Establishment and Characterization of Primary and Subsequent Subcultures of Normal Mouse Urothelial Cells

(cell culture / mouse urothelium / urinary bladder / differentiation / morphology)

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Abstract. In this study, we report a reliable technique for the harvest, cultivation and expansion of monoculture of NMU. The NMU were harvested by two methods, directly from the urothelium *in vivo* and indirectly from the urothelial outgrowths of bladder explant cultures. Primary cultures and subsequent subcultures were propagated in the mixture of media MCDB 153 and Advanced-DMEM, and conditioned medium. Primary urothelial cells required an initial plating density of 1×10^5 viable cells/cm^2 for survival, while passaged cells needed lower plating densities (1×10^4 viable cells per cm^2). The cultured cells were identified as urothelial by their epithelioid morphology and by the positive immunofluorescence labelling of tight junctional proteins, occludin and ZO-1, adherens protein E-cadherin and cytoskeletal protein cytokeratin 7. Markers of highly differentiated urothelial cells, cytokeratin 20 and uroplakins, were not expressed. Furthermore, the immunofluorescence labelling of occludin and cytokeratin 7 was not detected in later passages when urothelial cells replicated at a high rate. In spite of the use of conditioned medium derived from V79 fibroblast cell culture supernatant, the NMU in the primary cultures and subsequent subcultures expressed a basal/intermediate cell phenotype. In conclusion, we demonstrate that homogeneous long-term culture of NMU can be developed. Since powerful transgenic tools exist to manipulate the mouse genome, our findings should help design the mouse *in vitro* systems for studying the control mechanisms of urothelial cell proliferation, stratification and differentiation in health and disease.

In mammals, the epithelium that lines urinary bladder provides an effective barrier between the urine and the underlying connective tissue. This urothelium consists of three layers; the superficial umbrella cell layer that lines the luminal surface of the bladder, the underlying intermediate cell layer, and the basal cell layer that attaches the urothelium to the connective tissue substratum (Hicks, 1975). The latter two cell layers serve as progenitors for the umbrella cell layer. The large (up to 100 μm in length), highly differentiated umbrella cells have a specialized apical plasma membrane which is composed of multiple, thickened plaques of asymmetric unit membrane (AUM). The plaques are formed by interactions of four transmembrane proteins, uroplakins (Wu et al., 1990; Yu et al., 1994). Together with the highly resistant tight junctions the uroplakins contribute to the permeability barrier function of urothelium (Hu et al., 2002; Acharya et al., 2004).

In recent years, advances in the culture of bladder-derived urothelial cells and the development of different urothelial *in vitro* models have found a number of applications in studying urothelial development and function (Scriven et al., 1997; Truschel et al., 1999), how these functions are disrupted in diseases (Smith et al., 2001; Ehmann and Terris, 2002a), and how the urothelium is re-established during the wound healing process (Kreft et al., 2005). Moreover, many efforts are still expended to engineer the autologous cultured urothelium, which may, in the future, provide material for the surgical reconstruction of urinary bladder.

The cultures of animal urothelial cells provide useful material for study beside the cultures of human urothelial cells, especially when human tissue is not available. The monocultures of normal urothelial cells derived from rats (Zhang et al., 2001; Kurzrock et al., 2005), rabbits (Truschel et al., 1999) and pigs (Guhe and Föllmann, 1994; Ehmann and Terris, 2002b) have been described, but the cell culture model of mouse urothelial cells has not been developed yet. In the literature, the urothelial primary explant cultures of mouse bladder (deBoer et al., 1994; Sterle et al., 1997; Kreft et al., 2002, 2005) and the culture mixture of urothelial cells derived from foetal mouse ureter and fibroblasts (Bryant et al., 2001) have been described.

Therefore, our experiments were aimed at: a) establishing a simple, reliable, and reproducible method for the isolation and culture of normal mouse urothelial cells (NMU); and b) characterizing the cultures in terms of morphology, plating and growth characteristics and expression of antigenic markers associated with urothelium and urothelial differentiation.

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Abbreviations: AUM - asymmetric unit membrane, CK 7 - cytokeratin 7, CK 20 - cytokeratin 20, (M+A)-CM - (MCDB153 medium + Advanced-Dulbecco's modified essential medium)-conditioned medium, NMU - normal mouse urothelial cell, PBS – phosphate-buffered saline.

Material and Methods

Specimens and growth media

Animal experiments were approved by the Slovenian Veterinary Administration of the Ministry of Agriculture, Forestry and Food according to the Animal Health Protection Law and the Instructions for Granting Permits for Animal Experimentation for Scientific Purposes. Urinary bladders were obtained from adult male mice; strain NIH/OlaHsd (25–30 g).

The urothelial explant cultures of mouse bladder were grown in the (M+A) medium, consisting of equal parts of serum-free MCDB153 medium (Sigma, Taufkirchen, Germany) and Advanced-Dulbecco's modified essential medium (Advanced-DMEM) (Invitrogen, Gibco, Paisley, UK) and supplemented with 0.1 mM ethanolamine (Sigma), 0.1 mM phosphoethanolamine (Sigma), 15 μg/ml adenine (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 100 μg/ml streptomycin and 100 U/ml penicillin. The final Ca^{2+} concentration was 1.6 mM.

The primary cultures of NMU and the NMU subcultures were grown in the 1 : 1 mixture of (M+A) and conditioned medium (CM). The CM was prepared by incubating cultures of 90% confluent fibroblast cell line V79 in the Advanced-DMEM (Gibco) supplemented with 5% foetal bovine serum (Gibco) for 24 h. Thus fibroblast-conditioned media were collected and filtered through a 0.22 μm pore size filter. In the (M+A)-CM medium for the primary and subcultured NMU, the Ca^{2+} concentration was 1.0 mM. In all cultures the medium was changed on alternate days and the cultures were daily examined with the inverted microscope (Leica DM IL).

Preparation of urothelial explant cultures of mouse bladder

The urothelial explant cultures were prepared as described previously (Sterle et al., 1997; Kreft et al., 2002). In brief, the bladder was cut sagittally into halves and the region of trigone was excluded from further procedure. The urothelium and underlying lamina propria were separated from the submucosa and muscle layer mechanically using sterile forceps. In this study, the isolated mucosa was transferred directly onto 6-well culture inserts with 0.4 μm porous membranes (Cyclopore membrane, BD Falcon, Heidelberg, Germany) and oriented and spread out, so that the urothelium was on the upper side. The explants' size matched the size of bladder halves (20–35 mm^2). The culture medium (M+A) was introduced into the well containing the insert, so that the underside of the porous membrane was just in contact with the medium, with the tissue positioned at the air-liquid interface. The urothelial explant outgrowths were cultured for 14 days.

Urothelial harvest and primary culture of NMU

To determine the most efficacious technique for harvesting urothelial cells, two types of isolation methods were used: the direct and the indirect method. 1) With the direct method, the cells were isolated directly from the urothelium *in vivo*. The urothelial harvest *via* bladder eversion was used. After the whole bladder had been excised, the bladder neck was reinserted into the lumen, producing the "everted bladder ball" with the urothelial surface exposed. For the urothelial removal, everted bladders were immersed in collagenase IV (100 U/ml) (Sigma) at 37°C on a shaker for 60, 75, 90 or 120 min. After the incubation in collagenase IV, the bladders were placed in a 60-mm Petri dish containing the (M+A)-CM medium. The urothelial cells were gently scraped with a scalpel blade, collected, washed and seeded at different densities: 5×10^3, 1×10^4, 5×10^4, 1×10^5 and 2×10^5 viable cells/cm^2 on 24-well culture plates (BD Falcon, Heidelberg, Germany). 2) With the indirect method, the cells were isolated from 14 days old urothelial outgrowths. First the explants were removed, then the explant outgrowths were incubated in the animal origin-free proteolytic enzyme TrypLE™ Select (Gibco). Thus isolated urothelial cells were seeded at the same densities as the cells isolated by the direct method. The cells were counted in a haemocytometer chamber and viability was evaluated by trypan blue staining. The differences in the cell yield between the two isolation methods were evaluated with the Student's t-test. For immunofluorescence, 1×10^5 viable cells/cm^2 were plated onto 12-well culture inserts with 0.4 μm porous membranes and 0.9 cm^2 effective growth areas (Cyclopore membrane, BD Falcon).

NMU subculture

Just at confluency, the monolayers were incubated in TrypLE™ Select (Gibco) at 37°C for 5 min. The cells were resuspended in the (M+A)-CM medium, centrifuged at 200 *g* for 4 min and replated at different densities: 5×10^3, 1×10^4, 5×10^4 and 1×10^5 viable cells/cm^2 on 24-well culture plates (BD Falcon) or culture flasks (25 cm^2; TPP, Trasadingen, Switzerland). For immunofluorescence, 1×10^4 viable cells/cm^2 were plated onto 12-well culture inserts with 0.4 μm porous membranes and 0.9 cm^2 effective growth areas (Cyclopore membrane, BD Falcon).

Immunofluorescence of occludin, ZO-1, E-cadherin, CK 7, CK 20, uroplakins and vimentin

Cultures grown on the porous membranes were washed in PBS, pH 7.2, and fixed in absolute ethanol for 25 min at room temperature. The indirect immunofluorescence reaction was performed as described previously (Kreft et al., 2002, 2005). The panel of antibodies used was: occludin (rabbit polyclonal antibody, 71-1500, Zymed Laboratories, San Francisco, CA), ZO-1 (rabbit

polyclonal antibody, 61-7300, Zymed Laboratories), E-cadherin (mouse monoclonal antibody, clone: 34, Transduction Laboratories, Lexington, KY), cytokeratin 7 (CK 7) (mouse monoclonal antibody, clone: OV-TL 12/30, Dako, Glostrup, Denmark), cytokeratin 20 (CK 20) (mouse monoclonal antibody, clone: K_s20.8, Dako), uroplakins (rabbit polyclonal anti-AUM antibody, kindly provided by Prof. T.-T. Sun, New York University, School of Medicine), and vimentin (mouse monoclonal antibody, clone: Vim 3B4, Dako). Adequate secondary antibodies were conjugated with FITC (Sigma). For negative controls, the incubation with primary antibody was omitted or the specific primary antibody was replaced by a non-relevant antibody. The cultures were examined in a fluorescence microscope Nikon Eclipse TE 300.

Results

We have shown that using the direct or indirect isolation technique, normal mouse urothelial cells can be cultured reliably. The similarities and differences between the two types of the primary cultures of NMU and NMU subcultures are presented in Table 1.

There were significant differences ($P < 0.05$) in the cell yield between the direct and indirect isolation method. The number of viable cells isolated per animal and cell viability were higher when the urothelial cells were isolated indirectly *via* urothelial explant outgrowths than directly from urothelium *in vivo* (22×10^4 *vs.* 6×10^4 viable cells/animal; 58% *vs.* 33% cell viability). With the direct method, the optimal incubation time in collagenase IV was 75 minutes, which was shorter than the incubation time used for human urothelial samples (120 minutes) (Sugasi et al., 2000). In our study, the shorter incubation times resulted in the incomplete peel-off of urothelial cells, and the longer incubation times softened the urinary bladder to such a degree that the fibroblasts and smooth-muscle cells could be peeled-off together with the urothelial cells. The cell viabilities did not significantly change when shorter or longer incubation times with collagenase IV were used.

Obtaining the minimal plating density of viable cells per cm^2 was a crucial prerequisite for the establishment of primary cultures of NMU. We found out that the plating density of 1×10^5 viable cells/cm^2 was minimal for the establishment of the primary cultures of NMU. If this plating density was not reached, then the cultures failed to reach the confluence and the urothelial cells became senescent and degraded in 7-10 days. However, the plating efficiency in the primary cultures of NMU was comparable between the direct and indirect isolation method. At 24 h after seeding only few cells were attached on the culture plastic or porous membrane in both cases. The minimal plating density needed for the establishment of NMU subcultures was 1×10^4 viable cells/cm^2. The cells began to attach to the culture plastic or porous membrane already within an hour of seeding and after 24 h most of the cells were attached.

Although NMU in the primary cultures and subcultures were showing a characteristically epithelioid morphology with the phase-bright intercellular borders (Fig. 1), some differences between the primary cultures and subcultures were noticed. The primary cultures were grown as colonies of tightly packed polygonal cells (Fig. 1a, 1b), while the subcultures were grown

Table. 1. Comparison of primary cultures of NMU and NMU subcultures

		Primary cultures of NMU		NMU subcultures	
		Direct isolation	**Indirect isolation**	**Passages 1-2**	**Passages 3-7**
Growth medium		(M+A)-CM	(M+A)-CM	(M+A)-CM	
No. of isolated viable cells/animal		6×10^4	22×10^4		
Cell viability		33%	58%	74%	
Minimal seeding density		1×10^5 viable cells/cm^2		1×10^4 viable cells/cm^2	
Attached cells after 24 h		few	few	most	
Days to reach confluence		8-14	8-14	3-4	
Shape and size of cells		Various shapes and sizes		Polygonal shapes and uniform size	
Immunofluorescence[a]	occludin	++	++	+	–
	ZO-1	+++	+++	++	+
	E-cadherin	+++	+++	++	+
	CK 7	+	+	+	–
	CK 20	–	–	–	–
	uroplakins	–	–	–	–
	vimentin	–/+	–	–	–

[a] The immunofluorescence reaction was scored subjectively from - (negative) to +++ (strongly positive).

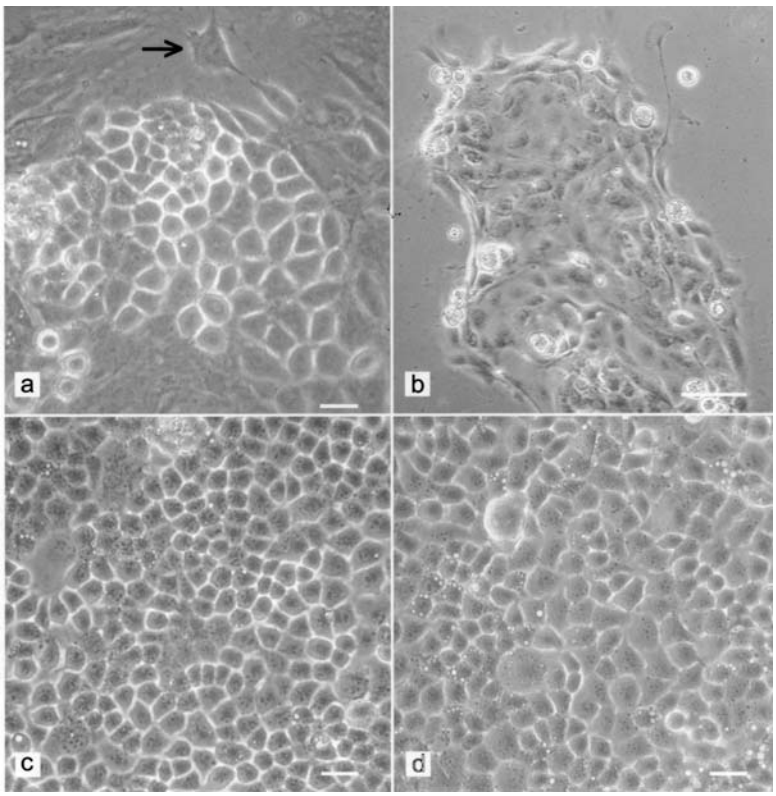

Fig. 1. Phase-contrast micrographs of normal mouse urothelial cells in primary cultures **(a, b)** and subcultures (4th passage) **(c, d)**. Note the morphological similarity between the urothelial cells in the subculture derived from the primary culture isolated directly from urothelium *in vivo* (c), and the urothelial cells in the subculture derived from the primary culture isolated indirectly *via* explant culture (d). Fibroblast (arrow). Scale bars, 20 μm in a, c, d; 50 μm in b.

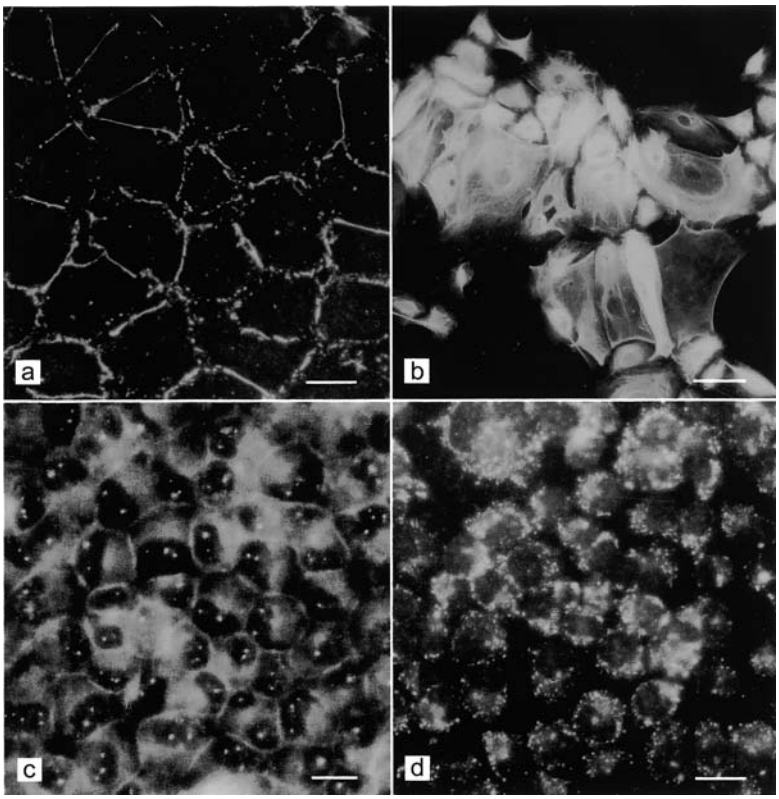

Fig. 2. Immunofluorescence of NMU subcultures: **(a, b)** 1st passage, and **(c, d)** 4th passage. (a) Punctate and patchy labelling of occludin is detected in the cell-cell junctions. (b) CK 7- positive NMU are detected. CK 7 is mainly organized in filaments. (c) ZO-1 is distributed in a continuous line around the cells. (d) E-cadherin is distributed in a punctuate and patchy line around the cells. The cells in the 4th passage are smaller than those in the 1st passage. Scale bars, 10 μm.

more equably over the growth area. The cells in the primary cultures were larger and more heterogeneous in shape than cells in the subsequent subcultures (Fig. 1c, 1d), and in primary cultures the cells needed 8-14 days to reach the confluence, while in subcultures the cells needed only 3-4 days for the equal growth area. Morphologically it was impossible to distinguish between NMU subcultures derived from the directly or indirectly established primary cultures. However, the cell morphology was a good indicator of the proliferative capacity of the culture. Slowly dividing or senescent cultures were made up of larger cells that showed a high

degree of morphologic heterogeneity. By contrast, highly proliferative cultures consisted of a characteristic small and morphologically homogeneous cell type. The NMU subcultures were expanded and maintained until the 7th passage and after that they were frozen.

The immunolabelling of primary cultures and subsequent subcultures (1st and 2nd passage) revealed that NMU were positive for occludin, ZO-1, E-cadherin and CK 7, thus confirming their epithelial origin. The immunofluorescence of tight junction protein occludin was punctate and patchy (Fig. 2a) in these passages, while in later passages it vas negative. Similarly, CK 7 was expressed and localized in filaments in the primary NMU cultures and first two passages of NMU subcultures (Fig. 2b), while in the later passages decreasing CK 7 immunolabelling was detected. On the contrary, the tight junction protein ZO-1 was distributed at the cell surface as a continuous line around the cells in all seven passages (Fig. 2c). E-cadherin, which specifies zonula adherens, was localized in the primary cultures and the first passages of subcultures as a continuous line around the cells, but in the later passages it was localized as a punctate line around the cells (Fig. 2d). The immunolabelling with antibodies against CK 20 and uroplakins revealed that neither CK 20 nor uroplakins were present in the primary cultures of NMU and NMU subcultures. Fibroblasts were occasionally seen in the primary cultures of NMU isolated directly from the urothelium *in vivo* (Fig. 1a), but their growth gradually diminished and in the subsequent subcultures the fibroblasts were never seen. Moreover, the immunofluorescence labelling of vimentin was negative in these subcultures. In the primary cultures of NMU isolated indirectly, fibroblasts were never seen and immunolabelling of vimentin was negative (Table 1).

Discussion

In this study, we have demonstrated that homogeneous long-term culture of NMU can be developed. Since there was no existing protocol for culturing the NMU, we established a simple, reliable, and reproducible method for the isolation and culture of NMU.

Obtaining the minimal plating density of viable cells per cm^2 was a crucial prerequisite for the establishment of primary cultures of NMU. In the literature there are many different data about the minimal plating density of urothelial cells (1×10^4 to 1×10^6 cells/cm^2) (Guhe and Fölmann, 1994; Southgate et al., 1994; Truschel et al., 1999; Sugasi et al., 2000; Zhang et al., 2001). We found out that the plating density of 1×10^5 viable cells/cm^2 was minimal for the establishment of the primary cultures of NMU. If this plating density was not reached, then the cultures failed to reach confluence and the urothelial cells became senescent and degraded. The number of viable cells isolated per animal was about four times higher when the urothelial cells were isolated indirectly *via* urothelial explant outgrowths than directly from urothelium *in vivo* (22×10^4 *vs.* 6×10^4 viable cells/animal). These results are consistent with our observation that, on average, from the initial 25 mm^2 halves of mouse bladders (explants), 100 mm^2 of three-to-four-layered urothelium was produced in 14 days. Therefore, approximately four times more urothelial cells could be harvested from one urinary bladder *via* explant culture than directly from one urinary bladder *in vivo*. However, the plating efficiency in the primary cultures of NMU was comparable between the direct and indirect isolation method. At 24 h after seeding only few cells were attached on the culture plastic or porous membrane in both cases. On the basis of our previous studies, the urothelial cells in the explant outgrowths form highly differentiated urothelium (Kreft et al., 2002), but the immunofluorescence labelling of microtubular protein β-tubulin and DNA labelling with DAPI detect only a few mitotic basal cells (Kreft et al., 2005). We suppose that the average number of isolated viable, attachable and proliferative urothelial basal cells per animal might be similar by both methods.

The minimal plating density needed for the establishment of NMU subcultures was 1×10^4 viable cells/cm^2. The cells began to attach to the culture plastic or porous membrane within an hour after seeding, and after 24 h most of the cells were attached. Thus, for subculture, the cells would tolerate much lower plating density than for the primary culture. Similarly, the subcultures of normal human urothelial cells survived even at as low plating densities as 2.5×10^4 cells/cm^2 (Southgate et al., 1994). Since the importance of stromal-epithelial interactions in the growth, morphogenesis, and differentiation of bladder tissue has been variously detailed in the literature (Howlett et al., 1986; Master et al., 2003), the lower plating densities of NMU might be expected when the substrata coated with the components of extracellular matrix are used.

Considering the fact that the continuous paracrine signals from the lamina propria are a prerequisite for normal growth and differentiation of urothelial cells (Kreft and Sterle, 2000; Zhang et al., 2001), we have used the conditioned culture medium (M+A)-CM that stimulated the growth of urothelial cells and inhibited the growth of fibroblasts. The fibroblasts were occasionally seen in the primary cultures of NMU isolated directly from the urothelium *in vivo*, but their growth gradually diminished and in the subsequent subcultures the fibroblasts were never seen. In the primary cultures of NMU isolated indirectly, fibroblasts were never seen. That additionally confirmed the urothelial purity of explant outgrowths and the inability of fibroblasts to survive in the (M+A)-CM medium. The immunofluorescence analysis of occludin, ZO-1, E-cadherin, CK 7, CK 20, and uroplakins showed that some markers associated with the urothelium and urothelial differentiation were disappearing through passages (occludin, CK 7),

some were never present (CK 20, uroplakins) and some were present through all seven passages (ZO-1, E-cadherin). These results are consistent with the finding that ZO-1 might be localized in the tight junctions of superficial umbrella cells as well as along the sites of cell-cell contact in all three epithelial cell layers (Acharya et al., 2004). The immunofluorescence of tight junctional protein occludin was punctate and patchy, much weaker than it is in the highly differentiated urothelium *in vivo* (Acharya et al., 2004) and *in vitro* (Kreft et al., 2002). Recently, we have noticed a comparable immunofluorescence labelling of occludin an hour after the urothelial full-thickness injury in the urothelial wound healing model (Kreft et al., 2005). This indicates that tight junctions in the NMU cultures were not fully developed. Since the immunolabelling with antibodies against CK 20 and uroplakins revealed that neither CK 20 nor uroplakins, both specific and sensitive markers of urothelial differentiation, were present in the primary cultures of NMU and NMU subcultures, we assume that the NMU in the primary cultures and subsequent subcultures expressed the basal/intermediate cell phenotype without the evidence of terminal differentiation. The addition of specific growth factors and the components of extracellular matrix might stimulate the proliferation and differentiation of NMU *in vitro.* The exact influence of specific growth factors and cell-to-matrix interactions on the differentiation process of NMU would require further investigation. Moreover, it seems that the conditioned medium derived from V79 fibroblast cell culture supernatant is not sufficient to induce the terminal differentiation of cultured NMU. Further studies will define whether species-specific fibroblast and/or smooth-muscle cell culture supernatant would be more appropriate for induction of the terminal differentiation of NMU cultures.

In conclusion, we have shown that using the direct or indirect isolation technique, normal mouse urothelial cells can be cultured reliably. The normal mouse urothelial cells can replicate at a high rate and can be extensively expanded in the culture if the minimal plating density of viable cells is reached. Since powerful transgenic tools exist to manipulate the mouse genome, we believe that the described cultures of normal mouse urothelial cells will improve and make easier further urinary bladder investigations.

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References

Acharya, P., Beckel, J., Ruiz, W.G., Wang, E., Rojas, R., Birder, L., Apodaca, G. (2004) Distribution of the tight junction proteins ZO-1, occludin, and claudin-4, -8, and -12 in bladder epithelium. *Am. J. Physiol. Renal Physiol.* **287,** F305-F318.

Bryant, P. L., Reid, L. M., Diala, E. S., Abbott, B. D., Buckalew, A. R. (2001) An in vitro model for murine ureteric epithelial cells. *In Vitro Cell Dev. Biol. Anim.* **37,** 395-398.

deBoer, W. I., Rebel, J. M., Vermey, M., deJong, A. A., van der Kwast T. H. (1994) Characterization of distinct functions for growth factors in murine transitional epithelial cells in primary organotypic culture. *Exp. Cell Res.* **214,** 510-518.

Ehmann, U. K., Terris, M. K. (2002a) Juxtacrine stimulation of normal and malignant human bladder epithelial cell proliferation. *J. Urol.* **167,** 735-741.

Ehmann, U. K., Terris M. K. (2002b) Long-term culture of porcine bladder epithelial cells. *In Vitro Cell Dev. Biol. Anim.* **38,** 137-141.

Guhe, C., Föllmann, W. (1994) Growth and characterization of porcine urinary bladder epithelial cells in vitro. *Am. J. Physiol.* **266,** F298-F308.

Hicks, R. M. (1975) The mammalian urinary bladder: an accommodating organ. *Biol. Rev.* **50,** 215-246.

Howlett, A. R., Hodges, G. M., Rowlatt, C. (1986) Epithelial-stromal interactions in the adult bladder: urothelial growth, differentiation, and maturation on culture facsimiles of bladder stroma. *Dev. Biol.* **118,** 403-415.

Hu, P., Meyers, S., Liang, F.-X., Deng, F.-M., Kachar, B., Zeidel, M. L., Sun, T.-T. (2002) Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. *Am. J. Physiol. Renal Physiol.* **283,** F1200-F1207.

Kreft, M. E., Sterle, M. (2000) The effect of lamina propria on the growth and differentiation of urothelial cells in vitro. *Pflugers Arch.* **440,** R181-R182.

Kreft, M. E., Romih, R., Sterle, M. (2002) Antigenic and ultrastructural markers associated with urothelial cytodifferentiation in primary explant outgrowths of mouse bladder. *Cell Biol. Int.* **26,** 63-74.

Kreft, M. E., Sterle, M., Veranič, P., Jezernik, K. (2005) Urothelial injuries and the early wound healing response: Tight junctions and urothelial cytodifferentiation. *Histochem. Cell Biol.* **123,** 529-539.

Kurzrock, E. A., Lieu, D. K., DeGraffenried, L. A., Isseroff, R. R. (2005) Rat urothelium: improved techiques for serial cultivation, expansion, freezing and reconstitution onto acellular matrix. *J. Urol.* **173,** 281-285.

Master, V. A, Wei, G., Liu, W., Baskin, L. A. (2003) Urothelium facilitates the recruitment and trans-differentiation of fibroblasts into smooth muscle in acellular matrix. *J. Urol.* **170,** 1628-1632.

Scriven, S. D., Booth, C., Thomas, D. F. M., Trejdosiewicz, L. K., Southgate, J. (1997) Reconstitution of human urothelium from monolayer cultures. *J. Urol.* **158,** 1147-1152.

Smith, B. A., Kennedy, W. J., Harnden, P., Selby, P. J., Trejdosiewicz, L. K., Southgate, J. (2001) Identification of genes involved in human urothelial cell-matrix interactions: implications for the progression pathways of malignant urothelium. *Cancer Res.* **61,** 1678-1685.

Southgate, J., Hutton, K. A. R., Thomas, D. F. M., Trejdosiewicz, L. K. (1994) Normal human urothelial cells in vitro: proliferation and induction of stratification. *Lab. Invest.* **71,** 583-594.

Sterle, M., Kreft, M. E., Batista, U. (1997) The effect of epidermal growth factor and transforming growth factor β1

on proliferation and differentiation of urothelial cells in urinary bladder explant culture. *Biol. Cell* **89,** 29-41.

Sugasi, S., Lesbros, Y., Bisson, I., Zhang, Y. Y., Kucera, P., Frey P. (2000) In vitro engineering of human stratified urothelium: Analysis of its morphology and function. *J. Urol.* **164,** 951-957.

Truschel, S. T., Ruitz, W. G., Shulman, T., Pilewski, J., Sun T.-T., Zeidel, M. L., Apodaca, G. (1999) Primary uroepithelial cultures. A model system to analyze umbrella cell barrier function. *J. Biol. Chem.* **274**, 15020-15029.

Wu, X.-R, Manabe, M., Yu, J., Sun, T.-T. (1990) Large scale purification and immunolocalization of bovine uroplakins I, II and III. Molecular markers of urothelial differentiation. *J. Biol. Chem.* **256,** 19170-19179.

Yu, J., Lin, J. H., Wu, X.-R., Sun, T.-T. (1994) Uroplakins Ia and Ib, two major differentiation products of bladder epithelium, belong to a family of four transmembrane domain (4TM) proteins. *J. Cell. Biol.* **125,** 171-182.

Zhang, Y. Y., Ludwikowski, B., Hurst, R., Frey, P. (2001) Expansion and long-term culture of differentiated normal rat urothelial cells in vitro. *In Vitro Cell Dev. Biol. Anim.* **37,** 419-429.