In Vivo Biocompatibility Testing of a Collagen Cell Carrier Seeded with Human Urothelial Cells in Rats

Lisa Daum[1], Sabine Maurer[1], Martin Vaegler[1] and Karl-Dietrich Sievert[1,2,3]*

[1]*Laboratory of Tissue Engineering, Department of Urology, Eberhard Karls University, Tübingen, Germany*
[2]*Department of Urology, Eberhard Karls University, Tübingen, Germany*
[3]*Department of Urology, University of Lübeck, Germany*

Abstract

Tissue-engineered, matrix-stabilized autologous urothelium is a new option for urethral reconstruction, particularly for patients for whom other autologous grafts are not available. *In vitro* engineering of urothelial tissue requires biomaterials as cell carriers that increase the stability of cell-based implants. The aim of this study was to investigate a new highly standardized, industrially manufactured bovine collagen type I-based cell carrier (CCC) for its suitability as a carrier matrix for human urothelial cells (HUC). As an *in vivo* biocompatibility test the behaviour and degradation of these implants was to be proven in a nude rat model.

Expanded HUC from tissue biopsies were phenotypically analysed by immuno¬cyto¬chemistry and seeded onto CCC. For *in vivo* application, CCC was seeded with PKH26-labelled HUC in high density and constructs were implanted onto the rectus abdominis muscle of nude rats. Integration, cell survival, and degradation of the urothelium-matrix-implants were studied after 1, 2, and 4 weeks. Immunohistological characterization of multilayered urothelium-matrix-constructs was performed for AE1/AE3 and p63 (epithelial phenotype), CK20 (differentiation), and E-Cadherin and ZO-1 (junction formation).

Immunocytochemical staining showed urothelial character of the isolated cells and the absence of fibroblasts and muscle cells. In twelve nude rats, urothelium-matrix-implants integrated well into the host tissue where no inflammation was observed. Immuno¬fluorescence analysis confirmed epithelial phenotype, adherence and tight junction formation homogeneous multilayer formation of stratified HUC cultures as well as urothelial phenotype and differentiation *in vitro* the CCC-matrix additionally revealed promising biocompatibility investigated in a nude rat model. Thus, this study demonstrates excellent suitability of CCC as a matrix for urothelial cells and recommends its use in a large animal model with regard to clinical application.

Introduction

Nowadays, there are only few therapeutic options for urethral reconstruction, e.g. for the treatment of urethral injuries or strictures. However conservative therapies are not sufficient for patients with severe urethral damage or for recurrent stricture incidence. The current gold-standard for complicated and serious urethral damage repair is the use of buccal mucosa which leads to favourable success rates [1,2]. Nevertheless some patients do not agree upon oral tissue retrieval or cannot provide an autologous graft due to donor side morbidity. Therefore different therapies are under investigation principally basing on the techniques of tissue engineering.

Different materials have been used for tissue replacement and combined with cells or used without seeding: Small intestinal submucosa (SIS) was one of the first materials which was investigated for urethral reconstruction and showed varying success. In a clinical study Sievert et al. observed a positive outcome in 9 of 13 patients and assessed that SIS grafts could be a beneficial option in selected cases of urethral stricture [3]. The limit of SIS lies in the length of the stricture and the condition of the urethral bed. Spongiofibrosis, inflammation or scarring constitute suboptimal prerequisites that result in healing complications and an adverse long-term outcome.

In rabbits acellular urethral matrix grafts were tested in a homologous and heterologous setting [4,5]. Blood vessel and smooth muscle ingrowth was observed already after ten days in homologous grafts. With regard to urodynamics and urethrography there was no significant difference between the two groups. Li et al. seeded acellular bladder matrix grafts (ABMG) with bone marrow mesenchymal stem cells (BMSC) and smooth muscle cells (SMC) for application in a homologous rabbit model [6]. The graft was implanted into the rabbit omentum for 2 weeks before urethral reconstruction. At 8 and 16 weeks after *in vivo* grafting, the tissue-engineered sheet was covered by multilayer urothelium, neovascularization was visible, and organized smooth muscle bundles were present. Retrograde urethrography verified the absence of diverticula or urethral stricture formation.

Currently the use of tissue-engineered buccal mucosa for urethroplasty is under investigation. In a clinical trial five patients suffering from lichen sclerosis related urethral stricture were treated with tissue-engineered artificial urethras based on buccal mucosa cells. Two of them experienced complications such as fibrosis and contraction during convalescence [7]. The remaining three patients had patent urethras although all required further instrumentation. A different tissue engineering principle was pursued by Raya-Rivera et al. [8]: A tissue biopsy was taken from five boys suffering from urethral defects and the muscle and epithelial cells were expanded and seeded

*Corresponding author: Karl-Dietrich Sievert, MD PhD, Universitat zu Lubeck, Lubeck, Germany, Tel: + 494515002271 E-mail: kd_sievert@hotmail.com

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onto tubularised synthetic scaffolds. Urethras were reconstructed with these tissue-engineered segments. Patient history, questionnaires, urine analyses, cystourethroscopy, cystourethrography, and flow measurements were performed regularly until 72 months after surgery to evaluate therapeutic success. Furthermore serial endoscopic cup biopsies were taken at 3, 12, and 36 months. Urinary flow rates and serial radiographic and endoscopic studies showed the maintenance of wide urethral calibres without strictures in addition to a normal appearing architecture of the grafts by 3 months after implantation. The authors therefore conclude that these tubularised urethras remain functional in a clinical setting for up to 6 years and can be used in patients who need complex urethral reconstruction.

In the present study a collagen type I-based matrix was seeded with human urothelial cells as a potential new therapeutic concept for urethral reconstruction, an alternative to buccal mucosa. For evaluation of *in vivo* biocompatibility, seeded matrices were implanted onto the rectus muscle of athymic nude rats and extracted tissues were evaluated histologically with aspects of integration, biocompatibility, localization, and degradation of the cell carrier.

Materials and Methods

Isolation and culture of human urothelial cells

Human ureter tissue samples were obtained from adult patients (aged 29-74 y; mean 52 y) undergoing open nephrectomy (local ethics committee approved). Tissue specimens were immediately placed in ice-cold transport medium [9]. HUC were isolated as previously described [10]. Briefly, excess fat and connective tissue were removed. Pieces of 1–2 cm^2 were transferred into stripping solution containing Hank's Balanced Salt Solution (HBSS)/1% ethylenediamine-tetraacetic acid (EDTA; Biochrom), buffered with 10 mM 2-(4-(2-hydroxyethyl) piperazine-1-yl)ethanesulfonic acid (HEPES; Invitrogen) and supplemented with 20 kIU/ml aprotinin (Bayer). After 3 h of incubation at 37°C, the urothelium was removed from the stroma. Isolated cells were washed in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor (both Invitrogen), and 30 ng/ml cholera toxin (List Biological Laboratories). Subsequently, cells were seeded into CellBIND culture flasks (Corning). Urothelial cultures were maintained at 37°C and 5% CO_2 in a humidified atmosphere. The culture medium was replaced every day. For further passaging, subconfluent monolayers were incubated in phosphate buffered saline (PBS) containing 0.1% EDTA (Biochrom) for up to 10 min at 37°C, followed by exposure to TrypLE Express (Invitrogen) for another 1-2 min. The detached cells were resuspended in culture medium and seeded into CellBIND culture flasks.

For stratification, HUC were seeded in high density with 3-4 x 10^5 cells/cm^2. Two days after seeding stratification was induced by adding $CaCl_2$ to the culture medium to a final concentration of 1.09 mmol/l.

Immunocytochemistry

For quality control the epithelial phenotype of the isolated cells was determined by expression of pankeratin while absence of fibroblasts and smooth muscle cells was confirmed by anti-fibroblasts and anti-α-smooth muscle actin (α-SMA) antibodies. Expanded HUC were seeded on chamber slides in a density of 5 x 10^4 cells/cm^2 and cultured until they reached 90% confluence. Cells were fixed by incubation in 3.7% paraformaldehyde. For pankeratin and α-SMA staining urothelial cells were permeabilised with 0.1% saponin solution. Immunocytochemistry

was performed with mouse monoclonal anti-pankeratin cocktail AE1/AE3 (Millipore), anti-fibroblasts monoclonal antibody clone TE-7 (Millipore), and anti-α-SMA antibody clone 1A4 (Sigma-Aldrich). Negative controls were performed omitting the primary antibody (Table 1). The EnVision+ System-HRP (DAB) kit (Dako) working with a streptavidin-biotin method was used for immunocytochemical staining. Binding to the primary antibody was detected as a brown colouration whereas negative cells only showed blue haematoxylin nucleus staining. The dried slides were mounted in glycerol gelatine (Merck), covered with a microscope cover slip and stored at room temperature.

Loading culture dishes with Collagen Cell Carriers

Sterile discs of the collagen cell carriers (CCC, 14 mm; Viscofan Bioengineering) were transferred into 24-well microplates and washed with pre-warmed DPBS (with Ca^{2+} and Mg^{2+}; Biochrom). The disks were adjusted and dried overnight in an operating laminar flow. After drying, the CCC attached firmly to the well bottom. Prior to cell seeding the dried CCC was equilibrated with KSFM at 37°C and 5% CO_2 for 30 minutes.

PKH26 labelling

PKH26 served as an *in vivo* tracking system to detect the implanted cells in the extracted urethral tissue after application of the seeded CCC. PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) including PKH26 cell linker and the corresponding diluent was used to stain HUC. Subconfluent urothelial monolayers were detached, the suspended cells were labelled with PKH26 according to the manufacturer's protocol (Sigma-Aldrich) and seeded on the cell carriers. The staining success was checked microscopically.

Study conditions of rat model

The rodent experiments were performed according to the approval of the Regional Administrative Authority of Tuebingen (CU 1/10) with athymic nude rats (Crl:NIH-Foxn1nu; Charles River Laboratories). Twelve female rats at the age of five weeks underwent surgery for the evaluation of biocompatibility of urothelium-CCC-constructs. They were kept in groups of four animals at a research animal facility of the University of Tuebingen. The animals were given vegetal commercial laboratory animal food and water ad libitum. Environmental enrichment measurements were undertaken. Prior to the beginning of the experiment, the animals were allowed to adapt to the surrounding conditions for one week.

Thawed HUC were cultured as described above, labelled by PKH26 and 4 x 10^5 cells/cm^2 were seeded onto CCC in a CellBIND 24-well plate (Corning Inc.). On stratification day eight urothelium-CCC-constructs were ready to be applied.

Surgery procedure

The animals were anaesthetised by intraperitoneal injection of xylazine/ketamine. In supine position the rectus muscle was exposed by a midline abdominal incision. The implants were washed in 0.9% NaCl, placed directly on the muscle tissue and covered with fascia and

Identification	Specificity	Clone	Dilution	Company
Epithelial phenotype	pankeratin	AE1/AE3	1/500	Millipore
Fibroblasts	cultured fibroblasts	TE-7	1/2000	Millipore
Smooth muscle cells	α-SMA	1A4	1/10000	Sigma-Aldrich

Table 1: Primary antibodies for quality control.

marked by 4 non-absorbable sutures. The fascia was adapted prior to the skin closure in buried suture. Carprofen was injected daily as an analgetic for the first three postoperative days.

Tissue retrieval

After the scheduled periods of one, two, and four weeks the animals were euthanised by intraperitoneal injection of pentobarbital. The tissue of the implantation site was removed spaciously for histological work-up. Tissue samples were directly placed in 18% saccharose solution as a cryoprotective agent and stored at 4°C overnight. The following day tissue samples were placed in tissue freezing medium, frozen in liquid nitrogen and then stored at -80°C until further processing.

Histological analysis

Cryostat sections (5 μm) of each tissue were processed and evaluated histologically and immunohistologically. For evaluation of *in vivo* biocompatibility haematoxylin/eosin (HE) staining was performed. Native tissue slides and HE stained slides were analysed microscopically with regard to the survival and integration of the implanted cells as well as the degradation of the CCC and potential inflammatory reactions. The applied cells were detected by red PKH26 fluorescence. Moreover tissue slides of each animal were immunohistologically investigated with the primary antibodies (Table 2) specific for pankeratin (Millipore), CK-20, E-Cadherin (both Dako), p63 (Dianova), and ZO-1 (Invitrogen). As secondary antibodies Cy2 conjugated donkey anti-mouse IgG F(ab')2 and goat anti-mouse IgG (H+L) were used (both Dianova). Negative controls were performed omitting the primary antibody. Processed slides were mounted in Immunoselect (Dianova) containing DAPI, and covered with cover slips. Tissues were assessed with regard to different fluorescence spectrums showing urothelial phenotype (green fluorescence) of the implanted cells (red fluorescence).

Results

Quality control

Immunocytochemistry was performed with antibodies for pankeratin (AE1/AE3) as a marker for epithelial phenotype, fibroblast surface antigen (TE-7), and α-SMA (1A4). Positive staining was evident by brown colour development of substrate chromogen. Negative controls did not show brown staining. All HUC seeded on chamber slides stained positive for AE1/AE3 and negative for both TE-7 and 1A4 (Figure 1). Thus, epithelial phenotype was confirmed by the specific staining characteristically for urothelial cells.

PKH26 staining

Success of PKH26 staining was confirmed by microscopic evaluation. A strong fluorescence signal was detected and the staining pattern was homogeneous and cell-associated (Figure 2). The CCC did not show autofluorescence.

Surgical intervention and health status

HUC were seeded on CCC and implanted on the rectus abdominis muscle of twelve nude rats (Figure 3A-3C). All animals survived anaesthesia and the respective experimental periods without incidents. They did not show any physical impairments or inflammatory reactions on the implantation site throughout the experimental period. The wound healing proceeded without apparent rejection reactions.

Macroscopic evaluation

Muscle tissue was extracted spaciously after euthanisation of the rats one, two, and four weeks after surgery. When extracted after one week, the implant could be assumed gleaming slightly on the rectus muscle. After two weeks the implant could be recovered as a white solid object resembling connective tissue in two animals (Figure 3D). In the other two animals the implant could not be detected macroscopically (Figure 3E). Four weeks after surgery the implant location was only detectable by the marking sutures.

Histological read-out

The seeded CCC was lying folded between the skin and the rectus muscle in eight animals and reached into the muscle tissue in four of them. It showed excellent integration into the host tissue in eight animals, while four revealed additional tissue formation around the implant. None of them showed inflammatory signs in the HE stained tissue sections. The CCC could be located easily, showing different states of degradation and different morphologies throughout the experimental period. The CCC was nearly completely degraded in two animals four weeks after application (Figure 4).

Immunofluorescence

HUC could be detected by red PKH26 fluorescence in all animals. Cryostat tissue sections of urothelium-CCC-constructs in rectus muscle of nude rats were characterised via immunofluorescence. Negative controls did not show fluorescence of Cy2 conjugates. None of the used primary antibodies showed unspecific binding to muscle and subcutaneous tissue surrounding the implant. Positive pankeratin expression was assessed at the implantation side one and two weeks after surgery (Figure 5B and 5C). Expression of CK-20, E-Cadherin, p63 and ZO-1 was equally verified one and two weeks after surgery (Figure 5D-5G).

Discussion

Collagen cell carrier (CCC) was tested for *in vivo* biocompatibility in rats by Rahmanian-Schwarz et al. in an unseeded implantation setting and showed good integration without scarring or inflammation and degradation over a period of 42 days [11,12]. Histological evaluation after 84 days revealed no evidence of encapsulation, scar formation, or long-term vascularization as well as inflammation.

In the present study histological analysis of extracted tissues proved excellent biocompatibility of human urothelial cells seeded on CCC in nude rats. The urothelium-matrix-constructs integrated well in the subcutaneous tissue and were almost completely degraded after four weeks. Neither severe inflammatory reactions were observed nor did any animal show adverse effects due to implantation of seeded CCC although slight additional tissue formation partly occurred when the transplants were placed deeply in the muscular tissue and moderate injury has been caused, which might be related to the surgical approach. Health status of all rats was not impaired at any time point of the experiment.

| Identification	Specificity	Clone	Dilution	Company
Epithelial phenotype	pankeratin	AE1/AE3	1/100	Millipore
Urothelial differentiation	p63	4A4	1/50	Dianova
Urothelial differentiation	cytokeratin 20	$K_s20.8$	1/50	Dako
Adherence junctions	E-cadherin	D33	1/100	Dako
Tight junctions	zonula occludens 1	ZO1-1A12	1/25	Invitrogen

Table 2: Primary antibodies for histological evaluation.
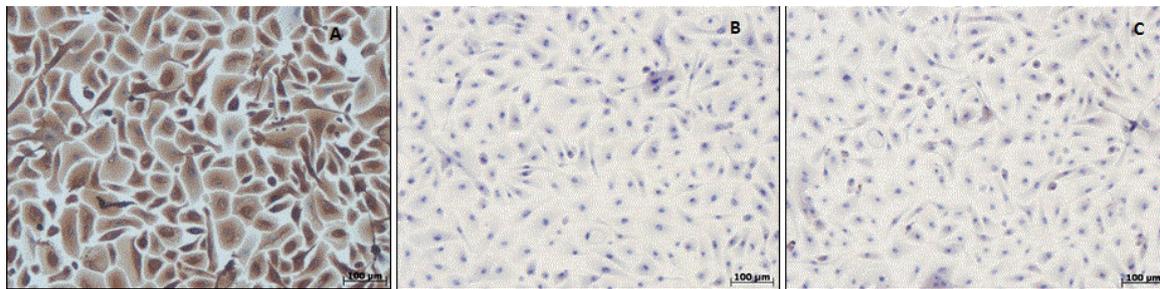
Figure 1: Immunocytochemical analysis for quality control of cultured urothelial cells. *In vitro* staining of characteristic markers for (A) urothelial cells (AE1/AE3), (B) fibroblasts (TE-7), and (C) smooth muscle cells (1A4) demonstrated a pure HUC population without cellular contaminants indicated by lacking brownish colouration following antigen binding.
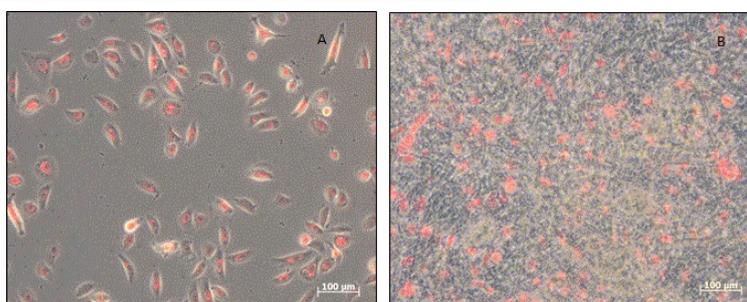
Figure 2: *In vitro* labelling of cultured urothelial cells. Suspension of cultured HUC were labelled with PKH26 and re-seeded on standard plastic surface (A) as well as on collagen cell carrier (B). Microscopic evaluation one day after seeding confirmed a strong fluorescence signal together with a cell-associated and homogeneous staining pattern.
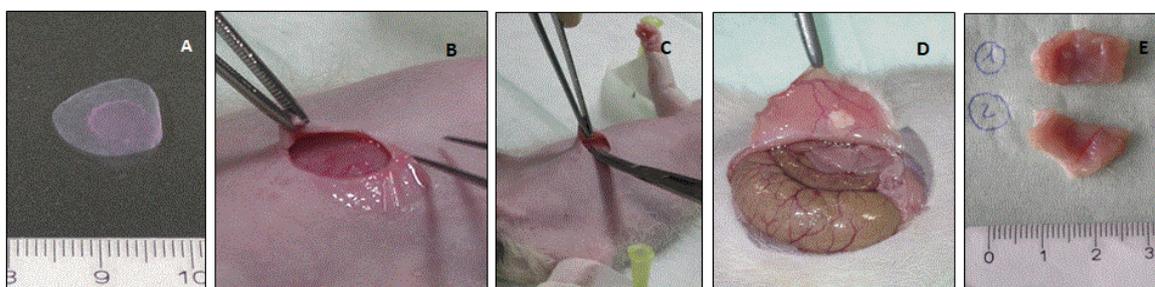

Figure 3: Surgical intervention and tissue harvesting. Collagen cell carrier (A) seeded with PKH26-labelled HUC prior to application onto the rectus abdominis muscle. Construct lying on the abdominal wound (B) prior to its positioning directly between muscle tissue and the associated fascia (C). Implantation site two weeks after surgery (D) with connective tissue formation (white spot). Tissue sample was harvested and divided into two pieces for further processing. Here, implant is not visible macroscopically one week after surgery (E).

In line with earlier investigations on HUC possible impurities of fibroblasts and smooth muscle cells of *in vitro* cultures could be excluded and verified the used protocol for isolation of urothelial cells from human tissue [13]. Labelling of expanded HUC for *in vivo* tracking prior to matrix seeding were demonstrated as homogenous and sufficient by their detection in histological analysis correspondingly.

Pattern of relevant markers for urothelial phenotype (CK-20, pankeratin, p63) and intercellular contacts (E-Cadherin, ZO-1) of implanted seeded constructs that would be in accordance to native urothelium were verified immunohistologically after one and two weeks post-op but found considerably reduced after four weeks. Expression profile changes of urothelial antigens could be caused by the untypical environment as well as the lacking of necessary stimuli as there are different pH, or the absence of urine-specific factors and urine streaming.

Regarding latest developments in clinical application of tissue engineering therapies for urethral reconstruction the use of buccal mucosa biopsy to seed an acellular biomaterial is an innovative approach. Requiring only small oral tissue biopsies the risk of donor site morbidity is minimized and *in vitro* manufacturing of urethral replacement tissue based on oral mucosa is successful [14,15]. Nevertheless there is a difference between oral and urothelial mucosa: It is questionable if squamous epithelium can display the same flexibility and a functional urine-blood barrier like urothelium does. Data of a large animal model proving feasibility and long-term effects after implantation are lacking. Therefore long-term assessment of human patients is essential for evaluation of this therapeutic approach.

Bioartificial urothelium is potentially superior due to its special properties being adapted to the urinary tract. Thus the evaluation of
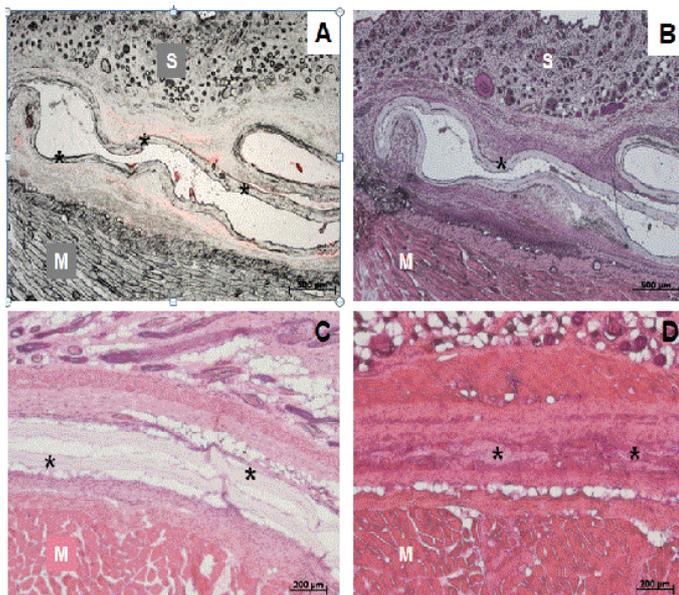
Figure 4: Histological evaluation of extracted tissues. Consecutive cryostat section one week after surgery (top row) showed seeded CCC (*) lying folded between skin and rectus muscle. Overlay of unstained section (A) demonstrated red fluorescent PKH26-labelling of seeded HUC. HE-stained section (B-D) revealed a continuous and intact matrix one (B) and two weeks post-op (C). After four weeks ongoing degradation process of CCC could be documented.
Note: * = CCC, M = muscle, S = skin.
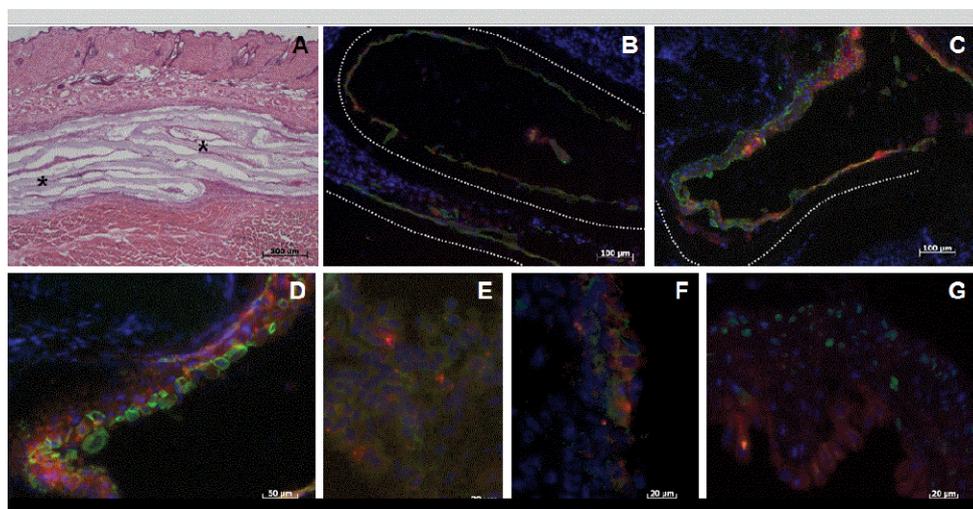
Figure 5: Immunofluorescence analysis of characteristic urothelial markers. HE-stained cryostat section after two weeks post-op showed seeded CCC (*) lying folded between skin and rectus muscle (A). Detected expression of pankeratin one (B) as well as two (C) weeks after surgery confirmed the epithelial phenotype of the red fluorescence-labelled HUC on CCC (dotted line). Positive expression of CK-20, E-Cadherin, ZO-1 and p63 (D-G) could be verified up to two weeks after implantation of the urothelium-matrix-constructs indicated by green fluorescence.

urethral reconstruction with tissue-engineered urothelium-matrix-constructs was analogously performed in an established stricture model of large animals. Here, we demonstrated that stratified human urothelial cells seeded on CCC transplanted on the rectus muscle of nude rats resulted in cell survival, potential tissue integration, and processing biodegradation of the CCC, without any sign of scaring, which are the main important aspects focusing a clinical application in the future.

Acknowledgments

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