Non-alcoholic steatohepatitis (NASH) is a complex multifactorial liver disorder with often a chronic and progressive course. NASH is characterized by dysfunction of hepatic lipid metabolism, chronic inflammation and ultimately liver damage. As NASH is a silent disease it frequently progresses to severe disease stages, such as advanced fibrosis and cirrhosis leading to liver failure and/or hepatocellular carcinoma. With the growing prevalence of NASH as one of the complications of a Western lifestyle, and fibrosis being the strongest predictor of adverse clinical outcomes, there is a high unmet medical need for drugs against NASH and liver fibrosis.

In order to identify novel targets for NASH-induced fibrosis a generic target discovery & evaluation workflow was developed. This workflow — presented using Keratin 8 (KRT8) as case study — uniquely integrates big data analysis in the fields of systems biology, network analysis, text- and data-mining to enable comprehensive evaluation and strategic selection of exploratory drug targets.

1. Systems Biology / Data Analysis

Fibrosis was induced in high-fat diet (HFD)-fed low-density lipoprotein receptor knockout (LDL−/−)Leiden mice, which develop NASH and hepatic fibrosis in the context of obesity, dyslipidemia, and insulin resistance, as is typical for NASH patients. Matrix deposition was quantified by labelling mice with deuterated water (D2O) for 7 days, and protein fractional synthesis rates were calculated using mass spectrometer proteomics analysis. In addition, RHAM was used to identify differentially Expressed Genes (DEGs) and subsequent top canonical pathways were analyzed. These data show, from 12 weeks onwards that the inflammatory, oxidative stress, and fibrogenic responses were activated. Clear modulation of pathways related to NASH and hepatic fibrosis was visible at week 5 after HFD treatment, as exemplified by expression changes of genes in lipid metabolism pathways and a strong activation of genes in the hepatic fibrosis/hepatic stellate cell activation and integrin signaling pathways.1

To identify key players in disease progression towards fibrosis, genes differentially expressed at both t=12 and t=24 were selected and combining with 0.5 matrix deposition were identified. This resulted in a set of 48 differentially expressed genes (HFD).

2. Network analysis

A subset of DEGs (signature genes) were translated into biological processes using enrichment strategies in the Ingenuity Pathway Analysis tool. The associated signature fibrosis genes (17) were subsequently selected for Path Explorer analysis to be directly linked to other signature genes and situated in the disease network. All genes in the pathways network have a differential expression fold change with a p-value < 0.05 relative to control. An inventory was made regarding the type of process related to NASH and fibrosis it participated in (e.g. inflammation, lipid metabolism, fibrosis, oxidative stress). Genes were subsequently ranked based on the number of processes it was active in, representing a 'gene hub' in the disease. This resulted in a top list of 48 fibrosis-related genes that were subjected to a quick scan of supporting evidence for a role in the disease. Based on this information, a top 10 drug target candidates were identified, including Keratin 8 (KRT8).

3. Target Evaluation KRT8

**BACKGROUND INFORMATION**

Keratin represent the largest subfamily of intermediate filaments (IFs), one of the major cytoskeletal proteins. They play important cell-specific roles in cytoskeleton from various mechanical and non-mechanical stresses. Epithelial keratins are subdivided based on isoelectric point in type I (basic) and type II (acidic). Type I and type II keratins exist as paired polymers: filaments that display a tripartite structure containing a conserved a-helical central rod domain flanked by less conserved b-termed head and C-termed tail domains. KRT8 is type II keratin that dimers often forms heteropolymers with type I keratin family (KRT18).

**Phosphorylation of KRT8**

Phosphorylation of KRT8/KRT18 is required for the regulation of KRT8/KRT18 filament organization, turnover, and interaction with other proteins. KRT8 phosphorylation is caused by cell cycle progression, exposure to various growth factors, or stress-activated kinases. Human KRT8 includes the three major in vivo phosphorylation sites (S23/S73/S431) that are conserved in mouse. S23 is phosphorylated under basal conditions, and S73/S431 are phosphorylated by stress activated protein kinases (SAPKs), such as p38, JNK, and p42 MAPK.1

**Human genetics**

- KRT8-cytoskeleton-disrupting variants were identified in patients with:
  - Total drug-induced liver disease
  - Hemorrhages / liver fibrosis development
  - Non-alcoholic liver disease
  - Cryptogenic liver disease

**Mouse genetics**

- KRT8 null mice:
  - Hepatocyte fragility 1
  - Liver injury 1 (microsystin-LR, concanavalin A, Fas antibody)

- KRT8 overexpression:
  - Mallory-Denk bodies
  - Increased expression or activation/stabilization of KRT8

**Biological function**

- KRT8 provides resistance to hepatic apoptosis by:
  - Blocking FAS targeting to the cell surface, preventing FAS-mediated apoptosis
  - Sequestering pro-apoptotic signals and death-promoting effector molecules
  - Modulating the TNF-dependent activation of iNOS and NFκB

**Prophylactic efficacy**

- KRT8 can control inflammatory responses by controlling NFκB signaling by:
  - Reducing TNFα
  - Inhibiting polyubiquitination of TRAF5
  - Inhibition of TLR-Mediated inflammatory response

**Preclinical efficacy**

- KRT8 can control inflammatory responses by controlling NFκB signaling by:
  - Reducing TNFα
  - Inhibiting polyubiquitination of TRAF5
  - Inhibition of TLR-Mediated inflammatory response

**Chances for first-in-class drug**

- Reduced progression of fibrosis
- Reduced progression of inflammation

**Required MoA**

- Increased expression or activation/stabilization of KRT8

**Drugability**

- No binding pocket for ligand binding; binding partners via protein-protein interactions

**Tool compounds**

- No known tool compounds

**Assay availability**

- No mechanistic of binding assays

- Functional readout: apoptosis by KRT8 staining / TUNEL assay

**Competitive landscape**

- No known drug targets

**Conclusions**

- A comprehensive and generic workflow has been developed that uniquely combines big data analysis in the field of systems biology, network analysis, text- and data-mining to identify, evaluate and rank novel targets based on predefined criteria.
- The text- and data-mining approach is being automated in a web-based interface to allow for more efficient evaluation of exploratory targets.
- Despite supporting evidence for a role of KRT8 in NASH/liver fibrosis from genetic, transgenic and biological function observations, the lack of compounds, tools, assays and the druggability / required mechanism of action make this a less attractive target to pursue in a drug-discovery setting.

**A discovery and evaluation workflow to identify novel targets for NASH-induced liver fibrosis**

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