Author’s response to reviews

Title: Expression of components of the urothelial cholinergic system in bladder and cultivated primary urothelial cells of the pig

Authors:

Dorothea Leonhäuser (dleonhaeuser@ukaachen.de)
Jasmin Kranz (jasminkranz@gmx.de)
Regina Leidolf (regina.leidolf@vetmed.uni-giessen.de)
Ulrich Schwantes (ulrich.schwantes@dr-pfleger.de)
Joachim Geyer (joachim.m.geyer@vetmed.uni-giessen.de)
Joachim Grosse (joachim.grosse1963@gmail.com)

Version: 2 Date: 22 Mar 2019

Author’s response to reviews:

Answer to the reviewer´s comments

Dear Prof. McDermott

Thank you for your questions concerning our manuscript titled “Expression of components of the urothelial cholinergic system in bladder and cultivated primary urothelial cells of the pig”

Regarding your first question:

1. What age were the animals that the bladders were taken from? Do they represent the aged population who typically experience bladder dysfunction?

The Göttingen Minipigs (GM) used for the animal studies were fully grown up, which means they were about two years old. German Landrace pigs (GL) are bred for meat production and thus get mature much faster. The Landrace pigs of which we used the urinary bladders were about six months old. Therefore, the animals used in the study do not represent the aged population. However, the Minipigs as well as the Landrace pigs are not supposed to develop any kind of bladder dysfunction without specific interventions.

To clarify these two points, we inserted the following text in the “Materials and Methods” section, page 5, lines 107-9:

Göttingen Minipigs (GM) were about two years old whereas the German Landrace (GL) pigs were six months old.

And in the “Discussion” section, page 13, lines 287-9, including two references concerning the growth development of the pigs:
Furthermore, the animals in this study were mature but not old (27, 28) and thus, do not represent the aged population which suffer from bladder dysfunctions.


2. There was a large decrease in mRNA expressions for some targets (e.g. OCTs and M1-3) but not others (e.g. M4 and M5). I think that the authors need a address in the discussion whether these targets are functionally relevant in the urothelium. What if anything is known.

Again, thank you for your inquiry. You were right that we needed to further address the functions of our analysed targets. We therefore added the following text passages into the “Discussion” section, page 12, lines 267-78:

Bexten et al. were able to show that, on the one hand, TrCL is a substrate of the aforementioned solute carriers OCT1 and OATP1A2 (uptake), but on the other hand also for the efflux carrier ABCB1 (19). The in vitro downregulation of the uptake transporters in combination with the still relatively high amount of the ABCB1 efflux carrier would lead to a non-physiological shift compared to the actual in vivo situation. This cultivation-related downregulation could also be observed for the muscarinic receptors, especially M2 and M3, which play a crucial role in the storing and voiding mechanisms of the bladder. M3 is known to mediate the contractile response and thus is addressed by the main muscarinic receptor antagonist TrCL (12). This has to be considered for uptake studies with anticholinergic drugs like TrCL. However, downregulation of M1-M5 has also been demonstrated for human urothelial cells in culture by Tyagi et al. (14).

Dear Sir or Madam,

Thank you, too, for your interest and the question on our study.
To strengthen the authors’ results and conclusions, I would recommend validation some of all of the receptors assayed by qPCR by IHC or IF.

Actually, we tried to establish some immunohistochemical stainings but mostly we had to use antibodies designed for human tissue. Nevertheless, we were able to establish two stainings for the relevant targets muscarinic acetylcholine receptor M2 and M3. We therefore added “Fig. 2” and changed the text of the “Materials and Methods” section on page 6, lines 131-49 and inserted a table on page 6, line 151 with the used primary antibodies.

Immunohistochemical staining of tissue and cells

Validation of the cell type was performed via immunohistochemistry. Therefore, confluent UC were detached with trypsin-EDTA (Life Technologies), washed with phosphate buffered saline (PBS) (Life Technologies) and fixed in 4% (w/v) phosphate buffered formaldehyde (Merck). After further centrifugation, supernatant formaldehyde was removed and the cells were mixed
with 3% (w/v) agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The agarose cell-hybrids were cooled for 3 min. in a fridge at 4°C. Native bladder tissue served as the control and was also used for staining of muscarinic receptors M2 and M3 and therefore fixed in phosphate buffered formaldehyde. The cell-hybrids and the native tissue were dehydrated, embedded in paraffin and cut into 3 µm sections.

Antigen retrieval of deparaffinised sections was performed using citrate-buffer (Zytomed Systems GmbH, Berlin, Germany) in a steamer for 30 min. Primary monoclonal and polyclonal antibodies (Tab. 1) were incubated for 1 h, and secondary antibody and chromogen development (DAKO Real EnVision HRP rabbit/mouse with DAB) were applied according to the manufacturer’s protocol. Counterstaining was performed using haemalaun (Merck). Staining was observed using a Leica DM6000B and integrated software Diskus (4.80.5909, Hilgers, Technisches Büro, Königswinter, Germany).

Tab. 1 Primary antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone number</th>
<th>Reactivity</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PanCK</td>
<td>AE1/AE3</td>
<td>Monoclonal mouse, anti-human</td>
<td>1:300</td>
<td>1 h</td>
<td>Dako GmbH, Hamburg, Germany</td>
</tr>
<tr>
<td>Anti-Muscarinic Acetylcholine Receptor 2</td>
<td>31-1D1</td>
<td>Monoclonal mouse, anti-human</td>
<td>1:100</td>
<td>1 h</td>
<td>Abcam, Cambridge, United Kingdom</td>
</tr>
<tr>
<td>Anti-Muscarinic Acetylcholine Receptor M3</td>
<td></td>
<td>Polyclonal rabbit, anti-pig</td>
<td>1:100</td>
<td>1 h</td>
<td>Abcam, Cambridge, United Kingdom</td>
</tr>
</tbody>
</table>

“Results”, page 9, lines 181-3:

The presence of muscarinic receptors M2 and M3, most relevant for storing and voiding of urine in the bladder, could also be visualized in the native urothelium of the German Landrace pig (Fig. 2a+b).

“Results”, page 9, lines 190-3:

Fig. 2: Immunohistochemical staining of muscarinic receptors in native bladder tissue and derived urothelial cells. a+c) Muscarinic receptor M2 and b+d) M3 could be visualized in a+b) native bladder tissue of the German Landrace pig but c+d) not in the derived urothelial cells at passage 1. Scale bar = 50 µm.

“Results”, page 10, lines 215-7:

The downregulation of muscarinic receptors M2 and M3 could also be visualized by immunohistochemical staining (Fig. 2c+d).
Please note, that we added Patrick Arndt as co-author, since the revised manuscript required the use of experimental work that he performed and analysed. Because of that and his revisions to the manuscript Mr. Arndt’s contribution grew overall significantly, so that listing him in the acknowledgment section would no longer be sufficient.