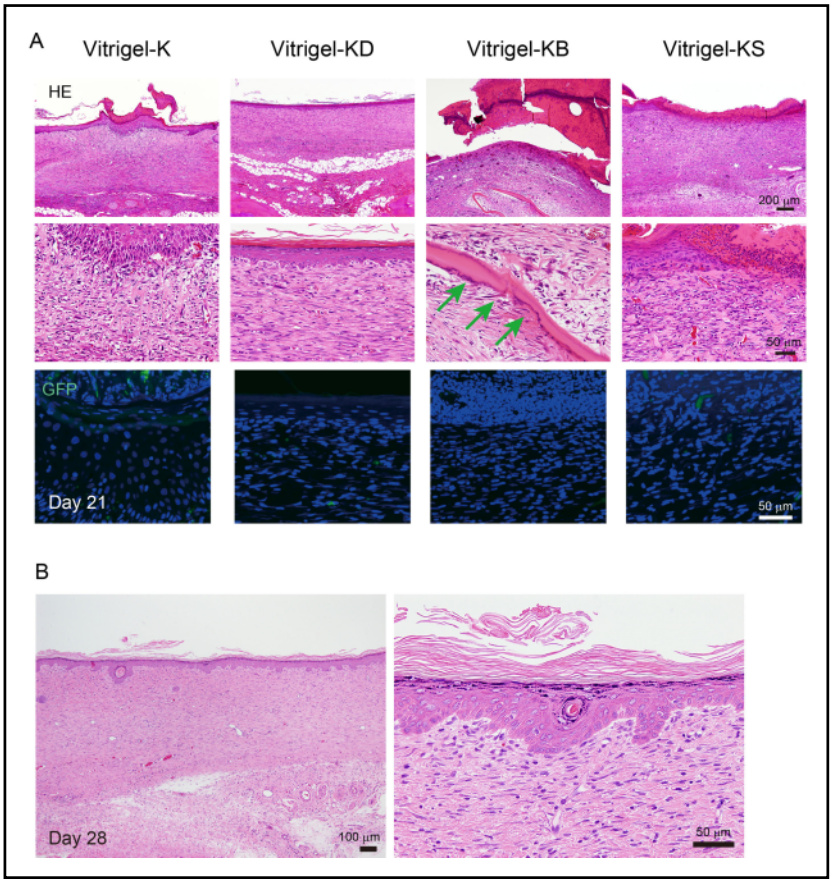


Fig.5 Histology of regenerated skin treated with cultured skin sheet

(A) Regenerative skin in the group transplanted with vitrigel-K or with vitrigel-KD shows a fully differentiated epidermal layer. In vitrigel-K transplanted group, dermal tissue displays disarrayed mesenchymal cells, while the arrangement of dermal mesenchymal cells in vitrigel-KD exhibits a horizontally ordered alignment. Wound tissue in vitrigel-KB and vitrigel-KS shows inflammatory granulation tissue in the regenerative dermis with crust. Remaining collagen vitrigel membrane is detected in vitrigel-KB (arrows). GFP-positive donor-derived cells are scant in regenerative tissue in every condition. (B) At day 28, regenerated skin in vitrigel-KB treated group shows a fully differentiated epidermal layer and hair follicle-like structure.

non-skin-derived mesenchymal cells on wound closure seemed to be somewhat poor, while skin sheet utilizing BMSCs reduced scar formation in regenerated skin. Contraction in the size of a wound is an important factor of the healing process. An exaggeration of this process leads to pathological contracture and results in deformities of the wound area[23]. In addition, inadequate connective tissue remodeling in the repair process leads to insufficient wound closure or hypertrophic scar. The myofibroblast is a key cell for the connective tissue remodeling that takes place during wound healing and fibrosis development. Myofibroblasts produce large amounts of ECM component, such as type I collagen, tanascin-C and SPARC[24]. Myofibroblasts arise from tissue fibroblasts through the effects of PDGF, TGF-$\beta$ and FGF-2 released by macrophages at the wound site, but they can also derive from bone marrow precursors known as fibrocyte[25], and from epithelial cells under the process of epithelial-to-mesenchymal transition[26]. Recently, several researchers have in fact expressed doubt about the potential of adult stem cells in tissue repair, and available reports present us with a "paradox". Mesenchymal cells originally attracted attention because of their stem cell-like properties, but frequently repair of injured tissues was seen without much evidence of either engraftment or differentiation[27-29]. However, the precise supporting function of mesenchymal cells including stem cells remains to be completely elucidated. Our findings suggest that mesenchymal cell types which are located in various organs possess a common and specific supporting effect on tissue regeneration, and there is a close relationship between these cell types and wound closure.

The fate of transplanted cells in a host remains to be clarified[30, 31]. Usual transplantation experiments are applied with immunodeficient rodent as a host animal. Immunodeficient animals, such as severe combined immunodeficient (scid), athymic (nude) mice and nude rats lack T cells but have normal natural killer (NK) cells[32, 33]. The NK cell activity in the nude rat is significantly higher than in their thymus-bearing littermates[34]. T and B cells mainly control the antigen-specific rejection and play either as effector, regulatory, or memory cells. Moreover, NK cells, endothelial cells, macrophages, or polymorphonuclear cells are also

important factors in transplant rejection[35]. The transplanted cells of skin sheet may be rejected by these immune cells and diminish in the wound, but the cells and collagen vitrigel transiently support and modulate the skin regeneration in the acute phase.

## Conclusion

In this review, we discussed the potential of clinical application of skin sheet utilizing a collagen vitrigel membrane in wide skin defect. Further research is needed, to be sure, but these findings suggest that cultured skin sheets will likely be a powerful tool in the treatment of severe skin defects and injuries. It is important that we researchers study, far more closely, the cellular kinetics of keratinocytes and mesenchymal cells in wound tissues of a recipient and define the clear roll of skin regeneration therapy.

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### Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

### References

1) Takezawa T, Ozaki K, Nitani A, Takabayashi C, Shimo-Oka T: Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. Cell Transplant. 2004; 13: 463-473.
2) Takezawa T, Aoki S, Oshikata A, Okamoto C, Yamaguchi H, Narisawa Y, Toda S, editors: A novel material of high density collagen fibrils: A collagen xerogel membrane and its application to transplantation in vivo and a culture chamber in vitro. In 24th European Conference on Biomaterials (Ed. International Proceedings Division), MEDIMOND, Bologne, 2012, pp181-185.
3) Takezawa T, Takeuchi T, Nitani A, Takayama Y, Kinooka M, Taya M, Enosawa S: Collagen vitrigel membrane useful for paracrine assays in vitro and drug delivery systems in vivo. J Biotechnol. 2007; 131: 76-83.
4) Takezawa T, Nishikawa K, Wang P-C: Development of a human corneal epithelium model utilizing a collagen vitrigel membrane and the changes of its barrier function induced by exposing eye irritant chemicals. Toxicol In Vitro. 2011; 25: 1237-1241.
5) McIntosh Ambrose W, Salahuddin A, So S, Ng S, Ponce Marquez S, Takezawa T, Schein O, Elisseeff J: Collagen Vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. J Biomed Mater Res B Appl Biomater. 2009; 90: 818-831.
6) Uchino T, Takezawa T, Ikarashi Y: Reconstruction of three-dimensional human skin model composed of dendritic cells, keratinocytes and fibroblasts utilizing a handy scaffold of collagen vitrigel membrane. Toxicol In Vitro. 2009; 23: 333-337.
7) Yamaguchi H, Kojima H, Takezawa T: Vitrigel-Eye Irritancy Test Method Using HCE-T Cells. Toxicol Sci. 2013; 135: 347-355.
8) Aoki S, Takezawa T, Oshikata-Miyazaki A, Ikeda S, Kuroyama H, Chimuro T, Oguchi Y, Noguchi M, Narisawa Y, Toda S: Epithelial-to-mesenchymal transition and slit function of mesothelial cells are regulated by the crosstalk between mesothelial cells and endothelial cells. Am J Physiol Renal Physiol. 2014; 306: F116-F122.
9) Sanders EJ: The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem Cell Biol. 1988; 66: 530-540.
10) Kratochwil K: Epithelial–Mesenchymal Interactions. eLS. 2013.
11) Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975; 6: 331-343.
12) Sun TT, Green H: Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. Cell. 1976; 9: 511-521.
13) Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA: Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science. 1989; 245: 752-755.
14) Florin L, Maas-Szabowski N, Werner S, Szabowski A, Angel P: Increased keratinocyte proliferation by JUN-dependent expression of PTN and SDF-1 in fibroblasts. J Cell Sci. 2005; 118: 1981-1989.
15) Yoshikawa T, Mitsuno H, Nonaka I, Sen Y, Kawanishi K, Inada Y, Takakura Y, Okuchi K, Nonomura A: Wound

therapy by marrow mesenchymal cell transplantation. Plast Reconstr Surg. 2008; 121: 860-877.
16) Witkowski JA, Parish LC: Histopathology of the decubitus ulcer. J Am Acad Dermatol. 1982; 6: 1014-1021.
17) Uccelli A, Moretta L, Pistoia V: Mesenchymal stem cells in health and disease. Nat Rev Immunol. 2008; 8: 726-736.
18) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284: 143-147.
19) Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH: Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002; 13: 4279-4295.
20) Aoki S, Toda S, Ando T, Sugihara H: Bone marrow stromal cells, preadipocytes, and dermal fibroblasts promote epidermal regeneration in their distinctive fashions. Mol Biol Cell. 2004; 15: 4647-4657.
21) Aoki S, Takezawa T, Uchihashi K, Sugihara H, Toda S: Non-skin mesenchymal cell types support epidermal regeneration in a mesenchymal stem cell or myofibroblast phenotype-independent manner. Pathol Int. 2009; 59: 368-375.
22) Aoki S, Kitajima Y, Takezawa T, Uchihashi K, Matsunobu A, Sugihara H, Toda S: Epidermal regeneration by keratinocyte-alien mesenchymal cell interactions. Inflamm Regen. 2010; 30: 428-433.
23) Aarabi S, Longaker MT, Gurtner GC: Hypertrophic scar formation following burns and trauma: new approaches to treatment. PLoS Medicine. 2007; 4: e234.
24) Darby IA, Hewitson TD: Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol. 2007; 257: 143-179.
25) Mori L, Bellini A, Stacey MA, Schmidt M, Mattoli S: Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. Exp Cell Res. 2005; 304: 81-90.
26) McAnulty RJ: Fibroblasts and myofibroblasts: their source, function and role in disease. Int J Biochem Cell Biol. 2007; 39: 666-671.
27) Prockop DJ: "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). Clin Pharmacol Ther. 2007; 82: 241-243.
28) Prockop DJ: Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. Mol Ther. 2009; 17: 939-946.
29) Ankrum J, Karp JM: Mesenchymal stem cell therapy: two steps forward, one step back. Trends in molecular medicine. 2010; 16: 203-209.
30) Gussoni E, Blau HM, Kunkel LM: The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat Med. 1997; 3: 970-977.
31) Weissman IL, Anderson DJ, Gage F: Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Ann Rev Cell Dev Biol. 2001; 17: 387-403.
32) Rolstad B, Fossum S, Bazin H, Kimber I, Marshall J, Sparshott S, Ford W: The rapid rejection of allogeneic lymphocytes by a non-adaptive, cell-mediated mechanism (NK activity). Immunology. 1985; 54: 127.
33) Clark EA, Shultz LD, Pollack SB: Mutations in mice that influence natural killer (NK) cell activity. Immunogenetics. 1981; 12: 601-613.
34) De Jong W, Steerenberg P, Ursem P, Osterhaus A, Vos J, Ruitenberg E: The athymic nude rat: III. Natural cell-mediated cytotoxicity. Clin Immunol immunopathol. 1980; 17: 163-172.
35) Moreau A, Varey E, Anegon I, Cuturi M-C: Effector Mechanisms of Rejection. Cold Spring Harb Perspect Med. 2013; 3: a015461.