Direct Induction of Layered Tissues from Mouse Embryonic Stem Cells: Potential for differentiation into Urinary Tract Tissue

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Abstract

The aim of this study was to induce organized layered tissues with characteristics of the urinary tract from embryonic stem (ES) cells alone. We seeded embryoid bodies (EBs) originating from mouse ES cells onto mono-layered collagen membranes and cultured them in four different media. Group 1 was grown in a mixed medium of keratinocyte serum-free medium (KSFM) and Medium 199, Group 2 in a mixed medium of KSFM and conditioned medium collected from 3T3 fibroblasts, Group 3 in an EB formation medium (control group), and Group 4 was KSFM only (control group). After 28 days, cultured tissues were transplanted into nude mice. Cultured tissues from Groups 1 and 2 formed four-layered structures comprising a stratified epithelium, a submucosal loose connective tissue layer, a smooth muscle cell layer identified immunohistochemically by α-smooth muscle actin, and a deep loose connective tissue layer identical to the adventitia. Immunohistochemistry showed that the epithelia were positive for cytokeratins. Tissues also expressed uroplakin as detected by reverse transcriptase/polymerase chain reaction. In contrast, specimens from Groups 3 and 4 demonstrated necrotic features. Uroplakin-positive (i.e., urothelium-like) cells

developed only in Group 2 in the transplanted culture tissues in nude mice. In addition to inducing organized, layered tissues from mouse ES cells directly *in vitro,* these findings demonstrate that tissues cultured in KSFM plus conditioned medium from 3T3 fibroblasts differentiate into luminal walls similar to those of urinary tract *in vivo*. These findings suggest a new approach to urinary tract regeneration.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocysts. These cells have an almost limitless capacity for self-renewal and are pluripotent (Odorico et al. 2001). When ES cells are cultured on feeder layers with leukemia inhibitory factor (LIF), they remain in an undifferentiated state (Shamblott et al. 2002). However, when transferred to a suspension culture without LIF, ES cells begin to differentiate and form 3-dimensional multicellular aggregates called embryoid bodies (EBs), composed of three embryonic germ layers. ES cells can differentiate into various cell types, e.g., hematopoietic cells, neural cells, cardiomyocytes, endothelial cells, and osteocytes (Itskovitz et al. 2000; Odorico et al. 2001; Shamblott et al. 2002; Thomson et al. 1998). ES cells and EBs are useful as investigational models in embryology and tissue engineering, and are a potential source of cells for transplantation therapy (Odorico et al. 2001; Reubinoff et al. 2000; Shamblott et al. 2002).

Many studies have shown that ES cells can be induced to form specific and uniform cell populations and/or fully differentiated “cells”, through the application of

growth factors and chemical compounds, extracellular matrices (ECMs), co-culture with somatic cells, conditioned mediums, and other methods (Heng et al. 2004; Schuldiner et al. 2000). However, few reports have focused on the induction of differentiated (organized) “tissues”.

In urology, the regeneration of urothelial tissues is an important subject. Investigations into the regeneration of urinary tract tissue by using harvested urothelial cells (UCs) and/or smooth muscle cells (SMCs) have been reported (Oberpenning et al. 1999; Zang et al. 2000). The regeneration of bladder muscle by using bone marrow-derived mesenchymal stem cells has also been described (Kanematsu et al. 2005).

Various culture media have been used to grow UCs. For instance, keratinocyte serum free medium (KSFM) is commonly used for UC cultures, whearas Medium 199 (M199) is used for the culture of SMCs (Baskin et al. 1993; Hutton et al. 1993). Long-term culture of UCs and epithelial stratifications are well supported by using 3T3 fibroblasts as feeders (Zang et al. 2001). In addition, natural collagen, a widely available biomaterial that is biodegradable, minimally immunogenic, and easy to process, is often used to support cellular attachment and promote proliferation and differentiation of

urinary tract cells (Marcovich et al. 2003; Sabbagh et al. 1998).

Few reports are available regarding the regeneration of urinary tract tissue from ES cells. We have conducted an investigation involving the use of mouse ES cells to induce the formation of organized tissues with the nature of urothelia, without the co-culture of somatic cells, in differentiation culture media followed by transplantation of cultured tissue into nude mice.

Materials and methods

Embryonic stem cell culture and formation of EBs

Mouse ES cells of the cell line 129/sv strain (Cell and Molecular Technologies, Phillipsburg, NJ, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 20% knockout serum replacement (Gibco), 100 μM nonessential amino acids (Gibco), 1 ㎜ sodium pyruvate, 100 μM 2-mercaptoethanol, penicillin (50 units/ml), streptomycin (50 μg/ml), and murine LIF (1000 units/ml) (Chemicon International, Temecula, CA, USA) on 0.1% gelatin-coated dish with STO (SIM [Sandoz inbred Swiss mouse] thioguanine-resistant ouabain-resistant) fibroblasts treated with mitomycin C at 37^{o}C in humidified air with 5% CO_2.

After maturation of colonies, ES cells were dissociated with 0.1% trypsin-EDTA, and suspended in EB formation medium consisting of Iscove's modified Dulbecco's medium (Gibco) supplemented with 20% fetal bovine serum (FBS), 100 μM

nonessential amino acids, 1 mM sodium pyruvate, 100 μM 2-mercaptoethanol, penicillin (50 units/ml), and streptomycin (50 μg/ml). For the formation of EBs, a suspension of 1.0 ml ES cells at 2×10^4 cell/ml were placed in a 1.5 ml polypropylene conical tube with a round bottom for 5 days, as previously described (Kurosawa et al. 2003) .

3T3 cell culture and conditioned medium

Swiss Albino transformed mouse fibroblasts (3T3 cells) were cultured in DMEM supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50 μg/ml). The conditioned medium was prepared by incubating cultures of 90% confluent 3T3 cells. The supernatant media were collected and filtered by polyvinylidine difloride membrane filters with 0.22 μm pore size (Millipore, Ireland).

Culture of EBs on collagen membrane

In 24-well plates, EBs were seeded onto mono-layered permeable collagen membranes (10 mm diameter) composed of purified type I athelocollagen (Koken, Tokyo, Japan) . The culture was maintained in EB formation medium for 3 days at 37^{o}C in an incubator with 5% CO_2, and then divided into four different media groups for up to 25 days. Groups 1 and 2 were grown in differentiation media. Group 1 in 1:1 defined KSFM (Gibco) and M199 (Gibco) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Group 2 in 1:1 defined KSFM and conditioned medium collected from 3T3 cells. Groups 3 and 4 were controls; Group 3 was grown in EB formation medium and Group 4 in KSFM only. Media were exchanged every 2 or 3 days.

Histological and immunohistochemical analyses

After being rinsed with phosphate buffered saline, cultured tissues were fixed with 4% paraformaldehyde at pH 7.4 and embedded with paraffin. Serial sections (3 μm) were obtained from each sample. Some sections were prepared for hematoxylin and eosin (H&E) staining.

For immunohistochemistry analyses, we used protein markers to detect epithelium, including pan-cytokeratin AE1/AE3 (CKAE1/AE3), cytokeratin 8 (CK8), cytokeratin 18 (CK18), uroplakin (UP) III, and Pax-2. UP III is the marker for urothelial differentiation, and Pax-2 is the marker for mesonephric duct differentiation. Smooth muscles cells were detected with alpha-smooth muscle actin (αSMA). Cell proliferation was evaluated by proliferating cell nuclear antigen (PCNA; correlated with DNA synthesis). The antibodies used in this study are listed in Table 1. The avidin-biotin complex detection (ABC) method (VECTASTAIN ABC kits ,Vector Laboratories, Burlingame, CA, USA) was used to localize significant markers histochemically. The treated samples were visualized using the diaminobenzidine reaction and counterstained with hematoxylin. Samples were observed with a light microscope.

Sections were incubated with a secondary antibody for the immunofluorescence

analyses (Table 1) and counterstained with 4, 6-diamidino-2-phenylindole (DAPI). They were observed with a confocal laser microscope (Leica DAS Microscopethe, Leica Microsystems GmbH, Wetzlar, Germany).

Transmission electron microscopy

EB-implanted collagen membranes were fixed with 2.5% glutaraldehyde, postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in a graded series of ethanol, and embedded with Epoxy restin. Ultrathin sections of the specimens were stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEM-1200; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Reverse transcriptase/polymerase chain reaction

To analyze gene expression, EBs (before implantation) and EB-implanted cultured tissues on collagen membranes were collected from each group on days 0, 3, 7, 10, 14, 21, and 28. Total RNA was extracted using an RNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacture's instructions. Complementary DNA was synthesized from 10 μl total RNA by using SuperScript II and RNase H^- reverse transcriptase. The resulting complementary DNA was amplified with Ex*Taq*. Expression of the genes *αSMA*, *hl-calponin*, and *smooth muscle myosin heavy chain* (*SM-MHC*) were assessed for smooth muscle differentiation, *UPII* for urothelial differentiation, and *Pax-2* for mesonephric differentiation. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous control. The primer sequences and annealing temperatures for the reverse transcriptase/polymerase chain reaction (RT-PCR) are summarized in Table 2. The amplified products were separated by electrophoresis on 1.5% agarose gels and stained with ethiduim bromide.

Transplantation of cultured tissue with collagen membrane to nude mice

This investigation conformed to the U.S. National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, revised in 1996).

Male nude mice (BALB*/c nu/nu*) at postnatal week 5 were used as recipients. At least 2 animals were used for each survival time in each group. All animals were purchased from SLC (Hamamatsu, Japan). The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium solution (0.04 mg/g body weight). On day 28, cultured tissue with attached collagen membrane from each media group was transplanted into a small subcutaneous incision in the back of each mouse. Collagen membranes without cells (i.e., nonseeded membranes) were also transplanted as controls. On days 14 and 28 post-transplantation, the anesthetized mice were killed and transplants were retrieved. Paraffin embedded sections were prepared, stained with H&E, and evaluated by immunohistochemistry for CKAE1/AE3, CK8, CK18, UPIII, Pax-2, αSMA, desmin, nestin, and PCNA by the methods described above (Table 1). Desmin was used for the detection of muscle components, and nestin was for neuronal

components.

We calculated the average proportions of each immunohistochemical positive areas in transplants on days 28 of Group 2 by computer digital softwares (Adobe Photoshop CS® 8.0 and NIH ImageJ 1.38w).

Results

Layered structure induced on collagen membrane

Seeded EBs attached to collagen membranes proliferated as two-dimensional outgrowths (Fig. 1a). On day 4, proliferation of αSMA^{+} cells was observed on the margins of the EBs (Fig. 1b). On day 10, some samples in Group 2 had smooth muscle contractions (i.e., peristaltic movements) observed on microscopy. Cultured EBs in Groups 1 and 2 differentiation media formed four-layered structures consisting of stratified epithelium and mesenchyme containing SMCs (Fig. 2a, b). Conversely, cultured tissues from Groups 3 and 4 (controls) demonstrated necrotizing tissues containing loose connective tissue and clusters of karyopyknotic cells (Fig. 2c, d). The PCNA expression was observed in Group 1 and 2, but was negative in Group 3 (Fig. 2e). In Group 4 (in KSFM only), cultured tissues demonstrated outgrowth for the first 1-2 weeks, but showed shrinkage and detachment thereafter. For this reason, we did not use this group for further analysis and transplantation.

Immunohistochemistry analyses revealing stratified epithelium were positive for CKAE1/AE3 (Fig. 3a) and CK8 (Fig. 3b), but negative for UPIII in all groups (Table 3). Group 2 alone had weakly positive CK18 samples (Fig 3c). In all samples, mesenchyme tissue had SMCs (i.e., αSMA^{+} cells) detected with H&E staining and immunohistochemistry; however they were more abundant in Groups 1 and 2 than in Group 3 (Fig. 3a, d).

Transmission electron microscopy images in Group 2 showed four organized layers consisting of epithelium, submucosal loose connective tissue, smooth muscle layer, and poorly developed loose connective tissue similar in appearance to adventitia (Fig. 4a). Epithelial cells had a large number of short microvilli on the apical area, cytoplasmic vesicles, mitochondria and glycogen granules in the cytoplasm, intercellular slits with some microvilli, and junctional spots forming in the intercellular area and basal lamina on their base (Fig. 4b). Submucosal loose connective tissue contained polymorphogenous cells with well-developed, rough-surfaced endoplasmic reticulum (rER) and rich collagenous fibrils (Fig. 4a). The third layer consisted of spindle-shaped cells that had microfilaments with dense bodies, rich glycogen granules,

rER, and mitochondria and that showed principal phenotypic features of myofibroblasts (Fig. 4c). Immunohistochemical analyses revealed that the spindle-shaped cells were αSMA^{+}. The fourth and last layer was loose connective tissue similar to the subserosal layer on the collagen membrane (Fig. 4a).

Expression of smooth muscle markers, *UPII*, and *Pax-2* in RT-PCR analysis

Expression of *αSMA* and *hl-calponin* mRNA was detected on day 7 in Groups 1, 2, and 3; *SM-MHC* was apparent on day 14 or later (Fig. 5). Expression of mRNA encoding *UPII* was observed on day 14 in the differentiation media (Groups 1 and 2), but was not found until day 21 or later in the control medium (Group 3). On the other hand, the expression of *Pax-2* gene was weak from day 7 to 21, and negative on day28 in Group 2. In contrast, these were expressed apparently in Grpup1. Results of the RT-PCR experiments are summarized in Table 4.

Transplanted Tissue

To induce the development of well-differentiated tissues, cultured samples were transplanted into nude mice on day 28. On post-transplantation day 28, transplanted tissues from all media groups formed teratomas. The teratomas contained various structures, such as ducts, vessels, muscles, and fibrous tissues (Fig. 6a, b). In the samples of the non-seeded collagen membrane, only some infiltrating cells were present on the membrane (Fig. 6c). The immunohistochemistry analyses showed αSMA^+ cells were localized near the ducts and $PCNA^+$ cells were dispersed diffusely in Group 2 samples (Fig. 7a, b). In Group 2, the apical part of the duct-like structure was positive for UPIII in day 28 samples (Fig. 7c). However, this structure had been unrecognizable on day 14 in Group 2. Samples in Groups 1 and 3 were negative for UPIII on days 14 and 28. The duct-like structure in Group 2 was also positive for CKAE1/AE3, CK8, and CK18 (Fig. 7d-f), but negative for Pax-2. The distribution of these structures in the specimen was limited.

The results of immunohistochemical staining of transplants were shown in Table 5. Many proliferating cells (positive for PCNA) and duct-like structures (positive for pan-cytokeratin) were seen, some of which were positive for both CK8 and UPIII (3 to 4 spots in each specimen). Muscle components were not abundant, and neuronal cells were rare.

Discussion

In this study, we have used differentiation media and collagen membranes to induce in vitro ES cells to differentiate and form organized layers similar to those of urinary tract tissues. The cultured and transplanted tissues have been histologically and immunohistochemically verified by using protein and genetic markers of urinary tract tissues. In vitro, distinct layered structures are found only in the cultures grown in differentiation media (i.e., Groups 1 and 2). Additionally, UP genes were expressed at an earlier time in these groups compared with the control group (Group 3). Because these results are reproducible, we believe that differentiation in these groups is not spontaneous. In contrast, little growth has been observed in the serum-free conditions of the second control group (Group 4). When tissues were transplanted to nude mice, urothelium-like structures developed only within the group originating from the 3T3-conditioned medium (Group 2). From these results, we speculated that, in addition to supplemented KSFM, serums and 3T3-conditioned medium might have some factors that induce layered structures composed of epithelia and mesenchyme containing SMCs

and that promote differentiation.

UCs are characterized ultrastructually by an apical membrane (also known as an asymmetrical unit membrane) composed of plaques, a high density of cytoplasmic vesicles, folded lateral membranes, and tight junctions connecting each cell (Lewis 2000). UPs proteins found in the plaques of the apical membranes of umbrella cells that form the waterproof barrier in the urothelium, are comprised of four major subunits (i.e., UPIa, UPIb, UPII, and UPIII), and are used as markers for the terminal differentiation of the urothelium (Sun et al. 1996). In general, UPIII antibody has been widely used in immunohistochemistry. On the other hand, the *UPIII* gene expression has also been detected in prostatic epithelium, although *UPII* gene expression has been recognized as being highly specific to the urothelium (Olsburgh et al. 2003). In this study, UP^+ cells have not been confirmed in vitro, in spite of the expression of the UP gene. The images obtained by transmission electron microscopy have demonstrated the presence of stratified epithelia, but the structures are not necessarily specific to urothelial tissue. However, immunohistochemical observations (Table 3) suggested that they had endodermal characteristics.

Cytokeratins are cytoskeletal proteins that serve as markers for the epithelium. These proteins have subtypes expressed in various tissues (Moll et al. 1982). For instance, CKAE1/AE3 are expressed in all epithelia, but CK8 and CK18 are expressed only in urinary bladder, intestines, and trachea; they are not found in epidermal tissue. Baskin et al (1996) have reported that CK8 and CK18 are expressed at 15 days of gestation in the rat bladder. On the other hand, Pax-2 represents the differentiation of mesonephric duct and nephron, ureteric bud, and its derivatives (Yamamoto et al. 2006). In view of these reports and our immunohistochemical results, the epithelium grown in differentiation culture has characteristics of the urinary tract or alimentary tract of endodermal origin, rather than an epidermis or mesonephros.

Group 2 is the only group in which transplanted tissues contain UP^+ cells, these cells being found in the upper most layer of the stratified mucosa of the duct and surrounded by the mesenchyme. The UP^+ cells stain on their apical areas, which is characteristic of UCs. Whether these UP^+ cells originate from the differentiated epithelium in vitro, and why they have not been confirmed in vitro, in spite of UP gene expression, remains unknown. Various factors may explain these phenomena, such as

the three-dimensional environment, blood supply, or many growth factors. Although ES cells are pluripotent and can differentiate into various cells, the conditions of the in vitro environment before transplantation are important to induce specific forms. Because only the transplanted tissues cultured in KSFM plus conditioned medium from 3T3 cells differentiated into UP^+ cells, we consider the development of UP^+ cells in vivo is not a spontaneous phenomenon.

Other investigators have also found the in vitro environment to be important. Frimberger et al. (2006) have shown that human embryonic germ (EG) cells grown in co-culture with UCs and SMCs exhibit better growth and migration of bladder cells compared with EG cells grown alone. Oottamasathien et al. (2007) have reported differentiation of UP^+ tissues from mouse ES cells, without teratoma formation, by transplanting collagen plagues seeded with ES cells mixed with mesenchymal cells fom rat embryonic bladder into the subrenal capsule of athymic mice. These studies demonstrated the contribution of bladder mesenchymal cells to the differentiation of urotheliua; however, Oottamasathien et al. (2007) have not used direct induction from ES cells alone, as in the present study.

Smooth muscle differentiation can be confirmed by various markers: for example, αSMA is an initially expressed marker, whereas calponin and smooth muscle myosin are expressed later followed by tissue differentiation (Owens 1995). Barker and Gomez (1998) report that αSMA^{+} cells in the urinary bladder begin to appear at 16 days of gestation, and that this phenotypic conversion (from mesenchyme to αSMA-expressing cells) is synchronous with epithelial stratification in rat models. Liu et al. (2000) have described the significance of mesenchymal epithelial interactions and the role of diffusible growth factors, such as keratinocyte growth factor and transforming growth factor α, in the differentiation of SMCs. In our study, spindle-shaped cells containing microfilaments resemble myofibroblasts, which are ultrastructural precursors of SMCs (Drake et al 2006). The results of our RT-PCR analysis suggest the differentiation of SMCs in the cultured tissues. However, SMC development is comparatively limited in the transplanted tissues. Further study is thus needed to evaluate the proliferation of SMCs in vivo and to investigate the relationship between the differentiation of epithelia and SMCs.

The components of the differentiation media used in our study have been

successfully used in other culture studies of urological tissues. Defined KSFM has been employed for the culture of keratinocytes and UCs. The medium contains epidermal growth factor, fibroblast growth factor, and insulin, and is thought to contribute to epithelial growth (Invitrogen 2001). In addition, the significance of fibroblasts for UC growth has been reported (Fujiyama et al. 1995). The long-term culture of UCs and epithelial stratifications have been reported to be well supported by using 3T3 cells as feeders, and 3T3-conditioned medium combined with KSFM has previously been used as a culture medium (Drewa et al. 2006; Ludwikowski et al. 1999; Zang et al. 2001**).**

Karbanova and Mokry (2002) evaluated the structure of EBs histologically, and reported the development of cystic structures consisting of outer cells with endodermal characteristics and inner cells including blood islands, mesenchymal cells with ECMs, and myogenic cells. Our results of the outgrowth of EBs on the mono-layered collagen membrane are similar. Mesenchymal cells (SMCs) are known to be able to synthesize ECMs, such as those containing collagen, fibronectin, and laminin, with collagen as the major component (Baskin et al. 1993; Marcovich et al. 2003**).** The mono-layered collagen membranes used in this study are useful for cell seeding and microscopic

observation during culture, and might have had some effects on the outgrowth and differentiation of ES cells. They also serve as carriers (delivery vehicles) for transplantation.

In conclusion, by using differentiation media and collagen in vitro, we have demonstrated the direct induction of organized layered tissues from ES cells. These tissues have characteristics similar to luminal organs of the urinary tract. Although additional exploration and quantitative analysis are essential to elucidate the mechanism of the differentiation and to prevent the development of teratomas during the induction of fully differentiated tissues, our findings may provide a new approach for the regeneration of urinary tract tissues.

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Figure captions

Fig. 1 a Implanted embryoid body (EB) showed outgrowth on day 4. **b** αSMA^{+} cells appeared in the margin on immunohistochemistry analysis (*boxed area* in **a**). *Bar* 50 μm

Fig. 2 a-c H&E staining of EBs on collagen membrane on 28 day after seeding **a** Group 1. **b** Group 2. **c.** Group 3. Note bundles of smooth muscle cells in **a**, **b** (*black arrows*). **d** An EB from Group 4 on culture day 14 is filled with karyopyknotic necrotizing cells. **e** PCNA-positive cells were present in epithelia in EBs on day 14 in Group 1. The collagen membrane support is seen *bottom* (*asterisks*) in **a**, **c**, **d**. *Bars* 50 μm

Fig. 3 a Immunofluorescent double-staining for CKAE1/3 (*green*) and αSMA (*red*) on day 28 of Group 2 (merged image; *blue* DAPI staining). **b** Immunohistochemical staining of EBs of Group 2 on day 28; stratified epithelia were positive for CK8 **c** EBs of Group 2 were also positive for CK18. **d** αSMA^{+} cells were present in mesenchyme on day 14. (*black arrows* bundles of smooth muscle cells) Collagen membrane supports

are seen *bottom* (*asterisks*) in **b**-**d**. *Bars* 50μm

Fig. 4 Transmission electron microscope images of Group 2 on culture day 28. **a** On the collagen membrane (*asterisk*), four-layered structures were observed that consisted of stratified epithelium (*epi*) with basal lamina (*black arrow-heads*) submucosal layer (*sm*), muscle layer (*m*), and adventitia (*adv*). **b** Epithelium with microvilli and folded intercellular slits (*white arrows*) with some junctional spots (*white arrow-heads*). **c** Smooth muscle layer with bundles of spindle-shaped cells with microfilaments in the mesenchyme (*black arrows*). *Bars* 5μm (**a**), 1 μm. (**b**, **c**)

Fig. 5 RT-PCR analysis of cultured samples. (*ES* embryonic stem cells for control, *EB* embryoid body before seeding, *3*, *7*, *10*, *14*, *21*, *28* embryoid body on relevant days after seeding. *P* mouse urinary bladder for positive control, *asterisk* mouse embryo used as positive control for *Pax-2*, *G1-3* samples obtained from Goups1, 2, and 3, respectively.) These results are summarized in Table 4.

Fig. 6 a Macroscopic appearance of transplants on 28 day (Group 2). *Bar* 5mm **b** H&E staining of transplanted sample from Group 2 (section of tissue shown in **a**). Duct-like structures were observed, although immature cells were also found. *Bar* 100 μm. **c** Non-seeded sample as a control (*arrow* collagen membrane). *Bar* 100μm

Fig. 7 Immunohistochemical staining showing localized αSMA^+ cells (**a**) and diffuse $PCNA^+$ cells (**b)**. Immunofluorescence revealed duct-like structures positive for UPIII (*green* in **c**), CKAE1/3 (*red* in **d**), CK8 (*green* in **e**), and CK18 (*red* in **f**), in serial sections counterstained with DAPI (*blue*). *Bars* 50μm

Table 1. Antibodies used in immunohistochemistry

Table 2. Primer sequences and annealing temperatures for RT-PCR analysis

Table 3. Results of immunohistochemical staining of control tissue and cultured samples

Table 4. Results of RT-PCR

Table 5. Results and proportion of immunohistochemical positive areas in transplants originating from Group2 on day 28