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Bioengineering and in utero transplantation of fetal skin in the sheep model: A crucial step towards clinical application in human fetal spina bifida repair

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Abstract: An intricate problem during open human fetal surgery for spina bifida regards back skin closure, particularly in those cases where the skin defect is much too large for primary closure. We hypothesize that tissue engineering of fetal skin might provide an adequate autologous skin substitute for in utero application in such situations. Eight sheep fetuses of four time-mated ewes underwent fetoscopic skin biopsy at 65 days of gestation. Fibroblasts and keratinocytes isolated from the biopsy were used to create fetal dermo-epidermal skin substitutes. These were transplanted on the fetuses by open fetal surgery at 90 days of gestation on skin defects (excisional wounds) created during the same procedure. Pregnancy was allowed to continue until euthanasia at 120 days of gestation. The graft area was analyzed macroscopically and microscopically. The transplanted fetal dermo-epidermal skin substitutes was well discernable in situ in three of the four fetuses available for analysis. Histology confirmed healed grafts with a close to natural histological skin architecture four weeks after in utero transplantation. This experimental study generates evidence that laboratory grown autologous fetal skin analogues can successfully be transplanted in utero. These results have clinical implications as an analogous procedure might be applied in human fetuses undergoing prenatal repair to facilitate primary skin closure. Finally, this study may also fertilize the field of fetal tissue engineering in general, particularly when more interventional, minimally invasive, and open fetal surgical procedures become available.

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Bioengineering and *in utero* transplantation of fetal skin in the sheep model: A crucial step towards clinical application in human fetal spina bifida repair

Short running title: *In utero* transplantation of bioengineered fetal skin

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Keywords: fetal surgery, in utero, transplantation, tissue engineered skin, spina bifida, fetal skin

Abstract

Background: An intricate problem during open human fetal surgery for spina bifida regards back skin closure, particularly in those cases where the skin defect is much too large for primary closure. We hypothesize that tissue engineering of fetal skin might provide an adequate autologous skin substitute for *in utero* application in such situations.

Material and Methods: Eight sheep fetuses of four time-mated ewes underwent fetoscopic skin biopsy at 65 days of gestation. Fibroblasts and keratinocytes isolated from the biopsy were used to create fetal dermo-epidermal skin substitutes (fDESS). These were transplanted on the fetuses by open fetal surgery at 90 days of gestation on skin defects (excisional

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wounds) created during the same procedure. Pregnancy was allowed to continue until euthanasia at 120 days of gestation. The graft area was analyzed macroscopically and microscopically.

Results: The transplanted fDESS was well discernable in situ in three of the four fetuses available for analysis. Histology confirmed healed grafts with a close to natural histological skin architecture four weeks after *in utero* transplantation.

Conclusion: This experimental study generates evidence that laboratory grown autologous fetal skin analogues can successfully be transplanted *in utero*. These results have clinical implications as an analogous procedure might be applied in human fetuses undergoing prenatal repair to facilitate primary skin closure. Finally, this study may also fertilize the field of *fetal* tissue engineering in general, particularly when more interventional, minimally invasive, and open fetal surgical procedures become available.

Introduction

Open spina bifida (myelomeningocele, myeloschisis) is a devastating congenital malformation with a worldwide incidence of 1:1000 (Copp et al., 2015). It is characterized by a complex tissue defect on the back through which a non-neurulated spinal cord protrudes and lays exposed. The leakage of cerebrospinal fluid through the defect (Bouchard et al., 2003; Paek et al., 2000) and the progressive damage of the exposed and thus unprotected spinal cord during gestation (Meuli et al., 1995) lead to a complex pattern of CNS anomalies along the cerebro-spinal axis resulting in a cluster of severe and lifelong handicaps (Copp et al., 2015; Meuli et al., 1995; Meuli & Moehrlen, 2013, 2014).

Open prenatal spina bifida repair (SBR) was shown to significantly reduce some of these impairments (Adzick et al., 2011) and has therefore become a valid treatment option for selected patients. However, despite its beneficial effects, open prenatal SBR as it is performed today is less than perfect. Not only is it associated with maternal and fetal risks (Adzick et al., 2011; Goodnight et al., 2019; Johnson et al., 2016), but also, results are not consistent. Therefore, future improvements, especially regarding operation techniques and timing, are warranted.

Doubtlessly, approaches based on tissue engineering harbour great potential in this respect. For instance, an autologous, tissue engineered skin substitute may be used to easily and readily close large defects where primary skin closure is not possible (in 20-30%), superseding the need of time-consuming and complicated skin mobilisation and flap construction (Mangels, Tulipan, Bruner, & Nickolaus, 2000; Meuli et al., 2017), or the implantation of acellular skin substitutes(Lapa Pedreira et al., 2018; Meuli et al., 2013; Papanna, Fletcher, Moise, Mann, & Tseng, 2016). It is also conceivable that such a tissue engineered skin "patch" could be used to cover a spina bifida earlier in gestation with minimal-invasive techniques, allowing spinal cord protection earlier in gestation and hence achieve better results with less invasiveness. As in burn patients, enrichment of the tissue engineered skin substitutes with stem cells might give it an extra edge (Chua et al., 2016).

The aim of this study was therefore to test whether laboratory grown autologous fetal skin can successfully be transplanted *in utero*. In prior sheep experiments, we already demonstrated that dermo-epidermal fetal skin substitutes (fDESS) can be engineered from a fetal skin biopsy (Mazzone et al., 2014). Also, we have shown that these substitutes can successfully be grafted in an *in vivo, ex utero* animal model (Mazzone, Pratsinis, Pontiggia, Reichmann, & Meuli, 2016). Now, the logical next step is to explore *in utero* fDESS transplantation in preparation for eventual clinical application of this treatment modality.

This study reports on our experience of transplanting laboratory engineered fetal skin *in utero* in the fetal sheep model.

Materials & Methods

Animal experiments

The animal experiments were approved by the local Committee for Experimental Animal Research (Nr 220/2012). Swiss alpine sheep were time mated after a one-week course of hormonal stimulation. At 65 days of gestation, a fetal skin biopsy was obtained as follows:

Anesthesia of the ewe was induced with Propofol (2-5mg/kg) and maintained with 1-3% Isoflurane. To ensure sufficient analgesia during surgery, a constant rate infusion of Remifentanyl (0.5µg/kg/min) was administered. Amoxicillin/Clavulanic acid was given as antibiotic prophylaxis. A small midline laparotomy was performed to expose the uterine horns. A 5mm endoscopic port and one or two 3mm working ports were introduced into the uterus and secured with purse string sutures. Carbon dioxide insufflation occurred with a pressure of 4mmHg. A fetal skin biopsy of approximately 1x1cm was harvested with endoscopic scissors. Amniotic fluid was replaced with pre-warmed normal saline 0.9% supplemented with 500mg cefazolin, and ports were removed. The purse string closure was secured if necessary with additional stiches. The laparotomy was closed in layers.

At approximately 90 days of gestation, transplantation of the autologous tissue engineered fetal skin substitute was performed. After anesthetizing the animal as described above, the uterus was re-exposed through an incision in the abdominal scar. The uterine horn containing the fetus was opened with electrocautery and the fetus was partially exposed through the hysterotomy. Two round shaped skin defects (2cm in diameter) were created. A brimmed silicon ring was inserted in each wound, with the brim underlying the skin edges and sutured in place. Inside one of the two rings, the autologous tissue engineered skin substitute was fixed to the wound ground with sutures or absorbable surgical staples and fibrin sealant (Tisseel, Baxter). The other excisional wound was left untreated (Figure 1). After replacing the amniotic fluid with pre-warmed normal saline 0.9% supplemented with 500mg cefazolin, the hysterotomy was closed in two layers. The laparotomy was closed in layers.

Buprenorphine was used for post-operative analgesia after both interventions. Ultrasound exams were performed before each procedure, a few hours after the procedure, and on the first postoperative day respectively. The first ultrasound examination was used to determine the number of living fetuses, the following ones to assess whether the fetuses were still alive and to document the amount of amniotic fluid. The ewes were observed and examined daily by the veterinarian team for clinical wellbeing and for signs of abortion. Finally, at 120 days of gestation, the animals were euthanized. The transplanted area was photographed and then excised for further analysis.

Cell isolation and expansion

As described before (Mazzone et al., 2014), biopsies harvested at 65 days of gestation were stored at 4°C up to 24 hours in (DMEM Gibco, Basel Switzerland) containing gentamycin 15 mg/ml (Gibco), Penicillin/Streptomycin 300 U/ml respectively 300 µg/ml (Sigma, Buchs, Switzerland), and Fungizone 250 ng/ml (Gibco). Cells were isolated by incubating the biopsies at 37° in 25 U/ml dispase (BD Biosciences, Allschwil, Switzerland) in Ham's F12 (Gibco) containing 5ug/ml gentamycin for 1-2 hours. Thereafter, the epidermis was separated mechanically from the dermis under a stereo microscope (Nikon SMZ1500, Nikon AG, Egg, Switzerland). The separated epidermis was further digested in pre-warmed 0.5% Trypsin/EDTA (Gibco) for 2 min at 37° and the so isolated keratinocytes (KC) were seeded and expanded in keratinocyte medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) supplemented with 5 µg/ml gentamycin in collagen type I-coated (BD biosciences, Basel, Switzerland) cell culture flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland). Fibroblasts (FB) were isolated from the dermis by incubating the dermis in 2mg/ml collagenase blend (Sigma) at 37°, 5% CO2 for about 45 min. FB were seeded and expanded in cell culture flasks with DMEM medium supplemented with 10% fetal calf serum (FCS), 4 mML-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 µg/ml gentamycin.

Cultures of fetal dermo-epidermal skin substitutes (fDESS)

Collagen-based hydrogels containing isolated ovine fetal FB at a concentration of $1.5x10^4$/ml were prepared in a transwell system (4.2cm2 six-well cell culture inserts with membranes of 3.0 µm pore size from BD Falcon, Basel, Switzerland). The mixture of acidic bovine collagen type I, buffer containing NaOH and ovine fetal fibroblasts was poured in the inserts and, after solidification, compressed plastically as described previously (Braziulis et al., 2012). After a cultivation time of 7 days in DMEM with 10% FCS, ovine fetal KC were seeded onto the surface of the hydrogel within siliconized polypropylene rings of 15mm

diameter at a density of approximately $2x10^5$-$4x10^5$ cells. The ring was removed 4 hours later. After seeding, culture was continued with modified Rheinwald and Green medium (RGM; three parts of DMEM and one part of Ham's F12, 5 mg/ml gentamycin, 1.4mM CaCl2, 0.4 mg/ml hydrocortisone, 5 mg/ml1 insulin, 2 nM triiodothyronine, 180 mM adenine, 10 ng ml/1 EGF (all from Invitrogen, Basel, Switzerland), 0.1nM choleratoxin (Calbiochem/VWR International AG, Dietikon, Switzerland) and 10% FCS (from Gibco)).

Medium changes were performed every 2-3 days. Approximately ten days after KC seeding, fDESS (Figure 2) were transplanted *in utero*.

Histological analyses

The resected areas were photographed, measured and embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen to -20° or fixed in PFA 4% and thereafter embedded in paraffin. Both were sectioned at 10µm using a microtome. Sections were stained with haematoxylin and eosin (H&E, Sigma) or used for immunofluorescence according to the protocol described by Pontiggia et al. (Pontiggia et al., 2009). Components of epithelial cells were visualized with following antibodies: K1 (ab93652, 1:100, Abcam), K10 (ab9026, 1:100, Dako), and E-cadherin (612130, 1:50, BD Biosciences). The basement membrane was visualized with antibodies against Laminin 1&2 (ab7463, 1:500, Abcam). Pictures of immunofluorescence staining were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope equipped with Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon NIS Elements 3.22.11). Images were processed with Adobe Photoshop Elements (Adobe Systems Inc, Germany).

Results

Four ewes were used for the experiments. Each of them had twins, therefore eight fetuses were available for skin grafting. The mechanical properties of the fDESS were

adequate for surgical handling: fDESS could easily be transferred from the six-well cell culture inserts to the fetus and sutured or stapled in place (Figure 1). All surgeries were accomplished without intraoperative complications. In the following course, two ewes had to be euthanized before reaching the end point of the experiment. In one ewe, fetal demise of one fetus was diagnosed on ultrasound one day after biopsy harvesting, while the other fetus was found dead two days after skin grafting. In the second ewe, signs of abortion were noticed four days after transplantation, ultrasound showed that both fetuses were dead and autopsy revealed a chorioamnionitis with ESBL (Extended Spectrum Beta-Lactamase) E. coli. The remaining two ewes were euthanized as planned at 120 days of gestation.

In the four fetuses available for analysis, all eight silicon rings were still in situ, but the brim had been expelled partially from under the skin in six cases and completely in one case. This was likely caused by marked wound contraction evident in all but two cases. The transplanted fDESS was well discernable in situ in three of the four fetuses. In one case, only a remnant of the former graft was present. The size of the fDESS had shrunk to about 20-25% of its original size. In one case, the fDESS was covered by an undefinable sheet-like structure that could be peeled off. The graft surfaces had a whitish, matt, and irregular appearance that did not recall a typical skin surface. In two cases, the silicon ring, although partially expelled, had clearly hindered the surrounding skin to get in contact with the fDESS. In the third case, fDESS was in contact with the surrounding skin. All control sites exhibited areas with open wounds. These macroscopic results are shown in Figure 3.

Histology sections stained with H&E revealed in all three remaining fDESS a two layer architecture consisting of a dermis and an epidermis. The epidermis was cell rich and consisted of up to 10-12 layers (Figure 4B).

Immunostaining confirmed the presence of a differentiated multilayer epidermis. Intracellular adhesion and thus stabilization of the epidermis was visualized by positive e-cadherin staining (Figure 6A). K1/K10 was found in a physiological, i.e. suprabasal location (Figure 6B/C), hence demonstrating some degree of differentiation (besides being a differentiation marker, K1/K10 is also known to provide epidermal stability). Positive staining

for laminin 1&2, a crucial component of the basement membrane, indicated robust dermo-epidermal anchoring. Further, laminin 1&2 staining also visualized the basement membrane of blood vessels, showing abundant graft neovascularization (Figure 6B/6C). In two cases (Figure 3A1/3A2), H&E and immunostaining confirmed that the silicon ring hindered epidermal ingrowth from the surrounding skin: fDESS were surrounded by a zone lacking KC (Figure 4C/6D) proving that the graft epidermis originated from the laboratory.

In the third case (not shown), although fDESS and autochthonous fetal skin seemed not to be in direct contact, histology revealed a discrete epidermal bridge between the two (not shown). The wound margins of most of the transplantation sites exhibited a minute outgrow of a multilayered epidermis on the wound surface (Figure 4C). Although all control sites showed an open wound surface, the epidermal outgrow from the wound edges varied in size (Figure 5C).

Discussion

The results described here provide evidence that an autologous, tissue engineered fetal skin substitute can be successfully transplanted *in utero*. To our knowledge, this has not been described so far. It is a critical finding as it represents an important step towards clinical application of tissue engineered fetal skin substitutes in fetal surgery for spina bifida. A number of issues call for a comment.

Favorably, our results show that in the fetal sheep model, grafted fetal skin substitutes heal and continue to feature a close to natural histological architecture up to 4 weeks after *in utero* transplantation although, theoretically, there are several reasons that may impede successful *in utero* skin grafting. Obviously, grafting in an intrauterine environment differs greatly from a standard, i.e. *ex utero,* grafting situation. Technical and biological points have to be considered. For instance, routine split or full thickness skin grafting requires a tie-over dressing or VAC-application (Schiestl et al., 2011; Stiefel, Schiestl, & Meuli, 2010) exerting pressure on the whole transplant over the first postoperative days so that a tight contact between graft and wound bed is warranted. This pressure is thought crucial for reliable graft

take. Yet, an effective tie-over is hardly possible in the fetal setting because of the frailty of fetal skin. Also, VAC-application appears technically impossible, as is application of a protective dressing. Further, amniotic fluid could penetrate between graft and wound bed and so hinder sufficient tissue contact. Finally, even with good contact, the low oxygen tension typically found in fetal tissues (Meschia, Cotter, Breathnach, & Barron, 1965) could negatively affect graft taking, as tissue oxygenation is crucial, at least during postnatal life, for adequate healing.

Interestingly, and in contrast to the above considerations, our own experience with the application of acellular dermal substitutes during human fetal SBR (Meuli et al., 2013) has taught us that skin substitutes do firmly attach and are integrated into the wound bed, however they are not epithelialized. This is confirmed by the work of other groups. Papanna et al. reported two cases of fetal myeloschisis repaired with cryopreserved human umbilical cord (Papanna et al., 2016). As noted by the authors, a surprising finding was the lack of epithelialization at birth, but then a rapid ingrowth of vascularization, epithelialization, and keratinization in the following 3-4 weeks. The presence of amniotic fluid as possible inhibitor of the healing process was discussed. Kohl et al. described their experience with the use of nonabsorbent polytetrafluoroethylene and collagen patches for fetoscopic spina bifida coverage: skin closure at birth was not noted (Kohl, 2014; Kohl et al., 2006). A bilaminar skin substitute over a cellulose patch was used by Lapa Pedreira et al. in large size defects during fetoscopic repair (Lapa Pedreira et al., 2018). In 5 of 13 patients, postnatal reoperations were needed (reasons were dehiscence at the repair site, cerebrospinal fluid leakage, wound infection), in the remaining 8 patients, the silicon layer of the bilaminar skin substitute detached itself spontaneously on average 25 $\pm$ 17.1 days after birth, thereafter, complete healing by secondary intention occurred. The lack of epithelization is an interesting observation, as it seems to be in contrast to the well described phenomenon of fetal scarless skin wound healing: early to midgestational fetal wounds heal rapidly with complete regeneration of dermis/ epidermis and without scar (Adzick & Longaker, 1992; Lo, Zimmermann, Nauta, Longaker, & Lorenz, 2012). However, the early period of scar

formation in human occurs around 24-weeks of gestation (Lorenz et al., 1992), i.e. when typically fetal spina bifida repair is performed.

The situation encountered during human fetal SBR differs from the one in these experiments. The acellular dermal substitutes used in the clinical setting are sutured to the skin edges of the surrounding skin, thus allowing FB and vascular ingrowth not only from the wound ground but also from the sides. This was demonstrated by the works of Lapa Pedreira. In the sheep model (Pedreira, Quintero, Acacio, Caldini, & Saldiva, 2011), a newly formed epidermis, starting from the wound edges, was observed to grow beneath the silicon layer of a bilaminar skin product. Further, in their clinical experience (Lapa Pedreira et al., 2018), a biocellulose patch was placed beneath the bilaminar skin substitute, therefore cells migrated mainly from the periphery to the center of the substitute. In our experiments, we chose to use a silicon ring to avoid contact between fDESS and surrounding skin. This was chosen to prove that the epidermis found on the transplanted fDESS was laboratory-grown skin. The use of the ring, however, brought along another problem: wound contraction (as noticeable by the expulsion of the silicon rings in Figure 3). It is known that sheep fetuses do close even larger skin defects, mainly by means of wound contraction (Brown, Keller, Pivetti, & Farmer, 2015; Kohl et al., 2003). The ring-associated, open wound around the graft may have favored wound contraction and fDESS shrinkage. Yet, when considering fDESS application clinically, grafts would benefit from being firmly fixed to the surrounding skin and therefore, the ring-associated problems, particularly shrinking, would not likely be present.

Although the macroscopical appearance is poor because of marked shrinkage, microscopy revealed a fDESS composed of a vascularized dermis and a well-stratified epidermis with an adult-like mature aspect. In contrast, the adjacent hair follicle bearing fetal epidermis shows only 2-3 layers of KC. Epidermal markers confirmed the multi-layered graft epidermis to be mature, despite the fetal origin of the cells and the *in utero* environment after transplantation. Of note, these “underwater” results are similar to “open air” results of fetal grafts transplanted on the back of rats (Mazzone et al., 2014; Mazzone et al., 2016) Possibly, the laboratory culturing procedure steers the evolving epidermis “irreversibly” towards an adult phenotype.

Regarding the clinical use of fDESS, two possible applications are envisioned. Of course, the most evident is grafting of large skin defects during open fetal SBR where primary skin suture is not possible (about 20% in our clinical experience of over 100 cases). Currently, in such situations, a skin substitute like e.g. Integra™ is implanted into the skin defect, especially if large. Here, postnatal healing by secondary intention over weeks or a ultrathin skin graft (Meuli et al., 2013) are needed to complete skin reconstruction. For smaller defects, performing pedicled transposition flaps is a suitable approach, even though it means an additional procedure that prolongs fetal operation time, and that, postnatally, might mandate cumbersome flap donor site management (Meuli et al., 2017). Taken together, we assume that autologous grafting with a close to natural fetal skin analog might be a more elegant and effective solution.

Finally, we hypothesize that fDESS could be used when operating endoscopically. Adequate, i.e. classical multilayer neurosurgical fetoscopic defect closure remains a big challenge, even in smaller lesions. Therefore, different acellular patches have been used for defect coverage, but have shown a high failure rate with patch detachment, insufficient spinal cord protection, persistent cerebrospinal fluid leakage, and the negative consequences associated with these problems (Joyeux et al., 2016). A robust, easy to handle autologous skin substitute like the fDESS presented here might prove to be superior.

However, the risk of infection represents a possible and serious limitation to the use of an autologous fDESS. In our experiments, at least one pregnancy was lost due to infection after transplantation. Even though infection rates in an experimental setting with an animal model might be higher than in clinical practice due to the different hygiene conditions, the risk of infection remains a threat for all tissue engineered products with living cells. In the clinical scenario, production of fDESS in a GMP (good manufacturing process) laboratory should allow to overcome the problem.

Conclusion

These experiments generate evidence that bioengineered autologous fetal skin analogues can successfully be transplanted *in utero* and that these skin substitutes show a near normal histology one month post grafting. Our results have a direct clinical impact as similar procedures may be used in human fetuses undergoing prenatal repair to realize primary skin closure. Finally, this study may also fertilize the field of fetal tissue engineering in general, particularly when more interventional, minimally invasive, and open fetal surgical procedures become available. However, the risk of infection caused by *in utero* application of bioengineered products represents a possible limitation.

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Conflict of interest statement

The author declare no conflict of interest

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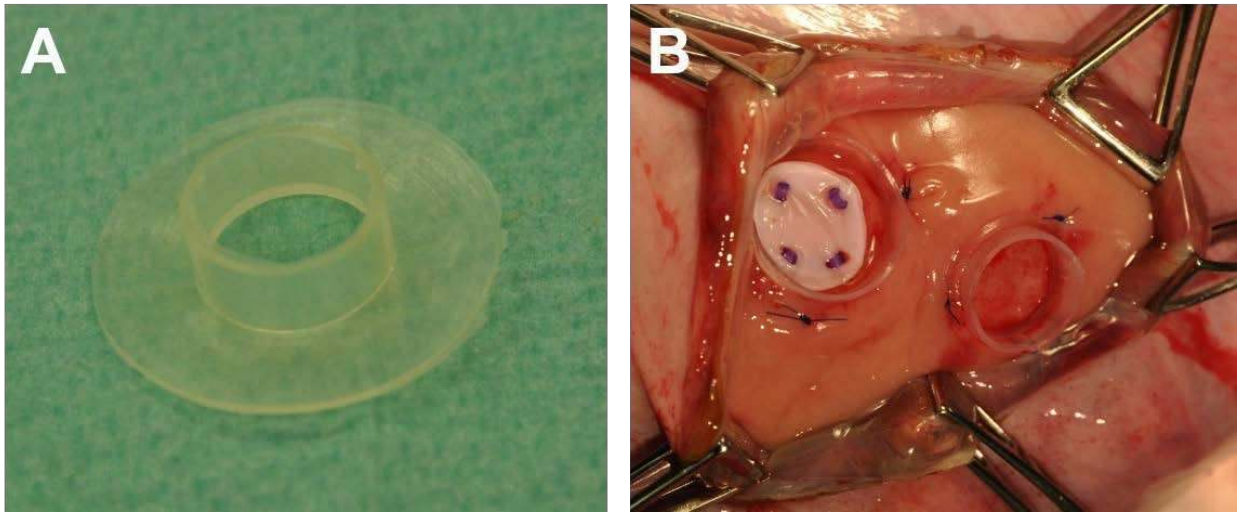

Figure 1. A: brimmed silicon ring. B: on the left: fetal dermo-epidermal skin substitute (fDESS) transplanted in a skin wound kept open by the inserted silicon ring. The silicon ring is sutured in place with the brim laying subcutaneously. The fDESS is hold in place with four absorbable surgical staples. On the right ringed control wound without fDESS

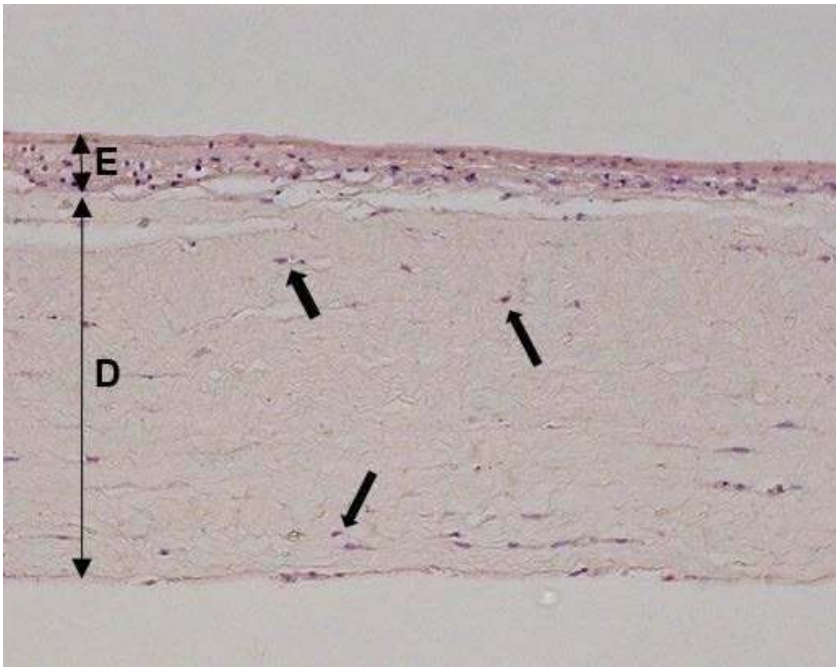

Figure 2: Collagen hydrogel containing fetal fibroblasts (arrows) approximately 10 days after fetal keratinocytes seeding. Four to five layers of keratinocytes can be noted. E: neoepidermis. D: neodermis

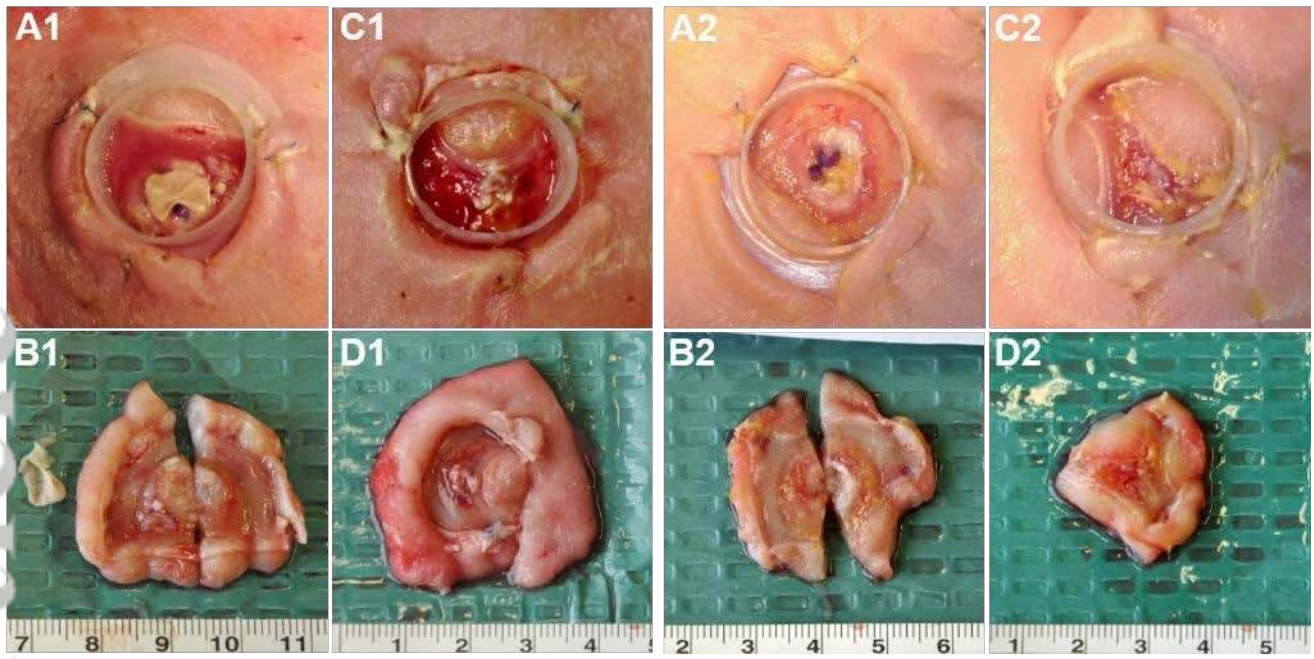

Figure 3: Macroscopic analysis of two of the 4 surviving fetuses. A1/A2: transplanted fDESS on the fetus. B1/B2: excised specimen of the fDESS without ring, with cut for histological analysis. C1/C2: Control wound on the fetus D1/D2: excised specimen of the control wound. Note the expulsion of the silicon ring, the wound contraction and the concomitant shrinkage of the fDESS. Both controls with open wound surface

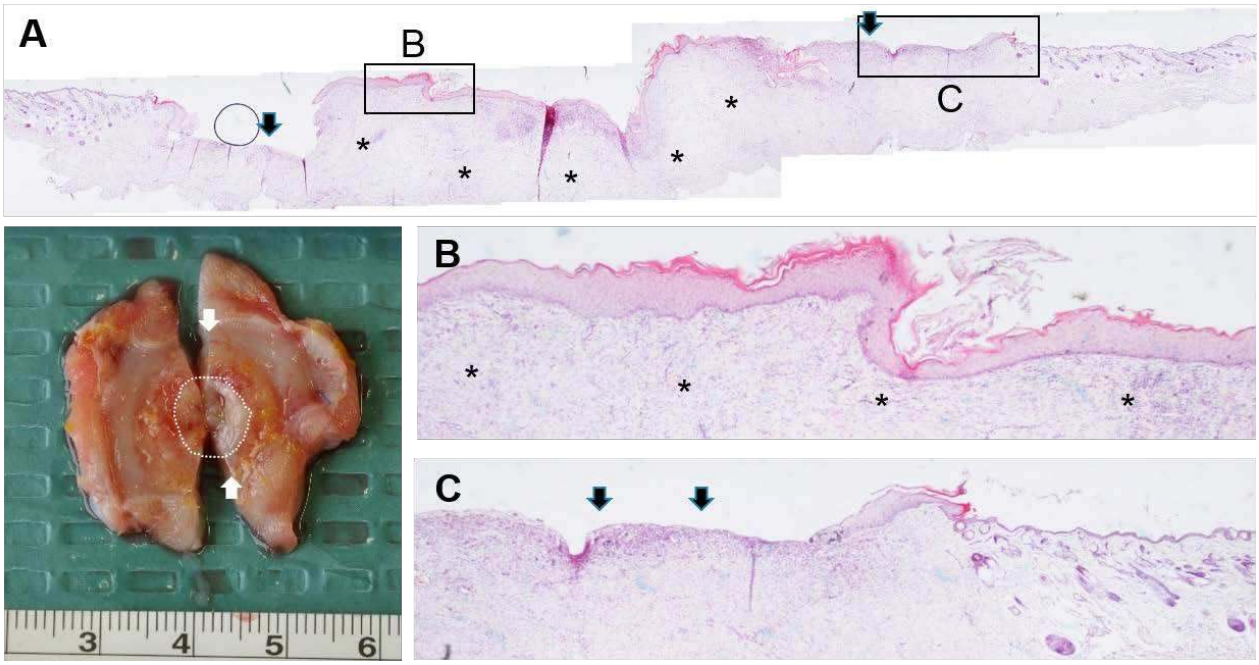

Figure 4: Histological analysis of the excised graft. The macroscopic picture shows the cut location for the histology slides, the graft is marked with a dotted line for better visualization A: H&E staining of the specimen. On the lateral borders, the hair follicle bearing fetal skin is visible. The graft (*) lays centrally and is surrounded by an epidermis-free area (arrows).B: Graft covered by a multilayered epidermis C: Epidermis free area around the graft. Note the multilayered epidermal «tongue» at the wound margin

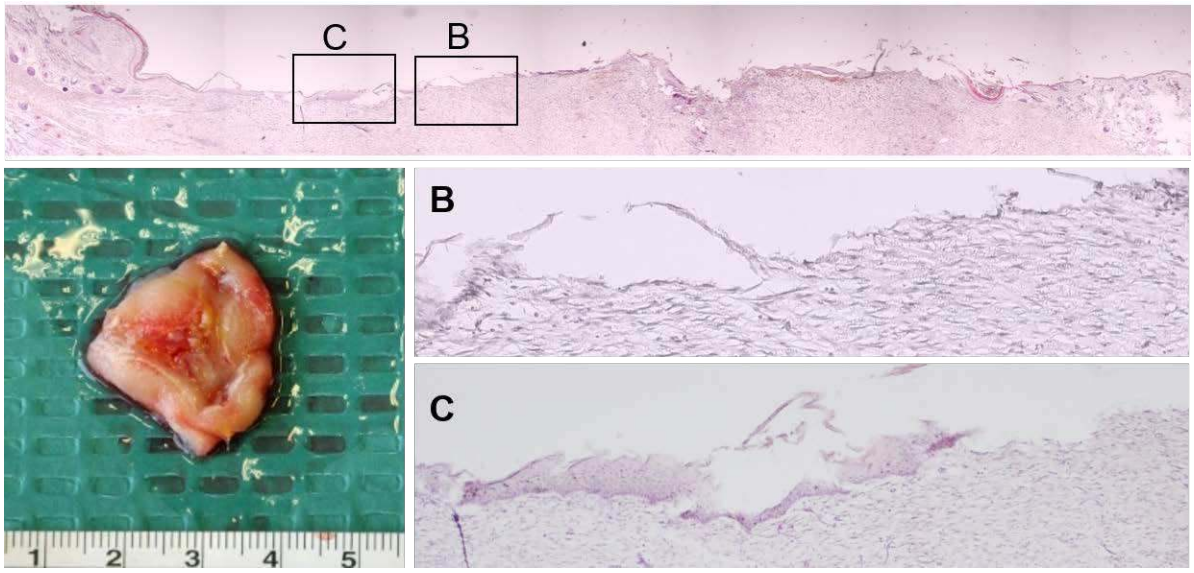

Figure 5. Control specimen. B: The wound surface is not covered by an intact epidermis. However, note the epidermal outgrow from the wound margins (C)

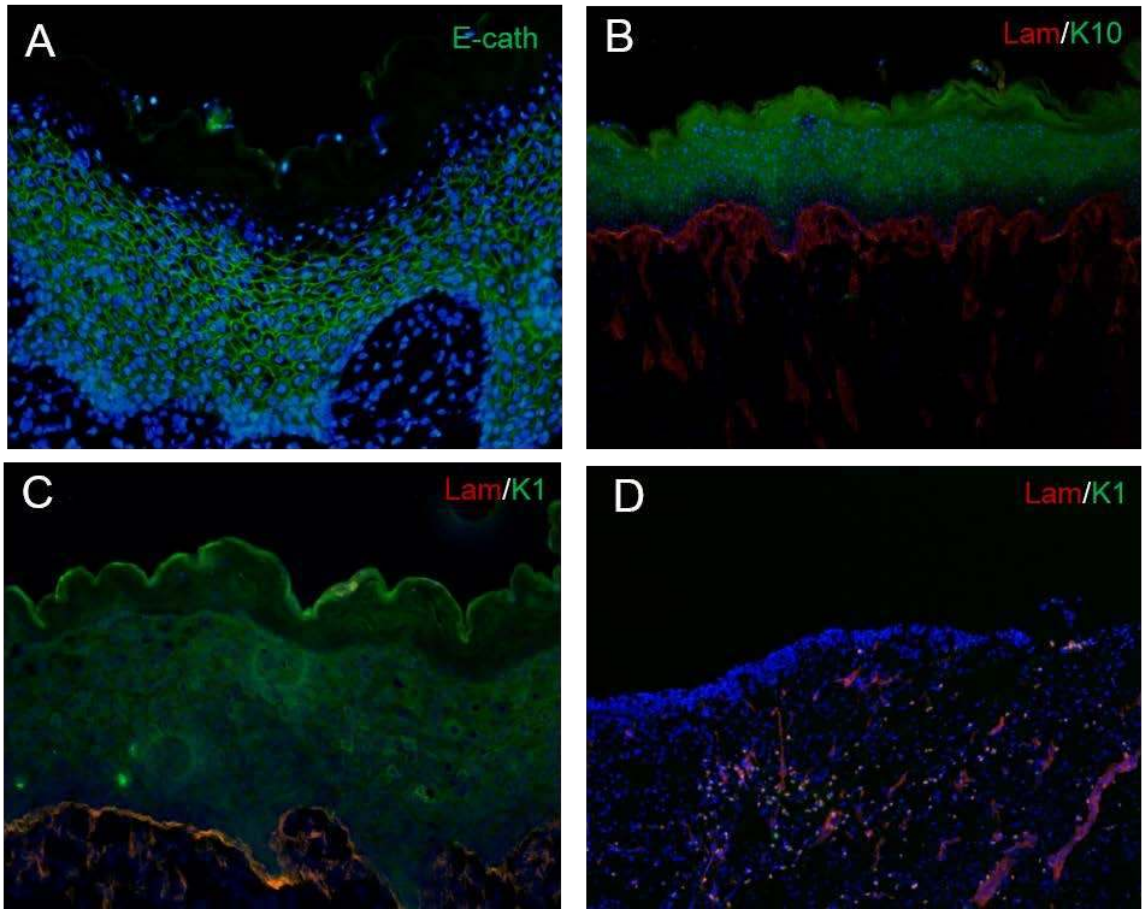

Figure 6. Immunohistochemistry of the graft (A-C) and of the ring area around the graft (D). A: positive staining for e-cadherin (FITC). B/C: correct suprabasal staining of the epidermis with K1 and K10 (FITC), positive laminin staining (TRITC) of the basement membrane inclusive blood vessels. D: negative K1 staining and laminin staining at the epidermal-dermal junction, positive laminin staining in blood vessels