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Studies on cellular and molecular mechanisms that drive normal and regenerative processes in the liver and pathological processes in Dupuytren's contracture

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Press

Department of Cell Biology, Institute of Molecular and Cell Biology,
University of Tartu, Estonia

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Supervisor: Viljar Jaks, MD, PhD
Department of Cell Biology
Institute of Molecular and Cell Biology
University of Tartu, Estonia

Opponent: Prof. Dr. Robert P. Coppes, PhD
Department of Cell Biology
Department of Radiation Oncology
University Medical Center Groningen
University of Groningen, the Netherlands

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LIST OF ORIGINAL PUBLICATIONS

I. **Viil J**., Klaas M., Valter K., Belitškin D., Ilmjärv S., and Jaks V. A unipotent label-retaining progenitor cell population resides within biliary compartment in mammalian liver. (2016), Manuscript.

II. Klaas M., Kangur T., **Viil J**., Mäemets-Allas K., Minajeva A., Vadi K., Antsov M., Lapidus N., Järvekülg M., and Jaks V. The alterations in the extracellular matrix composition guide the repair of damaged liver tissue. Sci. Rep. (2016), 6, 27398; doi: 10.1038/srep27398

III. **Viil J**., Maasalu K., Mäemets-Allas K., Tamming L., Lõhmussaar K., Tooming M., Ingerpuu S., Märtson A., and Jaks V. Laminin-rich blood vessels display activated growth factor signaling and act as the proliferation centers in Dupuytren's contracture. Arthritis Res Ther. (2015), 17:144

IV. Mäemets-Allas K., **Viil J**., and Jaks V. A Novel Inhibitor of AKT1-PDPK1 Interaction Efficiently Suppresses the Activity of AKT Pathway and Restricts Tumor Growth *In Vivo*. Mol Cancer Ther. (2015), 14(11): 2486–96.

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Ref I. Participated in study design, experimental work, data analysis and writing of the manuscript.

Ref II. Participated in experimental work and revision of the manuscipt.

Ref III. Participated in experimental work and writing of the manuscript.

Ref IV. Participated in experimental work, data analysis and revision of the manuscipt.

LIST OF ABBREVIATIONS

2-AAF	2-acetylaminofluorene
AFP	α-fetoprotein
ALB	albumin
BMP	bone morphogenetic protein
BrdU	5-bromo-2-deoxyuridine
C/EBPα	CCAAT-enhancer-binding protein
CC	cholangiocarcinoma
CCl_4	carbon tetrachloride
CDE	choline-deficient ethionine-supplemented
CK19	cytokeratin 19
CTGF	connective tissue growth factor
DC	Dupuytren's contracture
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
dox	doxycycline
DR	ductular reaction
ECM	extracellular matrix
EpCAM	epithelial cell adhesion molecule
FAH	fumarylacetoacetate hydrolase
FGF	fibroblast growth factor
FN	fibronectin
H2B-EGFP	histone H2B-enhanced green fluorescent protein
HCC	hepatocellular carcinoma
HGF	hepatocyte growth factor
HNF1β	hepatocyte nuclear factor 1 beta
HNF4α	hepatocyte nuclear factor 4 alpha
IGF-2	insulin-like growth factor-2
LLRC	liver label-retaining cell
LRC	label-retaining cell
MMP	matrix metalloproteinase
MSC	mesenchymal stromal cell
mTOR	mammalian target of rapamycin
NPF	normal palmar fascia
OPN	osteopontin
P	postnatal day
PCA	protein complementation assay
PDPK1	3-phosphoinositide-dependent protein kinase 1
PH	partial hepatectomy
PI3K	phosphatidylinositol 3-kinase
R26-rtTA	Rosa 26 promoter-driven reverse tetracycline-dependent transactivator
Rluc	*Renilla* luciferase
Sox9	Sex Determining Region Y-Box 9

tBDL	total bile duct ligation
tdTom	tdTomato
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of matrix metalloproteinase
TMX	tamoxifen
TNF	tumor necrosis factor
W	week

1. INTRODUCTION

The mammalian liver has a remarkable ability to restore its original size after substantial tissue loss by inducing massive cell proliferation. Despite the enhanced regenerative capacity, liver diseases have high mortality rate, and the numbers are increasing every year. Cirrhosis and primary liver cancer – the end-stages of chronic liver diseases – have only one treatment option – liver transplantation. Since the demand for donor organs surpasses their availability, development of alternative treatment methods has become increasingly important. In order to generate new therapeutics it is necessary to understand the cellular and molecular mechanisms that control liver regeneration and disease progression.

A common feature in many chronic liver diseases is the appearance of proliferative ductular structures (ductular reactions, DRs), and deposition of extracellular matrix (ECM). As the disease progresses, DRs and ECM gradually replace parenchyma, causing decline in liver functioning. In order to find ways to prevent or reverse these processes, it is important to identify the origin of DRs, understand their pathogenesis, and determine the impact of ECM components on cell behavior.

Tissue specific somatic stem cells are believed to be slowly cycling cells residing in unique niches. In many tissues these cells are crucial for tissue maintenance in normal conditions and for the recovery after injury, as they give rise to rapidly dividing transit amplifying cells, which replenish the lost cells. The identity of liver stem cells and their contribution in liver maintenance and regeneration is still under debate. Hence, the main objective of this thesis was to identify slowly cycling progenitor cells in an adult liver, and study their behavior in liver injury by using different liver injury mouse models. Next we examined which ECM changes are induced in response to different liver injuries and how these changes could affect liver regeneration. We also described the microenvironment that promotes cell proliferation and enhances fibrosis in Dupuytren's disease (DC). Although DC is not liver-related disease, fibrosis in all organs has similar histological features, and has similarities in cell signaling. Finally, we set out to identify small molecule compound that would inhibit AKT signaling pathway, which is commonly hyperactivated in liver cancers and other malignancies of various origin.

2. LITERATURE OVERVIEW

2.1. General overview of liver functions

The liver is the largest visceral organ of the body exhibiting both exocrine and endocrine properties [1]. It has more than 500 different functions that play major roles in the control of normal physiological processes of the entire organism. Some of the most important functions include bile secretion, glycogen storage, regulation of cholesterol levels and urea metabolism, drug detoxification, and secretion of various proteins. Constant exposure to environmental agents and toxins often results in liver diseases caused by severe changes in liver physiology and decreased functioning; and since the liver is involved in the regulation of a large number of pathways, it is no surprise that liver diseases are among the leading causes of deaths today [1].

2.2. Liver cell types and basic anatomy

Liver contains many different cell types, each one of them having their own particular role in their own particular niche. Proper hepatic functioning requires coordinated work of all of these cells. Two major cell types in the liver are hepatocytes and cholangiocytes (also known as bile/biliary duct cells), constituting ~ 70% and ~ 3% of the cell population, respectively. Other cell types include Kupffer cells (resident liver macrophages), hepatic stellate cells (also known as Ito cells), endothelial cells, sinusoidal endothelial cells and pit cells (liver-specific natural killers). While hepatocytes and bile duct cells are derived from endoderm, the rest of the cells are of mesodermal descent [1, 2].

The smallest functional unit of the liver, the lobule [3], consists of hepatocytes which are organized in plates and are lined by sinusoids that radiate toward the central vein situated in the middle of the hexagonal lobule (Figure 1A,B). The lobule can be divided into zones, based upon decreasing oxygen concentration: periportal (zone 1), transitional (zone 2), and pericentral (zone 3) (Figure1B) [4]. Each tip of the lobule contains a triad of vessels – a portal vein, bile duct and hepatic artery – the "portal triad". Blood enters the lobule via portal vein and hepatic artery, flows in sinusoidal capillaries, where venous and arterial blood mix together, and leaves through the central vein. Hepatocytes have direct contact with blood plasma in the sinusoidal space where they absorb toxins and metabolites [5]. Concomitantly, bile acids and salts secreted by hepatocytes move in the bile canaliculi toward bile ducts in an opposite direction to the bloodflow. Bile ducts are formed by the second largest cell population in the liver, the biliary epithelial cells. These cells control bile flow rate and its pH, and secrete water and bicarbonate [1].

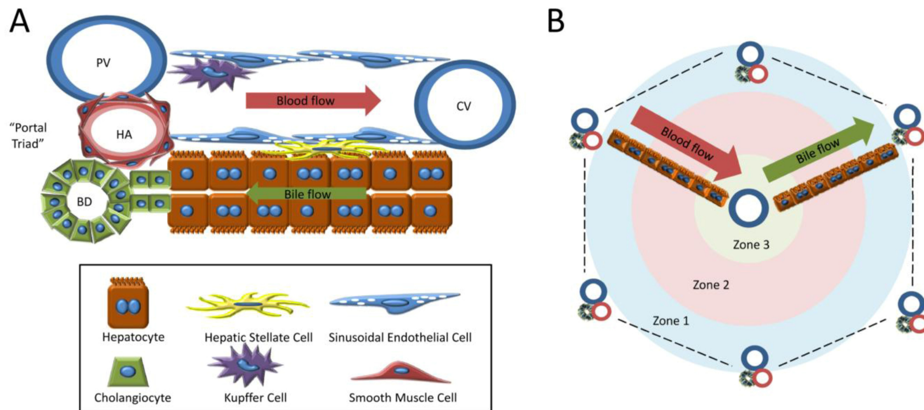

Figure 1. Architecture of the liver.
(A) Liver cell types and their organization in the liver. Blood enters the liver via portal vein (PV) and hepatic artery (HA), and flows in sinusoids toward the central vein (CV). Hepatocytes secrete bile that flows in bile canaliculi toward bile ducts (BD).
(B) Structure of the liver lobule. Hepatocyte cords radiate from CV (in the middle of the hexagonal lobule) toward portal triads. The direction of blood and bile flow, and lobule zones are also shown [4].

2.3. Embryonic development of hepatocytes and cholangiocytes

Hepatocytes and cholagiocytes originate from a common precursor, the hepatoblasts, which are derived from the definitive gut endoderm. At E9.5 of mouse development, the laminin-rich basal layer surrounding the hepatic endoderm disintegrates, and hepatoblasts migrate into septum transversum where they form the liver bud [1, 6–8]. Initially, bipotential hepatoblasts express fetal liver marker α-fetoprotein (AFP) as well as markers associated with both hepatocytes (hepatocyte nuclear factor alpha, HNF4α and albumin, ALB) and cholangiocytes (cytokeratin 19, CK19). As hepatoblasts begin to differentiate at ~E13.5 in mouse and 56–58 days after conception in human, the hepatic precursors adjacent to the portal veins increase their CK19 expression, decrease their HNF4α and ALB expression, and eventually differentiate into cholangiocytes. Concurrently, the hepatoblasts which are not in contact with portal veins and reside in the parenchyma, gradually differentiate into hepatocytes [9–11]. Although these differentiation processes start at the embryonic stage, the cells become fully mature only postnatally.

2.4. Signaling pathways in liver development

All processes in liver development, including bud formation, cell specification, differentiation, and maturation, involve coordinated signaling between cells as well as their environment. The onset of liver formation is dependent on the key signaling pathways controlled by fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) [12, 13]. It has been shown that if the signaling of either of them is blocked, liver formation is not induced [12, 14]. Homeodomain factor Hex and zinc finger transcription factors Gata4 and Gata6 are essential for liver bud formation and hepatoblast delamination [15–18]. Although Hex$^{-/-}$ mouse mutants are able to form initial liver bud, further bud development in these embryos is arrested and hepatoblasts are not able to invade the surrounding stromal tissue [17]. Similarly to Hex mutants, the liver development in Gata6$^{-/-}$ mouse embryos stops shortly after initial bud formation [18]. Hepatoblast delamination involves also rearrangement of the extracellular matrix (ECM), since the liver bud is surrounded by matrix consisting of laminin, nidogen, type IV collagen, fibronectin (FN), and heparan sulfate proteoglycans [19]. One of the transcription factors responsible for ECM remodeling is homeobox transcription factor Prox1 [20]. Prox1 downregulates E-cadherin in invading hepatoblasts and controls the expression of several ECM proteins and remodeling enzymes such as matrix metalloproteinase-2 (MMP-2) that are necessary for the disruption of the basement membrane and subsequent delamination [20–22].

Transcription factors, signaling pathways and corresponding signal transducers essential for hepatocyte differentiation and maturation include Wnt, Oncostatin M (OSM), glucocorticoids, hepatocyte growth factor (HGF), FoxA1/2, hepatocyte nuclear factor 1 alpha and beta (HNF1α and β), HNF4α, CCAAT-enhancer-binding protein (C/EBP) and HNF6 (reviewed in [5, 23]). Cholangiocyte differentiation and maturation is controlled by Notch, transforming growth factor beta (TGFβ), FGF and Wnt signaling that promote the expression of transcription factors needed for biliary fate (HNFβ1, Sox9 (Sex Determining Region Y-Box 9), and Oncostatin 1 and 2) and simultaneously suppress hepatogenic factors (HNF4 and C/EBP) (reviewed in [5, 23]). Pathways mentioned in this section represent only a subset of those that are known to play major roles in liver development. The entire network of factors is much more complicated and our understanding of this network is far from complete.

2.5. Liver in homeostasis and injury

Tissue homeostasis is achieved predominantly by two mechanisms: cell duplication and differentiation from stem/progenitor cells. For example, stem/progenitor cells provide a continuous supply of all cell lineages in the skin and intestine [24, 25]. Although these tissues have high cellular turnover rate, their

stem cell pool is able to maintain the cellular homeostasis and replenish lost cells after tissue damage throughout the entire life.

The liver on the other hand is a quiescent organ – only a small number of cells, 1 in 20,000 to 40,000 hepatocytes, are actively cycling in adult liver at any given time point [26]. Despite their slow cycling speed, the differentiated cells in the liver are believed to be capable of maintaining cellular homeostasis without any contribution from stem/progenitor cells. More than 30 years ago a "streaming liver" hypothesis was proposed, stating that new hepatocytes that are produced at the portal tract, move toward central vein, acquire new functions along the way, and populate the entire lobe over time [27]. This hypothesis gained support in 2011 by Furuyama *et al*., who showed in lineage tracing experiments that hepatocytes originate from Sox9+ bile duct cells and "stream" in a portal-to-centrilobular direction [28]. However, tamoxifen injection that was used to activate recombination in Sox9+ biliary cells, induces ectopic expression of Sox9 also in hepatocytes, therefore, one cannot exclude the possibility that new liver cells were actually derived from tamoxifen-induced Sox9+ hepatocytes [29]. As most studies contradicted the "streaming" hypothesis [30–32], it was generally accepted that liver homeostasis is maintained by simple replication of existing differentiated cells. Recently, a new mechanism of hepatocyte maintenance was demonstrated [33]. According to this concept, there is a population of cells – the hepatocyte stem cells – consisting of mainly diploid Axin2+ pericentral hepatocytes that can self-renew, have elevated proliferation rate, differentiate into more mature polyploid hepatocytes, and replace 40% of hepatocytes in centrilobular-to-portal direction in one year. This is similar to the "streaming liver" hypothesis: both concepts state that only a small subset of cells supplies new hepatocytes in liver homeostasis, and cell replacement involves unidirectional "streaming". However, cell migration in this new model is in opposite direction – from central vein to portal triad. Until these results are confirmed by others using independent methods, liver physiological maintenance is still considered to be supported equally by all hepatocytes.

Liver has also a remarkable regenerative potential along with a unique feature: the recovery mechanism is thought to be determined by the type of injury. After partial hepatectomy (PH), the liver mass is reinstated within 2–3 weeks by replication and hypertrophy of existing liver cells in rodents [34, 35]. In response to chronic liver injury when hepatocyte and/or cholangiocyte proliferation is abrogated, cells with oval-shaped nuclei, referred to as "oval cells" in rodents, and ductular reactions (DRs) in humans, accumulate typically around the portal areas in the liver [36–38]. These rapidly proliferating cells are considered to be facultative liver progenitor cells contributing to liver repair, since they can differentiate into both, hepatocytes and cholangiocytes [39–42]. Emergence of ductular reactions has been documented in a variety of human liver diseases [43], however, their origin, true nature, and role in liver regeneration and homeostasis are still unresolved.

2.6. Hepatic precursors in normal liver

Stem cells possess two unique characteristics that distinguish them from all other cell types: they are capable of self-renewal through numerous cell divisions and they can differentiate into a cell with more specialized functions [44]. Embryonic stem cells, which originate from the inner cell mass of the blastocyst, are pluripotent as they can generate any cell type in the body [45, 46]. Multipotent stem cells, such as adult stem/progenitor cells (also called somatic stem cells), which reside in specific niches in most adult tissues and participate in the maintenance of homeostasis in physiological and/or pathological conditions, sometimes after being inactive for long periods of time [47], can give rise to only a limited number of differentiated cell progenies.

Identifying and localizing somatic stem cells in the liver has proven to be a challenge as the liver is a relatively quiescent organ and does not need progenitor activity in normal conditions. Also, the lack of specific liver stem cell markers has hindered their characterization. One of the main focus points in liver stem cell research has been uncovering the origin of ductular reactions – the transit-amplifying cells – presumably derived from hepatic stem cells [48]. Ductular reactions occur in virtually all acute and chronic liver diseases of biliary and nonbiliary origin [48], and their contribution to liver regeneration has been consistently demonstrated in several species. Over time, various possible origins for ductular reactions have been proposed, including: 1) biliary duct system and canals of Hering, 2) mature hepatocytes, 3) bone marrow cells, and 5) mesenchymal stromal cells and hepatic stellate cells [26, 49].

2.6.1. Hepatic precursors in biliary duct system

Considering the appearance of ductular reactions in portal areas in severely injured livers, it is not surprising that biliary ducts and the canals of Hering (terminal branches of the biliary duct) have been thought to be the niche of hepatic precursors. Furthermore, many markers such as CK19, CK7, epithelial cell adhesion molecule (EpCAM), osteopontin (OPN), A6, and CD133, which are widely used to detect or isolate progenitor cells from injured liver, are also expressed by biliary cells in normal liver [49]. The canals of Hering are lined partly by hepatocytes and partly by cholangiocytes, and can be viewed as a bridge between the hepatocyte canalicular system and the biliary tree [38]. The presence of hepatic precursor cells in the canals of Hering has been described in postnatal and adult human livers [50, 51]. These cells were positive for EpCAM, CK19, and CD133 and also expressed low levels of albumin, albeit at a significantly lower level than mature hepatocytes. They differentiated into cholangiocytes and hepatocytes *in vitro* and EpCAM+ cells derived from postnatal liver where able to repopulate the liver of immunodeficient mice [50]. Tanimizu *et al*. showed also that EpCAM+ cholangiocytes isolated from neonatal mice converted into hepatocytes *in vitro* [52]. However, this capability

decreased gradually and was lost completely in adult mice. By comparing neonatal and adult cholangiocytes, they discovered that the levels of transcription factors important in hepatocyte differentiation such as HNF4α and C/EBPα were upregulated in neonatal cholangiocytes. Adult biliary cells, on the other hand, showed elevated expression of factors related to cholangiocyte differentiation. These results indicate that cholangiocytes lose their bidirectional differentiation potential during epithelial maturation. In contrast, Kamiya *et al.* demonstrated that adult stem cell population exists within cholangiocyte compartment even in 3-month-old mice [53]. After identifying CD13, CD133, EpCAM, and CD49f as markers expressed in liver precursors, they found that these cells can form colonies containing both, ALB+ hepatocytes and CK19+ bile duct cells, indicating that at least a subset of cholangiocytes may preserve their proliferative capability and bipotency. A comparative study with CD133+ cells that were isolated from normal adult liver (CD133+ cholangiocytes) and chronically injured liver (CD133+ cholangiocytes and ductular reactions) showed that although CD133+ cells from normal liver could form small colonies *in vitro*, they only expressed biliary marker CK7 [54]. CD133+ cells isolated from injured liver formed small and large colonies, where small colonies expressed only CK7, whereas cells in large colonies gradually differentiated and became either ALB+, CK7+, or ALB+CK7+. This shows that cholangiocytes are lineage-committed in normal liver, certain injuries, however, may induce some of them to proliferate and give rise to bipotential progenitors. Fate-tracing studies based on cholangiocyte-specific gene expression (OPN and hepatocyte nuclear factor 1 beta, HNF1β) also suggest that there are cells within biliary compartment which generate ductular reactions and these can differentiate into hepatocytes after certain liver injury [41, 55]. Other fate-tracing studies where CK19-Cre and HNF1β-Cre mice were used argue against this phenomenon, showing that although cholangiocytes give rise to DRs, new hepatocytes arise from pre-exicting hepatocytes by self-duplication and contribution from biliary-derived progenitor cell population is negligible in different injury models [56–58].

These examples suggest that although there are contradictory results when it comes to the differentiation potential of adult biliary cells and their progeny, evidence strongly suggest possible lineage-connection between cholangiocytes and facultative liver progenitor cells.

2.6.2. Hepatocytes as hepatic precursors

Cells within ductular reactions are variable in size and immunophenotype, ranging from 6 µm in diameter (size of the smallest cholangiocytes) to 40 µm (average size of hepatocytes); and express hepatic antigens such as HepPar1, ALB, and alpha-1-antitrypsin (AAT), in addition to biliary antigens [38]. Morphology of DRs depends largely on the type of liver disease or injury model. In addition, although ductular reactions predominantly appear peri-

portally, they can also be found around central vein, in a distance from the bile ducts [59]. These observations imply to the possibility of hepatocytes being the source for DRs, at least in certain injury conditions.

Indeed, *in vitro* studies have shown that hepatocytes can transdifferentiate into ductular cholangiocytes in three-dimensional organoid culture system [60] and hepatocyte-to-cholangiocyte/DR conversions have been demonstrated in chronically injured livers of transgenic mice [61–65]. However, the extent of transdifferentiation appears to be dependent on the mouse model, injury type, and method of identification. For example, in a lineage-tracing experiment with Alb-CreER^{T2}; R26R^{lacZ+} mice, over 60% of new ductular cells were derived from hepatocytes in response to chronic liver injury induced by DDC-treatment (3,5-diethoxycarbonyl-1,4-dihydrocollidine, DDC) [64], whereas only 2% of DRs were derived from hepatocytes using hepatocyte-specific MX dynamin-like GTPase 1 (Mx1)-Cre;R26R^{lacZ+} mouse model [63]. On the other hand, no hepatocyte-derived biliary cells were found in fate-tracing experiment with R26-EYFP mice, when hepatocyte-specific EYFP expression was induced with adeno-associated virus (AAV)8-Ttr-Cre construct [30]. Experiments with hepatocyte-chimeric fumarylacetoacetate hydrolase (FAH) mouse model have shown small contribution from hepatocytes [65].

In addition to the differences in the model systems, contrasting results can often be caused by different sets of antigens that investigators use to identify ductular reactions. OPN, Sox9, A6 and CK19 are some of the most well-known and used DR markers, however, their expression can vary greatly in different conditions. As demonstrated by Yanger *et al*., 48% of OPN+ cells but only 14% of CK19+ cells were derived from hepatocytes after DDC-treatment [61]. This discrepancy was even greater after bile duct ligation, when OPN+ cell number was 30 times higher than CK19+ cell number, while Sox9 and A6 expression levels fluctuated between these extremes. This shows that different types of injuries can induce different types of DRs with various immunophenotypes, and suggests that hepatocyte-to-cholangiocyte conversion is a stepwise process with OPN being the earliest and broadest indicator for conversion, and CK19 the marker for the differentiation end point. It is not yet known whether all hepatocytes are capable of transdifferentiation or if it is a characteristic of particular hepatocytes.

2.6.3. Bone marrow-derived hematopoietic stem cells as hepatic precursors

Bone marrow derived hematopoietic stem cells can give rise to many different cell types, however, data gathered so far suggest that they are not the source of hepatic progenitor cells. Experiments with FAH-deficient mice have shown that although transplanted bone marrow cells were able to restore liver function and FAH expression in hepatocytes, it was not through transdifferentiation into liver cells rather via fusion with host hepatocytes [66–68]. Furthermore, when bone

marrow from transgenic green fluorescent protein (GFP) mice was transplanted into immunodeficient and/or immune-competent mice, no evidence of bone marrow-derived liver specific cells were found in normal liver or after toxic liver injury [69]. These results correlate with data from rat experiments where it was demonstrated that hepatic progenitor cells and hepatocytes are not derived from bone marrow cells [70].

2.6.4. Mesenchymal stromal cells and hepatic stellate cells as hepatic precursors

Mesenchymal stromal cells (MSCs) are multipotent cells residing in the bone marrow, but can also be found in the connective tissues of most organs, including the liver. Although MSCs originate from the mesoderm, they have the ability to differentiate into cells of other lineages [71]. MSCs isolated from adipose tissue, bone marrow and umbilical cord-blood can differentiate into hepatocytes *in vitro* and *in vivo* [72–74] and since MSCs are relatively abundant and easily accessible, they show a great potential in treating liver diseases. It is not known whether transdifferentiation of extrahepatic MSCs into liver cells occurs also naturally in the body. Studies with liver-resident MSCs – hepatic stellate cells – have resulted in conflicting conclusions: there are reports supporting [75, 76] and refuting [77, 78] a role for hepatic stellate cell population as facultative stem cells. Since MSCs are plastic in their differentiation potential, one cannot rule out the possibility of mesenchymal stellate cells being the stem cell pool for epithelial liver cells. It has been suggested that there may be even a common organ-specific meso-endodermal precursor cell in an adult liver [79]. In a work by Conigliaro *et al*. it was demonstrated that liver precursors with novel immunophenotype (Sca1+CD34-CD45-ALB-) can give rise to either mesenchymal or epithelial subpopulations *in vitro*; and to hepatocytes and stellate cells *in vivo* [79]. It has to be noted, however, that these precursors were derived from fetal or neonatal mice. Hence, their existence and input in homeostasis and regeneration in mature liver remain to be discovered.

2.6.5. Quiescent stem cells in various compartments

Somatic stem cells are considered to have slow division rate in normal conditions, a feature that has been exploited in the label-retaining cell (LRC) assay to identify tissue specific stem cells [80–83]. In LRC assay, cells are first pulse-labeled with nucleoside analogues (such as 5-bromo-2-deoxyuridine, BrdU or 5-Ethynyl-2-deoxyuridine EdU) and the retention of nuclear label after a chase period indicates no or minimal proliferation. As the liver itself is quite quiescent organ, identifying quiescent adult liver stem cells has not been an easy task. For this reason LRC assay in combination with moderate hepatic injury, which should induce quiescent stem cells to proliferate, was used to locate BrdU-

retaining cells [84]. With this strategy four possible stem cell niches were identified: the canal of Hering, intralobular bile ducts, periductal “null” mononuclear cells (negative for OV-6, ALB, CD45 and desmin), and peribiliary hepatocytes [84]. Peribiliary hepatocytes were later hypothesized to be derived from the cells in the canals of Hering but the origin of “null” cells remained unclear. In another report, where LRC assay was performed in normal liver with a chase period of 23 months, the label-retaining BrdU+ cells were shown to locate periportally and very rarely pericentrally [85]. Again, “null” cells appeared after injury, however, in this report no BrdU-containig bile duct cells were identified. Since active DNA synthesis is the prerequisite for BrdU incorporation, it is possible that different injury methods used in these studies activated the proliferation in different cell compartments, which could explain somewhat different results. Although both these reports indicated that liver contained quiescent cells, their contribution to liver regeneration remained unresolved.

The inconclusive data regarding the origin, location, and contribution of hepatic progenitor cells point to the possible existence of multiple origins for these cells. One can speculate that the ability to supply new cells from diverse sources is the reason behind the amazing regenerative capacity of the liver. Future research will have to determine the exact mechanisms involved in liver maintenance.

2.7. Stem cell niche

In most postnatal tissues, stem cells are located in specialized microenvironments, i.e., niches, where they are maintained in optimal conditions and often in a relatively quiescent state. Neural and metabolic signaling, as well as interactions with other cellular and extracellular components within and outside the niche are crucial for their preservation and activation [86, 87]. Experiments in *C.elegans* and *D.melanogaster* have shown that germline stem cells need certain proximity to or direct contact with particular neighboring somatic cells to maintain their proliferative properties [88–90]. The importance of a specialized microenvironment is exemplified by the fact that upon depletion of endogenous germline stem cells, the empty niche can be populated by adjacent somatic cells, which are then stimulated to proliferate by the niche environment [91].

Organs with high cellular turnover rate such as hematopoietic system, skin, testis, and the gastrointestinal tract appear to have the most clearly defined stem cell niches [92]. But also other organs, which do not have as high degree of steady-state cell turnover, such as the brain, seem to have designated stem cell niches [92]. Defining the liver stem cell niche has proven to be a challenge, as the nature of resident liver stem cells has remained elusive, however, the niche and the signaling pathways that promote diverse processes in ductular reactions have been studied extensively.

The ductular reaction comprises several different processes, all contributing to the niche formation. These include accumulation of reactive progenitors,

infiltration of inflammatory cells, activation and accumulation of liver non-parenchymal cells in the vicinity of DRs, deposition and rearrangement of ECM, and activation of various signaling pathways [48].

2.7.1. Signaling in DRs

Lymphocytes and macrophages appear around DRs of injured and diseased livers in mice, rats and humans [93–96]. Inflammatory cells influence the behavior of progenitors in DRs by regulating the expression of different transcription factors and cytokines, and modulating the composition of matrix. Macrophages have been shown to control the proliferation, differentiation, and invasion of progenitor cells [95–98]. They produce cytokines like tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), TGFβ, and TNF-like weak inducer of apoptosis (TWEAK) that are important for the inititation and expansion of ductular reactions [98, 99]. Hepatic stellate cells and portal myofibroblasts, the main sources of liver extracellular matrix [100], are activated in injured livers and, in response, increase ECM deposition around DRs [101]. In addition, they produce a variety of bioactive molecules that promote progenitor proliferation or specify their differentiation pathway [102]. Increasing evidence suggest that macrophages, stellate cells, and myofibroblasts work together in creating a suitable microenvironment for liver regeneration [96]. Coordinated Wnt signaling from macrophages and Notch signaling from myofibroblasts are shown to be essential for determing the fate of DR cells [96, 103]. This discovery is not unexpected since activated Wnt and Notch signaling are common among different stem cell systems; and ectopic activation of Notch signaling has been shown to reprogram immature hepatocytes into biliary cells [104]. Boulter *et al*. demonstrated that Notch signaling drives the differentiation from bipotential progenitors into cholangiocytes after DDC-induced biliary liver damage [96]. In this type of injury the DRs are surrounded by layers of myofibroblasts that express Notch ligand Jagged-1, and by thick sheath of collagen I that prevents their contact with macrophages outside the niche. Following CDE-diet-induced hepatocellular injury, the accumulation of myofibroblasts is not as extreme and collagen I deposition is markedly decreased, allowing cell-cell contact between progenitors and macrophages. As a result, Wnt signaling from macrophages reaches progenitor cells, Wnt target Numb is upregulated and Notch becomes downregulated, forcing cells to suppress biliary differentiation and aquire hepatocyte specification [96]. Interestingly, Wnt activation in macrophages occured after phagocytizing hepatocyte debris, suggesting that macrophages adjust their Wnt signaling according to the condition hepatocytes are in. Wnt signaling has been shown to direct stem cell differentiation also in the epidermis [105] and skeletal muscle [106]. In hematopoietic system, however, the Wnt pathway supports stem cell proliferation [107] and in the intestinal epithelium it has dual role – proliferative and differentiative [108, 109]. Many other pathways implicated in DRs, including Hedgehog (Hh), nuclear factor-κB

(NF-κB), TGF-β/BMP, phosphatidylinositol 3-kinase(PI3K)/AKT, and Janus kinase/ signal transducers and activators of transcription (JAK/STAT) pathway, have been identified in other stem cell systems, as well, but the outcome of their activation can vary between tissues [48, 110], demonstarting that stem cell niches in different tissues are similar but not identical.

2.7.2. General overview of ECM

ECM is a complex and dynamic network, actively participating in organ development, maintenance and repair by modulating the production, arrangement and degradation of individual components. ECM not only provides the space, physical support and protection for the cells but also influences their behavior through cell-ECM connections and by regulating its physical properties such as stiffness, porosity, thickness and orientation [111, 112]. Powerful demonstration of the importance of ECM comes from the experiments with decellularized organs, in which only the ECM is left intact. When these ECM-scaffolds are inoculated with new cells, they are able to re-populate the organ and re-establishe its function [113].

The main structural ECM components include collagens, elastins, fibronectins (FNs) and laminins. Collagen, the most abundant protein in the ECM, gives strength to the tissue while elastins provide flexibility. Laminins, which are found in the basement membrane, and FN are both responsible for cell attachment to the matrix via integrins [114]. Proteoglycans contribute to the ECM assembly by filling the extracellular interstitial space in hydrogel form [112]. In addition to their structural properties, these proteins participate in cell migration, differentiation, inflammation, wound healing, and regulate numerous signaling cascades [114]. ECM maintenance and remodeling is controlled by matrix metalloproteinanses (MMPs) and tissue inhibitors of MMPs (TIMPs) [115]. Tight regulation of MMP and TIMP activities is essential for correct ECM homeostasis in tissues and their imbalance can lead to failures in organ functioning. The importance of ECM in normal physiology is illustrated by the fact that abnormal changes in ECM structure and quantity are prominent in many genetic and a wide variety of aquired human diseases [116]. These include cardiovascular, pulmonary, renal, intestinal, neurodegenerative, and connective tissue disorders [116]. Furthermore, tumor development is associated with altered ECM composition.

2.7.3. ECM in ductular reactions

Accumulative deposition of ECM (mostly collagen I) is also a hallmark of a progressive chronic liver disease [114]. The severity of the disease is in correlation with not only the increasing number of collagen producing myofibroblasts [117] but with the magnitude of progenitor cell activation and DR

response, as well [94, 118]. It is not yet clear whether the changes in the ECM are necessary for progenitor activation or are the expanding liver progenitors driving matrix remodelling and deposition. Experiments with the CDE dietary model suggest that myofibroblast activation and ECM deposition (collagens I and III) precede ductular reactions to provide a supportive niche for progenitor cell proliferation, migration, and differentiation [101]. However, it has been shown that in certain fibrotic conditions, hepatic progenitor cells could also induce collagen accumulation [119]. Leaving aside the order of these events, the fact that liver progenior cells are found outside the protective ECM cocoon very rarely [101] indicates that the ECM is a key player in DR niche system.

The ECM has an important part in all known stem cell niches, although its complexity varies considerably between different organs (reviewed in [111]). Since stem cells in different tissues have unique properties, the composition of ECM has to be adapted accordingly in order to protect and support their individual capabilities. During aging the niche-specific ECM composition changes, which may be one of the reasons why aged niches possess decreased ability in maintaining stem cell properties [113].

As described in the previous chapter, in the liver, the structure of ECM around DRs depends on the injury and regeneration program [96]. Moreover, the amount of ECM at various parts of single DR can differ greatly. For example, a fibrotic liver in a patient with alcoholic liver disease shows dense collagen deposition around biliary ends of the DR, but very little collagen around hepatocytic ends [120], meaning that cells with thin layer of ECM are exposed to different signaling molecules and factors than those embedded in thick ECM, which in turn may lead to different differentiation pathways. That could, to some extent, explain the cellular diversity within DR in terms of morphology and immunophenotype, as it has been shown that hepatocyte-like progenitors are mostly at the parenchymal border of DRs, and cholagiocyte-like cells at the portal border [48].

Although collagen has an important role in DR niche and is prominently expressed in fibrotic liver tissue, it is not the only ECM component that has an active influence on progenitor cell behaviour. Ductular reactions are also surrounded by laminin matrix [93]. In fact, progenitors need contact with laminins in the basement membrane in order to maintain their progenitor/biliary state; and laminin deposition is essential for DR expansion and for the inhibition of hepatocytic differentiation [41, 120, 121]. By contrast, suppressing laminin production yields less DRs and induces differentiation toward hepatocytes [41]. In a low dose of acetylaminofluorene (AAF)/ PH rat model, when progenitor cells lose contact with basement membrane (starting from the distal end of the DR), they simultaneously begin differentiating toward hepatocytes [122]. In addition, in alcoholic hepatitis, a disease with prominent laminin deposition, only biliary differentiation is observed [123]. *In vitro* studies have demonstrated that liver progenitor cells preserve their progenitor markers, up-regulate cholangiocyte specific genes, and down-regulate hepatocyte-specific gene C/EBPα when cultured on laminin but not on collagen I, IV, or fibronectin

(FN) [93]. On the contrary, FN matrix was shown to induce hepatocyte gene expression and differentiation toward hepatocytes [93], indicating that FN may be involved in hepatocyte fate determination.

Thus far, 16 laminin trimers have been identified [124] and not all laminins have the same effect on stem cells. For example, while laminin-511 supports the self-renewal and undifferentiated state of human and mouse embryonic stem cells, laminins-111, -332, and -411 do not have same competence [125, 126]. Laminin-322 has been shown to mediate osteogenic differentiation of mesenchymal stem cells [127], whereas laminin-111 induces neural fate [128]. Not much is known about different types of laminins and their roles in adult liver. Laminins-511 and-521 are expressed in normal liver while laminin-111 is transiently expressed after PH [129]. Laminin composition in chronic liver injury has not been extensively studied.

2.8. Liver progenitor cells and tumor development

Liver cancer is one of the most common cancers worldwide. Two types of primary liver cancers are hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). 80% of HCCs develop after long and persistent chronic liver injury, whereas CCs have an agressive nature and are often discovered in late stages [130]. There are many different risk factors associated with liver malignancies, such as hepatitis virus B and C, alcohol, diabetes, obesity, toxins, parasitic infections, hereditary conditions, etc. [131, 132], which could explain the high heterogeneity of liver cancers. HCCs are presumed to originate from hepatocytes and CCs from biliary epithelial cells, however, bipotential liver progenitor cells are also considered as possible contributors. The hypothesis of a common ancestor is supported by the fact that liver tumors often contain cells with heterogenous morphology, mixed immunophenotype of biliary and hepatocellular features, and a side population with progenitor cell profile [48, 133]. Even pure HCCs contain subpopulation of small cells with progenitor cell markers EpCAM, CK19, and AFP [134, 135]. Whether these intratumoral progenitors can be regarded as cancer stem cells is still an open question. However, hepatitis virus C patients with EpCAM+CK7+CK19+ intermediate cell foci in liver biopsies have been shown to possess higher risk of developing liver cancer [136], and overall CK19 expression in HCCs is associated with worse prognosis, higher rate of metastasis and cancer recurrence after liver transplantation [137]. Transdifferentiation is another possible mechanism for liver tumor development. Although CCs are presumed to arise from cholangiocytes or, possibly, liver progenitor cells, hepatocyte-specific lineage-tracing experiments by two independent groups have demonstarted that CCs could alternatively originate from mature hepatocytes through Notch-mediated transdifferentiation and AKT-mediated neoplasia [64, 138]. Considering the heterogeneity of liver tumors, it is reasonable to speculate that primary liver cancers could be derived from multiple sources.

2.9. Overview of AKT signaling pathway

AKT (also called protein kinase B, PKB) is a serine/threonine kinase involved in diverse processes, including cell survival, proliferation, and metabolism. AKT family members AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ are highly homologous despite being encoded by different genes on different chromosomes, and have overlapping or distinct functions, depending on the cellular context [139]. AKT is the central component in a pathway comprised of a myriad of proteins, some of which are tumor suppressors (eg. PTEN, FOXO) and others act as oncoproteins when overexpressed or activated (eg. eIF4E, PI3K) [140]. Hyperactivated AKT signaling has been associated with many diseases and malignancies in various tissues, underlining the importance of tight regulation of AKT activity in normal cell functioning [139]. In normal cells, AKT is maintained in an inactive unphosphorylated state [141]. Upon phosphorylation at Thr308 by PDPK1 (3-phosphoinositide-dependent protein kinase 1) AKT becomes active, however, for full activity phosphorylation at Ser473 is also required [141, 142]. A number of growth factors (GF) (eg. hepatic GF, insulin-like GF, fibroblast GF, nerve GF), cytokines, and stress stimuli (eg. hypoxia, heat shock, DNA damage, reactive oxygen species) are known to trigger AKT activation and subsequent translocation to different subcellular compartments, where it exerts its biological functions [143]. Depending on the stimulus, location, isotype, and context the activated AKT can induce cell cycle progression, proliferation and cell migration, as well as inhibit apoptosis, and control different metabolic processes [139, 140, 143].

2.9.1. AKT signaling pathway in liver diseases

In the liver, AKT not only participates in its normal functioning, but is also activated in response to cytokines and GFs released after tissue loss. AKT signaling pathway is believed to control liver regeneration by inducing the hepatocytes to move from quiescent state to proliferative state and by regulating the process of hepatocyte hypertrophy [144, 145]. As AKT is involved in glycose and lipid metabolism in the liver, dysregulated AKT is implicated in several metabolic liver disorders. For example, insulin resistance and type 2 diabetes are accompanied by abnormally low AKT levels [146], whereas hyperactivated PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is detected in fatty liver diseases and liver cancer [147, 148]. Nearly 40% of HCCs show elevated AKT2 levels [149], while active phosphorylated mTOR (pmTOR) is present in 15% of HCCs [150]. The levels of pmTOR and pAKT are also in a positive correlation with the expression of potential hepatic cancer stem cell markers CD133, CD90, and EpCAM [151]. Moreover, CD133+ HCC cancer stem cells with active pAKT are more resistant to chemotherapy compared to the tumor cells that lack CD133 [152], underlining the positive effect of AKT activation on cell survival. Hyperactivated PI3K/AKT/mTOR

pathway participates in tumor ECM remodeling by up-regulating the expression of MMP-9, a common cancer MMP involved in matrix degradation [153]. Since rearrangement of ECM is necessary for tumor cell invasion and metastasis, it is not surprising that activated AKT is also linked with enhanced invasiveness of cancer cells [154, 155]. On the other hand, the expression of PTEN, the inhibitor of AKT pathway, is reduced in 40% of HCCs [156] as are the levels of many proapoptotic AKT targets, such as BID, BAX, and P53 [143]. Since AKT activation and the results of aberrant AKT signaling are considered a common hallmark of cancer, components of this pathway have become attractive targets in anticancer drug design [140].

2.10. Dupuytren's contracture

Dupuytren's contracture (DC) is a progressive and irreversible fibrotic disease characterized by uncontrolled myofibroblast proliferation and formation of contractile nodules and collagen-rich cords in the palm of the hand [157], which cause bending of fingers (usually the forth and fifth digits) and gradual reduction in hand function.

DC occurs in three stages: proliferative phase, involutional phase, and residual phase [158]. The proliferative phase of DC is characterized by the proliferation of myofibroblasts and formation of nodules. In the involutional phase the disease advances, myofibroblasts align along the palmar fascia, resulting in the development of the cord and deposition of type III collagen. In the residual phase the myofibroblasts and nodules begin to disappear while the disease spreads further into fingers, the fibrous cords tighten and fingers contract towards the palm [159].

Surgical removal of the diseased tissue is the most common DC treatment method, however, surgery has several side effects and the recurrence for DC is quite common [160]. DC is most prevalent in Caucasian men of Northern European ancestry, and although the cause of this condition is not known, DC has been associated with alcoholism, diabetes mellitus, epilepsy, manual labor, smoking, and trauma [161]. A recent twin study showed that genetic factors play a major role in DC development, however, no particular genes have been confirmed to be responsible for DC [162].

Fibrosis is a pathological condition that can affect various tissues, including the liver, lungs, skin, kidneys, and cardiovascular system [163]. In DC, similarly to other fibrotic diseases, the excessive ECM (mostly collagens and fibronectins) is produced by increased number of myofibroblasts [158, 164]. It has been shown that proinflammatory cytokines such as tumor necrosis factor (TNF) [164], profibrotic cytokines such as TGF-β, and growth factors such as basic FGF (bFGF) [165–167], which are upregulated in DC tissue, induce proliferation and/or differentiation of myofibroblasts. Higher fibroblast density could explain increased collagen III/I ratio in DC, as it has been demonstrated that collagen I production is inhibited at high fibroblast concentration [168].

Inhibition of MMPs could also play a role in matrix deposition in DC. Indeed, a genome-wide analysis identified three MMPs – MMP1, MMP3, and MMP16 – that were significantly downregulated in fibroblasts derived from DC tissue [169]. There are several other modulators and pathways such as AKT and Wnt signaling pathways [169] that are implicated in DC pathogenesis, however, there is no overall consensus regarding the exact mechanism that is responsible for the onset and progression of this disease. The data gathered so far suggest that it is a combination of dysregulated cytokine and growth factor signaling along with altered ECM composition that induces and sustains DC progression.

3. AIMS OF THE STUDY

The aims of the study were as follows:

1. To identify, locate, and characterize quiescent liver LRCs, and determine their contribution in liver homeostasis and regeneration.
2. To identify the changes in the liver ECM after liver injury, and study the impact of these changes on cell proliferation.
3. To characterize the composition of ECM in Dupuytren's disease, and study the molecular mechanisms promoting the progression of this disease.
4. To identify the inhibitor of AKT1-PDPK1interaction and study its possible antitumor properties.

4. RESULTS AND DISCUSSION

4.1. The LRC identification strategy (Ref I)

In the LRC assay, slowly cycling cells are distinguished from rapidly proliferating cells by their ability to retain nuclear label. In the past, the identification of quiescent label-retaining adult liver stem cells has based on the incorporation of BrdU into DNA. This strategy, however, requires active DNA synthesis, which is in sharp contrast to the basic nature of quiescent stem cells. A way to force stem cells exit quiescence is to induce injury, an approach which has been used to label cells with BrdU in the liver. As the regeneration mechanism in the liver depends on the specific injury, the progenitor response can vary greatly between injuries, and generally the activation of differentiated cells and/or infiltration of extrahepatic cells is also involved. Therefore, cell labeling coupled with injury may not be the best method for identifying resident stem cells in the liver. On top of that, treatments required for BrdU visualization do not allow live cell isolation for further investigation.

Development of new genetic tools has made it possible to identify LRCs in uninjured liver. To locate quiescent liver stem cells in normal physiological conditions we used a bitransgenic mouse model in a pulse-chase experiment that enabled us to first, label liver cells in neonatal mice, then, monitor label retention and characterize LRCs in adult mice, and finally, study isolated cells *in vitro*. Bitransgenic mice were generated by crossing mice, which express histone H2B-enhanced green fluorescent protein (H2B-EGFP) under the control of doxycycline (dox) inducible tetracycline regulatory element (TRE) [170] with a mouse line harboring Rosa 26 promoter-driven reverse tetracycline-dependent transactivator transgene (R26-rtTA) [171] (Fig. 1a). When the offspring of this mating received dox from birth to postnatal day 5 (P5), 86% of CK19+ cholangiocytes and 98% of HNF4α+ hepatocytes expressed H2B-EGFP fusion protein on P5 (Fig. 1b,e). Such high level of transgene induction at the beginning of the experiment confirmed its suitability for LRC identification in subsequent chase phase. We administered dox straight after birth because we reasoned that massive liver growth and high proliferative activity in adolescent mice would induce rapid EGFP dilution, and we would be able to distinguish LRCs from the background much quicker, as opposed to adult mice in which liver cells proliferate infrequently (Suppl. Fig. 1a-b).

4.2. Identification of liver LRCs (LLRCs) (Ref I)

After 1 week of chase (W2), there were no significant changes in the number of H2B-EGFP expressing cells (Fig. 1e). There are two explanations for this phenomenon: first, although cells are highly proliferative at this stage of liver development, it is possible that dox was still being washed out from the system and transgene was induced in newly formed cells by residual dox. And second,

we could detect somewhat lower EGFP intensity at W2 when compared to P5 (Fig. 1d, Suppl. Fig 2b), indicating cell proliferation and EGFP dilution even if it was not accompanied by smaller EGFP+ cell numbers. A significant drop in EGFP intensity was seen at W3 (Fig. 1d, Suppl. Fig. 2a-b), but yet again the proportion of labeled CK19-positive cholangiocytes was not decreased and the percentage of HNF4α-positive hepatocytes was decreased by 8% (Fig. 1e). A significant change occurred at W7 when, for the first time, we could distinguish LRCs in bile duct-like structures from the background (Fig. 1d, Suppl. Fig. 2a-b), as the majority of hepatocytes had lost their labeling (decrease from 90% to 3%), whereas the majority of cholangiocytes had retained the label (from 86% to 71%) (Fig. 1e). From W7 to W15 the number of labeled hepatocytes continued to decrease (Fig. 1e) until only single EGFP+ hepatocytes could be found. In the cholangiocyte compartment, however, the EGFP positive cell population maintained its size, and we concluded that in the normal liver the LRCs reside in the bile ducts. Since the proliferation kinetics profile among cholangiocytes and hepatocytes was quite similar (Suppl. Fig. 1c-d), there are several possible scenarios that could explain how the biliary cells retain the EGFP label. One can speculate that while all hepatocytes divided during the chase period and thereby lost the label, in cholangiocyte compartment only a subset of cells proliferated repeatedly and the remaining cells stayed quiescent or divided less frequently. The other explanation is based on the "immortal DNA strand hypothesis", which proposes that in a certain type of asymmetric cell division chromatids are segregated between daughter cells according to the age of the template [172]. Older chromatids are sorted to the cell that becomes the stem cell and newly synthesized chromatids are sorted to the second daughter cell that is destined to differentiate. The existence of nonrandom template strand segregation has been demonstrated in intestinal [173], muscle [174], and neural stem cells [175]. In the liver, however, it appears that label retention is achieved, at least to some extent, by differential proliferation activity among biliary cells. We compared Ki67 expression in EGFP+ and EGFP- biliary cells and found that although EGFP+ cells proliferated actively in neonatal mice, their proliferative activity declined sharply at week 2 and remained lower than that of EGFP- cells until the end of the chase period (Suppl. Fig 3c). It has to be noted that changes in EGFP-H2B expression cannot be attributed solely to DNA duplication since a subset of H2B histones is also removed from nucleosome when replication is not present [176].

4.3. Characterization of LLRCs (Ref I)

4.3.1. LLRC immunophenotype

Potential bile duct-associated hepatic progenitors and bile duct cells share several common antigens [49]. As we had established that LLRCs in adult mice localized in the bile ducts, our next goal was to characterize LLRCs using

antibodies, which recognize biliary cells and which have been used in progenitor cell characterization. We found that in addition to CK19, LRCs also express EpCAM, CD133, increased levels of CD166, and A6 (Fig. 2c-f, Suppl. Fig. 5a-d). The parenchyma was essentially EGFP-negative with rare labeled hepatocytes (Fig. 2b). These results confirmed the biliary status of LRCs and provided us with a selection of cell surface antigens (EpCAM, CD133, CD166) that could be used for live cell isolation in later stages.

4.3.2. LLRC response to liver damage and participation in regeneration

4.3.2.1. LLRC are not activated in hepatocyte injury

The reparation mechanism that drives liver regeneration following injury is thought to be dependent on the type of injury. We used five different mouse models of liver injury to study the contribution of LLRCs in regeneration (Fig. 3). We found that in response to acute hepatocyte injury by single CCl_4 injection the LLRC compartment was not activated as the proliferative activity of EGFP+CK19+ cells remained at maintenance level (Fig. 4a,d) and there was no decrease in labeled cholangiocyte number after 2 weeks of recovery (Fig. 3b,g). To induce massive hepatocyte regeneration without hepatic toxins we performed 2/3 partial hepatectomy and again observed that the number of biliary LRCs remained essentially unaltered (Fig. 3c,g). A slight, yet statistically insignificant tendency toward decrease could be explained by the fact that not only hepatocytes but also other mature liver cell types proliferate after PH [177]. Since DRs are not induced after PH and previous studies have also shown that stem cell activation does not occur in this type of regeneration [177], it is unlikely that the LRCs, which lost their label during recovery, gave rise to new hepatocytes. Based on these observations we concluded that LLRCs are not needed for hepatocyte regeneration when hepatocytes are capable of re-establishing tissue homeostasis. Therefore, it would be interesting to study the behavior of LRCs in a situation where hepatocyte proliferation is suppressed. Indeed, several studies indicate that liver progenitors are activated in response to hepatolobular damage only when hepatocyte proliferation is compromised. In rats, this can be achieved by combining 2-acetylaminofluorene (2-AAF) administration with PH [178] or CCl_4 [179]. Both of these liver injury models have been shown to stimulate the expansion of oval cells, however, their contribution to hepatocyte regeneration and restoration of liver function has remained controversial [122, 180]. To block hepatocyte proliferation in mice a transgenic mouse model of Damaged DNA Binding protein 1 (DDB1) selectively deleted in hepatocytes has been generated. DDB1 deletion was shown to abolish hepatocyte self-renewal capacity which led to DR expansion and their differentiation toward hepatocytes [181].

4.3.2.2. LLRC are induced to proliferate in response to primary biliary injury

To study the behavior of LRCs in response to biliary injury, we either performed total bile duct ligation (tBDL), or fed the mice DDC or CDE diet. tBDL, DDC and CDE diet target not only cholangiocytes but other liver cell types as well. These widely used injury models mimic a variety of human liver diseases, including secondary biliary fibrosis, alcoholic and nonalcoholic steatohepatitis, metabolic liver diseases, sclerosing cholangitis, biliary fibrosis, and chronic cholestatic liver diseases [182]. Although no known mouse model corresponds exactly to specific disease, they are a valuable tool for understanding the underlying mechanisms of liver disease development [182]. tBDL, DDC and CDE diet all induced ductular reactions, infiltration of immune cells, and LRC activation. After DDC diet and tBDL, most bile ducts and DRs were EGFP negative (Fig. 3d-e) with only 6% and 10% of CK19-positive LRCs left, respectively (Fig. 3g), suggesting that these injuries induced LRC proliferation. Indeed, the expression of proliferation marker Ki67 was dramatically increased in the biliary compartment of injured livers when compared to low proliferation rate in normal adult livers (Fig. 4 b-d). Interestingly, in case of tBDL, the LLRCs proliferated more actively than non-LRCs, indicating a difference in their responsiveness and proliferative capacity in certain injury conditions. Although CDE diet-induced biliary damage did not cause as dramatic decrease in LRCs numbers as tBDL or DDC, the change was still significant (Fig. 3g). A number of DRs in CDE liver demonstrated weak EGFP signal (Fig.3 f), suggesting that they were derived from biliary LRCs. Overall, these results demonstrated that the activation of LRCs is needed after biliary injury, LRCs proliferate, and thereby lose EGFP-labeling.

In parallel with these observations, we noticed the appearance of CK19-negative cells with strong EGFP signal around DRs (Fig. 3d-f, Fig. 4b-c). Since H2B-EGFP transgene has been shown to be promiscuously expressed in bone marrow by hematopoietic cells without dox induction [183], we hypothesized that these CK19-EGFP+ cells might be of hematpoietic origin infiltrating into the site of injury. Immunostaining with pan-leukocyte marker CD45 confirmed that these cells were indeed infiltrating inflammatory cells and not hepatic cells (Suppl. Fig. 6a).

4.3.2.3. Differentiation potential of LLRCs

Loss of EGFP expression in actively proliferating LRCs made it impossible to determine their differentiation potential *in vivo*, as we would not have been able to distinguish normal liver cells from LRC-derived cells. Therefore, in order to study their multipotency and self-renewal properties, we isolated live cells from normal pulse-chased mouse livers by fluorescence-activated cell sorting (FACS) (Fig. 6a) and cultivated them *in vitro* (Fig. 6b). The ability to self-renew is a key feature of stem cells. To determine, whether liver LRCs possess

enhanced self-renewal capability, we compared the colony forming efficiency (CFE) of two biliary cell populations: EGFP+EpCAM+CD45- cells (LRCs) and EGFP-EpCAM+CD45-cells (non-LRCs). After two weeks of cultivation the LRCs formed colonies, whereas non-LRCs did not (Fig. 6d), suggesting that LRCs do indeed possess enhanced self-renewal capacity when compred to non-LRC biliary cells. However, LRCs did not show bilineal differentiation potential, as we could only detect CK19 single-positive colonies devoid of any HNF4α-expressing hepatocytes (Fig. 6e-g). These data suggest that although LLRCs possess enhanced self-renewal capacity, they are restricted to biliary lineage.

Our results corroborate with the study by Suzuki *et al*. where it was shown that although CD133+ biliary cells isolated from normal adult liver formed colonies, they did not differentiate into hepatocytes [54]. On the other hand, in a report by Okabe *et al*., it was shown that EpCAM+ cholaniocytes isolated from normal liver not only formed colonies as successfully as EpCAM+ cells isolated from DDC-injured liver (bile duct cells and DRs combined), but also differentiated into functional hepatocytes [40]. In this report, the differentiation was achieved by special differentiation medium, whereas we, and Suzuki *et al*. investigated "spontaneous" differentiation. One could argue that our unsuccessful differentiation was caused by the lack of differentiating supplements in the medium. However, in the same Suzuki *et al*. report, it was demonstrated that CD133+ cells isolated from DDC-treated livers did possess bipotency even without supplemented medium.

4.3.2.4. Gene expression profile of liver LRCs and non-LRCs

We then compared the transcriptional profile of the aforementioned sorted cell populations by RNA sequencing in order to find out what are the molecular features that discriminate LLRCs from non-LRCs. We found that out of 95 differently regulated genes, 20 were up-regulated and 75 down-regulated in LLRCs. Genes associated with tumorigenesis (*Spdya, Styk1, Fgr, Pbk, Palb2, Cxcl10, Tmc7, Mas1*) constituted almost half of the upregulated genes. This could be related to the enhanced self-renewal property of LLRCs we discovered previously. Interestingly, 5 of the upregulated genes are known to function in central nervous system but their function in liver is unknown. More than half of downregulated genes were related to plasma membrane or cell-cell contact formation. Several genes associated with vessel development (eg *Wnt2, Bmp4, Rspo3, Flt1, Tek*) [184] were also downregulated. The nature of downregulated genes suggests that LLRCs do not participate actively in the maintenance of biliary system in the normal liver.

4.4. Elucidating the contribution of biliary cells to liver maintenance and regeneration (Ref I)

We had discovered that about 30% of biliary cells participated actively in postnatal liver development and/or maintenance illustrated by the loss of EGFP label. At the same time, more than 70% of bile duct cells were quiescent or proliferated fewer times, and thereby retained their label, but were strongly activated upon biliary injury. Although *in vitro* experiments suggested that LLRCs do not possess bipotency, we could not verify this *in vivo* with the R26-rtTA-H2B-EGFP mouse model. Since LLRCs express CK19, we turned to a transgenic mouse model in which upon tamoxifen (TMX) injection the CK19 positive biliary cells and their progeny start to express tdTomato (tdTom), allowing us to trace the fate of labelled cells in liver maintenance and regeneration. We injected mice with TMX at P21 and traced the tdTom label up to 6 months (Fig. 5a-c). We found that during the homeostatic maintenance the CK19+ biliary cells give rise only to new biliary cells as we did not detect any other tdTom-labeled cell type with. To study the behavior of bile duct cells in liver regeneration we introduced 6 different types of liver injury and examined tdTom expression. tBDL, DDC diet, and CDE diet induced DRs, some of which were tdTom-positive, indicating that these cells originated from bile duct cells (Fig. 5d-f). As expected, not all DRs were tdTom-positive, since tdTom initial labeling efficiency in these experiments was much lower than 100%. However, we could not find any tdTom-labeled hepatocytes in these livers. We also did not detect any contribution from CK19+ compartment to hepatocyte recovery after PH (Fig. 5g), after short-term hepatocyte damage by single CCl_4 injection (Fig. 5h), or after long-term hepatocyte damage by repeated CCl_4 administration (Fig. 5i). Based on these results and data from H2B-EGFP mouse experiments, we concluded that LRCs in an adult liver represent a population of fully differentiated cells, which, compared to other biliary cells, have retained superior regenerative capacity due to overall lower proliferative activity during liver postnatal development and maintenance. Despite their enhanced self-renewal property, LLRCs have restricted differentiation potential and only take part in biliary cell regeneration.

4.5. Changes in liver ECM after liver damage (Ref II)

Liver regeneration involves not only cell proliferation but also remodeling of extracellular matrix [185]. As the components of ECM facilitate signal transduction between cells and their environment, rearrangements in ECM cause shifts in cell signaling which in turn lead to necessary changes in cell behavior. The correct ECM composition during liver tissue repair is achieved by coordinated balance between protein synthesis and degradation [186, 187]. Since dysregulated ECM production and degradation is implicated in many liver pathologies [187], knowledge about the changes in ECM and the molecular

mechanisms behind them could be used for the prevention and treatment of liver diseases.

4.5.1. ECM protein composition after liver injury

In order to study how acute CCl_4 injury and DDC-induced injury change liver ECM composition, we first decellularized and homogenized the liver samples, then performed proteomics analysis using nano-LC-MS/MS, and finally compared the protein contents of normal and damaged liver samples (Fig. 1A). We found that the levels of 32 ECM proteins were altered in CCl_4-treated livers (48h after injection), the levels of 22 proteins were altered in DDC-treated livers (fed for 2 weeks), and only 9 proteins were similarly up-or downregulated in response to both treatments (Fig. 1B), indicating that regeneration from different types of liver injuries required different ECM composition. Indeed, while DDC induced upregulation of collagen I and V and downregulation of collagen VI, in CCl_4 treated livers we detected significant downregulation of collagen IV and VIII (Fig. 1C). Upregulation of collagen I mRNA in DDC-treated liver has been shown before [188]. Deposition of FN and down-regulation of laminin chains α3, α5, and γ2 was detected in both liver injuries. Since CCl_4 is specifically toxic to hepatocytes it was no surprise that FN was up-regulated following CCl_4 treatment as FN has been shown to be necessary for hepatocyte survival in acute liver injury [189]. Antiapoptotic FN properties have been described also for ovarian and breast cancer cells, where adhesion to FN activated PI3K/AKT2 pathway, which ultimately led to chemoresistance of the tumor cells [190]. In addition, FN is also known to control the availability of active TGF-β in injured liver [191]. Imbalanced TGF-β signaling leads to enhanced stellate cell activation and fibrosis, whereas FN up-regulation prevents TGF-β overactivation and protects liver from excessive fibrosis [191], thereby giving the liver time to regenerate. In conclusion, FN deposition appears to have protective role in liver injury.

In addition to the changes in the composition of structural ECM proteins, we also identified several non-structural ECM components, which were deregulated in response to liver injury (Fig. 1C). CCl_4 injury induced up-and downregulation of numerous proteins, whereas DDC diet influenced the levels of only a few non-structural ECM proteins, indicating that different pathways or mechanisms might be involved in liver regeneration after these two injuries.

4.5.2. The changes in the elasticity and microarchitecture of ECM after liver injury

Elastin is usually accumulated in fibrotic and cirrhotic livers [192, 193] and is used as a marker to describe the chronicity of fibrotic change [194]. Although DDC diet is known to induce biliary fibrosis [195], we did not detect higher

elastin levels in the livers of DDC-fed mice. It is possible that 2-weeks of DDC feeding was not long enough to induce substantial fibrosis, as elastin deposition occurs only in the advanced stages of fibrosis [193]. In acute CCl_4 injury, the level of elastin was significantly decreased (Fig. 1C). Since elastin provides flexibility to the liver ECM, we hypothesized that CCl_4-treated livers would be stiffer than normal livers. We measured the stiffness of decellularized livers with atomic force microscope (AFM) indentation and found that, indeed, the ECMs isolated from the CCl_4-treated livers were significantly stiffer than ECMs of normal livers, whereas DDC-treated livers showed only slight increase in liver stiffness (Fig. 3G). Increasing evidence suggest that contact with stiffer matrix may facilitate activation of hepatic stellate cells and portal fibroblasts, and induce proliferation of active myofibroblasts [196]. In case of acute liver injury this activation would be short-term but in persistent injury conditions active myofibroblasts would produce increasing amounts of matrix proteins, which eventually would lead to fibrosis. This could explain why the increased liver stiffness precedes matrix accumulation and fibrosis [197].

Both liver injuries also induced remarkable disorganization of the ECM structure. In normal liver, the collagen was organized in wavy cords or tape shape fibers (Fig. 3A-B), whereas in injured livers we detected disarranged and branched reticular-type collagen fibers (Fig. 3C-F). Such dramatic changes in the ECM architecture suggest that the changes in its molecular makeup might play a part in this process. Since ECM stores numerous cytokines, growth factors, hormones, and enzymes, changes in its structure are likely to influence their availability. Altered composition, concentration, or activation of signaling molecules is a common feature in injured liver. The exact role of the structural alterations of the ECM in liver regeneration and disease, however, are yet to be determined.

4.5.3. Impact of structural ECM components on cholangiocyte and hepatocyte proliferation

Proteomics analysis provided us the information about the overall changes in ECM composition in response to liver injury. In order to determine the exact location of these changes in the liver, we performed immunofluorescence analysis and studied the expression and localization of structural ECM components in the livers of CCl_4-and DDC-treated mice (Fig. 2). FN was accumulated around portal areas and sinusoids in both damaged liver samples. In DDC-treated livers, collagen I and IV accumulated periportally, whereas in CCl_4-treated livers, we detected collagen I accumulation pericentrally and collagen IV reduction in pericentral sinusoids. We also studied the expression of proliferation marker Ki67 and found that proliferating cells concentrated around portal areas (Fig. 5C). Immunostaining results are discussed further in the context of the data from *in vitro* experiments in the next paragraph.

Since CCl_4 intoxication and DDC diet primarily affect different cell populations, and the respective changes in ECM composition according to proteomics analysis were different to a large extent, we hypothesized that individual ECM components may have different effect on hepatocytes and cholangiocytes. We isolated hepatocyte and cholangiocyte populations from normal livers and cultured them on dishes coated with FN, collagen I, or collagen IV. We found that FN and collagen I, which were up-regulated in DDC-treated mice, promoted the growth and proliferation of biliary cells more effectively than collagen IV (Fig. 4A-C). This is in good correlation with the findings that in DDC-treated liver, FN and collagen I accumulated around biliary ducts and ductular reactions, where the proliferative activity is the highest.

In contrast, the proliferation of hepatocytes was most prominent on collagen IV (Fig. 5A-B). Although the overall collagen IV expression was down-regulated in CCl_4-treated livers, its expression was retained at normal level around portal areas where the proliferative hepatocytes were located (Fig. 5C), correlating well with the results obtained in *in vitro* experiments.

Although FN was upregulated in CCl_4-treated livers, it had only a modest impact on hepatocyte proliferation. It has been shown previously that while FN helps hepatocytes to evade apoptosis it is not necessary for their proliferation [189]. In addition, FN plays an important role in regulating cell-to-cell and cell-to-matrix adhesion [198]. Since FN accumulated in the portal areas where new hepatocytes were generated, it is possible that in addition to facilitating pro-survival signals, FN enhanced the establishment of interactions between newly formed hepatocytes and neighboring cells and the ECM.

Results from *in vitro* experiments, immunofluorescence analysis, and proteomics analysis suggest that hepatocytes and cholangiocytes require specific niche composition for cell proliferation.

4.6. Molecular mechanisms that regulate cell proliferation in Dupuytren's contracture (Ref III)

Dupuytren's contracture is a widespread disease affecting both men and women, and although it has been extensively studied since its first description 3–4 centuries ago [199], the knowledge regarding DC pathogenesis is fragmented and the molecular mechanisms involved in DC pathogenesis are still poorly understood. Since DC is characterized by increased myofibroblast proliferation [158], our goal was to identify the molecular and structural components that support this process.

In order to study the molecular and cellular mechanisms sustaining the fibrotic processes in DC, we utilized immunofluorescence analysis and quantitative PCR (qPCR) methods and compared the gene and protein expression profiles of DC and normal palmar fascia (NPF) samples. First, we determined that the majority of proliferating cells in DC were smooth muscle actin (SMA)-positive myofibroblasts located in or in the vicinity of the blood vessels, which

were abundantly scattered throughout the DC tissue (Fig. 1a-f). A small proportion of proliferative endothelial cells indicated the formation of new blood vessels (Fig. 1f). Blood vessels in NPF samples were very rare and they did not contain proliferating cells (Suppl. Fig. S1a,c). Increased vascularisation in DC also caused higher ECM content in DC tissue samples, as laminins 411/421, 511/521, and collagen IV (components of the endothelial basement membrane [200]) and SMA were expressed in myofibroblast layer of the blood vessels (Suppl. Fig. S1b,d;S2,S3). Although we detected FN in both NPF and DC tissues, increased amounts of FN were expressed in blood vessel walls in diseased tissue (Suppl. Fig. S3n-p).

Angiogenesis is an important component in wound healing and a stimulator of fibrosis in several tissues [201]. Furthermore, it has been previously shown that stem cell niches in normal [202] and malignant [203] brain tissues, and in heart [204] contain small blood vessels that regulate stem cell self-renewal. As we had found that the majority of proliferative cells concentrated in and around blood vessels, we hypothesized that blood vessels could possess similar role in DC and thus maintain the uncontrolled cell proliferation. Since AKT signaling pathway has been proposed to play a role in DC [205] and AKT hyperactivation can cause abnormally high cell proliferation [140], we studied phosphorylated AKT (pAKT) presence in the blood vessels of DC and NPF samples. We detected pAKT in all the layers of blood vessels in DC tissues (Fig. 3a). Interestingly, in addition to blood vessels, pAKT was highly expressed in sweat gland ducts and acini (Fig. 3b).

Next we studied the protein and mRNA expression of growth factors that have been associated with DC development. These include pro-proliferative growth factors bFGF and insulin-like growth factor-2 (IGF-2) [167, 206], and pro-fibrotic connective tissue growth factor (CTGF) [207]. CTGF is a TGF-β downstream modulator involved in extracellular matrix synthesis [208]. CTGF is known to exert fibrotic activity in other tissues, as well. For example, CTGF mRNA is significantly upregulated in fibrotic and cirrhotic livers [209, 210]. Likewise, bFGF expression levels are also elevated in fibrotic livers [211], and increased IGF-2 expression have been reported in livers with fibrosis and HCC [212]. As expected, all three were up-regulated in DC tissues (Fig. 3d-f), however, each growth factor exhibited unique expression pattern. IGF-2 was expressed throughout the DC tissue (Fig. 3j-k). IGF-2 is known to induce cellular contractility in DC [206], which could explain its homogenous expression. bFGF was expressed in blood vessel endothelium (Fig. 3m-n), while CTGF was detected in sweat gland acini (Fig.3 g-h). The role of sweat glands in DC development is unknown, however, the expression of pAKT in ducts and acini, and CTGF presence in acini suggests their involvement in DC pathogenesis.

Overall, these results suggest that DC progression involves upregulation of growth factors that are also implicated in other fibrotic diseases. We propose that CTGF originating from sweat glands, bFGF secreted from blood vessels, and IGF-2 released from ECM synergistically induce the upregulation of AKT pathway. Increased AKT signaling in conjunction with the specific ECM

composition forms an environment near the blood vessels that supports myofibroblast proliferation and pathologic ECM production. At the moment, hand surgery is the prominent treatment for this disease. Inhibition of growth factor-induced AKT pathway might represent a new therapeutic strategy in DC treatment when less invasive methods are needed. Since these molecules are also overexpressed in other fibrotic and malignant diseases, similar approach could be considered for several diseases.

4.7. Identification of small-molecule inhibitors of AKT1-PDPK1 interaction (Ref IV)

AKT signaling is aberrantly upregulated in many different diseases and tumors [139]. Although there are a number of inhibitors that target AKT pathway [213], they exert broader kinase specificity and are thus relatively toxic, which prevents their clinical usage. Since AKT is activated through phosphorylation of Thr308 by PDPK1 that physically interacts with AKT protein [141], we aimed at finding the inhibitors of AKT pathway that target this interaction. For this purpose we used split *Renilla* luciferase (Rluc)-based protein complementation assay (PCA) [214]. First, we generated AKT1 and PDPK1 fusion proteins with complementary Rluc fragments. When these fusion proteins were expressed in cells their interaction was detected as Rluc activity. When this interaction was interrupted the Rluc fragments moved apart and the enzyme activity dis-appeared (Fig. 1A). We screened the NCI Diversity Set I small molecular compound library that contained 2000 chemicals, utilizing the PCA assay, and selected 36 chemicals for further evaluations (Fig. 1B). All 36 reduced AKT1-PDPK1 interaction by at least 50 % (Suppl. Table S5), but their toxicity stayed below 25 %.

In the following step we tested the ability of the selected chemicals to reduce AKT1 phosphorylation at Thr308 by western blot (Fig. 2A). We found that out of 36 chemicals only 4 significantly reduced AKT1 phosphorylation while having no effect on cell density and morphology (Suppl. Fig.S1A-D).

Next, we utilized *in situ* proximity ligation assay (PLA) to study the potency of the selected 4 compounds to disrupt the interaction between endogenous AKT1 and PDPK1 proteins in PC-3 prostate cancer cells with active AKT signaling pathway. We found that only one compound, NSC156529, inhibited this interaction (Suppl Fig. S2), as the number of AKT1-PDPK1 interaction sites was significantly decreased in cells incubated with NSC156529 compound when compared to control cells.

Treating cells with NSC156529 compound also inhibited AKT downstream anti-apoptotic and pro-proliferative targets such as pBAD, pGSK3β, pFOXO, and phosphorylated procaspase 9 (Fig. 3B). The anti-proliferative effect of NSC156529 was further illustrated by its ability to inhibit cell growth of malignant and normal cells *in vitro*. We detected dose-dependent cell growth inhibition of PC-3 prostate tumor cells, osteoblasts, and fibroblasts (Fig. 3C).

NSC156529 inhibited also cell growth of HEK293, HepG2, K07074, and H1299 cell lines (Suppl. Fig. S4 A-D).

In order to test the anti-proliferative properties of NSC156529 *in vivo*, we first established tumors in nude mice by injecting PC-3-EGFP prostate cancer cells under the dorsal skin of immunodeficient mice. When tumor size reached 29–32 mm^3 we proceeded with subcutaneous NSC156529 administration at concentrations 1 mg/kg, 5 mg/kg, or 10 mg/kg 3 times per week for 4 weeks (Fig. 4A). Control tumors were treated with vehicle only. We found that all NSC156529 concentrations inhibited tumor growth as measured with external caliper and an *in vivo* imaging device (Fig. 4B-C), and no adverse side effects such as weight loss or ulcerations were detected. We also measured alanine transaminase (ALT) and aspartate transaminase (AST) levels – indicators of liver damage – at the end of the experiment. We did not detect any significant increase, indicating that NSC156529 was not hepatotoxic (Suppl. Fig. S5). It has to be noted that while subcutaneous NSC156529 administration had no effect on mouse well-being, intraperitoneal administration caused severe irritation, suggesting a potential toxicity towards mucous membranes.

Immunohistochemical examination of tumors showed lower pAKT and pBAD levels in NSC156529-treated tumors when compared to vehicle-treated tumors (Fig. 5A-B), indicating that NSC156529 inhibited AKT signaling *in vivo.* NSC156529-treated tumors were also mitotically less active than control tumors (presumably in consequence of decreased AKT activity) (Fig. 5C-D), but apoptotic activity in these tumors was not significantly increased (Suppl. Fig. S6A-B). Since apoptosis appeared not to be the mechanism behind tumor growth reduction, we hypothesized that treatment with NSC156529 induced differentiation of PC-3 tumor cells, which otherwise exhibit the properties of poorly differentiated prostate adenocarcinoma [215]. Indeed, we found that treated tumors showed increased expression of CK15/17 and CK8/18 – markers of fully differentiated prostate epithelial cells [216] – suggesting that the antitumor effects of NSC156529 are potentially mediated by tumor cell differentiation (Fig. 6). Whether this process is mediated by sole repression of AKT signaling remains to be verified.

These results indicate that NSC156529 is a new small molecule compound that has potential in tumor treatment in different ways. For example, a combined therapy of pAKT inhibition by small molecule compound, followed by chemotherapy could be a possible treatment strategy for certain liver HCCs. It has been shown that liver HCCs contain stem cells harbouring active AKT signaling that makes them more resistant to chemotherapy [152] and can thus be the cause for cancer relapse. Hence, suppressing AKT activity before or during chemotherapy could potentially make the tumor stem cells more sensitive to chemotherapy. Furthermore, NSC156529 could be used to promote differentiation in malignancies with undifferentiated cellular background that normally have poor response to conventional antitumor treatments. Currently, the most studied and clinically used differentiating agent is retinoic acid, which shows good results in acute promyelotic leukemia treatment, but has shown only

limited effect in the treatment of solid tumors [217]. Differentiating agents that have potential in solid tumors include histone deacetylase inhibitors trichostatin A, vorinostat and depsipeptide, and peroxisome proliferator-activated receptor-γ (PPARγ) agonist trogitazone [218]. These compounds have shown promising results *in vitro* but in clinical settings they have low effect on tumor size, or severe side effects when used as a single agent to treat solid tumors [219]. However, vorinostat administration in combination with other chemotherapy drugs has demonstrated anti-cancer activity in patients with head and neck cancer and non-small cell lung cancer [220], indicating that differentiating agents have potential in tumor treatment when used in a form of combined therapy.

Since AKT pathway is also upregulated in fibrotic diseases, NSC156529 could potentially be beneficial in fibrosis treatment. For example, inhibition of AKT1 has been shown to inhibit fibrosis in interstitial lung disease [221]. Taken together, the small molecular compound NSC156529 is a new promising candidate for the development of novel anti-tumor and possibly anti-fibrotic therapeutics.

5. CONCLUSIONS

Most chronic liver diseases induce the accumulation of heterogenous cell populations – the ductular reactions. Although there is evidence that DRs are able to differentiate into cholangiocytes and hepatocytes, their origin is still under debate. It has been shown in other tissues that somatic stem cells are slowly cycling cells that can give rise to rapidly proliferating intermediate progenitor cells. We hypothesized that similar process could occur in the liver and therefore applied label-retaining cell assay to identify such cells in normal liver and study their behavior in liver injury.

Any type of liver injury is usually accompanied by the deposition of extracellular matrix. In case of chronic injury, ECM accumulates predominantly around DRs, forming a niche for regenerating cells. We studied in detail the ECM components that are up-or down-regulated in acute and chronic injury, and examined their effect on cell proliferation.

Progressive deposition of ECM in chronic liver injuries that persist for several years leads to fibrosis, cirrhosis and in many instances to liver cancer. The pathological processes and signaling pathways that induce fibrosis are relatively similar in different tissues. Since the exact signaling network behind the fibrotic disease Dupuytren's contracture had remained elusive, we studied the expression of some of the most important fibrosis-inducing components in DC tissue.

AKT signaling pathway is upregulated in several fibrotic diseases and in many tumors, including liver tumors. Thus, AKT inhibition is a promising strategy in fibrosis treatment and in tumor therapy. Our goal was to find an inhibitor of AKT1-PDPK1 interaction that would suppress AKT activity and tumor cell growth.

The main results of this thesis can be outlined as follows:

1. In an adult liver, the slowly cycling cells, identified as label-retaining cells (LRCs), reside in the biliary duct system. ~ 70% of bile duct cells retained nuclear label for at least 15 weeks.
2. LRCs represent a population of fully differentiated bile duct cells that participate in biliary cell regeneration in response to chronic biliary liver damage, but are not activated in liver injuries, when primarily hepatocyte regeneration is needed.
3. LRCs possess enhanced self-renewal properties, nevertheless are restricted in their differentiation potential to biliary lineage *in vitro*.
4. Fully differentiated CK19-positive bile duct cells give rise to ductular reactions and new bile duct cells, but do not differentiate into hepatocytes *in vivo*.
5. The cell type which is predominantly affected in specific liver injury appears to dictate, at least partially, which alterations in the ECM composition occur in injured liver. And v*ice versa*, distinct ECM components altered in injured

livers have diverging effects on the regenerative properties of different liver cell types.

6. Proliferating myofibroblasts that induce fibrosis in Dupuytren's contracture localize in close proximity to blood vessels, which form a supportive niche for sustained cell proliferation.
7. The bFGF, IGF-2, and CTGF that are capable of activating AKT signaling potentially synergize in creating a favorable microenvironment for DC progression.
8. Small molecule compound NSC156529 is a new inhibitor of AKT1-PDPK1 interaction and a new potential anti-tumor agent. NSC156529 reduces the activity of AKT signaling pathway, and growth of tumor cells in *in vitro* and *in vivo*.

SUMMARY IN ESTONIAN

Terve ja kahjustatud maksa regeneratsioonis ning Dupuytren'i kontraktuuri progressioonis osalevate rakuliste ja molekulaarsete mehhanismide uurimine

Maks on imetaja organismi suurim siseelund, millel on rohkem kui 500 erinevat funktsiooni. Maksa peamiste ülesannete hulka kuuluvad näiteks toksiliste ühendite kahjutustamine, toitainete lagundamine, sapi tootmine seedimise hõlbustamiseks, erinevate valkude sünteesimine ja metabolismiga seotud signaaliradade reguleerimine. Maksa tähtsus organismi normaalses talitluses tuleb kõige paremini esile maksatsirroosi (maksaparenhüüm on asendunud fibroosse armkoega) ja maksapuudulikkusega inimeste puhul, kelle maks ei suuda enam vajalikke protsesse läbi viia ning tihtipeale on nende ainsaks ravivõimaluseks maksasiirdamine. Lisaks maksa funktsioonide kadumisele tekivad maksatsirroosiga patsientidel sageli pahaloomulised maksakasvajad, mille esinemissagedus järjest kasvab. Kuna maksa siirdamine ei ole alati võimalik, on oluline välja töötada uusi alternatiivseid ravimeetodeid, mis takistaksid haiguse progresseerumist või pööraksid patoloogilised protsessid ümber. Võimalike ravisihtmärkide leidmiseks on vaja teada, millised rakud ja signaalirajad haiguse arengus osalevad.

Normaalsetes tingimustes on maksal väga hea regeneratsioonivõime, kuid järjepidev kahjustus kurnab maksa taastumise potentsiaali, mille tagajärjel tekivad häired selle organi töös. Enamiku krooniliste maksahaiguste peamisteks tunnusteks on eellasrakupopulatsioonide ilmumine maksakoesse ning rakuvälise maatriksi ümberkorraldumine ja sünteesi suurenemine (fibrootilise koe teke). Nende tunnuste esinemise tase on positiivses korrelatsioonis fibroosi astmega ning tihtipeale esinevad eellasraku omadustega rakud ka maksakasvajates. Eellasrakud võivad diferentseeruda nii hepatotsüütideks kui sapijuharakkudeks, kuid nende päritolu on praegusel hetkel veel lahtine.

Käesoleva töö üheks eesmärgiks oli välja selgitada, kas maksas esineb aeglaselt jagunevaid rakke ning kuidas need rakud osalevad maksa alalhoius ja taastumises. Kuna aeglane jagunemine on paljudele täiskasvanud organismi tüvirakkudele omane tunnus, otsustasime uurida selliste rakkude olemasolu maksas, kasutades transgeenset hiireliini, mis võimaldab detekteerida tuuma märke säilimist vähe jagunevates rakkudes. Leidsime, et hiire maksas paiknesid aeglaselt jagunevad rakud sapijuhades ning nad omasid diferentseerunud sapijuharaku tunnuseid. Need rakud ei osalenud maksa kahjustusejärgses taastumises, kui regeneratsiooniks oli peamiselt vajalik hepatotsüütide jagunemine. Samas sapijuharakkude kahjustuse korral märkega rakud aktiveerusid ja kaotasid jagunemise tagajärjel oma tuuma märgistuse ning samuti ilmusid maksakoesse eellasrakud. Sellest järeldasime, et maksa aeglaselt jagunev rakupopulatsioon osaleb sapijuharakkude, aga mitte hepatotsüütide regeneratsioonis. *In vitro* katsed hiirest eraldatud rakkudega näitasid, et kuigi aeglaselt jagunevate

sapijuharakkude eneseuuendamise võime oli parem kui sama hiire normaalse jagunemiskiirusega sapijuharakkudel, ei olnud nad võimelised hepatotsüütideks diferentseeruma. Sellest järeldasime, et täiskasvanud hiire maksa aeglaselt jagunevad rakud on omavad kõrgenenud jagunemise potentsiaali, kuid oma diferentseerumise võime poolest on nad unipotentsed.

Kuna maksahaigusi iseloomustab rakuvälise maatriksi ületootmine, uurisime proteoomi analüüsi abil kahjustusejärgseid muutusi maksa rakuvälises maatriksis. Leidsime, et lühiajaline CCl_4 manustamine (akuutne hepatotsüütide kahjustus) ja pikaajaline DDC manustamine (krooniline sapijuharakkude kahjustus) põhjustasid maatriksis erinevaid muutusi. Samuti avastasime, et erinevatel maatriksi komponentidel on hepatotsüütide ja sapijuharakkude proliferatsioonile erinev mõju.

Fibroosi uurimiseks kasutasime Dupuytren'i kontraktuuri patsientidelt eemaldatud fibroosse koe preparaate. Kuigi Dupuytren'i kontraktuur ei ole maksaga seotud haigus, on enamike fibrootiliste haiguste histoloogiline pilt ja peamised signaalirajad sarnased. Nägime, et vastupidiselt normaalsele koele sisaldas haige kude väga palju väikeseid veresooni, mille sees või läheduses paiknesid fibroosi põhjustavad prolifereeruvad müofibroblastid. Samuti avastasime, et haige koe erinevad komponendid sünteesivad erinevaid fibroosi ja proliferatsiooni soodustavaid molekule, moodustades haiguse arenguks sobiliku keskonna. Lisaks leidsime, et Dupuytren'i kontraktuuri progressioonis võib ka higinäärmetel tähtis roll olla.

Töö viimases osas keskendusime AKT1-PDPK1 interaktsiooni inhibiitori väljaselgitamisele. AKT signaalirada on ebanormaalselt aktiivne paljudes pahaloomulistes kasvajates, sealhulgas maksakasvajates. Kuna PDPK1 on üks AKT1 aktivaatoritest, siis eeldasime, et nende omavahelise interaktsiooni lõhkumine võiks vähendada aktiivse AKT valgu taset. 2000 analüüsitud ühendi seast leidsime ühe väikesemolekulaarse ühendi (NSC156529), mis inhibeeris AKT1-PDPK1 interaktsiooni, vähendas aktiivse AKT valgu ja tema sihtmärkvalkude taset ning pidurdas rakkude kasvu *in vitro* ja tuumori kasvu *in vivo*. Meie tulemused näitasid, et NSC156529 on ühend, millel võiks olla potentsiaali hüperaktiivse AKT signaalirajaga kasvajate-vastases ravis.

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PUBLICATIONS

CURRICULUM VITAE

Name: Janeli Viil
Date of birth: August 3,1983
Address: Department of Cell Biology,
Institute of Molecular and Cell Biology,
University of Tartu, Riia Street 23, 51010, Tartu, Estonia
E-mail: Janeli.Viil@ut.ee

Education:

1990–2002 Haapsalu Gymnasium, silver medal
2002–2005 University of Tartu, BSc studies in Natural Sciences, (Gene Technology), *cum laude*
2005–2007 University of Tartu, MSc studies in Natural Sciences, (Gene Technology)
2008–2016 University of Tartu, Phd studies in Molecular and Cell Biology

Professional employment:

2007–2015 Lab technician, Department of Cell Biology, University of Tartu
2010–2015 Lab technician, Competence Center for Cancer Research

Scientific work:

- Main research is focused on studying the role of progenitor cells and extracellular matrix in liver regeneration.
- Supervised two BSc and two MSc dissertations.
- Teaching “Practical Course in Cell Biology” (LTMR06)

List of publications:

1. Kadaja-Saarepuu L., Laos S., Jääger K., **Viil J**., Balikova A., Lõoke M., Hansson G.C., and Maimets T. CD43 promotes cell growth and helps to evade FAS-mediated apoptosis in non-hematopoietic cancer cells lacking the tumor suppressors p53 or ARF. Oncogene. (2008), 27(12): 1705–1715.
2. Balikova A., Jääger K., **Viil J**., Maimets T., and Kadaja-Saarepuu, L. Leukocyte marker CD43 promotes cell growth in co-operation with β-catenin in non-hematopoietic cancer cells. Int J Oncol. (2012), 41(1): 299–309.
3. **Viil J**., Maasalu K., Mäemets-Allas K., Tamming L., Lõhmussaar K., Tooming M., Ingerpuu S., Märtson A., and Jaks V. Laminin-rich blood vessels display activated growth factor signaling and act as the proliferation centers in Dupuytren’s contracture. Arthritis Res Ther. (2015), 17:144
4. Mäemets-Allas K., **Viil J**., and Jaks V. A Novel Inhibitor of AKT1-PDPK1 Interaction Efficiently Suppresses the Activity of AKT Pathway and Restricts Tumor Growth *In Vivo*. Mol Cancer Ther. (2015), 14(11):2486–96.
5. Klaas M., Kangur T., **Viil J**., Mäemets-Allas K., Minajeva A., Vadi K., Antsov M., Lapidus N., Järvekülg M., and Jaks V. The alterations in the extra-

cellular matrix composition guide the repair of damaged liver tissue. Sci. Rep. (2016), 6, 27398; doi: 10.1038/srep27398

6. Urgard E., Lorents A., Klaas M., Padari K., **Viil J**., Runnel T., Langel K., Kingo K., Tkaczyk E., Langel Ü., Maimets T., Jaks V., Pooga M., and Rebane A. Pre-administration of PepFect6-microRNA-146a nanocomplexes inhibits inflammatory responses in keratinocytes and in a mouse model of irritant contact dermatitis. Journal of Controlled Release (2016), doi: 10.1016/j.jconrel.2016.06.006.

ELULOOKIRJELDUS

Nimi: Janeli Viil
Sünniaeg: 3. august, 1983
Aadress: Rakubioloogia õppetool, Molekulaar-ja Rakubioloogia Instituut, Tartu Ülikool, 51010, Tartu, Eesti
E-mail: Janeli.Viil@ut.ee

Haridus:
1990–2002 Haapsalu Gümnaasium, hõbemedal
2002–2005 Tartu Ülikool, bakaluareuseõpe loodusteadustes, (Geenitehnoloogia), *cum laude*
2005–2007 Tartu Ülikool, magistriõpe loodusteadustes, (Geenitehnoloogia)
2008–2016 Tartu Ülikool, doktoriõpe Molekulaar- ja rakubioloogias

Erialane teenistuskäik:
2007–2015 Laborant, Rakubioloogia õppetool, Tartu Ülikool
2010–2015 Laborant, Vähiuuringute Tehnoloogia Arenduskeskus

Teadustegevus:
– Peamiseks uurimisteemaks on eellasrakkude ja rakuvälise maatriksi rolli väljaselgitamine maksa regeneratsioonis.
– Kahe bakalaureusetöö ja kahe magistritöö juhendaja või kaasjuhendaja.
– Bakalaureuseõppe aine "Rakubioloogia praktikum" juhendamine (LTMR06).

Teaduspublikatsioonid:
1. Kadaja-Saarepuu L., Laos S., Jääger K., **Viil J**., Balikova A., Lõoke M., Hansson G.C., and Maimets T. CD43 promotes cell growth and helps to evade FAS-mediated apoptosis in non-hematopoietic cancer cells lacking the tumor suppressors p53 or ARF. Oncogene. (2008), 27(12): 1705–1715.
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