CRITICAL REVIEW

Animal Models for Studies of Keloid Scarring

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Significance: Keloid scarring is a disfiguring fibroproliferative disorder that can significantly impair the quality of life in affected individuals. The mechanisms that initiate keloid scarring are incompletely understood, and keloids remain one of the most challenging skin conditions to treat. Keloids are unique to humans; thus, the lack of adequate animal models has hindered research efforts aimed at prevention and effective therapeutic intervention.
Recent Advances: In the absence of a suitable animal model, keloid researchers often rely on studying excised keloid scar tissue and keloid-derived cultured cells. Recently, *in vivo* models have been described that involve transplantation to mice of reconstructed skin containing keloid-derived fibroblasts and/or keratinocytes. These mouse–human hybrid animal models display some similarities with keloids and may enable investigation of novel therapies, although no model yet recapitulates all the features of human keloid scarring.
Critical Issues: Differences in skin physiology and modes of healing contribute to challenges in modeling keloids in laboratory animals. Furthermore, recent studies suggest that cells of the immune system contribute to keloid pathology. The need to use immunodeficient hosts for transplanted human keloid cells in recently described animal models precludes studying the role of the immune system in keloid scarring.
Future Directions: Future animal models may take advantage of humanized mice with immune systems reconstituted using human immune cells. Such models, when combined with grafted tissues prepared using keloid-derived cells, might enable investigation of complex interactions between systemic and local factors that combine to promote keloid scar formation and may aid in the development of novel therapies.

Keywords: keloid, animal model, scar, wound healing, extracellular matrix, fibrosis

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SCOPE AND SIGNIFICANCE

Keloid scarring is a disfiguring fibroproliferative disorder that can significantly impair the quality of life in affected individuals.[1–4] Keloids result from an abnormal wound healing response, which involves excessive and prolonged deposition of extracellular matrix (ECM), particularly collagen. Despite decades of research that has advanced our understanding of wound healing, the mechanisms that initiate keloid scarring remain poorly understood. In contrast to normal scars, which stabilize over time, keloid scars are exuberant fibrous growths that extend beyond the original wound boundary and tend to grow indefinitely (Fig. 1), often impairing range of motion and interfering with normal daily activities. Keloid scars are firm, dense, and

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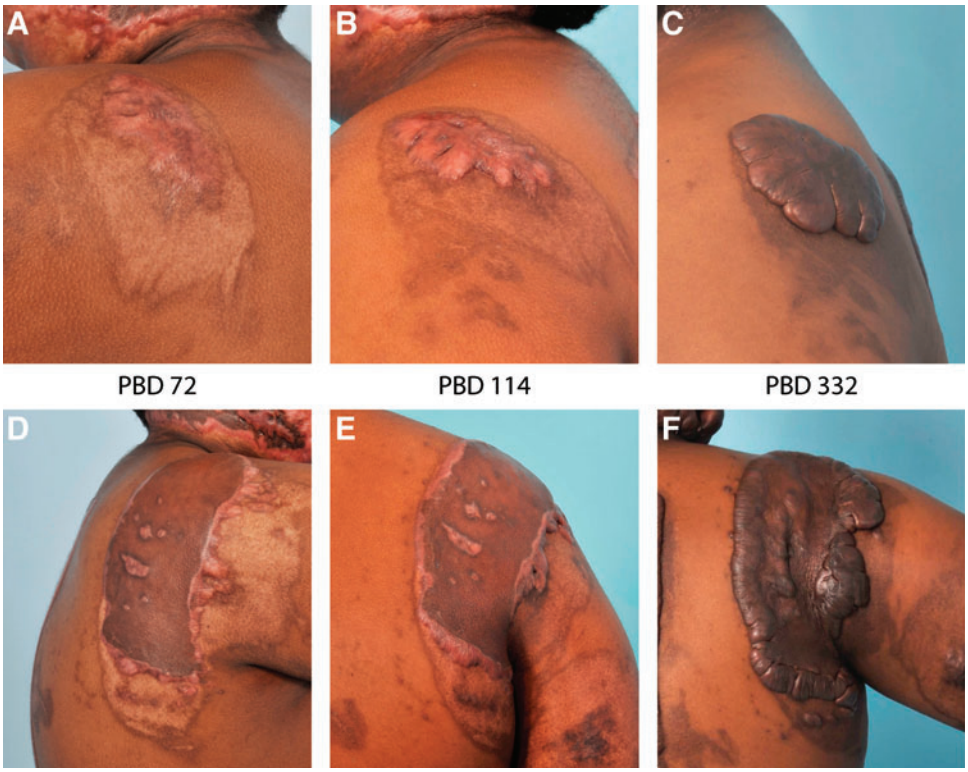

Figure 1. Keloid scar development. Shown are photos of the same patient illustrating the rapid development of keloid lesions over time after skin injury. The patient, an African American male who sustained a 15% total body surface area burn at 15 years of age, developed widespread keloid lesions in both grafted and ungrafted burn wounds, as well as donor sites used for autograft harvesting. **(A–C)** Images of patient's left shoulder, showing a healed partial-thickness burn wound that was not grafted. Note the rapid development of large keloid with typical bulging appearance. **(D–F)** Images of patient's right shoulder; this deeper burn wound was treated with split-thickness skin autograft. Note the development of keloid scarring around the skin graft and within the grafted area where wounds appear to have occurred. Photographs were taken at PBD 72 **(A, D)**, PBD 114 **(B, E)**, and PBD 332 **(C, F)**. PBD, postburn day. Color images are available online.

itchy and can be complicated by ulceration, bleeding, and infection.[5] Keloids share several features with hypertrophic scars (HTS), another form of abnormal scarring. Until relatively recently, keloids and HTS were considered different manifestations of the same abnormal scarring process; currently, however, the more widely accepted viewpoint defines keloids and HTS as separate entities that have some common features, but which follow different clinical courses.[6–8] Susceptibility to keloid scarring can run in families, suggesting a possible genetic predisposition in some keloid patients. Although recent studies have identified multiple loci that are strongly associated with keloid risk,[9–11] no single causative gene has yet been identified. Keloids are more common in populations with darkly pigmented skin, such as African Americans, but it is not yet known whether this is due to pigmentation or common genetic ancestry.[9] Furthermore, the possible influence of environmental factors on keloid risk has not been elucidated. Keloids can occur at any age but most commonly occur during the second and third decades of life[5]; it is not known whether this is due to hormonal influences or other factors. Although there are multiple therapies available for keloids, they remain one of the most challenging skin conditions to treat.[12,13] There is currently no universally effective treatment, and most therapies are successful for only a subset of patients and have limited long-term success, with high recurrence rates. Development of improved treatments will require a deeper understanding of the molecular mechanisms that cause healing wounds to progress to keloid scars, as well as appropriate preclinical models for evaluation of novel therapies. This review discusses the complexities of modeling keloid scarring, summarizes currently available animal models, and describes new technologies that may improve future preclinical keloid models.

TRANSLATIONAL RELEVANCE

There is a paucity of high-quality clinical research evaluating keloid therapies. The evidence

supporting many current therapies is based on low-quality studies, nonrandomized trials, and case reports, limiting the ability to make evidence-based decisions regarding therapy.[13,14] Ideally, effective therapies would be based on a detailed understanding of the underlying pathology and would be developed and tested using preclinical animal models. However, keloid scarring is unique to humans, complicating the use of animals as models for preclinical studies. This has hindered translational research efforts aimed at development of effective strategies for keloid prevention and treatment.

CLINICAL RELEVANCE

For functional analysis of the dynamic process of human wound healing, samples would ideally be obtained from uninjured skin and from wounds at multiple time points during healing. This is problematic for clinical studies of keloid scarring. How do researchers ethically sample normal tissue from a keloid patient if this will cause a new keloid to form at the biopsy site? If there is no family history or prior incidence of keloids, how can we predict who will form a keloid so that we can follow its early development and progression? For these reasons and others, animal models are critical.

OVERVIEW

Human versus animal wound healing

It is not known why humans form keloid scars, but animals do not. Keloid etiology is complex, and there are numerous physiological differences between humans and rodents, the most commonly used research animals, as well as differences in pathology and responses to injury.[15] Anatomically, rodent skin is thinner and is more loosely attached to the underlying fascial tissue. Skin contraction plays a larger role in wound healing in rodents, whereas granulation tissue formation and re-epithelialization constitute the primary mode of healing in human wounds.[16] Mechanical forces are known to regulate profibrotic gene expression in human fibroblasts, resulting in the upregulation of genes encoding ECM proteins, such as collagen and fibronectin, and profibrotic cytokines, such as transforming growth factor beta 1 (TGF-β1).[17] Differences in skin tension during wound healing may underlie, in part, the different physiological responses to wounding in human versus murine skin. Human skin, which is tightly adhered to the underlying fascia, experiences higher levels of mechanical stress during healing than murine skin, which is tightly attached to the panniculus carnosus but more loosely adhered to underlying fascia, resulting in protection from excess mechanical load during healing. Differences in density and size of hair follicles, which can contribute stem cells to aid in wound healing, as well as immunological differences also contribute to different wound healing processes in rodents and humans.[16,18] Additionally, the microbiome has been shown to influence wound healing, but there are differences in the microbiomes of mice and humans.[19,20] Despite these numerous differences, mice are commonly used in wound healing research, and although they can form thickened scars under certain experimental circumstances, keloids have never been observed in murine skin.

Fibrotic wound healing in animals

Pigs are increasingly used in wound healing studies, as pig skin more closely resembles human skin in thickness and hair density.[16,21,22] Some pig breeds develop raised scars resembling HTS under certain wounding conditions; thus, despite their relatively high cost, pigs have become a useful model for HTS. Deep wounds (full thickness or deep partial thickness) in the female red Duroc pig were shown to heal with characteristics similar to human HTS.[23,24] These include contracted, thickened, hyperpigmented scars with abnormal gene expression patterns similar to the alterations observed in human HTS, including increased TGF-β1 and collagen type 1 and decreased decorin, although the levels of expression varied over time after wounding.[24,25] Histologically, deep excisional wounds in the female red Duroc pig model display disorganized collagen fibers, collagen nodules, presence of myofibroblasts, and elevated mast cell counts similar to human HTS.[26] However, in the pig model, the timing of these features appears compressed relative to human HTS.[26] A recent innovation of this model from Powell's laboratory involves a full-thickness burn followed by grafting with meshed split thickness skin autograft in the female red Duroc pig, which was found to more closely model human burn scars compared with excisional wounds.[27,28] Unfortunately, features observed in human keloids but not HTS, such as continued growth beyond the wound margin, have not yet been reported in any porcine wound model.

The only animal, aside from humans, known to naturally develop extreme fibroproliferative scarring is the horse. Limb wounds in horses, in contrast to wounds in other areas of the body, can develop a

type of scarring known as exuberant granulation tissue (EGT) or "proud flesh," which is similar in some respects to human keloid scars.[29] Wounds on the distal limbs of horses are characterized by complications not encountered in trunk wounds, such as minimal soft tissue coverage around the wound, reduced blood supply, frequent movement of the injured limb, and greater risk of microbial contamination. Additionally, leg wounds have reduced contraction and slower rates of wound reepithelialization.[30] Loss of tissue in leg wounds is common, complicating primary closure and resulting in healing by secondary intention, which contributes to the deposition of excessive granulation tissue.[31] Similar to keloids in humans, EGT in horses is raised and extends beyond the wound bed, resembling benign tumor-like growths, and fibroblasts of EGT display overproduction of ECM.[31] In contrast to keloids, which form after wound reepithelialization is complete and have an intact (although aberrant) epidermis, EGT frequently occurs before completion of wound reepithelialization. The wound margins may display a hyperplastic epidermis, but ulceration of the central portion of the wound is common. As an animal model for human pathology, horses are less than ideal due to high costs for veterinary care and housing, long life span, large size, and paucity of species-specific reagents compared with rodent models. However, as the only other known species to exhibit extreme fibroproliferative healing, comparative studies of EGT and keloid scarring may provide insights that can ultimately benefit both species.

Experimental animal models of dermal fibrosis

Although keloid-like scarring has not been induced in rodent models to date, manipulation of the murine wound to reduce contraction and promote healing via reepithelialization, as in human wounds, can enable generation of scars exhibiting some features of human HTS.[15] To create murine wound healing model that more closely resembles human wound healing, Gurtner's laboratory created a model in which splints were fastened to the margins of excisional wounds in mice.[32,33] The splints counteracted wound contraction, increasing the role of reepithelialization in wound closure, more analogous to healing in human skin.[32] This group later used biomechanical loading devices for application of mechanical stress during healing of incisional wounds to mimic the forces experienced by human wound healing under tension.[34] Mechanical loading during the proliferative phase of healing resulted in scars more closely resembling human HTS, emphasizing the role of mechanical stress in HTS formation.[34]

A reproducible model of raised scarring was developed in rabbits[35] and has been subsequently utilized in numerous studies for preclinical analysis of anti-scar therapies. This model involves full-thickness excisional wounds (≥7 mm diameter) created on the ventral ear surface down to the level of cartilage, including removal of the perichondrium.[36] Wounds of this size and depth display delayed reepithelialization, resulting in thickened scars, similar to human HTS, which may persist for weeks to months.[36] The efficacy of silicone gel sheeting, a commonly used intervention for HTS and keloids, was tested in rabbit ear wounds and was shown to significantly reduce scar elevation in this model.[37] The mechanism of action appeared to involve increased epidermal hydration due to occlusion, which reduced the keratinocyte activation and decreased profibrotic paracrine signaling resulting in lower dermal ECM production.[38,39] However, when analyzed in numerous clinical trials, the benefits of silicone sheeting have been less clear.[40] This exemplifies important differences between preclinical studies in animal models and human clinical trials that complicate clinical translation of many promising therapies. Animal models can be highly uniform and reproducible, enabling preclinical studies with tightly controlled treatment regimens and strictly defined outcome measures. In contrast, human trials can be limited by subject heterogeneity, susceptibility to bias, and uncertainty regarding patient compliance. For anti-scar therapies, the numerous differences between humans and animals, detailed above, and inadequacies of current models further complicate clinical translation.

A mouse model involving orthotopic grafting of human skin was developed in Tredget's laboratory that has similarities to human HTS.[41,42] This model involves grafting of full-thickness or split-thickness normal human abdominal skin obtained from elective cosmetic surgery to full-thickness excisional wounds on the backs of athymic mice. Approximately 1 month after grafting, the human skin grafts harden and the upper layer peels off; by 2 months after grafting, the grafts appear reddish, firm, and raised compared with surrounding mouse skin, reminiscent of human HTS.[41] The hypertrophy was observed to be greater in grafts of full-thickness human skin compared with split-thickness skin; this was attributed to injury caused by dermatome preparation of the split-thickness skin, which may have initiated proinflammatory

profibrotic responses in the graft.[41] Similarities with human HTS included increased levels of collagen type 1 alpha 1 (COL1A1), TGF-β1, and connective tissue growth factor compared with normal human skin, as well as increased numbers of mast cells, macrophages, and alpha smooth muscle actin-positive cells.[41] In addition, whorled collagen fibers and decreased levels of decorin compared with normal skin were observed.[42] These studies demonstrate that under specific conditions, this chimeric model can recapitulate many of the features of HTS in a convenient small animal model, using a human tissue source that is more readily available than HTS or keloid scar.

It is important to note that none of these models mimic features that distinguish keloids from HTS, such as continued growth beyond the boundaries of the initial wound. This supports the concept that keloids and HTS represent distinct clinical entities and suggests that critical components present in humans, but not in animals, are missing from these models.

DISCUSSION: CURRENT STATUS OF KELOID-SPECIFIC MODELS

Keloid scar implantation models

Because keloid scarring is a uniquely human trait and, as described above, no single causative gene has yet been identified, keloid-specific models involve cells and/or tissue derived from keloid patients. Studies by Shetlar and colleagues dating back to the 1980s described implantation of keloid scar tissue into subcutaneous pockets in immunodeficient mice, with tissue integrity reportedly maintained from 60 to >240 days without rejection.[43,44] The implants vascularized quickly, and remodeling of the edges of the implants was observed in addition to the reduction of implant size over time.[44] A follow-up study also demonstrated a reduction in weight of implanted tissue over time, as well as a significant reduction in chondroitin-4-sulfate levels, particularly 80 days postimplantation.[45] Normal human skin implants also decreased in size but to a lesser degree than the scar implants. The authors speculated that the reduction in implant size over time might have been due to rejection, which may have been greater for the keloid tissue compared with normal skin,[45] although there was no histological analysis shown to support the immune rejection. Nevertheless, the authors asserted that the model could be useful for testing of therapeutic agents in relatively short-term studies. They subsequently tested the effects of oral pirfenidone, an antifibrotic drug, using this model; the weight of all implants decreased with time, as observed in prior studies, but weights of implants in the pirfenidone-treated animals were significantly lower than untreated controls.[46] They also tested injection of triamcinolone, which is a common treatment for keloid scars, but had difficulty in injecting the drug directly into the subcutaneously implanted keloid tissue. Subcutaneous injection near the implants caused a reduction in size of the tissue but also caused a decrease in body weight of the treated mice.[46] Another group of investigators undertook a similar study using mice harboring subcutaneous implants of deepithelialized keloid tissue pieces to investigate intralesional triamcinolone in addition to four other pharmaceutical agents.[47] In that study, the implants initially increased in size, and then decreased, with no differences observed among any of the treatment groups and controls. The authors of that study found that collagen organization of the implanted tissue was similar to the original tissue, but several of the implants were enclosed in a "pseudocapsule" that may have interfered with drug exposure.[47]

There are several limitations inherent to models that rely on implanted keloid scar tissue. Because they depend on the availability of freshly excised scar tissue, they are limited to studies of keloids treated by surgical excision, which are likely to be older larger scars. Thus, these studies cannot be used to investigate early events in the development of keloid scars or preventative strategies. Although not explicitly stated in all publications describing these models, they generally involve the implantation of dermal tissue with the epidermis removed[47]; thus, dermal–epidermal interactions cannot be studied. Furthermore, because only a small piece of keloid tissue can be implanted, any regional differences present in the keloid[48] but not represented by the implanted tissue may affect the results. These models involve the implantation of keloid tissue into subcutaneous locations[43–46] rather than orthotopic grafting, which may result in different responses to investigational therapies. Additionally, keloids are somewhat age-dependent, occurring most often in people between the ages of 10 and 30 years[5]; this age dependence and the potential role of hormonal status are not considered in these implantation models. Importantly, because immunodeficient host animals must be used to prevent the rejection of implanted human cells, the role of the immune system in keloid scarring cannot be studied. A more recent publication reported the implantation of deepidermalized keloid tissue into subcutaneous pockets

in immunocompetent animals.[49] The authors reported that keloid tissue pieces persisted for up to 4 months after implantation and expressed human genes, including TGF-β1 and vascular endothelial growth factor. Interestingly, xenogeneic skin grafts became necrotic within 5 days, in contrast to the subcutaneously implanted keloid tissue, suggesting that the subcutaneous pocket provided a privileged environment for engraftment.[49] Unfortunately, few experimental details were provided in that study, making replication by other investigators difficult.

Tissue-engineered keloid models

Several keloid models have been described that involve engineered tissues prepared using keloid-derived cells, which can be studied *in vitro* or *in vivo* after orthotopic grafting or implantation in mice. One of the first reports of organotypic culture to study keloid pathology involved a "raft" culture system, which is an artificial tissue consisting of fibroblasts embedded in a collagen gel with keratinocytes seeded on the surface.[50] In studies of raft cultures containing keloid or normal fibroblasts and normal keratinocytes, wounding of the rafts *in vitro* resulted in increased collagen deposition, with higher levels observed in rafts containing keloid fibroblasts compared with normal fibroblasts.[50] A similar study was performed in our laboratory utilizing engineered skin substitutes (ESS), which were originally developed as an adjunctive therapy for long-term wound closure in patients with very large full-thickness burns.[51] ESS are prepared using a bovine collagen-based scaffold seeded with primary fibroblasts and overlain with primary keratinocytes. During *in vitro* culture at the air–liquid interface, which promotes epidermal stratification, fibroblasts begin to remodel the dermal matrix and replace the bovine collagen in the dermal scaffold with newly synthesized human collagen.[52] Remodeling continues after transplantation to wounds, with peak expression of type 1 collagen occurring from 2 to 4 weeks after grafting.[52] When ESS were prepared using keloid-derived fibroblasts and keratinocytes, COL1A1 and COL1A2, as well as periostin (POSTN), a matricellular protein overexpressed in keloid fibroblasts,[53] were expressed at higher levels than in ESS composed of normal cells.[54] Wounding of keloid ESS *in vitro* resulted in increased deposition of newly synthesized collagen and increased POSTN expression compared with ESS containing normal fibroblasts and keratinocytes, suggesting that this model recapitulates a keloid-like cellular phenotype.[54]

To generate a keloid animal model, ESS were prepared using keloid-derived fibroblasts and keratinocytes or normal skin-derived fibroblasts and keratinocytes and, after the 2-week incubation period, ESS were grafted to 2×2 cm full-thickness wounds in immunodeficient Foxn1$^{nu-/-}$ mice. After 12 weeks, keloid ESS were significantly thicker than ESS prepared using normal cells and displayed densely packed, thick disorganized collagen fibers (Fig. 2). This engineered skin model was used to investigate the differences between fibroblasts isolated from different regions in keloid scars.[55] The dermal component of ESS prepared with keloid keratinocytes and fibroblasts from the deep reticular dermis of keloid scars was significantly thicker than the dermis of control ESS containing normal cells and expressed significantly increased COL1A1 levels. In contrast, ESS prepared with keloid keratinocytes and superficial, papillary dermal fibroblasts of keloid scars was not thicker than controls but significantly increased in area with time after grafting compared with control normal ESS and ESS prepared using deep keloid fibroblasts.[55] In all previous studies using non-keloid cells, the ESS contracted after transplantation to mice; the grafts generally contract to 40–60% of their original area by 6–8 weeks postgrafting, after which point the grafts area stabilizes.[56,57] ESS prepared using keloid keratinocytes and superficial keloid fibroblasts initially decreased in area, but at about 4 weeks after transplantation, the grafts increased in area, in some cases exceeding the area of the original graft.[55] This phenomenon was attributed to the loose-skinned nature of the mouse model; instead of bulging over the wound margin, as might occur in humans, which are tight-skinned, the grafts displace surrounding mouse skin in this loose-skinned model. The results suggest that this model recapitulates some of the features of keloid scars and can be used to investigate novel therapies for keloid scar suppression. The phenotypes observed with deep versus superficial keloid fibroblasts allowed us to propose a model that describes the different contributions of these cell populations in the development of bulging keloid scars (Fig. 3).[55] This model is consistent with the increased migration rate of keloid keratinocytes, which was observed in other *in vitro* studies.[53]

A similar model was described that utilized a plasma/fibrin gel as the dermal scaffold.[58] In this model, thrombin was added to human plasma containing dermal fibroblasts, and the mixture was dispensed into a polyethylene ring that served as a frame during polymerization. Keratinocytes were

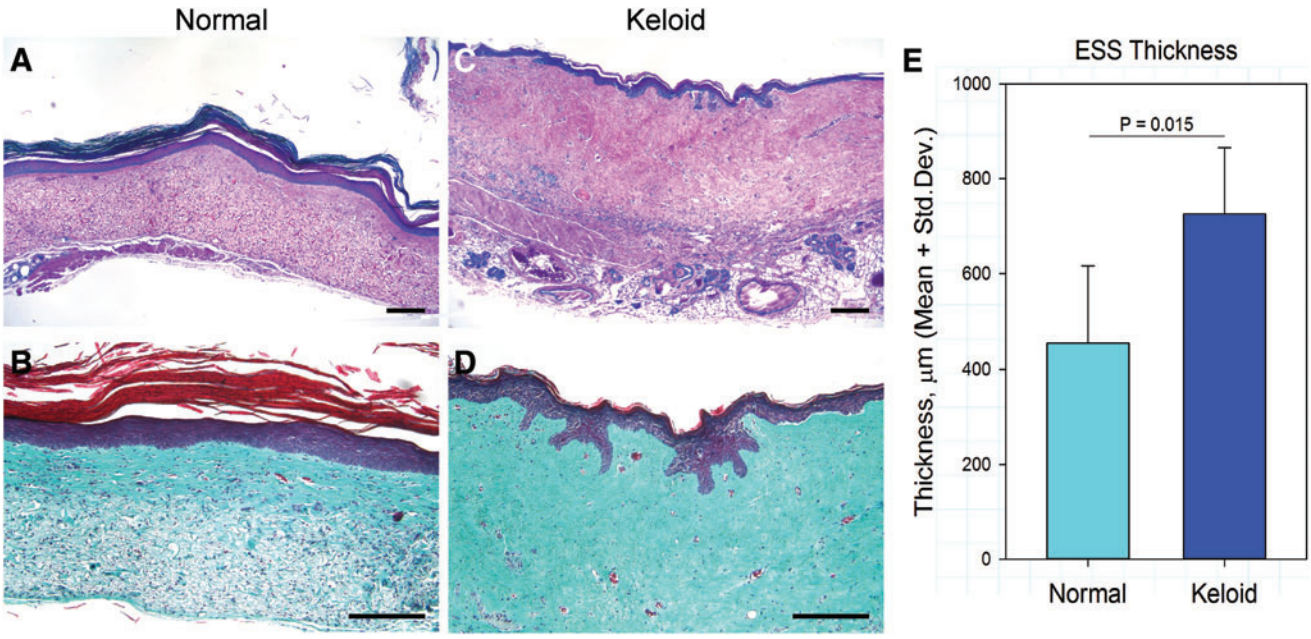

Figure 2. Normal and keloid ESS *in vivo*. **(A–D)** Shown are histological sections of normal **(A, B)** and keloid **(C, D)** ESS 12 weeks after transplantation to mice. Sections in the *top panels* **(A, C)** were stained with Tango stain, similar to hematoxylin; *bottom panels* **(B, D)** show Masson's trichrome-stained sections. Note the densely packed disorganized collagen fibers in the keloid ESS. Scale bars for all sections, 200 μm. **(E)** Quantitation of the total thickness of the grafted tissue demonstrated that ESS prepared with keloid cells were significantly thicker than ESS prepared using normal cells. ESS, engineered skin substitutes. Color images are available online.

seeded atop this structure, and the constructs were incubated *in vitro* for 2 weeks before transplanting to full-thickness wounds in immunodeficient mice.[58] The constructs were affixed to wounds by suturing through the polyethylene frame; the frames prevented initial contraction but detached 2 weeks after transplantation. The transplants were found to stably engraft for up to 18 weeks, and skin substitutes containing keloid fibroblasts and keratinocytes displayed increased collagen density

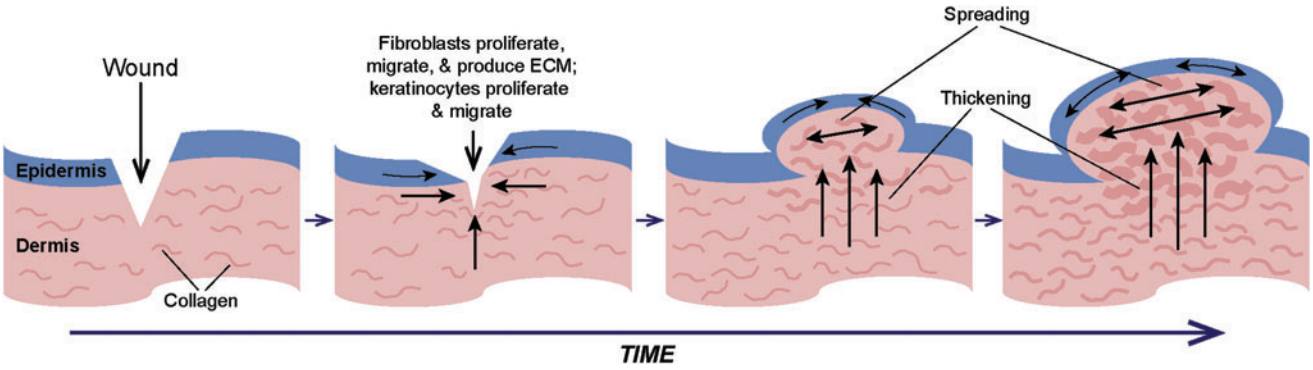

Figure 3. Model for the development of bulging keloid scars. Analysis of deep versus superficial keloid fibroblasts in engineered skin grafted to mice suggested that deep fibroblasts contribute to graft thickening, whereas superficial fibroblasts induce a spreading phenotype.[55] Other studies described the migratory phenotype of keloid keratinocytes.[53,68] Together, these observations contributed to the model illustrated here. Shown at the *left* is a schematic diagram of a cross-section of skin following a wound. During wound healing and over time, fibroblasts proliferate, migrate, and deposit ECM to form granulation tissue over which keratinocytes migrate to close the wound. For reasons that have yet to be identified, cells in scars that progress to keloids fail to respond to "stop" signals, and proliferation, ECM production, and migration continue unchecked. Continued production of ECM in fibroblasts in the deep dermis contributes to thickening of the lower dermis, while fibroblasts in the upper dermis exhibit a spreading phenotype, causing an increase in area. With increasing time after injury, the combination of deep dermal thickening and superficial spreading results in a bulging phenotype. Figure adapted with permission from Supp *et al.*[55] ECM, extracellular matrix. Color images are available online.

and increased size of collagen bundles compared with controls containing normal cells.[58] The keloid skin substitutes were thicker than normal controls and displayed increased expression of COL1A1 and plasminogen activator inhibitor-1. A unique feature of this model is that there is no exogenous collagen; all collagen deposited in the constructs is produced by the cells seeded within the skin substitute.[58]

More recently, a model was described that involved transplantation of keloid fibroblasts and keratinocytes to mice utilizing a chamber graft model.[59] In this model, human skin fibroblasts and keratinocytes were mixed in a chamber that was sutured to wounds in mice, and the cells self-organized into reconstituted skin analogs *in vivo*.[59] Keloid scar cells and "white" scar cells (presumably, nonkeloid scars) were compared with normal skin cells using this model.[59] The chambers were implanted in highly immunodeficient NOD/Shi-scid/IL-2RγKO mice (NOG mice), which lack all T cell, B cell, and NK cell activity and have reduced macrophage function. Reconstituted skin implants consisting of keloid-derived cells were significantly thicker at 12 weeks after implantation compared with those prepared using either "white" scar cells or normal cells.[59] In addition, the keloid reconstituted skin displayed a keloid-like disorganization of collagen fibers and expressed high levels of the protein versican. Unlike keloid scars in humans, invasion of surrounding tissue was not observed, and the authors speculated that this might be due to the features of the human skin microenvironment, such as tension, which are absent in the mouse model.[59] This is consistent with the results observed in our keloid ESS model, with keloid grafts displacing rather than growing over adjacent mouse skin.

Future prospects

Like the implantation models, the keloid engineered skin models are limited in that immunodeficient mice are required to enable engraftment of human cells *in vivo*. Thus, the role of the immune system in keloid scarring cannot be investigated using these models. There is mounting evidence of immune cell dysregulation in keloid scarring. Keloid scars have been found to have increased numbers of T cells, B cells, and mast cells compared with normal skin.[60–62] Additionally, "alternatively activated" (M2) macrophages were elevated in keloid scars compared with normal skin and normal scar tissue.[61,62] Macrophages in keloid scars were shown to express higher levels of interleukin 10, interleukin 12, and TGF-β1, consistent with the M2 phenotype.[62] M2 macrophages are considered to be anti-inflammatory and are associated with wound closure, in contrast to "classically activated" (M1) macrophages, which are proinflammatory and highly phagocytic and are associated with the early phases of wound healing.[63] M2 macrophages stimulate fibroblast proliferation, differentiation, and collagen production,[63] which not only promote wound closure but may also lead to excessive scarring if unchecked; studies suggest that this may occur in keloids.[61,62] In addition to differences in resident immune cell populations in keloid scars, circulating CD14+ monocytes, the precursors of macrophages, isolated from peripheral blood of keloid patients were found to stimulate the proliferation of fibroblasts to a greater degree than monocytes from nonkeloid controls.[64] These findings suggest that immune cell contributions to keloid pathology should not be overlooked in development and use of keloid animal models.

Even if immunocompetent mice could be used in keloid models, they would likely be inadequate due to numerous differences between the immune systems of humans and mice.[18] For example, the balance of neutrophils and lymphocytes is different, with human blood being very neutrophil-rich compared with mouse blood.[18] There are also numerous differences in T cell development, regulation, and activation between humans and mice.[18] Future keloid models might benefit from the use of "humanized" mice harboring components of the human immune system. Although several models that involve transplantation of human tissues to mice have been referred to as "humanized," more broadly this term has been used to describe immunodeficient mice that possess immune systems reconstituted with human cells.[65,66] These models have been used to study numerous human diseases, including infectious diseases and cancer.[65–67] Humanized mice are generated using severely immunodeficient recipients harboring multiple mutations that result in lack of adaptive immunity and compromised innate immunity; further immune suppression can be achieved using sublethal doses of radiation.[66] Multiple approaches can be used to reconstitute the recipient's immune system with human cells. The first involves injection of peripheral blood leukocytes, which results in rapid engraftment of T cells.[66] This model is most useful for short-term studies of T cell function due to the development of xenogeneic graft-versus-host disease (GVHD), which may occur in as little as 1–2 months. This may be an unsuitable period for studies of keloid scarring. In a second model, called the bone mar-

row/liver/thymus or "BLT" model, human fetal liver and thymus tissue pieces are implanted under the kidney capsule of immunodeficient mice, which are also injected with fetal liver hematopoietic stem cells.[67] This model permits the development of all human hematopoietic cell lineages, although these mice are also susceptible to GVHD. The use of these mice may be limited due to ethical considerations regarding the use of human fetal tissue in their generation; regulations regarding fetal tissue research vary, and this work may be prohibited by law in some locations. A third model involves intravenous infusion of CD34+ hematopoietic stem cells from bone marrow or cord blood, which enables engraftment of a virtually complete human immune system, including cells of the myeloid lineage.[66] Investigation of implanted native keloid tissue or engineered keloid grafts using mice with humanized immune systems might enable the identification of key immune cell populations involved in keloid pathology. Ideally, for studies of human keloid development, humanized mice would be reconstituted with hematopoietic stem cells from keloid-susceptible individuals (Fig. 4). The requirement for highly enriched hematopoietic stem cell populations for generation of fully humanized mice is currently an obstacle to adoption of this approach for studies of human keloid scarring. However, this is a rapidly progressing field, and

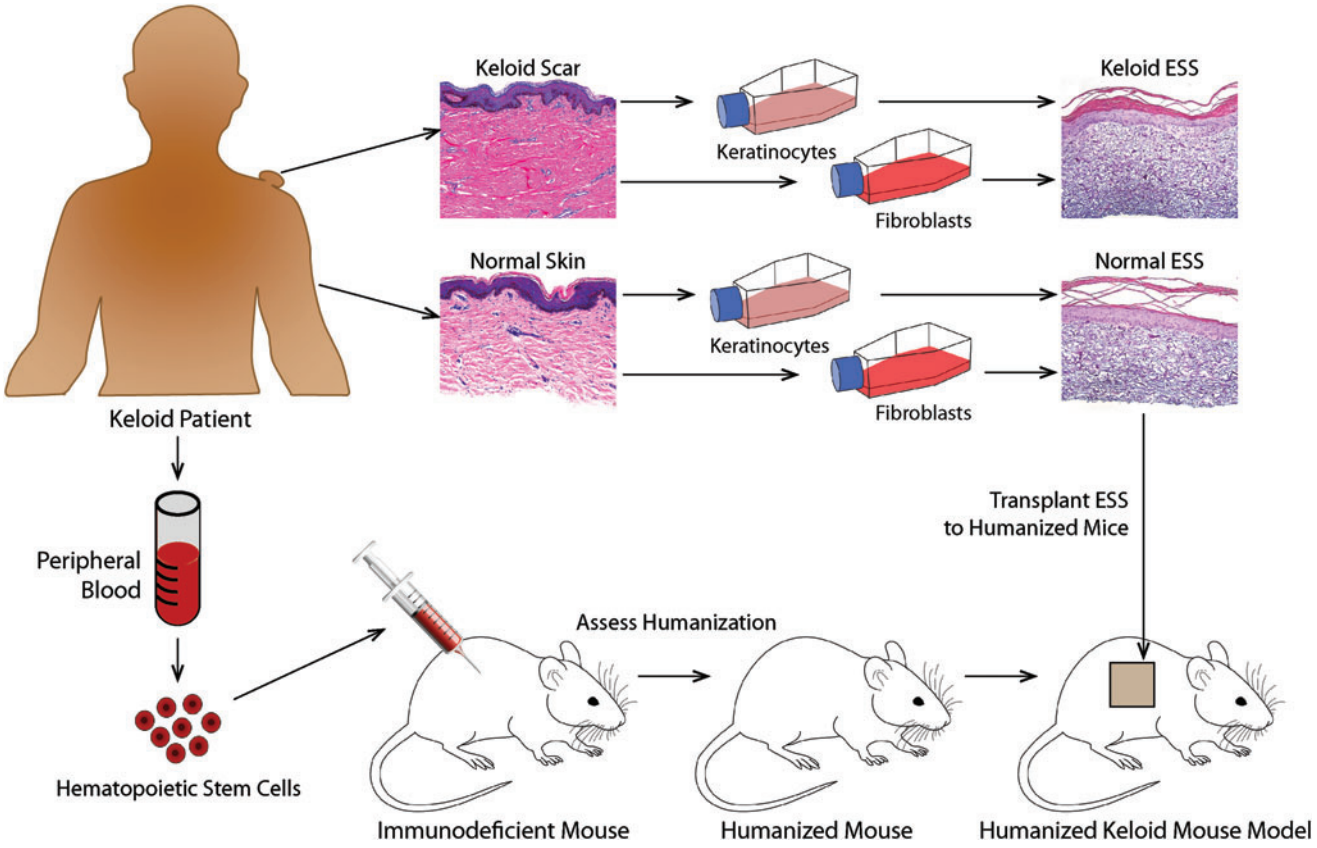

Figure 4. Use of humanized mice to study keloid scar development. This schematic diagram illustrates the potential use of humanized mice as hosts for grafting of ESS prepared using keloid-derived or normal skin-derived fibroblasts and keratinocytes. Humanized mice are prepared by injection of CD34+ hematopoietic stem cells into severely immunodeficient mice (see text for details). Resulting mice harbor immune systems reconstituted by human cells, enabling studies of the human immune response in a mouse experimental model. Grafting of ESS containing keloid-derived cells to humanized mice can permit investigation of the role of the immune system in keloid scar development, which is currently not possible using standard immunodeficient mouse hosts. ESS containing normal cells can be compared with ESS containing keloid-derived cells to determine the relative contribution(s) of skin cells and immune cells in keloid pathology. This diagram shows images of ESS prepared using primary keratinocytes and fibroblasts cultured from keloid scar and normal skin. Melanocytes[69] and microvascular endothelial cells[70] have also been used in preparation of ESS; thus, this model can be used to study the relative roles of numerous different cell types in keloid pathology. Comparison of mice humanized with keloid patient-derived hematopoietic stem cells versus normal donor stem cells can be used to identify specific components of the immune system involved in keloid development. Currently, isolation of sufficient numbers of hematopoietic stem cells from peripheral blood is an obstacle to implementation of such a model, but future developments aimed at expansion of this population and improved methods for stem cell recovery are expected to enable such studies in the near future. Color images are available online.

new developments and further refinements are expected that will increase the utility of humanized mice and enable their future use for keloid animal models. As we learn more about the role of the immune system from keloid clinical studies and from existing mouse models, we may be better equipped to engineer humanized mice using the most relevant immune cell components. In addition, continued research into the genetic basis for keloid scarring may uncover specific genes that predispose individuals to keloid scarring; this knowledge could then be incorporated into humanized mouse models using transgenic or gene-targeting technologies.

SUMMARY

Keloids remain a challenging problem for patients, clinicians, and researchers. Development of improved animal models will certainly benefit the keloid research community. In the meantime, the field can benefit from carefully designed preclinical studies involving cells and tissues isolated from keloid scars. As with clinical trials, careful study design is critically important for preclinical experiments involving tissue samples obtained from patients. Table 1 lists some aspects of preclinical studies that should be considered for the performance of well-designed *in vitro* and animal studies.

Despite some of the limitations outlined in this review, the models involving engineered or reconstituted skin containing keloid or normal cells can be used to investigate novel therapies for keloid suppression because different phenotypes are observed when grafts are prepared using keloid versus normal cells. However, because these models do not recapitulate all the features of human keloid scar, therapies developed using current models may not all translate to human studies. Raised scarring can be induced in some animal models, as detailed above, but these lesions do not display specific critical features of keloid scars, such as continued growth beyond the wound margin. Thus, there are significant factors specific to keloid pathology that have not yet been identified or modeled in animals. Currently, the best animal model to study keloid scarring is the human. High-quality well-controlled clinical trials for keloid therapies are required to unequivocally demonstrate the safety and efficacy of therapeutic interventions but unfortunately are lacking for many current treatment options.[14] Thus, many clinicians rely on anecdotal evidence or personal experience for selection of appropriate therapeutic approaches. Clinical researchers must invest the time and resources required to perform well-

Table 1. *Considerations for preclinical keloid studies*

Contact institutional review board (IRB) to determine if patient consent is required for collection of keloid scar tissue.
- Informed consent is required if protected health information (PHI) is collected or if patient can be identified by information collected.
- An IRB protocol is necessary if you are required to obtain informed consent, even if discarded tissue is collected.
- An IRB protocol may not be required if samples are de-identified, but check with your local IRB first to make sure.

Collect as much demographic and medical information on keloid patient as possible for every sample collected.
- Patient age, race, sex, general health, and single/multiple scars
- Scar etiology: cause, duration, and prior treatments
- Family history

Confirm keloid diagnosis before initiating experiments.
- View clinical photos
- Examine histological sections

Carefully document scar characteristics.
- Size, shape, thickness, pigmentation, ulceration, and infection
- Locations of biopsies

Include normal controls in experiments and use "matched" controls whenever possible.
- Ideally, nonlesional skin from keloid patient (although this may not be truly "normal" if patient predisposed to keloid formation).
- For unrelated normal skin controls, try to match age, race, sex, and body site.

Always use multiple biological replicates.
- "Biological" replicates are from different individuals; do not confuse with "technical" replicates.
  - Biological replicates help control for person-to-person variability.
  - Technical replicates help control for experiment-to-experiment variability.
- Perform a power analysis to ensure sample size is large enough to detect a significant difference if one exists.

For mouse studies, select mouse strain(s) carefully.
- Strain-specific differences may affect experimental outcomes.
- Outbred mice may exhibit more mouse-to-mouse variability in phenotype compared with inbred mice.

designed trials for keloid treatments; widespread adoption of any specific therapy must be supported by data, which can only be generated by careful evaluation in clinical trials.

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TAKE-HOME MESSAGES:

- Keloids are considered an extreme form of abnormal fibroproliferative scarring. Keloids can develop relatively rapidly after wounding in susceptible individuals. These lesions tend to grow indefinitely and extend beyond the boundary of the original wound. This distinguishes keloids from HTS, which do not extend beyond the original wound margin.
- Keloid scars are extremely resistant to treatment. Although many different treatment options currently exist, most are successful for only a subset of keloid patients, and recurrence rates for most therapies, including surgical excision, are very high. There have been relatively few well-designed controlled clinical studies for most keloid therapies. There is a need for the development and validation of effective therapeutic interventions and preventative strategies.
- Keloid scars are unique to humans. This may be due to differences in skin physiology, modes of wound healing, and immune system function between laboratory animals, such as rodents, and humans. Because animals do not get keloid scars, there is no accepted animal model for keloid scarring. This has hindered research aimed at understanding the underlying molecular mechanisms of keloid pathology and evaluation of novel therapies.
- Although wounds in some experimental animal models, including mice, rabbits, and pigs, can generate thickened scars under specific circumstances that resemble HTS, keloid scarring has not been observed in these animals. Horses develop a type of proliferative scarring, called EGT, which shares many features with human keloid scarring. However, horses are less than ideal animal models, due in part to prohibitive costs and paucity of species-specific reagents, and dissimilarities between keloids and EGT suggest differences in underlying pathologies.
- In the absence of an animal model, researchers have utilized keloid tissue samples and cultured primary cells isolated from excised keloids for preclinical research studies. Early keloid animal models involved subcutaneous implantation of keloid tissue into immunodeficient mice. These models were limited by availability of excised scar tissue, the requirement for immunodeficient mouse hosts to prevent immune rejection of human tissue, and subcutaneous location of implants.
- Current animal models involve the production of engineered skin-like tissues fabricated using keloid-derived fibroblasts and keratinocytes. After orthotopic grafting to mice, these engineered keloid tissues exhibit some features of human keloids, such as excess collagen production and thickening. However, they do not increase in size to the same extent as human keloids and do not extend beyond the original wound boundary. Like implantation models, the engineered skin models are limited by the requirement for immunodeficient mice to enable engraftment of human cells.
- Future keloid animal models may make use of humanized mice, which are genetically immunodeficient mice that have immune systems reconstituted using human cells. The use of humanized mice for grafting of engineered keloid tissues might one day enable the investigation of the relative contributions of the immune system and skin cells in keloid pathology.

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Abbreviations and Acronyms

COL1A1 = collagen type 1 alpha 1
ECM = extracellular matrix
EGT = exuberant granulation tissue
ESS = engineered skin substitutes
GVHD = graft-versus-host disease
HTS = hypertrophic scar
IRB = institutional review board
PBD = postburn day
PHI = protected health information
POSTN = periostin
TGF-β1 = transforming growth factor beta 1