Results on the *ABCC6* gene, the encoded protein and the associated genetic disorder, pseudoxanthoma elasticum (PXE):

Genom-evolution and genetic variability:

The completion of the Human Genome Project has brought the understanding that our genome contains an unexpectedly large proportion of segmental duplications. We have conducted an in-depth study of a subset of segmental duplications on chromosome 16. This poses the challenge of elucidating the consequences of recent duplications on physiology. We focused on PKD1 and ABCC6 duplications because mutations affecting these genes are responsible for the Mendelian disorders autosomal dominant polycystic kidney disease and pseudoxanthoma elasticum, respectively. We establish that duplications of PKD1 and ABCC6 are associated to low-copy repeat 16a and show that such duplications have occurred several times independently in different primate species. We demonstrate that partial duplication of PKD1 and ABCC6 has numerous consequences: the pseudogenes give rise to new transcripts and mediate gene conversion, which not only results in disease-causing mutations but also serves as a reservoir for sequence variation. The duplicated segments are also involved in submicroscopic and microscopic genomic rearrangements, contributing to structural variation in human and chromosomal break points in the gibbon. In conclusion, our data shed light on the recent and ongoing evolution of chromosome 16 mediated by segmental duplication and deepened our understanding of the history of two Mendelian disorder genes.

Population genetics and ABCC6 *as a genetic risk factor*

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.

Transcriptional regulation of human ABCC6

Although *ABCC6* is mainly expressed in the liver, the disease has dermatologic, ocular and cardiovascular symptoms. 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 tissue-specific expression pattern to the gene and with a putative hepatocyte nuclear factor 4α (HNF4α) binding site. Several lines of evidence suggested that HNF4α might indeed have a functional role in the regulation of *ABCC6* (e.g. ChIP-on-chip and siRNA analyses).We demonstrated that HNF4α is a master regulator of the expression of *ABCC6,* acts through the putative binding site and determines its tissue-specific expression. We also showed that HNF4α is inhibited by the activation of the ERK1/2 cascade. In conclusion we described the first physiological regulatory pathway of *ABCC6* expression showing that the ERK1/2-HNF4α axis has key role in the tissue-specific regulation of the gene.

Homology model building of the ABCC6 protein and structure-function studies

ABCC6 encodes a 1503 amino acid long ABC transporter, ABCC6/MRP6. The functional link between the impaired activity of the protein and the disease is not known. No high-resolution three dimensional structure of ABCC6 is available. However, building a three dimensional homology model of ABCC6 was made possible by the recent publication of high resolution crystalline structures of different ABC proteins. The structures representing the nucleotide-saturated, outward facing conformation show that the two nucleotide-binding (ABC) domains are in close proximity to each other in the characteristic head-to-tail orientation reflecting to the previously described "nucleotide sandwich dimer. Newly

recognized structural elements are the long "rigid" extensions of the transmembrane helices, called intracellular loops (ICL). Each half of the ABC proteins has two ICLs interacting with the ABC-domains. The coupling helices contact with their “own” as well as with the “opposite” ABC-domains, hence a special type of domain swapping can be recognized in the structure .

We have built a homology model of this transporter, and analyzed the distribution of the known 119 missense PXE-associated mutations within the predicted structure. Significant clustering of the missense mutations has been found at complex domain-domain interfaces: at the transmission interface that involves four intracellular loops and the two ABC domains as well as at the ABC-ABC interacting surfaces. The mutations affecting these regions are 2.75 and 3.53-fold more frequent than the average mutational rate along the transporter protein sequence. Though the functional link between the impaired activity of the protein and the disease is not known, our data provide a genetic proof of the importance of these domain-domain interactions in the ABCC6 transporter and in the PXE phenotype.

A novel animal model to investigate ABCC6: the zebrafish (Danio rerio)

This animal model has been developed in collaboration with research laboratories at Th. Jefferson University and at NIH (bot in the US).

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 (dpf) and the expression was specific to the Kupffer’s vesicles. The abcc6b expression was evident at 6 dpf and remained high up to 8 dpf, corresponding to embryonic kidney proximal tubules. Morpholinos were designed to both genes to block translation and to prevent premRNA splicing. Injection of the abcc6a morpholinos into 1-4 cell zebrafish embryos decreased gene expression by 54 to 81%, and induced a phenotype, cardiac edema and curled tail associated with death at around 8 dpf. 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 to embryos, with knock-down

efficiency of 100%. These results suggest that abcc6a is an essential gene for normal zebrafish development and provide novel insight into the function of ABCC6, the gene mutated in PXE. These results serve as basis of a novel knockdown animal model system. Our ongoing experiments show that the human mRNA is also capable of rescuing the morpholino phenotype opening avenues toward the in vivo investigation of the human protein and its mutant variants in this novel animal model system.

Invite reviews

As a result of our pioneering work on the molecular biology of PXE and ABCC6 we were invited to write a larger review entilted: "ABCC6 as a target in Pseudoxanthoma Elasticum" for the Current Drug Targets (IF: 4.20). This gave us an excellent opportunity to make a deep survey of all aspects of the disease, the gene and the protein. Most of the members of the group, graduate (PhD) students and postdocs participated in this work thus allowing them to develop skills in writing scientific reviews. The manuscript enjoyed highly favorable comments from the referees:

"This review provides an excellent and up-to-date overview of what is known on ABCC6, e.g. on gene structure, evolution, transcriptional regulation, protein structure and the potential physiological function of ABCC6." (Referee 1.)

"This is a valuable review, the first to take what is known about the pathogenesis of PXE and translate it into physiologic and pharmacologic mechanisms for targeted therapy. In that respect, it has succeeded." (Referee 2.).

In the paper we have reviewed that mutations in the *ABCC6* gene cause a rare, recessive genetic disease, pseudoxanthoma elasticum, while the loss of one *ABCC6* allele is a genetic risk factor in coronary artery disease. We also reviewed the information available on gene structure, evolution as well as the present knowledge on its transcriptional regulation. We gave 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 was also discussed. Finally, we analyzed 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 were also reviewed.

In an other review written in collaboration with our collaborators we have described that missense mutations in the GGCX gene, either in compound heterozygous state or digenic with a recurrent ABCC6 nonsense mutation (p.R1141X), have been identified in patients with PXE-like cutaneous findings and vitamin K-dependent coagulation factor deficiency. GGCX encodes a carboxylase which catalyses gamma-glutamyl carboxylation of coagulation factors as well as of matrix gla protein (MGP) which in fully carboxylated form serves as a systemic inhibitor of pathologic mineralisation. Collectively, these observations suggest the hypothesis that a consequence of loss-of-function mutations in the *ABCC6* gene is the reduced vitamin K-dependent gamma-glutamyl carboxylation of MGP, with subsequent connective tissue mineralisation. We've concluded that further progress in understanding the detailed pathomechanisms of PXE should provide novel strategies to counteract, and perhaps cure, this complex heritable disorder at the genome-environment interface. The paper was published in attracted Januray 2009 and attracted 13 citations in one year.

The Drosophila MRP shares the biochemical features of its human ABCC orthologues

DMRP, an ABC transporter encoded by the dMRP/CG6214 gene, is the Drosophila melanogaster orthologue of the "long" human multidrug resistance-associated proteins (MRP1/ABCC1, MRP2/ABCC2, MRP3/ABCC3, MRP6/ABCC6, and MRP7/ABCC10). In order to provide a detailed biochemical characterisation we expressed DMRP in Sf9 insect cell membranes. We demonstrated DMRP as a functional orthologue of its human counterparts capable of transporting several human MRP substrates like beta-estradiol 17-beta-D-glucuronide, leukotriene C4, calcein, fluo3 and carboxydichlorofluorescein. Unexpectedly, we found DMRP to exhibit an extremely high turnover rate for the substrate transport as compared to its human orthologues. Furthermore, DMRP showed remarkably high basal ATPase activity (68-75 nmol Pi/mg membrane protein/min), which could be further stimulated by probenecid and the glutathione conjugate of N-

ethylmaleimide. Surprisingly, this high level basal ATPase activity was inhibited by the transported substrates. We discussed this phenomenon in the light of a potential endogenous substrate (or activator) present in the Sf9 membrane.

Modulation of the activity of the human ABCG transporters by the membrane cholesterol

The ABCG5/ABCG8 heterodimer is a sterol transporter with the physiological role in the efflux of sitosterols from the enterocytes and cholesterol from hepatocytes. We hypothetised that cholesterol modulates the ATPase activity of the ABCG5/ABCG8 heterodimer. We have performed a study involving several other ABC transporters and found no modulation of ABCG5/ABCG8 activity. However, we have observed that mild cholesterol depletion of intact mammalian cells inhibited ABCG2-dependent dye and drug extrusion in a reversible fashion, while the membrane localization of the transporter protein was unchanged. Cholesterol enrichment of cholesterol-poor Sf9 cell membrane vesicles greatly increased ABCG2-driven substrate uptake, substrate-stimulated ATPase activity, as well as the formation of a catalytic cycle intermediate (nucleotide trapping). Interestingly, modulation of membrane cholesterol did not significantly affect the function of the R482G or R482T substrate mutant ABCG2 variants, or that of the MDR1 transporter. The selective, major effect of membrane cholesterol on the wild-type ABCG2 suggests a regulation of the activity of this multidrug transporter during processing or in membrane micro-domain interactions. The experimental recognition of physiological and pharmacological substrates of ABCG2, as well as the fight against cancer multidrug resistance may be facilitated by demonstrating the key role of membrane cholesterol in this transport activity.

Intramolecular rearrangments in ABCG2

In our previous study we found that the 5D3 monoclonal antibody shows a function-dependent reactivity to an extracellular epitope of the ABCG2 transporter. In the current experiments we have further characterized the 5D3-ABCG2 interaction. The effect of chemical cross-linking and the modulation of extracellular S-S bridges on the transporter function and 5D3 reactivity of ABCG2 were investigated in depth. We found that several

protein cross-linkers greatly increased 5D3 labeling in ABCG2 expressing HEK cells; however, there was no correlation between covalent dimer formation, the inhibition of transport activity, and the increase in 5D3 binding. Dithiothreitol treatment, which reduced the extracellular S-S bridge-forming cysteines of ABCG2, had no effect on transport function but caused a significant decrease in 5D3 binding. When analyzing ABCG2 mutants carrying Cys-to-Ala changes in the extracellular loop, we found that the mutant C603A (lacking the intermolecular S-S bond) showed comparable transport activity and 5D3 reactivity to the wild-type ABCG2. However, disruption of the intramolecular S-S bridge (in C592A, C608A, or C592A/C608A mutants) in this loop abolished 5D3 binding, whereas the function of the protein was preserved. Based on these results and ab initio folding simulations, we propose a model for the large extracellular loop of the ABCG2 protein.

Multidrug resistance and tyrosine kinase inhibitors

Nilotinib and dasatinib were high-affinity substrates of ABCG2, and this protein mediated an effective resistance in cancer cells against these compounds. Nilotinib and dasatinib also interacted with ABCB1, but this transporter provided resistance only against dasatinib. Neither ABCB1 nor ABCG2 induced resistance to bosutinib. At relatively higher concentrations, however, each TKI inhibited both transporters. We concluded that a combination of in vitro assays may provide valuable preclinical information for the applicability of novel targeted anti-cancer TKIs, even in multidrug-resistant cancer. The pattern of MDR-ABC transporter-TKI interactions may also help to understand the general pharmacokinetics and toxicities of new TKIs.

A cellular model system to study ABCA1

The ABCA1 protein plays a pivotal role in reverse cholesterol transport, by mediating the generation of HDL particles and removing cellular cholesterol. Both the proper expression of ABCA1 in the plasma membrane and the internalization along with apoA-I are required for function. Therefore, we developed a model system to investigate the

effect of clinically relevant drugs on the cell surface appearance of ABCA1. By retroviral transduction system, we established stable mammalian cell lines expressing functional and non-functional ABCA1 variants, tagged with an extracellular hemagglutinin epitope. After characterization of the expression, proper localization and function of different ABCA1 variants, we followed quantitatively their cell surface expression by immunofluorescent staining, using flow cytometry. As expected, we found increased cell surface expression of ABCA1 after treatment with a calpain inhibitor, and observed a strong decrease in plasma membrane ABCA1 expression upon treatment with a trans-Golgi transport inhibitor, Brefeldin A. We tested cholesterol level lowering drugs and other potential inhibitors of ABCA1. We demonstrated that ezetimibe affects ABCA1 cell surface expression only in the case of a functional ABCA1. Our model system allows a quantitative detection of cell surface expression of ABCA1, screening of substrates or specific inhibitors, and investigating transport regulation.

Method development: the eccPCR technique

One of the most important strategic steps in the design of quantitative PCR experiments is the choice of an adequate internal standard gene. A reliable standard gene is expected to show unchanged expression under all experimental conditions. If the mRNA level of the standard gene is altered during the experiment, small changes of target mRNA levels can be especially difficult to detect. Indeed, several studies reported that the expression level of commonly used internal standards (e.g., glyceraldehyde-3-phosphate dehydrogenase; GAPDH) may change considerably in response to various factors . Despite that, the expression of standard genes is rarely controlled, introducing thereby a risk of misinterpretation when analyzing real-time quantification experiments.

Several methods to avoid quantification errors due to the use of internal standards of variable expression have been developed recently. Nevertheless none of them solved the problem conclusively. We developed the external cell control PCR (eccPCR) method, an approach developed primarily for cell culture experiments. This techique controls all steps of sample preparation and overcomes the incertitude of normalization to internal standard genes. In conclusion, we developed and validated the eccPCR method to overcome the pitfalls of using single internal standard genes in qPCRs. We showed that

the eccPCR technique provides more reliable data than those obtained by normalization to frequently used internal standard genes. We demonstrated that eccPCR can detect expression induction as small as 50% and can be used on both adherent and nonadherent cells. This new method has several advantages over the existing normalization techniques because it offers an easy to perform, reliable, fast, and economic solution to the problem of normalization of qPCR. Finally, we have to emphasize that further development of the technique (normalization to DNA content instead of cell counting, inclusion of other external control cells, etc.) may considerably enlarge the field of application of the eccPCR assay.

Review article on the pharmacokinetic role of the multidug ABC transporters

As a result of our intensive work on the role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) we were invited to write a larger review to Drug Discovery Today (IF:6.62)

We have reviewed that ATP binding cassette (ABC) drug transporters play important role in cancer drug resistance, protection against xenobiotics, and in general in the passage of drugs through cellular and tissue barriers. We explored how human ABC transporters modulate the pharmacological effects of various drugs, and how this predictable ADME-TOX modulation can be used during the process of drug discovery and development. We provided a description of the relevant human ABC drug transporters and reviewed the models and assay systems that can be applied for the analysis of their expected drug interactions. The use of the in vitro, in vivo, in silico models, their combination, and the emerging clinical information were critically evaluated with respect to their potential application in early drug screening.

The review attracted very high interest: 33 independent citations in less than two years.