Final report

Several cell types exhibit a polarized arrangement of cellular components. These include dividing and migrating cells, as well as specialized cells such as neural cells, endothelial and epithelial cells that form physiological barriers, such as the airways, gut, liver, kidney, blood-brain-barrier, etc. Establishment and maintenance of cell polarity is an energy-intensive, complex process involving **trafficking machinery, various cargo molecules, cytoskeletal and junctional proteins, and regulatory mechanisms**. Our goal was to better understand the process in its context by identifying the elements involved and exploring the interrelationships between them. Initially, we focused on the **polarity of hepatocytes**, as these cells are important and interesting because of their crucial role in several physiological functions such as detoxification, drug metabolism and excretion, and because of their **peculiar cell polarity** by forming bile canaliculi from their apical membrane. As indicated in the progress reports, we have continuously adjusted our project in the light of the results obtained and adapted the plan to the results published in the field throughout the project. Thus, the project has been extended to investigate the **cell polarity formation in neural cells**, another cell type with important physiological functions and unique cell polarity. In general, our studies have focused on **identifying the factors responsible** for cell polarity formation and the **signaling pathways that regulate them**.

The project resulted in a total of **17 publications in international journals** with a cumulative impact factor of 98.22, in which the support of NKFIH was acknowledged. In addition, a completed manuscript containing the results of the project is under revision by a prestigious journal, while two other manuscripts are in preparation. During the course of the project, five further papers in international journals with a cumulative impact factor of 29.4 were published. With the indication of the funding, we have published 1 book chapter on the research topic and presented the project's results **at 17 national and 11 international conferences**. As instructed, conference presentations whose results were subsequently published in journals were not included in the list of publications. These relatively high numbers are partly due to the fact that the project was not carried out over 4 years, but over a longer period for the reasons detailed below. It is worth mentioning that during this period, **4 PhD students graduated summa cum laude**, whose dissertations were based in whole or in part on publications presenting the results of this project.

We have faced several challenges throughout the project. First, the reorganization of the research network of the Hungarian Academy of Sciences caused such a degree of uncertainty that two PhD students who would have played a key role in the project left the group and the research network. Because of the extra time required to recruit and train new students, I requested and

received a 1-year postponement of the project. In later years, restrictions due to the Covid-19 pandemic made experimental work difficult and even stalled. For this reason, I requested and received a further postponement of the project, which has also been approved.

Despite these difficulties, we have achieved most of our objectives, **the research was successfully completed and yielded a number of important findings**, which can be discussed in three different groups. Regarding the regulation of hepatocyte polarization, the biggest challenge was to find the proper cellular model. We applied hepatic cell lines (Huh7 cells), hepatic progenitor cells (HepaRG), primary hepatocytes isolated for rats, and hepatocyte-like cells (HLCs) differentiated from human pluripotent stem cells. Despite our best efforts, Huh7 cells failed to polarize, and mixed culture of cholangiocytes and hepatocytes obtained by a two-week long differentiation of HepaRG progenitor cells only result in partial polarization, i.e., hepatocytes form only spherical semicanaliculi. However, we have discovered that when HepaRG-derived hepatocytes are cultured under polarizing conditions, in a collagen sandwich configuration, these cells are also able to develop chicken wire-like, branching canalicular structures. Our gold standard has been primary rat hepatocyte culture, on which we have always validated the results obtained with cell lines, although the genetic manipulation of these terminally differentiated, non-dividing cells is rather limited. Animal housing and on-site hepatocyte isolation were not feasible due to changes in the conditions of the host institute, but this difficulty was overcome by obtaining and using commercially available rat hepatocytes isolated under standardized conditions. The fourth liver model we used in this project was HLCs generated from pluripotent stem cells by directed differentiation, which have the great advantage of being cells of human origin. However, they have the disadvantage of being labor and cost intensive to produce, taking 3-4 weeks. Our studies have led us to the discovery that **cell polarity is a critical issue for the applicability of stem cell-derived HLCs**, as transporters essential for liver function, such as ABCG2 and MRP2, are only expressed at the appropriate level and in the correct membrane compartment when HLCs are properly polarized, which can be achieved by using the polarizing culture conditions mentioned above (Török et al. 2020).

Later, we made efforts to generate stem cell derived HLC cultures of higher purity. Since terminally differentiated, mature hepatocytes do not proliferate, contaminating (undifferentiated, partially differentiated, or non-hepatic) cells, which still divide, overgrow hepatocytes after a short while, restricting the applicability of such cultures. (In order not to hinder future patenting, we report the results of this section without technical details). Previously genetically engineered pluripotent stem cells were differentiated into the liver lineage and the cell cultures were drug treated to remove contaminating non-hepatocyte-like cells. In this study, we demonstrated that either acute or chronic drug treatment **eliminated dividing cells from stem cell-derived HLC**

cultures, resulting in **improved hepatic character**, as hallmarked with increased albumin and urea secretion, as well as the elevated expression and function of the major hepatic drug-metabolizing enzyme CYP3A4 (manuscript in prep.)

Investigating the signaling pathways regulating hepatocyte polarity, we found that **not only LKB1, but PKA is also essential for canalicular network formation**. In our efforts to explore the subcellular localization of LKB1, we again faced challenges, as we were confronted with the inadequacy of commercially available antibodies. We tested a large number of antibodies, but none proved to be suitable for immunolocalization studies. We therefore pursued tagging approach instead. Unfortunately, the originally proposed method using split-GFP to detect LKB1 localization does not provide enough contrast due to the low signal-to-noise ratio, thus being insufficient to detect small differences. Similarly, the bioorthogonal labeling has not fulfilled our expectations – again due to the low signal-to-noise ratio. However, **a methodological paper** based on our development **on biorthogonal labeling** has been published (Török et al. 2021.) To overcome the problems above, we finally used a more traditional approach, namely epitope tagging. We introduced HA-tagged LKB1 into HepaRG progenitor cells using Sleeping Beauty transposon-based gene delivery, and established a stable cell line, which allowed us to specifically detect subcellular localization of LKB1.

We also investigated whether there is a crosstalk between Epac-LKB1- and PKA-dependent signaling pathways in the regulation of hepatocyte polarization. We demonstrated in several hepatic models, including HepaRG-derived human hepatocytes and rat primary hepatocyte cultures, that **specific activation of PKA results in elevated phosphorylation of LKB1** and its downstream effector AMPK. In addition, we showed that PKA activation significantly **accelerated formation of bile canaliculi** in terms of both length and complexity of canalicular network. To investigate whether this process is dependent on or independent of LKB1 activation, we used a siRNA knockdown (KD) approach. Employing an LKB1-siRNA adenovirus construct, we demonstrated effective silencing of LKB1 expressions in the applied hepatic models. We also showed that LKB1 KD prevents the stimulatory effect of PKA activation on canalicular development, suggesting that **PKA stimulates canalicular network formation through LKB1.** The results obtained here allowed us to map out a consistent regulatory pathway network that controls hepatocyte polarity (manuscript under review). In a currently established international collaboration, we are seeking to divulge the involvement of other kinases, such as PAR1.

An essential part of cell polarity is the targeted delivery of proteins (junctional molecules, membrane proteins) to the appropriate membrane compartment. The polarized trafficking of transporter proteins is of particular importance for the physiological functions of the liver. The

multidrug transporter **ABCG2 residing in the canalicular membrane of hepatocytes, as a key player in hepatic detoxification**, mediates the excretion of various endogenous and xenogeneic toxic into the bile. In other polarized cells, such as intestinal and renal epithelial cells, ABCG2 is responsible for the excretion of urate, the end product of purine metabolism. Therefore, its mutations are associated with hyperuricemia and gout, and even affects the tissue distribution, excretion and consequently the toxicity of various drugs in connection with its hepatic function. During the project, we studied the expression and trafficking properties of disease-causing polymorphic and mutant variants of ABCG2 (Q141K and M71V) and **demonstrated their impaired delivery to the plasma membrane** (Zámbó et al 2020). As conventional methods, such as immunolabeling, provide information only on the steady state distribution of the protein, leaving the dynamics of cellular routing unexplored, we adapted the so-called RUSH (Retention Using Selective Hooks) system to assess cellular trafficking of ABCG2 variants. Using this approach, we dissected the cellular routing and identified the specific defects of the disease-causing ABCG2 mutants, which were shown to be **partially retained in the endoplasmic reticulum and also impeded their delivery to the plasma membrane** (Bartos and Homolya, 2021). We also studied a peculiar, structurally unresolved cytoplasmic loop in ABCG2 potentially influences cellular trafficking of the transporter. Mutational analysis of this region revealed that **K360 is critical residue** whose deletion results in **accelerated trafficking to the plasma membrane**, while other residues including a potential phosphorylation site had no major effect cellular routing (Mózner et al. 2023).

The GFP-ABCG2-expressing MDCK cell line generated for the trafficking studies was also utilized in a collaborative work in which nucleotide binding was identified as a key regulator of ABCG2 conformational changes required for transport (Gyöngyi et al. 2023). It is known that the transport function of ABCG2 is modified by sterols through different sterol-sensing elements. Cryo-EM structural data on ABCG1, a close relative of ABCG2, were published during the project, and therefore we studied the localization and function of this transporter. Combining mutational analysis with published structural data, we have **identified the motifs crucial for proper localization and revealed the potential sterol-sensing element** in this and related transporters including ABCG5 and ABCG8, which are responsible for the biliary secretion of cholesterol in the liver (Hegyi et al. 2022). As a response to the challenges of the COVID-19 pandemic emerged during the project, we also studied the interactions of potential anti-COVID-19 drugs with ABCG2 variants (Mózner et al. GRC talk 2021). In a review article, we overviewed the key cellular pathways involved in the processing and trafficking of membrane proteins, with special focus on ABCG2 (Mózner et al. 2019). We also discussed in detail the structure-trafficking-function relationships of different ABCG2 variants (Sarkadi et al. 2020). Finally, I have proposed a **novel systematic classification of ABCG2 variants**, in which the variants are

categorized by theratypes, the specific strategies required for phenotype correction (Homolya, 2021). In the near future, we plan to investigate the trafficking and dimerization of ABCG2 and ABCG5/G8 disease-associated mutants.

As mentioned above, as an extension of our project, we have studied cell polarity development and regulation in another specifically polarized cell type, neural cells. In this case, too, the selection of a suitable model cell was a challenge, and here again, progeny cells differentiated from human pluripotent stem cells offered a solution. Here, we focused on the very first step in the cell polarity development of neural cells, the outgrowth of undifferentiated neurites from neural progenitor cells (NPCs). For this purpose, we first generated and characterized human pluripotent stem cell-derived NPCs (Szabó et al. 2020). The established method for the generation of NPCs has also been published in a methodological paper (Szabó et al. 2022). We then stably expressed GFP in these cells at high levels using transposon-based gene delivery and developed a high content screening-based method to reliably detect thin projections. Using this technique, we demonstrated a **pivotal regulatory role for the motor protein non-muscle myosin II in neural cell polarization** and showed that the **upstream ROCK1 signaling pathway controls the process**, whereas found no contribution of JNK signaling to neurite outgrowth regulation (Lilienberg et al. 2021). We also observed that the extracellular matrix components greatly influence neurite formation and demonstrated that **inhibitory effect of the restrictive environment can be overdriven by non-muscle myosin II inhibitors**. We also investigated how microglial cells influence NPCs' neurite development, and revealed **a complex, fine-tuned modulatory role for microglia** depending on their activation state (naïve, proinflammatory, or anti-inflammatory) as well as on the stage of neural cell differentiation (Lilienberg et al. 2022). In this study, we investigated not only neurite development of NPCs but also neural cells differentiating toward hippocampal dentate gyrus granule cells (DGGCs). In connection with this, we studied the effect of **typical and atypical antipsychotics** on neurite outgrowth of DGGCs and found that these drugs **promoted neurogenesis by influencing neurite outgrowth rather than changing cell survival or gene expression** (Jezso et al. 2024). Using these *in vitro* cellular models, we studied the molecular disease pathways connected to **de novo mutations with schizophrenia**. In contrast to the results obtained with antipsychotics, we found **no changes in neurite outgrowth** but marked glutamatergic dysregulation in schizophrenia patient-derived DGGCs (Tordai C. et al, 2024).

Using the experimental toolkit developed for our project, we have been involved in several collaborative projects, including studies focusing on subcellular events in polarized neurons, such as synaptic dysfunction of mitochondria in Alzheimer's disease (Győrffy et al. 2020), as well as mechanism of synaptic pruning (Kovács et al. 2021). In another collaboration, we demonstrated a

syndecan-4-dependent development of an asymmetrical front-to-rear calcium gradient in migrating myoblasts, which was a prerequisite for migration as it regulates assembly of focal adhesions and acto-myosin contractility (Becsky et al. 2020). Recently, we have investigated the role of extracellular vesicles in the development of calcium gradients in these cells (manuscript in prep).

In summary, the objectives of our project on the formation and regulation of specific forms of cell polarity have successfully completed; moreover, have been complemented by additional exciting new questions that have emerged during the implementation of the project. Several other stimulating queries have also been raised, offering perspectives for future research.