Network: Diseases Due to Environmental Factors

Project: Pathway Mapping

Philip Rosenstiel - Universitätsklinikum Schleswig-Holstein, Campus Kiel - p.rosenstiel@mucosa.de

Introduction

This shared scientific project provides functional characterization of genes from systematic disease gene finding efforts to all groups in the disease network. It has two parts:
i. once a disease susceptibility gene has been positionally defined, its unique regulation and function within the pathophysiological pathways has to be assigned. A full range of cell biology techniques including a central high-thoughput real-time PCR platform has been locally established during NGFN-1. The local set-up is used to understand the molecular epidemiology of disease using human disease tissues or to follow expression screening results in cell culture or tissue samples, respectively. Standardized cellular extracts from in vitro cell culture models of inflammation are available to the network partners.
ii. a systematic phylogenetic analysis identifies evolutionary conserved structure and function in a set of selected barrier genes and their variants occuring during evolution.
The overall aims are:

- To integrate novel disease genes into pathways and regulatory cascades within the specific pathophysiology
- To understand functional consequences of disease-associated sequence variants
- To unveil novel molecular targets for prevention and therapy of the underlying disorders

This project coordinates the application of novel high-throughput technologies (e.g. SNP-dependent alternative splicing analysis, MALDI-TOF-based phosphoproteomics, tissue arrays for expression profiling, generation of stable siRNA clones or HTS-Yeast-Two-Hybrid assays) generated within the chain of SMPs (DNA-RNA-Protein-Cell) in the context of gene families important for human barrier organ function.

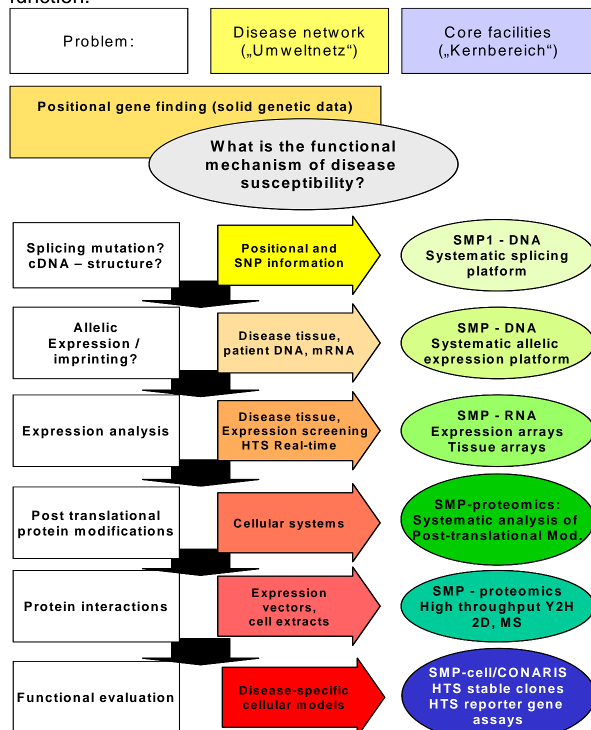

***Fig 1:** Flow chart of the standardized joint functional assignment strategies for disease-associated mutations.*

Results/Project Status

Characterization of NOD2/CARD15 as an intracellular PAMP receptor in intestinal epithelial cells

Genetic variations of NOD2, a member of a protein family containing a nucleotide binding and oligomerization domain, have been linked to susceptibility for Crohn´s disease (CD), a human chronic relapsing inflammatory bowel disease by gene finding efforts in NGFN-1. Our group for the first time described expression and transcriptional regulation of the NOD2 gene in intestinal epithelial cells (IECs), which are a primary element of the intestinal barrier (Rosenstiel et al. 2003). We could also demonstrate an upregulation of NOD2 in colonic epithelial cells under inflammatory conditions in vivo in biopsies from active CD patients. These findings have been independently replicated (Berrebi et al. 2003; Hisamatsu et al. 2003; Lala et al. 2003).
It could be demonstrated that overexpression of NOD2 sensitizes IECs to stimulation with muramyl dipeptide, leading to the secretion of the chemotactic cytokine IL-8 which may serve as a protective danger signal of the innate immune system. Interestingly, the disease-associated NOD2 variant SNP13 is deficient in this protective cytokine induction. Further bioinformatical studies performed together with the MPI-BIOINF have revealed structure-function relationships of NOD2 and other NOD proteins in health and disease (Albrecht et al., 2003 a, b, c).

Proteomics of NOD2/CARD15

Together with the proteomics group at the MPI-MG (Gobom/Lehrach), we have established a proteomic approach to describe protein patterns induced by NOD2-signalling pathways. HEK 293 cells were stably transfected with constructs for wildtype NOD2 or the SNP13 variant. Aim of the study was to compare the differential protein induction upon stimulation with NOD2 ligand muramyl-dipeptide and to identify specific differences between the wildtype- and the disease-associated SNP13-form. So far, 287 significantly regulated spots have been detected with a common set of spots induced in both variants and a subset of differentially regulated spots that are either reduced or increased in the disease-associated variant only. The spots have been analyzed by MALDI-MS/peptide mass fingerprinting and MS/MS techniques.

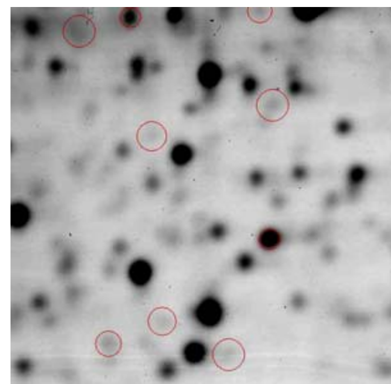

***Fig 2:** Section of a 2D Gel from stably NOD2 overexpressing HEK 293 cells stimulated with muramyl dipeptide for 4h. Red circles mark differentially regulated spots compared to the empty vector control. A total of 287 differential spots were picked and have been analyzed by MALDI-TOF.*

NGFN
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A complex pro-inflammatory program regulated by NOD2wt that encompasses a regulation of key genes involved in protein folding, DNA-repair, cellular redox homoeostasis and metabolism was observed both under normal growth conditions and after stimulation with MDP. Using the comparison of NOD2wt and disease-associated NOD2SNP13 variant, we have identified a proteomic signature pattern that may further our understanding of the influence of genetic variations in the NOD2 gene in the pathophysiology of chronic inflammatory bowel disease (Weichart et al.,JBC *in revision*).

The $^{Met}196^{Arg}$ Variation of human TNFR2 affects TNF-α-induced apoptosis by impaired NF-κB-signalling and target gene expression

Tumor necrosis factor-α (TNF-α)-induced signalling is pivotally involved in the pathogenesis of chronic inflammatory diseases. A polymorphism in the TNF receptor 2 (TNFR2) gene resulting in an inversion from methionine ($TNFR2^{196}$MET) to arginine ($TNFR2^{196}$ARG) at the amino acid 196 has been genetically associated with an increased risk for systemic lupus erythematosus and familial rheumatoid arthritis. The molecular effect of the mutation on the biological function of TNFR2 is still unclear, albeit the mutation does not affect the binding properties of TNFR2.
Stable transfectants carrying either variant of TNFR2 were generated in HeLa cells and embryonic fibroblasts from tnfr1/tnfr2 double deficient mice. Apoptosis was assessed using MTT assays and Annexin V FACS. NF-κB activity was investigated by reporter gene assays. TNFR2/TRAF2 association was determined using co-immunoprecipitation and confocal-laser-microscopy.

The study provides evidence that the mutation results in a significantly lower capability to induce NF-κB signalling downstream of TNFR2. Pre-triggering of TNFR2 with a receptor specific mutein leads to an enhancement of TNFR1-induced apoptosis, which is further increased in cells carrying the $TNFR2^{196}$ARG variant. In parallel, a diminished induction of NF-κB-dependent target genes conveying either anti-apoptotic or pro-inflammatory functions, such as cIAP1, TRAF1, IL-6 or IL-8 is observed (real-time PCR, Western blot, ELISA). The mutated form $TNFR2^{196}$ARG shows a lack of inducible TRAF2 recruitment upon TNF-α stimulation. Our data suggest a profound impairment of TNF-α signalling via the mutated TNFR2 variant at a level of the complexation of the adaptor protein TRAF2 (Till et al., 2005). The findings gain insight into basic mechanisms of TNFR2 signalling and suggest a common molecular principle for the involvement of the M196R TNFR2 mutation in the etiopathogenesis of different chronic inflammatory disorders.

Outlook

Cell biological exploration of inflammation processes and barrier disorders represents one of the most competitive areas in biomedical research. The approach taken in this project has a competitive advantage from its early link between positional gene findings, pathophysiological knowledge and core technologies generated within the SMPs of the NGFN. This synergism results in a substantial acceleration in assigning disease mechanisms to disease-associated mutations.

*Lit.: **1.** Costello, C.M., N. Mah, R. Hasler, P. Rosenstiel, G.H. Waetzig, A. Hahn, T. Lu, Y. Gurbuz, S. Nikolaus, M. Albrecht, J. Hampe, R. Lucius, G. Kloppel, H. Eickhoff, H. Lehrach, T. Lengauer, and S. Schreiber. 2005. Dissection of the inflammatory bowel disease transcriptome using genome-wide cDNA microarrays. PLoS Med 2:e199. **2.** Hellmig, S., S. Ott, M. Musfeldt, M. Kosmahl, P. Rosenstiel, E. Stuber, J. Hampe, U.R. Folsch, and S. Schreiber. 2005. Life-threatening chronic enteritis due to colonization of the small bowel with Stenotrophomonas maltophilia. Gastroenterology 129:706-712. **3.** Rosenstiel, P., J. Agnholt, J. Kelsen, V. Medici, G.H. Waetzig, D. Seegert, and S. Schreiber. 2005. Differential modulation of p38 mitogen activated protein kinase and STAT3 signalling pathways by infliximab and etanercept in intestinal T cells from patients with Crohn's disease. Gut 54:314-315; author reply 316-316. **4.** Rosenstiel, P., M. Fantini, K. Brautigam, T. Kuhbacher, G.H. Waetzig, D. Seegert, and S. Schreiber. 2003. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. Gastroenterology 124:1001-1009. **5.** Schreiber, S., P. Rosenstiel, M. Albrecht, J. Hampe, and M. Krawczak. 2005. Genetics of Crohn disease, an archetypal inflammatory barrier disease. Nat Rev Genet 6:376-388. **6.** Schreiber, S., P. Rosenstiel, J. Hampe, S. Nikolaus, B. Groessner, A. Schottelius, T. Kuhbacher, J. Hamling, U.R. Folsch, and D. Seegert. 2002. Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. Gut 51:379-385. **7.** Till, A., P. Rosenstiel, A. Krippner-Heidenreich, S. Mascheretti-Croucher, P.J. Croucher, H. Schafer, P. Scheurich, D. Seegert, and S. Schreiber. 2005. The Met-196 -> Arg variation of human tumor necrosis factor receptor 2 (TNFR2) affects TNF-alpha-induced apoptosis by impaired NF-kappaB signaling and target gene expression. J Biol Chem 280:5994-6004. **8.** Valentonyte, R., J. Hampe, K. Huse, P. Rosenstiel, M. Albrecht, A. Stenzel, M. Nagy, K.I. Gaede, A. Franke, R. Haesler, A. Koch, T. Lengauer, D. Seegert, N. Reiling, S. Ehlers, E. Schwinger, M. Platzer, M. Krawczak, J. Muller-Quernheim, M. Schurmann, and S. Schreiber. 2005. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. Nat Genet. **9.** Waetzig, G.H., P. Rosenstiel, A. Arlt, A. Till, K. Brautigam, H. Schafer, S. Rose-John, D. Seegert, and S. Schreiber. 2004. Soluble tumor necrosis factor (TNF) receptor-1 induces apoptosis via reverse TNF signaling and autocrine transforming growth factor-beta1. Faseb J. **10.** Waetzig, G.H., P. Rosenstiel, S. Nikolaus, D. Seegert, and S. Schreiber. 2003. Differential p38 mitogen-activated protein kinase target phosphorylation in responders and nonresponders to infliximab. Gastroenterology 125:633-634. **11.** Waetzig, G.H., D. Seegert, P. Rosenstiel, S. Nikolaus, and S. Schreiber. 2002. p38 mitogen-activated protein kinase is activated and linked to TNF- alpha signaling in inflammatory bowel disease. J Immunol 168:5342-5351. **12.** Wilms, H., P. Rosenstiel, J. Sievers, G. Deuschl, L. Zecca, and R. Lucius. 2003. Activation of microglia by human neuromelanin is NF-{kappa}B dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. Faseb J 17:500-502.*