Minced Skin for Tissue Engineering of Epithelialized Subcutaneous Tunnels

Magdalena Fossum, M.D., Ph.D.,¹ Baraa Zuhaili, M.D.,² Tobias Hirsch, M.D.,³ Malte Spielmann, M.D.,³ Richard G. Reish, M.D.,² Priyesh Mehta, B.S.,² and Elof Eriksson, M.D., Ph.D.²

We used minced, autologous skin for neoepithelialization of surgically created subcutaneous tunnels in a large animal model. Partial-thickness skin grafts were harvested from the back region of five 50–60 kg Yorkshire pigs. The skin was minced to $0.8 \times 0.8 \times 0.3$ mm particles. Silicone-latex tubes were covered with fibrin, rolled in minced skin, and placed in subcutaneous tunnels created in the abdominal area. For comparison, single cell suspensions of keratinocytes and fibroblasts in fibrin or fibrin only were transplanted on tubes. Tunnels were extracted after 14, 21, and 28 days for microscopic evaluation. All tubes transplanted with minced skin particles showed neoepithelialization. The epithelium was stratified and differentiated after 2 weeks *in vivo*, and the stratum corneum was directed toward the implanted tube. No epithelium formed from tubes transplanted with single cell suspensions, and only sparse keratinocytes could be detected by serial sectioning and immunostaining on day 14, but not later. No epithelial lining was found in tunnels with fibrin-only-coated tubes. Epithelial cysts could be found the first 2 weeks after transplantation in the minced skin group but not later. In conclusion, a minced skin technique could serve as a potential source for tissue engineering of tubular conduits for reconstructive purposes of the urethra and for cutaneous stomas for bladder catheterization, or intestinal irrigations. The method would have the advantage of being simple and expeditious and not requiring *in vitro* culturing.

Introduction

FULL-THICKNESS AND PARTIAL-THICKNESS skin grafting is routinely used for coverage of skin defects and is, in selected cases, used in the urogenital area such as in vaginal reconstructions or severe hypospadias.^{1,2}

Tissue for bladder augmentation or for a vesico-cutaneous stoma for bladder emptying requires properties such as high compliance, good blood supply, and availability. The small intestine is therefore considered a good tissue source for reconstruction in the urogenital system. Drawbacks include the need for laparotomy, risk of mucus and stone formations, and infections that can lead to repeated surgical interventions and significant morbidity.^{3,4} Recent long-term follow-up studies have reported an increased rate of malignancy in bladders augmented with gastrointestinal mucosa.^{5,6}

Autologous *in vitro* cultured keratinocytes can be used for regeneration of the skin if donor skin is lacking.^{7,8} Cultured urothelial cells have been used for augmentation of the urinary bladder and for reconstruction of the penile urethra.^{9,10} However, cell culturing *in vitro* is time consuming, laborious,

and expensive. In addition, it requires continuous quality controls and highly skilled personnel adhering to good laboratory practice.

The aim of this study was to create epithelialized tunnels that eventually could be used as conduits in the urogenital system either as a neourethra or as a stoma for catheterization. We wanted to investigate if minced skin particles can migrate, proliferate, and differentiate around a foreign body similar to the healing of a full-thickness wound transplanted with minced skin in a wet environment in a large animal model.^{11,12}

We have transplanted different tubes covered with minced skin as well as tubes covered with cells in single cell suspension and for comparison tubes without cells that were all placed in subcutaneous tunnels. Fibrin was applied to the tube to make the minced skin or single cells adhere.^{13–15}

Materials and Methods

The Harvard Medical Area Standing Committee on animals approved the study protocol. All procedures conformed with the regulations related to animal use and other

¹Division of Pediatric Urology, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden.

²Laboratory of Wound Healing and Gene Transfer, Division of Plastic Surgery, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts.

³Klinik für Plastische Chirurgie und Schwerbrandverletzte, BG Universitätskliniken Bergmannsheil, Ruhr Universität Bochum, Bochum, Germany.

FIG. 1. (A) Mincing device. (B) Detail of rotating cutting disks.

federal statutes. Female Yorkshire pigs (Parson's Farm, Hadley, MA), weighing 50–60 kg, were used in the studies.

Surgical procedures

After 12-h fasting, an intramuscular injection of 4–6 mg/kg tiletamine and zolazepam (Telazol; Fort Dodge Veterinaria, Vall de Bianya, Spain) and 2.2 mg/kg xylazine (Xyla-Ject; Phoenix, St. Josephs, MO) was administered for induction of anesthesia. General anesthesia was maintained with 2% isoflurane (Novaplus, Hospira, IL) via snout mask or endotracheal tube. Buprenorphine (0.001 mg/kg; Bedford Laboratories, Bedford, OH) followed by a transdermal patch releasing 12 µg fentanyl per hour for 72 h (Duralgesic, Janssen, Titusville, NJ) was used for postoperative analgesia. For infection prophylaxis, an intraoperative dose of cefazolin (1 g) was given intravenously and then daily 3 days postoperative by intramuscular injections.

After depilation, the skin was cleaned with soap and with successive applications of 10% povidone iodine scrub (Betadine; Purdue Products LP, Stamford, CT) and 70% isopropanol (Aaron Industries, Clinton, SC).

From the neck, an autologous 0.3–0.6 mm split thickness 10-cm² skin graft was harvested with a handheld dermatome. The grafted area was covered with moist protective dressing.

The pig was turned to a supine position for surgical exposure of the abdomen. Subcutaneous tunnels were created by blunt and sharp dissection between the subcutaneous fat and the panniculus carnosus, between two parallel 2-cm-long skin incisions. All tunnels were 3 cm long and located above the umbilicus and lateral to the mamillary glands.

Mincing of skin and preparation of tubes

The skin grafts were washed twice in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) and cut into 10×7.5 mm (75 mm²) squared pieces with a scalpel. The pieces were kept moist in DMEM until mincing and transplantation. Each square was minced sep-

arately immediately before transplantation. The mincing device had two handles, angled superiorly with a central array of 30 rotating cutting disks (Fig. 1). By placing the skin graft on a cutting board and running the device twice over the skin graft in perpendicular directions, approximately 115 small squared particles measuring 0.8×0.8×0.3 mm were obtained per 75 mm² skin graft (Fig. 2A).

Twenty-two Fr Silicon-latex Foley catheters (Silastic Brand Foley catheter; C.R. Bard, Covington, GA) were cut into 2-cm-long pieces, and each end was closed with 3–0 running nylon sutures (Ethlion; Ethicon, Sommerville, NJ) to isolate the space inside the tube from the outside and to minimize the risk of bacterial colonization of the construct. The middle 10 mm of the construct (approximately 220 mm²) was marked with a marking pen (Fisherbrand; Fisher Science Education, Hanover Park, IL). The 10 mm central (unmarked) part was covered with minced skin particles, single cells, or fibrin only (Fig. 2B).

For the transplantation of minced skin, a thin layer from a two-component tissue sealant (Tisseel VH; Baxter, Westlake Village, CA) was applied to the tube twice. The particles were evenly distributed over the central 10 mm of the tube construct for a 1:3 expansion rate.

Single cell suspension

For preparation of the single cell suspension, 75 mm² skin pieces were minced as previously described, and the particles were washed in PBS with penicillin and streptomycin (Invitrogen, Auckland, New Zealand) twice. After removal of PBS, the particles were suspended in 1:1 EDTA-Trypsin solution (Invitrogen) and stirred with a rotating magnet in 37°C for 20 min. The supernatant was then removed and centrifuged in 360 g for 5 min. This procedure was repeated three times. Pellet was resuspended in 0.5 mL DMEM and stored in 5°C until all pellets were resuspended and pooled in 0.5 mL equal amounts of DMEM and sealer protein concentrate (Tisseel VH; Baxter). For each tube, approximately 3 million cells (Phase hemacytometer, Neubauer; Hausser

FIG. 2. (A) Minced particles, 0.8×0.8×0.3 mm, on a cutting board. (B) Tube with minced skin particles, ready for transplantation.

Scientifics, Horsham, PA) from 115 minced skin particles were obtained by these means.

The central 10 mm of the silicon-latex tube was covered with a thin layer of thrombin solution (Tisseel VH; Baxter) to solidify the cell suspension enough for transplantation; the single cells in fibrinogen were aspirated to a syringe and then applied evenly over the tube through an injection needle.

Samples from the minced skin group and the single cells in solution (in fibrinogen and fibrinogen-thrombin solution, respectively) from each pig were cultured separately *in vitro* to ascertain the viability and proliferative capacity of the transplants. For all cultures serum-free keratinocyte medium (Invitrogen) and collagen I-coated culture dishes were used under standard conditions.

Transplantation of tubes

All tubes were placed in the prepared subcutaneous tunnels, and each end of the tube was sutured to the fascia of the panniculus carnosus with 4-0 nylon sutures (Ethilon; Ethicon), two in each end. The subcutaneous tissue was closed with 4-0 absorbable sutures (Vicryl; Ethicon) in two layers, and finally the skin was closed with interrupted 3-0 nylon sutures (Ethilon; Ethicon).

Forty-five tubes were transplanted to five pigs (10 + 10 + 9 + 9 + 8). Tubes transplanted with minced skin for biopsy after 2 and 3 weeks were distributed on all five pigs (2 + 1 + 1 + 1 + 2) and (1 + 2 + 1 + 1 + 2), respectively. Tubes for biopsy after 4 weeks were distributed on four of the five pigs (1 + 1 + 1 + 1 + 0). Tubes transplanted with cells in single cell solution and controls were distributed on all five pigs (3 + 3 + 3 + 3 + 2), respectively. Every week, when a sample was taken a tube was removed for biopsy from cases and controls. The tubes were randomly distributed, and their position was altered between pigs.

Study on different carrier materials

In a parallel study, 22 Fr pure silicone (Tygon; Saint-Gobian, Beaverton, MI) and 22 Fr latex tubes (Bard Foley Catheter; C.R. Bard, Covington, GA) were used to clarify if the material of the implanted tube had any effect on the regenerative capacity of the minced skin transplants. The two substances are composites of the previously used silicon-latex tubes and were therefore chosen. A total of 12 tubes were transplanted to two pigs, every other tube was latex or silicon. The placement of the tubes was distributed in a way that each material was equally distributed under the abdominal skin on the two animals (6 tubes/pig). Preparation of tubes and transplantation of minced skin particles as well as surgical procedures were identical to what have been previously described; biopsies were performed 10 days after transplantation.

Histology

Biopsies were fixed in 10% formalin solution, embedded in paraffin, and cut into 5- μ m-thick sections. During this procedure, tubes fell off because they did not adhere to the tissue. After rehydration, slides were stained with hematoxylin-eosin for routine histology and with Masson's trichrome for evaluation of neogenerated extracellular matrix.

For immunoperoxidase analysis, endogenous peroxidase activity was first blocked with 3% hydrogen peroxide in methanol for 10 min. For epitope enhancement, tissue sections were digested with bacterial protease type XXIV (Sigma Chemical, St. Louis, MO). Tissue specimens were evaluated with immunostaining of cytokeratins, laminin, and p63. Primary antibodies against various cytokeratins (pancytokeratins 1-7, 10, 13-16 and 19; DakoCytomation, Glostrup, Denmark, Cat# M0821, clone: MNF 116), laminin (DakoCytomation, Cat# Z0097), and p63 (Neomarkers, p63Ab-1 4A4, Cat # MS/1081/P; Thermo Fisher Scientific, Fremont, CA) were used, respectively.

FIG. 3. Cross sections of biopsies from tunnels transplanted with minced skin particles after 2 weeks *in vivo*, staining with Masson's trichrome. (A) Intact cross section of a biopsy, including superficial skin (S) and deep side of tunnel (PC) (original magnification, $\times 2$). (B) Cross section from superficial side of tunnel, from top; lumen (L), where the tube was originally placed, lined by a continuous new epithelium (E), granulation tissue (G), subcutaneous fat (F), native dermis (D), and native abdominal skin (S) (original magnification, $\times 40$). (C) Cross section from deep side of tunnel with panniculus carnosus (PC) at bottom. Transplanted dermis (asterisk) in minced skin particle still detectable in granulation tissue (G). New stratified squamous epithelium (E) lining the luminal surface (original magnification, $\times 100$). (D) Detail from (C). Color images available online at www.liebertonline.com/ten.

Rabbit-anti-mouse antibodies were used as secondary antibodies (G/2 system/AP, rabbit/mouse, DAKO Envision, Carpinteria, CA). All sections were developed using 3,3'-diaminobenzidine (Sigma Chemical) as substrate and counter stained with Mayer's hematoxylin.

Statistics

Means, standard deviation, intervals of confidence of 0.95, and paired two-sided Student's *t*-test were used to compare planimetric data from tissue sections evaluated under microscopy. The mean epithelialized area of four serial sections was calculated for each biopsy specimen. *p*-Values lesser than 0.05 were considered statistically significant. All measurements were performed in $\times 100$ magnification using a

built-in ruler in a Nikon Labophot light microscope (DSC optical services, Newton, MA).

Results

Tubes implanted with minced skin particles formed squamous epithelium in the subcutaneous tunnels (Fig. 3). The newly formed epithelium showed differentiation. Keratin formed toward the lumen where the implanted tube had been located. Transplantation with single cell suspension or control tubes with fibrin only did not lead to epithelialization (Fig. 4). However, after 2 weeks, some tissue sections (2/7) from the transplants with single cell solution demonstrated isolated groups (2–5 cells) of keratin-containing cells. These could not be found in later biopsies.

FIG. 4. Cross sections and routine histology with hematoxylin–eosin from biopsies at 4 weeks. (A) Illustrating new epithelium in tunnels transplanted with minced skin particles. The epithelium is squamous and stratified with keratin formation toward the lumen (original magnification, $\times 100$). (B) Normal abdominal skin for comparison (original magnification, $\times 100$). (C) Single cell suspension transplants. No epithelium could be found (original magnification, $\times 200$). (D) Control with fibrin only. No epithelium could be found (original magnification, $\times 200$). Color images available online at www.liebertonline.com/ten.

Immunostaining of the biopsy specimens demonstrated cytokeratin in the cytoplasm of cells forming the neoepithelium lining the subcutaneous tunnels. The proliferative epithelial basal cells were localized by immunostaining of protein p63, and the basal membrane was visualized by immunostaining of laminin (Fig. 5).

At 2 weeks, the native transplanted subepithelial dermal tissue could easily be distinguished from the newly generated granulation tissue (Fig. 3C, D). The transplanted minced tissue particles, containing both native epithelium and native dermis, were mainly oriented with the epithelium facing the lumen of the surgically created subcutaneous tunnel, that is, toward the tube. However, at this time point also subepithelial cysts (defined as lumen with epithelium separated from the organized epithelium surrounding the tube) could be found.

Transplanted minced skin specimens were evaluated over a time course of 2–4 weeks, and the neoepithelium was stable in respect to the area of epithelialization (Table 1). A trend suggesting that the percentage of epithelialized area decreased with prolonged time *in vivo* could not be verified statistically. Subepithelial cysts, as previously described, could only be found during the first 14 days *in vivo*, neither could the dermis of minced skin particles be distinguished from the surrounding subepidermal tissue after 14 days. There was no statistical difference between the thickness of the superficial and deep granulation tissue, respectively, at any time after the transplantation, and no correlation could be found between the thickness of the granulation tissue and the area with new epithelium (Table 1).

In some of the tissue sections, mononucleated cells infiltrating the neoepidermis and parakeratosis were found, suggesting an inflammatory reaction. In the same slides, pseudo follicles with mononucleated cells and giant cells were found in the underlying granulation tissue. These findings were not consistent, but more often found in biopsies taken after 2 weeks. Mononucleated cells and inflammation were also seen in controls.

Cells in the *in vitro* cultures from the minced skin group with fibrinogen and from the single cell suspension in fibrinogen and thrombin were viable and proliferative. Cells reached confluence as a monolayer in the bottom of the culture well. Cell morphology was typical for keratinocytes and for fibroblasts (Fig. 6).

The subcutaneous tunnels transplanted with minced skin on latex tubes had a significantly larger area of epithelialization (204 mm^2 , range $180\text{--}240 \text{ mm}^2$) than tunnels transplanted with minced skin on silicone tubes (127 mm^2 , range $10\text{--}200 \text{ mm}^2$) after 10 days *in vivo* (Fig. 7). No significant difference was found between the latex and silicone groups in respect to thickness of the granulation tissue formed between the native skin dermis and the neoepithelium (superficial granulation tissue) of the subcutaneous tunnel and the native muscle and the neoepithelium (deep granulation tissue), nor was a difference in thickness between the superficial and deep granulation tissue within the groups. Epithelial cysts, with epithelium not in continuation with the neoepithelium, were equally frequent in both groups.

Discussion

The surgical repair in reconstructive pediatric urology can be limited by shortage of tissue for reconstruction and

FIG. 5. Cross sections of minced skin specimens after 2 weeks *in vivo*. All sections counterstained with hematoxylin (original magnifications, $\times 100$). (A) Cytokeratin stain. (B) Protein p63 stain. (C) Laminin stain. Basal membrane of the neoepithelium (arrow) and basal membrane of capillary endothelium in granulation tissue are visualized (asterisks).

TABLE 1. THE AREA OF NEOEPITHELIALIZATION AT DIFFERENT TIME POINTS AFTER SURGERY (2–4 WEEKS)

<i>Minced skin transplanted tubes</i>			
Time after transplantation	2 weeks	3 weeks	4 weeks
Number of tubes	7	7	4
Area epithelialized, mm ² (range)	148 (55–220)	115 (22–132)	129 (95–220)
Percentage of area epithelialized	66%	57%	59%
Superficial granulation tissue, mm (range)	0.8 (0.5–1.25)	1.2 (1.0–1.5)	1.0 (0.9–1.1)
Deep granulation tissue, mm (range)	0.7 (0.05–1.6)	1.4 (1.0–2.0)	1.0 (0.9–1.2)
Number of cysts	2.1 (0–6)	Not found	Not found

No significant difference was seen in area of epithelialization after 4 weeks in comparison to 2 weeks after transplantation ($p=0.24$). Note that after 2 and 4 weeks some structures present with full neoeptithelialization (2/7 after 2 weeks and 1/4 after 4 weeks). Measurements of granulation tissue were made in two opposite directions, between the tube and the native skin (superficial) and between the tube and the muscle (deep), with no significant differences between groups ($p=0.2$ and $p=0.33$, respectively, comparing data from 4 and 2 weeks). Cysts were found 2 weeks after transplantation and not later.

difficulties in restoring the three-dimensional anatomy, especially in secondary and tertiary operations or in severe congenital cases of malformations with aplasia of the affected organ.

Early studies in wound healing and transplantation revealed that skin epithelium regenerate from the edges. In 1958, Meek described how the regenerative capacity of a skin biopsy could be expanded by cutting it into smaller pieces.¹⁶ For every transection, wound edges could be increased by 50% and so also its regenerative capacity.

To further develop the technique described by Meek, we have used a wet wound environment to enhance neoeptithelialization in full-thickness wounds.^{11,12} In these studies, wounds transplanted with minced skin particles healed faster than wounds transplanted with autologous cells (keratinocytes and fibroblasts) in single cell suspensions, and as well as wounds transplanted with cultured autologous keratinocytes that had been expanded *in vitro*.¹⁷ By using a wet environment, the minced skin particles can reorient and therefore be transplanted in a random fashion, that is, not depending on being placed with the epidermis side up. We therefore hypothesized that the same principle could be applied to a moist internal environment such as a subcutaneous space.

As shown in the full-thickness wound model, transplanted cultured keratinocytes migrate toward the surface of the granulation tissue as they proliferate and reepithelialize the wound.¹¹ Further, the studies previously referred to by Svensjö and collaborators clearly illustrate that the position of the transplanted keratinocytes or minced skin is of minor

importance because they will all migrate to the surface. In addition, the expansion rate of minced skin tissue is similar to that of transplanted cultured, proliferative cells in spite of the fact that the latter have been cultured for 2 weeks or more.

Bladder augmentation and urethral reconstruction with cell culturing techniques is today in the pipeline and could be of major clinical importance in the future.^{9,10} In our studies, we harvested cells by washing the bladder with saline, a noninvasive procedure that can be performed without sedation. We used acellular dermis as a carrier. In the studies by Atala *et al.*,⁹ cell harvesting from bladder biopsies was first performed, then smooth muscle tissue was separated from the bladder mucosa, and each cell type was cultured and expanded separately. After cell expansion, culturing proceeded on prefabricated bowl-shaped molds that were incorporated into the bladder 7 weeks after the initial bladder biopsy.

Cell expansion by *in vitro* culture is rewarding in respect to the expansion rate. Only cells able to proliferate are selected, and these cells continue to expand after transplantation back to the patient before contact inhibition and differentiation. The method is considered safe in respect to malignancy, and malignant transformation *in vitro* has never been reported without active manipulations of the genome. Skin malignancy in areas treated with cultured cells has been reported; however, chronic wounds and burn wounds have an increased risk for malignancy *per se*.¹⁸ Major disadvantages are high costs, time of culture, and laborious cell culture procedures precluding its use in the majority of surgical patients.

FIG. 6. Proliferative cells in primary culture, day 10 (original magnification, $\times 40$). (A) Cells seeded from cells in suspension. (B) Cells proliferating from minced skin particle (seen in right lower corner).

FIG. 7. Neoepithelialization 10 days after transplantation of minced skin particles on latex tubes compared to silicone tubes. Mean area of epithelialization expressed in mm² ($p=0.04$).

We wanted to avoid these drawbacks by transplanting small pieces of skin particles and obtain direct cell expansion *in vivo*. This study showed that the transplanted particles survived, proliferated, migrated, and differentiated. The method is simple and expeditious as tissue harvesting, preparation, and transplantation can be performed in less than 30 min. The phenotypic characteristics of the epithelium remained, supportive subepithelial connective tissue was generated, and the epithelium differentiated with an orientation of *stratum corneum* toward the implanted tube.

Fourteen days posttransplantation, the biopsies revealed epithelial cysts separated from the luminal surface; the cysts could not be found in later biopsies. In earlier wound healing studies,^{11,12} similar cysts could be found as the transplanted minced skin particles migrated from the bottom of the wound bed and through the granulation tissue to reconstitute the neoepidermis of the healed wounds. We suspect that the same course of events occur with the minced particles that by mechanical forces are buried in the subcutaneous fat within a certain distance from the implanted tube. The minced particles subsequently form cysts with the keratin facing the lumen and migrate in the granulation tissue to reach the surface around the implanted tube. By these means, the appearance of cysts is a transient state and part of the process of neoepithelialization.

In hypospadias surgery, growth of hairs in the neourethra can be bothersome when genital skin has been used in the repair. In this study, only partial-thickness skin biopsies were grafted, by these means only the epidermis and superficial dermis were minced, and we do not suspect any risk of hair formation in the neoepithelium. This was also supported by examining cross sections of the grafted 0.3 mm biopsies where no hair follicles could be detected.

All tubes included in the studies were initially manufactured for stenting or catheterization purposes. All tubes are routinely used in clinical urology and were purchased through medical healthcare suppliers.

Interestingly, however, when comparing different tube materials, latex was superior to silicon concerning neoe-

epithelialization after balloon embolization of arterial aneurysms in a dog model, silicone balloons gave rise to less fibroblast proliferation and lesser endothelialization than latex.¹⁹ For stenting of the biliary duct, it has previously been shown that latex has desirable properties such as rapid tissue tract formation.^{20,21}

We have not examined physical and mechanical material properties (i.e., hardness, elasticity, and surface quality) of these tubes, even though it might have a stronger impact on the regenerating epithelium than the substance *per se*.

Although the technique for mincing the skin is easy and can be successfully performed without previous surgical training, we encountered some problems in this porcine model that might have influenced the take of transplants in a negative way. Mainly, the weight of the pig (60–70 kg at the end of study) in combination with its abdominal resting position sometimes subjected the implanted transplants to pressure. The porcine model also involves a high exposure to contamination and risk for infections. Wound dressings and covering of the operated area in combination with antibiotics is of major importance to reduce the risk of infection.

In addition, when evaluating the pilot studies we found it imperative to immobilize the transplanted tubes at both ends to prevent movement or migration in the subepithelial fat. As indicated in Table 1, a loss of epithelium with prolonged time *in vivo* can be suspected. We believe that above-mentioned problems with the porcine model might influence long-term patency negatively, but also a connection of the tube to a hollow organ and the external body surface might be of major importance for epithelial patency. The problems could also be solved by placing the tubes on the back region of the pig or by using another animal model.

In conclusion, we have successfully developed a three-dimensional *in vivo* technique for creating epithelialized tubular structures. The method has a potential in creating catheterizable stomas to the urogenital canal or for intestinal irrigations. Future studies will focus on the incorporation of the epithelialized tunnel to the urinary bladder and the skin to serve as a conduit and the use of transplanted minced bladder mucosa instead of skin. In addition, different tube materials and transplantation to other soft tissues, for example, the great omentum, will be tested. The clinical application is promising, and the technique could be of major importance in a variety of clinical situations. It would markedly reduce the drawbacks associated with ordinary cell culturing procedures due to its simplicity, availability, and low cost.

Acknowledgments

The Sweden-America Foundation research grants, Hirsch research fellowship from Karolinska Institutet, research grants from Stockholm County Council, the Swedish Society for Medical Research, and NIH grant 5ROIGM51449 supported this study. We thank Laboratory Manager Cliff Chapman at Children's Hospital and Laboratory Technician Christine Lam at Brigham and Women's Hospital for the excellent histological preparations.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Elof Eriksson, M.D., Ph.D.

Laboratory of Wound Healing and Gene Transfer

Division of Plastic Surgery

Harvard Medical School

Brigham and Women's Hospital

75 Francis St.

Boston, MA 02115

E-mail: eeriksson@partners.org

Received: March 7, 2008

Accepted: December 5, 2008

Online Publication Date: March 11, 2009