ION HOMEOSTASIS AND TRANSPORT ARE REGULATED BY GENES DIFFERENTIALLY EXPRESSED IN PORCINE BUCCAL POUCH MUCOSAL CELLS DURING LONG-TERM CULTURE IN VITRO – A MICROARRAY APPROACH

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Abstract
The oral mucosa is a compound tissue composed of several cells types, including fibroblasts and keratinocytes, that are characterized by different morphology, as well as biochemical and metabolomic properties. The oral mucosal cells are the most important factors mediated between transport and drugs delivery. The changes in cellular ion homeostasis may significantly affect the bioavailability of administrated drugs and their transport across the mucous membrane. Therefore we investigated the expression profile of genes involved in ion transport and homeostasis in porcine buccal pouch mucosal cells. The oral mucosa was separated surgically and isolated enzymatically. The cells were examined during long-term in vitro culture (IVC). The cultured cells were collected at 7, 15 and 30 days of IVC and subsequently transferred to RNA isolation and next, the gene expression profile was measured using Affymetrix microarray assays.

In the results, we can extract genes belonging to four ontology groups: “ion homeostasis”, “ion transport”, “metal ion transport”, and “inorganic ion homeostasis”. For TGFbeta1 and CCL2, we observed up-regulation after 7 days of IVC, down-regulation after 15 days of IVC and upregulation again after 30 days of IVC. The ATP13A3, ATP1B1, CCL8, LYN, STEAP1, PDPN, PTGS2, and SLC5A3 genes showed high activity after day 7 of IVC, and in the days 15 and 30 of IVC showed low activity.

We showed an expression profile of genes associated with the effects of ion influence on the porcine normal oral mucosal cell development in IVC. These studies may be the starting point for further research into oral diseases and will allow for the comparison of the gene expression profile of normal and disease altered cells.

Running title: Ion homeostasis and transport in porcine buccal pouch mucosal cells

Keywords: porcine oral mucosal cells, ion homeostasis, ion transport, microarrays

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Introduction

The oral mucosa is a compound tissue composed of several cell types, including fibroblasts and keratinocytes, that are characterized by different morphology, as well as biochemical and metabolomic properties. Moreover, both of these cell populations displayed various capacities for proliferation and differentiation during primary in vitro culture (IVC). Therefore, it brings several difficulties in the process of monolayer and/or 3D primary culture of these cells in vitro. Our experiments indicated that the monolayer mucosal fibroblast and keratinocyte culture system require to develop advanced culture media and supplements that are necessary at the beginning of IVC when the „chemical“ first step separation must be achieved. However, the differential cellular morphology is the first indicator of proper cell separation before long-term primary culture. Our recent studies indicated that oral mucosal cells are also characterized by different potency to grow, proliferate and differentiate during both short- and, long-term primary culture [1, 2]. We found differential expression of proliferation markers such as Ki67, PCNA, and involucrin as well as differentiation markers such as MAP1B, DAB2, FN1, SOX9 and CXCL12 [3, 4]. The differentiation and stem-like cell specificity of mucosal cells were well defined in our recent microarray studies. These experiments also showed a huge regenerative capacity of oral mucosal cells, which was indicated by expression of genes involved in wound healing, neoangiogenesis, and vasculature development during primary IVC. However, the stemness plasticity and proliferation/differentiation capacity were recently intensively investigated in our studies (data not published), the biochemical and metabolomic features of mucosal cells are still not entirely known. However, our recent study first indicated the differential expression pattern of connexins (Cx36, Cx37, Cx40, Cx43, and Cx45) in porcine buccal pouch mucosal cells during long-term in vitro culture [2]. These experiments indicated the existence of functional GJC connections in mucosal cells as well as the association between connexin expression and proliferative capacity of cells, analyzed in real-time.

The function of oral mucosal cells, as the most important factors mediated between drug transport and delivery, is well defined. However, the inorganic ion metabolism still needs further investigations. Therefore in this study, we researched for genes expression profile involved in ion homeostasis and transport in porcine buccal pouch mucosal cells during long-term primary culture in vitro using microarray assays, for the first time.

Material and Methods

Animals

For this study, a total of 30 pubertal crossbred Landrace gilts bred on a commercial local farm were used. They had a mean age of 155 days (range 140 – 170 days), and the mean weight was 100 kg (95-120 kg). All of the animals were housed under identical conditions and fed the same forage (depending on age and reproductive status). The experiments were approved by the Local Ethical Committee of the Poznan University of Life Sciences, Poland (permission no. 32/2012, 30.06.2012).

Cell isolation and culture

After slaughter, samples of buccal pouch mucosa were obtained within 40 min and transported to the laboratory. The excised tissue was washed twice in Dulbecco’s phosphate buffered saline (D-PBS; Sigma Aldrich, Madison, USA). The surface of the buccal pouch was surgically removed using sterile blades. The cell suspension obtained from this digestion was filtered through a mesh to remove non-dissociated tissue fragments and then incubated with 0.05% collagenase I (Sigma Aldrich, Madison, USA) for 40 min at 37 °C in a shaking water bath. Isolated cells were washed three times by centrifugation (10 min at 300 g) with Dulbecco’s modified Eagle’s medium (DMEM; Sigma Aldrich, Madison, USA) supplemented with gentamicin (20 μg/mL) and 0.1% BSA. The epidermal side of the mucosa was gently scraped with a scalpel to detach the keratinocytes. The resultant cell suspension was filtered through a 400-micron mesh and then washed three times by centrifugation (10 min at 300 g) with supplemented DMEM. The final cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Sigma Aldrich, Madison, USA) and 100 U/ml penicillin G, 10 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cell viability was 90 to 95% as determined by trypan blue staining (Sigma Aldrich, Madison, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Once the keratinocyte cultures attained 70–80% confluence, they were passaged by washing with PBS, digested with 0.025% trypsin/EDTA (Cascade Biologics, Portland, USA), neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifuged, and resuspended at a seeding density of 2x10⁴ cells/cm². The culture medium was changed every three days.

Microarray expression analysis and statistics

Total RNA (100 ng) from each pooled sample was subjected to two rounds of sense cDNA amplification (Ambion® WT Expression Kit). The obtained cDNA was used for biotin labelling and fragmentation by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labelled fragments of cDNA (5.5 μg) were hybridized to the Affymetrix® Porcine Gene 1.1 ST Array Strip (48°C/20 h). Microarrays were then washed and stained according to the technical protocol using the Affymetrix GeneAtlas Fluidics Station. The array strips were scanned employing Imaging Station of the GeneAtlas System. Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The
quality of gene expression data was confirmed according to the quality control criteria provided by the software. The obtained CEL files were imported into downstream data analysis software.

All of the presented analyses and graphs were performed using Bioconductor and R programming languages. Each CEL file was merged with a description file. In order to correct background, normalize, and summarize results, we used the Robust Multiarray Averaging (RMA) algorithm. To determine the statistical significance of the analyzed genes, moderated t-statistics from the empirical Bayes method were performed. The obtained p-value was corrected for multiple comparisons using Benjamini and Hochberg’s false discovery rate. The selection of significantly altered genes was based on a p-value beneath 0.05 and expression higher than two-fold.

Differentially expressed genes were subjected selection by examination of genes involved in ionic distribution. The differentially expressed gene list was uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery) [5], where genes belonging to “inorganic ion homeostasis”, “ion transport”, “ion homeostasis” and “metal ion transport” gene ontology (GO) terms were obtained. Interactions between differentially expressed genes/proteins belonging to the inorganic ion homeostasis”, “ion transport”, “ion homeostasis” and “metal ion transport” GO terms were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes) [6]. The list of gene names was used as a query for an interaction prediction. The search criteria were based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression, and experimentally observed interactions. The results of these analyses generated a gene/protein interaction network where the intensity of the edges reflected the strength of the interaction score.

In order to further investigate the changes in studied GO terms, we performed the z-score (the number of up-regulated genes minus the number of down-regulated genes divided by the square root of the count) analysis using the GOplot package.

**Ethical approval**

The research related to animal use has been complied with all the relevant national regulations and instructional policies for the care and use of animals. Bioethical Committee approval no. 32/2012.

**Results**

Whole transcriptome profiling by Affymetrix microarray allows us to analyse the gene expression changes between 7, 15 and 30 days of buccal pouch mucosal cell culture. Using Affymetrix® Porcine

![Figure 1](image-url) **Figure 1** Heat map representation of differentially expressed genes belonging to “inorganic ion homeostasis”, “ion transport”, “ion homeostasis” and “metal ion transport” GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower expression). Log2 signal intensity values for any single gene were resized to Row Z-Score scale (from -2, the lowest expression to +2, the highest expression for single gene)
Gene 1.1 ST Array Strip we examined the expression of 12257 transcripts. Genes with a fold change higher than \( \text{abs}(2) \) and with a corrected p-value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 130 different transcripts.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of gene ontology biological process terms (GO-BP) that contain differently expressed transcripts. Up and down-regulated gene sets were subjected to DAVID searching separately and only gene sets where adj. p-value was lower than 0.05 were selected. The DAVID software analysis showed that the differentially expressed genes belonged to 56 Gene ontology groups. In this paper, we focused on “cation transport” and “cation homeostasis” GO BP terms. These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (Fig. 1). The gene symbols, fold changes of expression and corrected p values of that genes were shown in table 1. In order to further investigate the changes within chosen GO BP terms, we measured the enrichment levels of each selected GO BP terms. The enrichment levels were expressed as z-score and presented as circular visualization (Fig. 2).

Moreover, genes that formed one particular GO group can also belong to other different GO term categories in Gene Ontology Database. For this reason, we explore the gene intersections between selected GO BP terms. The relation between those GO BP terms was presented as well chart (Fig. 3) as well as heatmap (Fig. 4).

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>FC D15/D7</th>
<th>FC D30/D7</th>
<th>FC D30/D15</th>
<th>P Value D15/D7</th>
<th>P Value D30/D7</th>
<th>P Value D30/D15</th>
</tr>
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<td>0.101557</td>
<td>0.924613</td>
<td>0.009909</td>
<td>0.002855</td>
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<td>PTGS2</td>
<td>0.176579</td>
<td>0.327797</td>
<td>1.856379</td>
<td>0.033272</td>
<td>0.086183</td>
<td>0.447703</td>
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<td>0.33894</td>
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<td>0.05553</td>
<td>0.540232</td>
</tr>
<tr>
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<td>2.708448</td>
<td>0.040744</td>
<td>0.404391</td>
<td>0.235089</td>
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<tr>
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<td>0.291783</td>
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<tr>
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<td>1.837431</td>
<td>0.015016</td>
<td>0.216601</td>
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**Figure 3** The representation of the mutual relationship "Inorganic ion homeostasis", "ion transport", "ion homeostasis" and "metal ion transport" GO BP terms. The ribbons indicate which gene belongs to which categories. The genes were sorted by logFC from most to least changed gene. The logFC of these genes is represented by inner circle, which represent the logFC between D15/D7, D30/D7 and D30/D15 respectively.

**Figure 4** Heatmap showing the gene occurrence between "Inorganic ion homeostasis", "ion transport", "ion homeostasis" and "metal ion transport" GO BP terms.
tion method provided us with the molecular interaction network formed between protein products of studied genes (Fig. 5).

**Discussion**

Ion transport is a process that is important for the functioning of cells. The concentration of specific ions in the extracellular and intracellular environment is significant for cell development and survival. For demonstrating the role of ion homeostasis in the oral cavity, Luca et al. compared concentration of ions (Ca\(^{2+}\), Mg\(^{2+}\), Fe\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\)) in the serum of the patients with oromaxillofacial malignant cancers and for the patients that are showing no malignant cancer pathology [7]. They showed that changes in intracellular and extracellular homeostasis of these cations are involved in the pathogenic mechanisms of many diseases [7]. In oral mucosa, calcium concentration is significant for keratinocyte development. Rachow et al. demonstrated that the downregulation of occludin (OCLN) in keratinocytes results in decreased cell-cell adhesion, reduced susceptibility to induced apoptosis and altered Ca\(^{2+}\) homeostasis [8]. In other studies, Wu et al. showed effects of a calcium influx inhibitor, boxy amido-triazole (CAI) on the cell lines delivered from head and neck squamous cell carcinoma (HNSCC). The CAI blocks cellular proliferation, migration, and production of matrix metalloproteinases (MMPs) in HNSCC [9]. Their results suggest an important role for calcium in HNSCC development [9].

The above results show the influence of ions on maintaining the homeostasis, with its disturbance leading to the formation of lesions and diseases, which is why in our study we analysed genes belonging to ontological groups related to ionic distribution.

In the present study, we investigated the expression mRNA transcripts belonging to four ontology groups: (A) ion homeostasis, (B) ion transport, (C) metal ion transport, (D) inorganic ion homeostasis. In our microarray analysis, two genes (TGFB1, CCL2) showed decreased expression after 15 days of IVC and later increased expression after 30 days of culture. Others genes showed increased expression after 7 days of IVC and decreased mRNA expression level after 15 and 30 days of IVC. From analyzed genes, only PDPN and PTGS2 were present in one ontology group: ion transport. SLC5A3 was present in two ontology groups: ion transport and metal ion transport. Other genes belong to all four ontology groups.

Transforming growth factor beta 1 (TGFB1) is a peptide that controls proliferation and differentiation of cells. TGFB1 play a key role in epithelial proliferation [10]. It is secreted by epithelial cells, fibroblasts, macrophages, and platelets [10]. TGFB reduces airway epithelial proliferation *in vitro* [11] and stimulates mucosal remodelling *in vivo* [10]. Semlali et al. showed that cells in the asthmatic model released significantly more active TGFB1 than cells in the healthy model. Substitution of healthy fibroblasts by asthmatic fibroblast in the healthy model increased active TGFB1 release [11]. Results obtained by Semlali et al. and Mimura et al. suggest that TGFB is one of the major pathways involved in interactions between epithelial cells and fibroblasts [11, 12]. TGFB1 is also involved in immune homeostasis and can mediate in intracellular calcium level. TGFB1 inhibits aberrant T cell expansion by maintaining intracellular calcium concentration levels low enough to prevent a mitogenic response by calcium-independent stimulatory pathways [13].
Studies have shown that mast cells in mice may promote homeostasis of normal cells in the oral cavity, via production of TGFβ [14].

The second gene which exhibits high expression level after 30 day of IVC is chemokine (C-C motif) ligand 2 (CCL2). The role of CCL2 is often described in pulmonary fibrosis [15]. CCL2 may exert a profibrotic effect by inducing the expression of TGFβ and may promote the development of fibrocytes [15]. It was shown that CCL2 promotes the invasion of hepatocellular carcinoma (HCC) cells by the induction of epithelial-mesenchymal transition (EMT) [16].

High level of expression of two genes, TGFβ and CCL2, after day 7 of IVC may indicate the adaptation of the cells to the IVC conditions and may indicate the high level of proliferation of keratinocytes and fibroblasts in the IVC. After 15 days of IVC the expression level of these genes decreased and after day 30 of IVC microarray showed increased expression of TGFβ1 and CCL2. This may mean that the fibroblasts are the predominant cells in the IVC after 30 days.

Podoplanin (PDPN) and prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX2) showed expression on a high level after 7 days of IVC. PDPN is weakly expressed in mucous epithelial cells in the basal layer, and strongly in myoepithelial cells of salivary glands. This protein may be involved in the transition of epithelial cells into mesenchymal cells [17]. These results were confirmed by Renart et al. They described that PDPN is strongly expressed in tumour-derived cell lines and these cells promote cell migration and triggering of the epithelial-mesenchymal transition. The cells acquire fibroblast phenotype and increased motility [18].

The PTGS2 is associated with biologic events such as injury, inflammation, and proliferation [19, 20]. Boza et al. used ultraviolet (UV) treated and irradiated human oral mucosa fibroblasts. After irradiation, they observed inhibition of their proliferation and increased expression of PTGS2 [21]. It will be known that the expression of PTGS2 is associated with proliferative status of the fibroblasts. In quiescent fibroblasts, PTGS2 is up-regulated, being upregulated in proliferative cells of that type [21]. Boza et al. concluded that UV may have a carcinogenic effect through the increase of PTGS2 expression [21].

The role of PTGS2 is described in the case of different inflammatory diseases and is often associated with changes in the ion transport [19, 22].

In our study, we showed expression of different genes associated with the ion distribution. We presented the expression profile for normal porcine oral mucosal cells in long-term in vitro culture. The high expression level for analyzed genes at day 7 of IVC, compared to days 15 and 30, may be the result of an adaptation of cells to the in vitro conditions, as well as processes of proliferation and cell adhesion to the culture dish.

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Conflict of interest statement
The authors declare they have no conflict of interest.

References


