Final Report

of Research Conducted as OTKA NK Grant

"Complex study of the ABCC6 gene and the encoded protein: from transcriptional regulation of the gene associated to genetic diseases to structure-based mechanism of the transporter."

2010. 02.01 – 2014.01.31.

During the above period we have published 22 peer reviewed research papers in international journal with the combined impact factor of 87.8.

Our research activity focused on the *ABCC6* gene, mutations in the encoded transporter protein cause two monogenic diseases of soft tissue calcification, pseudoxanthoma elasticum (PXE) and general arterial calcification of on infancy (GACI). We have studied the protein and its disease-causing mutants, the transcriptional regulation of the gene and performed research related to the metabolite(s) of the transporter. We have also developed two *in vivo* model systems to study the function of the protein (zebrafish and mouse). We were invited to write reviews (4 papers) and organized three international conferences of ABCC6 research. We were also active in characterizing another ABC family member, ABCG2, playing crucial role in cancer multidrug resistance.

1/ The protein, its intracellular localization, metabolites, missense mutants, animal models (9 publications)

We have established a complex experimental strategy to determine the structural and functional consequences of disease-causing mutations in the human *ABCC6*. The major aim of our study was to identify mutants with preserved transport activity but failure in intracellular targeting. Five missense mutations were investigated: R1138Q, V1298F, R1314W, G1321S and R1339C. Using *in vitro* assays, we have identified two variants; R1138Q and R1314W that retained significant transport activity. All mutants were transiently expressed *in vivo*, in **mouse** liver via hydrodynamic tail vein injections. The inactive V1298F was the only mutant that showed normal cellular localization in hepatocytes while the other mutants showed mostly intracellular accumulation indicating abnormal trafficking. As both R1138Q and R1314W displayed endoplasmic reticulum localization, we tested whether 4-phenylbutyrate (4-PBA), a drug approved for clinical use, could restore their intracellular trafficking to the plasma membrane in MDCKII (Madin-Darby canine kidney (cell line)) cells and in mouse liver. The cellular localization of R1314W was significantly improved by 4-PBA treatment, thus potentially rescuing its physiological function. Our work demonstrates the feasibility of the *in vivo* rescue of cellular maturation of some ABCC6 mutants in physiological conditions very similar to the biology of the fully differentiated human liver and could have future human therapeutic application. (Le Saux et al, 2011)

We have tested 10 frequent disease-causing ABCC6 missense mutants for the transport activity by using Sf9 (*Spodoptera frugiperda*) cells, characterized the subcellular localization in MDCKII cells and in **mouse** liver, and tested the phenotypic rescue in **zebrafish**. We aimed at identifying mutants with preserved transport activity but with improper plasma membrane localization for rescue by the chemical chaperone 4-phenylbutyrate (4-PBA). Seven of the mutants were transport-competent but mislocalized in mouse liver. The observed divergence in cellular

localization of mutants in MDCKII cells versus mouse liver underlined the limitations of this 2D in vitro cell system. The functionality of ABCC6 mutants was tested in zebrafish, and minimal rescue of the morpholino-induced phenotype was found. However, 4-PBA, a drug approved for clinical use, restored the plasma membrane localization of four ABCC6 mutants (R1114P, S1121W, Q1347H, and R1314W), suggesting that allele-specific therapy may be useful for selected patients with PXE and GACI. (Pomozi et al, 2014)

The **zebrafish** (*Danio rerio*) has two ABCC6-related sequences. To study the function of abcc6 during zebrafish development, the mRNA expression levels were measured using RT-PCR and in situ hybridization. The abcc6a showed a relatively high level of expression at 5 days post-fertilization (d.p.f.) and the expression was specific to the Kupffer's vesicles. The abcc6b expression was evident at 6 hours post-fertilization (h.p.f.) and remained high up to 8 d.p.f., corresponding to embryonic kidney proximal tubules. Morpholinos were designed to both genes to prevent pre-mRNA splicing and block translation. Injection of the abcc6a morpholinos into 1-4 cell zebrafish embryos decreased gene expression by 54-81%, and induced a phenotype, pericardial edema and curled tail associated with death at around 8 d.p.f. Microinjecting zebrafish embryos with full-length mouse Abcc6 mRNA together with the morpholino completely rescued this phenotype. No phenotypic changes were observed when the abcc6b gene morpholino was injected into embryos with knock-down efficiency of 100%. These results suggest that abcc6a is an essential gene for normal zebrafish development and provide insight into the function of ABCC6, the gene mutated in PXE. (Li Q et al, 2010)

We performed immunofluorescent labeling of frozen mouse and human liver sections, as well as primary hepatocytes. We used several different antibodies recognizing human and mouse ABCC6. Our results unequivocally show that ABCC6 is in the basolateral membrane of hepatocytes and is not associated with the mitochondria, mitochondria-associated membrane, or the endoplasmic reticulum.
Our findings support the model that ABCC6 is in the basolateral membrane, mediating the sinusoidal efflux of a metabolite from the hepatocytes to systemic circulation. (Pomozi et al, 2013)

By using two models of infarction, nonischemic cryoinjury and the pathologically relevant coronary artery ligation, we confirmed a large propensity to acute cardiac mineralization in *Abcc6−/−* mice. Furthermore, when the expression of ABCC6 was reduced to approximately 38% of wild-type levels in *Abcc6+/−* mice, no calcium deposits in injured cardiac tissue were observed. In addition, we used a gene therapy approach to deliver a functional human ABCC6 via hydrodynamic tail vein injection to approximately 13% of mouse hepatocytes, significantly reducing the calcification response to cardiac cryoinjury. We observed that the level and distribution of known regulators of mineralization, such as osteopontin and matrix Gla protein, but not osteocalcin, were concomitant to the level of hepatic expression of human and mouse ABCC6. This study showed that the expression of ABCC6 in liver is an important determinant of calcification in cardiac tissues in response to injuries and is associated with changes in the expression patterns of regulators of mineralization. (Brampton et al, 2014)

We have performed a deep search of the available literature to find published data on the 40 ABC-transporter nsSNPs to delineate them with the published predictions. Comparing the predicted phenotype with the phenotype extracted from the published data reveals misprediction in roughly half of the cases (10/19) thus

demonstrating the inherent difficulties of rationalizing and predicting the functional impact of snSNPs. (Arányi et al, 2011)

The inability of the ABCC6 transporter to secrete its substrate into the circulation is the likely cause of PXE. Vitamin K plays a role in the regulation of mineralization processes as a co-factor in the carboxylation of calcification inhibitors such as Matrix Gla Protein (MGP). Vitamin K precursor or a conjugated form has been proposed as potential substrate(s) for ABCC6. We investigated whether an enriched diet of vitamin K1 or vitamin K2 (MK4) could stop or slow the disease progression in *Abcc6-/-* mice. *Abcc6-/-* mice were placed on a diet of either vitamin K1 or MK4 at 5 or 100 mg/kg at prenatal, 3 weeks or 3 months of age. Disease progression was quantified by measuring the calcium content of one side of the mouse muzzle skin and histological staining for calcium of the opposing side. Raising the vitamin K1 or MK4 content of the diet increased the concentration of circulating MK4 in the serum. However, this increase did not significantly affect the MGP carboxylation status or reduce its abnormal abundance, the total calcium content or the pathologic calcification in the whiskers of the 3 treatment groups compared to controls. Our findings showed that raising the dietary intake of vitamin K1 or MK4 was not beneficial in the treatment of PXE and suggested that the availability of vitamin K may not be a limiting factor in this pathology. (Brampton et al, 2011)

Vitamin K3 is an important intermediate during conversion of the dietary vitamin K1 to the most abundant vitamin K2 form. Here we examined the efflux of the glutathione conjugate of vitamin K3 (VK3GS) from the liver in wild type and *Abcc6-/-* mice, and in transport assays in vitro. We found in liver perfusion experiments that VK3GS is secreted into the inferior vena cava, but we observed no significant difference between wild type and *Abcc6-/-* animals. We overexpressed the human ABCC6 transporter in Sf9 insect and MDCKII cells and assayed its vitamin K3-conjugate transport activity *in vitro*. We found no measurable transport of VK3GS by ABCC6, whereas ABCC1 transported this compound at high rate in these assays. These results show that VK3GS is not the essential metabolite transported by ABCC6 from the liver and preventing the symptoms of pseudoxanthoma elasticum. (Fülöp et al, 2011)

It was suggested that adenosine is the transported substrate of ABCC6. Lack of adenosine tranport results in higher expression of non-tissue specific alkaline phosphatase. The high activity of this enzyme causes the breakdown of pyrophosphate, a known inhibitor of soft tissue calcification, thus causing PXE. We have established by biochemical transport as well as by liver perfusion experiments that adenosine is not transported by ABCC6, consequently it has no direct relevance to PXE. (Szabó et al, 2011)

2/ Transcriptional regulation, population genetics (3 publications)

Although PXE is a recessive disease, microscopic dermal lesions, serum alterations, and higher anecdotal incidence of stroke or CAD among carriers were reported. Here we investigated the association of the c.3421C>T loss-of-function mutation of ABCC6 and CAD and stroke. A previous study demonstrated the association of the c.3421C>T mutation with CAD; however, the frequency found in the control population was unexpectedly high, contradicting, thus, the prevalence of PXE. In the present study, genomic DNA from 749 healthy blood donors was used as control, while 363 and 361 patients suffering from stroke and CAD were investigated,

respectively. One carrier was found in our control group, which is in accordance with the reported prevalence of this mutation. No significant association was found between carrier status and stroke in our cohort. In contrast, a significant association of carrier status and CAD was observed (5/361 carriers: p = 0.016, odds ratio [OR] = 10.5). We propose that carriers of ABCC6 loss-of-function mutations benefit from CAD prevention therapy. (Köblös et al, 2010)

We investigated the transcriptional regulation of the gene and observed that hepatocyte growth factor (HGF) inhibits its expression in HepG2 cells via the activation of ERK1/2. Similarly, other factors activating the cascade also inhibited ABCC6 expression. We identified the ERK1/2 response element in the proximal promoter by luciferase reporter gene assays. This site overlapped with a region conferring the tissue-specific expression pattern to the gene and with a putative hepatocyte nuclear factor 4 alpha (HNF4alpha) binding site. We demonstrated that HNF4alpha regulates the expression of ABCC6, acts through the putative binding site, and determines its cell type-specific expression. We also showed that HNF4alpha is inhibited by the activation of the ERK1/2 cascade. In conclusion we describe here the first regulatory pathway of ABCC6 expression showing that the ERK1/2-HNF4alpha axis has an important role in regulation of the gene. (de Boussac et al, 2010)

We investigated the transcriptional regulation of the gene, using DNase I hypersensitivity assay followed by luciferase reporter gene assay. We identified three DNase I hypersensitive sites (HSs) specific to cell lines expressing ABCC6. These HSs are located in the proximal promoter and in the first intron of the gene. We further characterized the role of the HSs by luciferase assay and demonstrated the transcriptional activity of the intronic HS. We identified the CCAAT/enhancer-binding protein β (C/EBPβ) as a factor binding the second intronic HS by chromatin immunoprecipitation and corroborated this finding by luciferase assays. We also showed that C/EBPβ interacts with the proximal promoter of the gene. We propose that C/EBPβ forms a complex with other regulatory proteins including the previously identified regulatory factor hepatocyte nuclear factor 4α (HNF4α). This complex would account for the tissue-specific expression of the gene and might serve as a metabolic sensor. Our results point toward a better understanding of the physiological role of ABCC6. (Ratajewski et al, 2012)

3/ International scientific mettings organized and invited reviews (4 publications)

We have organized three international conferences on ABCC6 and on soft tissue calcification. Two meetings were held in Budapest (2011 and 2013), while one of them took place in Bethesda, MD, USA (2012). A major review-summary of the 2012 Bethesda Meeting was published covering the field from basic genetics to potential clinical applications.

We reviewed upon the invitation of *Current Drug Targets* the information available on gene structure, evolution as well as the present knowledge on its transcriptional regulation. We give a detailed description of the characteristics of the protein, and analyze the relationship between the distributions of missense disease-causing mutations in the predicted three-dimensional structure of the transporter, which suggests functional importance of the domain-domain interactions. Though neither the physiological function of the protein nor its role in the pathobiology of the diseases are known, a current hypothesis that ABCC6 may be involved in the efflux of

one form of Vitamin K from the liver is discussed. Finally, we analyze potential strategies how the gene can be targeted on the transcriptional level to increase protein expression in order to compensate for reduced activity. In addition, pharmacologic correction of trafficking-defect mutants or suppression of stop codon mutations as potential future therapeutic interventions are also reviewed. (Váradi et al, 2011)

We have summarized that ABCC6 deficiency is the primary cause for chronic and acute forms of ectopic mineralization described in diseases such as pseudoxanthoma elasticum (PXE), β-thalassemia, and generalized arterial calcification of infancy (GACI) in humans and dystrophic cardiac calcification (DCC) in mice. These pathologies are characterized by mineralization of cardiovascular, ocular, and dermal tissues. PXE and to an extent GACI are caused by inactivating ABCC6 mutations, whereas the mineralization associated with β-thalassemia patients derives from a liver-specific change in ABCC6 expression. DCC is an acquired phenotype resulting from cardiovascular insults (ischemic injury or hyperlipidemia) and secondary to ABCC6 insufficiency. Abcc6-deficient mice develop ectopic calcifications similar to both the human PXE and mouse DCC phenotypes. The precise molecular and cellular mechanism linking deficient hepatic ABCC6 function to distal ectopic mineral deposition is not understood and has captured the attention of many research groups. Our previously published work along with that of others show that ABCC6 influences other modulators of calcification and that it plays a much greater physiological role than originally thought. (Le Saux et al, 2012)

We have also reviewed the mechanisms of this restricted tissue-specific expression and the role of hepatocyte nuclear factor 4 α which is responsible for the expression pattern. Detailed analyses uncovered further regulators of the expression of the gene pointing to an intronic primate-specific regulator region, an activator of the expression of the gene by binding CCAAT/enhancer-binding protein beta, which interacts with other proteins acting in the proximal promoter. This regulatory network is affected by various environmental stimuli including oxidative stress and the extracellular signal-regulated protein kinases 1 and 2 pathway. We discussed the structural and functional consequences of disease-causing missense mutations of ABCC6. A significant clustering of the missense disease-causing mutations was found at the domain-domain interfaces. This clustering means that the domain contacts are much less permissive to amino acid replacements than the rest of the protein. We summarize the experimental methods resulting in the identification of mutants with preserved transport activity but failure in intracellular targeting. These mutants are candidates for functional rescue by chemical chaperons. The results of such research can provide the basis of future allele-specific therapy of ABCC6-mediated disorders like pseudoxanthoma elasticum or the generalized arterial calcification in infancy. (Arányi et al, 2013)

4/ Structure-function studies of ABCG2 (6 publications)

We contributed in developing a rapid in vitro assay to identify transport modulation by measuring the cell surface interaction of a conformation sensitive monoclonal antibody (5D3) with ABCG2 in intact cells. As documented, in conjunction with membrane ATPase, transport and cytotoxicity measurements, this assay provides a reliable estimate of concentration-dependent modulation of ABCG2 by

newly emerging pharmacophores. A high-throughput, 96-well plate assay platform is also provided. (Telbisz et al, 2012)

The PI3-kinase/Akt signaling axis has been implicated as a key element in regulating various cellular functions, including the expression and plasma membrane localization of ABCG2. We demonstrated that besides inhibiting their respective target kinases, the pharmacological PI3-kinase inhibitor LY294002 and the downstream mTOR kinase inhibitor rapamycin also directly inhibit ABCG2 function. In contrast, wortmannin, another commonly used pharmacological inhibitor of PI3-kinase does not interact with the transporter. We suggest that direct functional modulation of ABCG2 should be taken into consideration when pharmacological agents are applied to dissect the specific role of PI3-kinase/Akt/mTOR signaling in cellular functions. (Hegedűs et al, 2012a).

Numerous tumors depend on unregulated EGFR signaling, thus inhibition of this receptor by small molecular weight inhibitors such as gefitinib, and the novel second generation agents vandetanib, pelitinib and neratinib, is a promising therapeutic option. In the present study, we provide detailed biochemical characterization regarding the interaction of these EGFR inhibitors with ABCG2. We show that ABCG2 confers resistance to gefitinib and pelitinib, whereas the intracellular action of vandetanib and neratinib is unaltered by the presence of the transporter. At higher concentrations, however, all these EGFR inhibitors inhibit ABCG2 function, thereby promoting accumulation of ABCG2 substrate drugs. We also report enhanced expression of ABCG2 in gefitinib-resistant non-small cell lung cancer cells, suggesting potential clinical relevance of ABCG2 in acquired drug resistance. Since ABCG2 has important impact on both the pharmacological properties and anti-cancer efficiencies of drugs, our results regarding the novel EGFR inhibitors should provide useful information about their therapeutic applicability against ABCG2-expressing cancer cells depending on EGFR signaling. In addition, the finding that these EGFR inhibitors efficiently block ABCG2 function may help to design novel drug-combination therapeutic strategies. (Hegedűs et al, 2012b).

We aimed to decipher the role of hepatocyte growth factor (HGF) and the related kinase cascades on the expression of ABCG2 and the role of the different promoters in this process in the HepG2 human HCC cell line. We observed that HGF treatment increased the amount of ABCG2 on the cell surface in parallel with an increased ABCG2 transcription. ABCG2 mRNA expression was also increased by EGF, oxidative stress or activation of the aryl hydrocarbon receptor, while decreased by TGFb. Treatment with U0126, a specific inhibitor of the ERK1/2 cascade, prevented the HGF and the oxidative stress induced ABCG2 upregulation. We also show that the regulation of ABCG2 by various modulators involve specific alternative promoters. In conclusion, we demonstrate a unique role of the ERK1/2 cascade on ABCG2 modulation in HepG2, and the differential use of the alternative ABCG2 promoters in this cell line. This study reveals the molecular participants of ABCG2 overexpression as new potential treatment targets in HCC. (de Boussac et al, 2012)

We have established an efficient protocol for the purification and reconstitution of the functional ABCG2 protein. We found that the drug-stimulated ATPase and the transport activity of ABCG2 are fully preserved by applying excess lipids and mild detergents during solubilization, whereas a detergent-induced dissociation of the ABCG2 dimer causes an irreversible inactivation. By using the purified and reconstituted protein we demonstrate that cholesterol is an essential activator, whereas bile acids are important modulators of ABCG2 activity. Both wild-

type ABCG2 and its R482G mutant variant require cholesterol for full activity, although they exhibit different cholesterol sensitivities. Bile acids strongly decrease the basal ABCG2-ATPase activity both in the wild-type ABCG2 and in the mutant variant. These data reinforce the results for the modulatory effects of cholesterol and bile acids of ABCG2 investigated in a complex cell membrane environment. Moreover, these experiments open the possibility to perform functional and structural studies with a purified, reconstituted and highly active ABCG2 multidrug transporter. (Telbisz et al, 2013)

We have analyzed whether certain regions in this protein are involved in sterol recognition. We found that replacing ABCG2-R482 with large amino acids does not affect cholesterol dependence, but changes to small amino acids cause altered cholesterol sensitivity. When leucines in the potential steroid-binding element (SBE, aa 555-558) of ABCG2 were replaced by alanines, cholesterol dependence of ABCG2 activity was strongly reduced, although the L558A mutant variant when purified and reconstituted still required cholesterol for full activity. Regarding the effect of bile acids in isolated membranes, we found that these compounds decreased ABCG2-ATPase in the absence of drug substrates, which did not significantly affect substrate-stimulated ATPase activity. These ABCG2 mutant variants also altered bile acid sensitivity, although cholic acid and glycocholate were not transported by the protein. We suggest that the aforementioned two regions in ABCG2 are important for sterol sensing and may represent potential targets for pharmacologic modulation of ABCG2 function. (Telbisz et al, 2014)