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

Jennifer Venhorst, Lars Verschuren and Roeland Hanemaaijer

TNO, Zeist, the Netherlands

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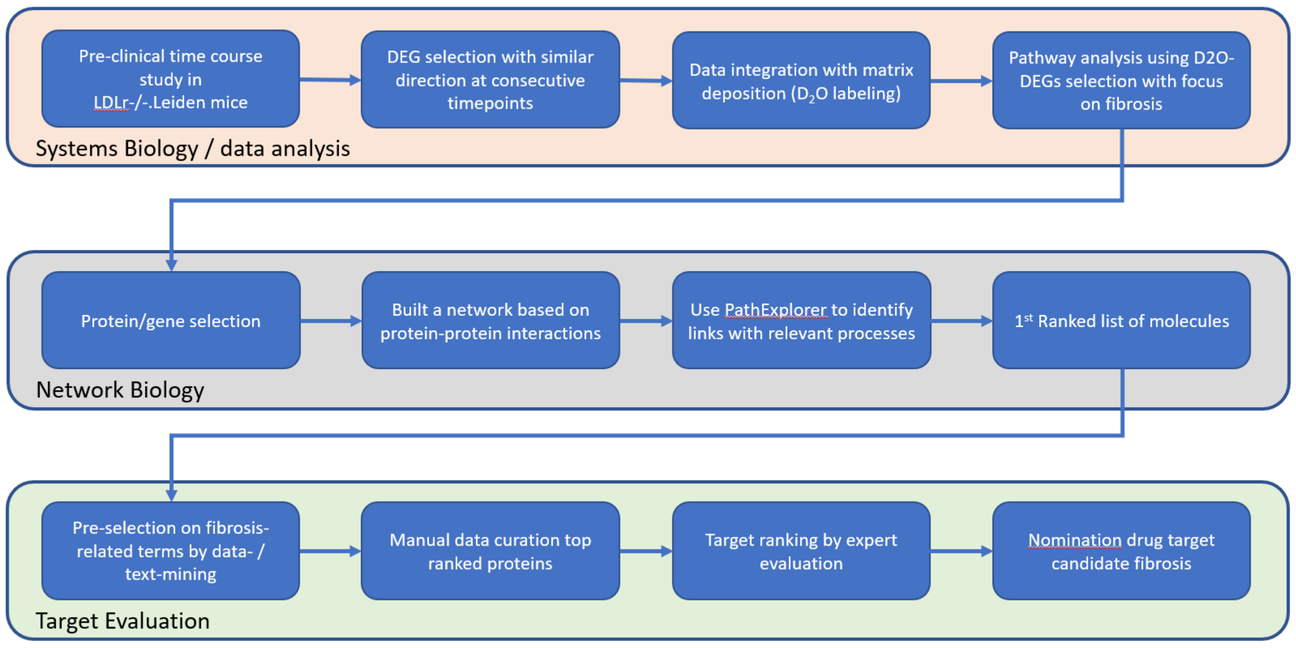
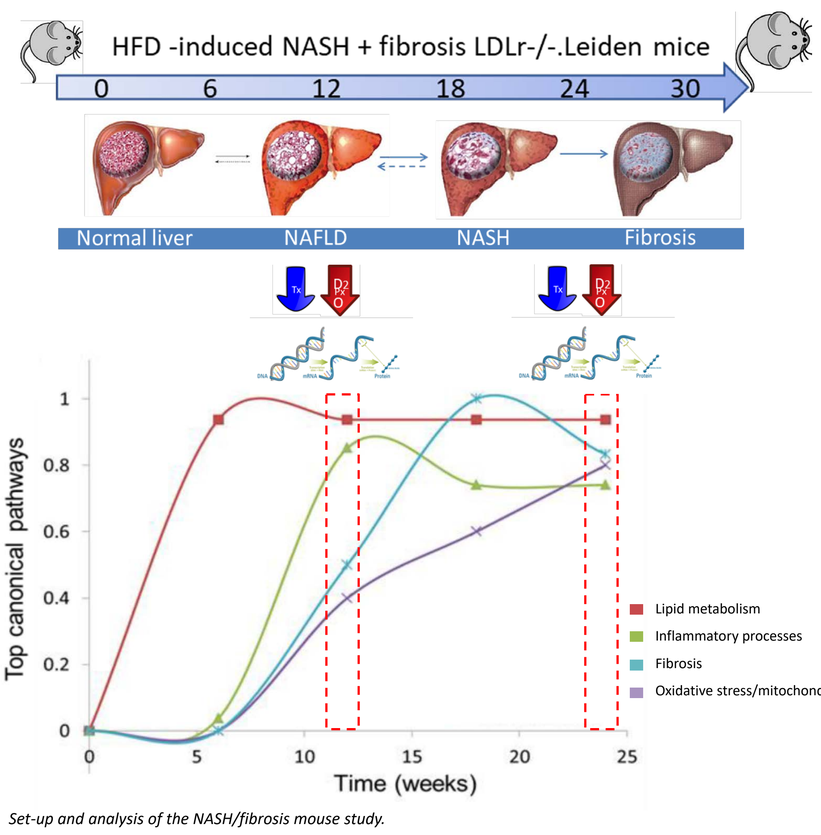
SUMMARY

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 serious 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 life style, 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



Set-up and analysis of the NASH/fibrosis mouse study.

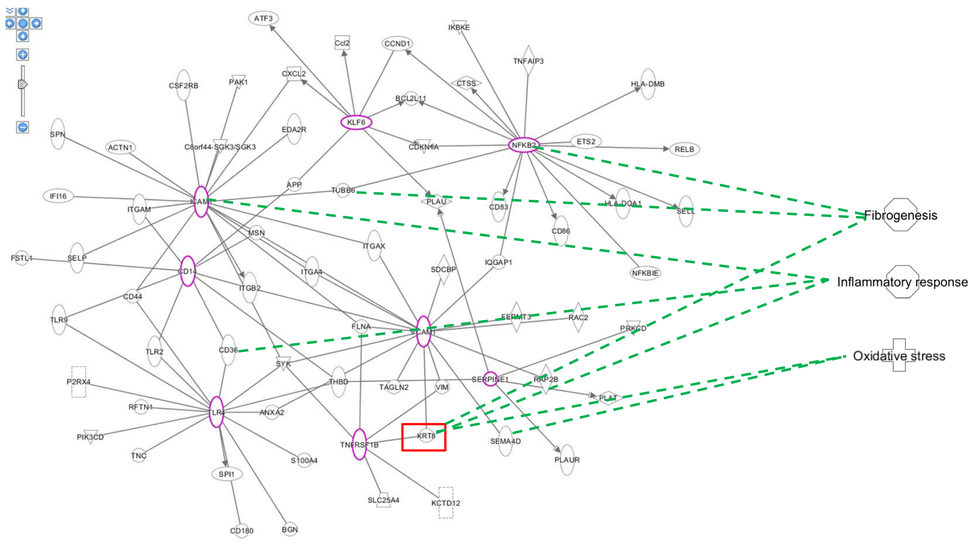
Fibrosis was induced in high-fat diet (HFD) fed low-density lipoprotein-receptor knockout (LDLr-/-.Leiden) mice, which develop NASH and hepatic fibrosis in the context of obesity, dyslipidemia, and insulin resistance, as is typical for NASH patients. Matrix deposition was quantified by labelling mice with deuterated water (D_2O) for 7 days, and protein fractional synthesis rates were calculated using mass isotopomer proteomics analyses. In addition, RNAseq was performed to identify Differential Expressed Genes (DEGs) and subsequent top canonical pathways were analyzed. These data show, from 12 weeks onwards that the inflammatory, oxidative stress, and fibrotic responses were activated. Clear modulation of pathways related to NASH and hepatic fibrosis was visible at week 24 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 correlating with D_2O matrix deposition were identified. This resulted in a set of 586 differentially expressed genes (DEGs).

[1] *A van Koppen et al. (2017) Uncovering a Predictive Molecular Signature for the Onset of NASH-Related Fibrosis in a Translational NASH Mouse Model. Cell Mol Gastroenterol Hepatol. 5(1):83-98.*

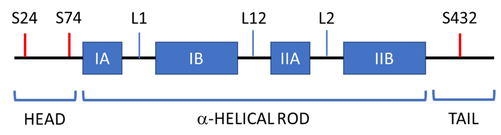
2. Network analysis

A subset of 586 DEGs (signature genes) were translated into biological processes using enrichment strategies in the Ingenuity Pathway Analysis tool. The associated Hepatic Fibrosis genes (17) were subsequently selected for Path Explorer analysis to be directly linked to other signature genes and visualized in the disease network. All genes in the pathways network have a differential expression fold change with a p-value < 0.05 relative to chow 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 40 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).



Results of the network analysis on DEGs that correlated with D_2O matrix deposition in HFD mice. Dotted green line is an illustration of the relation of genes in the network with NASH and fibrosis-related biological processes. Based on the number of connections to a relevant biological process a candidate ranking was generated. Indicated by a red box is one of the top ranked candidates, KRT8. Lipid metabolism is not shown.

3. Target Evaluation KRT8



Schematic structure of KRT8. The α-helical rod domain is sub-divided into four domains which are connected with three linkers. Conserved phosphorylation sites are shown.

BACKGROUND INFORMATION

Keratins represent the largest subfamily of intermediate filaments (IFs), one of the major cytoskeletal proteins. They play important cell-specific roles in cytoprotection from various mechanical and non-mechanical stresses. Epithelial keratins are subdivided based on isoelectric point in type I (acidic) and type II (basic). Type I and type II keratins exist as paired polymeric filaments that display a tripartite structure containing a conserved α-helical central rod domain flanked by less conserved N-terminal head and C-terminal tail domains. KRT8 is a type II keratin that amongst others forms heteropolymers with type I keratin 18 (KRT18).

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 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. p38 phosphorylates only S73 (Ku, 2006).

EVALUATION OF KRT8 AS A TARGET FOR NASH/LIVER FIBROSIS

Evidence that supports or weakens KRT8 as a potential drug target for NASH/fibrosis, as obtained from literature and data mining, is reported in the table below. Ranking colors (good – bad):

Criterium	Evidence	Evaluation
Expression	• Highly expressed in multiple organs on protein level • Adult hepatocytes only express KRT8 and KRT18 • Liver KRT8/KRT18 is increased in patients with biliary cirrhosis • Higher soluble KRT18 level in patients with NASH • KRT8 and KRT18 upregulated in patients with advanced liver fibrosis	😐

Human genetics	• KRT8 cytoskeleton-disrupting variants were identified in patients with:
	  • Fatal drug-induced liver disease
	  • Hemochromatosis / liver fibrosis development
	  • Non-alcoholic fatty liver disease (NAFLD)
	  • Cryptogenic liver disease
		🙂

Mouse genetics	• KRT8 null mice:
	  • Hepatocyte fragility ↑
	  • Liver injury ↑ (microsystin-LR, concanavalin A, Fas antibody)
	  • Steatosis ↑ (HFD)
	  • Liver hemorrhage with embryolethality / mild chronic hepatitis (strain dependent)
	• KRT8 overexpression:
	  • Mallory-Denk bodies*↑ (HFD or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC))
	• KRT8 mutants:
	  • Liver injury ↑ (microsystin-LR, concanavalin A, Fas antibody, acetaminophen, griseofulvin)
	  • Hepatocyte apoptosis (Fas stimulation)
		🙂

Biological function	• KRT8 provides resistance to hepatocyte apoptosis by:
	  • Modulating FAS targeting to the cell surface, preventing FAS-mediated apoptosis
	  • Sequestering pro-apoptotic signals and death-promoting effector molecules
	  • TNFR2 binding moderates the TNF-dependent activation of JNK and NFκB
	  • Regulating the shape and function of mitochondria
	• KRT8 controls inflammatory responses by controlling NF-κB signaling by:
	  • Binding TNFR2
	  • Inhibiting polyubiquitination of TRAF6 → negative regulation of the TLR-mediated inflammatory response.
		🙂

Preclinical evidence	• siRNA to KRT8 gene enhances FAS expression and FAS-induced apoptosis • Mice transgenic for KRT18 Arg90Cys pretreated with PKC412 were protected from liver injury and parenchymal hemorrhage as measured by histopathology and serum alanine aminotransferase after Fas-induced injury. PKC412 enhances the binding of KRT8 with NMHC-IIA which ameliorates the KRT18 mutation-induced disorganization of K8/K18 filaments.	☹
Required MoA	Increased expression or activation/stabilization of KRT8 with KRT18	☹
Druggability	No binding pocket for ligands; binding of 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 • Expression analysis	☹
Competitive landscape	No known development of KRT8 stimulators, KRT8/KRT18 stabilizers of expression enhancers • Chances for first-in-class drug	🙂

** MDBs represent protein inclusions in hepatocytes typically found in human steatohepatitis.*

4. 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 pre-defined 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.