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 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 (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.
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 Ca2+ 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 Ca2+ 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 flasks (25 cm²; TPP, Trasadingen, Switzerland). For immunofluorescence, 1 × 10⁵ viable cells/cm² were plated onto 12-well culture inserts with 0.4 µm porous membranes and 0.9 cm² effective growth areas (Cycloparen 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 × 10³, 1 × 10⁴, 5 × 10⁴ and 1 × 10⁵ viable cells/cm² on 24-well culture plates (BD Falcon) or culture flasks (25 cm²; TPP, Trasadingen, Switzerland). For immunofluorescence, 1 × 10⁴ viable cells/cm² were plated onto 12-well culture inserts with 0.4 µm porous membranes and 0.9 cm² effective growth areas (Cycloparen 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: Ks20.8, 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 cm² was a crucial prerequisite for the establishment of primary cultures of NMU. We found out that the plating density of 1×10⁴ viable cells/cm² 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 1×10⁴ viable cells/cm². 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

<table>
<thead>
<tr>
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<th>Primary cultures of NMU</th>
<th>NMU subcultures</th>
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<tr>
<td></td>
<td>Direct isolation</td>
<td>Indirect isolation</td>
</tr>
<tr>
<td>Growth medium</td>
<td>(M+A)-CM</td>
<td>(M+A)-CM</td>
</tr>
<tr>
<td>No. of isolated viable cells/animal</td>
<td>6×10⁴</td>
<td>22×10⁴</td>
</tr>
<tr>
<td>Cell viability</td>
<td>33%</td>
<td>58%</td>
</tr>
<tr>
<td>Minimal seeding density</td>
<td>1×10⁴ viable cells/cm²</td>
<td>1×10⁴ viable cells/cm²</td>
</tr>
<tr>
<td>Attached cells after 24 h</td>
<td>few</td>
<td>few</td>
</tr>
<tr>
<td>Days to reach confluence</td>
<td>8-14</td>
<td>8-14</td>
</tr>
<tr>
<td>Shape and size of cells</td>
<td>Various shapes and sizes</td>
<td>Polygonal shapes and uniform size</td>
</tr>
<tr>
<td>occludin</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ZO-1</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>E-cadherin</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>CK 7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CK 20</td>
<td>–</td>
<td>–</td>
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<tr>
<td>uroplakins</td>
<td>–</td>
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<tr>
<td>vimentin</td>
<td>–/+</td>
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a The immunofluorescence reaction was scored subjectively from - (negative) to +++ (strongly positive).
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

**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.
degree of morphologic heterogeneity. By contrast, highly proliferative cultures consisted of a characteris-
tic 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 subse-
quently 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 was negative. Similarly, CK 7
was expressed and localized in filaments in the primary
NMU cultures and first two passages of NMU subcul-
tures (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 uro-
plakins 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 immuno-
fluorescence labelling of vimentin was negative in these
subcultures. In the primary cultures of NMU iso-
lated indirectly, fibroblasts were never seen and
immunolabelling of vimentin was negative (Table 1).

Discussion

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

Obtaining the minimal plating density of viable cells
per cm² was a crucial prerequisite for the establish-
ment of primary cultures of NMU. In the literature there
are many different data about the minimal plating density
of urothelial cells (1 x 10⁴ to 1 x 10⁶ cells/cm²) (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 x 10⁵ viable
cells/cm² was minimal for the establishment of the pri-
mary 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 x 10⁴ vs. 6 x
10⁴ viable cells/animal). These results are consistent
with our observation that, on average, from the initial
25 mm² halves of mouse bladders (explants), 100 mm² of
three-to-four-layered urothelium was produced in 14
days. Therefore, approximately four times more urothe-
hal cells could be harvested from one urinary bladder
via explant culture than directly from one urinary blad-
ner in vivo. However, the plating efficiency in the pri-
mary cultures of NMU was comparable between the
direct and indirect isolation method. At 24 h after seed-
ing 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 out-
growths 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 estab-
lishment of NMU subcultures was 1 x 10⁴ viable
cells/cm². The cells began to attach to the culture plas-
tic 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 sub-
cultures of normal human urothelial cells survived even
at as low plating densities as 2.5 x 10⁴ cells/cm² (South-
gate 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 compo-
nents 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 occa-
sionally 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 NMU isolated indirectly via urothelial explant outgrowths
and the inability of fibroblasts to survive in the (M+A)-CM medium. The immunofluo-
rescence analysis of occludin, ZO-1, E-cadherin, CK 7,
CK 20, and uroplakins showed that some markers asso-
ciated 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 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 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**


