Wound healing in a fetal, adult, and scar tissue model: A comparative study

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ABSTRACT

Early gestation fetal wounds heal without scar formation. Understanding the mechanism of this scarless healing may lead to new therapeutic strategies for improving adult wound healing. The aims of this study were to develop a human fetal wound model in which fetal healing can be studied and to compare this model with a human adult and scar tissue model. A burn wound (10×2 mm) was made in human ex vivo fetal, adult, and scar tissue under controlled and standardized conditions. Subsequently, the skin samples were cultured for 7, 14, and 21 days. Cells in the skin samples maintained their viability during the 21-day culture period. Already after 7 days, a significantly higher median percentage of wound closure was achieved in the fetal skin model vs. the adult and scar tissue model (74% vs. 28 and 29%, respectively, $p < 0.05$). After 21 days of culture, only fetal wounds were completely reepithelialized. Fibroblasts migrated into the wounded dermis of all three wound models during culture, but more fibroblasts were present earlier in the wound area of the fetal skin model. The fast reepithelialization and prompt presence of many fibroblasts in the fetal model suggest that rapid healing might play a role in scarless healing.

Fetal and adult skin heal via different mechanisms.[1] In adults, healing of deep wounds often leads to excessive scarring. In contrast, early gestation fetal skin has the ability to heal wounds without a scar.[2,3] During fetal healing, tissue architecture and appendages are reconstituted and normal function is restored.[1,2] Understanding the mechanisms of this scarless healing may lead to new therapeutic strategies for improving adult wound healing. Fetal skin model systems are important in unraveling the mechanisms underlying this scarless wound healing. Several in vivo and in vitro models have been described in the literature.[4–7]

The possibilities to use in vivo human models are limited, due to obvious ethical considerations. As an alternative to human studies, animal studies are frequently performed to investigate fetal wound healing.[4,8,9] Animals commonly used in fetal wound healing studies are mice, rats, and sheep. Mice and rats are advantageous, because relatively large numbers of animals can be studied and genetically modified animals, such as nude mice, transgenic or knock-out animals, can be studied. However, the short gestation of rodents limits analysis to late in the third trimester and due to their small size, surgical manipulation is difficult.[5] Larger experimental animals, like sheep, permit fetal wound healing analysis throughout gestation, allow easier fetal surgical manipulation, and have a lower risk of abortion after fetal surgery. However, the difficulty of housing large animals and the high costs restrict the use of sheep as test animals. In addition, animal fetal skin has a different composition than human embryonic skin. For example, animal fetal skin contains more developing hair follicles and a higher chondroitin sulfate (CS) level than human fetal skin.[10] This makes extrapolation to the human situation difficult.

Because of the limitation of in vivo studies, there is a demand for well-controlled human, in vitro alternatives. In vitro models are generally rapid, less costly, can easily be standardized, and involve minimal ethical considerations compared with in vivo studies. The simplest in vitro model is wounding of fetal fibroblast monolayers.[6,7] In this model system, it is possible to study cell migration, proliferation, and protein synthesis. More complex models are three-dimensional models, which are commonly composed of a fetal fibroblast-populated collagen lattice with or without an epidermis.[11,12] These models can be used to study contraction, cell migration, cell–matrix interaction, and cell–cell interaction. The major disadvantage of in vitro models is that multiple cell types, inflammation, blood clotting, and physical factors, such as multiple cytokines or correct oxygenation, are not present. After all, in vivo models are far more complete than in vitro models, and for the short term cannot be banned from wound healing research completely.

α-SMA	α-smooth muscle actin
BrdU	Bromodeoxyuridine
CS	Chondroitin sulfate
DEJ	Dermo-epidermal junction
ECM	Extracellular matrix
Hsp47	Heat shock protein 47
K14	Keratin 14
K17	Keratin 17
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SKALP	Skin-derived antileukoproteinase

To circumvent some of the disadvantages of in vitro models, several researchers have developed an ex vivo explant model of fetal skin.[13–15] This model is based on the in vitro culture of excised fetal skin. All matrix and cellular elements, including developing appendages, are present in this model. However, most of these studies use animal skin for their explant model. Because there are differences between animal skin and human skin development,[16] extrapolation to the human situation can be difficult. In addition, the explants were only maintained in culture for a few days. Recently, we have developed an in vitro adult skin model that remained vital for a longer period of time and had many similarities to in vivo wound healing.[17]

The aims of this study are to develop a human fetal ex vivo wound model that can be cultured for a long period of time and to compare the fetal ex vivo wound model with a human ex vivo adult skin and scar tissue model, in order to differentiate important factors involved in regeneration and scarring.

MATERIAL AND METHODS

Burn wound model

This study was approved by the Medical Ethical Committee of Noord-Holland, Alkmaar, the Netherlands, and was performed in accordance with institutional guidelines. Tissues were collected after informed consent.

Human fetal skin was harvested from five fetuses at 17–22 weeks gestational age. Human fetal samples were derived from fully informed, consenting patients undergoing elective terminations of pregnancy. Human adult skin was obtained from five healthy donors undergoing abdominoplasty. Human hypertrophic scar (HS) tissue was derived from 10 patients during reconstructive surgery (more than 1 year postburn) and was confirmed to be hypertrophic by the plastic surgeon. Human fetal skin was excised from the fetal limbs and cut into pieces of 1 cm^2. From the adult skin and scar tissue, a 0.7 mm split thickness skin graft was harvested using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany) and this graft was also trimmed into pieces of 1 cm^2. A copper device (2 mm × 10 mm) attached to a HQ soldering iron (Niehoff, Denekamp, the Netherlands) was heated to 95 °C and applied for 10 seconds to the epidermis without exerting pressure. The temperature of the copper device was measured using a digital thermometer (Farnell InOne, Utrecht, the Netherlands). The burned skin samples were placed dermis down on a stainless steel grid and cultured at the air–liquid interface at 37 °C with 5% CO_2 in culture medium described previously by Coolen et al.[17] Parallel to the air-exposed cultures, burned fetal skin samples were cultured submerged under the same conditions. The medium was refreshed twice weekly.

Assessment of viability

To assess the cell viability throughout the culture period, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Cross-sectional biopsies were taken directly after burning, and after 7, 14, and 21 days of culture and they were incubated with (4 mg/mL; Sigma-Aldrich, St. Louis, MO) for 3 hours at 37 °C. To localize formazan, the biopsies were snap frozen in liquid nitrogen and sectioned at −20 °C.

Immunohistochemistry

Biopsies were fixed in either 4% formaldehyde or kryofix (50% ethanol, 3% PEG300) directly after burning and after 1, 7, 14, and 21 days of culture and processed for paraffin embedding. Sections (4 μm) were deparaffinized and rehydrated for hematoxylin & eosin (H&E) staining or immunohistochemical analysis by staining for keratin 17 (K17), keratin 14 (K14), skin-derived antileukoproteinase (SKALP), collagen type IV, laminin, α-smooth muscle actin (α-SMA), heat shock protein 47 (Hsp47), CS, and elastin (see Table 1 for specifications). Sections were incubated with primary antibodies, followed by incubation with secondary biotinylated rabbit anti-mouse or goat anti-rabbit polyclonal antibody (Dako, Glostrup, Denmark) for 1 hour at room temperature. Subsequently, the sections were stained with streptavidin–biotin–peroxidase complex system (Dako) according to the manufacturer's instructions. Peroxidase activity was detected with the 3,3¢ diaminobenzidine solution substrate. All sections were counterstained with hematoxylin. Negative controls were performed in the absence of primary antibody.

Table 1. Antibodies used in immunohistochemical analysis

Primary antibody	Clone	Dilution	Fixation	Source
Keratin 14	LL002	1 : 50	Formaldehyde	Novocastra, Newcastle Upon Tyne, UK
Keratin 17	E3	1 : 100	Kryofix	Dako
SKALP	TRAB2O	1 : 1600	Formaldehyde	Hycult Biotechnology, Uden, the Netherlands
Collagen type IV	CIV 22	1 : 250	Formaldehyde	Dako
Laminin	Polyclonal	1 : 50	Formaldehyde	MP Biomedicals
α-SMA	1A4	1 : 250	Formaldehyde	Dako
BrdU	MIB-1	1 : 50	Formaldehyde	Dako
Hsp47	M16.10A1	1 : 500	Formaldehyde	Stressgen, Vic., Canada
Elastin	BA-4	1 : 500	Formaldehyde	Sigma-Aldrich
CS	CS-56	1 : 500	Formaldehyde	Sigma-Aldrich

α-SMA, α-smooth muscle actin; BrdU, bromodeoxyuridine; CS, chondroitin sulfate; Hsp47, heat shock protein 47; SKALP, skin-derived antileukoproteinase.

Cell proliferation in the wound model

To evaluate cell proliferation in the burn wound model, the incorporation of bromodeoxyuridine (BrdU) was examined. The skin samples were incubated with 20 μM BrdU (Sigma-Aldrich) for 24 hours. The samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 μm) were deparaffinized, rehydrated, and pepsine (0.5%; Roche Diagnostics, Mannheim, Germany) treated for 15 minutes at 37 °C. The cellular DNA was denatured in 2 N HCl for 30 minutes and neutralized with 0.1 M Borax (Sigma-Aldrich). The sections were incubated with monoclonal anti-BrdU antibody (MP Biomedicals, Illkirch, France) and further processed as described above.

Scoring of immunoreactivity

All sections were evaluated for the presence and intensity of specific staining at the light-microscopic level (magnification ×200) at five randomly chosen regions. Staining was classified as negative (−), weakly positive (+/−), moderate (+), and strongly positive (++).

For Hsp47 and BrdU staining, the number of positive cells/mm^2 in the wounded dermis was calculated using NIS Elements (Nikon Instruments Europe B.V., Amstelveen, the Netherlands). In addition, the number of BrdU-positive keratinocytes per μm in the neo-epidermis was determined using NIS Elements.

Statistical analysis

All data are expressed as the median of at least three experiments carried out in duplicate. Because normal distribution of the data could not be assumed, statistical significance was determined by Mann–Whitney U for unpaired data or by Wilcoxon's signed-ranks test for paired data. p-values < 0.05 were considered statistically significant.

RESULTS

Cell viability in the air-exposed cultured fetal, adult, and scar tissue model

To determine cell viability in the unwounded part of the fetal, adult, and scar tissue model, MTT assay and histology were performed. The ability of the cultured and uncultured skin to convert MTT into the insoluble, blue formazan is shown in Figure 1A. Similar to uncultured skin, both the epidermal and dermal cells in the unwounded part of the fetal, adult, and scar tissue model were able to form formazan crystals after a 21-day culture period. This indicates that the cells were still viable after culture.

Figure 1B shows the histology of the uncultured fetal skin and of the unwounded part of the fetal skin model after 21 days of culture. In the cultured fetal skin, no vacuoles and no signs of pyknosis were visisble and the epidermis was still attached to the dermis. In comparison with the uncultured fetal skin, the cultured fetal skin contained more epidermal layers and had a stratum corneum. The histology of the unwounded part of the adult skin and scar tissue model after a 21-day culture period was similar to uncultured adult skin and scar tissue (results not shown). However, the number of stratum corneum layers was increased in cultured adult skin and scar tissue compared with uncultured skin.

In contrast to cells in the unwounded part of the fetal skin, cells in the complete burn wound were not able to convert MTT into formazan directly after burning

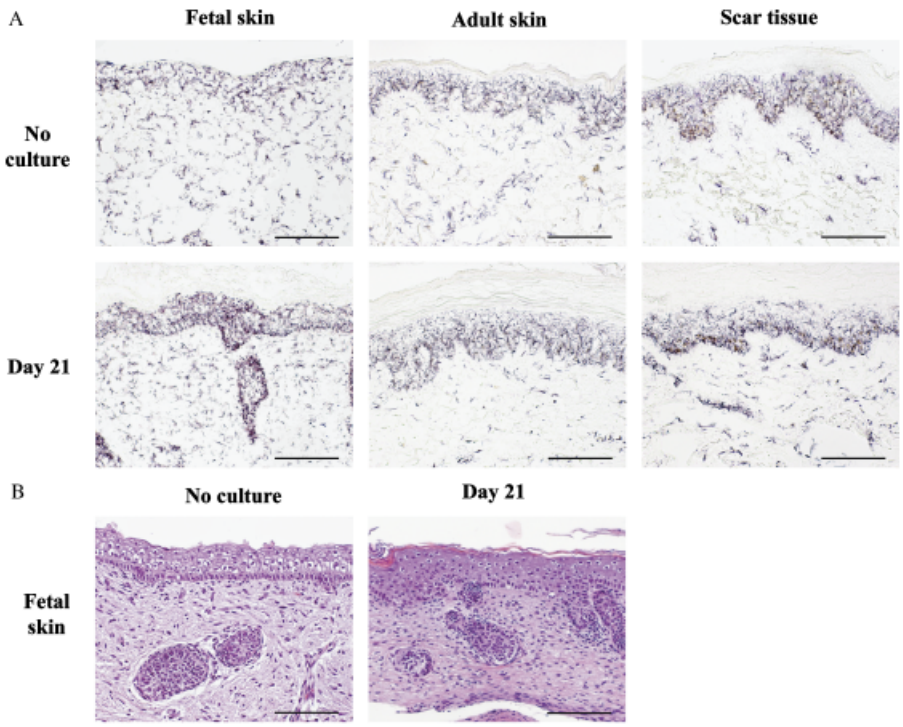

Figure 1. Viability of the air-exposed cultured fetal, adult, and scar tissue. (A) Presence of formazan after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in uncultured fetal, adult, and scar tissue and in unwounded fetal, adult, and scar tissue after 21 days of culture. In the in vitro models, the ability to form formazan crystals after a 21-day culture period was similar to uncultured skin. This indicates that the cells were still viable after culture. Scale bars: 100 μm. (B) Histology of uncultured fetal skin and of unwounded fetal skin after 21 days of culture. After 21 days of culture, no vacuoles or pyknotic cells were visible in the fetal skin model. Scale bars: 100 μm.

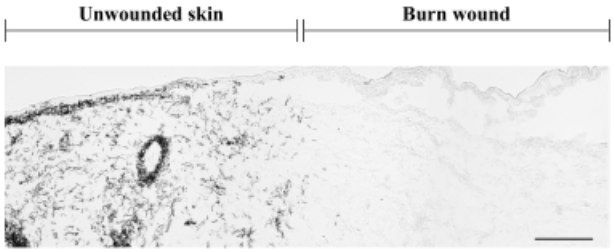

Figure 2. Presence of formazan in fetal tissue after incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution directly after burning. In unwounded fetal skin, formazan crystals were detected after incubation with MTT solution, indicating viable cells, but no crystals were found in the entire burn wound. Scale bar: 100 μm.

(Figure 2). This was similar in the adult skin and scar model (results not shown).

Histology of the air-exposed cultured fetal, adult, and scar tissue model

Immediately after infliction of the burn wound, both the epidermis and the complete thickness of the dermis were damaged. After 7 days of air-exposed culture, re-epithelialization occurred in the fetal wound model from both sides of the wound margins and progressed toward the wound centre (Figure 3A). In the fetal wound model, the newly formed epidermis first grew underneath and then over the old disrupted epidermis (Figure 3A, inset). After 7 days, the neo-epidermis covered 74% (range 36–88%) of the fetal burn wound (Table 2). After 14 days of culture, the median wound closure was 91% (range 63–93%) and after 21 days 100% (range 88–100%), but the neo-epidermis was detached from the dermis. In contrast to the fetal wound model, the neo-epidermis did not cover the wound area completely in the adult skin and scar model after 21 days of culture (52% (range 43–55%) and 42% (range 32–57%), respectively) (Table 2). In addition, the neo-epidermis of the adult skin and scar tissue model grew underneath the old burned epidermis, whereas the neo-epidermis of the fetal wound model grew over the burned epidermis (Figure 3B).

Presence of fibroblasts in the wound area

To examine the presence of fibroblast in the wound area, the sections were stained with anti-Hsp47. Hsp47 acts like a molecular chaperone during the biosynthesis and secretion of procollagen and is present in viable fibroblasts.[18]

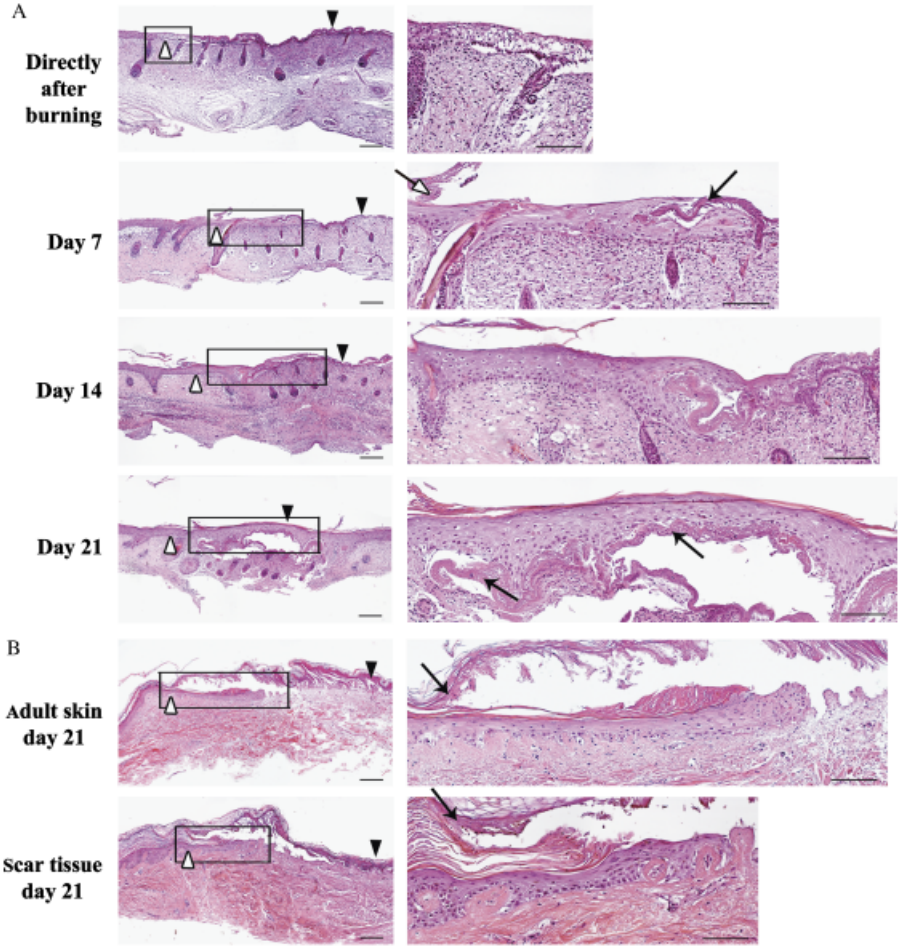

Figure 3. Histology of the air-exposed cultured fetal skin (A), adult skin, and scar tissue (B). (A): H&E staining of the fetal wound directly after burning, after 7, 14, and, 21 days of air-exposed culture. Open arrow heads indicate wound margins and closed arrowheads indicate the middle of the wound. Because of the lack of space, only half of the wounds are shown. Scale bars: 200 μm. Insets show the epithelial tongue in more detail. After 7 days of culture, the neo-epidermis first grew underneath (open arrow) and then over the old burned epidermis (closed arrow). After 21 days, the neo-epidermis covered the burn wound completely and grew over the old burned epidermis (closed arrows). Scale bars: 100 μm. (B): H&E staining of the adult skin and scar tissue model after 21 days of culture. Scale bars: 200 μm. Insets show the epithelial tongue in more detail. The neo-epidermis of the adult skin and scar model did not cover the burn wound completely and grew underneath the old burned epidermis (closed arrow). Scale bars: 100 μm.

Table 2. Median percentage of wound closure in the fetal, adult, and scar tissue model after 7, 14, and 21 days of culture

	7 days	14 days	21 days
Fetal skin model	74% (36–88%)	91% (63–93%)	100% (88–100%)
Adult skin model	28% (16–34%)*	39% (33–41%)*	52% (43–55%)*
Scar tissue model	26% (24–38%)*	36% (30–51%)*	42% (32–57%)*

*$p < 0.05$ vs. fetal wound model; Mann–Whitney *U*.
Values displayed are medians (range).

Directly after burning, no Hsp47-positive cells were visible in the wound area of the fetal, adult, and scar tissue model throughout the entire dermis (Figure 4A–C). Expression of Hsp47 was visible in the unwounded part of the skin models throughout the culture period (results not shown). During the culture period, the number of Hsp47-positive cells was increased in the wound area of the in vitro wound models (Figure 4G). In all three wound models, significantly more Hsp47-postive cells were detected in the wounded dermis after 21 days than after 7 days of culture. However, a higher number of fibroblasts was present in the wound area of the fetal wound model than in the wound of the adult and scar tissue model. In the fetal wound model, most Hsp47-positive cells were visible in the wounded fetal dermis close to the wound edges at day 21, but several fibroblasts were also detected in the central part of the fetal burn wound (Figure 4D). In the adult and scar tissue model, Hsp47-positive cells were observed throughout the wound area after 21 days of culture (Figure 4E,F).

Cell proliferation in the air-exposed fetal, adult, and scar tissue model

Proliferation of fibroblasts and keratinocytes in the burn wound model was evaluated by the incorporation of BrdU. At day 7 of culture, many basal and some parabasal keratinocytes were proliferating in the newly formed

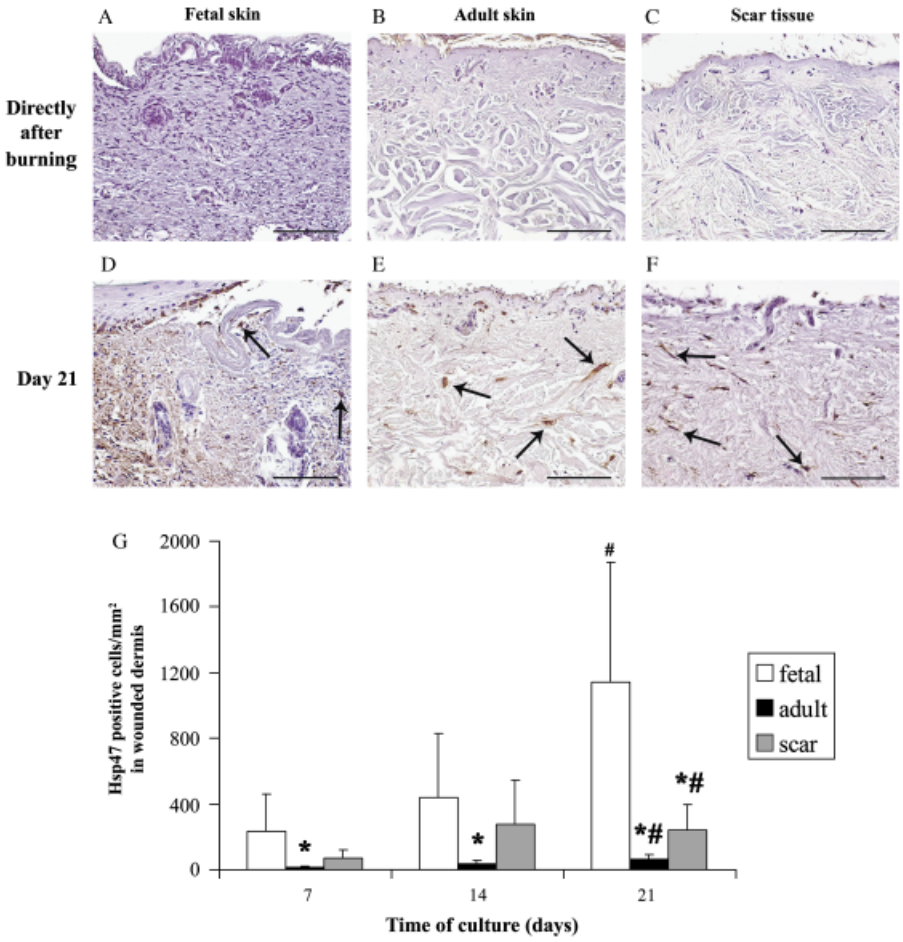

Figure 4. Presence of heat shock protein 47 (Hsp47) in the burn wound area of fetal, adult, and scar tissue model directly after burning (A–C) and after 21 days of air-exposed culture (D–F). Number of Hsp47 positive cells/mm^2 in the wounded dermis of the fetal, adult, and scar tissue model after 7, 14, and 21 days of air-exposed culture (G). Directly after burning, no expression of Hsp47 was found in the wound area of the fetal, adult, and scar tissue model (A–C). In the fetal wound model, most Hsp47-positive cells were visible in the wounded dermis close to the wound edges, but several fibroblasts were also detected in the central part of the fetal burn wound (arrows) at day 21 (D). In the adult skin and scar tissue model, Hsp47-positive cells were present throughout the dermis (arrows) at day 21 (E, F). Scale bars: 100 µm, *$p < 0.05$ vs. fetal wound model; Mann–Whitney *U*, $^{\#}p < 0.05$ vs. day 7; Wilcoxon's signed-ranks test.

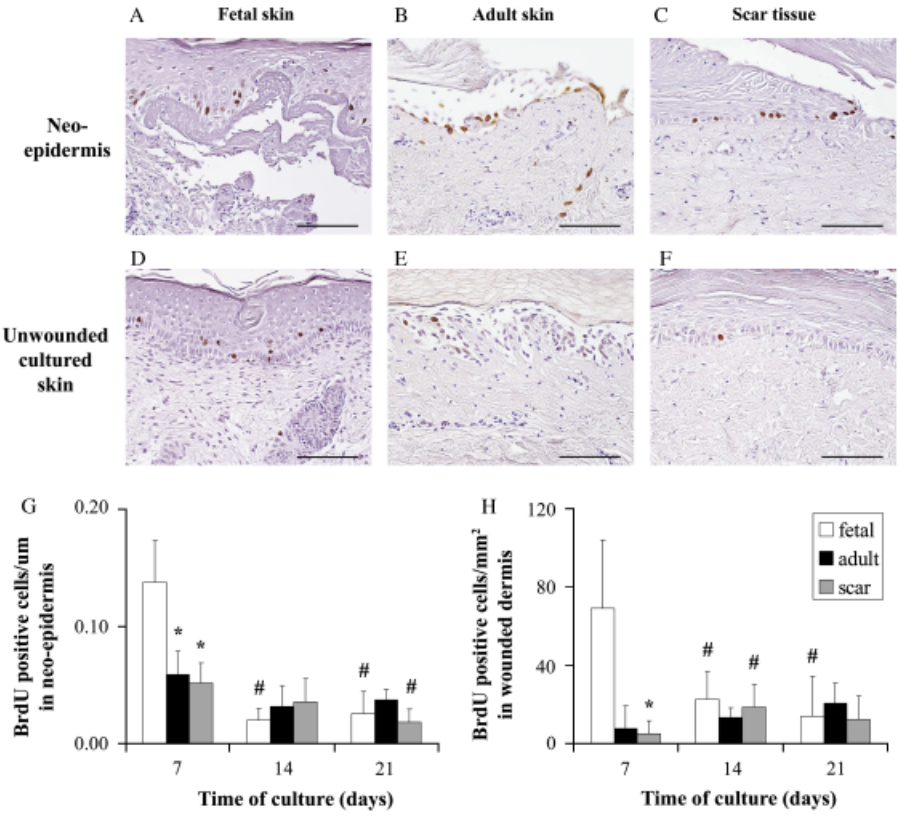

Figure 5. Cell proliferation in the fetal skin (A, D), adult skin (B, E), and scar tissue model (C, F) after 21 days of air-exposed culture shown by immunoperoxidase staining of bromodeoxyuridine (BrdU). Number of BrdU-positive keratinocytes per μm in the neo-epidermis (G) and number of BrdU-positive fibroblasts/mm^2 in the wounded dermis of the fetal, adult, and scar tissue model after 7, 14, and 21 days of air-exposed culture (H). Many proliferating cells were detected in the neo-epidermis of the fetal, adult, and scar tissue model after 21 days of culture (A–C). In the fetal wound model, many proliferating cells were also found in the unwounded cultured skin (D), but only a few proliferating cells were visible in unwounded cultured skin of the adult skin and scar tissue model (E, F). Scale bars: 100 μm, $^*p < 0.05$ vs. fetal wound model; Mann–Whitney *U*, $^\#p < 0.05$ vs. day 7; Wilcoxon's signed-ranks test.

epidermis of all three wound models. However, the neo-epidermis of the fetal skin model contained significantly more proliferating cells than the neo-epidermis of the adult and scar tissue model after 7 days of culture (Figure 5G). At day 21, many keratinocytes were still proliferating in the neo-epidermis of the fetal, adult, and scar tissue model (Figure 5A–C), but the number of proliferating keratinocytes as significantly decreased in the fetal and scar tissue model (Figure 5G). Different from the fetal wound model, only very few BrdU-positive fibroblasts were detected in the unwounded skin of the adult skin and scar tissue model throughout the culture period (Figure 5D–F).

In contrast to the adult and scar tissue model, many BrdU-positive fibroblasts were detected in the wounded dermis of the fetal skin model at day 7 (Figure 5H). During prolonged culture, the number of BrdU-positive fibroblasts was decreased in the wound area of the fetal skin model, whereas the number of proliferating fibroblasts was increased in the wound of the adult and scar tissue model.

Presence of activation markers in the new epidermis

To study abnormal epidermal differentiation and hyperproliferation, the presence of K17, SKALP, and K14 were determined. Table 3 summarizes the expression of these proteins in the fetal, adult, and scar tissue model after 21 days of air-exposed culture. The regenerated epidermis (i.e., neo-epidermis) of the fetal and scar tissue model contained more positive layers for K17, SKALP, and K14 than the uncultured skin of the fetal and scar tissue model. In the adult wound model, only the expression of SKALP and K14 was higher in the regenerated epidermis than in the uncultured adult skin. During culture, the presence of

Table 3. Presence of K17, SKALP, and K14 in uncultured skin and in the fetal, adult, and scar tissue model after 21 days of air-exposed culture

Wound model	Tongue tip	Regenerated epidermis	Unwounded cultured epidermis	Uncultured epidermis
Keratin 17				
Fetal	NA	++	+	+/–
Adult	+	–	–	–
Scar	++	+	+	–
SKALP				
Fetal	NA	+	+/–	–
Adult	+	+	–	–
Scar	+	+	+	–
Keratin 14				
Fetal	NA	++	+	+/–
Adult	++	+	+	+/–
Scar	++	++	++	+

K14, keratin 17; K17, keratin 17; NA, not applicable, because of complete reepithelialization; SKALP, skin-derived antileukoproteinase.

Table 4. Presence of basement membrane proteins in the dermo-epidermal junction (DEJ) of uncultured skin and in the DEJ of the fetal, adult, and scar tissue model after 21 days of air-exposed culture

Wound model	Tongue tip	Regenerated epidermis	Unwounded cultured epidermis	Uncultured epidermis
Collagen type IV				
Fetal	NA	+/–	++	+
Adult	–	+	++	++
Scar	+/–	+	++	++
Laminin				
Fetal	NA	+/–	++	+
Adult	+/–	+	++	++
Scar	+/–	+	++	++

NA, not applicable, because of complete reepithelialization.

K17, SKALP, and K14 was increased in the unwounded skin of the fetal and scar tissue model. In contrast, only the expression of K14 was enhanced in the unwounded skin of the adult tissue during culture.

Presence of basement membrane proteins

To assess the presence of basement membrane during wound healing, the sections were stained with antibodies directed against collagen type IV and laminin. A summary of collagen type IV and laminin expression in the different wound models is presented in Table 4. In uncultured fetal, adult, and scar tissues, collagen type IV and laminin was localized at the dermo-epidermal junction (DEJ) and in the basal lamina of capillaries. During air-exposed culture, the expression of the basement membrane proteins remained almost similar in the unwounded cultured skin. Strong expression of both collagen type IV and laminin was found in the DEJ of the wound margins of the fetal wound model; however, only a weak expression of these proteins was observed underneath the regenerated epidermis in the middle of the fetal burn wound. In the adult and scar tissue model, the basement membrane proteins were restored underneath the regenerated epidermis. Toward the leading epidermal edge (tongue tip), the deposition of the proteins became less intense.

Characterization of dermal components

To investigate the presence of blood vessels and myofibroblasts in the wound models, the sections were stained with anti–α-SMA. Expression of α-SMA was only observed in the blood vessels and not in the fibroblasts in uncultured fetal, adult, and scar tissues (Table 5). During the culture period, α-SMA remained only visible in the blood vessels.

To examine the presence of new extracellular matrix (ECM) components, the presence of CS and elastin was determined in the wound models. Table 5 demonstrates the expression of these proteins in the fetal, adult, and scar tissue model after 21 days of air-exposed culture. In both the fetal and adult skin model, the presence of CS remained similar during culture (Figure 6). Remnants of CS were still observed in the fetal burn wound directly after burning. In contrast to the fetal and adult skin model, expression of CS was increased in the burn wound and in the unwounded part of the scar tissue model after 21 days of culture.

Table 5. Presence of α-SMA, CS, and elastin in uncultured skin and in the fetal, adult, and scar tissue model after 21 days of air-exposed culture

Wound model	Burn wound directly after burning	Burn wound after culture	Unwounded cultured skin	Uncultured skin
α-SMA				
Fetal	–	–	+/–	+
Adult	–	–	+	+
Scar	–	–	+/–	+
CS				
Fetal	+	+	++	++
Adult	–	–	+/–	+/–
Scar	–	++	+	+/–
Elastin				
Fetal	–	–	–	–
Adult	++	++	++	++
Scar	+/–	+/–	+/–	+/–

NA, not applicable, because of complete reepithelialization.

Table 5 summarizes the expression of elastin in the fetal, adult, and scar tissue model. The presence of elastin was not changed during the culture period in all three wound models.

Submerged culture of the fetal wound model

Fetal skin is normally not exposed to air; therefore, we also cultured the fetal skin model under submerged conditions. Figure 7A shows the histology of the uncultured fetal skin and of the unwounded part of the fetal skin model after 21 days of submerged culture. Remarkably, the unwounded fetal epidermis was sloughed off during the submerged culture and a new epidermis grew underneath the shed fetal epidermis. This new epidermis contained less epidermal layers than the uncultured fetal epidermis and had a stratum corneum. Although the cultured epidermis appeared viable, some pyknotic cells were visible in the dermis. The MTT test also showed a reduced viability in the dermis of the submerged cultured model (results not shown).

Similar to the air-exposed cultured fetal wound model, reepithelialization occurred in the submerged cultured fetal skin model from both sides of the wound margins. After 7 days, the neo-epidermis covered a smaller part of the fetal burn wound than the neo-epidermis of the air-exposed cultured model (33% [range 20–38%] and 74% [range 36–88%], respectively), but this difference was not statistically significant ($p > 0.05$, Mann–Whitney U).

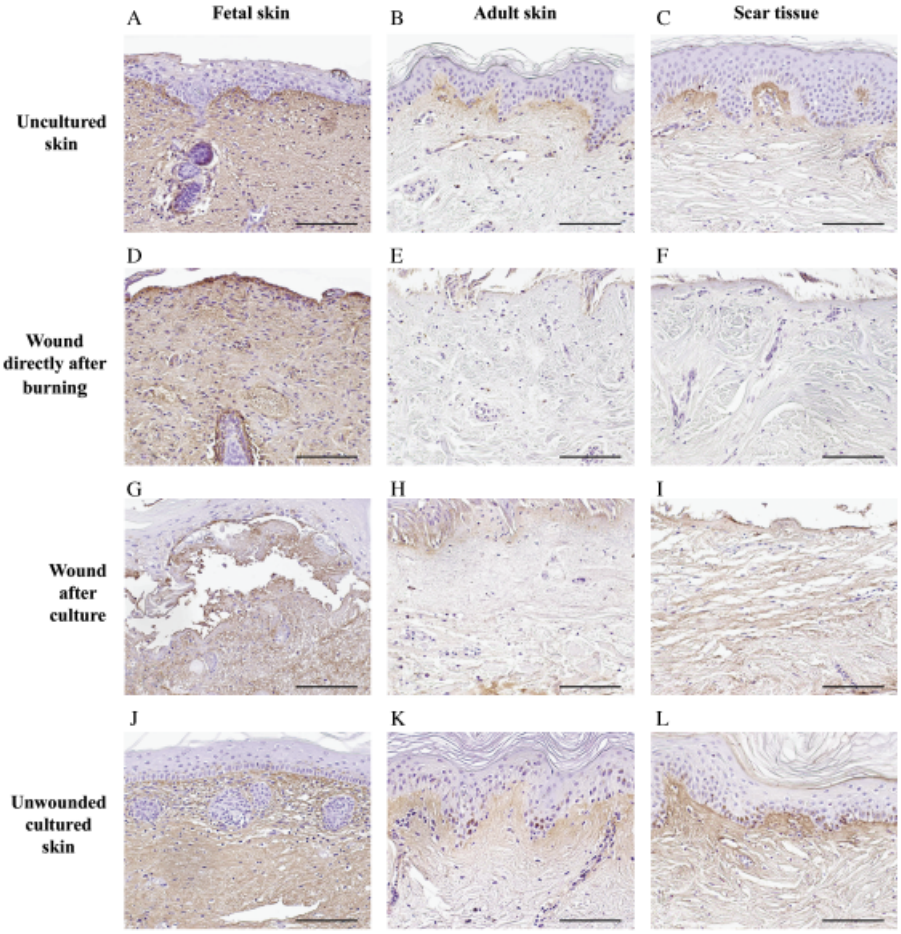

Figure 6. Presence of chondroitin sulfate (CS) in uncultured skin (A–F) and in the fetal, adult, and scar tissue model after 21 days of air-exposed culture (G–L). In fetal uncultured skin, CS was detected in the entire dermis (A) and was still present after wounding (D). After 21 days of culture, CS was detected in the entire burn wound (G) and in unwounded skin of the fetal wound model (J). In uncultured adult skin, CS was visible in the dermo-epidermal junction (DEJ) and around blood vessels (B), but it was not present directly after burning (E). After a 21-day culture period, CS was only slightly detected in the burn wound (H) and was still found in the DEJ and around the blood vessels in the unwounded cultured skin of the adult wound model (K). Similar to adult skin, CS was present in the DEJ and around the blood vessels in uncultured scar tissue (C) and it was not visible immediately after burning (F). After 21 days of culture, CS was present in the entire burn wound (I) and at the DEJ, around blood vessels and in some parts of the dermis in the unwounded skin of the scar model (L). Scale bars: 100 μm.

Comparable to the air-exposed cultured model, the neo-epidermis covered 100% (range 91–100%) of the burn wound after 21 days of culture (Figure 7B). The neo-epidermis of the submerged model grew underneath the old burned epidermis and remained attached to the underlying dermis, whereas the neo-epidermis of the air-exposed model grew over the burned epidermis and was detached from the dermis. In addition, the new epidermis of the submerged model contained less cell layers than the neo-epidermis of the air-exposed model.

Like the air-exposed cultured model, the expression of K17 and SKALP was increased during the submerged culture. However, the epidermis of the submerged model contained less positive layers for SKALP than the epidermis of the air-exposed model after 21 days of culture (results not shown). Identical to the air-exposed model, laminin was found in the DEJ of the wound margins in the submerged model, but it was only weakly detected underneath the regenerated epidermis in the middle of the burn wound.

DISCUSSION

In the present study, we developed a fetal wound model by wounding ex vivo fetal skin and subsequently culturing the skin samples. In order to differentiate important factors associated with regeneration and scarring, we compared the fetal wound model with a scar model and an adult skin model, which we have described previously.[17] The cell types normally present in fetal skin, adult skin, and scar tissue are present in the models and these cells remained intact and vital at least until day 21. Some investigations also showed prolonged culture of ex vivo fetal skin samples,[19,20] but in most studies ex vivo fetal skin was only maintained in culture for a few days.[14,15] It is possible that the life span of our skin explants was increased by use of an enriched culture medium.

The developed fetal wound healing model shows many similarities with in vivo epidermal wound healing. Re-epithelialization started directly after wounding and the burn wound was closed during the culture period. In addition, reepithelialization was faster in the fetal wound model, than in the adult skin models (i.e., adult and scar tissue model).[1,4,21] However, reepithelialization of the in vitro wound models occurred much slower than in vivo. Different from our fetal skin model, wounds are generally closed between 1 and 7 days in the fetus in the in vivo situation, depending on wound size.[4,8] It is possible that additional systemic growth factors or other variables are necessary for faster reepithelialization. The finding that wound closure was faster in the fetal than in the adult and

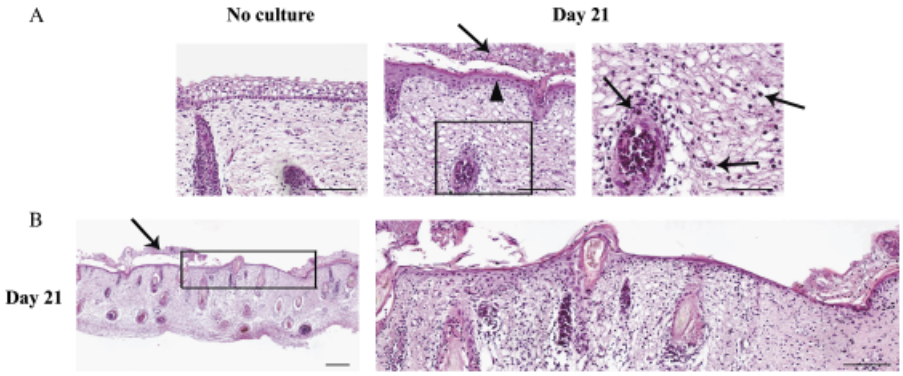

Figure 7. Histology of the submerged cultured fetal wound model. (A) H&E staining of uncultured fetal skin and of the unwounded fetal skin after 21 days of submerged culture. Remarkably, unwounded fetal epidermis was sloughed off (arrow) during submerged culture and a new epidermis (arrow head) grew underneath the shed epidermis. Scale bars: 100 μm. Inset shows the dermis in more detail. Pyknotic cells (arrows) were visible in the dermis after culture. Scale bar: 50 μm. (B) H&E staining of the wound area of the fetal wound model after 21 days of submerged culture. The neo-epidermis in the submerged model grew underneath the old burned epidermis (arrow). Scale bar: 200 μm. Inset shows the neo-epidermis in more detail. Scale bar: 100 μm.

scar tissue model suggests that an increased reepithelialization rate might partially be responsible for a better wound healing outcome. This is supported by the fact that delayed reepithelialization is associated with HS formation in burn patients.[22] The mechanism for this relationship in burn patients is not entirely understood, but it has been hypothesized that after complete reepithelialization, the inflammatory response is down-regulated and that keratinocytes decrease the production of collagen by fibroblasts.[23,24] The present study suggests that stimulation of early wound closure is a beneficial clinical strategy to decrease HS formation.

As in vivo, many proliferating cells were observed in the neo-epidermis of the in vitro wound models. However, the number of proliferating cells in the fetal neo-epidermis was decreased during prolonged culture. As reepithelialization was finished in the fetal wound from day 14, it appears reasonable that less proliferating cells were present in the fetal neo-epidermis during extended culture. Similarly, the number of BrdU-positive cells in the adult neo-epidermis did not decrease significantly during culture, probably because the adult wound was still not closed. Remarkably, the number of proliferating cells in the neo-epidermis of the scar model diminished after day 14, even though the wound was yet not closed. Possibly, the different composition of the dermal scar tissue might have accounted for this difference.

Restoration of the basement membrane was observed in the fetal, adult, and scar tissue model and this was identical to the in vivo situation.[4,25] However, a weak expression of basement membrane proteins was found underneath the regenerated epidermis in the middle of the fetal burn wound. In fetal in vivo healing, reepithelialization is not immediately accompanied by laminin and collagen type IV staining.[4] These basement membrane proteins are only visible in the DEJ some time after finishing reepithelialization. It is possible that a prolonged culture time is necessary for the fetal wound model to complete basement membrane restoration. Possibly, the weak expression of basement membrane proteins in the middle of the fetal wound may have accounted for the detachment of the fetal neo-epidermis from the underlying dermis. However, the growth of the fetal neo-epidermis over the old burned epidermis (see below) may also have resulted in the detachment of the fetal neo-epidermis from the dermis. Because of this event, the fetal neo-epidermis was only attached to the underlying dermis via the old burned epidermis. As the basement membrane between the burned epidermis and dermis was severely damaged, the old burned epidermis could disassociate from the dermis without difficulties.

The neo-epidermis of the fetal wound model exhibited an increased expression of SKALP, K17, and K14 upon wounding. This is similar to previous studies, which also showed an increased expression of proteins associated with the activation in the fetal wound edge.[26,27] In addition to the fetal neo-epidermis, the unwounded fetal skin was also activated during culture. As the fetal skin samples were cultured under nonphysiological conditions, i.e., exposed to air, it is reasonable that the unwounded keratinocytes were also activated.

Remarkably, the neo-epidermis grew over the old burned epidermis in the air-exposed cultured fetal wound model. This is dissimilar from the adult skin and scar tissue model, in which the epidermis grew underneath the burned epidermis. An explanation for this finding is elusive. The fact that reepithelialization in fetal wounds proceeds according to a different mechanism than in adult wounds might be involved. In adult healing, epithelial cells form lamellipodia and filopodia and they up-regulate various proteases in order to cut their way through the damaged dermis and blood clot.[28] In contrast, embryonic wounds heal by a process independent of either lamellipodial crawling or an adhesive substrate.[29] Fetal reepithelialization involves the formation of an actin cable in the basal marginal epidermal cells that may act as a purse-string to draw the wound margins together.[30] Possibly, the fetal mechanism of reepithelialization allows an easier growth of the neo-epidermis over the old burned epidermis. Another possible explanation for the this unusual finding might be the lower expression of basement membrane proteins underneath the regenerating epidermis (see above), thus causing a less effective binding of the keratinocytes to the DEJ.

As in vivo, fibroblasts were able to migrate into the wound area of all three models, but fibroblast migration occurred at a much slower rate than in vivo.[4] The lack of growth factors or other variables is possibly responsible for the retarded migration of fibroblasts in our wound models. In contrast to the adult skin and scar tissue model, a large number of fibroblasts was detected in the fetal

wound model at an early time point. This finding may suggest that the prompt presence of many fibroblasts in the wound area might be important in scarless healing. It allows the rapid production of ECM components, such as fibronectin and tenascin, which are important in fast reepithelialization and cell migration.[21]

Similar to the in vivo situation, proliferating fibroblasts were detected in the wound area of the fetal, adult, and scar tissue model. However, the number of proliferating cells decreased in the fetal wound area from day 7. Possibly, signals from the closed epidermis (present from day 14) may reduce fibroblast proliferation in the fetal wounded dermis.

No myofibroblasts were detected in the fetal, adult, and scar tissue model during in vitro culture. The finding that myofibroblasts were absent during fetal healing was in agreement with the in vivo situation. It has been shown by several studies that myofibroblasts were not present in early scarless fetal wounds.[8,31,32] In contrast, other studies demonstrated that myofibroblasts appear early but transitory in fetal healing.[33,34] Therefore, it is also possible that no myofibroblasts were detected in the fetal wound model, because these cells have already disappeared after 7 days of culture. In contrast to fetal healing, the absence of myofibroblast in the adult and scar tissue model was different from in vivo healing. Several studies demonstrated that both mechanical tension and pro-fibrotic growth factors, like TGF-β1 and PDGF, are important in promoting myofibroblasts.[35–37] As neither of these factors were present in the adult skin and scar tissue model, it appears reasonable that myofibroblasts were absent. Furthermore, it has been suggested that fibrocytes are the precursor cells for myofibroblasts.[38] Fibrocytes are thought to migrate from the bone marrow into the wound area, where they contribute to the myofibroblast population. As no bone marrow or blood flow was present in our wound models, fibrocytes could not have migrated into the in vitro wound area. Consequently, no fibrocyte-derived myofibroblasts could be formed in the wound model systems. Remarkably, no myofibroblasts were found in the uncultured HS tissue. As the HS samples were obtained from patients after more than 1 year postburn, it is possible that the myofibroblasts have already disappeared over time.[39] Moreover, myofibroblasts are usually located in deeper parts of the scar.[40] Therefore, it is also possible that no myofibroblasts were detected in the uncultured HS samples, because only the upper part (i.e., 0.7 mm) of the scar tissue was used for this model.

Despite the similarities, we also observed differences between embryonic in vivo healing and our fetal wound model. In contrast to the in vivo situation, we did not find histological signs of dermal regeneration in the fetal skin model. This suggests that in this model neither fibroblast activity nor epidermal cell-derived factors were sufficient to trigger the rapid dermal healing. However, Hsp47 was produced by fibroblasts in the wound area during culture (see above), indicating the synthesis of collagen in the wounded dermis.[18] In contrast, we were not able to show new production of CS in the fetal burn wound after culture. Although the dermis was severely damaged after burning, the monoclonal antibody against CS could still detect this protein in the fetal burn wound. Consequently, new deposition of CS could not be studied in the burn wound. Owing to lower amounts of CS in the scar tissue, no CS was detected in the wound area of the scar model directly after burning; therefore, new CS production could be shown in the scar tissue after culture. Similarly, we were not able to demonstrate new production of elastin in the adult wound models, because the monoclonal antibody against elastin could still detect this protein directly after burning.

Fetal skin is normally not exposed to air; therefore, we also cultured the fetal skin model under submerged conditions. The submerged cultured model contained less epidermal layers and was less viable than the air-exposed cultured model. In contrast to our model, Zeltinger et al. were able to maintain fetal skin in submerged culture.[19] Different culture conditions may account for this difference. In contrast to the air-exposed cultured model, the neo-epidermis grew underneath the old burned epidermis in the submerged cultured model. We cannot explain this phenomenon. The finding that the unwounded epidermis was shed during submerged culture suggests that the epidermis could easily detach from the dermis. Hence, the new epidermis could grow more easily underneath the damaged epidermis. Obviously, more research is necessary to investigate these findings in more detail.

In the present study, an in vitro fetal wound model is described that remained viable for a long period of time and is compared with similar models in adult skin and scar tissue. After 21 days of culture, the burn wound was completely closed in the fetal wound model and new fibroblasts had migrated into the wound. Compared with the adult and scar tissue model, reepithelialization was faster in the fetal skin model and more fibroblasts were present in the wound area at an early time point. These findings suggest that rapid healing might play an important role in scarless healing. The fetal skin model can be useful in studying different aspects of fetal wound healing. In addition, it can be manipulated to study the role of specific growth factors or other elements in scar formation.

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