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# Endothelial regulation of liver homeostasis and regeneration

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"Endothelial GATA4 controls liver fibrosis and regeneration by preventing a pathogenic switch in angiocrine signaling"

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# List of abbreviations

Acta2	actin alpha 2
ALT	alanine aminotransferase
Apln	apelin
αSMA	α-smooth muscle actin
AST	aspartate aminotransferase
ATAC-Seq	assay for transposase accessible chromatin sequencing
Axin2	axis inhibition protein 2
BMDLP	bone marrow derived LSEC progenitors
Bmp	bone morphogenetic protein
bp	base pairs
CaCl <sub>2</sub>	calcium chloride
c-CASP-3	cleaved Caspase 3
CCI4	carbon tetrachloride
CD	cluster of differentiation
CDAA	choline-deficient, I-amino acid-defined
cdh5	cadherin 5
cDNA	complementary DNA
CEC	continuous endothelial cell
ChIP	chromatin immunoprecipitation
ChIP-Seq	chromatin immunoprecipitation sequencing
CIMPR	cation-independent mannose 6-phosphate receptor
c-MAF	c-musculoaponeurotic fibrosarcoma oncogene homolog
Col15a1	collagen type XV alpha 1 chain
Col1a1	collagen type I alpha 1 chain
Col3a1	collagen type III alpha 1 chain
Col4a1	collagen type IV alpha 1 chain
Col4a2	collagen type IV alpha 2 chain
CP	crossing point
Cre	cyclization recombination
Ctrl	control
DAPI	4',6-diamidino-2-phenylindole
Des	desmin
dH <sub>2</sub> O	distilled water
ddH <sub>2</sub> O	double distilled water
DNA	deoxyribonucleic acid

dNTP	nucleoside triphosphate
DTT	1,4-Dithiothreitol
E	embryonic day
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EJ	exon junction
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
EndMT	endothelial-to-mesenchymal transition
eNOS	endothelial nitric oxide synthase
ERG	ETS-related gene
Esm1	endothelial cell specific molecule 1
et al.	et alia (and others)
FABP1	fatty acid binding protein 1
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
fl	floxed
FOG2	friend of GATA 2
fw	forward
Gak	cyclin G associated kinase
GATA4	GATA binding protein 4
GLDH	glutamate dehydrogenase
GSEA	gene set enrichment analysis
H&E	hematoxylin and eosin
HET	heterozygous
HGF	hepatocyte growth factor
HIER	heat induced epitope retrieval
HNF	hepatic nuclear factor
HRP	horseradish peroxidase

hrs	hours
HSC	hepatic stellate cell
IF	immunofluorescence
lgfbp5	insulin like growth factor binding protein 5
IHC	immunohistochemistry
IL	Interleukin
ISH	in situ hybridization
kb	kilo base
КО	knock out
Lamb1	laminin subunit beta 1
Lamc1	laminin subunit gamma 1
LMEC	lung microvascular endothelia cells
LoxP	locus of X-over P1
LSEC	liver sinusoidal endothelial cell
LYVE-1	lymphatic vessel endothelial hyaluronan receptor
MACS	magnetic activated cell sorting
MgCl <sub>2</sub>	magnesium chloride
mm2	square millimeter
mRNA	messenger RNA
Mrpl46	mitochondrial ribosomal protein L46
MSigDB	molecular signatures database
NAFLD	Non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
neg	negative
NO	nitric oxide
ORO	oil red O
Р	postnatal
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdgfb	platelet derived growth factor subunit B
Pdgfrb	platelet derived growth factor receptor beta
PFA	paraformaldehyde
PHx	partial hepatectomy
PIIINP	procollagen type III N-terminal propeptide
pos	positive
PRR	pattern recognition receptor

PSR	picrosirius red
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative Real-time polymerase chain reaction
rev	reverse
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
ROS	reactive oxygen species
Rspo3	R-spondin 3
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sema7a	semaphorin 7a
Smad	mothers against decapentaplegic
SNP	single nucleotide polymorphism
Sparcl1	secreted protein acidic and rich in cysteine -like protein 1
SPL	Smart Protein Layers
Srp72	signal recognition particle 72
SSC	saline sodium citrate
Stab2	stabilin 2
STM	septum transversum mesenchyme
TAE	tris-acetate EDTA
TEM	transmission electron microscopy
tg	transgene
TGF	transforming growth factor
TGS	tris glycine SDS
TNF	tumor necrosis factor
TSS	transcription start sites
U	units
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
Wnt2	Wnt family member 2
Wnt9b	Wnt family member 9B
wt	wild type

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## Abstract

Endothelial cells (EC) display heterogeneity in morphology and function to meet the specific requirements of the respective organ. EC are no passive cell layer, but rather control development, homeostasis, and regeneration by interacting with their microenvironment via angiocrine factors. A prime example of angiodiversity are the discontinuous and fenestrated capillaries of the livers, referred to as liver sinusoidal EC (LSEC). LSEC are characterized by fenestrations without diaphragm, lack of a basement membrane and expression of various scavenger receptors. They regulate functions such as liver growth, zonation, and regeneration by secreting specific angiokines including WNT2 and HGF. Recently, transcription factor GATA4 was identified as a master regulator of hepatic endothelial differentiation during development. The early embryonic Gata4 deletion in Stab2 positive LSEC causes capillarization of the endothelium, hypoplasia and fibrosis resulting in fetal anemia and late embryonic lethality. In order to investigate the function of Gata4 *in vivo* in the adult liver, Clec4g Cre deleter mice were used to generate adult Gata4<sup>LSEC-KO</sup> mice since analyses of Clec4gCre;Rosa26-YFP reporter mouse showed late embryonic (E17.5) Cre activity in CD31+ EC.

Gata4<sup>LSEC-KO</sup> mice were born at the expected mendelian ratio and were viable. However, Gata4<sup>LSEC-KO</sup> livers showed macroscopic irregularities, were significantly smaller as well as serum values indicated liver damage and metabolic alterations. Adult endothelial Gata4 deletion led to perisinusoidal liver fibrosis and increased collagen deposition. Gene expression profiling revealed 403 genes significantly dysregulated in LSEC of Gata4<sup>LSEC-KO</sup> mice and a LSEC-to-continuous endothelial transdifferentiation. Furthermore, capillarization of liver sinusoids was accompanied by the formation of a continuous basement membrane. LSEC restricted Gata4 deficiency caused impaired angiocrine signaling resulting in impaired metabolic zonation of the liver. In addition, profibrotic angiocrine factors were upregulated due to Gata4 deletion including stellate cell-activating mitogenic factor Pdgfb. For the first time LSEC were identified as main source of *Pdgfb* in Gata4<sup>LSEC-KO</sup> livers by duplex ISH and *Pdgfb* expression was amplified by activated MYC. Moreover, ATAC-Sequencing of Gata4<sup>LSEC-KO</sup> LSEC revealed that GATA4 mediated its repressive function by regulating chromatin accessibility. Analyses of regeneration after partial hepatectomy (PHx) revealed an impaired capability of regenerative hypertrophy caused by missing Wnt2 induction in the early phase of regeneration in Gata4<sup>LSEC-KO</sup> livers. In addition, liver regeneration following a single CCl4 injection was also impaired in Gata4<sup>LSEC-KO</sup> mice due to a delayed uptake and/or metabolization of CCI4.

These data indicate that GATA4 acts as a master regulator of sinusoidal endothelial differentiation in the adult liver, as endothelial deletion of Gata4 results in LSEC capillarization,

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profibrotic angiocrine signaling responsible for development of perisinusoidal liver fibrosis, and deficiency in the regenerative capacity of the liver.

## Zusammenfassung

Endothelzellen (EC) weisen eine Heterogenität in Morphologie und Funktion auf, um die spezifischen Anforderungen des jeweiligen Organs zu erfüllen. Sie sind keine passive Zellschicht, sondern steuern die Entwicklung, Homöostase und Regeneration, indem sie über angiokrine Faktoren mit ihrer Mikroumgebung interagieren. Ein Paradebeispiel für die Angiodiversität sind die diskontinuierlichen Kapillaren der Leber, die als sinusoidale EC der Leber (LSEC) bezeichnet werden. Charakteristika der LSEC sind ihre Fenestrationen ohne Diaphragma, das Fehlen einer Basalmembran und die Expression verschiedener Scavenger-Rezeptoren. Sie regulieren Funktionen wie das Leberwachstum, die Zonierung und die Regeneration, indem sie spezifische Angiokine wie WNT2 und HGF sezernieren. Kürzlich wurde der Transkriptionsfaktor GATA4 als Hauptregulator der hepatischen endothelialen Differenzierung während der Entwicklung identifiziert. Die frühe embryonale Deletion von Gata4 in Stab2-positiven LSEC verursacht eine Kapillarisierung des Endothels, Hypoplasie und Fibrose, was zu fetaler Anämie und später embryonaler Sterblichkeit führt. Um die Funktion von Gata4 in vivo in der adulten Leber zu untersuchen, wurden Clec4q-Cre-Deletionsmäuse verwendet, um adulte Gata4<sup>LSEC-KO</sup> Mäuse zu erzeugen, da Analysen der Clec4gCre;Rosa26-YFP-Reportermaus eine spätembryonale (E17.5) Cre-Aktivität in CD31+ EC zeigten.

Gata4<sup>LSEC-KO</sup> Mäuse wurden im erwarteten Mendelschen Verhältnis geboren und waren lebensfähig. Die Gata4<sup>LSEC-KO</sup> Lebern zeigten jedoch makroskopische Unregelmäßigkeiten, waren signifikant kleiner und auch die Serumwerte deuteten auf Leberschäden und Stoffwechselveränderungen hin. Die adulte endotheliale Gata4-Deletion führte zu einer perisinusoidalen Leberfibrose und einer erhöhten Kollagenablagerung. Die Erstellung von Genexpressionsprofilen ergab, dass 403 Gene in LSEC von Gata4<sup>LSEC-KO</sup> Mäusen signifikant dysreguliert sind und eine Transdifferenzierung von LSEC zu kontinuierlichem Endothel stattfindet. Desweitern wurde die Kapillarisierung der Lebersinusoide von der Bildung einer kontinuierlichen Basalmembrane begleitet. Ein Gata4 Mangel in LSEC führte zu einer Beeinträchtigung der angiokrinen Signalübertragung und somit zu einer gestörten metabolischen Zonierung der Leber.

Darüber hinaus wurden durch die Gata4-Deletion profibrotische angiokrine Faktoren hochreguliert, darunter der Sternzellen-aktivierende mitogene Faktor *Pdgfb*. Zum ersten Mal wurden LSEC als Hauptquelle von *Pdgfb* in Gata4<sup>LSEC-KO</sup> Lebern durch Duplex-ISH identifiziert und die *Pdgfb* Expression wurde durch aktiviertes MYC verstärkt. Darüber hinaus ergab die ATAC-Sequenzierung von Gata4<sup>LSEC-KO</sup> LSEC, dass GATA4 seine repressive Funktion durch Regulierung der Chromatin-Zugänglichkeit vermittelt. Analysen der Regeneration nach partieller Hepatektomie (PHx) ergaben eine beeinträchtigte Fähigkeit zur regenerativen Hypertrophie, die durch fehlende Wnt2-Induktion in der frühen Phase der Regeneration in

Gata4<sup>LSEC-KO</sup> Lebern verursacht wird. Darüber hinaus war die Leberregeneration nach einer einzelnen CCl4-Injektion in Gata4<sup>LSEC-KO</sup> Mäusen aufgrund einer verzögerten Aufnahme und/oder Metabolisierung von CCl4 ebenfalls beeinträchtigt.

Diese Daten deuten darauf hin, dass GATA4 als Hauptregulator der sinusoidalen Endothelialdifferenzierung in der adulten Leber fungiert, da die endotheliale Deletion von Gata4 zu einer Kapillarisierung von LSEC, einer fibrotischen angiokrinen Signalübertragung, die für die Entwicklung einer perisinusoidalen Leberfibrose verantwortlich ist, sowie zu einer Beeinträchtigung der Regenerationsfährigkeit der Leber führt.

## 1 Introduction

## 1.1 The blood vascular system

The two major circulatory systems in our body are the blood and lymphatic systems, which consist of vessels transporting blood and lymph through the body. The blood vascular system is used for the transport (e.g. nutrients, oxygen) and removal (e.g. carbon dioxide, waste products) of substances, homeostasis, hydraulic force generation, protection, ultrafiltration, thermoregulation and whole-body integration (Haber et al., 1995; Monahan-Earley et al., 2013). Blood vessels can be divided into arteries, arterioles, capillaries, venules and veins. Arteries and arterioles carry oxygen-rich blood and nutrients away from the heart and are connected with the capillaries. Through the walls of the capillaries, gases and nutrients as well as waste products are exchanged between blood and tissue. Deoxygenated blood is then transported through venules and veins back to the lungs for re-oxygenation.

Transcription factors involved in the developmental regulation of arterial, venous, and lymphatic differentiation have been identified in recent years. Notch signaling in blood vessels repress venous differentiation during embryonic vascular development and is therefore required for proper development of arteries (Lawson et al., 2001). Furthermore, You et al. revealed that during normal development, COUP-TII repressed Notch signaling and expression of arterial-specific genes in venous endothelium to maintain venous cell fate (You et al., 2005). In contrast, lymphatic differentiation is controlled by the homeobox gene PROX1 (Wigle and Oliver, 1999).

The morphology of these blood vessel types is heterogenous in different organs and show distinct organotypic characteristics reflecting their organ-specific functions. Furthermore, they also differ on hierarchical levels. Large blood vessels such as arteries and veins have the same basic structure consisting of three layers: Tunica intima, tunica media and tunica externa/tunica adventitia. The latter is the outer layer and consists of connective tissue anchoring the vessel in the environment. The tunica media – the middle layer – is composed of smooth muscle cells and elastic fibers and is thicker in arteries than in veins. It is the thickest layer and serves the stability and the regulation of the blood flow. The interior surface of blood vessels is lined by a cell monolayer – the endothelium – separating the lumen of the vessels from neighboring tissue. The tunica intima (inner layer) is formed from single-layered continuous EC with a basement membrane and is the thinnest layer. In contrast to the large blood vessels, capillaries only consist of an endothelial layer of cells with a varied distinct basement membrane. EC also differ in morphology and function along the vasculature to ensure that they are adapted to the specific requirements of different organs. This heterogeneity is referred to as "angiodiversity" and will be discussed in more detail in the next chapter.

In the embryogenesis mesodermal stem cells differentiate into hemangioblasts, which are precursors to both hematopoietic stem cells and angioblasts (endothelial progenitor cells). The embryonic vasculature forms by two fundamental processes: vasculogenesis and angiogenesis (Risau, 1997). Vasculogenesis involves the de novo formation of blood vessels from angioblasts in combination with the formation of a primitive vascular plexus. The migration of angioblasts to form the primary vascular network is controlled by a combination of regulatory mechanisms and factors (Schmidt et al., 2007). Vasculogenesis also includes the differentiation of angioblasts into EC. On the other hand, angiogenesis is defined as the growth of new capillaries from preexisting functional blood vessels. It occurs in utero and throughout life to guarantee a sufficient oxygen supply to all cells of the body. While some vascular beds result from a combination of vasculogenesis and angiogenesis, others arise exclusively through angiogenesis e.g. brain and retina (Potente and Mäkinen, 2017).

#### 1.1.1 Endothelial Heterogeneity

EC form the inner single cell layer called endothelium that lines the blood (and lymph) vessels. EC are highly divers in their morphology and function. In the 1950s and 1960s, electron microscopy studies revealed for the first time that EC have a structural heterogeneity (Florey, 1966). Thus, EC could be morphologically categorized into three types, which vary in terms of their permeability: 1. Continuous, non-fenestrated 2. Continuous, fenestrated and 3. Sinusoidal/Discontinuous (Figure 1) (Aird, 2007). It is assumed that endothelial heterogeneity is an evolutionary conserved core feature of the endothelium (Geraud et al., 2014; Monahan-Earley et al., 2013). This diversity enables EC to fulfill a wide variety of functions and is thereby optimally adapted to the needs of the respective vasculature of different tissues. Possible functions are for example control of vasomotor tone, scavenging and clearance, inflammatory process, hemostasis and angiogenesis. Another function is the regulation of permeability, in which EC differ greatly. The exchange of substances can be implemented either transcellularly or intercellularly through the endothelium. The heterogeneity in permeability may be explained by the presence or absence of transcellular pores (fenestrae), differential activity of transcytosis and/or differences in junctional properties (Aird, 2007). The EC are connected by two main types of cell-cell adhesion complexes: adherens junctions and tight junctions, which form a barrier controlling the paracellular transport.

#### Continuous, non-fenestrated endothelium

Barrier-forming, continuous, non-fenestrated endothelial cells are found in the vasculature of the brain (blood brain barrier), skin, heart, and lung (Aird, 2007). They all have in common that they have no fenestration and sit on a basement membrane, which major constituents are collagen IV and laminin (Timpl and Brown, 1996). Number and complexity of tight junctions in the continuous endothelium are high, wherefore the permeability is low. Water and small solutes can diffuse between EC, whereas larger molecules (e.g. glucose) pass through the EC monolayer via transcytosis. Transcytosis is mediated by transport proteins and caveolae, which are submicroscopic, flask-shaped invaginations of the plasma membrane. The number of caveolae is highest in the endothelium of the heart, lung, and skeletal muscle (Simionescu et al., 2002). In the central nervous system continuous endothelium is even more specialized: A large number of tight junctions enables a highly selective permeability that prevents the passage of most large molecules, drugs, and pathogens (Augustin and Koh, 2017). In the endothelium of the blood brain barrier caveolae are rare (Simionescu et al., 2002). Transcytosis is mediated by substrate-specific transporters that control transport of nutrients, energy metabolites, and other essential molecules (Zhao et al., 2015).



#### Figure 1: Three major types of EC.

(A) Continuous, non-fenestrated endothelial cells are present in most organs such as brain, skin and heart. The EC monolayer forms a barrier through which solutes are transported by controlled transcytosis. (B) Continuous, fenestrated capillaries are found in organs involved in filtration, secretion and increased transport processes. These EC have intracellular pores covered with a diaphragm to ensure a higher permeability. (C) Discontinuous or sinusoidal EC are found in the liver, spleen and bone marrow. Sinusoidal capillaries are characterized by larger intercellular gaps and an incomplete basement membrane. These EC possess also fenestrae without a diaphragm allowing a high permeability and extensive exchange of materials. EC, endothelial cell. Figure adapted from Augustin and Koh (Augustin and Koh, 2017). Reprinted with permission from AAAS.

#### Continuous, fenestrated endothelium

Continuous, fenestrated endothelium is especially found in endocrine and exocrine glands, kidney as well as gastric and intestinal mucosa. In these organs, a higher permeability of the endothelium is required to ensure increased transport (e.g. secretion) or filtration processes.

The fenestrated endothelium has similar to the continuous, non-fenestrated endothelium a basement membrane but relatively fewer caveolae. In addition, they possess fenestrae, 60-80 nm diameter transcellular pores, which participate in the trans-endothelial transport. The majority of fenestrae are provided with a non-membranous diaphragm across the opening. These diaphragms act as molecular filters and increase size selectivity of the endothelium (Aird, 2007). Water and small molecules (e.g. ions, sugars, small peptide hormones) can pass through the EC layer while the trans-endothelial transport of larger molecules is blocked (Stan et al., 2012).

#### Sinusoidal/ Discontinuous endothelium

Organs such as the liver, spleen, bone marrow, lymph nodes and several endocrine organs (e.g. pituitary gland) have a discontinuous, also referred to as sinusoidal, endothelium. Sinusoidal EC also possess fenestrations, which are larger in diameter and lack a diaphragm (Wisse, 1970). The underlying basement membrane is thinner and poorly formed and between the EC are gaps instead of pores. These properties enable a high permeability and extensive exchange even for large solutes such as plasma proteins (Augustin and Koh, 2017). As a prime example of endothelial heterogeneity, the liver sinusoidal endothelial cells (LSEC) are discussed in more detail in section 1.2.1.

The molecular, structural and functional differences of the EC reflect differences on the transcriptional and proteomic level. There are surprisingly few genes that are endothelial-specifically expressed throughout the vascular branch. One example is VE-cadherin (Cadherin 5 (Cdh5), CD144), a endothelial-restricted classical cadherin, which is the transmembrane component of endothelial adherens junctions (Lampugnani et al., 1992). In contrast, many more endothelial-specific genes demonstrate expression limited to partial quantities of EC (Aird, 2012). Furthermore, EC also differ temporally and spatially on their molecular level. Petzelbauer and colleagues were able to show in 1993 that E-selectin (ELAM-1, CD62) expression was induced by tumor necrosis factor (TNF) or IL-1 in dermal microvascular EC (Petzelbauer et al., 1993).

In the liver, only central vein EC and LSEC close to the central vein produce the Wnt ligands Wnt2 and Wnt9b, which are necessary to preserve metabolic zonation in the adult liver (Leibing et al., 2018; Preziosi et al., 2018; Wang et al., 2015).

The endothelial heterogeneity is thereby mediated by two forces – (epi-) genetics and environment. Because blood vessels are distributed throughout the body, EC are exposed to a variety of different biochemical (e.g. oxygen, cytokines) and biomechanical (e.g. shear stress) influences (Aird, 2007, 2012). EC are able to sense these environmental factors and respond. These various signal inputs lead to changes in posttranslational modification of

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proteins and/or induction of gene expression by transcription factors mediating the different EC phenotypes across the body (Aird, 2007). This explains why EC that are isolated from their native microenvironment and cultivated undergo phenotypic alterations (Geraud et al., 2010; Lacorre et al., 2004). On the other hand, Chi and colleagues demonstrated that the molecular distinction in transcriptional profiles between arterial and venous EC in culture is persistent and independent of environmental differences (Chi et al., 2003). These epigenetically fixed properties are mitotically stable and implicated mechanisms include deoxyribonucleic acid (DNA) methylation and histone modifications (Fish and Marsden, 2006).

#### 1.1.2 Cellular interactions in vascular niches

EC were perceived as passive barrier. However, it has been more and more recognized that EC establish an instructive tissue-specific vascular niche that actively control their microenvironment (Geraud et al., 2014; Rafii et al., 2016). They interact with neighboring parenchymal and stromal cells by deploying stimulatory and inhibitory growth factors, extracellular matrix molecules, transmembrane proteins and chemokines. These EC-derived, paracrine or juxtracrine growth factors are referred to as angiocrine factors (Butler et al., 2010). Therefore, EC play an important role in the maintenance of metabolism and homeostasis, organ development and in controlling tissue regeneration (Rafii et al., 2016). For instance, liver regeneration after partial hepatectomy is modulated by the angiocrine factors Wnt2 and hepatocyte growth factor (HGF), which are expressed by liver sinusoidal endothelium (Ding et al., 2010). Furthermore, the angiocrines MMP14 and epidermal growth factor (EGF)-like ligands are secreted by lung EC to promote alveolar regeneration (Ding et al., 2011). These two examples show that the EC within each organ express an unique combination of angiocrine factors to meet the requirements of that particular organ (Nolan et al., 2013).

As angiocrine factors are secreted to activate the proliferation of stem and progenitor cells after tissue injury, it is conceivable that this switch of the vascular niche have an instructive role in activating tumor-initiating cells, promoting tumor growth and metastasis (Butler et al., 2010; Nolan et al., 2013). However, a stable quiescent vasculature constitutes a niche that sustains tumor dormancy and tissue homeostasis (Ghajar et al., 2013; Nolan et al., 2013). Already in 1989 Paget advanced the "Seed and soil" hypothesis, which states that different primary tumors preferentially metastasize in certain organs – referred to as organotropic metastasis (Paget, 1989). For example, breast carcinomas have a preference to metastasize to bones, lungs, and brain. The now existing multitude evidence for this theory shows that the vascular niche exerts an important role. For instance, intercellular adhesion molecule (ICAM)-1 mediates liver metastasis in various cancers (Benedicto et al., 2017; Wohlfeil et al., 2019).

## 1.2 The hepatic vascular niche

The liver has a unique dual blood supply. Lipid droplet-rich blood coming from the portal vein and oxygen-rich blood from the hepatic artery converge into the hepatic sinusoids (Figure 2A). After flowing through the sinusoidal capillaries the blood exits via the central veins and finally drains into the vena cava inferior. This creates a gradient of oxygen and nutrients along the liver lobule leading to molecular and functional zonation of hepatocytes and LSEC.



Oxygen, Nutrients, Blood flow, Gluconeogenesis, Urea synthesis Beta oxidation, Size of fenestrations

#### Figure 2: Schematic representation of the liver sinusoid.

(A) Nutrient-rich blood from portal vein and oxygen-rich blood from hepatic artery flows through the hepatic sinusoids and drains into the central vein creating a gradient of nutrients and oxygen along the liver lobule. (B) The perisinusoidal space between the hepatocytes and the LSEC is referred to as space of Disse. Here HSC and microvilli of the hepatocytes are located. The resident macrophages of the liver are the Kupffer cells which can adhere to the LSEC. (C) The nutrient and oxygen gradient cause a zonation of hepatocytes and LSEC. Therefore, periportal LSEC possess larger fenestrae, while pericentral LSEC have smaller but more fenestrae. Latter secrete angiocrine Wnt signals which determine zonation of the hepatocytes leading to differential metabolic gene expression. HSC, hepatic stellate cell; LSEC, liver sinusoidal endothelial cell. Figure adapted from Koch et al. (Koch et al., 2021). (CC BY 4.0) https://creativecommons.org/licenses/by/4.0/

Based on this zonation the liver lobules can be subdivided into three distinct areas: periportal, midlobular and pericentral. In these various sections, LSEC secrete different angiocrines, possess diverse gene expression and also reveal structural differences, which are discussed in more detail in section 1.2.1. For example, periportal LSEC are CD36 high and express no CD32 and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), whereas CD32 is expressed brightly on midlobular LSEC and near the central vein (Strauss et al., 2017). Therefore, the liver vasculature is a prime example of organ-specific endothelial heterogeneity. Recent work showed that pericentral LSEC and central vein EC orchestrate liver zonation and self-renewal of hepatocytes by secreting Wnt morphogenes (Wnt2 and Wnt9b) as well as the amplifier of Wnt signaling R-spondin 3 (Rspo3) (Carmon et al., 2011; Halpern et al., 2017; Rocha et al., 2015; Wang et al., 2015). Hepatocytes of each zone possess differential metabolic gene expression and functionality as well (Trefts et al., 2017). Hepatocytes in the highly oxygenated periportal area express high levels of enzymes involved in gluconeogenesis, ureagenesis and beta oxidation, while pericentral hepatocytes take on tasks such as glycolysis, xenobiotic metabolism and bile synthesis (Figure 2C). Overall, more than 50 % of liver genes are significantly zonated (Halpern et al., 2017). Hepatocytes, the liver parenchymal cells, make up the largest part of liver volume. They possess microvilli, which protrude into the narrow region between LSEC and hepatocytes. This space is referred to as space of Disse. HSC/lto-cells, which are the pericytes of the sinusoids, reside here. HSC can occur in a quiescent or activated state. Quiescent stellate cells contain large lipid vacuoles, where vitamin A is stored. Liver damage leads to their activation causing the production and deposition of collagen, wherefor they play a key role in the initiation, progression, and regression of liver fibrosis (Zhang et al., 2016). Besides the hepatocytes, LSEC and HSC, Kupffer cells are one of the four major cell populations in the liver (Figure 2B). The Kupffer cells are the resident macrophages of the liver and play an important role in the innate immune response. They are located within the sinusoids at the endothelial surface, allowing them to efficiently phagocytize pathogens (Dixon et al., 2013).

#### **1.2.1 Morphology of liver sinusoidal endothelial cells**

LSEC are highly specialized discontinuous EC lacking a subendothelial basement membrane. The cytoplasm of LSEC contains numerous fenestrae, whose diameter and number differ depending on species and age (Le Couteur et al., 2008; Snoeys et al., 2007; Wisse et al., 1985). Fenestrations lack a diaphragm and are arranged in ordered linear arrays within large planar clusters, called sieve plates (Stan, 2007). In mammals, LSEC are the only EC with non-diaphragmed fenestrae without an underlying basal lamina (Sørensen et al., 2015). LSEC demonstrate a varying degree of fenestration depending on their localization within the liver.

Fenestrae are larger but the number of fenestrae per cell is smaller in the periportal region, while pericentral LSEC possess smaller but more fenestrations, allowing increased exchange of oxygen as the partial oxygen pressure drops across the lobule (Sørensen et al., 2015; Wisse et al., 1985). Fenestrae in LSEC are dynamic structures, which can change their diameters in response to the cellular environment (e.g. hormones, drugs or diseases) (Braet and Wisse, 2002).

LSEC can be regarded as selective sieve: soluble molecules, virus particles, lipoproteins and other nanoparticles with a diameter below that of the fenestrae can pass from the blood to the space of Disse, whereas larger particles (e.g. blood cells) cannot enter (Snoeys et al., 2007). This enables a direct interaction between blood cells (e.g. lymphocytes) and the microvilli of hepatocytes or stellate cells. On the other hand, LSEC exert important scavenger functions by clearing soluble macromolecular waste products from the blood circulation and have a high endocytic ability. Therefore, LSEC are well equipped with a strong lysosomal activity to enable the removal of waste products. In the early 1980s, it was described for the first time that LSEC play an important role in clearance of hyaluronan (Fraser et al., 1981). The clearance function is executed by different scavenger and other endocytosis receptors. Since 2017, scavenger receptors are categorized in 11 different classes (A to L) depending on structure and function (PrabhuDas et al., 2017). LSEC express for example scavenger receptor A (SR-A), SR-B1 and SR-B2/CD36, mannose receptor/CD206/SR-E3, stabilin 1 and 2 (STAB1/SR-H1 and STAB2/SR-H2), CD32b/FcyRII as well as LYVE-1 (Malovic et al., 2007; Politz et al., 2002; Prevo et al., 2001; Stegner et al., 2016). STAB1/2 are considered the primary scavenger receptors of LSEC, which mediate binding, uptake and metabolisms of a variety of ligands (e.g. hyaluronan) (McCourt et al., 1999; Pandey et al., 2020). Mice lacking both STAB1 and STAB2 exhibited premature mortality due to severe glomerular fibrosis and albuminuria, while they developed only a mild perisinusoidal liver fibrosis (Schledzewski et al., 2011). Thus, STAB1 and STAB2 are essential for the proper clearance of noxious molecules and preservation of the homeostasis of the liver and other distant organs. In human, hepatocarcinogenesis is accompanied by loss of expression of LSEC markers such as Stabilin-1 and -2, and the loss of latter was related to increased patient survival (Geraud et al., 2013).

In addition to their scavenger function, LSEC also possess effective immune functions. They detect microbial infection through various pattern recognition receptors (PRRs), serve as antigen presenting cells and provide with their surface a location for interaction with immune cells (Knolle and Wohlleber, 2016). As part of the innate immune system, these PRRs recognize pathogen associated or damage associated molecular patterns (Kawai and Akira, 2010). LSEC express various PRRs: first and foremost, the stabilins and the mannose

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receptor. Furthermore, they express various toll-like receptors and are able to produce cytokines such as IL-6 and TNF $\alpha$  (Sørensen et al., 2015; Wu et al., 2010).

In the liver, gut-derived pathogens that need to be eliminated first encounter LSEC of the periportal area as the first line of host defense. In this context, portal EC eliminate antigens themselves by means of the above-mentioned PRRs, but at the same time also possess the ability to actively orchestrate the localization of immune cells. This asymmetric distribution of myeloid and lymphoid immune cells leads to an immune zonation which optimizes host defense of the liver (Gola et al., 2021). As part of adaptive immunity, T lymphocytes must be activated by antigen presentation, which are bound by major histocompatibility complex (MHC) receptors. LSEC express MHC I receptors to present antigens that are synthesized intracellularly (e.g. those of viral origin) to CD8+ T cells as well as MHC II receptors presenting extracellular antigens to activate CD4+ T helper cells (Burgdorf et al., 2007). Thus, LSEC with their immune function make an important contribution to immune surveillance in order to maintain liver homeostasis.

As previously mentioned in section 1.2.1, LSEC exhibit heterogeneity among themselves due to their zonation. Therefore, they also differ in the expression of surface markers. For example, Endomucin (EMCN) serves as a marker for the pericentral LSEC as well as EC of the central vein, whereas LYVE-1 shows the highest expression in midzonal LSEC (Mouta Carreira et al., 2001; Walter et al., 2014). Thus, no unique specific LSEC marker exists, apart from their fenestrae without diaphragm in the absence of basement membrane (Poisson et al., 2017). LSEC also play an important role in angiocrine signaling in the liver and thereby control liver function, development, metabolism and regeneration (Matsumoto et al., 2001; Rafii et al., 2016). Recent studies showed that LSEC regulate the iron metabolism not only in the liver but in the whole organism through the secretion of the angiocrines bone morphogenesis protein (BMP)2 and BMP6 (Koch et al., 2017; Latour et al., 2017). Iron homeostasis is thereby directly affected by regulation of hepatocytic hepcidin and the knockout of either angiocrine in LSEC causes hepatic iron overload and extrahepatic iron accumulation (Kostallari and Shah, 2016; Malovic et al., 2007). As briefly mentioned previously, LSEC-derived Wnt ligands and Wnt signaling enhancer Rspo3 control hepatocyte zonation (Leibing et al., 2018; Rocha et al., 2015). Furthermore, EC around the central vein maintain pericentral hepatocyte progenitor cells by secretion of Wnt ligands which have the ability of self-renewal as well as proliferation to replace hepatocytes along the liver lobule during homeostatic renewal (Wang et al., 2015). The LSEC specific deletion of Wnt secretion mediator wntless (WIs) cause reduced liver growth and an impaired liver zonation in mice, while intrahepatic EC zonation is not affected (Leibing et al., 2018). The role of LSEC-derived angiocrines in liver regeneration will be discussed in more detail in section 1.4.2.

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Under physiological conditions growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) can stimulate mature LSEC to proliferate (DeLeve, 2013; LeCouter et al., 2003). Furthermore, intrahepatic or resident LSEC progenitors and bone marrow derived LSEC progenitors (BMDLP) also contribute to LSEC renewal (DeLeve, 2013). Both express cell markers CD133 and CD31 as well as the hematopoietic cell marker CD45 (Wang et al., 2012). BMDLP do not participate in LSEC renewal under normal conditions, while under pathological conditions both BMDLP and resident LSEC progenitors can act as a potential source of new vessels depending on the vascular fitness (Singhal et al., 2018; Wang et al., 2012). Damaged resident LSEC have a restricted proliferative capacity resulting in recruitment and incorporation of BMDLP for repairing the liver vasculature (Singhal et al., 2018).

#### **1.2.2.** Molecular regulators of liver sinusoidal endothelial cells

EC emerge from mesodermal progenitor cells to form a primary vascular plexus. Subsequently, further phenotypic and functional specialization of the endothelium is necessary to fulfill the various arterial, venous, hemogenic, and lymphatic functions. The specialization of all endothelial cell types requires exogenous signals and regulatory events (Marcelo et al., 2013). To identify molecular factors mediating LSEC specific differentiation, Géraud and colleges used the fact that cultured LSEC rapidly lose their characteristic morphology as well as some of their specialized functions suggesting that environmental factors play an important role (Geraud et al., 2010). Therefore, they compared the molecular programs of freshly isolated LSEC, lung microvascular endothelial cells (LMEC), and short-term cultivated LSEC. LSEC-specific genes of several categories (e.g. transcriptional regulators, angiocrine factors, scavenger receptors) were identified including transcription factor GATA binding protein 4 (GATA4) (Geraud et al., 2010). Further studies showed that GATA4 acts as master regulator for organ specific EC differentiation in the liver (Geraud et al., 2017). LSEC-restricted deletion of GATA4 results in dedifferentiation of LSEC and transformation of discontinuous to continuous endothelium, a process referred to as capillarization (Geraud et al., 2010).

The endothelial transcription factor ETS-related gene (ERG) also plays a crucial role in maintaining LSEC specification. LSEC specific ablation of ERG also causes dedifferentiation of the liver endothelium, characterized by down-regulation of endothelial lineage identity markers and up-regulation of mesenchymal markers (Dufton et al., 2017). ERG regulates the canonical transforming growth factor (TGF) $\beta$ / mothers against decapentaplegic (SMAD) signaling and thereby prevents development of liver fibrosis and endothelial-to-mesenchymal

transition (EndMT). In addition, ERG expression is lost in liver endothelium of patients with fibrotic liver disease (Dufton et al., 2017).

The Notch signaling has also an crucial impact on LSEC homeostasis. Endothelial Notch activation causes decreased fenestration, increased basement membrane, as well as LSEC dedifferentiation (Duan et al., 2018). In addition, EC-specific Notch activation leads to a down-regulation of the angiocrines Wnt2a, Wnt9b and HGF causing a compromised hepatocyte proliferation under both quiescent and regenerating conditions (Duan et al., 2018).

Recently a triple transcription factor combination of c-musculoaponeurotic fibrosarcoma oncogene homolog (MAF), GATA4 and MEIS homeobox 2 (MEIS2) has been identified as important regulators of the unique LSEC fingerprint (de Haan et al., 2020). Their combination showed a synergistic effect on the increase of LSEC signature genes such as G protein-coupled receptor 182 (de Haan et al., 2020).

Furthermore, Gómez-Salinero et al. analyzed the *in vivo* relevance of c-musculoaponeurotic fibrosarcoma oncogene homolog (c-Maf) as a LSEC-specific transcription factor, which orchestrates the acquisition of sinusoidal characteristics during maturation of unspecified liver capillaries (Gómez-Salinero et al., 2022). Postnatal deletion of c-Maf expression in Cdh5+ LSEC impairs hepatic sinusoidal differentiation and promotes pathophysiological retention of liver hematopoiesis. In addition, postnatal loss of c-Maf in LSEC increases the fibrotic liver damage induced by repeated carbon tetrachloride (CCl4) administration in adult mice. c-Maf overexpression induced the activation of a sinusoidal transcriptional program and sinusoid phenotype in human ECs *in vitro* (Gómez-Salinero et al., 2022).

All this suggests that probably not only a single transcription factor determines the specific molecular fingerprint of LSEC including endothelial zonation. In arteries, it has been shown that it is the combination of 8 different factors, which in a complementary and overlapping way are crucial for the arterial fingerprint (Aranguren et al., 2013). It is therefore likely, that a combination of different organ-specific transcription factors is also required to achieve full LSEC specification.

#### 1.2.3 Liver sinusoidal endothelial cells in health and disease

Under normal conditions, differentiated LSEC actively control their microenvironment to maintain organ development, homeostasis and tissue regeneration in the hepatic vascular niche. Furthermore, healthy LSEC prevent HSC activation and maintain them quiescent (Deleve et al., 2008). In LSEC, endothelial nitric oxide synthase (eNOS) constantly produces small amounts of nitric oxide (NO), essential for controlling intrahepatic sinusoidal vascular tone and blood flow (Iwakiri and Kim, 2015). eNOS is thereby activated by stimuli such as

sinusoidal blood flow, shear stress and VEGF, derived from hepatocytes and HSC. The NO produced by LSEC keeps HSC in an inactive state and maintains fenestrae of LSEC through activation of soluble guanylyl cyclase (sGC) (Deleve et al., 2008; Marrone et al., 2013; Xie et al., 2012). In addition to the VEGF stimulated NO pathway, maintenance of LSEC differentiation also requires an NO-independent signaling (Xie et al., 2012).

Since LSEC are the cell population in the liver first exposed to toxic stimuli, it is not surprising that they play an important role in liver disease initiation and progression through their capillarization, angiogenesis, angiocrine signals and vasoconstriction (Poisson et al., 2017). In pathological conditions, eNOS activity is diminished and causes a reduced NO production in LSEC. Thus, the VEGF stimulated NO-dependent signaling is impaired leading to capillarization of LSEC. Capillarization is also called dedifferentiation and describes the loss of LSEC markers and fenestrations associated with basement membrane synthesis as well as the loss of functions. LSEC dedifferentiation precedes onset of fibrosis in alcoholic liver injury, nonalcoholic steatohepatitis or toxic liver injury (DeLeve, 2015; Deleve et al., 2008; Horn et al., 1987). Liver fibrosis is described as the excessive accumulation of extracellular matrix proteins including collagen and is characteristic of most types of chronic liver diseases (Bataller and Brenner, 2005). Advanced liver fibrosis can progress into cirrhosis, which is a risk factor for developing hepatocellular carcinoma, portal hypertension and liver failure and often requires liver transplantation (Dhar et al., 2020). However, studies have shown that fibrosis can regress after removal of the fibrotic trigger (e.g. alcohol, toxin, obesity) (Dixon et al., 2004; Parés et al., 1986).

Dedifferentiated LSEC permit HSC activation accompanied with extracellular matrix deposition and is therefore an early event in the disease progression. Activated HSC express a variety of markers, such as  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), tissue inhibitor of matrix metalloproteinase-1, type I collagen and increased F-actin stress fibres (Deleve et al., 2008). Co-culture experiments demonstrated that restoration of capillarized LSEC to the differentiated phenotype accelerates reversion of activated to quiescent HSC and induced some apoptosis of activated HSC (DeLeve, 2015; Xie et al., 2012). Since LSEC are able to maintain HSC quiescent as long as they are differentiated, differentiated LSEC are gatekeepers of fibrosis (Deleve et al., 2008; Poisson et al., 2017). Capillarized LSEC showed a reduced expression of scavenger receptor STAB1, STAB2, LYVE-1 as well as CD32b causing an elevation of circulating hyaluronic acid in cirrhotic livers (Geraud et al., 2013; Tamaki et al., 1996).

Ding and colleagues demonstrated that divergent LSEC-derived angiokines either stimulate regeneration after acute liver injury or provoke fibrosis after chronic injury (Ding et al., 2014). Thus, after acute liver damage, CXCR7 is upregulated in LSEC and cooperates with CXCR4

to induce pro-regenerative transcription factor Id1 pathway and to trigger angiocrine-mediated regeneration. In contrast, in chronic injury model, constitutive fibroblast growth factor receptor-1 (FGFR1) signaling in LSEC perturbates the CXCR7 pathway leading to a shift of pro-regenerative response to a CXCR4-dominanted pro-fibrotic angiocrine signaling (Ding et al., 2014).

Furthermore, under pathological conditions like fibrosis and cirrhosis angiogenesis and vasoconstriction are promoted. Here, angiogenesis is characterized by increased mural coverage of vessels by contractile HSC (Thabut and Shah, 2010). The dedifferentiation of LSEC is accompanied by a switch from vessel co-option/intussusceptive angiogenesis to sprouting angiogenesis (Frentzas et al., 2016; Geraud et al., 2013). The correlation between these two processes, fibrosis and angiogenesis, remains to be elucidated, but it is suggested that portal myofibroblasts may play a role both by stabilizing newly formed vessels and by providing a scaffold for collagen deposition (Lemoinne et al., 2016).

#### **1.3 Transcription factor GATA4**

The GATA family of transcription factors consists of six paralogs (GATA1 – GATA6), which can be divided into two subfamilies, GATA1/2/3 and GATA4/5/6, based on their spatial and temporal expression patterns as well as their similarity. They are involved in a variety of physiological and pathological processes and are essential for the development of tissue derived from all three germ layers. GATA transcription factors are evolutionarily conserved among animals, plants and fungi (Tremblay et al., 2018). GATA factors possess two zinc finger domains that bind to the consensus DNA binding sequence (A/T)GATA(A/G), known as the GATA motif after which they are named. The carboxyl-terminal finger binds to the consensus GATA recognition sequence, while the second zinc finger promotes the interaction between GATA and specific DNA sequences through stabilizing the association with zinc finger protein cofactors (Gao et al., 2015; Trainor et al., 1996). The zinc finger motifs are more than 70 % conserved among the 6 GATA proteins, whereas the sequences of the amino-terminal and carboxyl-terminal regions, which encode transcriptional activation domains, exhibit lower similarity (Lentjes et al., 2016; Morrisey et al., 1997).

Transcription factors bind to specific sequences of DNA called enhancer or promotor regions and modulate the transcription of genes. However, a unique class of transcription factors called pioneer factors are able to bind within closed and condensed chromatin, trigger chromatin opening and recruit additional transcription factors binding to these DNA sequences. GATA transcription factors can also act as pioneer factors. For example, GATA4 and hepatocyte nuclear factor 3 (HNF3) were the first two pioneer factors shown to open compact chromatin by binding to histone H3 and H4 (Cirillo et al., 2002). Moreover, GATA factors can act in concert to regulate distinct subsets of genes (Charron et al., 1999).

#### 1.3.1 Expression profile of GATA4

In 1993, transcription factor GATA4 was described for the first time by Arceci and colleagues. They identified GATA4 by screening embryonic day (E) 6.5 murine embryo library with oligonucleotide probes corresponding to the conserved region of the zinc finger domains and demonstrated GATA4 expression in the heart, primitive endoderm and gonads as well as lung, liver and small intestine by northern analysis and in situ hybridization (ISH) (Arceci et al., 1993). Thus, GATA4 is expressed early in the post-gastrula embryo and is an important regulator in the development of the endoderm and mesoderm (Arceci et al., 1993; Heikinheimo et al., 1994; Rossi et al., 2001). To obtain further information regarding GATA4, global GATA4 loss-offunction studies were performed. Kuo et al. generated a homozygous null mutation of the GATA4 gene in murine embryonic stem (ES) cells (Kuo et al., 1997). Homozygous GATA4 deficiency resulted in an embryonic lethality between E8.5 and E10.5 and mutant embryos displayed defects in ventral folding, which is needed for normal cardiac morphogenesis, and heart tube formation (Kuo et al., 1997). However, analysis of cardiac development showed that GATA4 was not required for specification of endocardial cell lineages or cardiac myocytes (Kuo et al., 1997). Molkentin and colleagues conducted a similar study, but instead of mice on C57BL/6 x CD1 mixed background used by Kuo et al. they used C57BL/6 x Sv129 mice (Kuo et al., 1997; Molkentin et al., 1997). Here, homozygous GATA4 null mice arrested in development between E7.0 and E9.5 and lacked a central heart tube and foregut due to failure in lateral and ventral folding in embryogenesis (Molkentin et al., 1997).

Rojas and colleagues identified that GATA4 expression is downstream of BMP4 in the lateral mesoderm and septum transversum (Rojas et al., 2005). Furthermore, their studies revealed GATA4 as direct transcriptional target of Forkhead and GATA transcription factors in the lateral mesoderm (Rojas et al., 2005).

In order to investigate the role of GATA4 in the early development of the mammalian liver and pancreas, Watt et al. examined *Gata4-/-* ES cell-derived embryos, which arrested at around E9.5 (Watt et al., 2007). These mice did not allow analyses of GATA4 contribution in later stages of hepatic and pancreatic organogenesis. However, differentiation of the ventral foregut endoderm to form the parenchymal components of liver and ventral pancreas starts around E8.0 and could thus be examined. Gata4-/- embryos revealed a complete absence of the ventral but not dorsal pancreas, while the liver bud failed to expand, although the hepatic

endoderm was able to form a pseudostratified epithelial liver bud, and these embryos lacked septum transversum mesenchyme (STM) (Watt et al., 2004; Watt et al., 2007). However, Watt and colleagues also revealed that Gata4 expression maintained in the STM while it was no longer detected in the delaminating hepatoblast at E9.0 (Watt et al., 2007). Therefore, they hypothesized that the absence of the STM due to Gata4 deficiency accounted for the missing expansion of the liver bud. To analyse this hypothesis, GATA4 was conditionally inactivated in the STM and its derivates in another study (Delgado et al., 2014). Here, GATA4 knockout caused embryonic lethality around E13.5 and liver hypoplasia, while size and morphology of the hearts remained unchanged. Loss of GATA4 impaired liver growth and led to fetal hematopoiesis. By 13.5 GATA4 knockout livers displayed a distinct increase of the extracellular matrix (ECM) components laminin and collagen type IV associated with HSC activation inducing a fibrogenic process (Delgado et al., 2014). Furthermore, haploinsufficiency of GATA4 promoted liver fibrosis in adult mice after a short-term CCl4 treatment.

GATA4 also functions in combination with other transcription factors. In the heart, GATA4 interacts with cardiogenic homeodomain factor Nkx2-5 to regulate gene expression of downstream genes critical for early cardio genesis (Durocher et al., 1997; Sepulveda et al., 1998). Also, disruption of the interaction between GATA4 and the T-box protein TBX5 caused cardiac septal defects in humans (Garg et al., 2003). Furthermore, Crispino and colleagues demonstrated that proper coronary vascular development and cardiac morphogenesis depended on the interaction of GATA4 with friend of GATA 2 (FOG2) cofactor (Crispino et al., 2001). Thus, replacement of a single amino acid in GATA4, which impaired interaction with FOG2, led to embryonic lethality around E12.5 (Crispino et al., 2001). In the intestinal epithelium and liver expression of the rat fatty acid binding protein 1 (FABP1) was regulated by the specific interaction of GATA4 and hepatic nuclear factor 1 (HNF1) (Divine et al., 2004).

In humans, congenital heart diseases (CHDs), caused by abnormal formation of the heart or major blood vessels during the cardio genesis, are the most common birth defects worldwide (Yu et al., 2018). It was proven that mutations of some cardiac transcription factors lead to CHDs (Granados-Riveron et al., 2012; Salazar et al., 2011; Wang et al., 2011). Since GATA4 is a master regulator required for ventral morphogenesis and heart tube formation, in CDHs over 100 GATA4 heterozygous mutations have been reported, whereby a distinction must be made between deleterious sides and neutral sides (Yu et al., 2018). Some deletions in the GATA4 locus cause more severe forms of CHDs in patients, including septation defects, outflow tract alignment defects, dextrocardia, and pulmonary stenosis (Hirayama-Yamada et al., 2005; McCulley and Black, 2012; Okubo et al., 2004; Pehlivan et al., 1999; Sarkozy et al., 2005). With regard to the liver, Delgado et al. revealed that Gata4 expression was

downregulated in human liver samples from patients with advanced liver fibrosis and cirrhosis (Delgado et al., 2014).

#### 1.3.2 The functional role of GATA4 in endothelial cells

Since GATA4 plays an essential role in heart and foregut formation and a global GATA4 knockout resulted in early embryonic lethality, no conclusion could be drawn regarding the function of GATA4 during further heart development or its later function in other organogenesis (Kuo et al., 1997; Molkentin et al., 1997; Watt et al., 2004). Therefore, mouse studies were performed in which GATA4 was inactivated only in specific cell types. Studies of the function of GATA4 in endothelial cells of heart and liver are highlighted in this next.

#### In the heart

In addition to its expression in the myocardium, the endocardium and the endocardial cushions exhibit robust GATA4 expression (Heikinheimo et al., 1994). To elucidate the function of GATA4 within the endocardium and its derivatives, Rivera-Felician and colleagues inactivated GATA4 in endothelium and endothelium-derived cushion mesenchyme using a Tie2cyclization recombination (Cre) transgenic mouse (Rivera-Feliciano et al., 2006). GATA4 expression was shown to be required in endothelial-derived cells for proper atrioventricular maturation as well as for epithelial-to-mesenchymal transition (EMT) (Rivera-Feliciano et al., 2006). Endocardial cells at the atrioventricular canal undergo EMT to form the endocardial cushions. After their growing and fusion, the endocardial cushions forms the atrioventricular complex that divides the inlet into a left and a right valve. Based on GATA4 activity in the endothelium and mesenchyme of the atrioventricular valves it was assumed that this transformation was influenced by GATA4 activity. Mutants displayed decreased Erbb3 expression associated with impaired Erk activation, both essential for EMT in the atrioventricular cushions (Rivera-Feliciano et al., 2006). Thus, in Gata4 mutant embryos endothelium failed to undergo EMT and generate atrioventricular cushion mesenchyme. Furthermore, abrogation of GATA4 interaction with FOG within endothelial-derived cells caused an unseptated ventricular inlet (Rivera-Feliciano et al., 2006). Thus, GATA4 was essential for proliferation and remodeling of the atrioventricular cushion mesenchyme after its formation by EMT. Endothelial GATA4 deficiency caused fetal lethality around E12.5 due to these cardiac defects (Rivera-Feliciano et al., 2006).

#### In the liver

The molecular program of rat LSEC revealed in comparison of that from rat LMEC a GATA4 overexpression (Geraud et al., 2010), making GATA4 a potential candidate for an LSEC specific transcription factor. To pursue this hypothesis, Géraud et al. generated and used
#### Introduction

Stab2-Cre deleter mice to conditionally and selectively inactivate GATA4 in LSEC during development while endocardial GATA4 expression remained intact (Geraud et al., 2017). Deletion of GATA4 in LSEC caused fetal lethality between E15.5 and E17.5 and impaired liver development. Furthermore, the LSEC restricted inactivation of GATA4 led to capillarization of LSEC around E10.5 characterized by subendothelial basement membrane deposition, switch from discontinuous LSEC to continuous EC and increased VE-cadherin expression indicating an increased stability of adherens junctions (Geraud et al., 2017). This switch caused liver fibrosis and perivascular ECM deposition associated with HSC activation. Moreover, capillarization in GATA4 knockout mice impaired immigration of hematopoietic progenitor cells into the liver resulting in severe anemia around E14.5.



Figure 3: Graphical illustration of liver sinusoid microvasculature in control and Stab2Cre;Gata4 knockout livers.

Left side: Under normal conditions, GATA4 promotes discontinuous endothelial differentiation of EC accompanied by the lack of a basement membrane, weak cell-cell contacts, and transmigration of hematopoietic stem and progenitor cells into the liver parenchyma. Middle: GATA4 deficiency in LSEC results in the dedifferentiation/capillarization of the endothelium causing basement membrane formation, ECM deposition, HSC activation, and increased stability of adherens junctions. The resulting impairment colonization of hematopoietic progenitor cells during liver development led to a fatal anemia. ECM, extracellular matrix; HSC, hepatic stellate cell. Figure adapted from Geraud et al. (Geraud et al., 2017). (CC BY 4.0) https://creativecommons.org/licenses/by/4.0/

In general, GATA4 was identified as master regulator for organ-specific EC differentiation in the fetal liver and acquisition of organ specific vascular competence, which are essential for liver development (Geraud et al., 2017).

### 1.4 Liver regeneration

The regenerative capacity of the liver already played a role in the Greek mythology of Prometheus. In 1931, Higgins and Anderson first documented a method to reproducibly investigate liver regeneration after two thirds partial hepatectomy (PHx) of the liver in rats (Higgins et al., 1931). Besides PHx, the liver can also regenerate after being damaged by toxins or infections. Therefore, two main animal models are used to investigate liver regeneration, including surgical PHx, and chemical-induced hepatotoxic injury (e.g. CCl4), which will be described in more detail in the next chapters.

However, this feature is not a regeneration in the biological sense, i.e. reconstitution of lost structure. Since the process does not follow the same general steps of the true regeneration such as the blastema formation containing dedifferentiated cells it rather describes the compensatory hypertrophy (increase in cell size mediated by enlargement) followed by hyperplasia (increase in cell number mediated by proliferation) of remaining hepatocytes (Abu Rmilah et al., 2019; Fausto et al., 2006). Thereby, the remaining liver expands to compensate for the lost tissue until full liver size is restored and the metabolic needs of the organism are met. Thus, "liver regeneration" is a process of compensatory liver hyperplasia rather than liver regeneration but it is termed as this in the scientific literature.

The introduction of the technique of living donor liver transplantation showed that the human liver also possesses the capacity of regeneration (Kawasaki et al., 1992). Thereby the process of regeneration in laboratory animals and humans is remarkably similar (Fausto, 2001): in the first seven days after liver resection the remaining liver tissue massively proliferates, even though in humans the initial preoperative liver weight was reached by 60 days after surgeries (Marcos et al., 2000). Furthermore, the liver is mainly responsible for detoxification and is exposed to many potentially harmful substances. Fibrosis is a wound-healing response of the liver to many chronic injuries, of which viral infection, alcohol and non-alcoholic steatohepatitis are the most common (Pellicoro et al., 2014). After removal of the cause, the liver has the possibility to regenerate from early fibrosis. On the other hand, advanced liver fibrosis results in cirrhosis and liver failure and often the only available treatment is liver transplantation, because hepatocytes develop an aberrant capacity of regeneration.

Liver regeneration is a complex process, which requires innumerable intercellular interactions between different cell types, such as hepatocytes, endothelial cells, biliary epithelial cells (cholangiocytes) as well as HSC. These interactions are tightly regulated by a network of different signaling pathways and cytokines, including HGF, EGF, TNF, Wnt/ $\beta$ -catenin and Notch signaling, VEGF and TGF $\beta$  (Ishikawa et al., 2021). The angiocrine regulation of liver regulation will be elucidate in chapter 1.4.2.

In many injury models preexisting hepatocytes become activated to proliferate and newly form hepatocytes during repair (Malato et al., 2011; Yanger et al., 2014). Zhao et al. showed that, after acute CCl4 damage, peri-injury hepatocytes, which express Wht target gene axis inhibition protein 2 (Axin2) proliferated to repopulate the injured parenchyma (Zhao et al., 2019). After PHx, nearly all remaining hepatocytes enter one or two cell cycles to restore the lost liver mass with minimal contribution of liver progenitor cells to acute hepatocytes advances as a wave from periportal to pericentral zones of the liver, whereas glutamine synthetase (GS) positive hepatocytes around the central veins are the last ones to replicate (Rabes, 1977). Several studies revealed that hepatic progenitor cells are then activated to differentiate into hepatocytes under circumstances of severe hepatocyte injury such as chronic viral hepatitis, alcoholic liver cirrhosis or non-alcoholic steatohepatitis (NASH) (Libbrecht et al., 2000; Machado et al., 2015; Ray et al., 1993). Thereby, these progenitor cells in the periportal region have a bipotential ability and can transform into both mature cholangiocytes and hepatocytes.

Singhal and colleagues investigated the source of newly formed vessels during liver regeneration. They demonstrated that both preexisting liver EC and bone marrow-derived endothelial progenitor cells can act as potential source depending on vascular fitness (Singhal et al., 2018). In case the hepatic vasculature was irreversibly damaged resulting in restricting proliferative capacity of resident EC, bone marrow-derived progenitor cells were recruited and incorporated (Singhal et al., 2018). In contrast, when the liver endothelium was intact, the reconstitution of liver vasculature relied on remaining intact EC without contribution of progenitor cells.

### 1.4.1 Liver regeneration models

Multiple methods can be used to induce liver regeneration. The most common animal models to investigate the regenerative capacity of the liver are PHx and chemical-induced hepatotoxic injury models, which will be highlighted in the next two chapters, or pharmacological models. Liver tissue must be damaged by a certain extent. Thus, it is described that a tissue loss of more than 9-12 % of the original liver volume is required to elicit a regenerative response (Yamanaka et al., 1993).

### 1.4.1.1 Surgical Partial hepatectomy (PHx) model

The surgical removal of 70 % of the liver, referred to as partial hepatectomy, was first described by Higgins and Anderson in rats and has the benefit of a good toleration and reproducibility (Higgins et al., 1931). Furthermore, the procedure is not difficult and only requires basic surgical skills (Mitchell and Willenbring, 2008). The rodent liver is divided into five lobes: left lateral lobe, median lobe, which can be subdivided into left and right median lobe, right lobe and caudate lobe (Figure 4 A). In PHx the left lateral as well as the median lobe is removed under anesthesia (Figure 4 A, B). Following resection, the remaining liver tissue proliferates and expands to restore the original mass within 5-7 days, what allows the investigation of all regenerative processes (Abu Rmilah et al., 2019).

Following the PHx, liver regeneration can be divided into three distinctive phases: initiation or priming phase, proliferation phase and termination phase.





### Initiation/Priming phase

In the priming phase, activation of more than hundreds of genes are induced immediately after PHx, which directly or indirectly prepare hepatocytes to enter the cell cycle (Taub, 2004). This phase refers to the events occurring in the early period of 0-5 hours (hrs) after PHx (Michalopoulos, 2007). Due to PHx, hemodynamic changes occur, i.e., portal blood pressure increases in the residual tissue as well as exerting mechanical stress on the EC. Sokabe et al. revealed an increased activity of urokinase plasminogen activator (uPA) following mechanical stress (Sokabe et al., 2004). Therefore, as first biochemical change, a rise of uPA activity can be observed in the whole tissue 5 min after PHx. Urokinase is known to activate ECM

remodeling. During ECM remodeling, HGF bound by ECM is activated by uPA and excreted, resulting in an activation of c-Met/hepatic growth factor receptor within 30-60 min after PHx (Stolz et al., 1999). Simultaneously, EGF produced by Brunner's glands of the duodenum is produced, constantly enters the liver through the portal circulation and activates epidermal growth factor receptor (EGFR) (Olsen et al., 1985). Also, concentration of TNF $\alpha$  and interleukin 6 (IL6) increase in circulation. They are the most widely studied proinflammatory cytokines and are mainly produced by hepatic macrophages through the nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway (Tao et al., 2017). While these events unfold outside the hepatocytes,  $\beta$ -catenin and Notch-1 intracellular domain (NICD) translocate into hepatocyte nuclei 1-3 hrs and 15-30 min after PHx, respectively.

#### **Proliferation phase**

The second phase is referred to as proliferation phase and converts cells from G1 to mitosis. It can be subdivided into an induction phase and angiogenic phase. Former describes the proliferation of hepatocytes and cholangiocytes. It starts at the end of the priming phase, peaks at 36 hrs for the mouse and ends 72 hrs after PHx (Abu Rmilah et al., 2019). The angiogenic phase commences immediately after the inductive phase and continuous for 2-3 days (Sadri et al., 2015). It refers to the proliferation of the nonparenchymal cells (HCS, KC and hepatic EC) in response to hepatocyte-derived signals. The two most important factors for the initiation and enhancement of the proliferation phase are the growth factors HGF and EGF. They belong to the group of complete mitogens and exhibit direct hepatotropic effects, i.e., they could lead DNA synthesis in serum-free media in vitro and cause liver enlargement when injected in vivo (Tao et al., 2017).

Before proliferation, all cells firstly undergo regenerative hypertrophy. Therefore, hypertrophy dominates in the early phase of the regeneration, whereas regenerative hyperplasia starts gradually later. However, hyperplasia stands as the major mechanism contributing to liver mass restoration after PHx, with hypertrophy playing a transient role in the process (Marongiu et al., 2017).

#### Termination phase

To restrain the liver cell proliferation and correct an overshooting of regenerative response, the liver must go through a termination phase mediated by proliferation-inhibiting factors. Also this phase is as important as the other two phases, the mechanisms underlying the termination have not been well investigated yet. So far, the most well-known anti-proliferative factors are TGF- $\beta$  and related TGF- $\beta$  family members such as activin. TGF- $\beta$  is mainly secreted by nonparenchymal cells such as HSC, KC and platelets (Liu and Chen, 2017). Russel and colleagues demonstrated that administered TGF- $\beta$  inhibited the proliferative response to PHx

in rats (Russell et al., 1988). TGF- $\beta$  expression levels elevate at 2-3 hrs and peak at 72 hrs after PHx. During initiation phase, TGF- $\beta$  is found to be neutralized and its activity is blocked. Since only activated TGF- $\beta$  has the ability to inhibit cell proliferation, conversion from latent pro-TGF- $\beta$  into its active form takes place in the middle of the proliferation phase and is mediated by the indirect inhibitor of hepatocyte growth cation-independent mannose 6-phosphate receptor (CIMPR) (Liu and Chen, 2017; Schrum et al., 2001).

TGF- $\beta$  regulates proliferation through multiple mechanisms. On the one hand, its binding to transforming growth factor receptor leads to a phosphorylation of receptor-regulated cytoplasmic small mothers against decapentaplegic proteins, which accumulate in the nucleus (Liu and Chen, 2017). Here the Smads interact with DNA binding proteins and transcriptional regulators causing inhibition of cell cycle inducers and stimulation of cell cycle inhibitors (Miyazono et al., 2001; Ten Dijke et al., 2002). On the other hand, expression of TGF- $\beta$  increases the release of reactive oxygen species (ROS), essential mediators of apoptosis (Liu and Chen, 2017). ROS induce apoptosis of excess hepatocytes through a c-Jun-independent mechanism and thereby correct overshooting of proliferation (Samson et al., 2002).

Activin is a member of the TGF- $\beta$  superfamily and also has inhibitory effects on proliferation. It acts as an autocrine regulator of DNA synthesis in hepatocytes through the Smad pathway without competing with TGF- $\beta$  (Yasuda et al., 1993). Furthermore, it was shown that the administration of the activin-binding protein follistatin, which inhibits the activity of activin, promoted liver regeneration after PHx in rats (Kogure et al., 1996).

While hepatocytes are the first cells in the liver that undergo proliferation, cholangiocytes follow to enter the cell cycle in the inductive phase. Proliferation of LSEC and HCS starts 48 hrs after PHx, peaks during the angiogenic phase and terminates 5-6 days after PHx. During the regenerative process these different cell types interact with each other via mitogenic growth factors.

As described above, at the beginning of the regeneration proliferation of hepatocytes is stimulated by HGF, which is released and activated during ECM remodeling. The duplicating hepatocytes subsequently secrete different growth factors mediating interactions with the other nonparenchymal cells (Figure 5). On the one hand, they produce platelet-derived growth factor (PDGF), promoting HSC to enter the cell cycle (Pinzani, 2002). On the other hand, secreted TGF- $\alpha$  induces proliferation of the biliary epithelium. Hepatocyte-derived HGF-L mediates KC expansion and cause their production of more TNF and IL6 (Abu Rmilah et al., 2019). Furthermore, hepatocytes secrete growth factors mitogenic for EC, such as VEGF, Angiopoietins 1 and 2, FGF 1 and 2 as well as TGF- $\alpha$ , resulting in angiogenesis and restoration of the sinusoidal network (Ross et al., 2001; Sato et al., 2001; Shimizu et al., 2005).

KC and recruited macrophages also contribute to liver regeneration after PHx. Depletion of KC caused delayed proliferative response from hepatocytes due to missing cytokines and growth factors essential for initiating hepatocyte proliferation (Meijer et al., 2000; Sadri et al., 2015). The key cytokines are TNF and IL6, and mice deficient in either IL6 or TNF showed impaired hepatocyte proliferation 40 hrs after PHx and higher mortality (Figure 5) (Sadri et al., 2015). In addition, Yang *et al.* demonstrated that KC-derived Wnt ligands were essential for initiating hepatocyte proliferation in a timely manner (Yang et al., 2014).



**Figure 5:** Interaction between different hepatic cell types during liver regeneration after PHx. The regenerative process after PHx is precisely orchestrated and includes cooperation of parenchymal and non-parenchymal liver cells. During liver regeneration, the different hepatic cells interact with each other via mitogenic growth factors such as HGF, TGF and VEGF. FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PDGF, platelet derived growth factor; PHx, partial hepatectomy; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Figure adapted from Abu Rmilah et al. (Abu Rmilah et al., 2019).

After their activation by PDGF, HSC enter the cell cycle and essentially regulate the proliferation phase by producing Notch and HGF causing duplication of hepatocytes and biliary epithelial cells (Figure 5) (Michalopoulos and DeFrances, 1997). Furthermore, their production of the ECM proteins laminins establish the structural basis to rebuilt haptic lobules. HSC play a crucial role in the termination process by secreting TGF- $\beta$  that arrest regeneration once the

appropriate mass and function are achieved (Figure 5) (Michalopoulos, 2007; Michalopoulos and DeFrances, 1997).

As aforementioned, proliferating hepatocytes secrete mitogenic factors for EC including VEGF, FGF1 and 2, Angiopoietins, and TGF-α. During the angiogenic phase, EC proliferate and build new blood vessels from preexisting ones, a process called angiogenesis. Angiogenesis aims to re-establish the sinusoidal network in the liver and starts 2-3 days and ends 5-6- days after PHx (Michalopoulos, 2007). VEGF, considered as key regulator of angiogenesis, interacts with its receptor vascular endothelial growth factor receptor (VEGFR) on the surface of EC of existing blood vessels causing the release of proteolytic enzyme matrix metalloproteases (MMP) (Papetti and Herman, 2002; Senger and Davis, 2011). MMP lyse the collagen fibers and enable EC to migrate. Thereby ECM proteins support EC proliferation, survival and migration and provide a binding scaffold for a variety of cytokines that exert essential signaling functions during angiogenesis (Singhal et al., 2018). Afterwards, EC form a central lumen through which blood can flow followed by the recruitment of pericytes and vessel stabilization through intercellular junctions between EC (Papetti and Herman, 2002). How LSEC affect liver regeneration through angiocrines will be discussed in chapter 1.4.2.

### 1.4.1.2 Chemical-induced hepatotoxic injury models

Everything absorbed from the digestive tract passes through the liver before entering the circulation and other organs. Therefore, the liver is exposed to various substances and potential toxins and plays a crucial role in their metabolization and clearance. Thereby, toxins are converted into water-soluble metabolites in the liver to be excreted from the body. The detoxification is divided into two phases mediated by a variety of biotransforming enzymes, which catalyze the oxidation, reduction and hydrolysis (phase I) and/or conjugation (phase II) of functional groups on molecules (Grant, 1991). However, in phase I, some substances are converted into highly reactive metabolites, even more toxic because they bind to nucleic acids or proteins causing severe damage. Substances causing liver injury are called hepatotoxins and include CCl4, D-galactosamine, paracetamol or acetaminophen, isoniazid, thioacetamide, alcohol etc. Fortunately, the liver has the ability to regenerate from injury, which is crucial for survival of acute liver failure due to hepatotoxins. A better understanding of the underlying regeneration mechanisms could lead to the development of new therapies or identification of new biomarkers (Clemens et al., 2019). Hence, chemical-induced injury models have a great clinical relevance and are easier to execute than the PHx model. Otherwise, these models lack a high reproducibility as the outcome depends on administration mode, animal species, age and sex as well as doses. In these chemical-induced liver injury models acute liver failure is caused by various mechanisms such as apoptosis, reactive metabolite induced necrosis, TNF $\alpha$ -mediated inflammatory injury and immune mediated liver injury (Maes et al., 2016).

However, the underlying mechanisms of regeneration after chemical-induced hepatoxicity have been less studied so far, whereas liver regeneration after PHx has been thoroughly investigated. In the following section, a few chemical-induced liver injury models are described in more detail.

#### Carbon tetrachloride

Although CCI4 is a solvent for dissolving non-polar compounds and not a drug, its acute toxicity is commonly used in experimental animal models for inducing liver injury. The underlying injury and regeneration mechanisms have been investigated since the 1950s (Leevy et al., 1959). Even though CCI4 can be administered through inhalation, gavage and intraperitoneal (i.p.) injection, the latter is most common due to reproducibility, survival rates, safety and ease of performance (Scholten et al., 2015). The metabolization of CCl4 by cytochrome P450 2E1 (CYP2E1) leads to the formation of the trichloromethyl (CCI3\*) radical. This radical can in turn react with oxygen to form another highly reactive trichloromethylperoxy (CCI3OO\*) radical (Weber et al., 2003). These metabolites react with various biologically important substances such as nucleic acids, proteins, lipids and carbohydrates causing oxidative damage in hepatocytes. CCl4 injury leads to centrilobular necrosis and apoptosis of the pericentral hepatocytes, as CYP2E1 expression is zonated, within 48 hours of CCI4 administration (Clemens et al., 2019). CCl4-induced liver injury is accompanied by acute inflammatory response, which is dominated by polymorphonuclear leukocytes and macrophages infiltrating the liver to remove necrotic debris of hepatocytes (Mao et al., 2014). As a sign of liver injury, the amount of alanine transaminase (ALT) in the blood rises severely within 36 hrs after CCl4 administration in mice before falling again (Abu Rmilah et al., 2019). Following injury, hepatocytes surrounding the necrotic areas enter the cell cycle for repopulation. Zhao et al. demonstrated that these peri-injured hepatocytes express Wnt target gene Axin2 and thus respond to Wnt mainly secreted by endothelial cells (Zhao et al., 2019). As in PHx, Hgf is important for regeneration after CCI4 administration. Messenger ribonucleic acid (mRNA) expression of HGF as well as TGF- $\alpha$  show a peak at 12 hrs and 48 hrs after CCl4 administration (Webber et al., 1993). Treatment with an antibody against Hgf inhibits proliferation of hepatocytes by reduction of DNA synthesis after CCl4 injury (Burr et al., 1998). Similar to this study, Phaneuf et al. showed that hepatocyte-specific HGF knockout mice had a significantly reduced regenerative capacity after CCl4 treatment caused by a reduced DNA synthesis (Phaneuf et al., 2004). In contrast, liver regeneration in hepatocyte-specific EGFR KO mice was not impaired compared to WT mice (Scheving et al., 2015). However, EGFR-Met double KO as well as loss of Met alone lead to enhanced CCl4-induced necrosis and delayed liver regeneration (Scheving et al., 2015).

#### **D-galactosamine**

The amino sugar D-galactosamine is known as a hepatoxic substance and it induces cell death exclusively of hepatocytes since these are the only cells in the liver metabolizing D-galactosamine (Jaeschke et al., 1998). Its toxicity relies on the inhibition of ribonucleic acid (RNA) and protein synthesis caused by the depletion of uridine metabolites (Saracyn et al., 2015). D-galactosamine induced liver injury is associated with waste accumulation and systemic inflammation (Mao et al., 2014). Compared to the CCl4 model, regeneration processes occur at the same time, nevertheless, capacity of liver regeneration is impaired in this model (Abu Rmilah et al., 2019; Palmes and Spiegel, 2004). In contrast to the CCl4 model, mRNA expression of HGF and TGF- $\alpha$  differ in the liver following galactosamine administration: HGF expression peaks 24 hrs after galactosamine-induced injury, whereas TGF- $\alpha$  expression starts to increase after 48 hrs and peaks at day 5 (Webber et al., 1993). So it has been demonstrated that various growth factors increase in galactosamine hepatotoxicity models, but no studies have yet investigated whether or not they play a role in liver regeneration (Clemens et al., 2019; Okajima et al., 1990).

### **Paracetamol**

Paracetamol or acetaminophen is a well-known antipyretic and analgesic and its overdose is the major cause of acute liver failure in the western world. In hepatocytes, therapeutic amounts of paracetamol are converted into nontoxic metabolites by CYP2E1. However, in the event of overdosing the physiological metabolization reactions in the liver are saturated causing the accumulation of the toxic metabolite N-acetyl-p-benzoguinone imine (NAPQI) (Abu Rmilah et al., 2019). Under normal conditions, NAPQI is excreted after detoxification by glutathione into nontoxic conjugates in the mitochondria and cell cytosol. An overdose depletes the stores of glutathione and results in increased NAPQI levels causing oxidative stress and therefore also to mitochondrial dysfunction (Mitchell et al., 1973). Finally, this results in the breakdown of DNA and oncotic necrosis of hepatocytes (Gujral et al., 2002; Rotundo and Pyrsopoulos, 2020). In addition, cytokines excretion and KC activation ensue an acute inflammation. IL-6 expression levels increase 4 hrs after paracetamol overdose and James et al. demonstrated that in both IL-6 KO and tumor necrosis factor receptor KO mice hepatocyte regeneration was delayed after paracetamol toxicity (James et al., 2005; James et al., 2003). Furthermore, in mice  $\beta$ -catenine was already activated 1 hour after paracetamol-induced injury and thereby promote liver regeneration (Apte et al., 2009). Similar to PHx, activation of EGFR was observed within 15 min and peaked at 3-6 hrs after paracetamol overdose depended on the dosage (Bhushan et al., 2017). In addition, EGFR inhibitor study revealed a dual role of EGFR in both injury initiation and stimulation of subsequent compensatory regeneration after overdosing

(Bhushan et al., 2017). However, in comparison to other models, relatively less is known about the underlying regenerative processes of this hepatotoxic model (Mao et al., 2014).

### 1.4.2 Angiocrine regulation of liver regeneration

The liver possesses the unique ability of regeneration, in which the parenchymal and nonparenchymal liver cells precisely cooperate and interact with each other. LSEC are also involved in liver regeneration by orchestrating the secretion of paracrine growth factors and cytokines, the angiocrine factors (DeLeve et al., 2004). On the other hand, LSEC proliferation is also regulated by hepatocytes and other non-parenchymal cells (Lafoz et al., 2020). As mentioned in the previous chapter, angiocrine secretion in PHx model has been investigated in more detail than in chemical-induced liver injury models to date.

The activation of VEGFR-1 stimulates the release of HGF, which was identified as LSECderived angiocrine promoting hepatocyte proliferation (LeCouter et al., 2003). HSC and Kupffer cells also express Hgf but cannot compensate for the abrogation of endothelial Hgf (Zhang et al., 2020). Moreover, Ding et al. demonstrated in a PHx mouse model that in the early phase of liver regeneration the activation of VEGFR-2 on LSEC induce the upregulation of EC-specific transcription factor inhibitor of differentiation 1 Id1 (Ding et al., 2010). Upregulation of Id1 mediates a release of the pro-regenerative angiocrine factors HGF and Wnt2, which are required for hepatic proliferation. Microarray analysis revealed an upregulation of the angiocrine factors Hgf, Wnt2 as well as Bmp2 in the early phase of regeneration (2 days after PHx) (Nolan et al., 2013). In a further study, Ding and colleagues showed that even after acute liver injury, elicit by acetaminophen and CCI4 administration, increased Id1 expression also induces production of the angiocrines Wnt2 and HGF stimulating liver regeneration (Ding et al., 2014). However, acute liver damage first causes upregulation of CXCR7 in LSEC which, along with CXCR4, induces Id1 activation. Cooperation between CXCR7 and CXCR4 is enabled by stromal-derived factor-1 (SDF-1) and thereby stimulates pro-regenerative Id1 pathway in LSEC. In contrast, after chronic liver injury by repeated CCI4 injections FGFR-1 signaling counterbalanced CXCR7-Id1 response in LSEC and upregulated profibrotic CXCR4 (Ding et al., 2014). This predominance of the CXCR4 pathway over CXCR7-Id1 signaling caused proliferation and activation of HSC and compelled a pro-fibrotic vascular niche. Also in acute-on-chronic liver failure patients defects in CXCR7-Id1 dependent HGF expression lead to poor hepatocyte proliferation and impairs liver regeneration (Shubham et al., 2019). Furthermore, Hgf not only regulates hepatocyte proliferation, but Hgf and its receptor c-Met also control susceptibility to necrosis after PHx involving Deptor to prevent excessive organ damage (Zhang et al., 2020).

Notch signaling is revolutionarily highly conserved and it was recently shown that endothelial Notch activation caused LSEC dedifferentiation and damaged liver homeostasis (Duan et al., 2018). Consistently, CCl4-induced liver fibrosis was aggravated by Notch activation in LSEC. In addition, Notch activation downregulates several hepatocyte mitogens such as Wnt2, Wnt9b as well as Hgf modifying the angiocrine spectrum of LSEC remarkably and leading to a comprised proliferation of hepatocytes under regenerating conditions post-PHx (Duan et al., 2018). While expression of Wnt2a and Wnt9b is eNOS-sGC-dependent, expression of HGF is not.

The Angiopoietin 2 (ANGPT2)/TGF-β1/Tie2 axis is another pathway controlling liver regeneration. In resting endothelial cells ANGPT2 is an antagonist of receptor tyrosine kinase Tie2 and expressed at low levels (Augustin et al., 2009). In mouse PHx model expression of ANGPT2 in LSEC is biphasic. Initially, ANGPT2 expression is downregulated and recovers during the later angiogenic phase of regeneration (Hu et al., 2014). During the early inductive phase of liver regeneration after PHx, deletion of endothelial ANGPT2 expression leads to reduced TGF-B1 production. TGF-B1 is a potent inhibitor of hepatocyte proliferation. The downregulation of TGF-B1 enables liver regeneration by removing an angiocrine inhibitor mechanism of hepatocyte proliferation. During the later angiogenic phase of liver regeneration, LSEC reach their proliferation peak and concomitant ANGPT2 expression gradually recovers in LSEC. ANGPT2 re-expression upregulates the endothelial VEGFR-2/Tie2 axis in an autocrine manner and thereby controls LSEC proliferation. Thus, LSEC-derived ANGPT2 regulated proliferation of hepatocytes and LSEC as a spatiotemporal rheostat to efficiently restore liver structure and function after PHx (Hu et al., 2014). Duan et al. revealed that Notch activation in LSEC upregulates ANGPT2, which, in addition to the altered angiocrine profile of LSEC, might further compromises liver regeneration after PHx by affecting hepatocyte proliferation in the early inductive phase as well as angiogenesis in the later phase (Duan et al., 2018).

Due to partial hepatectomy, the liver has reduced volume, which causes a drastically increase of the blood flow speed. As the liver regeneration progresses blood flow speed gradually decreases again. Recently, Ishikawa et al. revealed that mechanical homeostatic signalling, including shear stress and portal pressure, to LSEC triggers initiation and termination of liver regeneration (Ishikawa et al., 2021). An increase in the blood flow rate caused by PHx results in drastic structural changes in sinusoidal volume and surface area 6 hrs after the surgery. At that time, when blood flow rate reaches its maximum, TGF- $\beta$ 1 expression is markedly downregulated in LSEC. Furthermore, Lorenz and colleagues demonstrated that endothelial

 $\beta$ 1 integrin and VEGFR3 translate blood perfusion by mechanotransduction into angiocrine signaling (Lorenz et al., 2018). Increasing perfusion rate at the beginning of liver regeneration following PHx causes mechanical stretch of LSEC. This activates  $\beta$ 1 integrin/VEGFR3 signaling, which is required for angiocrine production of HGF and thereby for proliferation of hepatocytes (Lorenz et al., 2018).

By contrast, Krüppel-like factor 2 (KLF2), a sheer stress-inducible transcription factor, induces an antiproliferative secretome, including activin A, which inhibits hepatocyte proliferation (Chen et al., 2014; Manavski et al., 2017). Genetic inactivation of KFL2 in LSEC results in augmentation of hepatocyte proliferation and, thus, reduced liver damage after CCl4-induced chronic liver damage (Manavski et al., 2017).

LSEC-derived angiokines such as Hgf, Wnt2 and TGF- $\beta$ 1, dynamically orchestrate the different phases of liver regeneration by spatiotemporally controlling proliferation of hepatocytes and LSEC by stimulatory as well as inhibitory effects. LSEC thereby respond to changes in the mechanical homeostasis due to liver injury and mechano-translate them into angiocrine signaling.

# 2 Aim of the thesis

The sinusoidal endothelial cells of the liver represent a prime example of angiodiversity with their unique morphology and function. LSEC interact with neighboring cells through angiocrine factors and are gatekeepers of their microenvironment by controlling development, homeostasis and liver regeneration. Recently, Gata4 was identified as a master regulator for organ-specific EC differentiation in the fetal liver (Geraud et al., 2017). The early embryonic deletion of Gata4 in LSEC causes fetal lethality and liver hypoplasia. In order to investigate the in vivo function of Gata4 during adulthood another EC-specific Cre deleter mouse was needed that would lead to late embryonic Gata4 deletion. Since Clec4g is expressed in LSEC and investigations of Clec4gCre;Rosa26-YFP reporter mouse revealed late embryonic (E17.5) Cre activity in CD31+ EC, this mouse was used to generate an adult Gata4<sup>LSEC-KO</sup> mice (Wohlfeil et al., 2019).

The first aim of this study was the comprehensive characterization of the *Clec4g*-icre<sup>tg/0</sup> x  $Gata4^{fl/fl}$  (donoted as Gata4<sup>LSEC-KO</sup>) mice to understand the role of Gata4 in adult LSEC.

The investigation of the regeneration capability of Gata4<sup>LSEC-KO</sup> livers was performed in the second part of this doctorate. Two animal models for liver regeneration were investigated: two-third PHx and CCl4-induced hepatotoxic injury.

# 3 Material and Methods

# 3.1 Material

# 3.1.1 Chemicals, buffer and reagents

### Table 1: Chemicals, buffers and reagents

Chemical/buffer/reagent	Cat. No.	Manufacturer
4',6-Diamidino-2-phenylindol, DAPI	D1306	Thermo Fisher Scientific
Agarose	A9539-500G	Sigma-Aldrich
Agencourt AMPure XP Beads	A63881	Beckman Coulter
Anti-CD146 MicroBeads	130-092-007	Miltenyi Biotech
Bovine Serum Albumin	10735086001	Sigma-Aldrich
CaCl <sub>2</sub>	CN92.2	Carl Roth
Carbon tetrachloride, CCl4	289116	Merck
Corn oil	SAFSC8267	Sigma-Aldrich
Dako AEC substrate chromogen	K3436	Agilent Technologies
Dako antibody diluent	S202230-2	Agilent Technologies
Dako aqueous mounting medium	S3025	Agilent Technologies
Dako fluorescence mounting medium	S302380-2	Agilent Technologies
Dako peroxidase solution	S2023	Agilent Technologies
DTT (1 M)	P2325	Life Technologies
Dulbecco's Phosphate Buffered Saline	14190169	Thermo Fisher Scientific
(DPBS)		
EcoMount	EM897L	Biocare Medical
EDTA (0.5 M, pH 8.0)	15575020	Thermo Fisher Scientific
Ethanol denatured	K928.4	Carl Roth
Ethanol ≥99.5 %, Ph. Eur., reinst	5054.3	Carl Roth
FcR Blocking Reagent, mouse	130-092-575	Miltenyi Biotec
Fetal Calf Serum (FCS)		Biochrom
FITC Streptavidin	405202	BioLegend
Formaldehyde (16 %), Methanol-free	11586711	Thermo Fisher Scientific
Formaldehyde solution (4 %)	P087	Carl Roth
GeneRuler DNA Ladder Mix	SM0333	Thermo Fisher Scientific
Gey's balanced salt solution	G9779	Sigma-Aldrich
Hematoxylin Gill I	3801501	Leica Biosystems
HIER citrate buffer pH 6.0	ZUC028-500	Zytomed Systems
Isofluran CP	1214	cp-pharma
Isopropanol	33539-2.5L-M	Sigma-Aldrich

Laemmli buffer	1610747	Bio-Rad Laboratories
Liquid nitrogen		Broser
Luminata Forte Western HRP substrate	WBLUF0500	Millipore
Methanol	32213-2.5L-M	Sigma-Aldrich
Mayer's hemalum solution	1.09249.2500	Merck
MgCl <sub>2</sub>	KK36.1	Carl Roth
Na-cacodylate		
Nancy 520	01494	Sigma-Aldrich
NEB Next High-Fidelity 2x PCR Master	M0541S	New England Biolabs
Mix		
Normal donkey serum (5 %)	017-000-121	Dianova
NP-40	74385	Merck
Nycodenz	1002424	Axis-Shield
Oil Red O	O0625-25G	Sigma-Aldrich
Oligo(dT)18 primer	SO131	Thermo Fisher Scientific
Paraformaldehyde, PFA (4 %)	0335	Carl Roth
Phosphate buffered saline (PBS)	A0964.9050	VWR International
Precision Plus Protein Dual Color	1610374	Bio-Rad Laboratories
Standard		
ProLong™ Gold Antifade Mountant	P10144	Thermo Fisher Scientific
Protease inhibitor cocktail (complete)	1183617001	Sigma-Aldrich
Protease inhibitor cocktail Set I	535142	Merck
Phosphatase inhibitor (PhosSTOP)	4906845001	Sigma-Aldrich
Rimadyl (Carprofen)	7202	WDT
RIPA lysis and extraction buffer	R0278	Sigma-Aldrich
Rotiphorese Buffer TAE	CL86-2	Carl Roth
Skim milk powder	70166	Sigma-Aldrich
SSC buffer (20X concentrate)	S6639	Sigma-Aldrich
TGS buffer (10X)	1610772	Bio-Rad Laboratories
Tris-HCI (1 M, pH 8.0)	15568025	Thermo Fisher Scientific
Tween® 20	P7949	Sigma-Aldrich

# 3.1.2 Instruments

### Table 2: Instruments

Instrument	Manufacturer	
Bath tube BT5	Julabo	
Calibration Check Microprocessor pH Meter HI221	Hanna <sup>®</sup> Instruments	
Centrifuge 5417 R	Eppendorf	
Centrifuge 6K15	Sigma	
ChemoStar Touch 21.5	Intas Science Imaging	
Cobas c311	Roche Diagnostics	
Cryotome CM3050S	Leica	
DS-Qi2 high definition monochrome camera	Nikon	
DS-Ri2 high definition color camera	Nikon	
Eclipse Ni-E motorized upright microscope	Nikon	
FACS Canto™ II	BD Biosciences	
GelStick Touch Imager	Intas	
HERAsafe	Heraeus	
HybEZ™ Oven	Advanced Cell Diagnostics	
Imaging Plate Scanner Micron	Ditabis	
Immersion thermostat Corio C	Julabo	
Infinite M 200 Pro	Tecan	
Intenslight Epifluorescence Illuminator		
Kelvitron T	Heraeus	
Lab Thermometer IP65 LT-101	TFA Dostmann	
MACS MultiStand	Miltenyi	
Magnetic Stirrer MR 3001 K	Heidolph Instruments	
Mupid-One Electrophoresis Chamber	Biozym	
NanoPhotometer <sup>®</sup> NP80	Implen	
Operating table type 12511	Medax	
pfm Cooling Plate 4100	pfm medical AG	
pfm Rotary 2006 EM	pfm medical AG	
pfm Waterbath 1000	pfm medical AG	
Precellys Evolution tissue homogenizer Bertin Technologies		
qTOWER 3 G touch thermal cycler	Analytik Jena	
Quadro MACS <sup>™</sup> Separator	Miltenyi	
Sartorius CPA623S	Sartorius	
Sartorius CPA225D	Sartorius	
Shaker DRS-12	neoLab	
Steamer MultiGourmet Typ 3216	Braun	

Thermoblock	Eppendorf
Thermocycler T100	Bio-Rad Laboratories
······································	
ThermoMixer C	Eppendorf
	Eppenden
Trans-Blot Turbo Transfer System	Bio-Rad Laboratories
Tubing Pump Ismatec	Cole-Parmer GmbH
Vortex Genie-2	Scientific Industries
Vortever KS 250 basic	IKA Werke
Water bath	lulaho
	501850
Zeiss FM 910	Carl Zeiss
	0411 20133

# 3.1.3 Consumables

#### Table 3: Consumables

Consumable	Manufacturer	
15 mL Falcon tubes	Greiner bio-one	
5 mL Round Bottom Polystyrene Tubes	Falcon	
50 mL Falcon tubes	Greiner bio-one	
96 well cell culture plate	Greiner bio-one	
BD Microlance needle 30G x 1/2	BD Biosciences	
Cell culture dish, 100/20 mm	Greiner bio-one	
Cell strainer 100 µm	BD Biosciences	
Cover Slips 24x60 nm	Engelbrecht GmbH	
CryoPure Tube 1.6mL white	Sarstedt AG & Co.KG	
Eppendorf Safe-Lock Tubes 1.5 mL	Eppendorf	
Eppendorf Safe-Lock Tubes 2.0 mL	Eppendorf	
Extra Thick Blot Filter Paper	Bio-Rad Laboratories	
Immune-Blot PVDF Membrane	Bio-Rad Laboratories	
LS Columns	Miltenyi	
Mash Polyester Monolen, 250 µm	neoLab	
Microtome blades, S35	Pfm medical	
Microvette® 500 μL Lithium-Heparin	Sarstedt AG & Co.KG	
Multiply®µStrip Pro mix.colour	Sarstedt AG & Co.KG	
precast polyacrylamide gel (4-20 %)	Bio-Rad Laboratories	
PVDF membrane	Bio-Rad Laboratories	
Rotilabo® CME syringe filters	Carl Roth	
Serological pipette, sterile	Greiner bio-one	
Single-use fine dosage syringe, 2-piece (1 mL)	B. Braun Melsungen AG	
Single-use syringe, 2-piece (20 mL)	B. Braun Melsungen AG	

SuperFrost®Plus Microscope slides	R. Langenbrinck GmbH
Surgical Disposable Scalpels	B. Braun Melsungen AG
SurPhob Low Binding Pipette tips	Biozym
Thermo Scientific <sup>™</sup> Nalgene <sup>™</sup> Rapid-Flow <sup>™</sup> Filter Unit	Thermo Fisher Scientific

# 3.1.4 Kits

### Table 4: Kits

Kit	Cat. No.	Manufacturer
Affymetrix GeneChip Mouse Gene 2.0	902119	Thermo Fisher Scientific
ST Arrays		
Bioanalyzer High Sensitivity DNA	5067-4626	Agilent
analysis kit		
ChIP DNA Clean and Concentrator Kit	D5205	Zymo Research
DC Protein Assay	5000112	Bio-Rad Laboratories
Hyaluronan DuoSet ELISA	DY3614-05	R&D Systems
innuMIX qPCR SyGreen Sensitive	845-AS-1310200	Analytik Jena
innuPREP RNA Mini Kit 2.0	845-KS-	Analytik Jena
	2040250m	
KAPA HotStart Mouse Genotyping Kit	KK7352	Sigma-Aldrich
Mouse procollagen III N-terminal	abx154546	Abbexa
propeptide ELISA Kit		
Nextera Index Kit	15055290	Illumina
NextSeq 500/550 v2.5 Kits	20022408	Illumina
Precellys Lysing Kit	P000918-LYSK0-	Bertin Technologies SAS
	А	
RNAscope 2.5 HD Red Kit	322350	Advanced Cell Diagnostics
RNAscope 2.5 HD Duplex Kit	322430	Advanced Cell Diagnostic
RNAscope Multiplex Fluorescent	323100	Advanced Cell Diagnostic
Reagent Kit v2		
Smart Protein Layers (SPL) Blue Kit	PR925	NH Dyeagnostics
Triglyceride Quantification	K622	BioVision
Colorimetric/Fluorometric Kit		
TruSeq ChIP library preparation kit	IP-202-1024	Illumina
TURBO DNA-free Kit	M1907	Thermo Fisher Scientific

# 3.1.5 Enzymes

### Table 5: Enzymes

Enzymes	Cat. No.	Manufacturer
Collagenase A	C2674	Sigma-Aldrich
Maxima Reverse Transcriptase	EP0752	Thermo Fisher Scientific
MNase	88216	Thermo Fisher Scientific
Nextera Tagment DNA Enzyme TDE	15027916	Illumina

# 3.1.6 Antibodies

# 3.1.6.1 Primary antibodies

### Table 6: Primary antibodies

Target	Clone	Host	Cat. No.	Supplier
CD3	17A2	rat	100202	BioLegend
CD31, APC conjugated	MEC 13.3	rat	551262	BD Biosciences
CD68	FA-11	rat	137002	BioLegend
Cleaved Caspase 3	N/A	rabbit	9661	Cell Signaling
с-Мус	9E11	mouse	sc-47694	Santa Cruz
				Biotechnology
Collagen type I	N/A	rabbit	R1038	Acris Antibodies
				GmbH
Collagen type III	N/A	rabbit	R1040	Acris Antibodies
				GmbH
Collagen type IV	N/A	rabbit	NB120-6586	Novus Biological
Endomucin	V.7C7	rat	14-5851-82	Thermo Fisher
				Scientific
Glutamine Synthetase	N/A	rabbit	G2781	Merck
Histone H3 (acetyl K27)	N/A	rabbit	ab4729	Abcam
ChIP Grade				
Histone H3 (trimethyl	N/A	rabbit	ab8580	Abcam
K4) ChIP Grade				
Ki67	SP6	rabbit	ab16667	Abcam
Lyve-1	N/A	goat	AF2125	R&D Systems
Lyve-1, PE conjugated	223322	rat	FAB2125P	R&D Systems
Podocalyxin	N/A	goat	AF1556	R&D Systems
Stabilin2 biotinylated	N/A	rabbit	AK2377	InVivo Biotechs
#3.1				
VE-cadherin	N/A	goat	AF1002	R&D Systems

# 3.1.6.2 Secondary antibodies

Target	Conjugate	Host	Cat. No.	Supplier
Anti-goat	Су™З	donkey	705-165-147	Dianova
Anti-goat	Alexa Fluor 647	donkey	705-605-147	Dianova
Anti-rabbit	Alexa Fluor 488	donkey	711-545-152	Dianova
Anti-rabbit	HEP	donkey	NA934	Merck
Anti-rabbit	HRP	goat	K4003	Agilent Technologies
Anti-rat	Cy™3	donkey	712-165-153	Dianova
Anti-rat	HRP	goat	AP136P	Merck

### Table 7: Secondary antibodies

# 3.1.7 Primers

Gene	-	Target Sequence	Supplier	
Mm Acto?	Fw:	CAGACATCAGGGAGTAATGGTTG	motabion international AC	
WITT ACIAZ	Rev:	GGCCACACGAAGCTCGTTAT		
Mm Anin	Fw:	ACTGCAGTTTGTGGAGTGCCA	metabion international AG	
	Rev:	CACTTGGCGAGCCCTTCAAT		
Mm Bmn2	Fw:	TGCTTCTTAGACGGACTGCG	motabian international AC	
	Rev:	CACGGCTTCTTCGTGATGGA	metablon international AG	
Mm Cd34	Fw:	CTTCTGCTCCGAGTGCCATT	metabion international AG	
	Rev:	AACTCCTCACAACTAGATGCTTCA	metablom international AG	
Mm Col15a1	Fw:	CACCATGACACACAGGAGGACC	metabion international AC	
	Rev:	GCCATAGCCGGTGGTAAAGG		
Mm Collal	Fw:	CAGGCTGGTGTGATGGGATT	metabion international AG	
Will Conar	Rev:	AAACCTCTCTCGCCTCTTGC		
Mm Col3a1	Fw:	GAGGAATGGGTGGCTATCCG	metabion international AG	
Will Colsa I	Rev:	GCGTCCATCAAAGCCTCTGT	metablom international AG	
Mm Col4a1	Fw:	CCAGGATGCAACGGTACAAA	motabion international AG	
	Rev:	AACGTGGCCGAGAATTTCAC		
Mm Colda2	Fw:	GGCGAGCCAGGGGTTA	metabion international AC	
WITT CO1482	Rev:	TGGATGCCTTTTAAGCCCAGATA		
Mm Des	Fw:	GAGGAGAGCAGGATCAACCTT	metabion international AG	
	Rev:	CTCTCCATCCCGGGTCTCAA		
Mm Esm1	Fw:	TGCAAAGACTGTCCCTATGGC	metabion international AC	
	Rev:	CCATCTCCGGATGCTGAGTC		

### Table 8: Primers for qRT-PCR analysis

	Fw:	CTGCCCACCAGGCATTTG		
Mm Gak	Rev: CCATGTCACATACATATTCAATGT		metabion international AG	
Mm Coto 4 (E 12/6)	Fw:	CCCCTACCCAGCCTACATGG	motobion international AC	
Min Gata4 (EJ2/6)	Rev:	CACCAGCAGGACCGAGATTG	metabion international AG	
Mm Gatad (E 14/5)	Fw:	ATGCATAGCCTTGTGGGGAC	motabian international AC	
	Rev:	GGCCTGGGAACCCCAT		
Mm Catad (E16/7)	Fw:	GCTCCATGTCCCAGACATTC	motabian international AC	
Will Gala4 (E30/7)	Rev:	ATGCATAGCCTTGTGGGGAC	metablon international AG	
Mm lafhn5	Fw:	CCTGCACCTGAGATGAGACAG	motabion international AG	
	Rev:	ACCAGCAGATGCCACGTTTG	metablom international AG	
Mm Lomb1	Fw:	TACTGTAAGCGCCTGGTGAC	motabian international AC	
	Rev:	CGGAGCAGCTATTGTTCAGC		
Mm / amc1	Fw:	AAGCTGAACAGCAGACCGC	metabion international AG	
	Rev:	TGATGGCGGGAATTCTCCTT		
Mm Mrnl/6	Fw:	GGGAGCAGGCATTCCTACAG	metabion international AG	
	Rev:	GGTCCGGTCATTTTTTTTGTCA		
Mm Myc	Fw:	TACAACACCCGAGCAAGGAC	metabion international AG	
	Rev:	GAGGCTGCTGGTTTTCCACT		
Mm Pdafh	Fw:	CTACCTGCGTCTGGTCAGC	metabion international AG	
	Rev:	GCTCAGCCCCATCTTCATCTAC		
Mm Pdafrb	Fw:	ATGGGTGGAGATTCGCAGGA	metabion international AG	
	Rev:	TCATAGCGTGGCTTCTTCTGCC		
Mm Sema7a	Fw:	ATCTCCGCCGTCTGGAAAG	metabion international AG	
	Rev:	CCGATGTTCACCGTGCG		
Mm Sparol1	Fw:	GCAACTCAAGGGAGCACAGT	motabion international AG	
wim Sparci1	Rev:	CACAAGCCTGATCTAGGATTTTTGC	metablom international AG	
Mm Srp72	Fw:	CACCCAGCAGACAGACAAACTG	motabion international AG	
	Rev:	GCACTCATCGTAGCGTTCCA		
Mm W/nt2	Fw:	GCCCTGATGAACCTTCACAAC	metabion international AC	
	Rev:	GGAGCCACTCACACCATGAC	The cabion international AG	
Mm W/nt9h	Fw:	GAGCGCTGTACTTGTGACGA	metabion international AC	
	Rev:	CCGCTCTTCACAGCCTTGAT		

# Table 9: Primers for genotyping

Gene		Target Sequence	Supplier	
Mm Clec4q_iCre	Fw:	AAGCTGAACAACAGGAAATGGTTC	motabion international AC	
Will Clec4g-ICle	Rev:	GGAGATGTCCTTCACTCTGATTCT		
Mm Gata4	Fw:	CCCAGTAAAGAAGTCAGCACAAGGAAAC	metabion international AG	
	Rev:	AGACTATTGATCCCGGAGTGAACATT		

 Table 10: Primers for recombination

Gene		Target Sequence	Supplier
Mm Gata4	Fw:	AACCTGAGCAGCTGATGACT	matchion international AC
	Rev:	CTAGACTATTGATCCCGGAGTG	metablom international AG

### Table 11: Primers for ChIP-qPCR

Gene		Target Sequence	Supplier
Mm Pdgfb	Fw:	GATGGTTCGTCTTCACTCGC	matchion international AC
exon1	Rev:	AGCTCTGCGCTTTCTGATCT	metablon international AG
Mm neg. Ctrl	Fw:	CACCCGTCTCATCAAAATCGC	matchion international AC
	Rev:	GGGGTCATGAAGCAGTGTCA	metablom methational AG

# 3.1.8 ISH probes

### Table 12: ISH probes

Probe	Catalog No.	Supplier
Mm-Axin2	400331	Advanced Cell Diagnostics
Mm-Bmp2-E3	427341	Advanced Cell Diagnostics
Mm-Cd34	319161	Advanced Cell Diagnostics
Mm-Cdh5-C2	312531-C2	Advanced Cell Diagnostics
Mm-Col1a1	319371	Advanced Cell Diagnostics
Mm-DapB (neg. Ctrl)	310043	Advanced Cell Diagnostics
Mm-Esm1-E3	411761	Advanced Cell Diagnostics
Mm-Hgf	315631	Advanced Cell Diagnostics
Mm-Igfbp5	425731	Advanced Cell Diagnostics
Mm-Myc	413451	Advanced Cell Diagnostics
Mm-Pdgfb	424651	Advanced Cell Diagnostics
Mm-Pdgfrb	411381	Advanced Cell Diagnostics
Mm-Pdgfrb-C2	411381-C2	Advanced Cell Diagnostics
Mm-Ppib I (pos. Ctrl)	313911	Advanced Cell Diagnostics
Mm-Rspo3	402011	Advanced Cell Diagnostics
Mm-Sparcl1	424641	Advanced Cell Diagnostics
Mm-Stab2	406611	Advanced Cell Diagnostics
Mm-Wnt2	313601	Advanced Cell Diagnostics
Mm-Wnt9b	405091	Advanced Cell Diagnostics

# 3.1.9 Software

### Table 13: Software

Software name	Manufacturer
Fiji ImageJ	Open source/National Institutes of Health
NIS-Elements AR 5.02	Nikon Instruments
GraphPad Prism 7	GraphPad Software, Inc.
SPL-LabImage 4.2.3	Kapelan Bio-Imaging
R	Open Source/R Core Team
Inkscape	Open Source/Inkscape Community
qPCRsoft	Analytik Jena
FlowJo V10	FlowJo, LLC
JMP Genomics 9	SAS Institute
LabImage 4.2.3	Kapelan Bio-Imaging

# 3.2 Methods

# 3.2.1 Animal experiments

All mouse experiments were approved by the animal ethics committee of the Regierungspräsidium Karlsruhe (I19-22/G158-17) and were conducted in accordance with national and international ethical guidelines.

# 3.2.1.1 Animal models

Female and male mice aged one day to 12 months were used for analyses. Mice were housed under pathogen-free conditions and were exposed to 12 hrs light and 12 hrs dark periods. They had free access to a standard rodent diet (V1534-000; Ssniff; Germany) and water. At the age of 28 days mice were weaned, marked by ear punch for identification, genotyped and kept gender separated. The following mice were used

### Table 14: Mouse strains

Short Name	Official Strain Name	Background
Clec4g-icre	C57BL/6N-Tg( <i>Clec4g</i> -icre)1.1Sgoe, MGI:6280453	C57BL/6N
Gata4 <sup>fl/f</sup>	STOCK Gata4 <sup>tm1.1Sad</sup> /J, JAX:008194	B6N; 129S1sv

Transgenic mice with *Clec4g* promoter-driven Cre expression (C57BL/6N-Tg(*Clec4g*icre)1.1Sgoe, MGI:6280453) were generated (Wohlfeil et al., 2019) and crossed with *Gata4*<sup>fl/fl</sup> mice (STOCK *Gata4*<sup>tm1.1Sad</sup>/J, JAX:008194) (Watt et al., 2004) bearing locus of X-over P1 (loxP) sites flanking exon 3 to exon 5 on both alleles. The resultant male mice with the genotype *Clec4g*-icre<sup>tg/0</sup> x *Gata4*<sup>wt/fl</sup> were mated with *Gata4*<sup>fl/fl</sup> females to generate the desired *Clec4g*-icre<sup>tg/0</sup> x *Gata4*<sup>wt/fl</sup> (denoted as Gata4<sup>LSEC-KO</sup>) mice. The functional Zinc-finger domain of Gata4 is missing in Gata4<sup>LSEC-KO</sup> due to homozygous recombination. Due to the mixed background we always used littermates bearing either the genotype *Clec4g*-icre<sup>0/0</sup> x *Gata4*<sup>fl/fl</sup> as controls.

### 3.2.1.2 Dissection and tissue preparation

Isoflurane anesthetized mice were sacrificed by cervical dislocation and the livers were removed and weight. Tail tips were collected and used to confirm the genotype (see page 47). The removed livers were macroscopically imaged, weighted, sectioned and either snap-frozen in liquid nitrogen or fixed in phosphate-buffered 4 % paraformaldehyde (PFA) solution at RT for two to seven days followed by dehydration and paraffin embedding.

# 3.2.1.3 Blood sampling and analyses

Mice were anesthetized with isoflurane and retrobulbar blood samples were taken during day cycle. Blood was centrifuged in lithium heparin tubes at 7000 x g for 7 min at RT. Plasma was

separated (upper phase) and alanine transaminase (ALT), aspartate transaminase (AST), glutamate dehydrogenase (GLDH), total protein, glucose, cholesterol, trigylcerides, creatinine and urea were determined in a Cobas c311 analyzer according to manufacturer's recommendations. Hyaluronan and procollagen III N-terminal propeptide (PIIINP) enzyme-linked immunosorbent assay (ELISA) were performed according to the manufacturer's instructions. All standards and samples were measured in duplicates using a TECAN plate reader.

### 3.2.1.4 Liver regeneration models

#### Partial Hepatectomy (PHx)

At the age of 8 weeks male Gata4<sup>LSEC-KO</sup> and control siblings were exposed to PHx by resection of 70 % of the liver following published methods (Mitchell and Willenbring, 2008). 30 min before the procedure mice received subcutaneously carprofen (5 mg/kg body weight) for analgesia. After anesthetization with inhaled isoflurane a midline abdominal skin and muscle incision was made to expose the xiphoid process. The left lateral lobe and the median lobe were ligated and resected. After closing the abdomen mice were placed on a warming pad until recovered from surgery. All experimental mice were regularly examined to recognize signs of pain, distress and discomfort. Following parameters were applied: no reaction, expression of pain during handling, pain when walking, self-isolation, permanent chewing, wound dehiscence, abnormal posture, paralysis, and over 20 % weight loss. As soon as a mouse reached the predefined limit of distress, the mice were immediately euthanized. Blood samples were taken from all recovered mice 48 h or 144 h after surgery under isoflurane anesthesia and sacrificed subsequently.

### Carbon tetrachloride (CCl4)-induced hepatotoxic injury

For acute CCI4-induced liver injury 8 weeks old male Gata4<sup>LSEC-KO</sup> and control siblings were used. Mice were treated with a single intraperitoneal (i.p.) injection of 100 µM of a 20 % solution of CCI4 dissolved in corn oil. Two or six days after CCI4 injection blood samples were taken and mice were sacrificed by cervical dislocation.

### 3.2.1.5 LSEC Isolation

Isolation was performed according to a modified protocol from Diehl et al. (Diehl et al., 2008). 12-14 weeks old mice were sacrificed by cervical dislocation and the abdomen was opened. The cava vein was cut after the portal vein was cannulated. Then, liver was perfused in situ via the portal vein with a 38 °C prewarmed 0,05 % collagenase in Ca<sup>2+</sup>deprived medium until all blood was flushed out. The liver was removed, weighed and the gallbladder dissected. Three minced livers were pooled in 15 mL GBSS and digested at 38°C for 25 min after addition of 150 µL collagenase for normal livers or 300 µL collagenase for fibrotic livers. Cell suspension was passed through a filter (250 µm mesh size) and washed twice with GBSS. Nonparenchymal cells were separated by a 19.3 % Nycodenz gradient (centrifugation at 1400 g for 25 min with no brake). After the cells were resuspended in 4 mL magnetic activated cell sorting (MACS)-Buffer, cell number was determined while washing. The resulting cell pellet was resuspended in 90 µL of MACS-Buffer per 10^7 cells, mixed with 10 µL Anti-CD146 MicroBeads per 10^7 cells and incubated for 15-20 min at 4°C. After a washing step the cells were resuspended with 1 mL MACS-Buffer, the LS separation column was rinsed with 3 mL MACS-Buffer and the cell suspension was applied on the column to separate the LSEC by MACS. The column was washed three times, removed from the separator and the magnetically labeled CD146+ LSEC were flushed out with 5 mL MACS-Buffer. Cell number was determined and the isolated LSEC were used either for assay for transposase accessible chromatin sequencing (ATAC-Seq) and chromatin immunoprecipitation (ChIP)-qPCR or to isolate RNA/protein and to determine the purity by flow cytometry.

#### Ca<sup>2+</sup>deprived medium

L-Aspartic acid	0.1 mM
Threonine	0.2 mM
Serine	0.3 mM
Glycine	0.5 mM
L-Alanine	0.6 mM
L-Glutamic acid	0.9 mM
L-Glutamine	0.9 mM
D (+) Glucose	20 mM
Fructose	20 mM
Sucrose	197 mM
KCI	3 mM
NaH <sub>2</sub> PO <sub>4</sub> -H <sub>2</sub> O	0.7 mM
MgCl <sub>2</sub>	0.5 mM
Hepes	10 mM
NaHCO <sub>3</sub>	24 mM

### 3.2.1.6 Perfusion and transmission electron microscopy (TEM)

Mice were anesthetized by i.p. injection of Ketamine/Xylazine and the abdomen and thoracic cavity were opened. The mice were perfused through the left cardiac chamber, first with PBS to remove the blood and second with fresh fixative for electron microscopy. Then, the livers were removed, dissected and liver lobes were kept in fixative.

Following steps were carried out in cooperation with the electron microscopy core facility (K. Richter) from the German Cancer Research Center:

Vibratome sections (200  $\mu$ m) of liver lobes were postfixed with either 1 % osmiumtetroxide or ferrocyanide reduced osmium (OsFeCN: 1 % OsO<sub>4</sub> in 1.3 % K<sub>4</sub>Fe(CN)<sub>6</sub>), dehydrated with and embedded in epoxy resin according to standard protocols. Ultrathin sections (60 nm) were stained with lead and uranyl and observed in a transmission electron microscope at 100 kV. Micrographs were taken with image plate scanner scanned at 15  $\mu$ m resolution.

# 3.2.2 Molecular biological methods

### 3.2.2.1 RNA isolation and cDNA synthesis

Total RNA was extracted from tissue or isolated LSEC with innuPREP RNA Mini Kit 2.0. RNase-free materials were used and surface was cleaned with an RNase spray.

For lysis either frozen pieces of livers were thaw on ice, homogenized with a Precellys tissue homogenizer in 450  $\mu$ L RL lysis buffer and centrifuged at max. speed for 1 min or up to 10<sup>6</sup> isolated LSEC were resuspended in 450  $\mu$ L RL lysis buffer and incubated for 5 min at RT. The genomic DNA was removed by centrifugation of the lysat at 11000 x g for 2 min with Spin Filter D. The filtrate containing the RNA was diluted with the equal volume of 70 % ethanol, added to Spin Filter R and centrifuged at 11,000 x g for 2 min. The RNA bound to the Spin Filter R was washed with 500  $\mu$ L washing solution HS and 750  $\mu$ L washing solution LS and ethanol waste removed subsequently by centrifugation (max. speed for 3 min). Afterwards the RNA was eluted with 50  $\mu$ L RNase-free water in a new tube and the RNA concentration and quality were measured. RNA was either stored at -80°C, used for complementary DNA (cDNA) synthesis or treated with TURBO DNA-*free* kit to remove contaminating DNA according to manufacturer's protocol if used for Microarray analysis.

The RNA was transcribed into cDNA using Maxima Reverse Transcriptase in accordance with manufacturer's protocol. 1  $\mu$ g RNA was used per reaction and reverse transcription was performed in duplicates per samples.

Reagent	Volume
RNA	1 μg in 12.5 μL Nuclease-free water
Oligo(dT) <sub>18</sub> primer	1 µL
dNTP Mix (10 mM)	1 µL
5X RT Buffer	4 µL
RiboLock RNase Inhibitor	0.5 μL (20 U)
Maxima Reverse Transcriptase	1 μL (200 U)
Total	20 µL

Reverse transcription was conducted at 50 °C for 30 min and terminated by heating at 85 °C for 5 min. cDNA was stored at -20 °C.

# 3.2.2.2 Genotyping and Polymerase chain reaction

DNA extraction from tissue and genotyping was conducted using the KAPA HotStart Mouse Genotyping Kit. DNA was extracted from either ear punches (1.5 mm<sup>2</sup>) or tail tips of 2 mm for genotype determination or confirmation.

Reagent	Volume
tissue (ear punch/tail tip)	
ddH <sub>2</sub> O	88 µL
10X KAPA Express Extract Buffer	10 µL
1 U/µI KAPA Express Extract Enzyme	2 μL
Total	100 µL

Lysis was performed at 75 °C for 10 min followed by heat inactivation at 95 °C for 5 min. DNA extract was diluted tenfold with 10 mM Tris-HCl (pH 8.0-8.5). For polymerase chain reaction (PCR) the following reaction mix was prepared with the designed primers (see Table 9):

Reagent	Volume
Template DNA	1 μL
ddH <sub>2</sub> O	3 μL
Forward primer (10 µM)	0.5 μL
Reversed primer (10 µM)	0.5 μL
2X KAPA2G Fast (HotStart) Genotyping Mix with	5 μL
dye	
Total	10 μL

The following cycle conditions were used:

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	15 sec	
Annealing	60 °C	15 sec	34
Polymerization	72 °C	15 sec	
Final extension	72 °C	1 min	1
Cooling	12 °C	×	1

PCR products were loaded on 1.5 % agarose gels prepared by dissolving agarose in (1X) trisacetate EDTA (TAE) buffer and addition of Nancy-520 fluorescent stain for double-stranded DNA. Gel electrophoresis was conducted at 100 V for 25 min. The agarose gel was subsequently stimulated with UV light in Intas GelStick Touch Imager, imaged and visualized DNA bands evaluated.

# 3.2.2.3 Quantitative Real-time polymerase chain reaction

To analyze RNA expression levels quantitative Real-time polymerase chain reaction (qRT-PCR) was performed using innuMIX qPCR SyGreen Sensitive on a qTOWER 3 G touch thermal cycler according to manufacturer's instruction. cDNA duplicates were diluted 1:10 with ddH<sub>2</sub>O per sample. The three reference genes cyclin G associated kinase (Gak), mitochondrial ribosomal protein L46 (Mrpl46) and signal recognition particle 72 (Srp72) were used for normalization (Hruz et al., 2011). In order to generate mRNA-specific primers, primer pairs span an exon-exon junction or needed to be separated by at least one intron (see Table 8).

Reagent	Volume
Template cDNA (1:10 diluted in ddH <sub>2</sub> O)	1 µL
ddH <sub>2</sub> O	4 µL
Primer premix (2 µM each)	5 µL
2X innuMix qPCR SyGreen Sensitive	10 µL
Total	20 µL

qRT-PCR was performed with following settings:

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	120 sec	1
Denaturation	95 °C	10-30 sec	- 40
Annealing and Detection	60 °C	30-60 sec	

The qRT-PCR data were analyzed with the qPCRsoft 4.0.8.0 software. To normalize expression values the Pfaffl method was used which depends only on amplification efficiency of the transcripts and  $\Delta$ CP (Pfaffl, 2001).

### 3.2.2.4 Microarrays

Total RNA was extracted from fresh isolated LSEC or liver tissue and contaminating DNA removed as described before. The Affymetrix core facility of the Medical Faculty Mannheim checked the RNA purity before performing the gene expression profiling using Affymetrix

GeneChip Mouse Gene 2.0 ST Arrays. Five independent samples were used per test group. The different gene expression profiles were analyzed with ANOVA using JMP.

Gene set enrichment analysis (GSEA) was conducted as further analytical method to interpret the gene expression data (Subramanian et al., 2005). Therefore, the gene expression profiling data were compared to the freely available molecular signature database (MSigDB) v6.2 hallmark gene set collection (Liberzon et al., 2015) by using R. 3.6.1 (R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2019) and 44 cluster Profiler 3.12.0 (Yu et al., 2012).

### 3.2.2.5 In situ hybridization

ISH was performed using RNAscope 2.5 HD Red, RNAscope 2.5 HD Duplex and RNAscope Multiplex Fluorescent v2 Kits on formalin-fixed paraffin-embedded (FFPE) tissue according to the manufacturer's instructions. ISH probes are listed in Table 12 and each time a positive and negative control were used. Tissue sections  $(3 \ \mu m)$  were backed at 60 °C for 1 h, subsequently deparaffinized in xylene (2 x 5 min) and 100 % ethanol (2 x 1 min) and air dried for 5 min at RT. Hydrogen peroxide was applied to each section for 10 min at RT followed by washing in dH<sub>2</sub>O. Target retrieval, protease treatment, hybridization steps of the probes and amplifiers as well as the detection of the signal differ between the kits (see below).

### RNAscope 2.5 HD Red

For target retrieval slides were submerged in boiling 700 mL 1X target retrieval solution for 30 min, then immediately transferred in dH<sub>2</sub>O followed by 100 % ethanol. Slides were air dried and tissue sections were surrounded with a hydrophobic barrier. The following day, protease plus were applied and incubated for 30 min at 40 °C. After two washing steps in dH<sub>2</sub>O the probes and controls were hybridized for 2 h at 40 °C followed by the hybridization of the amplifiers 1 to 6 according to the manufacturer's protocol. For signal detection 1 volume of RED-B was mixed with 60 volumes of RED-A, applied and incubated in a sealed tray for 10 min at RT. Slides were washed in dH<sub>2</sub>O and then counterstained with 50 % hematoxylin I for 90 sec, washed with tap water, 0.02 % ammonia water (10 sec) and dH<sub>2</sub>O before dried at 60 °C for 15 min. Slides were mounted with EcoMount and stained sections as well as negative and positive controls were examined with a bright field microscope.

### RNAscope 2.5 HD Duplex

The Braun steamer was filled with water to the maximum level. One slide holder with 200 mL of 1X target retrieval solution and one slide holder with 200 mL dH2O were placed in the steam bowl. The steamer was turned on and a digital thermometer was insert through a hole of the lid into the target retrieval solution making sure temperature reached at least 99 °C. Slides were acclimated for 10 sec in the heated dH2O and subsequently transferred into the target retrieval solution for 30 min. Afterwards slides were washed in dH<sub>2</sub>O, transferred to 100 %

ethanol before air dried. A hydrophobic barrier was drawn around each section and completely dried. During protease treatment for 30 min at 40 °C, the C2 probe was diluted 1:50 to the C1 probe. After washing the mixed probed as well as the controls were hybridized for 2 h at 40 °C. Slides were washed in 1X washing buffer and kept overnight in 5X saline sodium citrate (SSC) buffer at RT. The following day slides were washed and the amplifiers 1 to 6 were hybridized according to the manufacturer's instruction. The red signal was detected by applying the RED-A/RED-B (1:60) mixture for 10 min at RT. Slides were washed in washing buffer and the amplifiers 7 to 10 were hybridized. Green-B was mixed 1:60 with Green-A and applied for 10 min at RT in the dark. After washing for 5 min, slides were rinsed quickly in dH<sub>2</sub>O and counterstained with 50 % hematoxylin I for 30 sec and processed as described above (RNAscope 2.5 HD Red).

### RNAscope Multiplex Fluorescent v2

Deparaffinization of the slides, pretreatment, preparation of the C1 and C2 probe and hybridization was conducted as described for RNAscope 2.5 HD Duplex kit. Slides were kept overnight in 5X SSC buffer at RT. The next day slides were washed and amplifier 1, 2 and 3 were hybridized. The horseradish peroxidase (HRP)-C1 signal was developed and Opal<sup>™</sup>570 was assigned to the C1 channel. After the HRP-C2 signal was developed and C2 channel was matched to Opal<sup>™</sup>690, slides were washed and DAPI was incubated for 30 sec at RT. Slides were mounted with ProLong<sup>™</sup> Gold Antifade Mountant, dried overnight in the dark and then imaged with a fluorescence microscope.

C1 probe	Opal dye	Dilution in TSA buffer	C2 probe	Opal dye	Dilution in TSA buffer
Pdgfb	570	1:1250	Cdh5	690	1:800
Pdgfb	570	1:1250	Pdgfr-b	690	1:800

# 3.2.3 Biochemical methods

### 3.2.3.1 Protein isolation and quantification

### Protein isolation

Isolated LSEC were washed with PBS and cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) lysis and extraction buffer containing phosphatase and protease inhibitor. After incubation for 30 min on ice, samples were centrifuged at 14,000 x g for 10 min at 4 °C and the supernatants transferred to new tubes. Protein concentrations were either measured subsequently or samples were stored at -20 °C.

### Protein quantification

Protein concentrations were measured by using the colorimetric DC Protein Assay which is based on the Lowry method. The samples and the bovine serum albumin standard series ranging from 10 mg/mL to 0.16 mg/mL were prepared in duplicate. The absorbance at 750 nm was measured with a photometer and the standard curve was calculated. The protein concentrations were determined using the calculated standard curve.

# 3.2.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot

For normalization of target to total protein the Smart Protein Layers (SPL) technology was used (Faden et al., 2016). Here, total protein is labeled with SPL fluorophores before electrophoresis as loading control. Antibodies are listed in Tab. 6.

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were mixed with the SPL Reaction and Loading Mix and incubated at 95 °C for 5 min. The mix contains reducing agents and detergents to denature the proteins. Ready to use 4-20 % gradient polyacrylamide gels were placed in electrophoresis chambers filled with tris glycin sodium dodecyl sulfate (TGS) buffer. 15-50  $\mu$ g protein per sample and 12  $\mu$ L calibrator were loaded. The calibrator works as a fluorescent protein weight marker and also as specific standard to compare data derived from different experiments. Gel electrophoresis was conducted at 15 mA for 10 min and subsequently at 25 mA for approximately 30 min until the blue solvent front reached the end of the gel.

### Western Blot

Proteins were transferred to low fluorescent polyvinylidene fluoride (PVDF) membranes using a semi-dry blotting system from BioRad. The PVDF membrane was activated in methanol for about 30 sec and then placed in the blotting device together with filter paper and the gel. Proteins were blotted for 35 min at 0.4 A and the membrane subsequently blocked in 5 % skim milk (in PBS) for 1 h at RT. Primary antibodies were diluted in 5 % skim milk (in PBS) and the membranes were incubated at 4 °C overnight. After three washing steps in 1 % Tween (in PBS) and one in PBS membranes were incubated with secondary antibodies for 1 h at RT.

Detection of chemiluminescence of target protein and fluorescence of total protein and calibrator were performed with the ChemoStar Touch 21.5 Imager. Target signal was normalized to total protein using SPL-LabImage software

### 3.2.3.3 Hepatic triglyceride quantification

For quantification of hepatic triglyceride in livers the BioVision Triglyceride Quantification Colorimetric/Fluorometric Kit was used. 100 mg snap frozen liver tissue was homogenized in 1 mL 5 % NP-40 solution (in dH<sub>2</sub>O). Samples were slowly heated to 80-100 °C for 5 min and then cooled down to room temperature (RT). Heating was repeated to solubilize all triglyceride. Samples were centrifuged for 2 min at top speed to remove any insoluble material and supernatants were diluted 10-fold with dH<sub>2</sub>O. Triglyceride contents were measured followed the manufacturer's instruction of the triglyceride quantification kit.

### 3.2.3.4 Histological staining and histopathological analysis

### Sectioning of paraffin blocks

Paraffin blocks containing the FFPE tissue were cooled on a cooling plate and cut in tissue sections at 3 µm thickness using a microtome. Sections were transferred into a 42 °C warm water bath for smoothing and placed on microscope slides. Both a section of the KO and the control group were applied on each slide. After drying, the sections were stored at RT. Sections were dried at 60 °C for at least 2 h before staining.

### Histological staining

Histological staining including hematoxylin and eosin (H&E), Picrosirius red (PSR), Prussian blue and periodic acid-Schiff (PAS) were conducted in cooperation with the Pathological Institute of the Medical Faculty Mannheim (A. Marx) according to standard protocols.

To examine triglycerides and lipids an Oil Red O (ORO) staining was performed. Liver sections were freshly cut at 8  $\mu$ m thickness in a cryotome, placed on slides and dried for 1 h at RT. Sections were incubated for 5 min in ddH2O and then in 60 % isopropanol (in ddH<sub>2</sub>O) for 5 min. 0.5 g ORO were dissolved in 100 mL isopropanol and 60 mL of the solution were mixed with 40 mL ddH<sub>2</sub>O and filtered. Sections were stained in the ORO solution for 10 min, differentiated by briefly dipping in 60 % isopropanol. After washing in ddH<sub>2</sub>O, slides were counterstained for 90 sec in 50 % Mayer's hemalum solution (in ddH<sub>2</sub>O) and washed with tap water before mounting with aqueous mounting medium.

### Histopathological analysis

H&E stained sections were evaluated by an experienced liver pathologist. Parameters including inflammation, sinusoidal dilation, mitoses, binucleation and bile duct proliferation were assessed using a semiquantitative score ranging from 0 (none) to 3 (severe). To better describe the different levels of collagen deposition the score for fibrosis ranged from 0 (none) to 4 (cirrhosis).
### 3.2.3.5 Immunohistochemistry and Immunofluorescence

#### Preparation of FFPE sections

FFPE sections were cut and dried as described before (Page 52). Sections were deparaffinized in xylene three times for 5 min and rehydrated in a descending alcohol series ranging from 100 % to 70 % ethanol for 3 min each. After washing steps in dH2O and PBS, antigen retrieval was conducted in heat induced epitope retrieval (HIER) citrate buffer pH 6.0 at 95 °C for 45 min. Afterwards slides were cooled down for 20 min in the citrate buffer at RT and re-immersed in PBS for at least 3 min. Tissue sections were surrounded with a hydrophobic barrier followed by two washing steps in PBS for 3 min each.

#### Preparation of cryosections

Cryosections at 8 µm thickness were cut in a cryotome and air dried for 1 h at RT. Hydrophobic barriers were drawn around the tissue sections with a DAKO pen. The sections were fixed for 10 min in 4 % PFA (in PBS) at RT and washed with PBS. For immunofluorescence, sections were blocked with 5 % donkey serum (in PBS) for 30 min at RT.

#### Immunohistochemistry (IHC)

Tissue sections were blocked with peroxidase solution for 10 min at RT, washed in PBS and the primary antibody was incubated over night at 4 °C. After three washing steps in PBS, the HRP-conjugated secondary antibody was incubated for 1 h at RT followed by three washing steps. Sections were incubated with the AEC substrate for 5 to 30 min and counterstained with 1:2 diluted (in  $dH_2O$ ) and filtered Mayer's hemalum solution for 90 sec. Slides were rinsed in tap water and subsequently mounted with aqueous mounting medium. Antibodies are listed in Table 6 and Table 7.

#### Immunofluorescence (IF)

Primary antibodies were diluted in either antibody diluent or 1 % donkey serum (in PBS) and incubated over night at 4 °C. Sections were washed three times in PBS and the fluorochrome-conjugated secondary antibodies were incubated for 45 to 60 min at RT. After three washing steps in PBS sections were mounted with fluorescence mounting medium. Antibodies are listed in Table 6 and Table 7.

#### 3.2.3.6 Fluorescence-activated cell sorting

To analyze the purity isolated LSEC were stained at their surface and flow cytometry was performed by using a BD FACSCanto II. Therefor 5 x 10^5 LSEC were washed with fluorescence activated cell sorting (FACS) buffer (1 % FCS, 0.1 % NaN<sub>3</sub> in PBS) and then incubated with 50  $\mu$ L FcR Blocking Reagent (1:10 in FACS buffer) for 10 min at 4 °C. After washing with FACS buffer cells were stained with fluorochrome-conjugated antibodies (1:200 in FACS buffer) for 20 min at 4 °C in the dark. CD31 and/or Stab2 antibodies were used to identify LSEC while CD11b was used as Kupffer cell marker. LSEC were washed with FACS

buffer and fixed with 100  $\mu$ L 4 % PFA for 10 min at 4 °C in the dark. After washing stained LSEC were stored at 4 °C in the dark until analyzed by flow cytometry. Gata4<sup>LSEC-KO</sup> and control LSEC showed at least 95 % positivity for CD31 and/or Stabilin-2. FlowJo software was used to analyze the data. Antibodies are listed in Table 6.

### 3.2.3.7 Assay for transposase accessible chromatin sequencing

LSEC were isolated as described before followed by ATAC-Seq which was conducted in cooperation with the Anatomy and Developmental Biology Department (G. Dobreva) of the Medical Faculty Mannheim according to a modified protocol from Corces (Corces et al., 2017). 50,000 LSEC were resuspended in 50 µL cold lysis buffer and incubated on ice for 3 min. Cell lysis was stopped by addition of 1 mL resuspension buffer. After centrifugation for 10 min at 500 x g at 4 °C nuclei were incubated in 50 µL transposition mix for 30 min at 37 °C. After transposition fragmented DNA was purified using the ChIP DNA Clean and Concentration Kit. ATAC-Seq librariers were sequenced using the NEB High-Fidelity PCR Master Mix and the Nextera Index Kit. Quality of the libraries was analyzed using the Bioanalyzer High Sensitivity DNA analysis kit and the Agencourt AMPure XP Beads ensured library sizes between 150 and 1000 bp. Equimolar ratios of the libraries were mixed and sequenced on NextSeq550 platform using v2.5 chemistry.

#### Lysis buffer

Tris-HCI (pH 7.4)	10 mM
NaCl	10 mM
MgCl2	3 mM
Igepal CA-630	0.1 %
Resuspension buffer	
Tris-HCI (pH 7.4)	10 mM
NaCl	10 mM
MgCl2	3 mM
Transposition mix	
Nextera Tagment DNA Enzyme TDE	2.5 µL
2x transposition buffer	47,5 μL
Transposition buffer	
Tris-HCI (pH 7.6)	20 mM
MgCl2	10 mM
Dimethylformamide	20 %

### 3.2.3.8 Chromatin immunoprecipitation-qRT-PCR

Approximately 2 x 10<sup>6</sup> isolated LSEC were used for ChIP-qPCR analysis performed in cooperation with the Anatomy and Developmental Biology Department (G. Dobreva) of the Medical Faculty Mannheim according to the protocol from Gutin (Gutin et al., 2018). Fresh isolated LSEC were crosslinked with 1 % formaldehyde for 10 min at RT. By addition of 125 mM glycine crosslinking was stopped. After two washing steps with ice gold PBS cells were resuspended in 10  $\mu$ L Protease Inhibitor Cocktail and stored at -80 °C until further processing.

Before IP cells were lysed and treated with MNase to digest chromatin into nucleosomes or DNA-protein complexes. 4 µg anti c-Myc was added for IP and subsequently crosslinking was reversed. DNA purification was conducted and DNA was used for ChIP-qPCR analysis. Antibody is listed in Table 6.

## 3.2.4 Statistical analysis

Statistical analyses were performed with R.3.6.1 or GraphPad Prism. For comparisons of two samples either Welch's *t*-Test or Mann-Whitney *U*-test was used depending on a normal or not normal distribution. Using the Shapiro-Wilk test normal distribution was evaluated. Statistical testing of more than two samples was done by two-way ANOVA followed by Tukey's post hoc test. Logrank test was used to compare the survival distributions of two samples. Statistical significance was considered for p-values < 0.05. Error bars represent standard deviation (SD).

## 3.2.5 Image acquisition and processing

## 3.2.5.1 Image acquisition

Images were taken using the Nikon Eclipse Ni-E motorized microscope controlled by the NIS-Elements AR software. Fluorescence images were acquired using the Intensilight Epifluorescence Illuminator and the DS-Qi2 high-definition monochrome camera while bright field images were taken using the DS-Ri2 high-definition color camera. Fluorescence images taken as z-stacks were processed with background reduction, deconvolution and focusing.

## 3.2.5.2 Quantification of IF and ISH images

Three representative areas per section were chosen and images acquired. IF images were split into channels and the channel of interest were further processed by setting thresholds and analyzing either number of particles (> 30 pixels) or area. ISH images were taken in RGB format and were split into separate channels by performing color deconvolution with three defined regions (= colors) of interest. Threshold of the images was set displaying the region of interest and either the area or the number of particles (> 30 pixels) were determined.

## 3.2.5.3 Colocalization analysis

For colocalization analysis of duplex ISH sections, colors were defined by negativity for their complementary color. Therefor RGB images were split into their source channels (red, green and blue). In the red channel, area below a defined threshold was defined as blue signal. In the blue channel, area below a defined threshold was taken as red signal, while the green channel was not needed. The colocalization area of the blue and red signal was measured and analyzed by using the "Colocalization threshold" function in ImageJ. To determine colocalization in fluorescence ISH (FISH) sections, images were split into channels. Area above a defined threshold of the two channels of interest were taken and colocalization was analyzed by "Colocalization threshold" function in ImageJ.

## 5 Results

## 5.1 Characterization of Gata4<sup>LSEC-KO</sup> mice

## 5.1.1 Generation of Gata4<sup>LSEC-KO</sup> mice

To analyze the in vivo function of endothelial GATA4 in the adult liver, an EC-specific Cre deleter mouse was needed to induce a late embryonic Gata4 excision. Reporter analyzes of the *Clec4g-icre* driver mice displayed reporter activity in almost all LSEC at E17.5 (Wohlfeil et al., 2019).





(A) Breeding scheme and genotype distribution (n = 598). (B) Kaplan-Meier curve of control and Gata4<sup>LSEC-KO</sup> mice over 360 days (n = 18). (C) PCR of genomic DNA isolated from control (right column), heterozygous (left column) and Gata4<sup>LSEC-KO</sup> livers (middle). Primers flank Gata4 exons 3 and 5 (n = 5). (D-F) qRT-PCR analyses of isolated LSEC of Gata4<sup>LSEC-KO</sup> and control mice using primer pairs spanning (D) exon junction (EJ) 6/7, (E) EJ 4/5 and (F) EJ 2/6 (n = 5). <sup>ns</sup>  $P \ge .05$ ; \*\* P < .01; \*\*\*\* P < .0001; (B) Logrank test; (D, E, F) Mean ± SD, Welch's *t*-test. bp, base pairs; EJ, exon junction; fl, floxed; qRT-PCR, quantitative Real-time polymerase chain reaction; PCR, polymerase chain reaction; SD, standard deviation; wt, wild type. Figure modified from Winkler et al. (Winkler et al., 2020).

Therefore, the combination of the *Clec4g-icre<sup>tg/0</sup>* mice with *Gata4<sup>fl/fl</sup>* mice allowed specific genetic inactivation of GATA4 in adult LSEC. Clec4g-icre<sup>tg/0</sup> x Gata4<sup>fl/fl</sup> (Gata4<sup>LSEC-KO</sup>) mice were born at the expected Mendelian ratio (Figure 6A) and had a life expectancy similar to sibling controls (Figure 6B). In *Gata4<sup>fl/fl</sup>* mice, loxP sites spanned exon 3 to 5 containing the functional Zinc-finger domain. This domain was recombinated in Gata4<sup>LSEC-KO</sup> mice leading to the expression of non-functional Gata4. To prove the recombination of Gata4 in the genome of Gata4<sup>LSEC-KO</sup> mice, primers were designed binding left and right of the loxP sites. PCR analysis demonstrated a DNA construct of 2132 bp in control mice while the DNA construct of Gata4<sup>LSEC-KO</sup> mice was smaller than 400 bp based on the recombination (Figure 6C). qRT-PCR analysis of isolated LSEC demonstrated similar expression levels of the Gata4 region containing exon junction 6 to 7 (EJ6/7) in both groups (Figure 6D) while Gata4 mRNA containing exon junction 4 to 5 (EJ4/5) was significantly reduced in LSEC of Gata4<sup>LSEC-KO</sup> mice (Figure 6E). Furthermore, primers spanning exon junction 2 to 6 (EJ2/6) resulting from cre recombination at the loxP sites showed a highly significant increase of recombinated Gata4 in Gata4<sup>LSEC-KO</sup> LSEC (Figure 6F). Thus, Gata4 deficiency was demonstrated by both recombination PCR and gRT-PCR.

The susceptibility to animal experiments (e.g. regeneration after CCl4-induced hepatotoxic injury) can vary widely between mouse strains due to the genetic background (Scholten et al., 2015). Clec4g-icre mice were mated with Gata4<sup>fl/fl</sup> mice to generate Gata4<sup>LSEC-KO</sup> animals. As both mice strains have different backgrounds – Clec4g-icre mice are on a C57BL/6N background, while Gata4<sup>fl/fl</sup> mice have a mixed B6N;129S1sv background – littermate controls were used for all experiments and a genetic background analysis for one representative litter was performed. Here, single nucleotide polymorphisms (SNP) of the experimental mice were compared with SNP of the 129S1SvImJ and the C57BL/6N strains. Gata4<sup>LSEC-KO</sup> mice showed a 71.7 % similarity of SNP with 129S1SvImJ and a 67.4 % similarity with C57BL/6N mice (Table 15). These similarities were comparable to those of the control littermates (73.1 % 129S1SvImJ and 67.6 % C57BL/6N) (Table 15). The minor heterogeneity in the respective groups was also similar. It can be therefore excluded that the genotype had an influence on the experiments.

#### Table 15: Genetic background analysis of control, heterozygous and Gata4<sup>LSEC-KO</sup> mice.

Percent Match of single nucleotide polymorphisms of experimental mice to reference allelic profiles of 129S1SvImJ and C57BL/6N strains. fl, floxed; HET, heterozygous; KO, knock out; tg, transgene; WT, wild type. Table modified from Winkler et al. (Winkler et al., 2020).

Sample ID #	Experimental	icre	Gata4	Status	129S1SvImJ	C57BL/6N
	Group					
01	Gata4 <sup>LSEC-KO</sup>	tg/0	fl/fl	КО	70.9 %	67.3 %
02	Gata4 <sup>LSEC-KO</sup>	tg/0	fl/fl	КО	75.1 %	66.4 %
03	Gata4 <sup>LSEC-KO</sup>	tg/0	fl/fl	КО	70.2 %	68.5 %
04	Control	0/0	fl/fl	WT	73.4 %	68.6 %
05	not in experiment	tg/0	wt/fl	HET	73.6 %	64.9 %
06	Gata4 <sup>LSEC-KO</sup>	tg/0	fl/fl	КО	71.2 %	67.1 %
07	Control	0/0	fl/fl	WT	72.9 %	68.6 %
08	Gata4 <sup>LSEC-KO</sup>	tg/0	fl/fl	КО	71.3 %	67.5 %
09	Control	0/0	fl/fl	WT	70.7 %	71.5 %
10	Control	0/0	fl/fl	WT	75.3 %	61.7 %
11	not in experiment	tg/0	wt/fl	HET	71.2 %	68.5 %
12	parental (mother)	0/0	fl/fl	parental	76.7 %	56.0 %
13	parental (mother)	0/0	fl/fl	parental	78.2 %	56.4 %
14	parental (father)	tg/0	wt/fl	parental	65.0 %	80.2 %
			Mean WT Mean KO Mean Het		73.1 %	67.6 %
					71.7 %	67.4 %
					72.4 %	66.7 %

#### 5.1.2 Macroscopy and liver weight of Gata4<sup>LSEC-KO</sup> mice

Livers of both 3-month-old female and male Gata4<sup>LSEC-KO</sup> mice were macroscopically altered and showed signs of liver fibrosis (Figure 7A). The bodyweight of Gata4<sup>LSEC-KO</sup> animals was unchanged compared to control siblings in both sexes (Figure 7B), while liver weight and liver to body weight ratio were significantly reduced in Gata4<sup>LSEC-KO</sup> animals (Figure 7C, D).



Figure 7: Gata4<sup>LSEC-KO</sup> livers show macroscopic irregularities and have reduced liver weights. (A) Macroscopic images of livers of control and Gata4<sup>LSEC-KO</sup> mice. (B, C, D) (B) Body weight, (C) liver weight and (D) liver to body weight ratio of female (n = 13-16) and male (n = 9-10) control and Gata4<sup>LSEC-KO</sup> mice. <sup>ns</sup>  $P \ge .05$ ; \* P < .05, \*\* P < .01; \*\*\* P < .001; (B, C, D) Mean ± SD, Welch's *t*-test. SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

Measurements of liver values in the plasma revealed significant higher levels of ALT, AST and GLDH as well as a reduction of total protein in 3-month-old Gata4<sup>LSEC-KO</sup> mice indicating a liver damage with impairment of hepatocytes (Figure 8A-D). Furthermore, glucose, cholesterol, triglycerides and urea were significantly reduced in Gata4<sup>LSEC-KO</sup> animals while creatinine levels remained unchanged (Figure 8E-J).



#### Figure 8: Plasma levels indicate liver damage in Gata4<sup>LSEC-KO</sup> mice.

(A, B, C, D, E, F, G, H, J) Levels of (A) ALT, (B) AST, (C) GLDH, (D) total protein, (E) glucose, (F) cholesterol, (G) triglycerides, (H) urea and (J) creatinine in blood plasma of control and Gata4<sup>LSEC-KO</sup> mice (n = 12-19). <sup>ns</sup>  $P \ge .05$ ; \* P < .05, \*\* P < .01; \*\*\* P < .001; (A, B, D, F, H) Mean ± SD, Welch's *t*-test; (C, E, G, J) Mean ± SD, Mann-Whitney *U*-test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLDH, glutamate dehydrogenase; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

# 5.1.3 Histology anomalies and activated hepatic stellate cells in Gata4<sup>LSEC-KO</sup> mice

Standard staining of 3-month-old livers was conducted to examine the liver histology. PSR positive collagen fibers were markedly increased in Gata4<sup>LSEC-KO</sup> livers and revealed noticeable liver fibrosis with a perisinusoidal distribution pattern in the Gata4<sup>LSEC-KO</sup> livers (Figure 9A). The amount of red dye was significantly increased (Figure 9C) and the enhanced degree of fibrosis was also confirmed by a board-certified pathologist (Figure 9D). H&E staining demonstrated a reduced hepatocyte size as wells as increased levels of inflammation and sinusoidal dilatation (Figure 9B, E-G). Moreover, Gata4<sup>LSEC-KO</sup> livers showed enhanced mitoses and binucleated

hepatocytes (Figure 9H, J). So called "ductular reactions" are characterized by the proliferation of reactive bile ducts induced by liver disorders and are associated with liver fibrosis (Sato et al., 2019). These ductular reactions were observed more frequently in livers of Gata4<sup>LSEC-KO</sup> compared to control (Figure 9K). Further histological analysis by PAS and ORO demonstrated no changes, while Prussian blue staining revealed increased iron deposition in Gata4<sup>LSEC-KO</sup> livers (Figure 9L-N). Quantification of hepatic triglyceride levels did not show alterations (Figure 9O).





(A, B) (A) PSR and (B) H&E staining of livers (n = 6). (C) Quantification of PSR staining (n = 6). (D, E) Histological evaluation of (D) fibrosis and (E) inflammation in control and Gata4<sup>LSEC-KO</sup> livers (n = 6, 7). (F) Quantification of cells per mm<sup>2</sup> in livers (n = 6). (G, H, J, K) Histological evaluation of (G) sinusoidal dilatation, (H) mitoses, (J) binucleation and (K) bile duct proliferation (n = 6, 7). (L, M, N) (L) PAS, (M) ORO and (N) Fe staining of control and Gata4<sup>LSEC-KO</sup> livers (n = 6). (O) hepatic triglyceride levels in liver tissue of control and Gata4<sup>LSEC-KO</sup> mice (n = 5). Scale bars: 50 µm (A, B, L, M, N); <sup>ns</sup>  $P \ge .05$ ; \* P < .05, \*\* P < .01; (C, F, O) Mean ± SD, Welch's *t*-test; (D, E, G, H, J, K) Median, Mann-Whitney *U*-test. Fe, iron; H&E, hematoxylin and eosin; ORO, oil red O; PAS, periodic acid-Schiff; PSR, picrosirius red; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020). Postnatal (P) 1, P8 and 6-week-old Gata4<sup>LSEC-KO</sup> livers were investigated to exclude that the endothelial Gata4 deficiency impaired liver development. Therefore, livers were macroscopically examined, body and liver weight were measured as well as H&E and PSR staining were conducted. Gata4<sup>LSEC-KO</sup> mice had body weights comparable to control mice at any time in the development (Figure 10A, C, E). Liver weight was lower at P1 and after 6 weeks, whereas liver to body weight ratio was only significantly reduced in 6-week-old Gata4<sup>LSEC-KO</sup> animals (Figure 10A, C, E). Macroscopically, the P1 and P8 livers of Gata4<sup>LSEC-KO</sup> animals showed only minimal anomalies, while 6-week-old livers showed more obvious macroscopic irregularities, which were not as distinct as in 3-month-old mice (Figure 10B, D, F). Differentiation of hematopoietic stem cells starts in the fetal liver and continuous then in the bone marrow. Thus, H&E staining of both control and Gata4<sup>LSEC-KO</sup> livers showed multiple clusters of hematopoietic cells at P1. The amount of hematopoietic was noticeable reduced in control livers at P8, while it remained on a similar level in Gata4<sup>LSEC-KO</sup> livers compared to P1. 6-week-old livers of both genotypes demonstrated a highly organized liver architecture without any hematopoietic equivalent to the mature livers at 3-month age. P1 livers did not show histological signs of fibrosis upon PSR staining, whereas PSR levels slightly increased in P8 Gata4<sup>LSEC-KO</sup> livers (Figure 10A-D). The fibrosis continued to increase in the 6-week-old livers but remained below the values from 3-month-old Gata4<sup>LSEC-KO</sup> livers (Figure 10E, F). In summary, these results demonstrate that liver development is not affected by endothelial Gata4 deficiency – merely the relocation of hematopoiesis seems to be slightly delayed. Furthermore, liver fibrosis in Gata4<sup>LSEC-KO</sup> animals develops postnatal as the liver matures.



#### Figure 10: Gata4<sup>LSEC-KO</sup> mice show no defects in liver development.

(A, C, E) Analyses of body weight, liver weight, liver to body weight ratio and quantification of PSR staining of livers of control and Gata4<sup>LSEC-KO</sup> mice at the age of (A) P1 (n = 8, 16), (C) P8 (n = 4,8) and

(E) 6 weeks (n = 5,10). (B, D, F) representative macroscopic image, H&E and PSR staining of control and Gata4<sup>LSEC-KO</sup> livers of mice aged (B) 1 day, (D) 8 days and (F) 6 weeks. Scale bars: 0.5 cm (macroscopic images in B, D), 1 cm (macroscopic images in F), 50 µm (H&E, PSR staining); <sup>ns</sup>  $P \ge .05$ ; \* P < .05, \*\*\*\* P < .0001; (A, C, E) Mean ± SD, Welch's *t*-test. H&E, hematoxylin and eosin; P, postnatal; PSR, picrosirius red; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

To exclude that the phenotype of Gata4<sup>LSEC-KO</sup> mice aggravates with increasing age, livers of 6- and 12-month-old Gata4<sup>LSEC-KO</sup> and control mice were examined. The differences in liver weight as well as liver to body weight ratio have levelled out at the age of 6 month and also remained on comparable levels in 12-month-old mice (Figure 11A, C). Moreover, the phenotype of Gata4<sup>LSEC-KO</sup> livers regarding histology and microscopy did not deteriorate with ageing (Figure 11B, D). Therefore, further analyzes were conducted at the age of 3 month.





(A, C) Analyses of body weight, liver weight, liver to body weight ratio and quantification of PSR staining of livers of control and Gata4<sup>LSEC-KO</sup> mice aged (A) 6 months (n = 5-9) and (C) 12 months (n = 5-9). (B, D) macroscopic liver image, H&E and PSR staining of livers of control and Gata4<sup>LSEC-KO</sup> at the age of (B) 6 months and (D) 12 months. Scale bars: 1 cm (macroscopic images), 50 µm (H&E, PSR staining); n<sup>s</sup>  $P \ge .05$ ; \* P < .05, \*\* P < .01; (A (body weight, liver weight, liver to body weight ratio), C (body weight, liver weight)) Mean ± SD, Mann-Whitney *U*-test; (A (PSR), C (liver to body weight ratio, PSR) Mean ± SD, Welch's *t*-test. H&E, hematoxylin and eosin; PSR, picrosirius red; SD, standard deviation.

As shown before, deposition of collagen fibers was significantly increased in Gata4<sup>LSEC-KO</sup> livers (Figure 9A, C). Immunohistochemical staining confirmed increased deposition of collagen I, III and IV in Gata4<sup>LSEC-KO</sup> animals (Figure 12A-C). PIIINP is generated during synthesis of collagen III and can be regarded as indicator for liver fibrosis (Gudowska et al., 2017). Elevated PIIINP levels were measured in plasma of Gata4<sup>LSEC-KO</sup> mice (Figure 12E). In liver fibrosis, HSC get activated into a myofibroblast like phenotype that is proliferative and fibrogenic (Lee

and Friedman, 2011). Hence, HSC are the major producers of ECM in normal and fibrotic livers. By measuring expression levels of fibrosis markers like *Actin alpha 2 (Acta2), Col1a1, Col3a1, desmin (Des)* and platelet derived growth factor receptor beta (*Pdgfrb*), activation of HSC was demonstrated (Figure 12F). Also, ISH of HSC marker *Pdgfrb* revealed an increased number of HSC in Gata4<sup>LSEC-KO</sup> livers compared to controls (Figure 12D, G).



Figure 12: Activated HSC are the main source of collagens in Gata4<sup>LSEC-KO</sup> livers.

(A, B, C, D) Representative immunohistochemical staining of collagen (A) I, (B) III and (C) IV and (D). ISH of *Pdgfrb* in control and Gata4<sup>LSEC-KO</sup> livers. (E) Analysis of PIIINP in plasma of control and Gata4<sup>LSEC-KO</sup> mice by ELISA (n = 12). (F) qRT-PCR analysis of stellate cell activation marker genes *Acta2, Col1a1, Col3a1, Des* and *Pdgfrb* in whole liver lysates of control and Gata4<sup>LSEC-KO</sup> livers (n = 6). (G) Quantification of *Pdgfrb* ISH from (D) (n = 6). Scale bars: 50 µm (A, B, C, D); <sup>ns</sup>  $P \ge .05$ ; \* P < .05; (E) Mean ± SD, Mann-Whitney *U*-test; (F, G) Mean ± SD, Welch's *t*-test. Acta2, actin alpha 2; Col1a1, collagen type I alpha 1 chain; Col3a1, collagen type III alpha 1 chain; Des, desmin; ELISA, enzyme-linked immunosorbent assay; HSC, hepatic stellate cells; ISH, in situ hybridization; PIIINP, procollagen type III N-terminal propeptide; qRT-PCR, quantitative Real-time polymerase chain reaction; Pdgfrb, platelet derived growth factor receptor beta; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

## 5.1.4 Apoptosis, proliferation and inflammation in Gata4<sup>LSEC-KO</sup> livers

Immunofluorescence staining of proliferation marker Ki-67 revealed a significant increase of proliferating cells in Gata4<sup>LSEC-KO</sup> livers (Figure 13A, B). Co-IF demonstrated partial coexpression with EC marker Podocalyxin. However, there were also hepatocytes that were Ki67-positive. Moreover, significantly more apoptotic cells – primarily hepatocytes – per mm<sup>2</sup> of liver could be detected in Gata4<sup>LSEC-KO</sup> mice using apoptosis marker cleaved Caspase 3 (c-Caspase-3) (Figure 13C, D).





(A, B, C, D) Immunofluorescent staining of (A) proliferation marker Ki-67 and (C) apoptosis marker cleaved Caspase 3 (c-Caspase-3) with endothelial marker Podocalxyin in livers and quantification of (B) Ki-67 and (D) c-Caspase-3 in control and Gata4<sup>LSEC-KO</sup> livers (n = 6). (E, F) (E) IF and (F) quantification of T-lymphocytes marker CD3 with co-staining for Cadherin-5 (n = 6). (G, H) (G) Immunohistochemical staining and (H) quantification of macrophage marker CD68 (n = 6). Scale bars: 100 µm (A, C), 50 µm (E, G); \*\* P < .01, \*\*\* P < .001; (B, D) Mean ± SD, Mann-Whitney *U*-test; (F, H) Mean ± SD, Welch's *t*-test. Figure modified from Winkler et al. (Winkler et al., 2020).

Further analysis demonstrated that the increased inflammatory infiltrates of Gata4<sup>LSEC-KO</sup> livers contained CD3+ T-lymphocytes (Figure 13E, F). Also, an increased CD68+ area was observed in Gata4<sup>LSEC-KO</sup> livers indicating an elevated number of macrophages (Figure 13G, H).

### 5.1.5 LSEC-to-continuous endothelial cell transdifferentiation

To investigate Gata4 mediated gene expression changes in LSEC, gene expression profiling was performed using Affymetrix microarrays. Therefore, LSEC were isolated from Gata4<sup>LSEC-KO</sup> and control mice by density gradient centrifugation followed by magnetic activated cell sorting (adapted from (Diehl et al., 2008)) and purity of isolated LSEC was verified by FACS analysis. Gene expression profiling revealed 403 genes significantly dysregulated in LSEC of Gata4<sup>LSEC-KO</sup> mice compared to controls (Figure 14). Analyzing gene sets of LSEC and continuous EC showed a downregulation of discontinuous/sinusoidal genes (e.g. Wnt2, Maf) and an induction of continuous EC-associated genes (e.g. Cd34, Emcn) (Figure 14). This switch indicates a LSEC-to-continuous endothelial cell transdifferentiation in Gata4<sup>LSEC-KO</sup> mice



#### Figure 14: Endothelial Gata4 deficiency causes LSEC-to-CEC transdifferentiation.

Gene expression profiling data of isolated LSEC from control and Gata4<sup>LSEC-KO</sup> mice. 403 significant differentially regulated genes (*Padj* < 0.05) are demonstrated in the heat map (n = 5). P = 0.0200 (LSEC gene set – green marks), P = 0.0011 (CEC gene set – red marks); Fisher's exact test. CEC, continuous endothelial cells; LSEC, liver sinusoidal endothelial cells. Figure modified from Winkler et al. (Winkler et al., 2020).

and that Gata4 is a driver for discontinuous and sinusoidal differentiation of LSEC also in the adult liver.

#### 5.1.6 Sinusoidal capillarization and formation of a basement membrane

Capillarization of liver sinusoids is characterized by the lack of LSEC fenestration as well as the development of a basement membrane and is accompanied by alterations in gene expression in LSEC. Therefore, ISH, western blot analysis and co-immunofluorescent staining





(A, B, C) ISH of (A) LSEC marker *Stab2* and (B) continuous EC marker *Cd34* or (C) immunofluorescent staining of Endomucin (continuous EC) and LYVE-1 (LSEC) in control and Gata4<sup>LSEC-KO</sup> livers. (D, E) Quantification of (D) *Stab2* ISH in (A) and (E) *Cd34* ISH in (B) (n = 6). (F) ELISA for hyaluronic acid in blood plasma of control and Gata4<sup>LSEC-KO</sup> mice (n = 12). (G) Quantification of Endomucin in (C) (n = 5). (H) Western blot analysis and quantification of LYVE-1 in primary LSEC of control and Gata4<sup>LSEC-KO</sup> mice (n = 6). Scale bars: 50 µm (A, B), 100 µm (C); \* P < .05, \*\* P < .01, \*\*\* P < .001; (D, E, G, H) Mean ± SD, Welch's *t*-test; (F) Mean ± SD, Mann-Whitney *U*-test. EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; ISH, in situ hybridization; LSEC, liver sinusoidal endothelial cell; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

were conducted to investigate the expression of LSEC- and continuous EC-associated markers. ISH displayed a significant reduction of the LSEC marker *Stab2* in Gata4<sup>LSEC-KO</sup> livers (Figure 15A, D). Stab2 is a scavenger receptor important for clearance of circulating hyaluronan. Enhanced hyaluronic acid levels were measured in the plasma of Gata4<sup>LSEC-KO</sup> mice, confirming the significant *Stab2* reduction (Figure 15F). The down-regulation of LSEC marker Lyve1 was also determined by staining and western blot analysis in these mice (Figure 15C, H). However, expression of continuous EC markers *Cd34* and Endomucin were significantly increased in Gata4<sup>LSEC-KO</sup> livers (Figure 15B, C, E, G). These results further confirm capillarization of the sinusoids in livers of Gata4<sup>LSEC-KO</sup> mice.

Transmission electron microscopy showed the formation of a subendothelial basement membrane in hepatic microvessels of Gata4<sup>LSEC-KO</sup> mice and the loss of endothelial fenestrae also confirmation of the sinusoidal capillarization (Figure 16B). Furthermore, in the space of Disse deposition of collagen bundles were observed as well as a significant reduction of microvilli of hepatocytes (Figure 16A).



## Figure 16: Endothelial *Gata4* deficiency causes the loss of endothelial fenestrae and the formation of a subendothelial basement membrane.

(A, B) Representative TEM overviews and details of control and Gata4<sup>LSEC-KO</sup> livers (n = 2,3). Scale bars: 3 µm. Red arrows: endothelial fenestrae. Red arrowheads: subendothelial basement membrane. Col, collagen; MV, microvilli; SoD, space of Disse; TEM, transmission electron microscopy. Figure modified from Winkler et al. (Winkler et al., 2020).

## 5.1.7 Angiocrine signaling and metabolic zonation in Gata4<sup>LSEC-KO</sup> mice

Since LSEC regulate neighboring cells by LSEC-specific angiokines e.g. Hgf, Wnt-ligands and Bmp2, ISH were conducted to investigate if angiocrine signaling is impaired in Gata4<sup>LSEC-KO</sup> livers. ISH of the Wnt-ligands *Wnt2* and *Wnt9b* revealed a reduction of their expression in Gata4<sup>LSEC-KO</sup> livers (Figure 17A, B, C, D). Axin2 is a transcriptional target of β-catenin dependent Wnt signaling and in line with the *Wnt2* and *Wnt9b* reduction pericentral hepatocytes expressed less *Axin2* in Gata4<sup>LSEC-KO</sup> livers (Figure 17E, F). Furthermore, the secretion of Wnt-ligands is required for metabolic liver zonation (Leibing et al., 2018). Glutamine synthetase (GS) is regularly expressed in hepatocytes adjacent to central vein EC and can be regarded as marker of the metabolic liver zonation. In Gata4<sup>LSEC-KO</sup> livers GS expression was significantly reduced, indicating impaired metabolic liver zonation (Figure 17G, H). On the other hand, endothelial pericentral marker *Rspo3* expression was slightly increased (Figure 17J, K) while *Hgf* expressed by LSEC, was also downregulated in Gata4<sup>LSEC-KO</sup> livers (Figure 17N, O) (Geraud et al., 2010).



## Figure 17: Endothelial *Gata4* deletion leads to impaired angiocrine signaling and disturbed metabolic liver function.

(A, B, C, D, E, F, G, H) ISH and quantification of (A, B) *Wnt2*, (C, D) *Wnt9b* and (E, F) *Axin2* as well as (G, H) immunohistochemical staining and quantification of Glutamine synthetase (GS) in control and Gata4<sup>LSEC-KO</sup> livers (n = 6). (J, K, L, M, N, O) ISH and quantification of (J, K) *Rspo3*, (L, M) *Hgf* and (N, O) *Bmp2* (n = 6). Scale bars: 50 µm (A, C, E, G, J, L, N); <sup>ns</sup>  $P \ge .05$ ; \* P < .05, \*\* P < .01, \*\*\*\* P < .0001; (B, D, F, H, K, M, O) Mean ± SD, Welch's *t*-test. Hgf, hepatic growth factor. Figure modified from Winkler et al. (Winkler et al., 2020).

Moreover, RNA was extracted from whole livers of control and Gata4<sup>LSEC-KO</sup> mice and gene expression analysis were performed for further investigations. GSEA of these microarray data revealed that several metabolic pathways e.g. fatty acid metabolism, bile acid metabolism, oxidative phosphorylation and xenobiotic metabolism were suppressed in Gata4<sup>LSEC-KO</sup> livers (Figure 18). Furthermore, GSEA confirmed previous results by showing that apoptosis as well as inflammatory response was activated in Gata4<sup>LSEC-KO</sup> livers in comparison to control livers (Figure 18).



#### Figure 18: Endothelial Gata4 deficiency causes changes in several pathways.

GSEA of microarray data from whole liver lysates of control and Gata4<sup>LSEC-KO</sup> mice (n = 10,11). GSEA, Gene set enrichment analysis. Figure modified from Winkler et al. (Winkler et al., 2020).

#### 5.1.8 Identification of profibrotic angiocrine factors

As mentioned earlier, gene expression profiling yielded 403 significantly dysregulated genes in *Gata4* deficient LSEC (Figure 14). Among them we found known angiocrine factors as well as extracellular matrix genes. Moreover, "novel" angiocrine factors such as platelet derived growth factor subunit B (Pdgfb), insulin like growth factor binding protein 5 (lgfbp5), secreted protein acidic and rich in cysteine-like protein 1(Sparcl1) and endothelial cell specific molecule 1 (Esm1), known to be involved in liver fibrogenesis, were identified. Dysregulation of these genes of interests in LSEC of Gata4<sup>LSEC-KO</sup> mice were confirmed by gRT-PCR. Angiocrine factors apelin (ApIn), Esm1, Igfbp5 and Pdgfb were significantly upregulated in Gata4<sup>LSEC-KO</sup> LSEC (Figure 19A). Apln as well as Pdgfb are angiogenesis related genes and their upregulation confirmed GSEA of whole liver samples showing an activation of angiogenesis associated genes (Figure 18). However, expression of angiocrine factors Bmp2 and Wnt2 known to regulate the function of hepatocytes were reduced (Figure 19A). Wnt9b downregulation was demonstrated by ISH (Figure 17C, D) but could not be confirmed by qRT-PCR from Gata4<sup>LSEC-KO</sup> LSEC compared to control LSEC (Figure 19A). For qRT-PCR analysis all isolated hepatic EC were used, so that a reduction of Wnt9b, which is only expressed by pericentral LSEC and central vein EC, could get lost. In line with the pro-fibrotic switch in Gata4<sup>LSEC-KO</sup> LSEC, extracellular matrix genes such as collagens (Col15a1, Col4a1 and Col4a2), the laminins (Lamb1 and Lamc1), Sem7a and Sparcl1 were significantly upregulated in Gata4 deficient LSEC (Figure 19B). The expression of the fibrosis candidates was also visualized by ISH and the quantification of the ISH confirmed the altered gene expression in Gata4<sup>LSEC-KO</sup> livers. (Figure 19C-K). ISH of *Pdgfb* (Figure 19C) revealed a homogeneous distribution pattern comparable to the endothelial *Bmp2* expression (Figure 17N). To identify the source of *Pdgfb* in Gata4<sup>LSEC-KO</sup> livers, multiplex fluorescent ISH were conducted. Therefore, co-expression of Pdgfb and EC marker Cdh5 as well as HSC marker Pdgfrb was analyzed.





(A, B) ) qRT-PCR analysis of (A) angiocrine factors and (B) extracellular matrix genes in murine LSEC of control and Gata4<sup>LSEC-KO</sup> mice (n = 5). (C, D, E, F, G, H, J, K) ISH and quantification of (C, D) *Pdgfb*, (E, F) *Sparcl1*, (G, H) *Esm1* and (J, K) *Igfbp5* in control and Gata4<sup>LSEC-KO</sup> livers (n = 6). Scale bars: 50 µm (C, E, G, J); \*\* P < .01, \*\*\*\* P < .001, \*\*\*\* P < .0001; (A, B, D, F, H, K) Mean ± SD, Welch's *t*-test. Esm1, endothelial cell specific molecule 1; Igfbp5, insulin like growth factor binding protein 5; ISH, in situ hybridization; LSEC, liver sinusoidal endothelial cell; Pdgfb, platelet derived growth factor subunit B; qRT-PCR, quantitative Real-time polymerase chain reaction; SD, standard deviation; Sparcl1, secreted protein acidic and rich in cysteine like protein 1. Figure modified from Winkler et al. (Winkler et al., 2020).



#### Figure 20: LSEC are the main source of Pdgfb in Gata4<sup>LSEC-KO</sup> mice.

(A, B) Fluorescence ISH of *Pdgfb* with (A) EC marker *Cdh5* or (B) stellate cell marker *Pdgfrb* in control and Gata4<sup>LSEC-KO</sup> livers. (C) Quantification of colocalization area of *Pdgfb* and *Cdh5* or of *Pdgfb* and *Pdgfrb* in relation to *Pdgfb*+ area (n = 6). Scale bars: 100 µm (A, B); \*\*\*\* P < .0001; (C) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. Cdh5, cadherin 5; EC, endothelial cells; ISH, in situ hybridizationPdgfb, platelet derived growth factor subunit B; Pdgfrb, platelet derived growth factor receptor beta. Figure modified from Winkler et al. (Winkler et al., 2020).

Fluorescence ISH demonstrated that *Cdh5*+ endothelial cells are the main source of *Pdgfb*, while there was nearly no co-expression with *Pdgfrb*+ cells in Gata4<sup>LSEC-KO</sup> mice (Figure 20A, B). Quantification confirmed the significant colocalization of *Pdgfb* and *Cdh5* in Gata4<sup>LSEC-KO</sup> livers (Figure 20C).

In contrast, Duplex-ISH demonstrated that *Sparcl1* was expressed by both *Cdh5*+ EC and *Pdgfrb*+ HSC in Gata4<sup>LSEC-KO</sup> mice (Figure 21A-C). Upregulation of *Col1a1* was demonstrated by IHC and qRT-PCR previously (Figure 12A, F) and ISH revealed an increased number of *Pdgfrb* expressing HSC in Gata4<sup>LSEC-KO</sup> livers (Figure 12D, G) indicating HSC activation. In line with these results, Duplex-ISH showed that *Pdgfrb*+ HSC were the main source of *Col1a1* by distinct colocalization (Figure 21D-F).



#### Figure 21: Angiocrine factor Sparcl1 is expressed by LSEC and stellate cells.

(A, B) Duplex ISH of *Sparcl1* with (A) EC marker *Cdh5* or (B) stellate cell marker *Pdgfrb* in control and Gata4<sup>LSEC-KO</sup> livers. (C) Quantification of colocalization area of *Sparcl1* and *Cdh5* or of *Sparcl1* and *Pdgfrb* in relation to *Sparcl1*+ area (n = 6). (D, E) Duplex ISH of *Col1a1* with (D) *Cdh5* or (E) *Pdgfrb* in control and Gata4<sup>LSEC-KO</sup> livers. (F) Quantification of colocalization area of *Col1a1* and *Cdh5* or *Col1a1* and *Cdh5* or *Col1a1* and *Pdgfrb* in relation to *Col1a1*+ area (n = 6). Scale bars: 50 µm (A, B, D, E); \*\*\* P < .001; \*\*\*\* P < .001; (C, F) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. Cdh5, cadherin 5; Col1a1, collagen type I alpha 1 chain; EC, endothelial cell; ISH, in situ hybridization; Pdgfrb, platelet derived growth factor receptor beta; SD, standard deviation; Sparcl1, secreted protein acidic and rich cysteine like protein 1. Figure modified from Winkler et al. (Winkler et al., 2020).

#### 5.1.9 Activation of transcription factor Myc and its downstream targets

Among the 403 identified genes with altered gene expression in Gata4<sup>LSEC-KO</sup> LSEC were also several transcription factors (Figure 22A). Four of them were significantly downregulated (e.g. *Maf*) while 13 transcription factors were upregulated in LSEC of Gata4<sup>LSEC-KO</sup> mice (e.g. *Myc* and *Id1*) (Figure 22A). Moreover, GSEA of hallmark gene sets revealed altered transcriptomic processes in LSEC of Gata4<sup>LSEC-KO</sup> livers (Figure 22B). Besides angiogenesis-associated genes, MYC target genes were strongly activated in Gata4<sup>LSEC-KO</sup> isolated LSEC (Figure 22B). Further investigations confirmed increased endothelial Myc expression by ISH and qRT-PCR in Gata4<sup>LSEC-KO</sup> livers (Figure 22C-E).



## Figure 22: Endothelial *Gata4* deletion leads to activation of transcription factor *Myc* and its downstream tragets.

(A) Gene expression profiling data of isolated LSEC from control and Gata4<sup>LSEC-KO</sup> mice. Heat map of significant differentially regulated transcription factors (n = 5). (B) Gene set enrichment analysis (GSEA) of microarray data from isolated LSEC of control and Gata4<sup>LSEC-KO</sup> mice using MSigDB hallmark gene set collection (n = 5). (C, D) (C) ISH and (D) quantification of transcription factor *Myc* in control and Gata4<sup>LSEC-KO</sup> livers (n = 6). (E) qRT-PCR analysis of *Myc* in primary LSEC of control and Gata4<sup>LSEC-KO</sup> mice (n = 6). Scale bars: 50 µm (C); \*\* P < .01; \*\*\*\* P < .0001; (D, E) Mean ± SD, Welch's *t*-test. ISH, in situ hybridization; LSEC, liver sinusoidal endothelial cell; MSigDB, molecular signatures database; qRT-PCR, quantitative Real-time polymerase chain reaction; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020). In line with this, Enane and colleagues demonstrated that GATA4 antagonized MYC in hepatocytes (Enane et al., 2017). Furthermore, it has been described that Myc interacts with inflammatory mediators and plays thereby a central role in fibrosis, cirrhosis and liver cancer (Liu et al., 2015).

#### 5.1.10 Alterations in chromatin accessibility in Gata4<sup>LSEC-KO</sup> LSEC

To investigate how endothelial Gata4 controls gene expression in LSEC, LSEC of Gata4<sup>LSEC-KO</sup> and control mice were isolated and assay for transposase-accessible chromatin sequencing (ATAC-Seq) was performed to analyze chromatin accessibility. ATAC-Seq demonstrated increased chromatin accessibility at multiple gene loci in Gata4<sup>LSEC-KO</sup> LSEC.

ATAC-Seq on the *Myc* and *Pdgfb* gene loci were compared with genome tracks of GATA4 ChIP-Seq and histone marker H3K4me3 and H3K27Ac ChIP-Seq (Figure 23A, B). H3K4me3 is a histone modification highly enriched at active promoters near the transcription start sites (TSS) while H3K27Ac is associated with the upregulation of genes and is regarded as an active enhancer mark. Mapping these data demonstrated that *Myc* and *Pdgfb* gene loci might be directly bound by GATA4 and showed also increased chromatin accessibility in Gata4<sup>LSEC-KO</sup> LSEC (Figure 23A, B).

Combination of the gene expression profiling and ATAC-Seq data demonstrated significantly more ATAC-Seq reads around the TSS of genes significantly upregulated in Gata4<sup>LSEC-KO</sup> LSEC indicating a highly increased chromatin accessibility for these genes (Figure 23C). In contrast, chromatin accessibility was not altered for genes significantly downregulated in Gata4<sup>LSEC-KO</sup> LSEC (Figure 23C). These results suggest that GATA4 mediates its repressive function in contrast to its activating function on chromatin level.

MYC binding sites at the promoter region of the Pdgfb gene were identified by combining published MYC ChIP-Seq data of the liver (Kress et al., 2016a) and the ATAC-Seq peaks differentially regulated in LSEC of Gata4<sup>LSEC-KO</sup> livers (Figure 23B). This indicates that MYC might directly activate Pdgfb transcription in Gata4<sup>LSEC-KO</sup> LSEC. To confirm this assumption LSEC of control and Gata4<sup>LSEC-KO</sup> mice were isolated and MYC ChIP-qPCR was conducted and revealed an increased binding of MYC at the *Pdgfb* promotor in Gata4<sup>LSEC-KO</sup> LSEC compared to control LSEC (Figure 23D) indicating a direct amplification of *Pdgfb* expression by MYC in Gata4<sup>LSEC-KO</sup> livers. In line with this, Myc has recently been suggested to be a nonlinear amplifier of expression, acting universally at active genes (Nie et al., 2012).



Figure 23: Endothelial GATA4 controls gene expression by regulating chromatin accessibility.

(A, B) Genome tracks of GATA4 ChIP-Seq (Zheng et al., 2013), MYC ChIP-Seq (Kress et al., 2016b), histone markers H3K4me3 and H3K27Ac ChIP-Seq in LSEC as well as ATAC-Seq on (A) *Myc* and (B) *Pdgfb* locus in control and Gata4<sup>LSEC-KO</sup> LSEC. (C) ATAC-Seq reads mapped 5 kb around TSS of (upper panel) up regulated and (lower panel) down regulated genes in Gata4<sup>LSEC-KO</sup> LSEC. (D) MYC ChIP-qPCR of *Pdgfb* promotor in control and Gata4<sup>LSEC-KO</sup> LSEC (n = 3). \* P < .05; (N) Mean ± SD, Welch's *t*-test. ATAC-Seq, assay for transposase accessible chromatin sequencing; ChIP-Seq, chromatin immunoprecipitation sequencing; kb, kilo base; LSEC, liver sinusoidal endothelial cells; Pdgfb, platelet derived growth factor subunit B; qPCR, quantitative polymerase chain reaction; SD, standard deviation; TSS, transcription start sites. Figure modified from Winkler et al. (Winkler et al., 2020).

## 5.2 Functional role of endothelial Gata4 in liver regeneration

## 5.2.1 Liver regeneration after partial hepatectomy

In Gata4<sup>LSEC-KO</sup> livers hepatocytes showed a reduced cell size as well as elevated apoptosis accompanied by increased proliferation indicating injured hepatocytes. In addition, *Wnt2* was among the most strongly down-regulated angiocrine genes in Gata4 deficient LSEC and is known to contribute to liver regeneration. Based on these findings it has been hypothesized that endothelial Gata4 deficiency might impair liver regeneration. To analyze the regenerative capacity of Gata4<sup>LSEC-KO</sup> livers, two-thirds PHx were performed by removing the median and left liver lobes in 8-week-old male mice. In this model regenerative hypertrophy dominates in the early phase (up to day 3-4) and is mediated by enlargement of remaining hepatocytes,





(A) Experimental outline of PHx-induced liver regeneration in control and Gata4<sup>LSEC-KO</sup> mice. (B) Kaplan-Meier curve of control and Gata4<sup>LSEC-KO</sup> mice after PHx (n = 15). (C) Body weight of control and Gata4<sup>LSEC-KO</sup> (n = 4-7). (D, E) (D) liver weight and (E) liver to body weight ratio of control and Gata4<sup>LSEC-KO</sup> mice 48 hrs and 144 hrs after PHx (n = 4-7). \* P < .05, \*\* P < .01, \*\*\* P < .001; (B) Logrank test; (C, D, E) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. hrs, hours; PHx, partial hepatectomy; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020). while regenerative hyperplasia starts gradually at later time points and is characterized by hepatocyte proliferation. Gata4<sup>LSEC-KO</sup> and control livers were compared 48 hrs and 144 hrs after PHx to investigate both features (Figure 24A).

After PHx more Gata4<sup>LSEC-KO</sup> mice met the predefined criteria of euthanasia compared to control animals and did so in the early phase of regeneration indicating impaired regenerative hypertrophy (Figure 24B). Body weight of both control and Gata4<sup>LSEC-KO</sup> animals were similar at the time of the PHx and decreased a little 48 hrs after surgery. However, control animals were able to reach their initial bodyweight 144 hrs after PHx, while it was significantly lower in Gata4<sup>LSEC-KO</sup> mice (Figure 24C). Liver weight had nearly doubled between 48 hrs and 144 hrs after PHx in control mice whereas Gata4<sup>LSEC-KO</sup> animals failed to do so (Figure 24D). This was also reflected in the liver / body weight ratio: control animals were able to significantly increase the ratio 144 hrs after PHx on contrast to the Gata4<sup>LSEC-KO</sup> mice (Figure 24E). The reduced liver weight as well as liver / body weight ratio in Gata4<sup>LSEC-KO</sup> animals 144 hrs after PHx indicate an impairment of liver regeneration caused by endothelial Gata4 deficiency.

To investigate if the degree of liver fibrosis in Gata4<sup>LSEC-KO</sup> animals was correlated with survival after PHx, liver lobes of Gata4<sup>LSEC-KO</sup> mice were harvested 0 hrs, 48 hrs and/or 144 hrs after PHx, the extent of liver fibrosis was determined by quantifying the PSR+ area in liver sections and a regression analysis was conducted (Figure 25A-D). Thereby the degree of liver fibrosis was not correlated with the survival after PHx in control and Gata4<sup>LSEC-KO</sup> animals (Figure 25C, D).



**Figure 25:** Survival and extent of liver fibrosis does not correlate in Gata4<sup>LSEC-KO</sup> mice after PHx. (A, B) (A) Picrosirius red (PSR) staining and (B) quantification of PSR staining of control and Gata4<sup>LSEC-KO</sup> livers 0 hrs, 48 hrs and 144 hrs after PHx (n = 4-7). (C, D) Statistical correlation of PSR amount (liver fibrosis) with survival after PHx in control and Gata4<sup>LSEC-KO</sup> mice (n = 4-7). Scale bars: 50 µm; \*\*\*\* (A); P < .0001; (B) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test; (C, D). hrs, hours; PHx, partial hepatectomy; PSR, picrosirius red; SD, standard deviation. Figure modified from Winkler et al. (Winkler et al., 2020).

#### 5.2.1.1 Impact of endothelial Gata4 deletion on regenerative hypertrophy

Gata4<sup>LSEC-KO</sup> mice that did not recover from PHx did so in the early phase of regeneration, which is largely dependent on hypertrophy of hepatocytes. To analyze if the capability of compensatory hypertrophy was impaired in Gata4<sup>LSEC-KO</sup> livers, cell size of hepatocytes was determined by measuring cell numbers per area 0 hrs, 48 hrs and 144 hrs after PHx. Hepatocytes in control livers undergo clearly hypertrophy: the number of cells per mm<sup>2</sup> significantly decreased from roughly 3,000 cells per mm<sup>2</sup> 0 hrs after PHx to 2,300 cells per

mm<sup>2</sup> 48 hrs after PHx and then further to only approximately 2,100 cells per mm<sup>2</sup> 144 hrs after partial resection (Figure 26A, B). In contrast, in Gata4<sup>LSEC-KO</sup> mice the hepatocyte size remained similar (~ 4,000 cell per mm<sup>2</sup>) at all three time points (Figure 26A, B). This indicates that hepatocytes of Gata4<sup>LSEC-KO</sup> mice failed to undergo compensatory hypertrophy. A mere delay in the capability of regenerative hypertrophy can be excluded, as a delay should result in increased size of hepatocytes 144 hrs after partial resection.



## Figure 26: Hepatocytes are not able to undergo regenerative hypertrophy in Gata4<sup>LSEC-KO</sup> mice after PHx.

(A) Representative H&E staining of control and Gata4<sup>LSEC-KO</sup> livers 0 hrs, 48 hrs and 144 hrs after PHx. (B) Quantification of liver density in (Figure 27B) by counting cells per mm<sup>2</sup> (n = 4-7). Scale bars: 50 µm (A); \*\* P < .01, \*\*\* P < .001, \*\*\*\* P < .0001; (B) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. H&E, hematoxylin and eosin; hrs, hours; PHx, partial hepatectomy. Figure modified from Winkler et al. (Winkler et al., 2020).

#### 5.2.1.2 Impact of endothelial Gata4 deletion on regenerative hyperplasia

To test if endothelial Gata4 deficiency impairs also the capability of regenerative hyperplasia, cell proliferation was analyzed by measuring proliferation marker Ki-67 positive cells per area 0 hrs, 48 hrs and 144 hrs after PHx. As in the 3-month-old animals (Figure 13A, B), the number of proliferating cells in the livers of 8-week-old Gata4<sup>LSEC-KO</sup> mice was already increased at the time of PHx and remained increased also 48 hrs and 144 hrs after partial resection compared to controls (Figure 27A, B). However, both Gata4<sup>LSEC-KO</sup> and control mice showed a significant increase in proliferating cells after 48 hrs while proliferation declined again 144 hrs after PHx (Figure 27A, B). Furthermore, the number of apoptotic hepatocytes were analyzed by counting c-CASP-3 positive cells per area. Only minimal numbers of apoptotic cells were detected in

control mice at all three time points while the number of apoptotic hepatocytes which was already increased in Gata4<sup>LSEC-KO</sup> mice before PHx remained on a similar level 48 hrs and 144 hrs after partial resection (Figure 27C, D). These results indicate no defect in regenerative hyperplasia in Gata4<sup>LSEC-KO</sup> livers.





#### 5.2.1.3 Angiocrine factors in Gata4<sup>LSEC-KO</sup> mice during liver regeneration

Wnt/β-catenin signaling is an important driver of liver regeneration starting to operate within 1-3 hrs after PHx (Russell and Monga, 2018) and HGF plays a key role in the proliferative phase during liver regeneration (Liu et al., 1994). *Hgf* expression was unchanged in 3-month-old Gata4<sup>LSEC-KO</sup> mice (Figure 17K, N), while *Wnt2* was significantly down-regulated in Gata4 deficient LSEC (Figure 17A, E). Hence, these angiocrine factors were investigated during liver regeneration. *Hgf* expression increased 48 hrs after PHx in both control and Gata4<sup>LSEC-KO</sup> livers (Figure 28A, B). *Hgf* declined afterwards in control mice, while its expression continued to increase 144 hrs after partial resection in Gata4<sup>LSEC-KO</sup> livers (Figure 28A, B) indicating that endothelial *Gata4* deficiency does not impair *Hgf* induction after PHx. Quantification of ISH demonstrated that *Wnt2* expression was significantly increased 48 hrs after PHx in control mice, while this induction of Wnt2, characteristic of the early phase of liver regeneration, was clearly impaired in Gata4<sup>LSEC-KO</sup> animals (Figure 28C, D). This impaired Wnt2 induction is likely to affect the capability of regenerative hypertrophy and also leads to an impaired regeneration in the Gata4<sup>LSEC-KO</sup> mice.



#### Figure 28: Endothelial Gata4 deletion impairs induction of angiocrine factor Wnt2 after PHx.

(A) Quantification of *Hgf* in (B) (n = 4-9). (B) ISH of *Hgf* in control and Gata4<sup>LSEC-KO</sup> livers 0 hrs, 48 hrs and 144 hrs after PHx. (C) Quantification of *Wnt2* in (D) (n = 4-7). (D) ISH of *Wnt2* in control and Gata4<sup>LSEC-KO</sup> livers 0 hrs, 48 hrs and 144 hrs after PHx. Scale bars: 100 µm (B, D); \* P < .05, \*\* P < .01, \*\*\* P < .001; \*\*\*\* P < .0001; (A, C) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. Hgf, hepatic growth factor; hrs, hours; PHx, partial hepatectomy. Figure modified from Winkler et al. (Winkler et al., 2020).

#### 5.2.2 Liver regeneration after chemical induced injury by CCI4

Since the liver has the capability to regenerate also after a chemical-induced injury, liver regeneration following a single CCl4 injection was analyzed as a second regeneration model in Gata4<sup>LSEC-KO</sup> mice. Therefore, 8-week-old male Gata4<sup>LSEC-KO</sup> and control mice were
intraperitoneally administered a single CCl4 dose and livers were investigated 48 hrs and 144 hrs after injection. CCl4 induces acute liver injury after its breakdown by cytochrome P4502E1 (CYP2E1). As expression of CYP2E1 is zonated, CCl4 injury leads to necrosis and





(A, B, C) Quantification of necrotic area per mm<sup>2</sup> (A) 48 hrs and (B) 144 hrs after CCl4 administration or (C) number of necrotic foci 144 hrs after CCl4 in (D) (n = 5-8). (D) H&E staining of control and Gata4<sup>LSEC-KO</sup> livers 48 hrs and 144 hrs after CCl4 injection. (E) Quantification of PSR staining in (F) (n = 8). (F) PSR staining of control and Gata4<sup>LSEC-KO</sup> livers 48 hrs and 144 hrs after CCl4 administration. Scale bars: 100 µm (D, F); \* P < .05, \*\* P < .01, \*\*\* P < .001, \*\*\*\* P < .0001; (A, B, C) Mean ± SD, Mann-Whitney *U*-test; (E) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. CCl4, carbon tetrachloride; H&E, hematoxylin and eosin; hrs, hours; PSR, picrosirius red; SD, standard deviation.

apoptosis of pericentral hepatocytes within 48 hrs of CCI4 administration. To investigate the necrotic area around the central veins and numbers of necrotic foci, H&E staining were conducted. Centrilobular hepatocellular necrosis was clearly visible in both control and Gata4<sup>LSEC-KO</sup> livers 48 hrs after CCI4 administration, whereby the area in the control mice was significantly larger (Figure 29A, D). In contrast, necrotic foci as well as necrotic area were significantly increased in Gata4<sup>LSEC-KO</sup> livers compared to control livers 144 hrs after CCI4 treatment (Figure 29B, C, D). PSR+ area remained on an elevated level in Gata4<sup>LSEC-KO</sup> livers in comparison to control livers at both time points (Figure 29E, F). Since CCI4-induced bridging fibrosis occurs only after repeated administration, it is not surprising that both livers demonstrated no signs of CCI4-induced bridging fibrosis 48 hrs and 144 hrs after a single CCI4 dose.

Zhao and colleagues investigated the role of the Wnt/β-catenin signaling pathway in liver regeneration following acute liver toxicity and observed that CCl4 injury induced expression of the Wnt target gene Axin2 in mid-lobular hepatocytes encircling the necrotic area 3 days after CCl4 injury (Zhao et al., 2019). These Axin2+ cells proliferate to repair local injury. Since endothelial cells are the major source of Wnts after CCl4 administration and *Wnt2*, *Wnt9b* as well as *Axin2* were significantly down regulated in *Gata4* deficient LSEC, *Axin2* expression after CCl4 injection was analyzed by ISH. Both control and Gata4<sup>LSEC-KO</sup> livers demonstrated *Axin2* expression in few hepatocytes adjacent to the necrotic border 48 hrs after CCl4 injury (Figure 30A, B). In Gata4<sup>LSEC-KO</sup> livers Axin2 expression was slightly increased 144 hrs after CCl4 administration, while the number of pericentral Axin2+ hepatocytes was significantly 3-fold increased in control livers. The downregulation of Wnt ligands in Gata4<sup>LSEC-KO</sup> could lead to a delay in liver regeneration.



Figure 30: Downregulation of Wnt signaling in Gata4<sup>LSEC-KO</sup> livers seems to impair liver regeneration after CCI4 injection.

(A) Quantification of *Axin2*+ area in (B) (n = 5-6). (B) ISH of *Axin2* in control and Gata4<sup>LSEC-KO</sup> livers 48 hrs and 144 hrs after a single CCl4 injection. Scale bars: 100  $\mu$ m; \*\* *P* < .01, \*\*\* *P* < .001; (A) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. CCl4, carbon tetrachloride; hrs, hours.

Since CYP2E1 is the major P450 cytochrome metabolizing CCl4, IF staining of CYP2E1 was conducted. As mentioned before, it is expressed in pericentral hepatocytes. Therefore, IF staining of both control and Gata4<sup>LSEC-KO</sup> livers demonstrated that CYP2E1 expression is significantly reduced 48 h after CCl4 injection (Figure 31). CYP2E1+ hepatocytes were only shown adjacent to the border of necrotic area in control livers, while low CYP2E1 signal was detected in pericentral hepatocytes of Gata4<sup>LSEC-KO</sup> mice (Figure 31). This suggests that either CCl4 uptake or its metabolization or even both is impaired in Gata4<sup>LSEC-KO</sup> livers. Central veins were lined with rings of CYP2E1+ hepatocytes in both livers 144 h after CCl4 administration, indicating the repair of the local CCl4-induced injury (Figure 31).



**Figure 31: Endothelial** *Gata4* deletion seems to impair uptake and/or metabolization of CCI4. Representative immunofluorescent staining of CYP2E1, discontinuous EC marker LYVE-1, continuous EC marker EMCN and DAPI in control and Gata4<sup>LSEC-KO</sup> livers. Scale bars: 100 µm. CCI4, carbon tetrachloride; EC, endothelial cell; EMCN, Endomucin; hrs, hours.

To analyze proliferation in Gata4<sup>LSEC-KO</sup> and control mice, staining of proliferation marker Ki-67 was conducted 48 hrs and 144 hrs after CCl4 administration and Ki-67 positive cells per area were measured. The number of proliferating cells was increased in Gata4<sup>LSEC-KO</sup> livers 48 hrs after CCl4 injection, followed by a significant decline 144 hrs after CCl4 application (Figure 32A, B). Control mice also showed an increased proliferation 48 hrs following CCl4 administration, although the increase was not as pronounced as compared to Gata4<sup>LSEC-KO</sup> mice. Both, control and Gata4<sup>LSEC-KO</sup> livers, demonstrated a comparable proliferation rate 144 hrs after CCl4 injection in relation to 3-month-old mice (Figure 13A, E).

Previous studies have shown that treatment with CCl4 results in apoptotic damage of the liver including the activation of c-CASP-3, a marker of apoptosis (Schattenberg et al., 2012). Therefore, apoptosis was investigated by measuring c-CASP-3 positive cells per area. Since CYP2E1 expressing hepatocytes are metabolizing CCl4, CCl4-induced liver injury results in apoptosis of hepatocytes in the pericentral zone. Only few apoptotic cells were counted in control mice at both time points (Figure 32C, D). On the other hand, the number of apoptotic hepatocytes around central veins was significantly increased in Gata4<sup>LSEC-KO</sup> mice 48 hrs after





(A) Quantification of proliferation marker Ki-67 in (B) (n = 7-8). (B) Immunofluorescence staining of Ki-67, endothelial marker Podocalyxin and DAPI in control and Gata4<sup>LSEC-KO</sup> livers 48 hrs and 144 hrs after CCl4 injection. (C) Quantification of apoptosis marker c-CASP-3 in (D) (n = 4). (D) Immunofluorescence staining of c-CASP-3, Podocalyxin and DAPI in control and Gata4<sup>LSEC-KO</sup> livers 48 hrs and 144 hrs after CCl4 administration. Scale bars: 100 µm (B, D); \* P < .05, \*\* P < .01, \*\*\* P < .001; (A, C) Mean ± SD, two-way ANOVA followed by Tukey's post hoc test. C-CASP-3, cleaved Caspase 3; CCl4, carbon tetrachloride; hrs, hours; SD, standard deviation.

CCl4 treatment. These results indicate that apoptosis is still present in Gata4<sup>LSEC-KO</sup> animals, while it is already terminated in controls.

## 6 Discussion

## 6.1 Characterization of Gata4<sup>LSEC-KO</sup> mice

In order to analyze the function of endothelial Gata4 in the adult liver, a suitable LSEC-specific Cre deleter mouse was necessary that show late embryonic or postnatal reporter activity in LSEC and would leave endocardial Gata4 expression intact. By using an endothelium-subtype-specific Stab2Cre mouse LSEC-restricted Gata4 deletion from E12.5 onwards caused late embryonic lethality and therefore this deleter mouse was not suitable to investigate the function of Gata4 in LSEC in the adult liver (Geraud et al., 2017). However, *Clec4g-Cre<sup>tg/wt</sup>;Rosa26:eYFP<sup>fl/wt</sup>* reporter mice displayed reporter activity from E13.5 on and at E17.5 in LSEC and central vein EC (Wohlfeil et al., 2019). In addition, all microvascular EC but no other cell types were YFP-positive in the adult liver. Therefore, the combination of the *Clec4g-icre<sup>tg/0</sup>* mice with *Gata4<sup>fl/fl</sup>* mice allowed adult inactivation of GATA4 in LSEC.

Initially, the fetal liver is the main site of hematopoiesis. From E16.5 on, the embryonic liver gradually transitions to a primary organ of metabolism while hematopoietic stem cells migrate to bone morrow (Zaret, 2002). After birth, the key source of energy shifts from glucose from umbilical cord blood to breast milk lipids, sequentially causing significant adaption of the liver to the marked changes in nutritional environment (Ehara et al., 2015). This adaption is followed by a period of intense proliferation and differentiation of hepatocytes around postnatal day 3 resulting in a rapid increase in liver size (Haber et al., 1995). 7 days after birth, the liver shape with its five lobules has been formed and performs several metabolic functions such as xenobiotic metabolism (Ober and Lemaigre, 2018). In order to exclude, that the late embryonic deletion of Gata4 in LSEC impairs liver development, livers of Gata4<sup>LSEC-KO</sup> and control mice were investigated at postnatal day 1 and 8 as well as 6 weeks after birth. These analyzes exhibit that solely hematopoietic cells remain slightly longer in the postnatal liver (P8). To identify how long the relocation of hematopoiesis is delayed in Gata4<sup>LSEC-KO</sup> mice, livers would have to be investigated at further time points between P8 and 6 weeks. However, since all lobes of the livers are formed at day 8 and 6 weeks after birth and Gata4<sup>LSEC-KO</sup> livers have a normal liver architecture, it can be assumed that endothelial Gata4 deficiency does not impair liver development.

PSR staining demonstrated that liver fibrosis in Gata4<sup>LSEC-KO</sup> animals develops postnatal as the liver matures. Furthermore, it has been demonstrated that the level of fibrosis as well as histologically and microscopically the phenotype of Gata4<sup>LSEC-KO</sup> mice do not aggravate with increasing age. Therefore, examination of the livers was performed at 3 months of age, since the phenotype is fully developed at this age and does not deteriorate with time.

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It was shown for the first time that adult Gata4-deletion in LSECs leads to capillarization of liver sinusoids with impaired angiocrine signaling resulting in perisinusoidal liver fibrosis. The liver endothelium has been described as crucial gatekeeper for liver homeostasis interacting with its microenvironment via secreted angiocrine factors (Geraud et al., 2014; Rafii et al., 2016). In addition, DeLeve and colleagues demonstrated that dedifferentiation of the endothelium is a critical step in fibrogenesis and is prior to hepatic fibrosis (DeLeve, 2015). Therefore, it is not surprising that the other three major cell types of the liver are also affected by dedifferentiation of LSEC and the impaired angiocrine signaling. GSEA of whole liver lysates of Gata4<sup>LSEC-KO</sup> livers revealed that several gene sets are impaired.

Hepatocytes, as the most abundant cell type of the liver were effected by endothelial loss of GATA4. In Gata4<sup>LSEC-KO</sup> livers, hepatocytes do not only show alterations in metabolic liver zonation, but also metabolic pathways are impaired. Elevated transaminase values indicate hepatocyte damage and hepatocytes have a reduced size, impaired proliferation and undergo apoptosis more often. Dedifferentiated LSEC in Gata4<sup>LSEC-KO</sup> mice lost the capability to maintain HSC quiescent and, as differentiated LSEC are described as gatekeepers of fibrosis, this leads to HSC activation, deposition of ECM and collagens and thus promoting fibrogenesis (Poisson et al., 2017). McGuire et al. suggested that loss of fenestrations may be related to the accumulation of interstitial collagens in the space of Disse (McGuire et al., 1992). So that capillarized LSEC and activated, collagen-producing HSC stimulate each other in a selfperpetuating manner and thereby further promoting fibrosis (Marrone et al., 2016). Furthermore, hyaluronic acid (HA), one of the main components of the ECM, influences cell proliferation and migration. Within the liver, HA is mostly synthesized by HSC and then predominantly cleared by receptor mediated endocytosis in LSEC in an efficient homeostasis (Tangkijvanich et al., 2003). KC facilitate this balance, as their depletion impairs HA uptake by LSEC (Deaciuc et al., 1993). Since Stab2 as important scavenger receptor for clearance of circulating hyaluronan is reduced in Gata4<sup>LSEC-KO</sup> mice, it is not surprising that HA plasma levels are elevated. It was not investigated if HA levels in plasma of Gata4<sup>LSEC-KO</sup> mice correlates with the extend of fibrosis (PSR+ area) of the livers, since it was shown that serum HA level increases with the development for liver fibrosis (Halfon et al., 2005). In addition, more CD68+ macrophages were observed in Gata4<sup>LSEC-KO</sup> livers. In parallel, the other cell types of the liver also affect each other. For instance, apoptotic hepatocytes form apoptotic bodies, which once captured by HSC and KC contribute to their activation (Canbay et al., 2003; Jiang et al., 2009). In summary, capillarization of the liver endothelium due to Gata4 deletion distinctly affects the other cells of the liver and significantly impairs liver homeostasis, whereby loss of differentiation of LSEC is the preceding event in this mouse model.

DeLeve et al. revealed that capillarization of liver endothelium precedes liver fibrosis and that the reversal of capillarized LSEC to differentiated LSEC promotes reversion of activated HSC to quiescence, accelerates regression of fibrosis and prevents progression of cirrhosis (DeLeve, 2015). Therefore, drugs designed to target LSEC and ameliorate their phenotype by selectively modifying their dysregulated pathways seems to be a promising approach. Directly targeting LSEC enables a high specificity and bioavailability in LSEC in addition with low drug dosage and reduction of side effects. Since LSEC are the first parenchymal cells in the liver exposed to substances in the blood, they have the advantage of being easily accessible by blood. Additionally, LSEC express a variety of endocytic and scavenger receptors and therefore provide multiple potential targets. In NASH, LSEC also exhibit a pro-inflammatory phenotype and demonstrate enhanced expression of adhesion molecules. Blocking of these molecules or their ligands is efficiently used to control inflammation in NASH models and inhibitors against adhesion molecule VAP-1 have entered already clinical trials (Wang and Peng, 2021). Furthermore, nanocarriers have emerged as a promising miRNA delivery tool (Chen et al., 2015). Thus, miRNA loaded nanoparticles which targeted LSEC reduced murine colon cancer metastasis to the liver (Marguez et al., 2018). In a liver regeneration model, bone marrow-derived LSEC progenitors injected in rats through the peripheral vein engrafted, expanded in the liver, secreted HGF and thus promoted hepatocyte proliferation and liver regeneration (Wang et al., 2012).

In this mouse model capillarization of liver endothelium is caused by Gata4 deficiency in LSEC. In addition, Gata4 expression was dramatically reduced in advanced hepatic fibrosis and cirrhosis in humans (Delgado et al., 2014). So, maintaining Gata4 expression in LSEC and thereby keeping LSEC in a differentiated condition is an auspicious strategy to prevent or attenuate liver fibrogenesis.

GSEA of isolated LSEC of Gata4<sup>LSEC-KO</sup> and control livers revealed that more gene sets are activated due to endothelial Gata4 deletion than suppressed (10 vs. 3), indicating that Gata4 has a distinct repressive effect. In 2017, Geráud et al. also showed that GATA4 repressed expression of more continuous EC-associated genes than induced expression of LSEC-associated genes in GATA4-transgenic human umbilical vein endothelial cells (Geraud et al., 2017). Further investigations showed that genes significantly upregulated in Gata4<sup>LSEC-KO</sup> LSEC have a highly increased chromatin accessibility suggesting that during homeostasis Gata4 functions as a direct repressor of these genes in LSEC by suppressing chromatin opening. On the other hand, significantly upregulated genes do not display alteration on chromatin level indicating that Gata4 mediates its activating function in other ways. Transcription factors with the ability to directly bind condensed chromatin as well as allowing the binding of other transcription factors, chromatin modifiers, and nucleosome remodelers

and thus positively and negatively impact transcription of genes are described as pioneer factors (Zaret and Carroll, 2011). As early as 2002, Cirillo et al. showed that GATA4 is a pioneer factor capable to bind and open highly compacted chromatin and also competent for gene activity during hepatogenesis (Cirillo et al., 2002). These findings indicate GATA4 to act as a pioneer factor mediating downregulation of genes by suppressing chromatin opening also in LSEC.

Furthermore, anti-Myc ChIP-qPCR was conducted to examine if MYC binds the promotor region of *Pdgfb* and revealed an increased Myc binding to Pdgfb in Gata4<sup>LSEC-KO</sup> LSEC compared to control LSEC. This indicates an enhancement of Pdgfb expression in Gata4<sup>LSEC-KO</sup> livers by MYC, which in turn is itself upregulated since the loss of endothelial Gata4 causes an increased chromatin accessibility at the *Myc* locus. In line with this, Nie et al. described Myc as nonlinear amplifier of expression that acts universally at active genes (Nie et al., 2012).

de Haan and colleagues identified that a combination of TF c-MAF, GATA4, and MEIS2 lead to increase of LSEC signature genes and thus are important transcriptional regulators of the unique LSEC fingerprint (de Haan et al., 2020). However, Gene expression profiling data of isolated LSEC from control and Gata4<sup>LSEC-KO</sup> mice revealed that endothelial Gata4 deficiency results in a downregulation of TF Maf, suggesting that Maf expression is regulated by Gata4. Furthermore, the Maf gene has multiple Gata4 binding sites, also indicating that Maf could be a direct downstream target of Gata4. Therefore, it would be interesting to generate a mouse with adult endothelial Maf deletion to investigate which effects exhibited by the Gata4<sup>LSEC-KO</sup> mouse are mediated by downregulation of Maf by Gata4. Based on its midzonal expression, it is unlikely that Maf influences liver zonation. Gómez-Salinero et al. generated a mouse model, in which c-Maf was selectively deleted in adult Cdh5+ cells of the liver upon tamoxifen treatment at postnatal week 4 and analyzed at postnatal week 8 (Gómez-Salinero et al., 2022). Similar to the Gata4<sup>LSEC-KO</sup> livers, c-Maf deletion lead to decreased expression of sinusoidal genes such as Stab2, Lyve 1 as well as Wnt2. Nevertheless, analysis of the microstructure of sinusoids did not show an absence of fenestrations after c-Maf deletion and analysis of the zonation markers E-cadherin and Cyp2E1 also did not reveal changes in the portal to centrolobular zonation (Gómez-Salinero et al., 2022). Induced c-Maf deficiency during postnatal liver development revealed an increased in hematopoietic cells in H&E staining similar to Gata4<sup>LSEC-KO</sup> livers of mice aged 8 days. These findings indicate that c-Maf is required for the maintenance of liver sinusoidal identity while other pathways regulated by Gata4 might control zonation, fibrosis and unique structural modeling of LSEC. To confirm these assumptions, the generation of a conditional c-Maf mouse by using the Clec4g-Cre deleter mouse would be essential as endothelial c-Maf deficiency would start in LSEC at the same time as in the

Gata4<sup>LSEC-KO</sup> livers. Nevertheless, GATA4 is required for liver development and maintenance of liver homeostasis in the adult and therefore can be referred to as master regulator of hepatic microvascular specification (Geraud et al., 2017).

PDGF is a polypeptide growth factor which effectively promotes cell division and proliferation (Ying et al., 2017). PDGF is the most potent mitogenic factor involved in stimulating HSC proliferation, differentiation, and migration and is thus a key mediator of hepatic injury and fibrogenesis in vivo (Kocabayoglu et al., 2015). In addition, blocking PDGF signaling inhibits HSC activation and reduces liver fibrosis development (Kim et al., 2012; Ogawa et al., 2010). In healthy liver, expression of PDGF receptors is low in HSC, but markedly increases during injury (Pinzani et al., 1996). Czochra and colleagues demonstrated that the conditional overexpression of PDGF-B in the liver caused HSC activation and induced perisinusoidal liver fibrosis (Czochra et al., 2006). In addition, freshly isolated HSC proliferate upon PDGFB stimulation in vitro (Wilhelm et al., 2016).

For the first time, it was shown by multiplex fluorescent ISH that LSEC are also a source of *Pdgfb* in Gata4<sup>LSEC-KO</sup> mice. To confirm that PDGFB secreted by LSEC is the major angiocrine factor for HSC activation and formation of perisinusoidal liver fibrosis in Gata4<sup>LSEC-KO</sup> livers, further analyses would be necessary. Using an antibody against Pdgfb in Gata4<sup>LSEC-KO</sup> mice, could be a possibility to investigate if HSC activation is PDGFB mediated. The use of a tyrosine kinase inhibitor such as imatinib could be an alternative to target PDGF signaling, since PDGFR- $\beta$  belong to receptor tyrosine kinases. However, the mechanism of action of these inhibitors is not specific for PDGF signaling wherefore side effects are more likely. Studies with HSC-targeted imatinib showed an inhibition of phosphorylated PDGFR- $\beta$  expression resulting in anti-fibrotic effects while cytotoxicity was reduced compared to conventional imatinib (El-Mezayen et al., 2017).

Furthermore, the generation of a genetic Gata4, Pdgfb double KO mice with a Clec4g-Cre driver would be an option to investigate the GATA4/PDGFB/PDGFRβ axis. But also the Pdgfb KO mice with deletion in Clec4g+ cells could help to clarify whether LSEC-derived Pdgfb mediated HSC activation as soon as the mice are challenged e.g. with CCl4.

Another way to examine the GATA4/PDGFB/PDGFRβ interaction could be *in vitro* by cell culture experiments. Unfortunately, co-culture experiments involving LSEC are challenging since LSEC dedifferentiate and lose their characteristics morphology in cell culture shortly after cell extraction (Geraud et al., 2010). There have been attempts to defer, stop or restore the dedifferentiation process of LSEC *in vitro* (Bravo et al., 2019; Di Martino et al., 2019). For instance, Di Martino et al. demonstrated that new fenestrae with smaller diameter appeared 6 days after isolation following treatment with the actin-depolymerizing agent cytochalasin D (Di Martino et al., 2019). Although new insights have been gained regarding dedifferentiation

of LSEC in vitro, the main mechanism(s) behind the loss of fenestrae remains unknown to date. However, it can be assumed that capillarization mechanism of LSEC in Gata4<sup>LSEC-KO</sup> livers differs markedly from the dedifferentiation program *in vitro*.

It is still unclear which upstream mechanisms regulate Gata4 expression itself. However, it was previously shown that expression of GATA4 was significantly decreased in LSEC of *Bmp9*-KO mice (Desroches-Castan et al., 2019). Moreover, the addition of BMP9 to primary cultures of LSEC maintained the expression of Gata4, indicating Gata4 as possible target regulated by BMP9 *in vitro*. On the other hand, Drzewiecki et al. showed that continuous EC of GIMAP5-deficient livers did not express GATA4 (Drzewiecki et al., 2021). Furthermore, GSEA revealed that the expression of Gata4-dependent liver endothelial cell regulated genes were markedly downregulated in GIMAP5 deficient CD45-CD31+ cells, indicating an upstream position of GIMAP5 regarding GATA4. Nevertheless, further investigations are necessary to clarify the precise regulation of GATA4.

Non-alcoholic fatty liver disease (NAFLD), one of the most common causes of chronic liver disease, affects up to 30 % of the adult population (Fernando et al., 2019). It can progress into its severe form: NASH. In NASH, inflammation causes hepatocytes death and apoptosis. In addition, HSC get activated and lead to progressive collagen deposition and thus to fibrosis. Determining the severity of fibrosis is critical for patients with chronic liver disease as it predicts long-term clinical outcomes. Fibrosis typically starts in the perisinusoidal region and extends to other areas of the lobule as the disease progresses (Singh et al., 2019). The liver fibrosis caused by adult LSEC-restricted Gata4 deficiency resembles the perisinusoidal fibrosis in NASH. Winkler et al. revealed that microarray data from LSEC isolated from choline-deficient, I-amino acid-defined (CDAA) diet-fed mice showed a notable overlap of up- and downregulated (LSEC-specific) genes compared to Gata4<sup>LSEC-KO</sup> LSEC including Myc and Pdgfb (Winkler et al., 2020). In addition, GATA4 itself was significantly downregulated in LSEC from CDAA-fed mice. Furthermore, endothelial Gata4 deficiency led to an aggravation of perisinusoidal liver fibrosis in CDAA-fed Gata4<sup>LSEC-KO</sup> mice (Winkler et al., 2020). These findings indicate that GATA4 serves as a protective factor in the development of dietaryinduced perisinusoidal fibrosis. Therefore, the Gata4<sup>LSEC-KO</sup> mice could be used to gain further insights into fibrogenesis in NASH patients.

In 2014, Delgado et al. showed that Gata4 expression was markedly reduced in fibrosis and cirrhosis in humans (Delgado et al., 2014). Furthermore, analysis of single cell RNA-sequencing data revealed that in human cirrhotic livers EC underwent a transdifferentiation: MYC target genes including PDGFB were enriched, while GATA4 target genes and GATA4

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itself were reduced (Winkler et al., 2020). These results indicate a similarity between the underlying endothelial alterations in mice and humans during liver fibrosis/cirrhosis.

### 6.2 Regeneration

Nonparenchymal cells in the liver, including macrophages, stellate cells and LSEC are central to coordinating the regenerative process. Particularly, LSEC secret several angiocrine factors such as Wnt2 and Hgf and thus regulate liver regeneration. Since previous experiments have shown that certain angiocrine factors are influenced by endothelial Gata4 deletion in the liver, it is not surprising that liver regeneration is impaired in Gata4<sup>LSEC-KO</sup> mice. In addition to the impaired angiocrine signaling, deposition of ECM was shown to impact liver regeneration. Failure to degraded collagen I specifically affects the resolution of the fibrotic response and is associated with diminished hepatocyte regeneration (Issa et al., 2003). Furthermore, remodeling of ECM is required for hepatic progenitor cell-mediated regeneration (Kallis et al., 2011).

#### 6.2.1 Partial hepatectomy

To investigate the regenerative capability of Gata4<sup>LSEC-KO</sup> livers, PHx was conducted. First and foremost, it is remarkable that significantly more Gata4<sup>LSEC-KO</sup> animals met the predefined criteria of euthanasia and this exclusively in the first 3 days after PHx. This early phase of regeneration is dominated by regenerative hypertrophy, indicating that endothelial Gata4 deletion causes an impaired ability of hypertrophy. The extend of fibrosis does not seem to have an effect on survival, as there is no correlation between the degree of PSR+ area and survival. However, the capability of hepatocytes to increase in size and therefore undergoing regenerative hypertrophy was markedly impaired in Gata4<sup>LSEC-KO</sup> livers during regeneration. Miyaoka et al. showed that hypertrophy of hepatocytes was also maintained 14 days after PHx even after recovery of liver weight (Miyaoka et al., 2012). A mere delay in the ability of hypertrophy seems unlikely, since no increase in size of hepatocytes was detectable even 144 hrs after PHx.

Investigation of angiocrine Wnt2 showed that induction of Wnt2 in LSEC of Gata4<sup>LSEC-KO</sup> mice was impaired 48 hrs after PHx, as Wnt2 expression did not significantly increase compared to control mice. Wnt signaling is an important driver of liver regeneration in the early phase: secreted Wnt ligands act on hepatocytes by promoting expression of target genes, such as cell-cycle regulator cyclin D1, which leads to hepatocyte proliferation as early as 6 hrs post-PHx (Nelsen et al., 2001; Russell and Monga, 2018). Again, no delay but impairment can be assumed, since no change in Wnt2 expression is seen even after 144 hrs after surgery. Hence, impaired Wnt2 induction in Gata4<sup>LSEC-KO</sup> LSEC could cause the impairment of regenerative hypertrophy.

Several studies have shown that HGF/c-Met signaling pathway is indispensable for efficient liver regeneration (Huh et al., 2004). Shortly after PHx, Hgf secretion of LSEC increases and activates JAK/STAT3, PI3K/Akt/NF-KB and Ras/Raf pathways, initiating cell proliferation (Li et al., 2018). Hgf expression is not affected by Gata4 deletion in LSEC of 3-month-aged mice and an increase of Hgf was detectable in both, control and Gata4<sup>LSEC-KO</sup> livers 48 hrs post-PHx. In control mice, Hgf expression declined 144 hrs after PHx, while Hgf levels in Gata4<sup>LSEC-KO</sup> mice remained elevated, indicating that regeneration is impaired in Gata4<sup>LSEC-KO</sup> animals despite Hgf induction. This together with the fact that proliferation was not impaired in Gata4<sup>LSEC-KO</sup> mice suggests that regenerative hyperplasia is obviously not affected by endothelial Gata4 deficiency.

#### 6.2.2 Chemical-induced hepatotoxic injury model

To investigate the regenerative capability of control and Gata4<sup>LSEC-KO</sup> mice, CCl4 was once intraperitoneally injected to induce liver damage. Application of CCl4 is a key model in liver research and has been widely used in several mouse studies so far (Scholten et al., 2015). The great advantages of this model are its robustness, good survival rates, reproducibility and ease of administration. The susceptibility towards CCl4-induced liver injury varies between mouse strains due to genetic background, e.g. BALB/c inbred mice are most sensitive to CCl4-mediated fibrosis induction (Scholten et al., 2015). Even the mouse strains BL6J and BL6N differ in susceptibility to CCl4 damage: BL6J mice exhibited a more severe degree of oxidative stress and fibrosis in the liver than the BL6N mice after repeated CCl4 administration (Kawashita et al., 2019). The Clec4g-Cre mice are on C57BL/6N background, while the Gata4-floxed mice have a mixed background (B6N; 129S1sv). To rule out a difference in genetic background and thus on the susceptibility towards CCl4 between control and Gata4<sup>LSEC-KO</sup> mice, littermates were used as control animals in all experiments. Furthermore, genetic background analysis revealed a minor, comparable heterogeneity in control and Gata4<sup>LSEC-KO</sup> mice, respectively, so that an equal susceptibility to CCl4 can be assumed.

In order to induce liver damage and subsequent regeneration by a single CCl4 application, an appropriate dosage is crucial. At the same time, the dosage must not be too high, so that the survival of the animals is guaranteed. C57BL/6 inbred mice have an intermediate response to CCl4-induced liver injury (Scholten et al., 2015). Therefore, the dosage of 1 mL/kg was used, which is a rather high dosage compared to other CCl4 models with mice that have a pure C57BL/6 background (Kawashita et al., 2019).

#### Discussion

Investigations of CCI4-treated control and Gata4<sup>LSEC-KO</sup> mice 48 hrs and 144 hrs after administration revealed an impaired uptake and/or metabolization of CCI4 in Gata4<sup>LSEC-KO</sup> livers 48 hrs after CCI4-injection. At this timepoint, CYP2E1 positive pericentral hepatocytes were detectable. These hepatocytes metabolize CCI4 causing oxidative damage which results in necrosis and apoptosis around central vein (Clemens et al., 2019). Several pericentral apoptotic c-CASP-3 positive cells were detectable in Gata4<sup>LSEC-KO</sup> livers, whereas metabolism of CCI4 and the resulting apoptosis was completed in control mice 48 hrs after CCI4 application. Staining of CYP2E1 as well as c-CASP-3 at further time points would be interesting to see when metabolization and apoptosis in Gata4<sup>LSEC-KO</sup> animals begin and when these processes are completed in comparison to control mice. On the other hand, necrotic area around central veins was larger in control compared to Gata4<sup>LSEC-KO</sup> mice 48 hrs after CCI4 administration. Here, analyses at later time points could demonstrate whether necrosis increases later in Gata4<sup>LSEC-KO</sup> livers.

Liver fibrosis causes diminished blood flow throughout the liver. In addition, it was demonstrated that CCl4-mediated fibrosis in rats results in metabolic alterations in 36 pathways, including xenobiotic metabolism via cytochrome P450 and drug metabolism pathways (Dong et al., 2016). Therefore, fibrosis in Gata4<sup>LSEC-KO</sup> livers could be involved in the delay in uptake and/or metabolization of CCl4 since fibrosis was shown to reduce blood flow and impairs metabolization by CYP enzymes.

Recent studies showed that loss of Wnt production from LSEC leads to a delay in the proliferative response of peri-injury hepatocytes after injury (Zhao et al., 2019). In addition, Axin2 expression is first lost but is reactivated in peri-injury hepatocytes 3 days after CCl4-mediated injury during liver regeneration. Then these Axin2 positive hepatocytes proliferate to repair local injury. In LSEC of 3-month-old Gata4<sup>LSEC-KO</sup> mice *Wnt2* and *Wnt9b* are downregulated and *Wnt2* induction was impaired in Gata4<sup>LSEC-KO</sup> animals after PHx. These findings indicate that a delay in proliferation of midlobular hepatocytes bordering the damaged local tissue is feasible in Gata4<sup>LSEC-KO</sup> animals.

Zhang et al. demonstrated that Hgf controls susceptibility to necrosis after PHx via the Hgf/c-Met axis involving Deptor to prevent excessive organ damage (Zhang et al., 2020). Analysis of 3-month-aged Gata4<sup>LSEC-KO</sup> livers showed no difference in Hgf expression compared to control livers and Hgf induction was not impaired after PHx. On the other hand, Hgf stimulation did not appear to directly induce Deptor mRNA expression in vitro, indicating no direct regulation by Hgf (Zhang et al., 2020). Therefore, investigation of Deptor expression during regeneration after CCl4 administration would be interesting as reduced Deptor expression

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could be an explanation for the increased necrotic foci in Gata4<sup>LSEC-KO</sup> mice 144 hrs after CCl4 injection.

Taken together, these experiments provided new insights into the *in vivo* relevance of endothelial Gata4 in the adult liver and the impact of Gata4 deletion on liver phenotype especially regarding fibrosis, dedifferentiation, angiocrine signaling and regeneration. Here, it was demonstrated that loss of hepatic endothelial Gata4 deletion resulted in pro-fibrotic angiocrine signaling including MYC-mediated Pdgfb induction on chromatin level. In addition, liver fibrosis in Gata4<sup>LSEC-KO</sup> mice resembled the type of liver fibrosis also seen in dietary-induced NASH-associated perisinusoidal liver fibrosis in mice. As endothelial GATA4 expression was also reduced in human fibrosis/cirrhosis causing transdifferentiation of the endothelium, dysregulation of GATA4 might also contribute to the development of human liver fibrosis. Therefore, the GATA4/MYC/PDGFB/PDGFR $\beta$  axis represents a promising therapeutic target to prevent and/or treat perisinusoidal liver fibrosis.

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