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Generation and characterization of NeoHepatocytes from alcoholic patients and healthy controls for autologous cell therapy

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In Germany over 3.5 Million patients suffer from chronic liver disease, with clinical manifestations from fibrosis to cirrhosis and hepatocellular carcinoma. The main causes of liver fibrosis in industrialized countries include chronic HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH). Liver transplantation is the only available treatment for end stage liver disease. Fundamental limitation of this therapy is donor organ scarcity. This raises a growing interest in new therapeutic options, e.g. transplantation of hepatocyte-like cells (NeoHepatocytes) generated from peripheral blood monocytes. Aim of the here presented thesis was to generate and characterize NeoHepatocytes from patients with alcoholic liver damage for autologous therapy. Thereby, focusing especially on drug metabolism and TGF-β signaling, which is critically required for progression of chronic liver disease.

NeoHepatocytes were generated from peripheral blood monocytes of alcoholic patients and healthy controls and then compared to primary human and mouse hepatocytes. Urea and glucose formation as well as liver enzymes LDH, ALT, and AST were measured at different time-points of differentiation. Expression of hepatocyte marker genes was examined by RT-PCR, Western blot and immunofluorescent staining. Expression and activity of Phase I and II drug metabolizing enzymes, e.g. cytochrome P450 iso-forms 1A1, 2D6, 3A4, glutathione-S-transferase A1 and M1, was analyzed by RT-PCR, Western blot and fluorescence based enzyme assays. Toxicity of various compounds was measured by LDH release into the culture supernatant. Expression profile of the TGF-β signaling pathway was analyzed by RT-PCR, Western blot and reporter assays. Fat droplets were visualized by Oil Red O staining.

The yield of monocytes from patients ($2.2 \pm 0.8 * 10^7$ cells/ml blood) and controls ($2.5 \pm 0.9 * 10^7$ cells/ml blood) was comparable. There was no significant difference in the isolation and cultivation of the cells. After 12 to 15 days in differentiation medium, the generated NeoHepatocytes form a confluent layer with cell-cell contact displaying the typical hexagonal shape of hepatocytes. Basal urea formation of NeoHepatocytes was slightly lower than in human hepatocytes. However, glucose levels in the culture supernatant were similar. Membrane leakage of liver enzymes LDH, AST, and ALT was lower in NeoHepatocytes. Although albumin expression remained very low, other hepatocyte markers, e.g. Cytokeratin 18, transferrin and alcohol dehydrogenase 1, increased significantly. Monocytes and programmable cells of monocytic origin (PCMOs) expressed only some of the investigated drug-metabolizing enzymes. Throughout differentiation NeoHepatocytes showed increased expression of all metabolizing enzymes investigated, resulting in a stable basal activity after approximately 15 days. Fluorescence based activity assays indicated that NeoHepatocytes and primary hepatocytes have comparable enzyme kinetics. However, the basal activities were significantly lower in NeoHepatocytes compared to primary hepatocytes. Incubation of cells with 3-methylcholanthrene and Rifampicin markedly increased CYP1A1/2 and CYP3A4 activity, which could be selectively blocked by Nifedipine, Verapamil, Ketoconazol and Quercetin. Fat accumulation was induced by treatment with insulin, TGF-β and ethanol in NeoHepatocytes and hepatocytes, but not before differentiation. TGF-β signaling was comparable between NeoHepatocytes and hepatocytes, merely expression of Smad1 and 3 was reduced (~ 30 and ~ 60 %). This was confirmed by Western blot. Upon stimulation with TGF-β, NeoHepatocytes display activation of the signaling pathway via phosphorylation of Smad1, 2, and 3. Nuclear localization of phosphorylated Smads 1, 2, and 3 after stimulation with TGF-β was confirmed by immunofluorescent staining and adenoviral luciferase reporter assays. Decreased Smad3 signaling resulted in reduced expression of TGF-β regulated genes, e.g. CTGF, fibronectin und collagen.

We could successfully generate NeoHepatocytes from patients with alcoholic liver disease, which had a comparable quality to healthy controls. Our data reveal similarities in expression, activity, induction and inhibition of drug-metabolizing enzymes, when comparing NeoHepatocytes and primary

hepatocytes. This offers great perspectives for NeoHepatocytes to be used not only for cell therapeutic approaches, but also as an alternative for measuring bio-activation of substances. Furthermore, reduced Smad3 expression in NeoHepatocytes and subsequently deceased expression of extracellular matrix may lead to a proliferation advantage after transplantation. Thus, this method can help patients to bridge the waiting time for a suitable organ.