Final report on the research project:

"Regulation of ABC transporter protein expression during human pluripotent stem cell differentiation"

1. Background, scientific questions of the project

In this project we were planning to investigate the complex regulation of the expression and function of medically important human ATP-Binding Cassette (ABC) transporters in human stem cell lines and during directed stem cell differentiation. In order to do this, we planned to use advanced molecular biology and cell biology methods, including transposon-based and the CRISPR-Cas9 technology. Detailed analysis of the ABC protein function and expression was connected to generate reporter cell lines both from embryonic (ES), induced pluripotent (iPS) and cancer stem (CS) cells, and study the effects of cell differentiation, physiological and pathological conditions, including the effects of drugs.

During the four years of the work the scope of this research project needed a major groundwork effort for establishing cell biology (including stem cell culturing and differentiation) and molecular genetic (including a variety of CRISPR-based technologies and PCR-based evaluation) methodologies. We also had to acquire extended knowledge how to examine genetic and epigenetic regulation of membrane transporters – in these cases mRNA and protein expression levels may be entirely different and processing of membrane proteins may significantly contribute to regulation. This requirement made the actual project work wider in the sense of establishing which methods are most efficient for exploring transporter regulation, but also, more focused on a few given transporters.

The originally selected transporters for this study were the ABCG2 and ABCC6 proteins, while in the actual project the research has been focused on the ABCG2 protein, and on the technology improvements which made possible to investigate the regulation of this multidrug and uric acid transporter both in normal and tumor stem cells. The ABCG2 transporter is involved in major human diseases, thus a better understanding of its physiological and stress-induced regulation, as well as the effects of polymorphisms and mutations may significantly improve medical diagnostics and interventions. ABCG2 resides in the plasma membrane and in polarized cells in the apical (luminal) membranes and is working as an ATP-dependent xenobiotic exporter. ABCG2 has a promiscuous capacity of recognizing and extruding a large number of transported substrates - including many hydrophobic toxic compounds, amphiphilic positively or negatively charged agents, as well as practically water-soluble molecules. This protein is a physiologically important member of the xenobiotic defense systems in our body, and a major player in cancer drug resistance. Interestingly, the human ABCG2 is also an efficient uric acid transporter, and mutations or polymorphic variants of this transporter are causative in the development of gout. Based on clinical collaborations this latter aspect became an important area of the present research project.

In the human body high level ABCG2 expression is observed in the liver (bile canalicular membranes) and the kidney (proximal tubular luminal membranes), while other key sites for ABCG2 expression are the barrier forming tissues, including the luminal side of intestinal epithelial cells, and the endothelial cells of the blood-brain barrier. ABCG2 is preferentially expressed in both pluripotent stem cells (see Sarkadi et al, 2010, Stem Cells, 28: 174-176), and in the hematopoietic progenitors. ABCG2, causing a side-population phenotype of a Hoechst dye uptake, is also considered as a marker for cancer stem cells, resulting in chemotherapy resistance.

Regarding ABCC6, mutations in the gene coding for this protein result in multiorgan mineralization disorders, and in most cases cellular trafficking problems of ABCC6 seem to be the source of reduced function. During the preparation of the project proposal and in the first steps of this project we performed a detailed mRNA expression study in human embryonic stem cells and cancer stem cells (see Apáti et al, Expert Opin Drug Metab Toxicol. 2016;77-92), and the data showed that there is practically no ABCC6 expression in the human pluripotent cell types. In addition, since another research group in the Institute (led by Andras Varadi) in collaboration with us focused on the functional and regulatory aspects of ABCC6, in the present project we have not performed studies with ABCC6 in the pluripotent cells.

For the expression of the ABCG2 protein we have documented the presence of three different promoters and mRNA transcripts. However, the exact promoter and enhancer regions have not been experimentally explored, and the determination of the tissue-dependent functional ABCG2 expression has been an unresolved problem. The potential role of naturally occurring ABCG2 variants, their expression, processing and function has not been explored, and the application of normal and cancerous human stem cells for studying the regulation and trafficking of ABCG2 provided an unprecedented possibility. In the present project we achieved significant advancements in these areas.

In addition, we have explored the regulatory elements and the related polymorphic and mutant variants, as well as potential epigenetic regulators of various membrane transporters, including ABC and P-type transporters. A common goal in these studies was to find disease-related regulatory regions, based on GWA studies and protein expression data. We were especially successful in these studies related to the ABCG2 transporter and the plasma membrane calcium transporter (PMCA4b) protein.

II. Key results of the project

1. Expression of the ABCG2 transporter covalently tagged with fluorescent eGFP proteins in normal human pluripotent cells (hPSC) and in a cancer stem cell model (A549 cells)

Our previous studies established that the N-terminally eGFP-tagged ABCG2 is fully functional and properly trafficked to the plasma membrane (Orban et al, 2009, Stem Cells, **27**: 1077-1087). By applying a high efficiency Sleeping Beauty (SB) transposon method, we have generated hPSCs stably expressing GFP-tagged ABCG2 and its mutant variants. We have studied the effects of this construct on basic stem cell growth and differentiation, as well as the transporter protein localization. In these constructs the expression was driven by a CAG promoter, which we found to be active both in stem cells and in many differentiated tissues, thus we could follow ABCG2 expression in various stem-cell derived tissues.

In our published study (Erdei et al, Plos One, 2018) we have overexpressed eGFP-ABCG2, driven by a constitutive (CAG) promoter, in HUES9 human embryonic stem cells. Stem cell clones were generated to express the wild-type and a substrate-mutant (R482G) eGFP-ABCG2 variants, by using the Sleeping Beauty transposon system. We found that the stable overexpression of these transgenes did not change the pluripotency and growth properties of the stem cells, nor their differentiation capacity to hepatocytes or cardiomyocytes. ABCG2 overexpression provided increased toxin resistance in the stem cells and protected the derived cardiomyocytes against doxorubicin toxicity. These studies documented the potential of a stable ABCG2 expression for engineering toxin-resistant human pluripotent stem cells and selected stem cell derived tissues. Moreover, the R482 variant provided a better protection of stem-cell derived cardiomyocytes, than the wild-type ABCG2 protein.

These experiments, although did not reflect the endogenous regulation of the ABCG2 transporter protein, allowed important localization, functional and trafficking studies during early normal tissue differentiation. In as yet unpublished studies, we have also generated A549 lung cancer cells expressing the eGFP-ABCG2 fusion protein, driven by a constitutive (CAG) promoter. Since the A549 cells are rapidly growing, can be induced to differentiate, and show multidrug resistance (express relatively high levels of ABCG2), they are often regarded as cancer stem cell models. The generated A549 cell model has also provided an important control for the endogenous regulation of the ABCG2 protein in the CRISPR-based experiments (see section 4).

Parallel with these studies we have explored the various forms of the ABCG2 mRNA expressed in stem cells and in differentiated cell types (Sandor et al, BBA 2016). We aimed to explore the functional role of the various 5' untranslated exon variants (5' UTRs) and provide a comprehensive characterization of four ABCG2 mRNA variants with different exon 1 sequences. In addition, we investigated drug inducibility, stem cell specificity, mRNA stability, and translation efficiency of these variants. Although certain variants (E1B and E1C) are considered as "constitutive" mRNA isoforms, we have shown that chemotoxic drugs significantly alter the expression pattern of distinct ABCG2 mRNA isoforms. When examining human embryonic stem cell lines, we provided evidence that variant E1A has an expression pattern coupled to undifferentiated stem cell stage, as its transcript level is regulated parallel to mRNAs of the Oct4 and Nanog pluripotency marker genes. When

characterizing the four exon 1 variants we found no significant differences in terms of mRNA stabilities and half-lives of the isoforms. In contrast, variant E1U showed markedly lower translation efficiency both at the total protein level or regarding the functional presence in the plasma membrane. Taken together, these results indicate that the different 5' UTR variants play an important role in cell type specific regulation and fine tuning of ABCG2 expression.

2. Examination of the potential role of transporter variants in hemopoietic differentiation and metabolic diseases

ABCG2 has a potential role in protecting progenitors against the effects of heme and the accumulation of exogenous or endogenous toxic compounds. The relatively frequent polymorphic and mutant ABCG2 variants in the population may significantly alter disease conditions and pharmacological effects, and low-level or non-functional ABCG2 expression may increase individual drug toxicity and result in hyperuricemia and gout. In order to explore this question, we have performed collaboration studies with clinicians (Gyula Poor and Marton Palinkas, ORFI) treating gout patients, and examined the role of natural ABCG2 polymorphisms and mutations in the expression levels and gout development. In the first set of studies (Zambo et al, Sci Rep. 2018) we found that ABCG2 expression in the erythrocyte membranes of gout patients was significantly reduced. By genetic screening based on protein expression, we found a relatively frequent, novel ABCG2 mutation (ABCG2-M71V), which, according to cellular expression studies, causes reduced protein expression, although with preserved transporter capability. In these experiments eGFP expression, and eGFP-tagged ABCG2 variants have also been examined. Interestingly, ABCG2-M71V expression *in vitro* could be corrected by therapeutically relevant small molecules. These results suggested that personalized medicine should consider this newly discovered ABCG2 mutation, and genetic analysis linked to protein expression provides a new tool to uncover clinically important mutations in membrane proteins.

When studying the cellular expression, trafficking, and function of a further nine naturally occurring polymorphic and mutant variants of ABCG2, a comprehensive analysis of the membrane localization, transport, and ATPase activity, as well as retention and degradation in intracellular compartments was performed (see Zambo et al, Cell Mol Life Sci. 2019). Among the examined variants, R147W and R383C showed expression and/or protein folding defects, indicating that they could indeed contribute to ABCG2 functional deficiency. These studies and the applied methods should significantly promote the exploration of the medical effects of these personal variants, promote potential therapies, and help to elucidate the specific role of the affected regions in the folding and function of the ABCG2 protein.

In addition to the expression and trafficking experiments, in collaboration with the group of Tamas Hegedus, we performed detailed in silico modelling and molecular dynamics simulations for the ABCG2 protein and its variants. Based on the recently published atomic level structures we looked for the potential effects of mutations in the generated models, moreover, extended the experimental details of the protein structures by modelling of the unresolved parts of the molecule (see Laszlo et al, PLoS One. 2016 Oct 14;11). In the case of the M71V mutant, causing an incomplete folding and trafficking, we found an altered dynamics of the mutant protein (see Zambo et al, Sci Rep, 2018). The generated mutation database (Tordai et al, Database (Oxford). 2017) significantly helped to assess the effects of mutations in protein folding, trafficking and function. We have recently submitted a detailed analysis of the functional and trafficking effects of the ABCG2 polymorphisms and mutations on the plasma membrane expression of the transporter (Mozner et al, Cells, 603314, accepted for publication).

Closely related to these studies we have examined the potential epigenetic regulation of the plasma membrane calcium ATPase (PMCA4b), highly expressed in erythroid cells and also in human red blood cells. Interestingly, we found several healthy volunteers showing significantly reduced expression of RBC-PMCA4b. Reduced PMCA4b levels correlated with a lower calcium extrusion capacity in these erythrocytes. When exploring the potential genetic background of the reduced PMCA4b levels, we found no missense mutations in the ATP2B4 coding regions, while a formerly unrecognized minor haplotype in the predicted second promoter region closely correlated with lower erythrocyte PMCA4b protein levels. In recent GWA studies, SNPs in this ATP2B4 haplotype have been linked to reduced mean corpuscular hemoglobin concentrations (MCHC), and to protection against malaria infection. Our data (see Zambo et al, Cell Calcium, 2017) suggest that an altered

regulation of gene expression is responsible for the reduced RBC-PMCA4b levels that is probably linked to the development of human disease-related phenotypes.

3. Development of new functional assays for ABC multidrug transporters

In parallel with the molecular biology-based analysis we have decided to develop advanced flow cytometry methods to follow the transport function of the ABCG2 membrane protein. An efficient fluorescence-based method was expected to greatly facilitate any functional studies in stem cells and in their differentiated offspring. Therefore, we screened numerous compounds which could be transported by the ABCG2 protein and the related multidrug transporters. In the first set of these studies we found and published (Nerada et al, Cytometry, 2016) that DCV, a DNA-binding fluorescent compound is an excellent substrate of the ABCG2 protein and the application of this dye allows the application of high-content screening methods to follow ABCG2 expression and function.

In our further efforts to find useful transporter tracking fluorescent compounds we found a heavy metal chelator fluorescent dye, PhenGreen, which proved to be a high affinity substrate of ABC multidrug transporters, especially of the ABCG2 transporter. In our published paper (Szabo et al, PLoS One. 2018), we have examined the potential interactions of three key human ABC multidrug transporters with PhenGreen diacetate (PGD), a cell permeable fluorescent metal ion indicator. The non-fluorescent, hydrophobic PGD rapidly enters the cells and, after cleavage by cellular esterases, in the absence of quenching metal ions, PhenGreen (PG) becomes highly fluorescent. We found that in cells expressing functional ABCG2, ABCB1, or ABCC1 transporters, cellular PG fluorescence is strongly reduced. This fluorescence signal in the presence of specific transporter inhibitors is increased to the fluorescence levels in the control cells. The PG accumulation assay is a new, unique tool for the parallel determination of the function of the ABCG2, ABCB1, and ABCC1 multidrug transporters. Since PG has very low cellular toxicity, the PG accumulation assay also allows the selection, separation and culturing of selected cell populations expressing either of these transporters. In addition to this publication, we have applied for a patent protection in a Hungarian (P1700432) and an international (PCT/HU2018/050046) patent application already published and under approval (see https://patents.google.com/patent/WO2019081957A1/en) with an extension for diagnostic applications. Moreover, this patent has already been a subject of a contract between our Research Centre and a biotech company regarding a commercial utilization for research and diagnostics.

4. Development of new CRISPR/Cas9 methods for studying the regulation of ABCG2 in human normal and cancerous stem cell lines

A major effort in the research work in this project has been devoted to a detailed analysis of cellular ABCG2 protein expression regulation by developing a new CRISPR/Cas9 methodology in various human normal and cancerous stem cell lines. Since this proved to be an unexpectedly difficult task, most of the results of these studies are currently under publication or have not been published yet. Therefore, here I provide a more detailed description of the methods and the results concerning these studies.

Genome edited reporter cells are useful in investigating gene regulation and visualizing protein activity in live cells but require precise targeting to preserve native regulatory regions. In this project we have developed two different reporter systems for assaying the regulation of the ABCG2 expression in its native genomic environment.

4.a. Studies in an A549 cancer stem cell model.

Expression of the ABCG2 multidrug transporter is a marker of cancer stem cells and a predictor of recurrent malignant disease. Understanding how human ABCG2 expression is modulated by pharmacotherapy is crucial in guiding therapeutic recommendations and may aid rational drug development. The key results of this study are under publication, submitted to British Journal of Cancer, in 2019: *Kovacsics D. et al., "Precision-engineered reporter cell lines reveal ABCG2 regulation in live lung cancer cells*". In this paper we describe a fluorescent reporter assay that allows the noninvasive assessment of ABCG2 regulation in human lung adenocarcinoma cells. Using CRISPR-Cas9 gene editing coupled with homology-directed repair, we targeted an eGFP coding sequence to the translational start site of ABCG2, generating ABCG2 knock-out and *in situ* tagged ABCG2 reporter cells. Using the engineered cell lines, we have shown that ABCG2 is upregulated by a number of anti-cancer

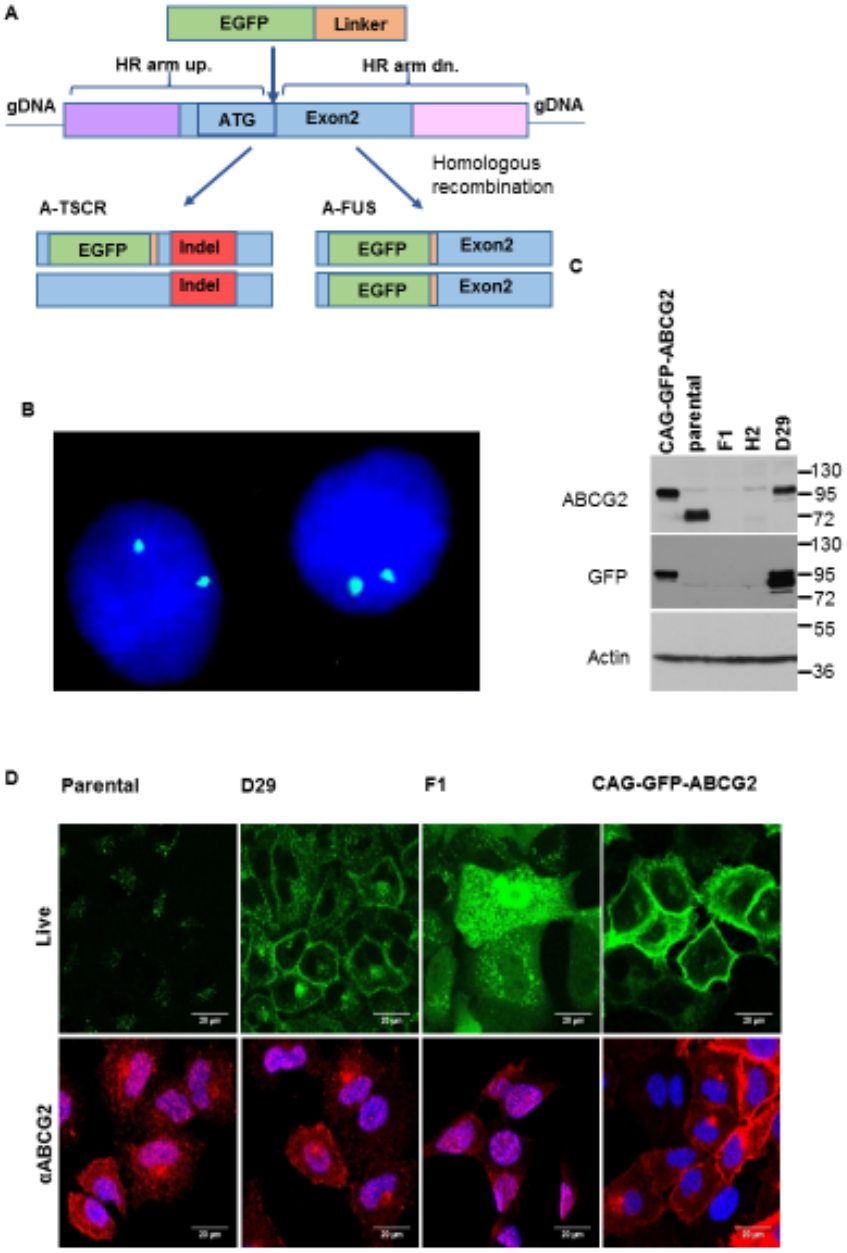
medications, HDAC inhibitors, hypoxia-mimicking agents and glucocorticoids, supporting a model in which ABCG2 is under the control of a general stress response.

Hereby I include some of the key results of this paper (only the key Figures are presented here). First, we documented the successful g**eneration of A549 reporter cell lines to study the regulation of ABCG2 expression.** A549 cells are colony-forming, self-renewing cells, capable of terminal differentiation. We chose A549 cells to modify the ABCG2 locus because they express moderate levels of ABCG2 and have preserved homology-mediated DNA repair mechanisms. Because A549 cells are hypotriploid, we performed chromosome mapping and FISH to confirm that A549 cells have two copies of chromosome 4 (Figure 1 B). To modify the ABCG2 locus, A549 cells were co-transfected with a plasmid expressing a guide-RNA-targeted wild type SpCas9, and a plasmid delivering the donor DNA template for HR. The HR donor was designed to insert a promoterless eGFP in frame with the translation initiation site (ATG) of the *ABCG2* gene. Cultures stably expressing eGFP were sorted for eGFP positivity, cloned and verified using Sanger sequencing.

As shown in Figure 1 A, cell lines reporting *ABCG2 transcription* **(A-TSCR)** have one copy of eGFP inserted at the target location, and biallelic indel mutations in the ABCG2 coding sequence near the targeted PAM site (del(chr4:89061079-89061080) on the GFP allele and ins(chr4:89061082C) on the non-GFP allele). Chromosome mapping and FISH showed no changes in chromosome number in the clones. Whole exome sequencing (WES) of the generated cell lines revealed no major off-target effects attributable to CRISPR/Cas9 engineering. While WES revealed a possible partial duplication of the target site, no WT ABCG2 sequence was detected at any allele frequencies. The **A-TSCR** cell lines had no measurable levels of ABCG2 protein expression, examined via 5D3 cell surface antibody labeling, mitoxantrone (MX) extrusion or Western blotting (Figure 1C), and the eGFP fluorescence was entirely intracellular (Figure 1D).

Figure 1.

A. Schematic showing the CRISPR-Cas9-engineering of A549 cells to generate **A-TSCR** (eGFP knock-in / ABCG2 knock-out), and **A-FUS** (eGFP knock-in / ABCG2wt) cells.
B. Representative FISH image of A549 cells using centromere probes for chromosome 4. All examined A549 cell lines (parental, F1, H2, D29) were disomic for chromosome 4.
C. Western blot analysis of parental and engineered A549 cells to detect ABCG2 and eGFP expression. Parental cells express the unmodified ABCG2 protein (glycosylated, at a mol. mass of about 70 KDa). The molecular mass of the EGFP-ABCG2 fusion protein is about 100 KDa. Cells engineered to express the native promoter-driven, tagged ABCG2 (D29) expressed slightly less ABCG2 than the parental cells. Cells overexpressing a CAG-promoter driven eGFP-ABCG2; however, showed ten times the normal ABCG2 expression.
D. Confocal microscopy images of the parental and engineered A549 cells. Upper panel: live cells analyzed for green fluorescence. Lower panel: cells stained with the anti-ABCG2 antibody BXP21 and an anti-mouse secondary antibody labeled with Alexa 647 (red fluorescence). First column: parental A549 cells expressing wild type ABCG2, second column: D29 cells expressing eGFP-ABCG2 driven by the native promoter, third column: F1 cells expressing eGFP driven by the native ABCG2 promoter but no ABCG2, fourth column: A549 cells expressing a CAG-promoter driven eGFP-ABCG2 protein.



As a second step to investigate ABCG2 regulation at the protein level, we generated A549 cells expressing an eGFP-ABCG2 fusion protein. We generated **A-FUS** cells by targeting a promoterless eGFP and a linker to the translation initiation site of the ABCG2 gene but, unlike for A-TSCR, we included a silent mutation in the HR donor to prevent further Cas9 cleavage of the recombinant genomic region. Since the eGFP-ABCG2 fusion protein was shown to be fully functional and properly membrane localized in the overexpression model (see section 1), the engineered cell line was expected to report the combined transcription, translation, and membrane insertion of the transporter. Cells stably expressing eGFP and cell surface ABCG2 (measured by 5D3 antibody staining), were sorted by flow cytometry and cloned. As an additional verification step prior to Sanger sequencing, clones were double-checked for ABCG2 and eGFP expression via confocal microscopy. Based on a detailed characterization, we selected a single, stable, **A-FUS** cell line (D29), with a correctly targeted eGFP on both *ABCG2* alleles (see Figure 1A). Western blotting demonstrated that the eGFP-tagged ABCG2 protein is uniquely present in the **A-FUS** cells (see Figure 1C) and membrane localization of the fusion protein was confirmed by confocal microscopy (Figure 1D) and 5D3 labeling. As before, we used chromosome mapping and FISH to ensure

that chromosome number was unaffected and exome sequencing to ascertain that there were no major off-target effects.

Transcriptional regulation of ABCG2 was examined using two different **A-TSCR** (eGFP knock-in, ABCG2-KO) reporter cell lines, named A549-F1 and A549-H2. Hormone- and drug-regulation of *ABCG2* transcription was assessed after 24 hours of drug treatment by measuring eGFP fluorescence in live **A-TSCR** cells. Parental A549 cells were treated in parallel to correct for changes in autofluorescence. During the 24-hour period, there was no significant cell death caused by most treatments. In the case of cytotoxic chemotherapeutics (e.g. mitoxantrone, platinum drugs), ABCG2 expression was assayed 48 hours post treatment, after 24 hours of recovery in fresh media. The specificity of ABCG2 activation was examined using A549 cells stably overexpressing a CAG promoter-driven eGFP-ABCG2 or a CMV-driven eGFP, as controls.

Using the **A-TSCR** cell lines, we found that numerous pharmacological agents, including known inducers of ABCG2, produced a major up-regulation of eGFP expression (Table 1, Figure 2). For example, several histone deacetylase inhibitors (HDACi), used as anticancer and anti-inflammatory agents, produced a 5- to 8-fold increase in eGFP reporter expression. Sodium butyrate and valproic acid, corresponding to their relatively high HDAC inhibitory concentrations, evoked this effect at millimolar concentrations. In contrast, the clinically applied HDAC inhibitor SAHA (suberoylanilide hydroxamic acid, Vorinostat), used in NSCLC as co-treatment, caused a profound induction of ABCG2 transcription at clinically relevant doses (Figure 2B).

It is well established that ABCG2 expression can be upregulated in response to chemical stressors. As glucocorticoid (GC) signaling is one of the major stress response system in humans, we were interested in investigating whether ABCG2 expression may be under the control of the glucocorticoid receptor (GR). Using the **A-TSCR** cells, we found that the glucocorticoid hormone cortisol (hydrocortisone, HC) (Figure 2C), as well as the synthetic glucocorticoids, dexamethasone (DEX) (Figure 2D) and methylprednisolone (MTP), induced a strong activation of eGFP expression, with an efficiency corresponding to the relative affinities of these glucocorticoids to the glucocorticoid receptor. The glucocorticoid-induced eGFP expression was fully inhibited by low concentrations of mifepristone (MIF) (Figure 2C-D), a steroid GR antagonist which binds with nanomolar affinity to the GR but not the mineralocorticoid receptor.

Figure 2. Regulation of eGFP expression in A-TSCR *ABCG2* reporter cell lines by HDACi, GCs and anti-cancer drugs.

The engineered A549 cells were drug-treated for 24 hours and analyzed for eGFP expression at 24 (A-D) or 48 (E-H) hours. Data represent the means from two to three independent experiments (parallels as indicated). Error bars represent SD. For additional data on drug effects see Figure EV2.

A. Effect of sodium butyrate (NaB), 4-phenyl butyrate (4PBA) and valproic acid (VLP) on eGFP expression in F1 cells (n = 3 experiments in duplicate).
B. Effect of suberoylanilide hydroxamic acid (SAHA, Vorinostat) on eGFP expression in F1 and H2 cells (n = 2 experiments in duplicate).
C. Effect of hydrocortisone (HC) and hydrocortisone+mifepristone (HC+MIF) on eGFP expression in F1 and H2 cells. MIF, a glucocorticoid receptor antagonist, strongly inhibits HC-induced eGFP expression (n = 2 experiments in triplicate).
D. Effect of dexamethasone (DEX) and dexamethasone+mifepristone (DEX+MIF) on eGFP expression in F1 and H2 cells. MIF, a glucocorticoid receptor antagonist, strongly inhibits DEX-induced eGFP expression (n = 3 experiments in triplicate).
E. Effect of mitoxantrone (MX) on eGFP expression in F1 and H2 cells (n = 2 experiments in triplicate).
F. Effect of cisplatin (CIS) and carboplatin (CPL) on eGFP expression in F1 and H2 cells (n = 3 experiments in triplicate). While cisplatin strongly increases eGFP expression in F1 and H2 cells the related durg carboplatin has no effect on reporter protein expression over a wide range of concentrations.
G. Effect of vinblastine (VLB) and vinorelbine (VNR) on eGFP expression in F1 and H2 cells (n = 3 experiments in triplicate). Both VLB abd VNR strongly increase eGFP expression, although at different concentrations.

H. Effects of erlotinib and etoposide on eGFP expression in F1 cells (n = 3 experiments in duplicate). While etoposide strongly increases eGFP expression in F1 cells, erlotinib does not affect reporter protein expression over a wide range of concentrations.

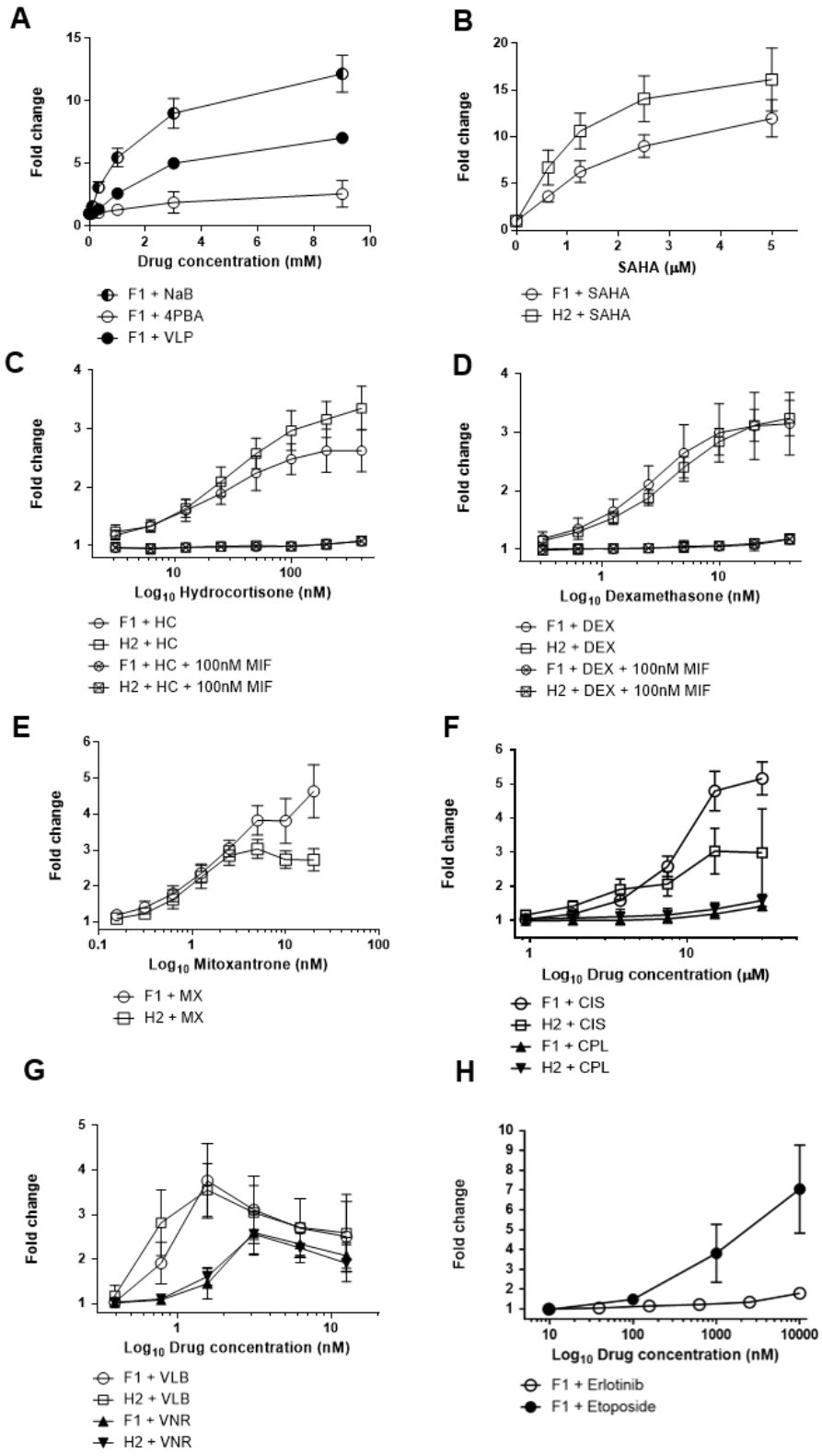


Figure 3. Analysis of eGFP-ABCG2 fusion protein expression in the A-FUS reporter cell line confirms ABCG2 regulation by HDACIs, chemotherapeutics and GCs

The engineered A549 cells were drug-treated for 72 hours and analyzed for eGFP-ABCG2 expression by flow cytometry. Unless otherwise stated, data represent the means from three independent experiments performed in triplicate, +/- SD.

A. Effect of NaB, 4PBA and VLP on eGFP-ABCG2 expression in D29 cells.
B. Effect of MX and GC on eGFP-ABCG2 expression in D29 cells.
C. Effect of HC and HC+MIF on eGFP-ABCG2 expression in D29 cells. MIF, a glucocorticoid receptor antagonist strongly inhibits the eGFP-ABCG2 expression induced by HC.
D. Effect of methylprednisone (MTP) and methylprednisone+mifepristone (MTP+MIF) on eGFP-ABCG2 expression in D29 cells. MIF, a glucocorticoid receptor antagonist strongly inhibits the eGFP-ABCG2 expression induced by MTP.
E. Cell surface labeling of ABCG2 in A549 parental and D29 cells treated with 10 nM DEX, 100 nM MIF or, 10 nM DEX + 100 nM. Left panel: changes in eGFP fluorescence in D29 cells. MIF does not affect the basal expression of eGFP in the engineered cell line but significantly inhibits DEX-induced eGFP expression. Right panel: indirect labeling (5D3+Ko143, Alexa 647) of surface ABCG2 in parental A549 and D29 cells. MIF does not affect the basal levels of surface ABCG2 in either the parental or the eGFP-ABCG2 fusion protein-expressing cells but significantly inhibits DEX-induced ABCG2 expression in both cell lines.

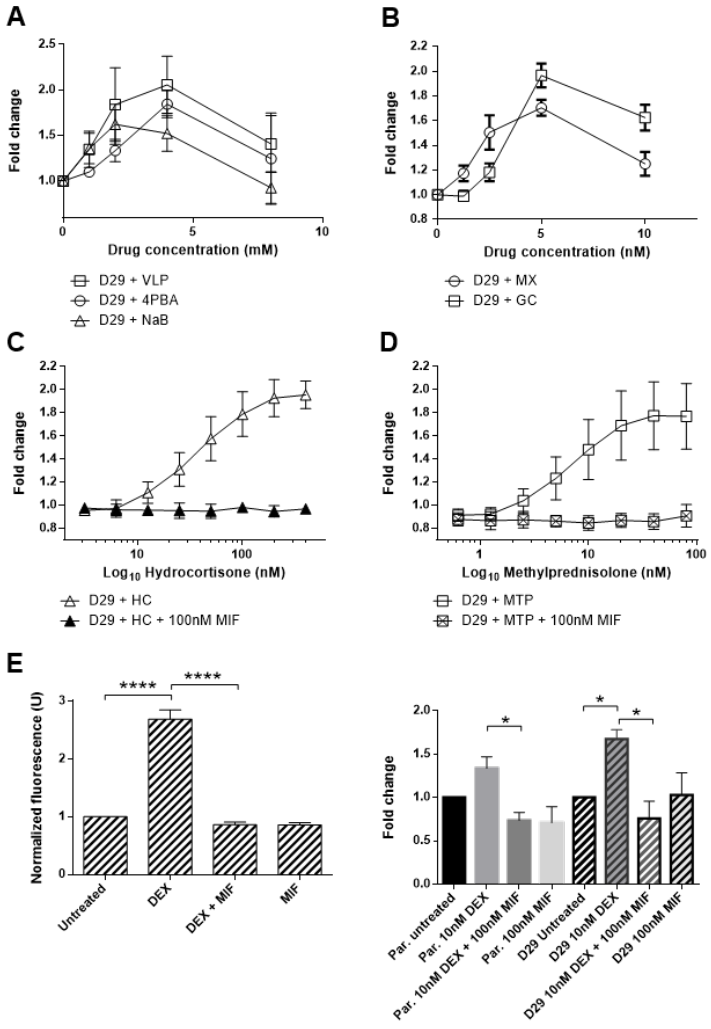


Table 1. Drug-induced expression of eGFP in A-TSCR (eGFP knock-in, ABCG2 knock-out) and A-FUS (eGFP-ABCG2) A549 reporter cells.

	eGFP knock-in, ABCG2 knock-out (A-TSCR) cells, F1 clone		**A-FUS (eGFP-ABCG2) cells, D29 clone**		**mechanism of action**
Drug treatment	**maximum fluorescence increase (fold change)**	**approx. EC50 (uM)**	**maximum fluorescence increase (fold change)**	**approx. EC50 (uM)**	
SAHA (Vorinostat)	8-15	1.5	1.3+/- 0.05	1.5	HDAC-inhibitor
Na-butyrate	8-10	1,500	1.6 +/- 0.1	1,000	HDAC-inhibitor
Valproic acid	4-5	800	2.1 +/- 0.1	1,500	HDAC-inhibitor
4-PBA	2-4	1,000	1.8 +/- 0.1	1,500	HDAC-inhibitor
Hydrocortisone	3.2 +/- 0.3	0.05	2.1 +/- 0.12	0.003	GCR-agonist
Dexamethasone	3.3 +/-0.4	0.004	1.8 +/- 0.3	0.002	GCR-agonist
Methylprednisolone	3.2 +/- 0.2	0.005	1.8 +/- 0.25	0.008	GCR-agonist
Cisplatin	4.8 +/- 0.3	0.8	2.2 +/- 0.1	0.5	Alkylating agent
Paclitaxel	5.5 +/- 0.2	0.025	1.3 +/- 0.1	0.005	Tubulin inhibitor
Gemcitabine	7.5 +/- 0.4	0.02	2.0 +/- 0.1	0.003	Nucleoside analog
Mitoxantrone	5.4 +/- 0.2	0.003	1.7 +/- 0.1	0.003	Topo II inhibitor

As a summary, by using CRISPR-Cas9 gene editing coupled with homology-directed repair, we targeted an eGFP coding sequence to the translational start site of ABCG2, and successfully generated ABCG2 knock-out and *in situ* tagged ABCG2 reporter cells. Using the engineered cell lines, we have shown that ABCG2 is upregulated by a number of anti-cancer medications, HDAC inhibitors, hypoxia-mimicking agents and glucocorticoids, supporting a model in which ABCG2 is under the control of a general stress response. This model system, as to our knowledge, the first fluorescence-based reporter assay to study the regulation of an ABC transporter based on the endogenous, native genetic environment.

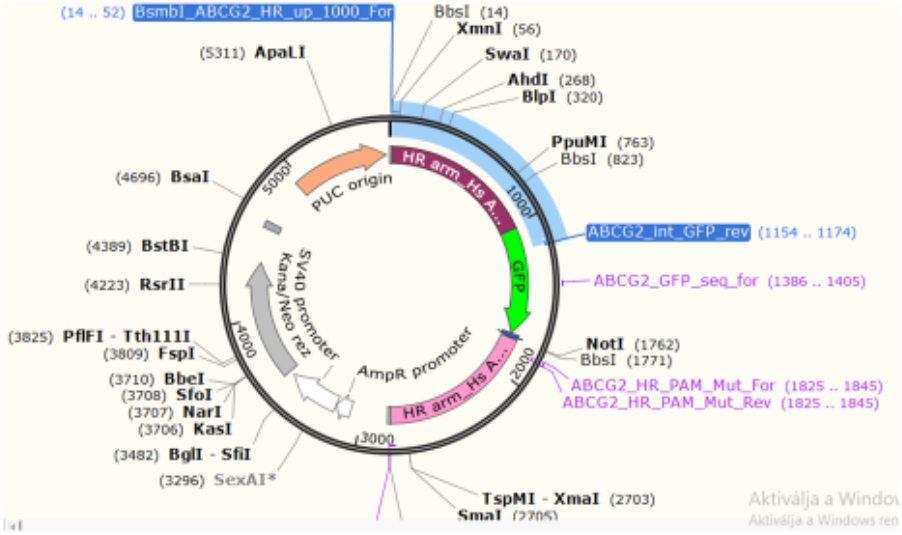
4.b. Studies in human pluripotent (ES and iPS) cell models.

Based on our experience in the A549 cancer stem cells, we attempted to generate genetically engineered human pluripotent stem cell lines in which the full-length normal ABCG2 promoter drives the expression of GFP-ABCG2 (an N-terminally tagged, fully functional fusion protein, as documented in section 1). Since ABCG2 is normally expressed in human pluripotent stem cells, selection of the genetically modified clones could be expected to be performed by following GFP-ABCG2 or GFP expression and using cell sorting and/or clone selection in fluorescence microscopy.

As shown in Figure 4, we have used a modified HR-donor plasmid, in which the PAM sequences were mutated, for the pluripotent stem cell experiments. I addition, the PCR methods to show the proper homologous incorporation of the eGFP fusion constructs have been devised (see inserts).

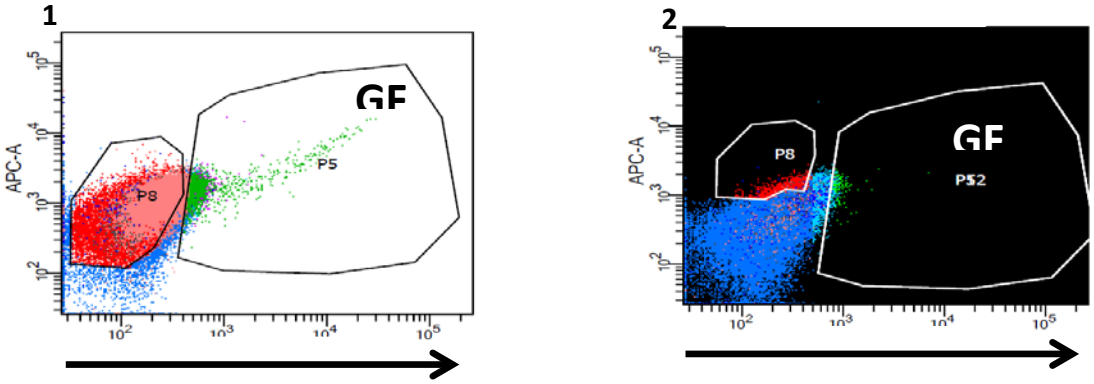
Figure 4. The HR donor plasmid and the PCR setup applied in the pluripotent stem cells.

Primer Name	Sequence	Primer Code	AT*(°C)	Product length
G2integrUP_1_For	CCGCCATTCCGTGATTTTGG	50	66-61	1487 bp
ABCG2_int_GFP_rev (starting at nt 149 in EGFP)	GTGCAGATGAACTTCAGGGTC	24	0.2 C decr./cycle	



For the gene-editing studies we have selected two human pluripotent stem cell lines, the HUES9 ES cells, already used in our laboratory, and the XCL1 induced pluripotent stem cells, which were obtained from the F. Gage laboratory, Scripps Institute. As it turned out, the major problem with this approach was the relatively low transfection efficiency of the hPS cells, and the relatively low and heterogenous ABCG2 expression levels in these cells. To overcome this problem, we have used a double-label sorting technology of the stably transformed cells, by using both the green fluorescence and the cell surface labeling of the ABCG2 protein by a monoclonal (5D3) anti-ABCG2 antibody (see Fig. 5). Thus, only the properly transduced, eGFP-ABCG2 expressing cells could be selected, which resulted in an extremely low number of sorted, gene-edited cells.

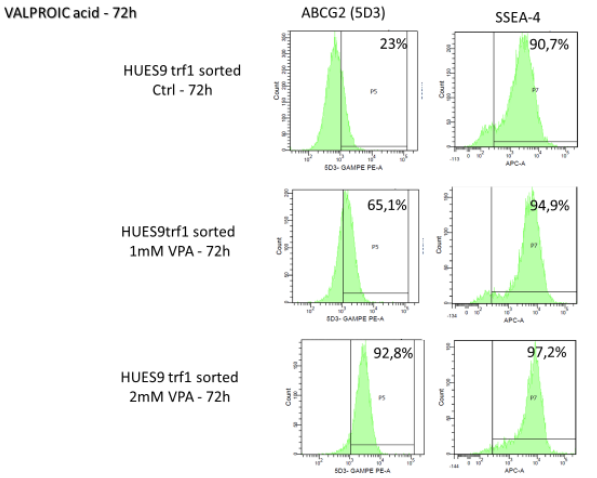
Figure 5. Sorting of the stably modified HUES9 cells after 14 days of transfection. Ordinate: APC fluorescence (5D3 mAb), abscissa: eGFP fluorescence



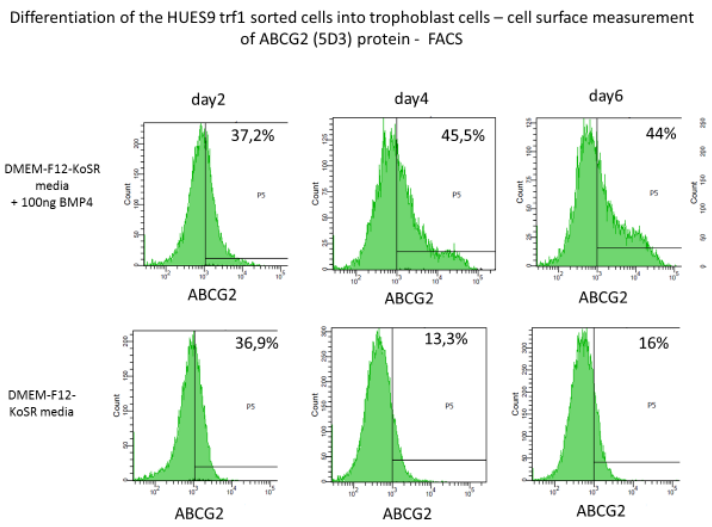
In order to overcome this problem, we have started to explore potential treatments of the stem cells which significantly increase ABCG2 expression while do not result in cell differentiation, thus the pluripotent state (represented by the SSAE-4 pluripotency marker) is preserved. A shown in Figure 6A, 72 hours treatment with 2 mM Valproic acid significantly (from 27 to 93%) increased ABCG2 expression, while the cells preserved their SSEA-4 pluripotency marker. Another approach in this regard was the induction of trophoblast differentiation from the hPS cells, which within 6 days greatly increased ABCG2 expression (Figure 6B)

Figure 6. Treatment of the HUES9 Cells with Valproic acid (A) or differentiation to trophoblasts (B), in order to increase ABCG2 expression.

A.

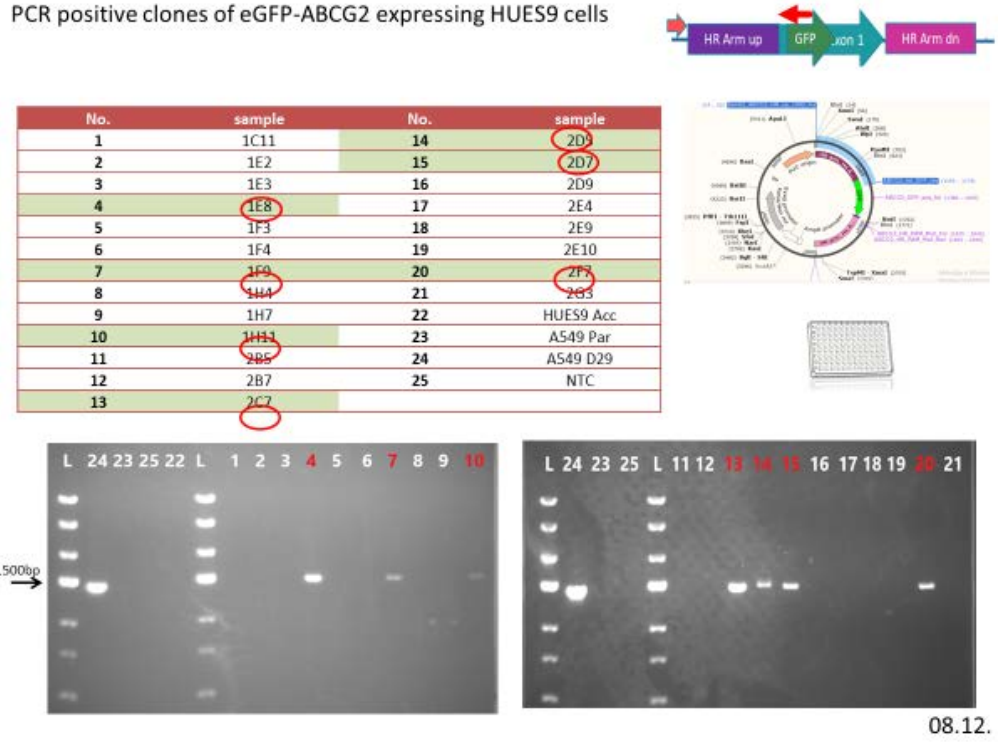


B.



Based on these experiments and the following sorting (as shown in Figure 5), we have prepared single-cell clones of the gene-modified hPS cells and performed PCR studies to find clones which contain the properly inserted eGFP-ABCG2 coding sequences. As shown in Figure 7, we obtained seven cloned cell lines with the properly inserted construct.

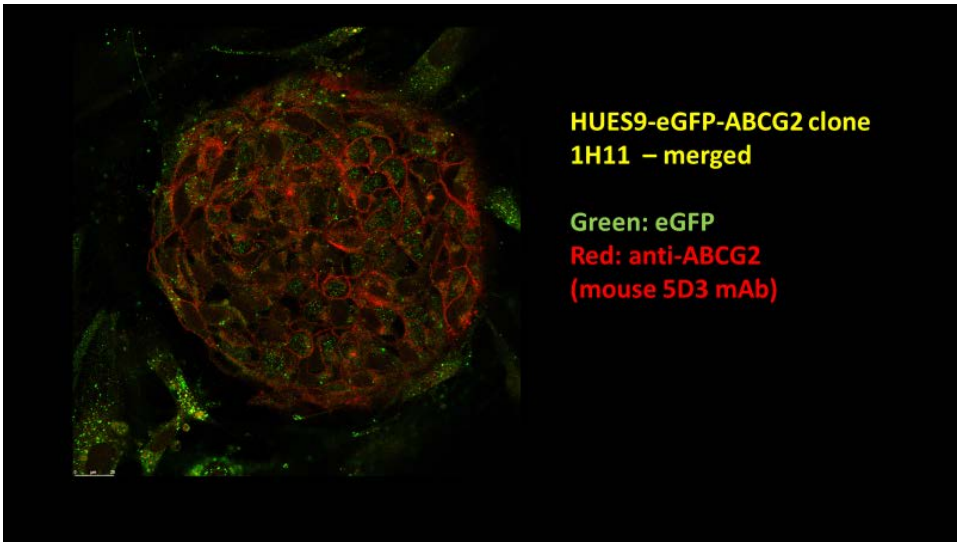
Figure 7. PCR studies on single-cell derived HUES9 cell clones for studying stable insertion.



No.	sample	No.	sample
1	1C11	14	2D9
2	1E2	15	2D7
3	1E3	16	2D9
4	1E8	17	2E4
5	1F3	18	2E9
6	1F4	19	2E10
7	1F9	20	2F7
8	1H4	21	2G3
9	1H7	22	HUES9 Acc
10	1H11	23	A549 Par
11	2B5	24	A549 D29
12	2B7	25	NTC
13	2C7		

Finally, we have started to investigate the *in situ* fluorescence of the expressed eGFP-ABCG2 in the cell populations obtained from the selected clones. As shown in Figure 8, the first promising results show a combined eGFP and ABCG2 expression in some of these preparations.

Figure 8. Confocal microscopy picture of a HUES9 clone stably modified to express eGFP-ABCG2



As a final step of the generation of these cell lines, several methods will have to be used to exclude off-target gene modifications and to ensure the preservation of the original genomic status of the genome-edited stem cell lines (including PCR, FISH and whole exome sequencing analyses), as performed for the gene-edited A549 cells.

III. Summary of the results

In this project we have investigated the complex regulation of the expression and function of medically important human membrane transporters. To explore these questions, we have performed detailed studies of the genetic and epigenetic regulation of an ABC transporter and the plasma membrane calcium pump. Among the ABC transporter we focused on the ABCG2 protein, which provides protection against the harmful accumulation of toxic compounds. An increased expression of ABCG2 in cancer cells results in multiple drug resistance, while a reduced function of this protein causes gout. We have used advanced molecular biology and cell biology methods, including transposon-based and CRISPR-Cas9 based gene editing technologies. Detailed analysis of the ABCG2 protein function and expression was connected to generate reporter cell lines both from normal human pluripotent stem (hPS) and cancer stem (CS) cells, and to study the effects of pathological conditions, including the effects of drugs. Regarding the calcium pump, we found a new, erythropoiesis-specific regulation of the transporter. The combined studies provide a new technology platform for investigating membrane protein regulation and should lead to a better understanding of the cellular regulatory and trafficking mechanisms of membrane transporters. The information obtained in this project provides important clues for further medical diagnostics, pharmacological research and potential therapy to fight major human diseases.

IV. Communications in international journals as a result of this NKFIH/OTKA project:

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3. Apáti A, Szebényi K, Erdei Z, Várady G, Orbán TI, **Sarkadi B**: The importance of drug transporters in human pluripotent stem cells and in early tissue differentiation, EXPERT OPIN DRUG MET 12: (1) 77-92, 2016. **IF: 3.027**
4. Klawitter S, Fuchs NV, Upton KR, Muñoz-Lopez M, Shukla R, Wang J, Garcia-Cañadas M, Lopez-Ruiz C, Gerhardt DJ, Sebe A, Grabundzija I, Merkert S, Gerdes P, Pulgarin JA, Bock A, Held U, Witthuhn A, Haase A, **Sarkadi B**, Löwer J, Wolvetang EJ, Martin U, Ivics Z, Izsvák Z, Garcia-Perez JL, Faulkner GJ, Schumann GG: Reprogramming triggers endogenous L1 and Alu retrotransposition in human induced pluripotent stem cells, NAT COMMUN 7: 10286, 2016 **IF: 12.124**
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9. Sándor S, Jordanidisz T, Schamberger A, Várady G, Erdei Z, Apáti Á, **Sarkadi B**, Orbán TI. Functional characterization of the ABCG2 5' non-coding exon variants: Stem cell specificity, translation efficiency and the influence of drug selection. Biochim Biophys Acta. 2016 Jul;1859(7):943-51. doi: 10.1016/j.bbagrm.2016.05.007. Epub 2016 May 15. PubMed PMID: 27191194. **IF:4.446**
10. Zámbó B, Várady G, Padányi R, Szabó E, Németh A, Langó T, Enyedi Á, **Sarkadi B**. Decreased calcium pump expression in human erythrocytes is connected to a minor haplotype in the ATP2B4 gene. Cell Calcium. 2017 Jul;65:73-79. doi: 10.1016/j.ceca.2017.02.001. Epub 2017 Feb 3. PubMed PMID: 28216081. **IF: 3.513**
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13. Szabó E, Türk D, Telbisz Á, Kucsma N, Horváth T, Szakács G, Homolya L, **Sarkadi B**, Várady G. A new fluorescent dye accumulation assay for parallel measurements of the ABCG2, ABCB1 and ABCC1 multidrug transporter functions. PLoS One. 2018 Jan 17;13(1):e0190629. doi: 10.1371/journal.pone.0190629. eCollection 2018. PubMed PMID: 29342177; PubMed Central PMCID: PMC5771559. **IF: 2.766**
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Toxicol. 2019 Jan;15(1):61-75. doi: 10.1080/17425255.2019.1558207. Epub 2018 Dec 17. PubMed PMID: 30526128**. IF: 3.487**

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17. Várady Gy, Szabó E, Kulin A, Zámbó B, Mózner O, Pálinkás M, Poór Gy and **Sarkadi B**. Potential Diagnostic Application of the Quantitative Assessment of Red Blood Cell Membrane Protein Expression. Thromb Haemost Res. 2019; 3(2): id1024.
18. Orsolya Mózner, Zsuzsa Bartos, Boglárka Zámbó, László Homolya, Tamás Hegedűs, and **Balázs Sarkadi**. Cellular processing of the ABCG2 transporter – potential effects in gout and drug metabolism. Cells, cells-603314, accepted for publication, **IF 5.656**

Cumulative impact factor: 74.807

Submitted, under review:

Daniella Kovacsics, Anna Brózik, Borbála Tihanyi, Zsolt Matula, Adrienn Borsy, Nikolett Mészáros, Edit Szabó, Eszter Németh, Ábel Fóthi, Boglárka Zámbó, Dávid Szüts, Ervin Welker, György Várady, Tamás I. Orbán, Ágota Apáti, and **Balázs Sarkadi**. Precision-engineered reporter cell lines reveal ABCG2 regulation in live lung cancer cells. British Journal of Cancer, TH-2019-4276 2019.

Book chapter:

Szakács G, Hegedűs T, **Sarkadi B**: Inborn Errors of the Cellular Expression and Localization of ABCG2 and ABCB6. A Database for ABC Transporter Mutations, In: Anthony George (szerk.) (szerk.) ABC Transporters - 40 Years on 2016. Cham (Svájc): Springer International Publishing, 2016. pp. 341-355.

Patent application:

Fluorescent dye accumulation assay**.** Inventors**:** Edit SZABÓ, Dóra KOVÁCS-TÜRK, Ágnes TELBISZ, Nóra KUCSMA, Tamás HORVÁTH, Gergely SZAKÁCS, László HOMOLYA, Balázs SARKADI, György VÁRADY - WO2019081957A1 - PCT/HU2018/050046**,** 2017-10-25 Priority to HU1700432, published and under approval - https://patents.google.com/patent/WO2019081957A1/en

Personal notes on the conditions of this research project

The NKFIH-OTKA grant provided an important support for the work described in this project, while the conditions of the use of this support were less than optimum. In the first year of the project the host Institute, the Research Centre for Natural Sciences, HAS, went practically bankrupt, and for more than six months all purchase orders and employment of new research participants were stopped or strongly reduced. In addition, in the final year of the project, due to the attempted transfer of the Academy Institutes to a governmental body, again all new enrollments, supply orders and external contracts were blocked for about six months. Due to the strict enforcement of the Hungarian public servant low, all post-doc participants on a fixed-term grant support and reaching a five-year employment, were forced to leave the Institute. These conditions resulted in a large fluctuation of the participants, and the PhD and MSc students remained the main force behind the experimental work. The forced, early publication of some results for supporting the students' thesis works is one of the explanations for the high number, but relatively low impact publications of our results. On the positive side, this project supported to obtain the successful diplomas of three PhD students and two MSc and two BSc students.