The mesenchymal regulation of ductal-driven liver regeneration

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This dissertation is submitted for the degree of

Doctor of Philosophy

Queens’ College September 2018
Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as specified in the text.

Also, it is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution.

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September 2018
Abstract

Liver epithelial cells – hepatocytes and bile duct cells – intermingle with a microenvironment of endothelial cells, macrophages and mesenchymal cells to form the functional unit of the tissue. In chronic or severe liver injury, when hepatocyte proliferation is compromised, ductal cells become activated into bipotential progenitors to replace lost epithelium. This process can be recapitulated in vitro by growing hepatic ductal cells under defined extracellular matrix and growth factors, which generates 3D epithelial ‘liver organoids’ that resemble adult tissue, yet lack stromal cell components (Huch et al., 2013). In this dissertation, we compared the capacity of two broad hepatic stromal cell populations, hematopoietic/endothelial (H/E) and mesenchymal (Msc) cells, to behave as a nurturing ‘niche’ of the ductal epithelium. In the absence of exogenous growth factors, primary Msc but not H/E cells support ductal cell proliferation and organoid formation in vitro. A cell surface marker screen of the Msc fraction showed labelling of up to 20% of the cells by the stem cell antigen 1 (SCA1). In vivo, SCA1⁺PDGFRα⁺ mesenchymal cells localise periportally, closely surrounding biliary duct cells, and co-expanding with them during damage-induced regeneration. Isolated SCA1⁺ mesenchymal cells express key pro-regenerative factors (Hgf, Rspo1/3, Fgf7), and support liver organoid formation independently of cell-to-cell contact. Mesenchyme-sustained organoids resemble those grown in standard medium, although they are biased towards a more mature ductal cell lineage. Liver organoids can in turn support the expansion of SCA1⁺ Msc cells in vitro, suggesting a positive feedback loop of growth. However, physical contact from the SCA1⁺ Msc cells can be cytostatic for the ductal cells depending on the ratio between the two cell populations. Interestingly, the mesenchymal-to-ductal ratios that permit and inhibit ductal proliferation in vitro recapitulate the ratios observed between the two populations in vivo, during the different phases of liver regeneration. Our findings underscore how the relationship between the ductal epithelium and its mesenchymal microenvironment regulates tissue regeneration, and provide avenues for the development of organotypic liver cultures to model epithelial/mesenchymal interactions in vitro.
External contributions

The details about external contribution to the work presented in this dissertation are as follows:

- Dr. Alessio Cantore and Michela Milani (Dr. Luigi Naldini lab, Italy) performed the lentiviral injections in mice in Figures 4.16 and 4.17.
- Timo Kohler (Dr. Florian Hollfelder and Dr. Kevin Chalut labs, University of Cambridge) performed the microgel encapsulations in Figures 5.8-5.14
Acknowledgements

I’d like to thank Meri for allowing me to pursue this project despite initially warning me it was ‘the difficult one’. When I first arrived in the lab she provided me with careful supervision, which I am very grateful for; but even more so for letting me work independently then onwards. I’m also thankful for her openness to discuss any concerns of mine, both science-related and not.

I’m thankful to the team of the Welcome Trust Stem Cell and Medicine PhD programme– Austin Smith, Brian Hendrich and Jo Jack. My masters’ year was instrumental in my training as a scientist and taught me to handle tough (but constructive) criticism. Beyond that, the numerous ‘PhD days’, talks and poster presentations of the programme most certainly helped me improve my skills in scientific speaking. I’ve had plenty more of scientific mentorship since my undergraduate years and it’s difficult to name all the people that have contributed to my education. A few names deserve a special mention. Thank you to Prof. Salvador Moncada for teaching me about Peter Medawar and the scientific method. Thank you so so much to Thilo Hagen. It was him who first taught me to work independently and completely de-mystified the process of publishing for me. Not many undergraduate students are allowed to write their own papers and I am extremely grateful to him for that. Along those lines, thank you to Michaela Frye, who showed me that, sometimes, 8 weeks of good research and your PI’s scientific integrity can leave a mark. My PhD also involved very frequent and long trips to the sorting facility. I’m thankful to Andy Riddell and Annie Hoxwell for their patience whenever I had numerous samples and 4-way sorting experiments. Thank you also to Richard Butler from the Gurdon Institute Imaging facility for designing several FIJI plug-ins for me and for bearing with my little knowledge in programming. And a special thanks to Bernhard Strauss for letting me use his spinning disk microscope for the live imaging in Chapter 5.

Research is tough and experiments fail frequently. What I believe makes a great difference in the day-to-day is a good lab environment. I was lucky to work in a lab full of friends who I truly appreciate and to whom I am extremely grateful. To
Mikel (aka sadness, diabeto), thank you for hot numbers and the coffee chats, the comedy ‘on the edge’, the beautiful violin plots. To Giovanni, thank you for adventure, ‘capicolo’, the Hulk, Lana/Future Islands. To Mikel and GM together, thank you for always enjoying Hey Ya as much as I do (or at least pretending that you did). To Luigi, thank you for the niche/stem cell talks, uvitas and all the concerts. To Flami (Gretch), thank you for being the calming big smile in the morning; Wednesdays are now very special to me thanks to you and Karen. To Nikitas, I cherish a lot the OncoID times and I’m really glad we came together as a team of friends. To Nicole, my desk/bench buddy, thank you for Blue Monday and all the laughs. Some people have left the Huch lab since I started my PhD but I remember them as part of the family, in particular I’d like to mention Chris, Laura, Daisy, Olga and Cristina. To Timo, needless to say, without you Chapter 5 of my thesis would be a lot less cool. You were an angel during my writing up period and I will always appreciate the headphones, the coffee breaks (espressos or not), raspberries, gallo pinto, the list goes on. The following are a few other people who I have crossed during my academic years, and who I think of very fondly: Natalie Ng, Sarita Menegatti, Ziva Brencic, Istvan Kovacs and Aleck Jones.

And last but not least, my family and loved ones. To Elise, you have been my strength since day 1 of the PhD. Weekends fly by yes, but they were incredibly important to me. Thank you for helping me grow into the strong person that I am today. And to my parents, Sofía, Andrés, Cons, Gael, Zoe, Gabo, Ale y Alonso. Being far from home is one of the most difficult things I have ever done, but you have been nothing but supportive and knowing how proud you are of me makes it all worthwhile. This is for all of you.
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Chapter 1

Introduction
1.1 Liver anatomy and physiology

Akin to its size – ranking as the largest internal organ of the body and second largest after the skin – the liver performs a prominent role in regulating whole-body homeostasis. Liver functions include metabolism of dietary compounds, defence against xenobiotics, regulation of blood glucose levels by glycogen storage, and secretion of bile, serum proteins and blood clotting factors. Such versatile physiology is the product of well-designed histoarchitecture and multicellular cooperation within the tissue.

![Liver Lobule Diagram]

Figure 1.1. The liver lobule. The liver histoarchitecture can be sub-divided into hexagon-shaped units known as lobules, which contain triads of portal veins (PV), hepatic arteries (HA) and biliary ducts (BD) at the periphery and a central vein (CV) at the centre.

At a simple glance, the landscape of the adult liver tissue is remarkably simple compared to other endoderm-derived organs, containing only a few anatomical landmarks to orientate the observer. Almost regardless of the angle of sectioning, the tissue can be subdivided into multiple “lobule” units centred around a hepatic venule (also aptly named central vein) and surrounded by triads of portal arteries, portal veins and bile ducts (Matsumoto et al., 1979); a tissue-uniformity referred to as “isotropic parenchyma” (Matsumoto and Kawakami, 1982) (Figure 1.1).

Hepatocytes and cholangiocytes (or bile duct cells) constitute the two main epithelial cell types of the lobule, which intermingle with a great variety of stromal cells to ensure correct liver physiology. Hepatocytes are by far the most abundant parenchymal cell, accounting for approximately 60% of the liver’s total cell number (Gebhardt, 1992) and 80% of its volume (Blouin, Bolender and Weibel, 1977), thus fuelling at times the erroneous perception of cellular homogeneity within the tissue. Hepatocytes are arranged in one-cell thick anastomosing plates or laminae of cords.
that span the portovenous distance of the lobule and face sinusoidal cells from their basolateral surface (MacSween, Anthony and Scheuer, 1987). Being the workhorse of the tissue, these cells are endowed with customised machinery – e.g. specific organelle content and expression of metabolic/cytochrome enzymes – to perform a wide range of functions. Their workload is nonetheless distributed; hepatocytes across the lobule have evolved remarkable location-based heterogeneity concerning the uptake, storage and transformation of biological molecules (Figure 1.2). First proposed by Jungerman and Sasse in 1978 upon studying carbohydrate metabolism (Jungermann and Sasse, 1978), liver zonation refers to the allocation of opposing metabolic pathways across the portovenous axis of the tissue (Gebhardt, 1992).

**Figure 1.2. Metabolic zonation of the liver.**
Hepatocytes (pink) make up the bulk of the liver parenchyma and are functionally specialised along the porto-venous axis of the lobule due to the concentration gradient of oxygen and nutrients arriving from the portal circulation. Zone 1 hepatocytes are closest to the portal vein and carry out oxidative phosphorylation, gluconeogenesis, β-oxidation and urea synthesis; whilst zone 3 hepatocytes are nearer to the central vein and specialise in glycolysis, lipogenesis, ketogenesis, glutamine synthesis and bile secretion. Zone 2 hepatocytes present intermediate phenotypes.

At the portal area, oxidative phosphorylation, glycogen synthesis (from lactate), gluconeogenesis, β-oxidation, cholesterol biosynthesis and urea production occur; whereas closer to the central vein there is glycogen synthesis (from glucose), glycolysis, lipogenesis, ketogenesis, bile acid synthesis and glutamine synthesis. Various enzymes of the cytochrome P450 family also localise perivenously, which may account for tissue-restricted toxicity to certain xenobiotics (Baron et al., 1986). A classic example is that of carbon tetrachloride (CCL₄), which is metabolised to the highly reactive trichloromethyl radical (CCl₃*) species by cytochrome P450 2E1 (CYP2E1). Given that CYP2E1 is only expressed by pericentral hepatocytes, CCl₃*-induced necrosis is restricted to these cells (Manibusan, Odin and Eastmond, 2007).
One of the main functions of hepatocytes is to produce bile, a yellowish green lipid surfactant that aids in fat digestion, and which is secreted into minute tubular spaces known as bile canaliculi between the tight junctions of neighbouring hepatocytes (Rogers and Dintzis, 2012) (Figure 1.3). At the hepatocyte-ductular interface lies the canal of Hering, a channel in which hepatocyte-lined canaliculi drain into the terminal cholangioles (Figure 1.3).

Figure 1.3. Bile canaliculi and the biliary duct network. Hepatocytes secrete bile into minute tubular spaces termed bile canaliculi (green shading), which drain into ductal cell-lined terminal cholangioles. A vast network of ductal conduits expands throughout the liver to transport the bile to the gall bladder. The Canal of Hering is a zone at the interface between the hepatocyte cords and the terminal cholangioles from where facultative progenitors are thought to arise.

Extending from here, a complex tree of conduits lined by biliary epithelial cells form interlobular, septal, and major ducts that run in parallel with branches of the portal vein and hepatic artery – the previously alluded portal triads (Figure 1.1). Intrahepatic ducts eventually coalesce into extrahepatic ones that lead to the gall bladder, where bile is stored and concentrated, ready to be funneled into the duodenum after food ingestion (Strazzabosco and Fabris, 2008). The ductal epithelium is also responsible for bile production, accounting for 40% of total bile in humans (Strazzabosco and Fabris, 2008) despite themselves only constituting 3% of the hepatic cell numbers. Ductal cells fluidise and alkalinise bile through a battery of secretory and absorptive processes (e.g. using osmolytes like Cl⁻ / HCO₃⁻) in order to meet physiological requirements (Prall and LaRusso, 2000). There is morphological – and likely functional– heterogeneity amongst ductal cells: columnar bile-modifying cells with a low nucleus/cytoplasmic ratio constitute the enlarged sections of the biliary tree (Benedetti et al., 1996); whereas the smaller, almost cuboidal, cells nearest to the canal of Hering have been attributed with inflammatory reactivity and cell-fate plasticity under specific stimuli (Sell, 1990).
The liver is a richly perfused organ, invariably receiving ~25% of the cardiac output through two main vascular systems: the hepatic artery (HA) and the portal vein (PV), the latter of which drains from the mesenteric, gastric, splenic, and pancreatic veins (Lautt, 2009). The high pressure, well-oxygenated arterial blood mixes with the partially de-oxygenated, but nutrient rich, portal blood as they enter the sinusoidal network that irrigates the liver parenchyma. Blood-borne nutrients and oxygen are thus progressively used up by cells across the perivenous distance, until the blood drains out into the central vein. This nutritional gradient accounts, at least in part, for the streamlined metabolic zonation of the lobule (Gebhardt, 1992) (Figure 1.2).

Considering the well vascularised nature of the liver, it is not surprising that arterial, venous and sinusoidal endothelial cells (LSECs) make up a sizeable percentage of the total hepatic population. LSECs line the microvasculature of the liver and comprise up to 20% of all liver cells (Poisson et al., 2017); they differ from other capillaries in the body because of the presence of small openings or fenestrae between adjacent cells – first described by Wisse in 1970 – which endow the sinusoids with high permeability and facilitate nutrient exchange with the surrounding parenchyma (Wisse, 1970; Braet and Wisse, 2002) (Figure 1.4).

Amongst the liver mesenchymal cell pool, hepatic stellate cells (HSCs) – formerly known as Ito or fat-storing cells – are the most numerous (5%) and most extensively studied for their collagen-depositing role during fibrogenesis (Hasegawa, Wallace and Friedman, 2015). In homeostasis, they have star-shaped cell bodies and are recognisable for their high intracellular content of vitamin A droplets. Stellate cells reside within a perisinusoidal area known as the space of Disse, from which they extend multiple processes to contact both LSECs and hepatocytes (Wake, 1995). Venous- and arterial-lining smooth muscle cells control vascular tone (Burkel, 1970; Dong, Ichimura and Sakai, 2010), whereas portal fibroblasts are found in the peribiliary space and their role in homeostasis and injury-response remains poorly understood (Wells, 2014) (Figure 1.4).

Liver-resident macrophages (Kupffer cells) localise intra-sinusoidally and constitute 15% of the total liver cell population (Bouwens et al., 1986). They represent the first line of defence against foreign agents absorbed from the
gastrointestinal tract –detoxifying and/or eliminating them–, but are also professional scavengers for in situ degenerated cells and mediate inflammation (Ju and Tacke, 2016). Other cells of the hematopoietic lineage that are either anchored in or transiting the liver include natural killer (NK) cells – first described as Pit cells (Bouwens et al., 1987) –, NK-T lymphocytes and dendritic cell precursors (Prickett, McKenzie and Hart, 1988; O’Connell et al., 2000; Crispe, 2003) (Figure 1.4).

Despite being linked to hepatocyte heterogeneity, the concept of liver zonation extends to cells of the stroma too. Sinusoidal wall porosity, as determined by the size of the fenestrae, increases along the portovenous axis, coupled to a change in endothelial cell ultrastructure (Wisse et al., 1985; Horn, Henriksen and Christoffersen, 1986). Kupffer cells are most numerous periportally (Bouwens et al., 1986), yet pericentral ones are more phagocytic (te Koppele and Thurman, 1990), likely due to the pH gradient across the lobule. In contrast, HSCs show a discreet preferential accumulation towards the central vein (Horn et al., 1988). Although much of this work has been based on descriptive electron microscopy, more recent studies are beginning to elucidate a molecular divergence in the portal-to-venous stroma. As an example, CV but not PV endothelium is a key source of the WNT agonist RSPO3, which helps to sustain GS⁺ hepatocytes (Rocha et al., 2015). These regional differences may contribute not only to homeostatic physiology but also to regeneration and disease susceptibility. Many pathological events in the liver show a considerable degree of zonal preference (McCuskey and Sipes, 2010).
1.2 Cellular turnover of the tissue: development, homeostasis and regeneration

1.2.1 Development

During embryogenesis, the anterior-posterior patterning of naïve endoderm gives rise to an epithelium with foregut, midgut and hindgut identities; the ventral segment of the former contains precursors for the commitment towards liver tissue and its hepatobiliary system, but also other organs including the pancreas, lung and thyroid (Zorn and Wells, 2009). The lineage choice of these precursors – which occurs from ~E8.0 to E9.5 – relies on a gradient of fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) derived from the closely apposed cardiac mesoderm and septum transversum mesenchyme (STM) (Gualdi et al., 1996; Jung et al., 1999; Rossi et al., 2001) (Figure 1.5). Based on differential cell proximity, these signals induce expression of lineage-specific transcription factors to determine the fate of one endodermal tissue versus the other (Zaret, 2008; Zorn and Wells, 2009). Nascent hepatic progenitors – collectively referred to as ‘hepatoblasts’ – have the potency to generate the two main epithelial cell types of the liver: hepatocytes and cholangiocytes (Miyajima, Tanaka and Itoh, 2014), and become identifiable based on the upregulation of key liver-determining transcription factors (Hepatocyte nuclear factor 4 alpha, Hnf4a) (Kyrmizi et al., 2006) and proteins such as α-fetoprotein (AFP) (Spear et al., 2006) and albumin (Cascio and Zaret, 1991). Moderate doses of FGF favour expression of the albumin gene (Alb) and patterning into hepatic fate, whereas too high and too low levels tilt the balance towards lung/thyroid and ventral pancreas/duodenum progenitors, respectively (Deutsch et al., 2001; Serls et al., 2005). Whilst the cardiac mesoderm expresses a battery of FGF factors around the onset of hepatogenesis (Fgf1, Fgf2, Fgf8, and Fgf10) (Parlow et al., 1991; Crossley and Martin, 1995; Zhu et al., 1996; Jung et al., 1999; Kelly, Brown and Buckingham, 2001; Cai et al., 2003), there is likely to be downstream redundancy amongst them via activation of the MAPK pathway (Miller et al., 2000; Calmont et al., 2006). The requirement for FGF is clear nonetheless, and is evolutionarily conserved in zebrafish, chick and Xenopus (Chen et al., 2003; Zhang et al., 2004; Shin et al., 2007).
As if to make hepatic specification fool-proof, high BMP signalling can also bias precursor fate towards the hepatic lineage at the expense of pancreatic development (Rossi et al., 2001; Chung, Shin and Stainier, 2008); this combinatorial effect of FGF/BMP signalling is peculiar, but has precedent in other developing tissues of the embryo like the tooth (Maas, 1998). Bmp4 null mouse embryos exhibit a morphological delay in liver bud formation, but while loss of Bmp4 alone does not prevent Alb induction in the endodermal progenitor pool, addition of the BMP inhibitor, Noggin, does (Rossi et al., 2001). Lineage tracing experiments have elegantly shown that Bmp2 – originating from the lateral plate mesoderm (LPM) – may account for this, as it also favours hepatic over pancreatic specification of the endoderm and therefore is redundant with Bmp4 in this context (Chung, Shin and Stainier, 2008).

The WNT signalling pathway has also been implicated in hepatic patterning of the embryo, although with a more complex, time-dependent, role than FGF and BMP. In early-somite Xenopus embryos, WNT signalling is antagonised (e.g. via secreted frizzled-related protein 5, Sfrp5) in the anterior endoderm to maintain foregut identity and to relieve inhibition of the hepatopancreatic-specifier Hhex; the converse situation takes place on the posterior endoderm, where high WNT signalling drives intestinal differentiation by obstructing foregut fate (McLin, Rankin and Zorn, 2007; Li et al., 2008) (Figure 1.5). At a later developmental stage, exogenous activation of WNT signalling leads to enlargement of the liver bud (McLin, Rankin and Zorn, 2007); despite seeming paradoxical, these results agree with studies in zebrafish wherein WNT signalling (Wnt2bb of mesodermal origin) specifies liver fate and promotes hepatoblast proliferation (Ober et al., 2006; Goessling et al., 2008). The reiteration of WNT signals, with distinct cellular outcomes across time, is a noteworthy feature of liver organogenesis (Nejak-Bowen and Monga, 2008).

The development of the liver diverticulum (or liver bud) begins at E9.5 in the mouse when hepatoblasts delaminate and migrate as cords into the adjacent STM (Zaret, 2002); this is an epithelial-to-mesenchymal transition (EMT)-like process that relies on close-range interactions with the mesenchyme (Le Douarin, 1975; Houssaint, 1980), expression of matrix metalloproteinases (MMPs) (Margagliotti et
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*al., 2008* – by both the epithelium and the STM – as well as the presence of endothelial cells despite the absence of blood flow (*Matsumoto et al., 2001*) (*Figure 1.5*).

At later stages, immature ‘mesothelial’ cells surrounding the liver parenchyma secrete mitogens such as hepatocyte growth factor (HGF), midkine, and pleiotrophin (*Onitsuka, Tanaka and Miyajima, 2010*), which induce hepatoblast expansion, whereas mature Thy1+ mesenchymal cells drive hepatoblast maturation by direct cell-to-cell contact as shown in a co-culture system (*Hoppo et al., 2004*). Embryos lacking *H2.0-like homeobox* (*Hlx*), a transcription factor enriched in visceral mesenchyme, exhibit impaired liver expansion – with only 3% of the normal cell count by E12.5 – despite successful generation of the liver diverticulum (*Hentsch et al., 1996*). There is thus strong evidence supporting the requirement of mesenchymal-to-epithelial interactions during liver ontogeny, as is the case for many other embryonic tissues (*Ribatti and Santoiemma, 2014*).

During mid-gestation the liver serves transiently as a site of haematopoiesis, a period in which hepatic epithelium and blood cells interact closely. Blood-cell (CD45+) derived Oncostatin M (OSM), a member of the interleukin-6 (IL-6) family, is required for hepatocyte maturation *in vivo* (*Kamiya et al., 1999*) and is commonly used for *in vitro* differentiation protocols from primary-isolated or induced pluripotent stem cell (iPSC)-derived hepatoblasts (*Suzuki et al., 2003; Si-Tayeb et al., 2010*). Interestingly, immature hepatic progenitors sustain haematopoiesis via secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) (*Hata et al., 1993*), but stop doing so as they differentiate – shown by *in vitro* co-cultures (*Kinoshita et al., 1999*). This highlights a feed-back loop of cell-to-cell communication that ensures epithelial differentiation but also the timely arrest of haematopoiesis. Hepatocytes undergo a further round of maturation at the peri- and postnatal stages of development, when anatomical (vasculature)-based zonation is established; a notable case is that of glutamine synthetase, which becomes restricted to perivenous hepatocytes (1 to 3 cell layers) in the last days before birth (*Gaasbeek Janzen et al., 1987*), while the heterogeneity in enzymes of the carbohydrate pathway stabilises 1 week postnatally (*Burch et al., 1963*). The precise signals driving this process are still largely uncharacterised.
In mice, the specification of bile duct cells from hepatoblasts occurs at ~E15 in the immediate vicinity of the portal vein, on cue from periportal mesenchymal cells. Nascent ductal cells can be identified by osteopontin (OPN) and the SRY-related HMG box transcription factor 9 (SOX9), the latter of which controls the timing of biliary tubulogenesis (Antoniou et al., 2009). Activation of Notch signalling via the Jagged1 (mesenchymal) – Notch2 (epithelial) axis is essential for ductal maturation (Figure 1.5); this is evident in Alagille syndrome patients carrying mutations in the JAG1 and NOTCH2 genes who suffer from intrahepatic biliary defects (Alagille et al., 1987; Li et al., 1997; McDaniell et al., 2006), as well as in multiple loss-of- and gain-of-function studies in mice (McCright, Lozier and Gridley, 2002; Hofmann et al., 2010). Besides directing progenitor commitment towards the ductal fate, Notch signalling regulates bile duct morphogenesis; constitutive Notch activation in hepatoblasts (AFP-Cre, RosaNCID) leads to more numerous but also ectopic (mid-lobular) formation of tubules (Zong et al., 2009). Juxtaposed to this, constitutive deletion of the Notch effector, hes family bHLH transcription factor 1 (Hes1), completely abrogates tubular formation of intrahepatic biliary ducts (Kodama et al., 2004).

Signalling via transforming growth factor beta (TGFβ) has also been implicated in ductal-fate specification. Using a transgenic mice harbouring an activin/TGFβ reporter (CAGA12/GFP), Clotman and colleagues showed the presence of a TGFβ gradient emanating from the portal tract of the developing liver (Clotman et al., 2005). At E15.5, there is widespread TGFβ1 expression in the liver, while Activin A, TGFβ2 and TGFβ3 are specifically enriched in the periportal mesenchyme (Antoniou et al., 2009). High periportal TGFβ signalling drives ductal commitment of the hepatoblasts; whereas towards the parenchyma, the Hepatocyte Nuclear Factor-6 (HNF-6) and One-cut-2 (OC-2) transcription factors counteract TGFβ signalling to allow commitment into the hepatocyte lineage (Clotman et al., 2005; Antoniou et al., 2009) (Figure 1.5). In addition, Sox9-deficient livers exhibit delayed ductal maturity and are characterised by abnormal expression of the TGFβ receptor type II (Antoniou et al., 2009).
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Figure 1.5. Liver organogenesis is modulated by the microenvironment. Around E8.5, the foregut endoderm becomes specified into hepatic epithelium by a gradient of FGF and BMP signals secreted by the apposed cardiac mesoderm and septum transversum mesenchyme (STM). Foregut identity itself is maintained through blockade of WNT signaling (e.g. via the WNT inhibitor Sfrp5). At E9.5, liver progenitors (hepatoblasts) delaminate from the liver bud structure in a process that requires matrix metalloproteinases (MMPs) and the presence of both mesenchymal and endothelial cells. At E15, commitment of hepatoblasts towards the biliary fate (SOX9+) is regulated by Notch2-Jagged1 interactions with the portal mesenchyme and by a portal-derived gradient of TGFβ. TGFβ signalling is blocked in parenchymal hepatocytes through the transcription factors HNF-6 and OC-2.

1.2.2 Homeostasis

The wear and tear of tissues across time invariably entails loss and/or aging of cells that the organism must replace in order to preserve histoarchitecture and function – a process referred to as tissue homeostasis. The timeframe and mechanisms through which this renewal occurs do vary considerably from cell to cell and across development.

In contrast to the rapid events of its organogenesis, the healthy adult liver is a slow cycling tissue in which no more than 0.5% of cells are dividing at any given time point (MacDonald, 1961). This paucity of cell proliferation differs significantly from endodermal organs like the small intestine and the stomach (Barker, Bartfeld and Clevers, 2010), and resembles that of the pancreas (Finegood, Scaglia and Bonner-Weir, 1995). Despite being bonafide progenitors during development, hepatoblasts are not believed to persist postnatally, and the homeostatic turnover of liver cells does not rely, or does so minimally, on a dedicated pool of adult stem cells; instead, the liver epithelium is maintained by the rare self-replication of existing hepatocytes and ductal cells (Miyajima, Tanaka and Itoh, 2014), without any mixing of the lineages. Still, this paradigm has been speckled with decade-long controversies, likely due to
the well-established ability of the liver to regenerate via mechanisms that may include facultative stem cell activation and reprogramming (see section 1.2.3).

Dating back to 1985, one of the most contested theories concerning hepatic homeostasis is that of the ‘streaming liver’, which suggests that hepatocytes become replenished from a periportal source within less than a year –as determined by tritiated thymidine tracing, much like a stream would flow across the portovenous distance of the lobule (Zajicek, Oren and Weinreb, 1985). Furuyama’s lineage tracing from the Sox9 locus –which claimed to be ductal-specific– exacerbated the debate, as it supported a streaming-like model of homeostasis but with the duct epithelium at the top of the hierarchy (Furuyama et al., 2011). Recent studies have nonetheless shown that periportal SOX9+ hybrid hepatocytes, not ductal cells, expand dramatically and repopulate the liver, although only in the context of damage (Font-Burgada et al., 2015); whilst Sox9-based tracing of embryogenic progenitors, marking both interlobular bile ducts and periportal hepatocytes in adulthood, showed no evidence of a continuous hepatic cell supply under steady-state (Carpentier et al., 2011). Further support against ductal-derived hepatocytes in homeostasis was obtained using an OPN-CreER mouse line (Español-Suñer et al., 2012). In the converse approach, Malato and colleagues have marked almost the entirety of the hepatocyte compartment (>99%) and found no dilution of the labelling index over time, thus concluding that hepatocytes are renewed from pre-existing ones in the absence of damage (Malato et al., 2011).

Could a unipotent hepatocyte progenitor exist in homeostasis then? At least not periportally. Work from the Nusse lab points towards a pericentral, and diploid, self-renewing Axin2+ hepatocyte population capable of robust liver repopulation and exhibiting a high proliferative index (Wang et al., 2015). This is at odds with in vitro experiments in which GS+ pericentral hepatocytes proliferate less than their GS- counterpart, irrespective of a wide range of mitogenic stimuli (Gebhardt et al., 1986). The answer could lie in the in vivo microenvironment, as Axin2+ hepatocytes rely on WNT secretion (Wnt2 and Wnt9b) from the central vein endothelium to maintain their high rates of proliferation (Wang et al., 2015).
Still, the dynamics of this cell expansion come across as rather exceptional, considering that ‘neutral’ tracing experiments in the liver have demonstrated slow and location-blind homeostatic cell replacement. For instance, Bralet and colleagues marked hepatocytes at near-to-clonal level using retroviral-delivered β-galactosidase after partial hepatectomy (so as to allow viral infection). One year after the procedure, the pattern of positive cell distribution—which encompassed periportal, midlobular and pericentral zones—was unmodified compared to day 3 and day 15 post-labelling, while the non-dilution of the positive clusters bespeaks of a slow rate of cell replacement in the tissue (Bralet et al., 1994).

A recent study by Lin et al., has rekindled the idea of a stem cell/progenitor population in the homeostatic liver (Lin et al., 2018). The group found rare, but whole-lobule interspersed hepatocytes with elevated expression and activity of telomerase reverse transcriptase (Tert), a key trait of stem cell populations in other tissues (Montgomery et al., 2011; Schepers et al., 2011; Itzkovitz et al., 2012; Pech et al., 2015). In the absence of damage, TertCreERT2 reporter mice showed a gradual increase in labelled hepatocytes (from ~3% to ~30%) after a year of tracing; with cell replenishment in all zones of the lobule, and potency towards GS− and GS+ hepatocytes according to clonal analyses. Expression of Tert on traced hepatocytes—assessed by single molecule RNA fluorescence in situ hybridization—was comparable between 3 days and 1 month, but decreased after a year, which could be indicative of a switch from self-renewal to asymmetric cell division. This finding comes after decades of evidence disproving the existence of homeostatic liver stem cells, and thus calls for further scrutiny.

1.2.3 Regeneration

Contrary to the customary turnover of cells that accompanies tissue functioning, acute and/or chronic injuries may disturb a tissue’s steady-state and awaken diverse wound healing mechanisms. The latter are shaped according to evolutionary pressure; and there is perhaps no better example than that of the liver: a metabolic hub under constant exposure to both foreign and endogenous damaging agents.
Bona fide regeneration is the ability to recreate original tissue architecture and function after damage without leaving a scar (Gurtner et al., 2008) (Figure 1.6). Far from mythological contrivance, this mechanism is present in nature yet varies dramatically across metazoan species (Sánchez Alvarado, 2000) and with age (Timchenko, 2009). Lower vertebrates like axolotls and salamanders can seamlessly regrow limbs after amputation; mammals share a similar ability during prenatal development but lose most of it in adulthood. Adult injuries tend to be repaired as opposed to regenerated, replacing functional tissue parenchyma with a meshwork of extracellular matrix (ECM). The liver is one of the few organs in the mammalian body that defies this paradigm, as it can regenerate efficiently from a wide range of physical and toxic injuries (Michalopoulos, 2007), although not indefinitely (Figure 1.6). The process of regeneration following an acute insult is characterised by a dynamic multicellular response involving pro-inflammatory cell recruitment, in situ mesenchymal cell activation, ECM remodelling and epithelial cell expansion to replenish lost numbers (Taub, 2004). This is however a transient reaction; switching-off mechanisms are embedded within the process of wound healing because the same
pathways that promote regeneration, when overstimulated (as in chronic injury), progressively drive scarring and degeneration of the tissue in a process known as fibrosis (Cordero-Espinoza and Huch, 2018) (see section 1.2.4).

1.2.3.1 The epithelial perspective

The relative mitotic quiescence of the liver epithelium is deceiving: if challenged, the hepatic tissue displays a remarkable capacity for regeneration and can reinstall homeostasis via several mechanisms.

1.2.3.1.1 The mighty hepatocyte: regeneration from surgical injury

Reminiscent of limb regrowth in amphibians, up to 70% of the liver can be surgically resected – a process known as partial hepatectomy (PHx) – and the organ will grow back to its original size through compensatory hypertrophy and proliferation of both the epithelium and the stroma (Michalopoulos and DeFrances, 1997; Miyaoka et al., 2012) (Figure 1.6B). Much like in homeostasis, the contribution of liver stem/progenitor cells to this process appears to be negligible (Michalopoulos and DeFrances, 1997). Reinstating 70% of lost hepatic tissue entails, in large part, generating a substantial new amount of hepatocyte mass; this has fuelled a fascination in the field concerning the remarkable, almost unlimited, expansion potential of hepatocytes: serial PHx and transplantation studies estimate that hepatocytes can repopulate 5-6 times the liver while undergoing at least 69 cell doublings or a $7.3 \times 10^{20}$-fold expansion (Simpson and Finckh, 1963; Overturf et al., 1997). As one of the earliest described models of liver injury (Higgins and Anderson, 1931), PHx has sculpted much of our understanding of wound healing in this organ (Michalopoulos, 2007); however, the hepatectomised liver should not be considered injured nor “damaged”, regeneration occurs from the unscathed lobe(s) as a result of the organ’s ability to sense insufficient size. At no point do necrosis and inflammation contribute to the process (Luedde, Kaplowitz and Schwabe, 2014). The PHx procedure is thus non-physiological – exposure to a surgeon’s scalpel is extremely rare for the average person; clinical relevance can be argued for live-donor transplants and tumour resections, but is of less consequence to high burden chronic liver pathologies like non-alcoholic fatty liver disease and cirrhosis (Lim and Kim, 2008). Still,
inadvertently, PHx studies are believed to have contributed to the commonly held prejudice that stem/progenitor cells are dispensable for liver regeneration.

1.2.3.1.2 ‘Oval cells’: a damage-associated hepatic population

Many adult tissues rely on facultative epithelial progenitors to compensate for tissue damage, in what has been hypothesized as a reiteration of developmental mechanisms (Jensen et al., 2005; Fancy et al., 2011). As proposed by Zipori, “stemness might be a transient and reversible trait that almost any cell can assume given the correct trigger (niche)” (Zipori, 2004).

Pioneering work dating back to the 1950s first described the appearance of small, proliferative, oval-shaped cells in adult rat livers with early stage carcinomas induced by ethionine or 2-acetylaminofluorene (2-AAF) (Farber, 1956). This observation, referred commonly as ductular reaction, held true for various other chemical/carcinogen-based models of liver damage, particularly those that inhibited hepatocyte proliferation (Petersen, Zajac and Michalopoulos, 1998). Oval cells sparked interest due to their mixed cell-state features: expressing a combination of biliary, hepatocyte and developmental markers (e.g. keratin 19 (krt19), γ-glutamyl transpeptidase, AFP) (Bird, Lorenzini and Forbes, 2008), and showing capacity to pulse-chase into the hepatocyte lineage (Evarts et al., 1987, 1989) – with some contradictory reports (Tatematsu et al., 1984; Gerlyng et al., 1994) – despite their suspected biliary cell ancestry. Evidence for the latter stems from their periportal location, branching from the Canals of Hering as ductular structures (Paku et al., 2001), as well as from their impaired proliferation after destruction of the biliary tree by 4,4’-methyleneedianiline (Petersen et al., 1997). A different model, perhaps just semantically (Zipori, 2004), posits that oval cells represent a transiently amplifying compartment derived from the activation of periductular progenitor cells (Dabeva and Shafritz, 1993). This was exciting, considering that ductular reactions characterise virtually all chronic liver pathologies and correlate with disease severity (Roskams and Desmet, 1998; Lowes et al., 1999; Roskams, Libbrecht and Desmet, 2003).
A better understanding of the oval cell-of-origin was assumed to come with the power of mouse genetics. Morphologically comparable cells have been detected in mice livers using alternative hepatotoxic regimens such as the choline-deficient ethionine-supplemented (CDE) diet and administration of the carcinogens 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or carbon tetrachloride (CCl₄) (Akhurst et al., 2001; Preisegger et al., 1999; Pritchard and Nagy, 2010). Unfortunately, these murine regimes do not block hepatocyte proliferation as effectively as the rat models (Wang, Foster, et al., 2003); 2-AAF in particular cannot be used in mice because they are unable to biotransform this compound into its damaging form (DeBaun et al., 1968). This is far from trivial, and may account for much of the difficulty in proving the existence of bipotential ductal progenitors in the mouse.

1.2.3.1.3 Probing the existence and potency of liver progenitors

The experimental precedent of the rat, as well as the expression of biliary duct markers in ‘oval cell’ equivalents of mice and humans (Conigliaro, Brenner and Kisseleva, 2010) suggested, but not proved, ductal ancestry of these putative progenitors. More recently, this knowledge has been harnessed to isolate ductal marker–enriched (e.g. EpCAM⁺ MIC1-1C3⁺, CD133⁺) primary cells or whole ductal tree fragments from the liver, which can be expanded in vitro as 2D monolayers or 3D organoid cultures that self-renew and/or maintain potency towards the ductal and hepatocyte lineage (Schmelzer et al., 2007; Kamiya et al., 2009; Okabe et al., 2009; Dorrell et al., 2011; Huch et al., 2013) (see section 1.3). In conjunction, studies of in vitro clonogenicity and differentiation potential have built the strongest case in favour of a ductal-derived bipotential stem/progenitor cell in the liver, but have been criticised by some since ex vivo culturing can induce artefactual cellular plasticity (Raff, 2003). Accordingly, efforts have shifted slightly towards classical in vivo assays of ‘stemness’, including repopulation capacity upon transplantation, as well as the more rigorous genetic-based lineage tracing.

Wang and colleagues performed a chimeric liver study wherein wild-type hepatocytes were transplanted into fumarylacetoacetate hydrolase (Fah)-deficient mice (with >90% repopulation efficiency), thus generating genetically distinct fractions of hepatocytes and all other hepatic cells; subsequent damage of these livers...
resulted in the expansion of progenitor cells of apparent non-hepatocyte origin (Fah-/). In addition, they demonstrated that isolated progenitors were competent to differentiate into large hepatocyte nodules following competitive transplantation (Wang, Foster, et al., 2003). Notwithstanding, issues such as purity of the transplanted cells and cell fusion may confound the interpretation of repopulation studies like this one, as was demonstrated in transplantations of bone marrow cells once purported to give rise to hepatocytes (Wang, Willenbring, et al., 2003).

Owing to lineage-specific CreER mouse lines generated in the last 10 years, it is almost irrefutable now that cholangiocyte-derived cells can expand in the context of regeneration, as shown by tracing from the Sox9, Opn, Hnf1β and Krt19 loci (Furuyama et al., 2011; Español-Suñer et al., 2012; Rodrigo-Torres et al., 2014; Schaub et al., 2014). Far more controversial has been the potency of these cells to replenish the hepatocyte parenchyma, which may in turn depend on the damaging model. Through a nearly exhaustive labelling of the hepatocyte compartment, Malato and colleagues found 1-2% newly born hepatocytes of non-hepatocyte ancestry in CCl₄ damaged livers (Malato et al., 2011) –whilst ruling out such phenomenon in homeostasis. In contrast, Furuyama and colleagues demonstrated extensive tracing throughout the hepatocyte parenchyma using the presumed biliary-specific Sox9-CreER allele across a range of injury models (Furuyama et al., 2011) (Figure 1.7); this argued for a much more prominent role of ductal-sustained regeneration that did not fit the estimates of Malato et al., 2011. But the strategy of Furuyama et al. may have unintentionally picked up on a hybrid hepatocyte population, found also periportal, which expresses low levels of Sox9 and excels at regenerating the injured liver (Font-Burgada et al., 2015). Using a different Sox9-CreER line and clonal-density labelling, Tarlow et al. found a much more conservative (<1%) ductal contribution towards the regeneration of hepatocytes (Tarlow, Finegold and Grompe, 2014). Fittingly, 2.45% biliary-derived hepatocytes were reported in Opn-CreER transgenic mice damaged chronically with the CDE diet, but not with DDC nor CCl₄ (Español-Suñer et al., 2012); whilst Rodrigo-Torres et al., argued for a contribution of just under 2% when damaging Hnf1β-CreER mice also with CDE but not DDC (Rodrigo-Torres et al., 2014) (Figure 1.7).
1.2 Cellular turnover of the tissue: development, homeostasis and regeneration

In parallel, there have been genetic fate-tracing studies from loci believed to be progenitor-specific, as is the case of forkhead box L1 (Foxl1) and leucine rich repeat containing G protein coupled receptor 5 (Lgr5) (Figure 1.7). The notion of progenitor specificity relies on the lack of, or minimal, expression of these markers in the healthy liver epithelium (both in cholangiocytes and hepatocytes), juxtaposed to a clear expression within the damage-induced progenitor cells. Both Foxl1\(^+\) and Lgr5\(^+\) cells expand following liver damage and exhibit bipotentiality in vivo and in vitro (Sackett et al., 2009; Shin et al., 2011; Huch et al., 2013). Instead of cellular identity, ‘progenitor-specific’ genes may reflect the molecular changes that accompany the process of de-differentiation and/or activation of stem-like features (Huch and Dollé, 2016). Lgr5 is a WNT target gene and common marker of adult stem cells which rely on high WNT signalling for their self-renewal (Barker, Tan and Clevers, 2013). In agreement with this, ductal fragments from healthy liver – expanded under defined growth medium including WNT agonists– self-renew in vitro as liver organoids with high Lgr5 expression (Huch et al., 2013).

![Figure 1.7: The contested existence of ductal liver progenitors.](image)

Multiple CreloxP methods of lineage tracing have been used to probe the existence of ductal progenitors in the adult damaged liver. The most remarkable, but controversial, proof of ductal-driven regeneration has come from the Sox9-CreER model by Furuyama et al., wherein significant hepatocyte replenishment was detected. This has since been contested using several ductal-specific alleles including a different Sox9-CreER as well as Opn-CreER and Hnf1\(\beta\)-CreER. These methods reported a small percentage (1-3\%) of ductal-derived hepatocytes, which fitted with the ~2\% of non-hepatocyte tracing observed in AAV8-Ttr-Cre labelled livers. Tracing from the Lgr5-CreER and Fox1-CreER loci, which label proliferating progenitors after damage, also supports potency towards the hepatocyte lineage but does not prove ductal ancestry. No tracing was detected with mesenchymal alleles (Pdgfrb, Sm22). In contrast, hepatocytes display a remarkable ability to regenerate themselves, as shown using Sox9CreER, AAV8-Ttr-Cre and AAV8-TGB-Cre. Grey arrows and text: negligible or non-detected tracing into hepatocytes. Thickness of arrows reflects the amount of tracing into hepatocytes. Brown hepatocytes: damaged/necrotic.
The liver field is no stranger to controversy. The existence of ductal-derived progenitors was questioned anew with a couple of studies from the Willenbring and Stanger labs in 2014. After refining their formerly published strategy of hepatocyte labelling (by coupling AAV8-Ttr-Cre with the more sensitive reporter R26-RFP, instead of R26-YFP) (Malato et al., 2011), the Willenbring group detected only 0.4% hepatocytes of non-hepatocyte origin and found negligible (<0.1%) hepatocyte regeneration using mouse lines of ductal (Krt19-CreER) and mesenchymal (Pdgfrb-CreER and Sm22-CreER) specificity exposed to the same chronic damage regime as in Español-Suñer et al. 2012 (Schaub et al., 2014). On that same year, Yanger and colleagues used a comprehensive list of pulse-chasing protocols – encompassing the labelling of cholangiocytes (Krt19-CreER), ductal progenitors (DDC or CDE followed by Krt19-CreER tracing), hepatocytes (AAV8-TBG-Cre) and rapidly proliferating cells (CDE or DDC followed by labelling with thymidine analogues) –, to convincingly demonstrate that regenerated hepatocytes arise from self-duplication (Yanger et al., 2014) (Figure 1.7).

Almost foretelling the controversy of the ensuing years, ‘true oval cell responses’ had already been reported to arise only when hepatocyte proliferation is inhibited (Petersen, Zajac and Michalopoulos, 1998). Comparably, in the clinic, ductular responses are observed in patients with chronic liver disease when hepatocytes are mostly senescent and cease to proliferate (Wiemann et al., 2002). It is thus emerging that ductal progenitor potency should be reassessed with models that stringently block regeneration from the hepatocyte compartment. Conditional deletion of transformed mouse 3T3 cell double minute 2 (Mdm2) in up to 98% of hepatocytes, which causes them to senesce, activates a vigorous ductal progenitor response that correlates with the full recovery of liver function in mice (Lu et al., 2015) (Figure 1.8). Similar results have been observed in zebrafish livers after extreme hepatocyte loss (Choi et al., 2014; He et al., 2014), which may reflect environmentally forced plasticity to cope with damage (see below) (Figure 1.8). On the other hand, mature hepatocytes have been shown to undergo reversible ductular metaplasia in chronically damaged livers, regenerating up to 60% of their lost cell numbers, which suggests that part of the progenitor pool could originate from a small number of hepatocytes as an injury escape mechanism (Tarlow et al., 2014). The
1.2 Cellular turnover of the tissue: development, homeostasis and regeneration

phenomenon of hepatocyte-to-ductal reprogramming occurs spontaneously during liver damage but can be enhanced through modulation of the Notch, Hippo and TGFβ signalling pathways (Jeliazkova et al., 2013; Yanger et al., 2013; Yimlamai et al., 2014; Schaub et al., 2018).

Recently, using lineage tracing approaches, Raven and colleagues have unequivocally shown that ductal progenitors contribute to the regeneration of the hepatocyte lineage in murine livers with impaired hepatocyte proliferation, caused by both p21 overexpression and loss of integrin β1 (Itgb1) (Raven et al., 2017) (Figure 1.8). Remarkably, when there is severe chronic damage, biliary cells may bypass the ‘intermediate’ progenitor state and directly convert into hepatocytes within the ductule structure in the absence of proliferation (Deng et al., 2018). This transdifferentiation process is not due to blockage of hepatocyte cycling as in the models of Lu et al. 2015 and Raven et al. 2017, and no ‘oval’ cell marker expression is detected (Figure 1.8).

![Figure 1.8. Biliary duct cells regenerate the liver when hepatocytes cannot.](image)

The contribution of ductal cells towards hepatocyte regeneration has been re-assessed using models in which there is significant hepatocyte loss or hepatocyte proliferation is impaired. The former has been done in zebrafish coupled to fate-tracing from the Tp1CreER locus, a Notch responsive element enriched in ductal cells. In mice, transplantation of ductal progenitors into livers with senescent hepatocytes (induced via knock-out of Mdm2) repopulated the hepatocyte parenchyma. 25% of non-hepatocyte tracing was also observed in damaged livers with hepatocyte-specific loss of Itgb1, which impairs cell proliferation. Tracing from the Krt19-CreER locus demonstrated that ductal-derived hepatocytes amount to 15% in livers where p21 overexpression causes hepatocyte senescence. Ductal cells can also transdifferentiate into hepatocytes after severe chronic damage without the need to genetically impair hepatocyte proliferation.

One concept which is able to reconcile much of the recent data in the liver regeneration field is that of cellular plasticity, whereby the epithelial cell compartments of the liver may altruistically interconvert onto one another to repair damage as needed – either through a facultative progenitor state or via direct
transdifferentiation (Kopp, Grompe and Sander, 2016; Michalopoulos, 2018). The concept of cellular plasticity has not been ascribed exclusively to the hepatic epithelium, yet it is possible that the evolutionary pressure applied by continuous metabolism of toxic waste has made it a more frequent and obvious necessity in this organ. This subject is comprehensively reviewed elsewhere (Aloia, Mckie and Huch, 2016; Kopp, Grompe and Sander, 2016).

1.2.3.2 The stromal perspective

Re-epithelization is the ultimate goal of the regenerative response; however, no epithelial cell, mighty as it be, can re-establish homeostasis unaided. The recovery from epithelial-specific injuries relies on auxiliary responses by nondamaged stromal cells that become activated in situ and/or get recruited from the bloodstream (Gurtner et al., 2008) (Figure 1.9). Borrowing from the stem cell field, this is an extension of the concept of 'niches': specialised micro-environments capable of influencing stem cell fate decisions like self-renewal and differentiation. But unlike other epithelial tissues with well-defined stem-niche duos (Mesa, Rompolas and Greco, 2015), the liver has lagged considerably in this respect, likely due to the relentless questioning of the stem cell itself.

Genetic mouse models and in vitro culturing efforts at the heart of the cell-of-origin debate have incidentally uncovered a multitude of paracrine molecules essential for liver regeneration (Erker and Grompe, 2008). The majority of these are thought to be of stromal origin —for instance WNT/RSpondin (Ding et al., 2010; Boulter et al., 2012; Rocha et al., 2015), HGF (Matsumoto and Nakamura, 1991; Ding et al., 2010; Chen et al., 2012) and fibroblast growth factor (FGF) (Takase et al., 2013); highlighting the absolute necessity of studying hepatic wound healing as a joint endeavour between stromal and epithelial cells. It is fascinating that many aspects of the regenerative microenvironment —both the cells and their paracrine signals— are conserved across various models of liver injury despite the distinct sources of epithelial replacement (e.g. hepatocytes, cholangiocytes, ‘oval’/ductal progenitors) (Michalopoulos, 2007; Duncan, Dorrell and Grompe, 2009). This hints at evolutionary adapted, and possibly redundant, pathways to ensure tissue regeneration by whatever means necessary: cell cycle entry, transdifferentiation and/or activation of
facultative stem cells. Notwithstanding, our knowledge in this field remains convoluted; the spatiotemporal dynamics and the cell source/recipient combinations of many regenerative pathways have been poorly defined.

1.2.3.2.1 Mesenchymal cell activation

The multicellular events that drive wound healing are notoriously well understood in the epidermis, particularly concerning the role of mesenchymal cells. In this tissue, soon after inflammation, resident fibroblasts adjacent to the wound or recruited from the bone marrow are induced to secrete ECM to generate a scar (Gurtner et al., 2008). This mesenchymal activation entails a phenotypic switch of the fibroblasts into myofibroblasts, highly contractile cells that lay down matrix (predominantly collagen) and deform it to progressively bring the edges of the wound together (Tomasek et al., 2002). In the latter phases of healing, auxiliary stromal cells including the myofibroblasts themselves retract or become apoptotic (Desmoulière et al., 1995), but epithelial–mesenchymal interactions are thought to continue to ensure tissue stability (Szabowski et al., 2000).

Taking lessons from the skin, mesenchymal cell activity has also been assessed in the context of hepatic regeneration, but even more so during pathological fibrogenesis (see section 1.2.4). Injury-induced myofibroblasts are thought to have diverse origins in the liver, the most prominent being HSCs and portal fibroblasts (Figure 1.9). Bone marrow–recruited monocytes may also differentiate into ECM-producing “fibrocytes” as part of the inflammatory response, but transplantation studies suggest that their contribution to the development of hepatic fibrosis is minimal compared to that of tissue-resident mesenchymal cells, for which they have been given less attention (Kisseleva et al., 2006; Brenner et al., 2012).

The transition from quiescent “cell X” (be it HSC, portal fibroblast, or monocyte) to active myofibroblast-like phenotype is a malleable process wherein the chronicity of the damage stimuli may generate a mixed spectrum of mesenchymal cell signatures (Schmitt-Gräff et al., 1994). While HSC-derived myofibroblasts have been reported to revert to quiescence readily (Kisseleva et al., 2012; Troeger et al., 2012), myofibroblasts originating from portal fibroblasts are locked in a more
mature/committed state and seem to die by apoptosis instead of transitioning back (Guyot et al., 2007, 2010). Myofibroblast heterogeneity may thus be crucial for understanding the balance between regeneration and fibrosis.

i. Hepatic stellate cells

Almost expectedly, the question of mesenchymal aid in the liver has swivelled attention to the HSC lineage, the most abundant mesenchymal cell of the tissue. Their widespread perisinusoidal location may indeed endow these cells with a competitive advantage – acting swiftly at multiple sites of injury – as they consistently respond to diverse types of damage such as DDC, CCl₄, and acetaminophen (Shen et al., 2011; Mederacke et al., 2013). Seminal observations established a spatiotemporal link between perisinusoidal ‘lipocytes’, now recognised to be HSCs, and collagen fibres in injured livers (McGee and Patrick, 1972; Kent et al., 1984). Following tissue damage, the vitamin A-rich quiescent HSCs indeed transition into proliferative, contractile cells that meet all the criteria of the myofibroblast-like phenotype. Their cytoskeleton bears witness to this: microfilament bundles – including α smooth muscle actin (αSMA), a marker for activated myofibroblasts – accumulate intracellularly, suggesting competence for contraction (Schmitt-Gräff, Chakroun and Gabbiani, 1993; Enzan et al., 1999) (Figure 1.9). In addition, these cells become equipped with a repertoire of fibrillar collagens including type I and III (Maher and McGuire, 1990), and express a number of mitogenic factors for epithelial cells, including HGF (Maher, 1993; Chen et al., 2012) (Figure 1.9). Still, much of this mechanistic evidence remains circumstantial, and the modes through which HSC-derived myofibroblasts contribute to regeneration, not fibrosis, are not well understood.

Non-genetic methods to inhibit HSC activation in vivo – including gliotoxin and l-cysteine – prevent normal regenerative responses of both hepatocytes and ‘oval’ cells in injured livers (Pintilie et al., 2010; Shen et al., 2011), although these have been criticised for their lack of cell specificity. A study by Kalinichenko et al. showed that haploinsufficiency of forkhead box f1 (Foxf1) – a transcription factor enriched in the septum transversum and HSCs throughout ontogeny – diminishes αSMA⁺ cell expansion and collagen deposition following CCl₄ injury, concomitant with an increase in pericentral hepatocyte apoptosis (Kalinichenko et al., 2003). Chen and
colleagues have also highlighted the dynamicity of the HSC ‘niche’: these cells switch from secreting pro-proliferative (HGF) to anti-proliferative (TGFβ) factors at different time points following CCl₄ administration (Chen et al., 2012), a biological rheostat that may ensure the timely progression and arrest of regeneration.

A big caveat of many HSC focused studies is the difficulty in proving the identity of these cells unequivocally. Even some of the most commonly used, markers like glial fibrillary acidic protein (GFAP) and Collagen-α1, are not exclusive to the HSC lineage and label other mesenchymal and even non-mesenchymal cell types of the liver (Mederacke et al., 2013; Lua et al., 2016); likewise, HSC ancestry should not be assumed for myofibroblasts based on co-expression of these markers, and the field would benefit from cell-specific CreloxP tools for fate tracing and ablation. Mederacke and colleagues have recently made advances on this respect by generating a CreloxP mouse in which 99% of HSCs are labelled from the lecithin-retinol acyltransferase (Lrat) locus (Mederacke et al., 2013). Similarly, both quiescent and injury-induced HSCs are labelled efficiently with a Pdgfrb-Cre line (Henderson et al., 2013).

ii. Portal fibroblasts

The portal fibroblast population sparked interest rather recently following reports from diseased livers in which peribiliary matrix-embedded myofibroblasts presented a signature inconsistent with HSC ancestry (Knittel et al., 1999; Cassiman et al., 2002). Although the ontogeny of portal fibroblasts and HSCs is thought to be shared – tracing back to STM-derived mesothelial cells (Asahina et al., 2011) – portal fibroblasts exclusively localise to the portal tract of the liver lobule, and their activation is seemingly biased to ‘injuries’ of the biliary epithelium. This may include malignancies like cholangiocarcinoma, which differs histologically from hepatocellular carcinoma with respect to the presence of abundant fibrous stroma (Tadashi et al., 1996). Tuchweber et al. compared the kinetics of mesenchymal cell expansion in rats following bile duct ligation (BDL), an obstructive model of cholestasis (Rodríguez-Garay, 2003); soon after injury, portal fibroblasts nearly doubled the proliferative index of HSCs (31% vs 18% at 72h post injury) and detection of αSMA-expression was restricted to the stroma adjacent to proliferating ductal cells, not the HSCs of the lobule (Tuchweber et al., 1996). A similar abundance
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of suspected non-HSC-derived myofibroblasts (based on lack of Desmin expression) was confirmed by ischemic injury of the bile ducts, although no positive lineage tracing was performed (Beaussier et al., 2007). The specificity and sensitivity of reported portal fibroblast markers like fibulin 2, elastin, IL-6 and NTPDase 2 need to be thoroughly validated (Wells, 2014); at present, studies remain ambiguous in their cell-lineage definition and instead appeal to the positional bias of the portal fibroblast compared to HSCs (Figure 1.9).

Consistent with the myofibroblast phenotype, periportal fibroblasts in the BDL model do synthesise ECM proteins like collagen I, IV, procollagen III, elastin and tenascin, as well as tissue inhibitor of metalloproteinase 1 (Timp1); although they appear to do so prior to myofibroblastic differentiation (as assessed via αSMA staining) (Desmoulière et al., 1997). Several of these matrix proteins – tenascin and collagen IV in particular– have been implicated in bile duct development and in intrahepatic cholangiocarcinoma in humans (Terada and Nakanuma, 1994). Moreover, collagen IV is one of the major constituents of Matrigel, the matrix required for the in vitro expansion of ductal progenitors into organoids, as discussed in section1.3.1.

Selected publications suggest that, beyond their injury-induced expansion and matrix deposition, portal mesenchymal cells engage with the biliary epithelium through paracrine signalling. Takase and colleagues showed that, in DDC-damaged livers, periportal thymus cell antigen 1 (Thy1)+ mesenchymal cells (encompassing portal fibroblasts but also HSCs) induce the activation and proliferation of murine liver progenitors through FGF7 secretion (Figure 1.9); and forced overexpression of this factor alone is sufficient to stimulate progenitor emergence in healthy livers (Takase et al., 2013). Years before, Sackett and colleagues had alluded to a similar niche of portal fibroblasts for Foxl1+ labelled ductal progenitors, although no molecular link had been established then (Sackett et al., 2009). In chronic models of liver damage, periportal Jagged1+ myofibroblasts direct progenitor differentiation towards the ductal lineage (Boulter et al., 2012) (Figure 1.9), a process that recapitulates the Notch-mediated interaction between hepatoblasts and portal mesenchyme in embryogenesis (see section 1.2.1). The model of Boulter and colleagues is appealing because it proposes that progenitor fate-decisions in
1.2 Cellular turnover of the tissue: development, homeostasis and regeneration

Adulthood are modulated according to the local microenvironment (Boulter, Lu and Forbes, 2013). If that is the case, peribiliary mesenchymal cells should be carefully studied as the putative ‘niche’ of ductal-derived progenitors.

Figure 1.9. Hepatic regeneration involves activation of mesenchymal cells. The process of hepatic wound healing involves an early inflammatory response, wherein tissue-resident macrophages and recruited monocytes detect epithelial necrosis and secrete pro-inflammatory cytokines. Mesenchymal cells (orange) in the tissue, mainly HSCs and portal fibroblasts (PF), become activated and differentiate into myofibroblasts (MF, magnified cell), contractile αSMA⁺ cells that deposit and remodel ECM to aid in wound closure. Activated HSCs secrete a variety of mitogenic growth factors (e.g. HGF) and are the main contributors to liver fibrosis regardless of aetiology. Activated PF expand periportally in cholestasis and similar ductal-specific injury models. Periportal Thy1⁺ mesenchymal cells secrete FGF7 to activate the ductal progenitors (green round cells) after injury, whilst mesenchymal Jagged1 promotes biliary fate commitment of the ductal progenitors. PF-MF: portal fibroblast-derived myofibroblast, HSC-MF: HSC-derived myofibroblast.

iii. In vitro isolation and culture of primary mesenchymal cells

Our understanding of hepatic mesenchymal cells, both in homeostasis and in injury, has been revolutionised by the ability to culture them ex vivo. For this, livers are typically perfused in situ or digested after harvest with tissue-disrupting enzymes like collagenase, as is customary for most other primary hepatic cell isolations. HSCs can be efficiently sorted out via density gradient separation, a method that takes advantage of their buoyancy caused by high content of intracellular vitamin A (Friedman and Roll, 1987); the caveat is a bias towards isolation of quiescent HSCs, given that HSC-derived myofibroblasts lose their lipid droplets during the process of differentiation (Friedman, Wei and Blaner, 1993). Portal fibroblasts, on the other hand, may be extracted through a modified protocol of cholangiocyte isolation (Kruglov, Jain and Dranoff, 2002) and by outgrowth from dissected biliary fragments (Uchio et al., 2002; Kinnman et al., 2003). At the expense of cellular yields, many of these techniques have been adapted to mice livers and now incorporate fluorescence
activated cell sorting (FACS) to ensure higher cell purity. It is clear that for both HSCs and portal fibroblasts, in vitro culture leads to the progressive acquisition of myofibroblastic morphology and marker expression (e.g. \( \alpha \)SMA), a process that is regulated by TGF\( \beta \), platelet-derived growth factor (PDGF) and substrate stiffness (Kinnman et al., 2003; Li et al., 2007). In addition, several stable cell lines of hepatic mesenchyme have also been developed to bypass the need for primary cell isolation (Herrmann, Gressner and Weiskirchen, 2007; Fausther et al., 2015), but demand extra caution due to the major alterations inherent to the process of cell immortalisation.

1.2.4 Fibrosis and its resolution

Regeneration and fibrosis share a common cascade of cellular and molecular interactions that bifurcates as a result of the chronicity of the damage (Cordero-Espinoza and Huch, 2018). At the heart of this lies the ability to deposit matrix, but also to remodel it and remove it in order to ensure recovery of the tissue (Issa et al., 2003; Kallis et al., 2011). Fibrosis occurs when ECM proteins accumulate in excessive amounts, leading to scarring that distorts the normal layout and stiffness of the tissue. As the injury becomes chronic, the once-functional hepatic parenchyma is overtaken by an acellular mesh of connective tissue – mostly collagen and elastin fibers – whose progressive cross-linking restrains access to degrading enzymes and makes scar resolution increasingly difficult (Ramachandran and Iredale, 2012).

An important mechanism of hepatic fibrogenesis is the accumulation of ECM-producing myofibroblasts in the tissue. Given that both portal fibroblasts and HSCs could fulfil the criteria of bona fide pro-fibrotic cells in the context of hepatic damage, the field has deliberated extensively about the pathogenic contribution of each cell-type. Mederacke and colleagues proposed HSCs as the dominant contributors of matrix deposition in liver fibrosis, independent of its aetiology (Mederacke et al., 2013), which is currently the most accepted view; there is however growing support for the portal fibroblast as a ‘first responder’ after injury, particularly that of the biliary epithelium (Wells, 2014).

For many years tissue fibrosis was considered to be a degenerative disease with no possibility of regression. A seminal study by Okazaki and Maruyama in 1974
1.2 Cellular turnover of the tissue: development, homeostasis and regeneration

was the first to show collagenase activity in fibrotic livers, hinting at the feasibility of disease resolution under certain contexts (Okazaki and Maruyama, 1974). Since then, the liver has provided exceptional evidence of the plasticity of this process, where even advanced fibrotic tissues are capable of reacquiring homeostatic traits (Ellis and Mann, 2012). The mechanisms for this are complex and out of the scope of this dissertation (Henderson and Iredale, 2007; Ramachandran and Iredale, 2012); however, they include pro-fibrotic cell clearance by immune cells (Radaeva et al., 2006; Glässner et al., 2012; Hammerich et al., 2014), reversal to quiescence (Kisseleva et al., 2012; Troeger et al., 2012) or senescence of the myofibroblast population (Krizhanovsky et al., 2008).

1.3 In vitro cultures of liver progenitors

Despite certain discrepancies in the methods of isolation, bipotential hepatoblast populations can be extracted from the developing embryo and grown in vitro (reviewed in (Miyajima, Tanaka and Itoh, 2014)). Pluripotent stem cell cultures (ESC or iPSC) can also be directed to differentiate into hepatoblast-like cells by recapitulating the soluble factors of the embryonic microenvironment in vitro in a timely and step-wise manner (Si-Tayeb et al., 2010; Touboul et al., 2010; Dianat et al., 2014; Ogawa et al., 2015; Sampaziotis et al., 2015) (Figure 1.10). Embryonic progenitors can be pushed towards a committed hepatocyte or cholangiocyte fate, but they typically remain immature compared to the adult tissue, particularly concerning the great degree of hepatocyte specification (i.e. zone specific signatures) that occurs postnatally (Baxter et al., 2015); these types of cultures are thus ideal for studying hepatic patterning and morphogenesis during development, but are less useful models of adult hepatic epithelium.

The expansion of adult hepatic cells has historically proven more challenging. In stark contrast to their robust regenerative capacity in vivo, hepatocytes fare poorly in vitro, both in terms of survival and retention of key metabolic functions (Shan et al., 2013), precluding their use for disease modelling and cell-based therapies (Figure 1.10). One alternative solution has been the holy grail of the adult stem/progenitor cell. Whilst elusive in vivo, cells fulfilling the requirements of: a) clonogenic with high expansion potential, b) able to differentiate into the hepatocyte and cholangiocyte
lineages, and in some cases c) capable of repopulating the liver after transplantation; do exist in vitro (Miyajima, Tanaka and Itoh, 2014) (Figure 1.10). Interestingly, most of such cells have been sorted using classical ductal markers including EpCAM (Okabe et al., 2009), CD133 (also known as prominin 1) (Kamiya et al., 2009) and the MIC1-1C3 antigen (Dorrell et al., 2011) from both damaged and healthy livers; the latter of which suggests that the extraction of cholangiocytes from their local niches and/or the in vitro conditions in which they are grown induces the activation of facultative progenitors.

During embryogenesis, the puzzle for building hepatic epithelium requires two key pieces: a competent endodermal cell source, but also stromal-derived signals for tissue specification. Having the ductal lineage as a common epithelial denominator in the adult, a natural leap forward was to attempt to faithfully recapitulate in vitro the signals that promote physiological tissue function and histo-architecture in vivo. A history of culturing hepatocytes as spheroids and via gel entrapment already hinted
at the relevance of three-dimensional organisation for correct liver-specific function (Meng, 2010). Huch et al. made a significant advancement in this respect by generating adult-derived self-organising 3D hepatic organoids in medium conditions that recapitulate in vivo regenerative pathways (Huch et al., 2013) (Figure 1.11).

### 1.3.1 Liver organoids

Seminal work from Sato and colleagues in 2009 first showed the isolation of single Lgr5+ adult stem cells from the small intestine and their expansion into near-physiological self-renewing 3D epithelial structures – coined ‘organoids’; these cultures contained a mixture of progenitor and mature functional cell types and were maintained in virtue of the faithful recapitulation of key signals from the endogenous stem cell niche (Sato et al., 2009). The simple and well-defined nature of the culture technology, juxtaposed to the complexity and long-term stability of the 3D epithelial structures, has quite literally revolutionised the field of in vitro adult stem cell cultures. Not surprisingly, this technology has since been adapted to grow epithelial stem/progenitor cell populations, both murine and human, from diverse endodermal-derived organs including colon, stomach, pancreas and liver (Barker et al., 2010; Sato et al., 2011; Huch et al., 2013, 2015; Stange et al., 2013; Boj et al., 2015), as well as non-endodermal tissues like the endometrium (Turco et al., 2017).

The signalling pathways that sustain endoderm-derived organoids are astonishingly conserved, with the Rspondin 1 (RSPO1)-LGR5 axis (agonist of WNT signalling) at the core of it all (de Lau et al., 2014). It was thus the recognition of WNT signalling as a key driver of liver regeneration and the identification of damage-induced bipotential Lgr5+ cells in the adult mouse liver that encouraged the idea of growing these cells as 3D organoids (Apte et al., 2007; Huch et al., 2013). Single Lgr5+ cells can be isolated and expanded in Matrigel™ – an extracellular matrix containing collagen and laminin- with the support of a medium rich in mitogens (FGF, epidermal growth factor, EGF; HGF) and the LGR5 ligand RSPO1 (Figure 1.11). As the cells proliferate, they self-organise into 3D single-layer epithelial structures of cystic morphology that express a mixture of progenitor (Lgr5, Tacstd2), ductal (Krt7, Krt19) and hepatocyte (Ttr, Hnf4a) markers. Placing healthy biliary ducts under the same culture conditions leads to the formation of identical organoids (Huch et al., 2013);
whilst this has been achieved for single EpCAM$^+$ cells – but not hepatocytes – from healthy human livers simply by supplementing the medium with an activator of cAMP signalling and an inhibitor of TGFβ signalling (Huch et al., 2015). This reinforces the idea that, at least in vitro, it is the ductal compartment that contains facultative progenitor potential; and highlights a conservation of progenitor biology across species in the liver. In support of this, canine and rat liver organoids have also been generated more recently (Nantasanti et al., 2015; Kuijk et al., 2016).

As if retaining memory of their cell-of-origin, liver organoids exhibit a molecular signature that is biased towards the biliary fate (Huch et al., 2013). Hepatocyte maturation is possible but does not occur spontaneously; it requires removal of proliferative stimuli (WNT agonists) and modulation of key signalling pathways that vary slightly according to species. This is unlike intestinal organoids, wherein the whole spectrum of differentiated cell types from the crypt-villus unit are produced without exogenous manipulation (Sato et al., 2009), but does resemble other organoid systems, even those of rapidly cycling tissues like the stomach (Barker et al., 2010). In the mouse liver organoids, addition of dexamethasone in combination with inhibition of Notch and TGFβ signalling – both of them crucial for biliary fate specification in the embryo – drives differentiation towards the hepatocyte lineage (Huch et al., 2013). The biliary fate may thus be the ‘default pathway’ for these bipotential progenitors, and hepatocyte commitment requires the blockade of biliary-sustaining signals. Following differentiation, the organoids express mature hepatocyte markers (Cyp3a11, Fah, G6pc and Alb) but also function as such. The gross majority of cells become competent for low-density lipoprotein (LDL) uptake and accumulate glycogen; they secrete albumin and display detoxifying capacities based on cytochrome P450 activity (Huch et al., 2013) (Figure 1.11). Although these functions outcompete hepatocyte cell lines (HepG2), they are still subpar compared to freshly isolated hepatocytes. Organoid-derived hepatocytes can also prolong the life span of Fah-/ mice despite poor engraftment in vivo (Huch et al., 2013) and pathologies with hepatocyte specific-phenotypes may be modelled in human organoids, as is the case of α1-antitrypsin (A1AT) deficiency (Huch et al., 2015), which manifests clinically as protein aggregates of A1AT within the endoplasmic reticulum of hepatocytes (Lawless et al., 2008).
A remarkable feature of all adult liver organoid cultures to date, especially considering the historical difficulties of primary liver cell culture, is their prolonged capacity for self-renewal: a single cell can generate $10^6$ cells in ~5-6 weeks (a population doubling rate of 48–60h) and continue to expand for up to 1 year (Huch et al., 2013, 2015). Despite this high number of cell divisions, organoid cells do not show signs of transformation even at late passage. Sequencing of human organoid cells soon after isolation and following 3 months of clonal expansion detected only one synonymous base substitution, a remarkable level of genomic stability (Huch et al., 2015). This contrasts greatly with iPSC-derived organoid cultures, which exhibit aneuploidy, chromosomal alterations and up to 1,058–1,808 de novo base substitutions that are mostly remnants of the process of somatic reprogramming (Cheng et al., 2012).

**Figure 1.11. Organoids as a novel 3D model to expand liver progenitors.** Healthy biliary duct fragments or damage-induced single Lgr5+ progenitors can be isolated from the liver and expanded in a 3D matrix of Matrigel and a defined growth factor medium that mimics key signalling pathways of liver regeneration (WNT agonist Rspo1, HGF, EGF and FGF10). The cells expand and self-organise into 3D single-layer epithelial structures of cystic morphology called ‘organoids’, which express a combination of progenitor (Lgr5) ductal (Krt19) and hepatocyte (Hnf4a) markers. Addition of dexamethasone in combination with inhibition of Notch (DAPT) and TGFβ signalling (A3801) commits the organoids to a more mature hepatocyte fate; they express markers like Alb, Cyp3a11 and Fah, but also secrete albumin, uptake LDL and store glycogen.

**1.3.2 Co-culturing of liver progenitors and stroma**

Adult-derived liver organoids are an excellent resource for modelling normal/pathological liver physiology and, in particular, regenerative processes inherent to the epithelial parenchyma (Hindley, Cordero-Espinoza and Huch, 2016). Yet the term ‘organoid’ is a bit of a misnomer, in that the adult liver is not only composed of epithelial cells but also a wide variety of stromal cells that aid in its
architecture and function, both in homeostasis and after injury (refer to section 1.2.3.2). *In vivo*, complete epithelial cell maturation from progenitor cells is also thought to require the support of non-parenchymal cells (Boulter, Lu and Forbes, 2013), which means that this level of multicellular complexity cannot be studied fully using the liver organoid technology as it stands today.

The directed differentiation of ESC/iPSC into hepatic cells often does encompass multicellular crosstalk because endodermal patterning is not 100% efficient, and thus, the emergence and/or maturation of bipotential hepatoblasts benefits from the presence of additional cell lineages that have co-differentiated from the pluripotent stem cells of origin. As an example, the directed differentiation of human ESCs-derived endodermal cells co-generates vascular endothelial growth factor receptor 2 (VEGFR2) hepatic progenitors and VEGFR2 mature hepatic cells, where the former population supports the maturation of the latter (Goldman *et al.*, 2013). Guye and colleagues have also shown that through heterogeneous induction of the visceral endoderm transcription factor, *Gata6*, all three germ layers co-emerge from a culture of human iPSCs and self-organise into a liver bud-like structure, including mesenchymal, endothelial and haematopoietic stromal cells (Guye *et al.*, 2015).

The approach of Guye et al. is elegant in terms of the minimal intervention required for multicellular co-emergence and self-organisation; however, these are stochastic events that are difficult to reproduce consistently *in vitro*. Takebe and colleagues have shown that iPSC-derived hepatoblasts can also be co-cultured with exogenously added human mesenchymal stem cells (hMSCs) and human umbilical cord endothelial cells (HUVECs) in a 3D culture system (Takebe *et al.*, 2013). This gives rise to multicellular embryonic liver bud structures that recapitulate some aspects of hepatogenesis *in vitro* and that can further mature when ectopically transplanted *in vivo* (Takebe *et al.*, 2013). In contrast, similar organotypic cultures with adult-derived liver epithelium have been less successful thus far, likely due to the higher diversification of stromal cells in the adult organ (e.g. HSCs, portal fibroblasts, LSECs, Kupffer cells and lymphocytes) and the difficulty of expanding them *in vitro*.
1.4 Aims

Tissue regeneration is a cooperative and timely process that relies on epithelial and stromal cell responses. Following chronic damage or when hepatocyte proliferation is inhibited, biliary duct cells expand *in situ* as progenitor-like cells and replenish the lost epithelium, a process guided by stromal-derived signalling. Nonetheless, stromal-to-epithelial interactions are inherently complex to study *in vivo*, and whilst major advances have been made to culture hepatic epithelium *in vitro* – including 3D liver organoids – current models have not been exploited to assess the ability of hepatic stromal cells to regulate epithelial cell fate as well as to pursue organotypic hepatic cultures. Specifically, in this dissertation I aim to:

a) Identify candidate hepatic stromal populations that behave as a supportive ductal cell ‘niche’ during regeneration, making use of the process of liver organoid formation *in vitro* as a model of the transition that ductal cells undergo from homeostasis to regeneration. We hypothesise that functional ‘niche’ cells should be enriched in pro-regenerative mitogens and support robust organoid growth *in vitro* when co-cultured with homeostatic ductal cells (*Figure 1.12-a1*). Candidate stromal cells, and their paracrine signals, will then be validated *in vivo* using animal models of liver damage (*Figure 1.12-a2*).

b) Generate chimeric liver organoid cultures that incorporate not only hepatic epithelium but also stromal cells identified in a) in an attempt to recreate the complexity of physical cell-cell interactions *in vivo*. To facilitate this, mouse and organoid reporter lines will be established to keep track of and inducibly ablate the different cell populations *in vitro* (*Figure 1.12-b*).
**Figure 1.12 Aims of the dissertation.**

**a1** *In vitro* screening of a niche population for DC

1. To screen for hepatic stromal cell populations that support ductal cell (DC) expansion *in vitro*. Hepatic stromal cell fractions will be sorted from healthy mice and co-cultured with DC to assess for enhanced 3D organoid formation *in vitro*, which models the proliferative transition of DC during the regenerative response.

2. To validate any putative niche population identified in a1) using an experimental mouse model of liver damage.

**b** Chimeric co-cultures

The aim is to generate chimeric organoids that incorporate both DC epithelium and stromal cell types within a single structure.
Chapter 2

Materials and Methods
2.1 Mouse line generation and maintenance

All mouse experiments were regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB). Mice were kept under standard handling procedures in a pathogen-free environment with a 12 h day/night cycle. Sterile food and water were given to the animals *ad libitum*. The mouse strains used in this study are summarised in Table 2.1. Hybrid lines containing two alleles were generated by breeding two different stock strains with each other and then crossing the f1 generation to obtain homozygous pups when possible. In the case of the *Sox2-Cre, nGFP* and *Sox2-Cre, mGFP* lines, the respective parental lines *nTnG* and *mTmG* were bred with *Sox2-Cre* mice; resulting in Cre-mediated excision of the *tdTomato* cassettes and in-frame expression of *GFP* since early on in embryonic development. After confirming successful recombination via GFP fluorescence in tissue biopsies, the f1 pups were bred further to verify germ line transmission.

2.2 Genotyping

Mouse earclips were digested overnight with DirectPCR lysis reagent (VIAGEN, 102-T) and Proteinase K (NEB, P8107S) at 60°C. Their DNA was amplified through polymerase chain reaction (PCR) using the GoTaq G2 Flexi DNA polymerase (Promega, M780B) and allele-specific primers detailed in Table 2.2 to determine zygosity at relevant gene loci. PCR products were analysed via DNA gel electrophoresis.

2.3 Liver damage by DDC administration

8-12 week old mice were transferred to individual wheat-free cages and were fed with diet pellets supplemented with 0.1% DDC (3,5-diethoxycarbonyl-1,4-dihydrocolidine) (Custom Animal diets, LLC). The diet was provided *ad libitum* for the duration of the experiment (5 days), after which the mice were either sacrificed or switched back to normal chow diet to allow recovery. Weight loss was monitored throughout the course of the diet and was not allowed to surpass 25%.
2.4 Lentiviral production and administration

PGK.GFP and PGK.GFP.142T lentiviral vectors (LVs) were produced by transient transfection in 293T cells and concentrated by ultracentrifugation as previously described (Dull et al., 1998). The titre of the viruses – expressed as transducing units (TU)/ml– was calculated by infecting a defined number of 293T cells with serial dilutions of each virus and analysing the percentage of GFP fluorescent cells by flow cytometry. Vector administration to mice was performed via tail vein injections. PGK-GFP LVs were injected into pups (2 days old) at 1 x 10⁷ - 2.5 x 10⁸ TU/pup, whereas adult mice (7 to 10 weeks old) were injected with PGK.GFP.142T at 1 x 10⁹ - 3 x 10⁹ TU/mouse. This work was carried out by Dr. Alessio Cantore and Michela Milani in the lab of Dr. Luigi Nadini, Milan.

2.5 Liver ductal tree isolation and fluorescence activated cell sorting (FACS)

Livers were harvested from 8-10 week old male mice and washed thoroughly in phosphate buffer saline (PBS) to remove non-resident cell populations. The tissue was placed on a petri dish to dissect out the gall bladder and to mince it with a razor blade until small pieces of ~1 mm³ were obtained. Enzymatic digestion was carried out for 3-4 hours at 37°C and constant shaking with a solution (25 ml/liver) containing 0.0125% (mg/ml) collagenase (SIGMA, C9407), 0.0125% (mg/ml) dispase II (GIBCO, 17105-041) and 1% foetal bovine serum (FBS) (GIBCO) in DMEM/Glutamax (GIBCO, 31966-021) supplemented with Heps (Invitrogen, 15630-056) and Penicillin/Streptomycin (Invitrogen, 15140-122). Halfway through the incubation period, the tissues were mechanically disrupted by pipetting, spun down at 100 g for 5 min, and incubated with fresh enzymatic solution. The progress of the digestion was monitored regularly by aliquoting small volumes of the solution and inspecting whether ductal tree fragments were visible under the microscope. After 3-4 hours, the duct-enriched tissue was digested into single cells by incubating with 5 ml of TrypLE 5x (Gibco, A12177-01) for 10 min. The solution was filtered twice through a 40 μm strainer and cells were quantified using a haemocytometer. Cells were collected into polypropylene FACS tubes (Falcon, 352063), blocked with 2% FBS.
DMEM/Glutamax for 10 min at 4°C and incubated with fluorophore-conjugated antibodies (Table 2.3) for 30 min at 4°C, at a concentration of 1 μl of antibody/1 x 10^6 cells in 1 ml of 1% FBS DMEM/Glutamax. After two washes in 1% FBS DMEM/Glutamax, cells were sorted with a MoFlo 3 machine (Beckman Coulter) and collected into eppendorf tubes containing AdDMEM/F12 (GIBCO, 12634-010) with 10 μM ROCK inhibitor (Ri) (Y-27632, AbMole). Organoid-forming ductal cells were sorted as EpCAM+CD45-CD11b-CD31+; whereas stromal fractions were EpCAM− and positive/negative for additional markers as detailed in results.

2.6 Organoid and mesenchymal cell maintenance

All cells were maintained in a humidified incubator at 37°C and 5% CO₂. Organoids were cultured in AdDMEM/F12 (Invitrogen) medium containing Hepes, Penicillin/Streptomycin, Glutamax (Invitrogen, 35050-068), 1% B27 (Invitrogen, 17504-044), 1% N2 (Invitrogen, 17502-048) and 1.25 mM N-acetylcysteine (Sigma-Aldrich, A9165) –referred to as Basal medium–, which was further supplemented with 10 nM gastrin (Sigma-Aldrich, G9145), 50 ng/ml mEGF (Invitrogen, PMG8043), 5% RSP01 conditioned medium (homemade), 100 ng/ml FGF10 (Peprotech, 100-26), 10 mM nicotinamide (Sigma-Aldrich, N0636) and 50 ng/ml HGF (Peprotech, 100-39) –referred to as expansion medium (EM). Following isolation, EpCAM+ cells were cultured in EM supplemented with 30% WNT conditioned medium (WNT CM) (homemade) and 25 ng/ml Noggin (Peprotech, 120-10C) for 3 days and were then switched to standard EM. Other medium compositions were created by removing specific growth factors mentioned above. In all experiments where cells were seeded as single cells (e.g. after sorting), 10 μM of ROCK inhibitor (Ri) was included in the medium to promote cell survival. Organoids were passaged at a 1:3 ratio once a week or when fully grown through mechanical dissociation –repeated pipetting with a narrowed glass Pasteur pipette– and were re-seeded in fresh matrigel bubbles. SCA1+ and SCA1− mesenchymal cells were cultured in Basal + WNT CM medium and were passaged at 1:3 and 1:2 ratios, respectively, through enzymatic digestion with TrypLE Express (Gibco, 12605-010) for 5 min at 37°C.
2.7 Co-cultures and conditioned medium

2.7.1 Within-matrigel drop co-culture

Freshly sorted ductal (EpCAM⁺CD45⁻CD11b⁻CD31⁻) and stromal cell populations were mixed unless stated otherwise in a 1:5 ratio (5000:25000 cells), spun down at 300 g for 5 min and seeded into three-dimensional droplets (25 μl) of matrigel on 48 well plates (Starlab, CC7682-7548). Cultures were maintained in Basal + WNT CM medium and analysed at d8-d10. Of note, this culture method resulted in limited physical contact between stromal and ductal cells.

2.7.2 Transwell co-culture

Freshly sorted mesenchymal cells were seeded on the bottom of 24 transwell-fitting plates (Corning, 3470) and cultured in Basal + WNT CM medium for 5-7 days until reaching 80-90% confluency. Freshly sorted EpCAM⁺ cells were then seeded on top on cell-impermeable transwell inserts within a 25 μl drop of matrigel. Both the top and bottom compartments of the transwell were maintained in Basal or Basal + WNT CM medium and analysed at d8-d10 after ductal cell seeding.

2.7.3 Conditioned medium

Sorted mesenchymal cells were expanded (up to passage 2 or 3) on cell culture-treated plates in Basal + WNT CM medium. When reaching 80-90% confluency, cells were washed twice with PBS and fresh Basal + WNT CM medium was added for conditioning. After 48 h, the medium was collected, spun down at 500 g for 10 min to remove any cell debris and stored at 4°C as ready-to-use conditioned medium.
2.8 Cell-cell aggregation

2.8.1 Encapsulation-based cell aggregation

Ductal and mesenchymal cells were co-encapsulated into microgels using a microfluidic flow focusing device (FFD) previously described (Anna, Bontoux and Stone, 2003) (Figure 2.1). Chips were designed to contain two separate inlets for the loading of two distinct cell populations (in aqueous phase), one inlet for the continuous phase (fluorinated oil HFE-7500 containing 0.3% PicoSurf surfactant) and one outlet. To maximize cell-to-cell proximity, the cross geometry of the chip where droplet formation occurs was limited to a width of 70 μm and a height of 75 μm. EpCAM+ ductal cells and SCA1+ mesenchymal cells were isolated from nGFP and nTnG mice, respectively or viceversa, and were expanded in vitro as detailed in Section 2.6. Each population was then harvested through TrypLE digestion at 37°C -10 min for the organoids, 5 min for the SCA1+ mesenchyme-, passed through a 40 μm cell strainer and resuspended as 0.75 x 10^6 cells/50 μl of Basal + WNT CM + Ri medium. The ductal and mesenchymal cell suspensions were mixed with 3% SeaPrep® agarose solution in a volume ratio of 1:1 (both at 37 °C) and were loaded onto two 100 μl glass syringes connected to tubings, which were in turn affixed to the two aqueous phase inlets of the FFD. A second syringe was prepared containing the continuous phase. The flow rates for the production of mono-dispersed micro gels were controlled using syringe pumps (neMESYS). A flow rate of 3 μl/min was used for the aqueous phase and a flow rate of 30 μl/min for the continuous phase. The resulting microgel/cell emulsion was collected in an ice-cooled test tube and was demulsified into 200 μl of Basal + WNT CM medium. For live imaging of the cells, μ-slide 8 well dishes (ibidi, 80826) were layered with 130 μl of ice-cold matrigel/well and 10-15 μl of the microgel/cell suspension was seeded within each well. The cultures were maintained in Basal + WNT CM medium. This work was carried out in collaboration with Timo Kohler from the labs of Dr. Florian Hollfelder and Dr. Kevin Chalut, University of Cambridge.
2.8.2 2D matrigel cell aggregation

As outlined in Section 2.5, EpCAM+ ductal cells and SCA1+ mesenchymal cells were isolated from fluorescently labelled mice and were either used directly for aggregation or expanded in vitro prior to being aggregated (stated in each case). Cells were harvested via TrypLE digestion and mixed in the following mesenchyme-to-ductal cell ratios: 0:1, 0.1:1, 0.5:1, 1:1, 5:1 and 10:1. After mixing, cells were spun down at 300 g for 5 min and were seeded on top of a 2D-layer of solidified matrigel covering the bottom of the well. Medium of choice (EM + WNT CM or Basal + WNT CM) was then added on top. The total number of ductal cells used was 5,000 or 10,000 and the matrigel layer 50 μl or 100 μl, when seeding onto 96-well or 48-well plates, respectively. We observed that, contrary to the within-matrigel drop co-culture, this method promotes mesenchyme-to-ductal cell aggregation very efficiently.

2.9 Colony formation and organoid size quantification

Organoid formation efficiency was quantified by counting the total number of lumen-containing organoid structures inside the matrigel bubble after 8-10 days in culture and normalising it by the total number of EpCAM+ cells seeded (typically
5000). Corrections were made to account for any ‘contaminant’ EpCAM+ cells present in the niche fraction (see Results). Organoid size was quantified with a custom-made Fiji macro designed by Richard Butler.

2.10 Tissue processing and cryostat sectioning

Tissues expressing fluorescent reporters were imaged with a stereoscope prior to being processed. Livers were washed in PBS, diced with a razor blade and fixed overnight in 10% formalin whilst rolling at 4°C. Small intestines were flushed once with PBS to remove their faecal content and then with formalin 10%; overnight fixation was carried out as for the livers. Tissues were washed thrice with PBS to remove any traces of the fixative and were incubated with 30% sucrose PBS for 24-48 h whilst rolling at 4°C. Liver pieces (up to 3 per mould) or short sections of the small intestine were placed into cryomoulds (Sakura, 4566), layered with OCT compound (VWR, 361603E) and frozen on dry ice. Tissue blocks were either stored at -80°C or cut with a Leica CM-3050S cryostat into 8 μm sections that were mounted onto SuperFrost Plus slides (VWR, 631-0108) for further staining.

2.11 Immunostaining, confocal imaging and cell counting

Cells were fixed with ice-cold 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, 15713-S) for 30 min and blocked/permeabilised for 2h in a buffer – referred to as PBSTD - containing 0-1% Triton X-100 (SIGMA, T8787), 2% dimethyl sulfoxide (SIGMA, D8418), 1% bovine serum albumin (BSA, A8806) and 2% donkey serum (SIGMA, D9663) diluted in PBS. Triton concentration was optimised according to the subcellular localisation of the protein of interest: 0% for membrane-bound, 0.3% for cytoplasmic and 1% for nuclear. Primary antibodies were added overnight at 4°C in 1:100-diluted PBSTD buffer and were washed three times prior to adding secondary antibodies for 2h at room temperature in PBS-0.05% BSA. Nuclei were counterstained with Hoechst 33342 (Thermo Scientific, H3570) for 10 min. Frozen tissue sections generated as in Section 2.10 were stained similarly, ignoring the initial fixation step. For the complete list of primary and secondary antibodies used refer to Table 2.3 and Table 2.4, respectively. Images were acquired using a confocal microscope (Leica SP5) and processed using Volocity software.
2.11 Immunostaining, confocal imaging and cell counting

(PerkinElmer) or ImageJ software. Cell populations were quantified using the ImageJ plugin ‘cell counter’.

2.12 EdU incorporation

EdU incorporation experiments were performed using the Click-iT® EdU Alexa Fluor® 594 or 647 Imaging Kit (C10339, Life Technologies) according to the manufacturer’s protocol. Cells were incubated for 16h with 10 μM EdU in their respective culture medium, after which they were fixed in 4% PFA for 30 min, permeabilised with 0.5% Triton X-100 for 20 min and incubated with freshly prepared 1X Click-iT™ EdU cocktail (Life Technologies) for 30 min at room temperature. Nuclei were stained with Hoechst 33342 (Life Technologies) for 15 min.

2.13 qRT-PCR

Total RNA was extracted from cells using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, 12204-01) according to the manufacturer’s protocol; including a 15 min digestion step with DNase to remove traces of genomic DNA. The RNA (50-250 ng) was reverse-transcribed with the Moloney Murine Leukemia Virus reverse transcriptase (M-MLVRT) (Promega, M368B) and was amplified using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The list of primers used for qRT-PCR is summarised in Table 2.5. Gene expression levels were normalised to the housekeeping gene Hprt1.

2.14 RNA sequencing and bioinformatic analysis

Liver ductal tree isolations were performed as in Section 2.5. For the sequencing of duct-associated stroma, populations were sorted from the healthy livers of two littermates as: ductal (EpCAM+ CD45- CD11b- CD31-), hematopoietic/endothelial (CD45+ CD11b+ CD31+ EpCAM-) and mesenchymal-enriched (CD45- CD11b- CD31+ EpCAM-), all of which were further sorted based on SCA1 staining (SCA1+ and SCA1-). For the sequencing of cells in culture, mesenchymal (SCA1+CD45-CD11b-CD31-EpCAM-) cells from two littermates were lysed as t=0 controls or were cultured on the bottom of 24 transwell-fitting plates (50000
cells/well) for 7 days in Basal + WNT CM medium; after which they were either cultured alone or together with freshly sorted ductal (EpCAM+ CD45− CD11b− CD31−) cells from two additional littersmates seeded on a cell-impermeable transwell insert (5000 cells/matrigel bubble) (as in Section 2.7.3) for 15 days in Basal + WNT CM medium. As controls, ductal cells from the same preparation were lysed at t=0 or seeded in EM without any mesenchymal cells and collected at day 15. Total RNA was extracted from all samples with the Picopure RNA Extraction Kit according to manufacturer’s instructions (including DNAse digestion).

RNA libraries were prepared using Smartseq2 (Picelli et al., 2014) and were sequenced on an Illumina HiSeq 4000 instrument in single read mode at 50 base length. FastQC (version 0.11.4) was used for initial quality control of the reads. Sickle (version 1.33) and Trim Galore (0.4.4) were used for quality and adapter trimming of the raw reads respectively. Reads were then mapped to the GRCh38/mm10 UCSC reference genome using STAR aligner (version 2.5.0a). Raw counts were generated using featureCounts (version 1.6.0) software and includes all exons for a gene from the mm10 UCSC GTF file (http://genome.ucsc.edu/cgi-bin/hgTables). RPKMs were generated with raw counts and gene lengths reported by featureCounts package. RPKMs were used for clustering and PCA analysis using ‘hclust’ and ‘prcomp’ R Core Packages. Heatmaps were prepared based on scaled RPKM values using the Clustvis software (https://biit.cs.ut.ee/clustvis/). Genes at RPKM <1.0 were considered non-expressed and were excluded from the analyses.

2.15 Time-lapse imaging and video processing

Time-lapse imaging of cells was carried out at 37°C and 5% CO2 for 24h-periods. For quantification purposes, we used a 20x air lens on a confocal spinning-disk microscope system (Intelligent Imaging Innovations, Inc. 3i) comprising an Observer Z1 inverted microscope (Zeiss), a CSU X1 spinning disk head (Yokogawa), and a QuantEM 512SC camera (Photometrics). Imaging was performed at 15 min intervals, with a z-step of 7 μm, a z-range of 100 μm and laser power of up to 20%. For higher image resolution, we used a 10x air lens on a Zeiss 710 confocal microscope.
2.15 Time-lapse imaging and video processing

and imaged at 15 min intervals, with a z-step of 9 μm, a z-range of 100 μm and 1024 x 1024 bidirectional scanning. Videos were generated with the Slidebook6 software and were analysed with the ImageJ software.

2.16 Flow cytometry

Single cell suspensions were prepared via trypsinisation (as detailed in Section 2.8.1), blocked in 2% FBS DMEM/Glutamax and stained with fluorophore-conjugated antibodies (Table 1) diluted in 1% FBS DMEM/Glutamax for 30 min at 4°C. Antibody staining was omitted in cells encoding fluorescent reporters. Cells were washed once in 1% FBS DMEM/Glutamax and were resuspended in 1% FBS DMEM/Glutamax containing DNAse at 0.1 mg/ml (Sigma Aldrich, DN25) for analysis with a BD Fortessa machine. Unstained or non-fluorescent cells were used as controls for setting up gating parameters.

2.17 Viability/cytotoxicity assay

Cell viability was assessed using the Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (30002-T, Biotum). Matrigel-embedded cells were washed twice in PBS and were incubated with 2 μM calcein AM/4 μM EthD-III for 30 min at room temperature as indicated in the manufacturer’s protocol. The staining solution was then replaced with fresh medium and the cells were imaged using a confocal microscope (Leica, Sp5). The calcein and EthD-III dyes were excited using the 488 and 568 lasers respectively. The percentage of live and dead cells was quantified using the ImageJ software.
2.18 Statistics

Data were analysed using the Mann–Whitney non-parametric test. P<0.05 was considered statistically significant. P<0.05 (*), P<0.01 (**), P<0.001 (***) , P<0.0001 (****). Calculations were performed using the Prism 6 software package.

Table 2.1 List of mouse strains

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Allele ID</th>
<th>Allele full name</th>
<th>Allele type</th>
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</thead>
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<td>nTnG</td>
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<td>mTmG</td>
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<tr>
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Table 2.2 List of genotyping primers and PCR conditions

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<td>AAA GTC GCT CTC AGT TGT TAT</td>
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<td>94°C 30s, 55°C 30s, 72°C 30s Mutants: 320 bp and 608 bp Wild-type: 608 bp and 496 bp</td>
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### Table 2.3 List of primary antibodies

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### Table 2.4 List of secondary antibodies

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### Table 2.5 List of qPCR primers

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Chapter 3
Identification of primary liver stromal populations that support organoid formation \textit{in vitro}
3.1 Isolation of primary ductal cells and associated stromal populations in the liver

The liver tissue may be subdivided into histological subunits known as lobules, which span from the portal triad – encompassing the portal vein (PV), hepatic artery (HA) and biliary duct (BD) – to the central vein (CV) area. In between, hepatocytes are arranged in linear cords and are irrigated by a capillary network of liver sinusoidal endothelial cells (LSECs) (Figure 3.1A).

![Figure 3.1. The cellular microenvironment of the biliary duct epithelium in the liver. A) Schematic depicting a cross section of the liver lobule, which spans from the central vein to the portal triad. The biliary duct epithelium lies next to the portal vein and is surrounded by a mixture of endothelial, hematopoietic and mesenchymal cells. B) Immunostainings of healthy liver frozen sections with antibodies against biliary duct cells (OPN, PCK), endothelial cells (CD31), hematopoietic cells (CD45, F4/80) and mesenchymal cells (Desmin). PV: portal vein, CV: central vein, BD: biliary duct.]

Given that biliary duct cells can be activated upon damage into a progenitor-like state, and that a niche cell is defined by its proximity to the progenitor compartment, we were interested in studying the stromal cells that reside around the biliary duct network. In homeostasis, the most proximal microenvironment of ductal cells – labelled by osteopontin (OPN) or pancytokeratin (PCK) – is composed of
3.1 Isolation of primary ductal cells and associated stromal populations in the liver

leukocytes (CD45+), liver resident macrophages or Kupffer cells (F4/80+), endothelial cells lining the portal vein (CD31+) and mesenchymal cells lining both the vein and the biliary duct itself (Desmin+) (Figure 3.1B).

To assess the putative niche potential of these diverse cell populations, we sought to isolate them from the tissue and study them ex vivo. Previous work from our laboratory had established a method of primary biliary tree isolation to grow liver organoids in vitro (Huch et al., 2013). Because the isolation procedure unavoidably entails the co-extraction of biliary associated stroma – previously regarded as ‘contaminant’ cells– we adapted the protocol to now preserve this non-epithelial compartment instead of discarding it. In accordance, murine livers are first digested enzymatically with a collagenase and dispase mixture until enriching for biliary duct fragments and a mixture of stromal cells. After further digestion with TrypLE, a final suspension of single cells is FACS-sorted using fluorophore-conjugated antibodies (Figure 3.2A).

Figure 3.2 Isolation strategy of primary biliary and stromal cell populations from the homeostatic liver.
A) Schematic depicting the process of primary liver cell isolation. Livers are harvested from mice, washed thoroughly and digested enzymatically with collagenase and dispase in order to enrich for biliary ducts and their associated stroma. The tissue is further processed into single cells and FACS–sorted with cell-type specific antibodies. B) Live cells were gated based on their forward and side scatter profile (R1), and were sorted as ductal cells (DC; EpCAM-CD45-CD11b-CD31+), hematopoietic/endothelial cells (H/E; EpCAM-CD45+CD11b+CD31+) and mesenchymal cells (Msc; EpCAM-CD45-CD11b-CD31+).
Dead cells and debris are excluded through forward and side scatter gating, and cells are parsed out according to their expression of a panel of lineage-specific cell surface markers: EpCAM, CD45, CD11b and CD31. As in Huch et al., EpCAM⁺ CD45⁻, CD11b⁻, CD31⁻ cells correspond to the ductal cell (DC) population (Figure 3.2B, green gate) (Huch et al., 2013). We defined the remaining cells as putative niche populations and divided them into two broad categories: CD45⁺CD11b⁺CD31⁺EpCAM⁻ cells of the hematopoietic/endothelial (H/E) lineage (Figure 3.2B, blue gate) and CD45⁻CD11b⁻CD31⁻EpCAM⁻ cells, hypothesised to be predominantly mesenchymal (Msc) from lineage exclusion (Figure 3.2B, orange gate).

Figure 3.3 Biliary-associated stroma can be subdivided into hematopoietic/endothelial (H/E) and mesenchymal (Msc) cell populations. A) qPCR analysis of lineage-specific (DC, H/E, Msc) genes expressed by the sorted populations in Fig3.2B. 2ΔCt values are normalised to the housekeeping gene Hprt and are represented as fold change relative to the DC fraction. N=2 independent mice, 2 technical replicates per mouse, error bars denote mean ± SD. The H/E fraction is enriched in markers of the hematopoietic (Ptprc, Ilgam, Adgre1) and endothelial (Pecam1, Cdh5, Flt4, Kdr) lineage; the Msc population expresses many mesenchymal-associated genes (Acta2, Thy1, Desmin, Gfap, Vimentin) but also some endothelial and hepatocyte markers. B) Brightfield pictures of the sorted cells in Fig3.2B following in vitro culture (3D matrigel, EM medium, d7). The DC fraction gives rise to organoids, whereas the H/E grows predominantly as small round cells and the Msc population as larger fibroblastic-looking cells.
The cellular identities of the DC, H/E and Msc populations were confirmed through qPCR analysis of a battery of lineage-specific genes (Figure 3.3A) and cell morphology after in vitro culturing (Figure 3.3B). As expected, the DC population expresses the ductal marker Krt19—but not any stromal genes—and gives rise to 3D epithelial organoids in vitro. H/E cells exhibit high expression of hematopoietic-specific genes like Ptprc (CD45), Itgam (CD11b) and Adgre1 (F4/80), as well as endothelial genes such as Pecam1 (CD31), Cdh5 (VE-cadherin), Kdr (VEGFR2), and Flt4 (VEGFR3); morphologically the H/E fraction grows as colonies of small spherical cells. The Msc population expresses various mesenchymal genes including Acta2 (αSMA), Thy1, Desmin, Vim and Gfap; yet also the endothelial markers Pecam1 and Cdh5 (to a lesser extent than H/E cells) and Alb (Albumin), a hepatocyte-specific gene. Although this suggests a percentage of contaminating cells in the Msc fraction, the population grows predominantly with large and fibroblast-like morphology, indicating prevalence of the mesenchymal lineage in vitro.

Whilst our organoid culture medium (EM) allows the long-term expansion of DC-derived progenitors, in vitro growth of primary cells is challenging and can be greatly influenced by medium composition. We thus tested the viability of the primary H/E and Msc lineages in various medium compositions (Basal, Basal + WNT CM, Basal + 3% FBS and EM) using a live-dead assay that was first validated in human mesenchymal stem cells (hMSCs) (Figure 3.4).

![Figure 3.4 Validation of the cell viability assay](image)

**Figure 3.4 Validation of the cell viability assay.** Viability staining of live or heat-killed hMSCs incubated with various concentrations of Calcein (green) and EthD-III (red), which label live and dead cells respectively. The concentration of 4μM calcein AM / 8μM EthDII was selected for further experiments.

At d2 of culture, H/E cells survive best in Basal + WNT CM and Basal + 3% FBS medium, with close to 40% of live cells; although by d7 survival drops and Basal +
WNT CM proves the best medium with only 20% survival (Figure 3.5A,B). The Msc cells overall survive better than the H/E fraction and grow best in Basal + WNT CM both at d2 and d7 with ~40-50% survival (Figure 3.5A,B).

3.2 Co-culturing ductal cells with mesenchymal or hematopoietic/endothelial populations enhances organoid formation

In vivo, a niche cell that supports ductal-driven regeneration would be expected to modulate ductal cell behavior in terms of activation, proliferation and/or differentiation. We thus assessed the niche potential of the H/E and Msc cells based on their ability to support the formation of DC-derived organoids in vitro. Freshly sorted cells were cultured as follows: DC alone (5000), H/E or Msc cells alone (25000) and DC + H/E or Msc cells co-cultured together (5000:25000) (Figure 3.6A). When grown in the standard organoid medium (EM), the DC fraction generates organoid structures at an expected mean efficiency of 3.6% and a median organoid area of
0.0024 mm². Co-culturing DC with the stromal populations, particularly the Msc cells, showed a trend towards increased organoid formation efficiency (up to 5.9%) (Figure 3.6B,C). The median organoid area was significantly augmented (Figure 3.6D) and organoids were characterised by a higher percentage of proliferative cells as judged from EdU incorporation (Figure 3.6E,F). Of note, organoid growth was detected in the H/E or Msc only controls at an efficiency of 0.175% and 0.38%, respectively. This is likely due to contaminating DC cells from the sort (~3% verified by purity check, data not shown) and was corrected for in subsequent co-culture quantifications.

Figure 3.6 Establishing co-cultures between ductal cells and H/E or Msc populations. A) Schematic of co-culture strategy: 5000 freshly sorted DC are either cultured on their own or with 25,000 primary H/E or Msc cells within a matrigel bubble in EM. H/E or Msc cells (25,000) are also cultured on their own as controls. B) Brightfield pictures of organoids formed within the matrigel droplets after 8 days of culture. C) Quantification of organoid formation efficiency in B). Results are expressed as percentage of organoids formed with respect to total DC seeded (5000). In the case of the H/E and Msc fractions, organoid formation was calculated based on 250,000 cells seeded. N=3 mice, 2 technical replicates per mouse, error bars denote mean ± SD. D) Quantification of organoid area (mm²) in B). N=3 mice, 2 technical replicates. Values are plotted on a log scale and are represented as violin plots, which denote population density. The box plots within represent the median + upper and lower quartiles; the lines denote the range of values and the dots are outliers defined to be greater than 1.5 interquartile range (IQR) from the median. E) EdU immunostaining of DC cultured alone or with H/E or Msc. F) Quantification of the percentage of EdU⁺ cells/organoid in E). N=1 mouse, 2 technical replicates per mouse. Violin plots were drawn as in D). p<0.0001 (****), p<0.001 (**), p<0.01 (*), p<0.05 (ns).
Immunostaining of the co-cultures highlights the co-existence, and at times, physical proximity between the different cell lineages in vitro (Figure 3.7). Organoids of cystic morphology and positive for the epithelial marker E-cadherin intermingle with CD45+ and F4/80+ cells from the H/E fraction or αSMA+ Msc cells. Notably, no CD31+ cells were detected, suggesting that the endothelial subpopulation of the H/E fraction does not survive in vitro and thereby does not contribute to sustaining organoid formation.

**Figure 3.7 Ductal and stromal cells co-exist in vitro.** EM-grown co-cultures at day 9 of culture stained with antibodies against epithelial (E-cadherin), hematopoietic (CD45, F4/80), endothelial (CD31) and mesenchymal (αSMA) proteins.

The expansion and self-renewal of ductal progenitors in vitro relies on a defined cocktail of growth factors supplemented to the culture medium: RSPO1 (LGR5 ligand and WNT agonist), the mitogens EGF, FGF10 and HGF; and WNT3A-conditioned medium following DC isolation (Figure 3.8A). Activation of the WNT pathway and signalling through receptor tyrosine kinases is likewise essential for liver regeneration in vivo (Apte et al., 2007; Ishikawa et al., 2012; Takase et al., 2013). At the mRNA level, Msc cells express Hgf, Fgf7 (part of the Fgf10 subfamily), Rspo1 and Rspo3; while H/E cells express Wnt2 and Rspo3 but very little of the other factors (Figure 3.8B). Expression of a key battery of pro-regenerative cytokines, as in the case of the Msc cells, could provide niche support for the ductal progenitors in vitro, but this is likely to be masked when culturing in a growth factor saturated medium such as EM (Figure 3.6). Co-cultures were thus performed in Basal + WNT CM medium (devoid of EGF, FGF10, HGF and RSPO1), a more stringent culture condition that deters organoid growth unless exogenous support (i.e. a functional niche cell) is
3.2 Co-culturing ductal cells with mesenchymal or hematopoietic/endothelial populations enhances organoid formation provided, and has the added benefit of enhancing H/E and Msc cell survival according to previous results (Figure 3.5). Under these conditions, the DC + Msc co-culture exhibits a significant 3-fold increase in organoid formation efficiency and 2.25-fold increase in organoid area compared to the DC control (Figure 3.8C-E); in contrast, co-culturing with the H/E fraction leads to no improvement in organoid growth. A similar trend was observed in Basal medium supplemented with 3% FBS but not WNT (Figure 3.8D), as well as in Basal medium only, although the latter with considerably lower levels of organoid formation (<0.5% for DC + Msc co-cultures) (Figure 3.8C,D).

Figure 3.8 Msc cells support organoid formation in the absence of exogenous growth factors. A) Schematic of the growth factor composition of the organoid medium, EM. B) qPCR analysis of growth factor gene expression in homeostatic DC, H/E and Msc populations. 2ΔCt values are normalised to the housekeeping gene Hprt and are represented as fold change relative to the DC fraction. N=2 mice, 2 technical replicates per mouse, error bars denote mean ± SD. C) Brightfield pictures of organoid formation after 8 days of culture in Basal or Basal + WNT CM. D) Quantification of organoid formation efficiency in C) and in Basal + 3% FBS medium. N=3 mice, 2 technical replicates per mouse, error bars denote mean ± SD. Results are expressed as percentage of organoids formed with respect to total DC seeded (5000) and are corrected for any contamination from the stromal fraction. E) Quantification of organoid area (mm²) in the Basal + WNT CM cultures from C). N=2 mice, 2 technical replicates per mouse. Violin plots were drawn as in Figure 3.6D. p<0.0001 (***) p<0.001 (**), p<0.01 (*), p>0.05 (ns).
Chapter 3 Identification of primary liver stromal populations that support organoid formation in vitro

In vivo, stromal cells may cooperate with each other to indirectly modulate progenitor behaviour. This does not appear to be the case between H/E and Msc cells, at least in vitro under Basal, Basal + WNT CM or Basal + 3% FBS culture medium, given that the triple DC + H/E + Msc co-culture does not enhance organoid formation compared to DC + Msc (Figure 3.9).

![Figure 3.9 Triple co-culture between DC, H/E, Msc cells does not further enhance organoid formation. A) Brightfield pictures of organoid formation after 8 days of culturing DC, DC + H/E, DC + Msc and DC + H/E + Msc in Basal + WNT CM or Basal + 3% FBS medium. B) Quantification of organoid formation in A), including controls in Basal medium. N=2 mouse, 2 technical replicates per mouse.](image)

The organoid-supportive effect of the Msc population appears to be dose-dependent. In experiments where increasing number of Msc cells are co-cultured with fixed numbers of DC (Figure 3.10A), a non-linear surge in organoid formation is observed, reaching a mean efficiency of up to 8.5% even in Basal medium (Figure 3.10B,C). This non-linear response could suggest a beneficial effect from culturing numerous Msc cells together in terms of their own survival, proliferation and/or cytokine secretion, although this remains to be formally investigated.
3.2 Co-culturing ductal cells with mesenchymal or hematopoietic/endothelial populations enhances organoid formation

**Figure 3.10** Msc cells support organoid formation in a dosage-dependent manner. A) Design of the co-culture with increasing numbers of Msc cells (25000, 50000 or 100000) and a fixed number of DC (5000). B) Brightfield pictures of organoid formation after 11 days of culturing in Basal medium. C) Quantification of organoid formation in B). N=2 mice, 2 technical replicates per mouse, error bars denote mean ± SD.
Chapter 4

SCA1 labels organoid-supportive mesenchymal cells that surround biliary ducts and expand following liver damage.
4.1 SCA1+ mesenchymal cells surround biliary ducts and expand after damage

*In vitro* assays from Chapter 3 identified Msc cells as an organoid-supportive niche, drawing interest in studying this population further. However, the Msc sorting strategy was based on negative marker selection (EpCAM-CD31-CD45-CD11b-) (*Figure 3.2B*), and although bulk RNA analysis showed enrichment of classical mesenchymal markers in these cells, 'contaminant' non-mesenchymal markers were also detected. It was thus necessary to find a mesenchymal surface marker to fine-tune the sorting of Msc cells and corroborate their identity.

With this aim, we searched for commercially available flow cytometry antibodies against mesenchymal stromal cells and found a panel recognising CD140a (PDGFRα), CD140b (PDGFRβ), Stem Cell Antigen 1 (SCA1), CD44 and CD29; which have been previously used to characterise fibroblasts in the heart (Furtado *et al.*, 2014). Validation of these markers by qPCR confirmed their expression in the Msc fraction –Cd29 and Ly6a (SCA1) being the most highly expressed– yet also showed their promiscuity at labelling other cell populations such as H/E and DC (*Figure 4.1A*). By flow cytometry, CD140a and CD140b labelled only a minimal percentage of Msc cells, whereas CD44 was predominantly expressed by H/E cells (20%). SCA1 and CD29, on the other hand, labelled 20% and 80% of the Msc cells respectively; the sheer abundance of CD29 staining in all the populations (76% of H/E cells, 100% of DC) questioned its value as a useful marker for refining the Msc sorting (*Figure 4.1B*). Considering that the whole panel of antibodies had been validated using mouse embryonic fibroblasts (MEFs) as positive controls (*Figure 4.2A*), the apparent lack of expression of CD140a and CD140b in the Msc population was unexpected. Inferring from the mRNA analysis, this might be due to a combination of low levels of protein abundance and low antibody sensitivity, given that higher antibody concentrations do detect up to 16% of CD140a+ cells in the liver (*Figure 4.2B*).
4.1 SCA1⁺ mesenchymal cells surround biliary ducts and expand after damage

Figure 4.1 Identification of cell surface markers in the Msc population. A) qPCR analysis of mesenchymal genes in H/E, Msc, DC and total liver cells. 2ΔCt values are normalised to the housekeeping gene Hprt. N=1 mouse, 2 technical replicates, error bars denote mean ± SD. B) Flow cytometry profile of the markers in A) amongst the H/E, Msc and DC populations. Results are expressed as univariate histograms depicting the relative fluorescence intensity of each fluorophore (x-axis) vs the number of detected cell events (y-axis). The percentage of stained cells is summarised for each population (N=1 mouse).
Chapter 4 SCA1 labels organoid-supportive mesenchymal cells that surround biliary ducts and expand following liver damage

Having found SCA1 as a putative marker for the Msc population, we wished to verify the existence of SCA1+ liver mesenchymal cells in vivo. Immunostaining of liver tissue sections shows SCA1 positivity localising exclusively at the portal tract of the lobule, not the central vein (Figure 4.3A), whereas Vimentin+ stroma is found on both areas. To identify mesenchymal populations more readily, we employed the Pdgfra-H2B-GFP knock-in reporter line (Hamilton et al., 2003), which has been previously used to assess fibrogenic responses in the liver (Hayes et al., 2014). SCA1 labels a subpopulation of mesenchymal cells (PDGFRα+) that closely associate with biliary ducts (Figure 4.3B-1), in contrast, SCA1-PDGFRα+ cells are found spread throughout the liver parenchyma (Figure 4.3B-2). Peribiliary SCA1+ cells are negative for αSMA, unlike pericytes wrapping around the portal vein and hepatic artery; but they do co-express CD34 and in some cases Elastin, a marker of portal fibroblasts (Figure 4.3C).
Amongst the other stromal cells of the portal tract, a population of SCA1+ CD31+ endothelium lines the portal vein; the surrounding LSECs marked by VEGFR3 are negative for SCA1, as is the case for F4/80+ macrophages (Figure 4.3D).

**Figure 4.3** SCA1 labels a subpopulation of periportal mesenchymal cells. **A)** Immunostaining of the portal tract and central vein areas of WT livers with mesenchymal/stromal (SCA1, Vimentin) and epithelial (OPN) markers. SCA1+ cells localise exclusively to the portal tract of the liver lobule, where the biliary duct epithelium (OPN*) is confined. **B)** Immunostaining of Pdgfra-H2B-GFP mouse livers with SCA1 and OPN antibodies. SCA1+ PDGFRα+ cells (B1) reside in close proximity to the ductal epithelium (OPN*), whereas SCA1 PDGFRα+ cells (B2) are spread throughout the liver parenchyma. **C,D)** Immunostaining of WT livers with SCA1, CD34, mesenchymal (αSMA, Elastin), endothelial (CD31, VEGFR3) and hematopoietic (F4/80) markers. The F4/80 and SCA1 stainings were compared in consecutive (c) sections as both antibodies were raised in the same host (rat). Biliary-associated SCA1+ cells co-express CD34 (yellow asterisk) and in some cases Elastin (C2, yellow asterisk vs C1 arrow), while they stain negative for αSMA as well as endothelial and hematopoietic markers (yellow arrows).
Chapter 4 SCA1 labels organoid-supportive mesenchymal cells that surround biliary ducts and expand following liver damage

The vicinity of SCA1+ PDGFRα+ cells to the biliary duct epithelium speaks in favour of these cells as a putative niche population. Periportal liver stromal cells including Thy1+ mesenchyme (Takase et al., 2013) have been shown to expand as a response to tissue damage, and thus draw the question of whether SCA1+ Msc cells could behave similarly. Liver damage can be induced in mice by supplementing their diet with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), a xenobiotic that inhibits heme metabolism in hepatocytes and leads to their death (Magnus, Roe and Bhutani, 1969). Accordingly, we utilised a protocol in which mice are fed with a DDC-supplemented diet for 5 days and are subsequently allowed to recover with normal diet for 7 days (Figure 4.4A). This experimental model leads to a well-documented expansion of periportal ductal progenitors (OPN+) which we indeed observe (Figure 4.4B,D); and interestingly, these cells are closely accompanied by a population of SCA1+PDGFRα+ mesenchymal cells (Figure 4.4A). Already at d5 post-diet administration, numerous mesenchymal SCA1+ cells co-expressing Vimentin, yet not the classic myofibroblast marker αSMA, are detected at the periphery of biliary ducts (Figure 4.4C). Whilst the total number of SCA1+PDGFRα+ cells per portal vein increases from DDC d0 to DDC d12, this is not the case for SCA1+ PDGFRα+ cells (Figure 4.4D). Moreover, there is an increase in the ratio of SCA1+ PDGFRα+ cells / OPN+ ductal cells from DDC d0 to DDC d12 (Figure 4.4E). Together, these data confirm the close association that exists, in homeostasis and in damage, between SCA1+ Msc and DC/progenitors, and heightens the interest in studying this mesenchymal microenvironment further.
4.1 SCA1+ mesenchymal cells surround biliary ducts and expand after damage

[Image: Timeline of the DDC model of liver damage and regeneration. Mice are administered DDC in their diet for a period of 5 days and are then allowed to recover for 7 days.]

4.2 Molecular characterisation of primary SCA1+ mesenchymal cells in homeostasis

To better understand the molecular make-up of liver mesenchymal cells, we isolated SCA1+ Msc and SCA1- Msc cells from homeostatic livers following a strategy modified from Figure 3.2B to include SCA1 discrimination (Figure 4.5A) and analysed their global gene expression through RNA sequencing; SCA1+ and SCA1- cells from the...
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H/E and DC lineages were also included as controls. Unsupervised clustering analysis of the 6 populations separates them into two major branches: an epithelial DC branch and a stromal branch that is further subdivided into the H/E and the Msc lineages (Figure 4.5B). Interestingly, SCA1 expression defines two subgroups within the Msc and H/E fractions respectively, whilst this is not the case for the DC cells, as samples cluster by biological replicate rather than by SCA1 expression (SCA1+ or SCA1−) (Figure 4.5B). In line with this, the demarcation of SCA1 staining in the FACS plots is not very clear for the DC compared to the H/E and Msc (Figure 4.5A); this is also reflected at the mRNA level as there is only a mild enrichment of Ly6a (SCA1) expression in SCA1+ DC compared to SCA1− DC (Figure 4.6A).

Figure 4.5 SCA1+ and SCA1− Msc cells are molecularly distinct from each other and from SCA1+/− DC and SCA1+/− H/E cells. A) FACS strategy for the isolation of hepatic primary DC (SCA1+, SCA1−), H/E (SCA1+, SCA1−) and Msc (SCA1+, SCA1−) populations. B) Hierarchical clustering of gene expression from the populations in A) sequenced by RNAseq (N=2 mice, the replicates are denoted as 1 and 2). The three cell lineages (DC, H/E, Msc) are molecularly distinct and SCA1 expression defines two clear subgroups within the Msc and H/E lineages respectively.
4.2 Molecular characterisation of primary SCA1+ mesenchymal cells in homeostasis

We assessed the expression of lineage-specific genes across the 6 populations and represented them as a heatmap (Figure 4.6A). Compared to the SCA1- Msc cells and the other lineages, the SCA1+ Msc fraction is enriched in several classical fibroblast/mesenchymal genes including Eln, Pdgfra, Pdgfrb, Vim, Col1a1 and Col1a2; as well as more lineage-fluid genes like Cd34 and Thy1, the latter of which has been used to identify both hepatic mesenchyme and ‘oval’ cells in the liver (Petersen et al., 1997; Takase et al., 2013).

Figure 4.6 Expression profile of lineage specific genes in the SCA1+ and SCA1- fractions of the H/E, Msc and DC populations. A) Gene expression heatmap (RPKM values) of mesenchymal, H/E, DC and hepatocyte (HC) genes across the H/E (SCA1+, SCA1-), Msc (SCA1+, SCA1-) and DC (SCA1+, SCA1-) populations sequenced in Figure 4.5. B) qPCR validation of selected genes from A). 2∆Ct values are normalised to the housekeeping gene Hprt. N=2 mouse, 2 technical replicates, error bars denote mean ± SD.
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The SCA1·Msc fraction expresses many of the aforementioned genes but to a lower extent, and is particularly enriched in Acta2 and Des. Similar to Figure 3.3A, some hepatocyte (HC) contamination is found within the SCA1·Msc fraction (sorted using negative selection), judging from the expression of HC-specific genes like Alb and Ttr.

The H/E cells express all the expected genes of their lineage, although interestingly, there is a separation of endothelial- (Pecam1, Cdh5, Flt-4) and hematopoietic- (Itgam, Prtprc) specific genes in the SCA1+ and SCA1- fractions respectively. The cluster of DC-specific genes (Krt19, EpCAM, Sox9, Cdh1, Krt7 and Prom1) is selectively upregulated in the DC fraction (SCA1+ and SCA1-), and no other H/E, Msc or HC-specific genes (with the exception of Hnf4a) appear to be expressed by DC. The above RNaseq pattern of gene expression was validated by qPCR with primers against a selected panel of lineage-specific genes (Figure 4.6B).

Niche cells may modulate DC behaviour through paracrine signalling. We thus assessed the 6 populations for expression of growth factors and/or cell-bound ligands from regeneration-relevant signalling pathways (WNT, FGF, HGF, EGF, Notch, TGFβ, etc). Unsurprisingly, the DC fraction does not express the vast majority of these factors, as it relies on exogenous sources for their supply. Some exceptions are Wnt7a and Wnt7b, particularly expressed on the SCA1+ cells, Fgf12 and Tgfb2, enriched more on the SCA1- cells, as well as Wnt5b, Dll1 and Tnf, expressed on both (Figure 4.7A,B). Compared to the other stromal populations, Msc SCA1+ cells express the greatest number of signalling molecules from the selected list. Notable factors are: Hgf, various Fgf genes (including Fgf7), Igf1, Igf2, the WNT agonists Rspo1 and Rspo3, the WNT ligands Wnt5a, Wnt6, Wnt9a and Wnt9b, but also WNT inhibitors Wif1, Dkk2, Dkk3; Notch ligands and receptors like Jag1 and Notch1, as well as Tgfb1, Tgfb3, Il6 and Cxcl12. The latter two genes show the highest level of expression in the Msc SCA1+ cells, followed by Dkk3, Rspo3 and Tgfb3 (Figure 4.7B). Il6 is of particular interest because it is a reported marker of portal fibroblasts in the liver (Wells, 2014). The Msc SCA1- fraction shares expression, although diminished, of many of the above genes; while there are other genes like Fgf1 that it uniquely overexpresses. The H/E cells, particularly the SCA1+ subpopulation, are enriched in WNT agonists and ligands such
as Rspo3 (expressed more than in the Msc SCA1+ cells), Wnt2 and Wnt9b, but also other molecules like the Notch ligand Dll4 and the pro-inflammatory Tnf. As in Figure 4.6B, selected genes from the RNAseq were validated by qPCR. Although overall concordance is observed, the relative levels of gene expression vary for some genes, as is the case for Hgf, which is more highly expressed than Rspo3 (Figure 4.7C).

**Figure 4.7** Expression profile of growth factors and cell-bound ligands in the SCA1+ and SCA1- fractions of the H/E, Msc and DC populations. A) Gene expression heatmap of growth factors and cell surface ligands of various signalling pathways across the H/E (SCA1+, SCA1-), Msc (SCA1+, SCA1-) and DC (SCA1+, SCA1-) populations sequenced in Figure 4.5. B) Plot of RPKM values of the genes in A). Each dot represents the mean of two biological replicates. C) qPCR validation of selected genes from A). 2ΔCt values are normalised to the housekeeping gene Hprt. Pooled H/E cells (purple bars) instead of H/E Sca1+ and H/E Sca1- subpopulations (light blue and dark blue bars, respectively) were used for screening expression of the genes Rspo1, Wnt2, Wnt5a and Wnt9b. N=1 mouse, 2 technical replicates, error bars denote mean ± SD.
4.3 Primary SCA1+ mesenchymal cells can be expanded in vitro

The enrichment of pro-regenerative paracrine signals within the SCA1+ Msc – including agonists of the ERK/MAPK and WNT pathways – portrayed these cells as a niche population worthy of study. Due to the lack of suitable mouse models to study their role in vivo (see Section 4.7), we took the alternative approach of characterising the crosstalk between these cells and DC/progenitors ex vivo. For this, it was paramount to achieve a certain level of expansion of the SCA1+ Msc cells as primary cultures.

**Figure 4.8 In vitro expansion of primary Msc SCA1+ cells.** A) Schematic of primary SCA1+ Msc cell isolation and in vitro passaging at a 1:3 ratio. B) Brightfield images of SCA1+ Msc cells at p1, p2 and p5 of culture on plastic or matrigel in Basal + WNT CM medium.

We isolated SCA1+ Msc as in Figure 4.5A and tested their growth capacity upon serial passaging (in a 1:3 ratio) on either plastic or matrigel as the culture surface.
4.3 Primary SCA1+ mesenchymal cells can be expanded in vitro

(Figure 4.8A). Basal + WNT CM was chosen as the growth medium based on the enhanced survival of Msc cells in it (Figure 3.5). On plastic, SCA1+ Msc cells can be expanded for up to 5 passages, after which they become senescent-looking and arrest growth, as expected from a primary cell population. In contrast, matrigel allows very little expansion of SCA1+ Msc cells and they cannot be cultured beyond 2 passages (Figure 4.8B).

Beyond expanding the SCA1+ Msc cells in vitro, we wished to ascertain whether their original cell identity remained unaltered with time in culture. To investigate this, we collected SCA1+ Msc cells for RNA sequencing immediately after sorting and after 15 days of in vitro culture in Basal + WNT CM medium (Figure 4.9A). SCA1+ Msc cells retain expression of the gross majority of mesenchymal-specific genes, many of which become upregulated, as is the case of Ly6a itself but also Pdgfra, Pdgfrb, Acta2, Col1a1 and Col1a2 (Figure 4.9B); notably, the latter three genes are expressed at relatively high levels (Figure 4.9C). Gene down-regulation is only observed for the case of Eln, Cd34, and mildly for Vim. Hepatocyte- and H/E-specific genes, with the exception of Cdhh5, also become downregulated in culture (Figure 4.9B). This is likely due to non-mesenchymal contaminating cells – present in the SCA1+ Msc fraction at t=d0 – that do not survive or become outcompeted in vitro. Exemplifying this are Alb and Ttr, hepatocyte-specific genes that transition from well-expressed at t=0 to non-detectable in culture (Figure 4.9C). The expression of growth factors and cell-bound ligands in SCA1+ Msc cells is relatively more susceptible to change upon in vitro culture (Figure 4.10). A fraction of the genes augment expression compared to t=0, as is the case of Fgf2,7,18, Rspo1,3, Igf1,2, Wnt5a,9a, Dkk2,3, Dll4 and Cxcl12. De novo expression is much more rare, Fgf9 is one such case, although only low levels are detected (Figure 4.10B,C). Loss of expression – to different degrees – is observed for Hgf, Wnt2,2b,6,9b,11, Wif, Jag1, Dll1, Notch1,2, Il6, Cxcl10 and Tgfβ1, with the most dramatic changes detected in Cxcl10 and Il6 (Figure 4.10B,C). Whilst all other genes in our list remained relatively stable, differential expression should be assessed in the future at the whole-transcriptome level to thoroughly characterise the identity of in vitro cultured Msc SCA1+ cells.
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Figure 4.9 Lineage identity of Msc SCA1+ cells after in vitro culturing. A) Strategy of collection and RNA sequencing of freshly isolated (t=d0) and in vitro cultured (t=d15) SCA1+ Msc cells. B) Gene expression heatmap of mesenchymal, H/E, DC and hepatocyte genes in Msc SCA1+ cells sequenced at d0 vs d15 in culture. DC at d0 vs d15 are also included as controls (N=2 mice, mean RPKM values were plotted). C) Plot of RPKM values of the genes in B). Each dot represents the mean of two biological replicates.
4.3 Primary SCA1+ mesenchymal cells can be expanded in vitro

Figure 4.10 Expression profile of growth factors and cell-bound ligands in Msc SCA1+ cells after in vitro culturing. A) Strategy of collection and RNA sequencing of freshly isolated (t=d0) and in vitro cultured (t=d15) SCA1+ Msc cells. B) Gene expression heatmap of growth factors and cell surface ligands of various signalling pathways in Msc SCA1+ cells sequenced at d0 vs d15 in culture. DC at d0 vs d15 are also included as controls (N=2 mice, mean RPKM values were plotted). C) Plot of RPKM values of the genes in B). Each dot represents the mean of two biological replicates.
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4.4 SCA1+ mesenchymal supports organoid formation in vitro via soluble factors

Similar to Section 3.2, we aimed to perform co-culture assays with the SCA1+ subpopulation of the Msc cells to determine their organoid-supportive potential in vitro. We cultured the cells within the same 3D matrigel droplet (Figure 4.11A) or separately using a transwell system with a porous cell-impermeable membrane (Figure 4.11C). As previously observed, the DC + Msc co-culture enhances organoid formation efficiency compared to DC alone (Figure 4.11A,B); interestingly, this effect is significantly pronounced when co-culturing with the SCA1+ Msc cells, but not the SCA1- Msc nor the H/E SCA1+ fractions (Figure 4.11A,B).

![Figure 4.11 Co-culturing of DC with Msc SCA1+ cells enhances organoid formation. A) Same droplet co-cultures between freshly sorted DC (5000 cells) and stromal cells -H/E SCA1+, Msc SCA1+, Msc SCA1- or Msc – (25000 cells) in Basal + WNT CM medium. B) Quantification of organoid formation efficiency in A). N=3 mice, 2 technical replicates per mouse, error bars denote mean ± SD. C) Transwell co-cultures in Basal + WNT CM medium between freshly sorted DC (5000 cells, top insert) and Msc SCA1+ expanded in vitro for 7 days (bottom well). A control with DC cultured alone in standard EM is also included. D) Quantification of organoid formation efficiency in C). N=3 mice, 2 technical replicates per mouse, error bars denote mean ± SD. In both types of co-culture (A,C), Msc SCA1+ cells promote robust organoid formation. p<0.0001 (***) p<0.001 (**), p<0.01 (**), p>0.05 (*), p<0.05 (ns).](image-url)
4.4 SCA1+ mesenchyme supports organoid formation in vitro via soluble factors

Even when physically separated from the DC, the SCA1+ Msc cells sustain organoid formation at an efficiency (4.4%) that closely matches the one in the mixed co-culture as well as that of DC grown alone in the growth factor-saturated EM (Figure 4.11C,D). In the presence of the SCA1+ Msc cells, organoid growth is observed even in Basal medium lacking WNT CM (2.5% efficiency), which precludes any confounding contribution from serum factors present in the WNT CM (Figure 4.11C,D). As a fortuitous observation, we also noticed that the SCA1+ Msc cells grow more densely when co-cultured with DC in Basal medium compared to cultured on their own (Figure 4.11C), leading to further investigation in Figure 4.14.

The transwell co-cultures suggested that secreted factors and not cell-bound molecules mediate the paracrine effect of SCA1+ Msc cells on organoid growth. We strove to reinforce this idea making use of the Pdgfra-H2B-GFP reporter line, as it would allow us to refine the sorting strategy of the mesenchyme (Figure 4.12A). Whilst both PDGFRα+SCA1+ Msc and PDGFRα+SCA1− Msc cells can be expanded in vitro and retain PDGFRα expression, the latter population grows comparatively slower and splitting ratios must be adjusted accordingly (see Methods); in contrast, the PDGFRα−SCA1+ Msc fraction does not grow at all under our in vitro conditions (Figure 4.12B). We incubated the in vitro expanded cells (PDGFRα+SCA1+ Msc and PDGFRα+SCA1− Msc) with Basal + WNT CM medium for 48h, thus generating conditioned medium (CM) rich in mesenchymal-secreted factors for testing on freshly sorted DC (Figure 4.12C). Conditioned medium from PDGFRα+SCA1+ Msc cells supports the greatest organoid formation from DC, with a mean efficiency of 3.9%, which is significantly higher than that of PDGFRα+SCA1− CM (2.8%) (Figure 4.12D,E), and is roughly equivalent to the organoid formation sustained by SCA1+ Msc in Figure 4.11. In contrast, non-conditioned Basal + WNT CM medium only sustains organoid growth of around 1% (Figure 4.12D,E). These experiments further suggest that in vitro expansion of SCA1+ (PDGFRα+) Msc cells does not alter their ability to sustain organoid formation.
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![Image of CD31, A5.11b-PECy7, EpCAM-APC, SCA-PE, and PDGFRα-GFP](image1)

Figure 4.12 Conditioned medium from Msc PDGFRα+ SCA1+ cells supports organoid formation. A) Sorting strategy of Pdgfrα-H2B-GFP mouse livers to isolate different Msc fractions: PDGFRα+SCA1+, PDGFRα-SCA1, and PDGFRαSCA1+. B) Brightfield and fluorescence pictures of expanded (p=2) mesenchymal populations (PDGFRα+SCA1+ Msc, PDGFRα+SCA1+ Msc) sorted in A). The PDGFRα SCA1+ Msc cells did not grow in vitro. C) Schematic of the experimental design: expanded Msc fractions are incubated with fresh Basal + WNT CM medium for 48h and the resulting conditioned medium (CM) is added to freshly sorted DC. D) Organoid formation from DC cultured with unconditioned medium (Basal + WNT CM), Msc PDGFRα SCA1+ CM or Msc PDGFRα SCA1- CM. E) Quantification of organoid formation efficiency from D). N=3 mice, 2 technical replicates per mouse, error bars denote mean ± SD. Organoid formation is best supported by CM from Msc PDGFRα SCA1+ cells. p<0.0001 (****), p<0.001 (**), p<0.01 (*), p>0.05 (ns).
4.5 Molecular characterisation of mesenchyme-supported organoids

Beyond morphology, we wished to characterise the molecular identity of mesenchyme-supported organoids to determine if they indeed recapitulate classical EM-grown cultures. We designed a strategy to compare DC by RNAseq at d0 of sorting and after 15 days of transwell culture alone in EM or in co-culture with SCA1+ Msc cells in Basal + WNT CM medium (Figure 4.13A). Of note, control organoids grown alone in Basal + WNT CM displayed minimal growth and could not be sequenced. Based on hierarchical clustering, organoids supported by SCA1+ Msc distance themselves molecularly from DC at t=d0 and instead resemble organoids cultured in EM (Figure 4.13B).

Notwithstanding, the mesenchyme and EM-supported organoids do exhibit certain key differences. Whilst the progenitor marker Lgr5 becomes upregulated in EM cultures relative to t=d0 DC, this is not the case for mesenchyme-supported organoids, at least at d15. qPCR analysis of an earlier co-culture (time point 72h) suggests that Lgr5 is in fact expressed, although at lower levels than in the EM organoids (Figure 4.13D), whilst Lgr5 was undetectable in control DC at t=d0 and SCA1+ Msc cells (Figure 4.13D). When compared to EM-grown organoids and even freshly sorted DC, mesenchyme-supported organoids are also enriched in a panel of differentiated ductal cell markers such as Krt19, Aldoa, Cilc3, S100a6, S100a11 and more mature markers like Krt7. Conversely, they exhibit decreased expression of some hepatocyte-specific genes like Ttr, Rbp4 and C3 (Figure 4.13C). The enrichment of differentiated ductal markers --and diminished Lgr5 expression- could be suggestive of a more mature ductal phenotype in the mesenchyme-supported organoids. Evidence supporting this is that the organoids do not withstand serial passaging, as shown in Figure 4.13E.
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Figure 4.13 Mesenchyme-supported organoids resemble EM-grown organoids. A) Schematic of co-culture for RNA sequencing: freshly sorted DC are collected at t=0 or cultured alone in standard EM or in Basal + WNT CM medium with Msc SCA1+ cells on the bottom of the transwell. After 15 days, the organoids formed are sequenced. B) Hierarchical clustering of gene expression from DC (t=d0), organoids (EM, t=d15) and organoids cultured with Msc SCA1+ (t=d15) sequenced by RNAseq (N=2 mice). C) Gene expression heatmap of progenitor and differentiated (DC, hepatocyte) markers across the populations in B). D) qPCR analysis of Lgr5 expression in DC at t=0 of isolation, at t=72h of EM culture or at t=72h of co-culture with Msc SCA1+ cells. Msc SCA1+ cells are included as negative control. N=1, 2 technical replicates, error bars denote mean ± SD. E) Brightfield pictures of DC serially passaged in unconditioned medium (Basal + WNT CM) or Msc SCA1+ CM.
4.6 Liver organoids support the growth of mesenchymal SCA1+ cells

A timely observation from Figure 4.11C led us to believe that the paracrine signalling between SCA1+ Msc cells and ductal cells may be bidirectional instead of just mesenchyme-derived. We designed a similar experiment as in Figure 4.12C, except that this time CM was generated from organoids incubated in Basal medium without WNT CM (Figure 4.14A). Conditioning cells included liver organoids at high and low density, and two technical replicates of low-density small intestinal (S.I.) organoids; after the 24h-period, CM was collected and the cells were assessed for viability to ensure quality of the CM (Figure 4.14A). Both the conditioning liver and S.I. organoids contained a percentage of live cells comparable to their respective EM-cultured controls (Figure 4.14B), suggesting that Basal culture for 24h did not adversely affect their cell viability.

![Figure 4.14 Conditioned medium from liver organoids supports Msc SCA1+ cell growth (part 1). A) Strategy to generate conditioned medium (CM) from liver and small intestinal organoids at different seeding densities. B) Quantification of organoid cell viability after conditioning. N=1, 2 technical replicates. Organoids grown in standard EM are included as positive controls.](image)

To test the CM from liver and control S.I. organoids, we added it to freshly split SCA1+ Msc cells – previously maintained in their standard Basal + WNT CM medium – and assessed mesenchymal cell numbers 24h later (Figure 4.14C). Remarkably, whilst CM from S.I. organoids did not appear to provide any benefit in growth compared to non-conditioned Basal controls, liver organoid CM did: SCA1+ Msc cell numbers increased by 2.8 and 2.2 fold when cultured in high-density and low-density liver CM, respectively (Figure 4.14D). Morphologically, SCA1+ Msc cells looked healthier and
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only displayed their typical elongated shape when cultured in liver organoid CM (Figure 4.14E).

![Figure 4.14](image)

**Figure 4.14** Conditioned medium from liver organoids supports Msc SCA1+ cell growth (part 2). C) Strategy to test organoid CM: Msc SCA1+ cells are split at a 1:3 ratio and are cultured for 24h with control or organoid CM generated in Figure 4.14A. D) Quantification of Msc SCA1+ cell numbers after culturing as in C) with Basal unconditioned medium, liver organoid CM (from organoids at high and low density) and Small Intestinal (S.I.) organoid CM (from organoids at low density). Counts of Msc SCA1+ cells are normalised to Basal control and expressed as relative numbers. N=1, 2 technical replicates. E) Brightfield pictures showing the morphology of Msc SCA1+ cells after culturing in conditions of D). F) Flow cytometry analysis of EdU+ Msc SCA1+ cells after culturing in conditions of D). Dens.= organoid density at the time of conditioning in Figure 4.14A.

The increase in SCA1+ Msc cell numbers could be caused by a boost in proliferation, survival or both. To begin to discern between these possibilities, we evaluated SCA1+ Msc proliferation using EdU incorporation as a proxy. An average of 6.3% of SCA1+ Msc cells were positive for EdU when incubated in Basal medium, compared to 10.7% and 10.6% in high- and low-density liver organoid CM, respectively. Although this would seem to suggest a pro-proliferative mode of action of the liver organoid CM, the CM from the S.I. organoids supported a similar average of 9.25% of EdU+ SCA1+ Msc cells (Figure 4.14F) despite not promoting SCA1+ Msc
4.6 Liver organoid cells support the growth of mesenchymal SCA1⁺

It is thus unlikely that liver organoids sustain SCA1⁺ Msc growth only via mitogens, and pro-survival factors may also be involved. Notwithstanding, these conclusions must be tested further in light of the lack of biological replicates.

In an independent experiment, we confirmed the enhanced growth of SCA1⁺ Msc cells in the presence of liver organoid CM, and found that it was almost comparable to culturing in the standard Basal + WNT CM; this was evident from both the morphology and the relative number of SCA1⁺ Msc cells (Figure 4.14G,H). Despite being preliminary, these results point towards a soluble signal crosstalk between liver SCA1⁺ Msc and ductal/organoid cells.

4.7 Strategies for the *in vivo* ablation of SCA1⁺ mesenchymal cells

*In vivo* loss-of-function studies are necessary to formally prove a regulatory role of SCA1⁺ Msc cells in ductal-driven regeneration. We deem best an experimental approach in which the whole cell population is ablated, considering the rich battery of signalling molecules that these cells express (Figure 4.7). The ideal strategy would thus entail crossing a CreERT2 mouse line under the control of a SCA1⁺ Msc cell specific-promoter with the inducible diphtheria toxin receptor mice (Buch *et al.*, 2020).
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2005), such that temporally controlled administration of diphtheria toxin (DT) would result in specific ablation of the CreERT2-expressing population, i.e. the SCA1+ Msc cells. Despite Ly6a expression not being exclusive to the SCA1+ Msc population, we investigated the suitability of a Ly6-Cre transgenic mouse generated by Mainardi and colleagues for our approach (Mainardi et al., 2014). As a first step, we crossed the Ly6-Cre line with a Rosa26-stop-tdTomato reporter mouse in order to assess the extent of transgene expression in the liver (Figure 4.15A). Macroscopic examination of the tissue revealed extensive tomato fluorescence throughout the liver and the small intestine (Figure 4.15B), as well as other examined organs like the kidney, the spleen and the stomach (data not shown). Such a widespread pattern of tdTomato labelling is suggestive of early embryonic expression of the Ly6-Cre transgene and consequently precludes the use of this mouse line for specifically ablating hepatic SCA1+ Msc in the adult. As an alternative, we are planning to test the suitability of a Pdgfra-CreERT2 mouse line (Rivers et al., 2008). In addition to Pdgfra being a more exclusive mesenchymal marker than Ly6a (Figure 4.6A), the use of a tamoxifen-inducible Cre should provide temporal control for targeting mice in adulthood only.

In parallel to CreloxP/iDTR-based ablation strategies, we explored the possibility of delivering lentiviral gene constructs to the liver in collaboration with the laboratory of Dr. Luigi Naldini (Figure 4.16, Figure 4.17). Although we made use of a ubiquitously expressed PGK-GFP lentiviral vector for preliminary experiments, the final strategy would consist on delivering a SCA1+ Msc-specific Cre vector into the
Rosa26-iDTR mice. Young pups (P2) were first injected with a low viral dose (1x10^7 TU/pup) of PGK-GFP and were analysed for GFP fluorescence 4 weeks later (Figure 4.16A). Macroscopically, very little fluorescence was detected in the injected livers (Figure 4.16B). H/E cells were the most targeted cell lineage (close to 6% of GFP+ cells), whereas DC and SCA1+ Msc cells had minimal GFP expression (less than 0.5%) (Figure 4.16C,D).

**Figure 4.16 Targeting of the liver with low titre lentiviruses.** A) Schematic depicting the delivery of low titre (1x10^7 TU/pup) GFP-expressing lentiviruses (PGK-GFP LV) into P2 pups and analysis at 4 weeks. B) Brightfield and epifluorescence images of uninjected and LV-injected livers. Minimal GFP fluorescence is observed in the treated livers. C) Flow cytometric analysis of LV-injected livers and quantification of the percentage of GFP+ cells within the H/E, Msc and DC fractions. N=3 mice, error bars denote mean ± SD. Msc SCA1+ GFP+ cells represent less than 1% of all Msc SCA1+ cells.

Given the poor targeting of SCA1+ Msc cells, we tested two new approaches: a higher viral titre in pups (2.5x10^8 TU/pup) and injection of adult mice with an immune-evading vector PGK-GFP.142T at two different titres (1x10^9 and 3x10^9) (Figure 4.17A,B) (Schmitt et al., 2010). Already at the whole-mount level, livers were more evidently fluorescent compared to Figure 4.16B (Figure 4.17C). Isolated bile ducts did not appear to be fluorescent themselves but contained associated GFP+ cells of different morphologies between the pup and the adult (Figure 4.17D).
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Figure 4.17 Targeting of the liver with high titre lentiviruses. A) Delivery strategy of high titre (2.5x10^8 TU/pup) GFP-expressing lentiviruses (PGK-GFP LV) into P2 pups and analysis at 4 weeks. B) Delivery strategy of low (1x10^9 TU/adult) and high titre (3x10^9 TU/adult) immune-evasive GFP-expressing lentiviruses (PGK.GFP.142T) into 8-week adults and analysis at 10 weeks. C) Brightfield and epifluorescence images of uninjected and LV-injected livers of pups and adults. Hepatic GFP fluorescence is much more evident than in the livers of Figure 4.16. D) Brightfield and epifluorescence images of isolated biliary ducts. GFP+ biliary-associated stromal cells of different morphologies are observed between the injected pups and adults. E) Quantification of the percentage of GFP+ cells within the respective H/E, Msc SCA1+ and DC fractions of the liver. N=3 pups, N=2 adults with medium viral titre, N=1 adult with high viral titre; error bars denote mean ± SD. The highest percentage of Msc SCA1+ GFP+ cells is ~15%. F) Immunostaining of pup and adult LV-injected livers with SCA1 and OPN antibodies. Periportal GFP+ SCA1+ Msc cells can be found in the pup livers (arrow and magnified insert), whereas adult livers exhibit altered histology and widespread parenchymal GFP positivity.
Quantification of GFP expression in the pups continued to show H/E cells as the most infected lineage, this time with more than 20% of GFP+ cells. SCA1+ Msc cells showed an improved mean of 13.3% GFP positivity, compared to the 3.9% of GFP+ DC. Of note, the percentage of GFP+ cells decreases with time in all lineages (8 weeks vs 4 weeks), likely due to cellular turnover (Figure 4.17E).

In adults, lentiviral targeting is more skewed towards the H/E lineage (> 30% GFP+ cells) regardless of the dose, whereas the percentages of GFP+ SCA1+ Msc and GFP+ DC at the higher dose are comparable to those of the pups at 4 weeks post-injection (Figure 4.17E). Notably, 2 out 3 of the high-dose injected adults died during the course of the experimental procedure. Liver tissue sections of the surviving animal exhibited widespread parenchymal GFP+ cells and altered histology reminiscent of a damaged liver (Figure 4.17F). In contrast, the livers of the pups appeared normal and contained, amongst other GFP+ cells, periportal GFP+ SCA1+ Msc cells. Still, the percentage of SCA1+ Msc targeting remains very low, making it insufficient for in vivo cell ablation.
Chapter 5

Ductal cell expansion is regulated by contact from SCA1\(^+\) mesenchymal cells in a ratio-dependent manner.
Chapter 5 Ductal cell expansion is regulated by contact from SCA1+ mesenchymal cells in a ratio-dependent manner

5.1 Generation of mouse and organoid tools to study cell aggregation

Evidence in Chapter 4 highlights the ability of SCA1+ Msc cells to sustain organoid growth via secreted paracrine factors \textit{in vitro}. Yet, in the homeostatic liver, SCA1+ Msc cells are found in the immediate vicinity of DC, at times physically wrapping around the biliary duct epithelium (Figure 4.3B-1), raising the question of whether direct cell-cell contact could contribute to the crosstalk between these two cell populations.

A critical prerequisite for contact-based experiments is the ability to unequivocally distinguish and follow the spatial coordinates of the two cell populations in time (DC and SCA1+ Msc). To do so, we chose to utilise cells indelibly marked with different fluorescent reporters, taking advantage of the \textit{Rosa26-nTnG} and the \textit{Rosa26-mTmG} mouse lines. The \textit{Rosa26-nTnG} allele consists of a CMV enhancer/chicken beta-actin core promoter, a \textit{loxP}-flanked nuclear \textit{tdTomato} cassette and a nuclear \textit{GFP} cassette inserted in the endogenous \textit{Rosa26} locus (Figure 5.1A) (Prigge \textit{et al.}, 2013). In the absence of \textit{Cre} mediated recombination, the \textit{tdTomato} gene—not the \textit{GFP}—is constitutively expressed and is effectively targeted to the nucleus by the C-terminus SRm160 domain (Figure 5.1A).

We analysed the livers of \textit{Rosa26-nTnG} mice by flow cytometry according to our standard protocol (Figure 3.2B) and gated for Msc and DC cells. As expected, both populations were almost exclusively \textit{tdTomato}+ –95% of Msc cells and 99.2% of DC—while there was a negligible percentage of \textit{GFP}+ cells (Figure 5.1B). What this signifies is that both nuclear \textit{tdTomato}-labelled DC and Msc cells can be effectively isolated from these mice when necessary (e.g. Figure 5.7). In addition, given that DC are capable of long-term \textit{in vitro} expansion as organoid cultures (Huch \textit{et al.}, 2013), we isolated \textit{tdTomato}+ DC and generated a line of liver organoids constitutively expressing nuclear \textit{tdTomato}+ for use in further experiments (Figure 5.1C). Epifluorescence of the organoids confirmed the nuclear pattern of \textit{tdTomato} expression and a lack of \textit{GFP} fluorescence (Figure 5.1C).
5.1 Generation of mouse and organoid tools to study cell aggregation

Figure 5.1 Analysis of Rosa26-nTnG livers and generation of nuclear tdTomato reporter organoids. A) Schematic explaining the Rosa26-nTnG mouse line, which exhibits constitutive nuclear expression of the tdTomato reporter in all cells. B) Flow cytometry analysis of tdTomato fluorescence in the DC and Msc populations of Rosa26-nTnG livers. C) Establishment of a Rosa26-nTnG liver organoid line from tdTomato+ DC sorted in B). The pattern of tdTomato fluorescence appears to be nuclear and no green fluorescence is observed.

Being loxP-flanked, the nuclear tdTomato cassette of the Rosa26-nTnG can be excised through Cre-mediated recombination, leading to in-frame expression of the newly created Rosa26-nGFP allele. We thus crossed the Rosa26-nTnG line with a Sox2-Cre transgenic mouse aiming to generate another reporter line for our cultures; we hypothesised that the early developmental expression of Sox2 (Wood and Episkopou, 1999) would result in nearly ubiquitous Cre-mediated recombination of the Rosa26-nTnG allele in most tissues of the mouse (Figure 5.2A). Earclip biopsies of F1 pups are a simple and accessible means of assaying recombination. In Figure 5.2B, representative earclips from Rosa26-nTnG and Sox2-Cre, Rosa26-nGFP pups display tdTomato and GFP fluorescence respectively. Recombination was also evident in the livers of Rosa26-nGFP mice, although with different degrees of efficiency according to the cell lineage: 22.6% of Msc cells were GFP+ and 0.9% were tdTomato+, while 84.8% of DC were GFP+ and 14.5% were tdTomato+ (Figure 5.2C). Sectioned livers confirmed the above results, as the tissue predominantly displays GFP+ cells and only a handful of tdTomato+ cells (Figure 5.2D). As done in Figure 5.1C, we sorted GFP+ DC and established Rosa26-nGFP organoids that exclusively express nuclear GFP (Figure 5.2E).
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Figure 5.2 Analysis of Sox2-Cre, Rosa26-nGFP livers and generation of nuclear GFP reporter organoids. 
A) Breeding strategy between Rosa26-nTnG and Sox2-Cre mice. In the F1 pups, Sox2Cre-mediated excision of the tdTomato cassette should give rise to constitutive nuclear expression of the GFP reporter in most tissues of the mouse. B) Epifluorescence pictures of ear dips from the F1 pups. The Sox2-Cre, Rosa26-nGFP pup expresses GFP but not tdTomato. C) Flow cytometry analysis of GFP and tomato fluorescence in the DC and Msc populations of Rosa26-nTnG livers. D) Tissue section of a Rosa26-nTnG liver displaying predominantly GFP+ cells and some residual tdTomato+ cells. E) Establishment of a Sox2-Cre, Rosa26-nGFP liver organoid line from GFP+ DC sorted in C. The pattern of GFP fluorescence appears to be nuclear and no tdTomato fluorescence is observed.

A main advantage of nuclear reporter lines like the ones generated above is the straightforward quantification of individual cell numbers, assuming that mononuclear cells are utilised. One drawback, however, is the lack of visibility of cellular outlines and membrane processes. A complementary model thus lies in the Rosa26-mTmG allele, which essentially represents the membrane counterpart of the Rosa26-nTnG construct—containing a CMV enhancer/chicken beta-actin core promoter, a loxP-flanked membrane-targeted tdTomato cassette and a membrane-
targeted GFP cassette inserted in the Rosa26 locus (Muzumdar et al., 2007) (Figure 5.3A). Analysis of a Rosa26-mTmG liver showed the totality of DC labelled with tdTomato fluorescence, whilst 75% of Msc cells were tdTomato+ and 25% were unlabelled (Figure 5.3B). We generated organoids expressing the membrane tdTomato reporter (Figure 5.3C) and crossed the Rosa26-mTmG and Sox2-Cre mouse lines to obtain F1 pups with membrane GFP expression (Figure 5.4A). Earclip-based genotyping of the first litter has shown cases of successful recombination (Figure 5.4B). The liver of these mice will thus be analysed as in Figure 5.2 and membrane GFP+ organoids will be established.

In vivo, damage-induced bi-potential progenitors are labelled by the stem cell marker Lgr5 (Huch et al., 2013). Although mesenchyme-sustained organoids express Lgr5 (Figure 4.13D), the spatial resolution of Lgr5+ cells within the organoid is unknown, which becomes of particular relevance in the face of contact-based cocultures. Given the lack of good antibodies against LGR5, we generated Lgr5-CreERT, Rosa26-stop-tdTomato mice by breeding Lgr5-CreERT2 (Huch et al., 2013) with the Rosa26-stop-tdTomato reporter (Figure 5.5A) and derived an organoid line from these mice. Administration of 4-hydroxytamoxifen (4-OHT) to the organoids is expected to
switch on expression of the tdTomato reporter in Lgr5+ cells (Figure 5.5B). Indeed, we observed that while DMSO controls showed no fluorescence, 4-OHT-treated organoids (at both 1 µM and 10 µM) contained scattered tdTomato+ cells at 24h, whose numbers increased when the culture was left to grow for 8 days (Figure 5.5C).

Figure 5.4 Analysis of Sox2-Cre, Rosa26-mGFP livers. A) Breeding strategy between Rosa26-mTmG and Sox2-Cre mice. In the F1 pups, Sox2-Cre-mediated excision of the tdTomato cassette should give rise to constitutive membrane expression of the GFP reporter in most tissues of the mouse. B) Epifluorescence pictures of ear clips from the F1 pups. The Sox2-Cre, Rosa26-mGFP pup expresses GFP but not tdTomato.

Beyond fluorescent reporter lines, we wished to generate tools to modulate the presence of the mesenchymal and epithelial fractions within the co-cultures. With that aim, we have crossed Rosa26-stop-iDTR mice with Ly6-Cre and Rosa26-CreERT2 mice respectively (Figure 5.6A,B), and have already obtained heterozygous pups for both genotypes (Figure 5.6C,D). The rationale for these crosses is to isolate primary liver DC (Rosa26-CreERT2, Rosa26-stop-iDTR) and SCA1+ Msc cells (Ly6-Cre, Rosa26-stop-iDTR) that can be inducibly ablated during in vitro co-culturing. Due to time constraints, these experiments will be carried out after submission of this dissertation.
5.1 Generation of mouse and organoid tools to study cell aggregation

Figure 5.5 Generation and validation of Lgr5-CreERT2; Rosa26-Stop-tdTomato reporter organoids. A) Breeding strategy between Rosa26-Stop-tdTomato and Lgr5-CreERT2 mice. F1 pups containing both alleles (Lgr5-CreERT2, Rosa26-Stop-tdTomato) were selected for liver harvest and organoid generation. B) Schematic of the pulse-treatment of Lgr5-CreERT2, Rosa26-Stop-tdTomato organoids with 4-hydroxytamoxifen (4-OHT) for 24h. Lgr5-expressing cells are expected to start expressing the tdTomato reporter. C) Brightfield and fluorescence images of DMSO or 4-OHT (1 μM and 10 μM) pulse-treated organoids at 24h and 8 days following treatment. Lgr5+ cells are labelled with tdTomato fluorescence and expand with time.

Figure 5.6 Generation of mouse lines to inducibly ablate cell populations. A) Breeding strategy between Rosa26-Stop-iDTR and Ly6-Cre mice to generate F1 pups heterozygous for both alleles (Ly6-Cre, Rosa26-Stop-iDTR). B) PCR-based genotyping of an F1 litter from A). Representative gel electrophoresis showing that pups ‘d’ and ‘e’ have inherited both the Ly6-Cre allele (top panel, mutant band) and the Rosa26-Stop-iDTR allele (bottom panel, mutant band). C) Breeding strategy between Rosa26-Stop-iDTR and Rosa26-CreERT2 mice to generate F1 pups heterozygous for both alleles (Rosa26-CreERT2, Rosa26-Stop-iDTR). D) PCR-based genotyping of an F1 litter from C). Representative gel electrophoresis showing that all pups have inherited both the Rosa26-CreERT2 allele (top panel, mutant band) and the Rosa26-Stop-iDTR allele (bottom panel, mutant band).
5.2 Using microfluidics to promote aggregation between organoids and SCA1+ mesenchymal cells

Our *in vitro* 3D Matrigel co-cultures (Figure 4.11A) were unsuitable for assaying cell-cell contact due to the large volume of matrix in which the cell populations were cultured, resulting in only spontaneous-and rare-meeting of the two cell types. To circumvent this problem, we tested microfluidics as a means of encapsulating cells into smaller-sized gels. The hypothesis being that confining cells within smaller volumes would increase chances of cell-cell contact.

The experimental set-up consists in dissociating cultures (organoids and SCA1+ Msc cells) into single cells, resuspending them in a matrix of choice – low-melting point agarose for ease of manipulation– and feeding the cell/matrix mixture into custom-designed microchips (Figure 5.7A,B). Two-inlet chips were designed to load the organoid and SCA1+ Msc cells separately and to mix them within the chip (Figure 5.7B), control organoid cells are loaded alone and mixed with an equivalent volume of empty matrix (Figure 5.7A). Microgel droplets are formed on the chip...
5.2 Using microfluidics to promote aggregation between organoids and SCA1+ mesenchymal cells through non-miscibility, when the cell/matrix solution is injected with flowing oil (refer to Figure 2.1 in Materials and Methods). Given that the dimensions of the chip channels determine the size of the microgels formed, we limited their diameter in order to generate small microgels and thus maximise the likelihood of contact between any co-encapsulated cells. Theoretically, the mixing of cells in a 1:1 ratio should give rise to microgels containing at least one cell of each type; in practice, however, multiple permutations are observed as depicted in Figure 5.7B (scheme), and the majority of gels contain no cells.

Once demulsified, the cell-containing microgels can be manipulated as desired. We seeded them into 8 μ-well ibidi dishes layered with either agarose or matrigel as 3D matrix (Figure 5.8A). As with our standard co-cultures in Basal + WNT CM medium (Figure 4.11), robust organoid formation is supported only in the presence of SCA1+ Msc cells (Figure 5.8B). Moreover, despite choosing agarose as a matrix for encapsulation, it was clear that the organoids do not grow well in it and require seeding into Matrigel instead (Figure 5.8B). Of utmost importance was the fact that, within the cultures, we managed to find chimeric organoid structures containing both epithelial (GFP+) and mesenchymal (tdTomato+) cells (Figure 5.8C-1). The layout of the chimeras is reminiscent of the spatial arrangement between ductal and SCA1+ mesenchymal cells in vivo (Figure 5.8C-2), whereby the mesenchymal cell(s) are found on the outer surface of the biliary epithelium, never on the inside.
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Figure 5.8 Aggregation of liver organoid and Msc SCA1+ cells via microfluidic-based encapsulation. A) The cell-containing agarose gels generated in Figure 5.7 were embedded within agarose or matrigel layers in μ-slide 8 well ibidi dishes for imaging. B) Organoid formation of cells seeded as in A) and cultured for 8 days in Basal + WNT CM. Organoids only grow robustly in matrigel and in the presence of the Msc SCA1+ cells. C1) Confocal image (single z-stack) of a chimeric 3D structure from B) (Matrigel) containing aggregated organoid cells (nGFP) and Msc SCA1+ cells (ntdTomato). C2) Confocal image (single z-stack) of a zoomed-in biliary duct structure from a Pdgfra-H2B-GFP mouse liver containing ductal cells (OPN, white) and mesenchymal (PDGFRα, red) SCA1+ (green) cells. Nuclei are labelled by Hoechst (blue).

The generation of ductal/mesenchymal chimeras via microfluidic encapsulation is inefficient (<25%); accordingly, we opted to locate and follow aggregated structures via in vivo time-lapse imaging. Moreover, because the vast majority of ductal cells never become competent to re-form organoids after single cell seeding (efficiency is rarely above 10%), we filmed structures from day 4 to day 5 of seeding, when the process of organoid formation (if any) has already begun. In a microfluidic-based co-culture between GFP+ organoid and tdTomato+ SCA1+ mesenchymal cells, we noticed that non-chimeric organoids (GFP+ only) augment in size as time progresses and maintain a standard cystic morphology (Figure 5.9, blue arrowheads), as expected from the positive effect of SCA1+ Msc-derived mitogenic factors; in contrast, chimeric organoids (where ductal GFP+ and mesenchymal tdTomato+ cells contact each other) appear to shrink over time and exhibit an altered morphology (Figure 5.9, orange arrowheads).
Using microfluidics to promote aggregation between organoids and SCA1+ mesenchymal cells

When studied systematically, it seems that organoid growth dynamics are indeed altered upon contact with SCA1+ mesenchymal cells, and this is dependent on the ratio of mesenchymal to ductal cells per structure (Figure 5.10). We followed a series of organoid structures and categorised them according to ranges of SCA1+ Msc/organoid cell ratios: 0, >0-0.1, >0.1-1, >1-10, >10-100. Immunofluorescent images at d4 and d5 show a very evident inverse correlation between SCA1+ Msc/organoid cell ratio and organoid growth (Figure 5.10A). When an organoid structure does not contact the mesenchyme or only contacts a small ratio of these cells (0-0.1), nGFP+ organoid cells double in numbers from d4 to d5 (Figure 5.10B). On the other hand, organoid cell numbers do not tend to increase at mesenchymal ratios higher than 0.1 (Figure 5.10B). A very similar trend was observed for organoid area (Figure 5.10C). Of note, chimeric structures with ratios of 1-10 or 10-100 are less represented in the quantifications because they are rare in culture. SCA1+ Msc cells do not appear to proliferate within the chimeric structures, as their numbers remain relatively steady for all SCA1+ Msc/organoid ratios (Figure 5.10D). This remains to be verified for periods longer than 24h, as the mesenchymal cell-cycle may be longer than the ductal one.
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Figure 5.10 High ratios of contacting Msc SCA1+ cells inhibit organoid growth. A) Video stills of organoid structures with increasing ratios of aggregated Msc SCA1+ to organoid cells (ranges of 0, >0.01, >0.1, >1, >10, >10-100) filmed from d4 to d5 of culture in Basal + WNT CM medium. B,C,D) Quantification of the organoid structures filmed in A) in terms of the change in number of GFP+ organoid cells (B), change in organoid area (C) and change in number of tdTomato+ Msc SCA1+ cells (D) from d4 to d5. Results are expressed as fold change of d4. N=2 independent experiments, median ± IQR is plotted. High ratios of Msc SCA1+ to organoid cells hinder organoid growth.

We hypothesised that the contact-based reduction in organoid growth in Figure 5.10 is at least partly due to a diminished rate of ductal cell proliferation. We assayed this through staining of EdU incorporation, which very clearly demonstrates a proliferative dichotomy between mesenchyme contacted (GFP+ + tdTomato+) and non-contacted (GFP+) organoid structures (Figure 5.11A). The latter contain an average of 50% of EdU+ cells, whereas this is almost halved for structures containing 0-0.1 SCA1+ mesenchymal/organoid cells; higher ratios of mesenchymal cells completely abolish ductal cell proliferation (Figure 5.11B).
5.2 Using microfluidics to promote aggregation between organoids and SCA1+ mesenchymal cells

Figure 5.11 High ratios of contacting Msc SCA1+ cells inhibit ductal proliferation. A) EdU immunostaining of non-chimeric (nGFP+) and chimeric (nGFP+tdTomato+) organoids from the same well of a d5 co-culture. B) Quantification of the percentage of EdU+ organoid cells according to the ratio of aggregated Msc SCA1+ to organoid cells. High ratios of Msc SCA1+ to organoid cells reduce ductal cell proliferation N=2, median ± IQR is plotted. OC: organoid cell.

Figure 5.12 High ratios of contacting Msc SCA1+ cells induce non-cystic organoid morphology. A) Confocal images (single z-stacks) of chimeric organoids displaying cystic morphology with single layer epithelium (left panel) vs non-cystic morphology with pseudo-stratified epithelium (right panel). B) Quantification of organoid morphology according to the ratio of aggregated Msc SCA1+ to organoid cells. High ratios of Msc SCA1+ to organoid cells induce non-cystic morphology with pseudo-stratified epithelium. N=2, mean percentages are plotted.

The proliferative defect of the mesenchyme-contacted organoids is accompanied by an altered epithelial morphology that is particularly conspicuous in single z-stack confocal images (Figure 5.12A). Traditionally, liver organoids are characterised by a single layer epithelium and cystic-like morphology containing a lumen. Organoids containing a low ratio of SCA1+ mesenchymal cells preserve this traditional epithelial morphology (Figure 5.12A, left panel); whereas an often pseudo-stratified epithelium is commonly observed in chimeras of higher mesenchymal ratios (Figure 5.12A, right panel). As before, results were quantified and validated according to the ranges of SCA1+ Msc/organoid cell ratios (Figure 5.12B).
Because dying or overgrown organoids may exhibit altered morphology as that of the chimeras, we wished to assess whether mesenchymal contact induces epithelial apoptosis. We first evaluated the quality of cleaved caspase 3 staining—a marker of apoptosis—in 3D matrigel embedded cultures, given that antibody penetration can be hindered by the matrigel matrix. For this we used healthy organoids and organoids treated with the apoptosis-inducing drug camptothecin (CPT); as expected, cleaved caspase 3 staining was observed in CPT-treated but not healthy Ki67+ organoids (Figure 5.13A), thus validating the staining. In the contact-based co-culture, we did detect cleaved caspase 3+ cells within the chimeras (Figure 5.13B), seemingly more frequently in those with higher number of mesenchymal cells. This was nonetheless a preliminary experiment and formal quantifications are pending.

![Figure 5.13 Contact from Msc SCA1+ cells may induce apoptosis. A) Validation of the cleaved caspase 3 immunostaining in healthy vs camptothecin (CPT, 10μM, 24h) treated organoids. Cells positive for cleaved caspase 3 are only observed in the CPT-treated organoids. B) Immunostaining of chimeric (nGFP+ tdTomato+) organoids of a d5 co-culture with Ki67 and cleaved caspase 3 antibodies.](image)

Altered organoid morphology may also bespeak of a differentiation switch: the epithelium of progenitor-enriched organoids differs from that of more columnar mature ductal cells and cuboidal hepatocytes. Because of this, we wished to evaluate the differentiation status of chimeric organoids in culture. The low efficiency of chimera formation meant that whole-well RNA analysis of the co-cultures could not be carried out; instead, we devised a strategy in which individual organoids were handpicked based on their morphology (cystic vs non cystic), scored for tdTomato
fluorescence and sorted in the following 4 populations: cystic chimera, cystic non-chimera, non-cystic chimera and non-cystic non-chimera (Figure 5.14A). Most abundant amongst the picked organoids were cystic non-chimeras and non-cystic chimeras, as shown in Figure 5.14B. Gene expression analysis of the picked structures showed very variable results amongst biological replicates. Chimeric organoids, particularly non-cystic ones, showed a tendency towards increased hepatocyte gene expression – *Glu1, Ttr* and *Hnf4a*, but not the more mature marker *Alb*, which was not detected. In contrast, the progenitor and ductal markers, *Tacstd2* (TROP2) and *Krt19* respectively, appeared to be expressed more in the cystic chimeras (Figure 5.14C). The pronounced variability in gene expression is likely reflective of the low number of picked organoids per biological replicate (see Figure legend), and call for a more in-depth transcriptomic analysis.

Figure 5.14 Gene expression analysis of aggregated organoids. A) Strategy for sorting chimeric and non-chimeric organoids in a mixed co-culture as in Figure 5.9: structures are mouth pipetted based on their morphology (cystic, non-cystic), sorted according to tomato fluorescence and collected for RNA. A total of 8 cystic chimeric organoids, 35 cystic non-chimeric organoids, 33 non-cystic chimeric organoids and 9 non-cystic non-chimeric organoids were collected from 2 independent experiments. B) Representative images of a picked cystic non-chimeric organoid (left) and a non-cystic chimeric organoid (right). C) qPCR analysis of the organoids from A,B. 2ΔCt values are normalised to the housekeeping gene *Hprt* and are expressed as fold change relative to the cystic non-chimeric organoids (green dots). N=2, except for non-chimera non-cystic organoids (blue dots, N=1); mean ± SD values are plotted.
5.3 An alternative 2D matrigel co-culture system to promote cell-cell aggregation

A key disadvantage of the microfluidic-based co-cultures in Section 5.2 is the lack of control on the cell-to-cell ratios that aggregate with each other, not to mention the low efficiency of aggregation. In addition, the technique relies on high cell concentrations, which is often difficult to achieve for freshly sorted primary populations like SCA1+ Msc and DC. For this reason, we developed a new contact-permissive co-culture method wherein limited cell numbers (as low as 5000 from each population) are seeded in 96-well plates on a 2D layer of matrigel (Figure 5.15A). In the immediate hours after seeding, tdTomato+ organoid and nGFP+ SCA1+ mesenchymal cells are found randomly interspersed on the 2D matrix, yet by day 2, self-organised 3D aggregates containing both cell types are observed (Figure 5.15A). At a 1:1 ratio of cell mixing, this method generates chimeras at much higher efficiency compared to microfluidics (almost 100%). Because of this, we can study the effect of mesenchymal-to-ductal cell contact at the whole population level and not on an organoid per organoid basis.

![Figure 5.15 An alternative 2D matrigel co-culture method to promote cell-cell aggregation.](image)

Using this novel method, we performed experiments in which increasing numbers of PDGFRα+SCA1+ Msc (nGFP+) are seeded with a fixed number of sorted DC (unlabelled) or organoid cells (OC, nuclear tdTomato+) (Figure 5.16A). The cells were cultured in Basal + WNT CM, or in EM supplemented with WNT CM in an effort to boost SCA1+ Msc survival. In the absence of mesenchyme (0:1 ratio), organoid
formation is more efficient when starting the culture with OC than DC, as expected since organoid cells are highly proliferative, and in EM + WNT CM than in Basal + WNT CM medium, given that exogenous growth factors are supplemented in the former (Figure 5.16A first column, Figure 5.16B). In Basal + WNT CM, a co-culturing ratio of 0.1:1 (PDGFRα+ SCA1+ Msc to DC) –where mesenchymal contact is rare (Figure 5.16A)– leads to enhanced organoid formation efficiency compared to when DC are seeded alone. Yet, remarkably, this is gradually reversed with increasing ratios of mesenchyme (>0.5:1) until nearly abolishing organoid growth (>1:1) (Figure 5.16B, top panel). At these higher ratios, there is instead substantial growth of the GFP+ mesenchymal fraction (Figure 5.16A).

Figure 5.16 The ratio of Msc SCA1+ to DC cells determines the fate of organoid growth.

A) A 2D matrigel co-culture in EM + Wnt CM or Basal + Wnt CM medium between increasing numbers of Msc PDGFRα+ SCA1+ cells (GFP+) and 5000 organoid cells (Tomato+) or DC (uncoloured) to achieve cell-to-cell ratios of 0:1, 0.1:1, 0.5:1, 1:1, 5:1. Representative pictures of organoid formation at d8 are shown. DC: ductal cell, OC: organoid cell. B) Quantification of organoid formation efficiency in A) from DC + Msc SCA1+ aggregates (top panel, N=2) and OC + Msc SCA1+ aggregates (bottom panel, N=1).

Whilst the trend in decreased organoid formation at high ratios of mesenchyme-to-DC (>1:1) is conserved in EM + WNT CM, there is no clear benefit of co-culturing with a low mesenchymal ratio (0.1:1) in this growth-factor rich medium.
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(Figure 5.16A,B). Overall, the above observations (in both media compositions) hold true for co-cultures between PDGFRα+ SCA1+ Msc and OC, although the peak in organoid formation at the 0.1:1 ratio in Basal + WNT CM is much less discernible for OC (Figure 5.16B, bottom panel). It should be emphasised that the mesenchymal inhibition on organoid growth is reliant on both cell-cell ratios and physical contact/proximity, as previous transwell experiments (Figure 4.11B) performed at a 10:1 SCA1+ Msc-to-DC ratio robustly sustained, never inhibited, organoid formation.

![Figure 5.17 The ratio of Msc SCA1+ to DC cells also determines the fate of organoid growth. A) A 2D matrigel co-culture in EM + WNT CM medium between increasing numbers of Msc PDGFRα+ SCA1+ cells (GFP+) and 5000 DC (tdTomato+). Representative pictures of organoid formation at d8 are shown. B) Quantification of mean organoid formation efficiency in A) (N=1).](image)

As an additional assay, contact-permissive co-cultures (as in Figure 5.16) were performed between DC and PDGFRα+ SCA1+ Msc in EM + WNT CM medium (Figure 5.17). To our surprise, a very similar pattern of organoid growth inhibition was observed at high ratios of SCA1+ Msc cells, suggesting that this may be a general property of hepatic mesenchymal cells instead of being PDGFRα+ SCA1+ Msc-specific.

*In vivo* experiments in Chapter 4 had shown temporal fluctuations in the absolute numbers, and ultimately cell-to-cell ratios, of PDGFRα+ SCA1+ Msc relative to DC/progenitors following liver damage. These results became of particular interest in light of the ratio-dependent modulation of organoid growth by the PDGFRα+ SCA1+ Msc cells *in vitro*. We thus projected, as a colour coded gradient, the ratio-dependent (PDGFRα+ SCA1+ Msc/DC) levels of organoid growth *in vitro* (Figure 5.16B, Basal + WNT CM) onto the *in vivo* ratios of PDGFRα+ SCA1+ cells / OPN+ ductal cells at DDC d0 and DDC d12 (Figure 4.4). *In vivo*, the transition from DDC d0 to DDC d12 is accompanied by a higher median ratio of PDGFRα+ SCA1+/ OPN+ cells (0.53), which according to our *in vitro* assays, falls within a less permissive range of organoid
formation (Figure 5.18) and could be a mechanism to terminate regeneration (see Discussion). It would be of interest to investigate if the organoid-sustaining ratio of 0.1:1 is observed at any intermediate time-points between homeostasis and DDC d12.

**Figure 5.18 In vitro and in vivo ratios of Msc SCA1⁺ to DC cells can be correlated to infer ductal cell proliferation.** The *in vitro* ratio-dependent levels of organoid formation in the co-culture between PDGFRα⁺ SCA1⁺ cells and DC in Basal + WNT CM (from Figure 5.16B) were projected as a colour-coded gradient (green=minimal organoid formation, red=maximal organoid formation) onto the *in vivo* ratios of PDGFRα⁺ SCA1⁺ cells/OPN⁺ ductal cells (from Figure 4.4) at DDC d0 and DDC d12. At DDC d12, a median ratio of 0.53 is reached, which corresponds to a less permissive rate of organoid growth *in vitro* compared to DDC d0.
Chapter 6

Discussion
6.1 Isolation and co-culture of primary ductal cells with associated stromal populations in the liver

The process of wound healing is a cooperative endeavour that requires timely interactions between the epithelium and the cells of the stroma, regardless of the affected tissue. In the liver, ductular cell expansion is a common feature of human pathologies and recent publications have highlighted the potency of these cells in replenishing tissue parenchyma following chronic damage or when hepatocyte proliferation is impaired (Lu et al., 2015; Raven et al., 2017; Deng et al., 2018). Whilst the stroma has been implicated in the activation and cell-fate commitment of ductal progenitors (Boulter et al., 2012; Takase et al., 2013), the cell identity, molecular signature and spatiotemporal dynamics of a regenerative ‘ductal niche’ have not been fully characterised, partly due to the difficulty of assessing niche potential in vivo.

Making use of tissue microdissection and explant culture techniques – commonplace in the field of chick embryology – Nicole Le Douarin performed a series of seminal experiments to elucidate the mesoderm-to-endoderm signalling that underlies hepatic development (Le Douarin, 1968, 1975). This contributed to a widespread assessment of ‘stromal guidance’ in hepatogenesis, either via similar co-culture assays between purified hepatic cell populations or via robust step-wise protocols for the directed differentiation of hepatocytes and cholangiocytes from pluripotent stem cells (refer to Section 1.2.1 and 1.3). Our approach of primary cell isolation and ex vivo co-cultures between adult duct epithelium and stroma aimed to assess ‘niche’ potential as it has been done in the embryo, but using organoid formation as a functional readout of regeneration. A key difference between our strategy and Le Douarin’s is the isolation of cells via tissue digestion and FACS sorting as opposed to microdissection, which removes key information about the spatial coordinates of the cells within the tissue. Hepatic stroma may co-fractionate with ductal cells in “non-parenchymal cell” isolation protocols not because of spatial proximity but because of their comparable cell size – in contrast to the much larger hepatocytes (Wang, Foster, et al., 2003); this is particularly cumbersome for circulating hematopoietic cells that do not reside long-term within the tissue but are very abundant in this highly vascularised organ (Lautt, 2009). The identification of
6.1 Isolation and co-culture of primary ductal cells with associated stromal populations in the liver

the Msc fraction as a putative ductal niche in Chapter 3 thus required us to validate the co-localisation of these two cell populations in situ, as was done in Chapter 4.

Compared to the embryo, the adult liver displays a greater diversification of stromal cell populations: hepatic stellate cells, portal fibroblasts, liver sinusoidal cells, arterial and venous endothelial cells, macrophages, NK cells, T cells, etc; not to mention the heterogeneity that exists within each population. Our reductionist approach of H/E (EpCAM⁺CD45⁺CD31⁺CD11b⁺) vs MSc (EpCAM⁻CD45⁻CD31⁻CD11b⁻) is thus limited in that organoid-supportive fractions may be masked within the bulk populations, either due to insufficient cell numbers or inhibitory effects from one fraction to another. For instance, immune cells may induce 'tolerance' amongst each other and suppress pro-inflammatory signalling thought to be important for ductal cell expansion (Knolle and Gerken, 2000). Moreover, our assay assumes that cell survival and behaviour in vitro are equivalent to the physiological context, despite the variable of culturing in 3D matrigel and Basal + WNT CM medium; but these conditions are evidently not optimal for all cell subpopulations given the underrepresentation of CD31⁺ endothelial cells in vitro. Accordingly, our co-culture assay cannot rule out the capacity of the H/E fraction to sustain ductal cell expansion, but does highlight this role for the Msc population.

6.2 SCA1⁺ mesenchymal cells surround biliary ducts and expand after damage

In many respects, the pinpointing of mesenchymal cells as being ductal cell-supportive in the adult liver was not surprising given the co-dependency between mesodermal and endodermal lineages throughout the ontogeny of this organ (refer to Section 1.2.1 on Development). Interestingly, our surface marker screen identified that the stem cell antigen 1 (SCA1) labels a percentage of the Msc fraction, though not exclusively, and that SCA1⁺ mesenchymal (PDGFRα⁺) cells are localised periportally in the liver, in very close proximity to the biliary ducts.

SCA1 (or lymphocyte antigen 6A-2/6E-1) is a member of the multi-gene Ly6 family comprised of highly homologous, cross-hybridising genes tightly linked on
mouse chromosome 15; and which are thought to have arisen from gene duplication events (LeClair et al., 1986, 1987; Shevach and Korty, 1989). Adding to the complexity, SCA1 is encoded by two strain-specific allelic variants at the Ly6a locus – Ly6a.1 or Ly6e.1 and Ly6a.2 – (Palfree, Dumont and Hammerling, 1986) that differ by only two amino acids yet show dramatic differences in cell-type specific expression (Spangrude and Brooks, 1993). A human orthologue of SCA1 does not exist; in fact, a 500 kilobase region of the murine chromosome 15 was lost from mouse to rat speciation, deleting Ly6a together with five additional Ly6 members (Holmes and Stanford, 2007). This has fuelled the belief that SCA1 function, if any (see below), has been made redundant during evolution.

SCA1 was first identified more than 40 years ago in thymus-resident and peripheral lymphocytes (Yutoku, Grossberg and Pressman, 1974; McKenzie, Cherry and Snell, 1977), but rose to fame as the phenotypic marker-of-choice for isolating bone marrow-repopulating hematopoietic stem cells in conjunction with other lineage antigens (Spangrude, Heimfeld and Weissman, 1988; Spangrude et al., 1989). Its expression has since been linked to multipotent bone marrow mesenchymal stem cells (BM-MSC) (Baddoo et al., 2003; Meirelles Lda and Nardi, 2003; Sun et al., 2003), as well as progenitor populations in the musculoskeletal system (Torrente et al., 2001; Qu-Petersen et al., 2002; Tamaki et al., 2002), prostate (Burger et al., 2005; Xin, Lawson and Witte, 2005), skin (Toma et al., 2001; Fernandes et al., 2004), heart (Oh et al., 2003), mammary gland (Welm et al., 2002), lung (Bender Kim et al., 2005) and even liver (Petersen et al., 2003; Dorrell et al., 2008).

Our studies do confirm expression of SCA1 on liver ductal cells, but suggest a broader hepatic staining pattern that includes mesenchymal (PDGFRα+), endothelial (CD31+) and hematopoietic cells (CD45+) – but not macrophages (F4/80+) – exclusively at the portal tract. Freshly isolated LSECs, which in situ are spread across the liver lobule, express SCA1 constitutively and upregulate it upon challenge with tumour necrosis factor α (TNFα) (Luna, Paez and Cardier, 2004). Although this would seem to be at odds with the portal tract specificity we report for SCA1, it may simply indicate a gradient of expression, where the highest protein levels of SCA1 demarcate periportal cells. This is in some ways reminiscent of the lobule zonation discussed in Section 1.1, and it would be interesting to explore whether high oxygen
concentrations or specific signals from the portal vein circulation regulate SCA1 expression in homeostasis. Alternatively, shared SCA1 expression amongst some of these cells could reflect a common developmental origin (see Section 6.3). The anatomical coordinates of SCA1+ PDGFRα+ mesenchymal cells, residing in exquisite proximity to the biliary ducts, coupled to their expression of Elastin and Il6 match at least partially the description of portal fibroblasts (Wells, 2014). SCA1 had not been previously reported as a marker of these cells and its specificity could be of great interest for the field, given that non-periportal PDGFRα+ cells – likely corresponding to HSCs – show no detectable SCA1 expression, and the distinction between these two cell lineages has proven historically difficult (Wells, 2014).

Considering the widespread use of SCA1 as a phenotyping marker for progenitors, it is somewhat surprising that its biological function remains shrouded in mystery. Ly6 family members are glycosyl phosphatidylinositol-anchored proteins (GPI-AP) that localise to lipid rafts of the plasma membrane and can from complexes with tyrosine kinases (Štefanová et al., 1991). Most famously, cross-linking of SCA1 by specific monoclonal antibodies provokes T lymphocyte activation and proliferation, whilst SCA1 inhibition prevents it (Malek et al., 1986; Flood, Dougherty and Ron, 1990). Although a tentative SCA1 ligand was once suggested (English et al., 2000), the identity of this protein remains unknown and no evidence so far supports a traditional ligand-receptor signalling activity for SCA1. Ly6a (SCA1) null mice are viable and display no overt phenotype in homeostasis besides minor hematopoietic lineage skewing (Stanford et al., 1997; Ito et al., 2003); they do however exhibit stress or age-related phenotypes including osteoporosis (Bonyadi et al., 2003; Holmes et al., 2009) and reduced muscle size in older animals (Mitchell et al., 2005). Interestingly, Holmes and colleagues showed that osteoclastogenesis was defective in the bone marrow due to stem cell-intrinsic effects but also because of reduced stromal support towards osteoclast differentiation (Holmes et al., 2009). Loss of SCA1 has also been linked with defective ECM production/remodelling both in bone and muscle (Bonyadi et al., 2003; Kafadar et al., 2009); which in turn associates with impaired regeneration in the latter. Based on the above, it would be of interest to examine whether the presence of SCA1 in the periportal mesenchyme is biologically relevant or simply a convenient marker for cell isolation. A partial answer to this may be obtained from
the RNAseq analysis of SCA1+ vs SCA1- populations, but will undoubtedly require additional loss-of-function studies. Of note, another GPI-AP member (Štefanová et al., 1991), Thy1, labels portal mesenchyme in the liver (Takase et al., 2013), which means there could be functional redundancy between SCA1 and Thy1 if they do label the same cell population.

Following liver damage, the absolute number of periportal SCA1+ PDGFRα+ – but not SCA1- PDGFRα+- mesenchymal cells increases along with the ductal cell fraction. Although we propose this is an expansion of the homeostatic SCA1+ PDGFRα+ cells, two other alternatives are possible:

a) SCA1+ PDGFRα+ cells, potentially bone marrow-derived, are brought into the tissue through the portal circulation as a damage response mechanism. Chimeric mice transplanted with *Col1a1-GFP* reporter bone marrows and then subjected to bile duct ligation contain GFP+ 'fibrocytes' (co-expressing Cd45+) within the liver parenchyma (Kisseleva et al., 2006), although a SCA1+ PDGFRα+ signature has not been reported so far for fibrocytes.

b) SCA1 expression may be upregulated in resident SCA1- cells, particularly the SCA1-PDGFRα+ fraction. For this to be the case, SCA1+PDGFRα+ cells may also have to migrate closer to the portal area, as they localise more intra-lobularly compared to the SCA1+PDGFRα+ cells according to our results.

The second scenario is worthy of attention given that SCA1 expression appears to be tuneable in the context of injury. In LSECs, *in vitro* challenge with the pro-inflammatory cytokine TNFα leads to increased SCA1 expression (Luna, Paez and Cardier, 2004), while extract from crushed muscle upregulates SCA1 in cultured myoblasts, both reversibly and independently of cell proliferation (Kafadar et al., 2009). To formally prove the *in situ* expansion of SCA1+PDGFRα+ cells in our system, we require evidence of the proliferative capacity of these cells after damage (e.g. assessed via EdU incorporation) and, ideally, confirmation via *Cre* -based lineage tracing. The former has been technically challenging due to the inability to co-stain SCA1, a surface-bound protein, with nuclear markers like EdU/Ki67. In addition, currently available mesenchymal *Cre* lines (e.g. *Ly6-Cre, Pdgfra-CreER, Sm22-
6.2 SCA1+ mesenchymal cells surround biliary ducts and expand after damage

*CreER*) would most likely label other populations apart from SCA1+ PDGFRα+ cells (see more on Section 6.7).

Regardless of their provenance, it is clear that SCA1+ PDGFRα+ cells do increase their numbers periportally as a consequence of tissue damage, and this alters their ratio with respect to the ductal cells. In muscle, SCA1+CD31- fibro-adipogenic progenitors (FAPs)– with bipotency towards fibrocytes and adipocytes– reside in close proximity to myogenic progenitors (MP) and proliferate upon damage. FAPs expand from d2-d3 after damage, nearly equalling MPs in number, but by day 5 the FAP:MP ratio has returned to pre-damage (Joe et al., 2010). Our model differs in that, by day 7, the SCA1+ PDGFRα+: ductal cell ratio does not return to homeostasis and begs the question of whether at later timepoints it would. Likewise, earlier timepoints are of interest in order to sample the dynamics of cell expansion immediately after damage (see more on Section 6.8).

6.3 Molecular characterisation of primary SCA1+ mesenchymal cells in homeostasis

Despite reportedly labelling ductal progenitors, the lack of separation between SCA1+ and SCA1- ductal cells in the unsupervised clustering analysis of gene expression suggests that SCA1, on its own, does not demarcate a molecularly distinct subpopulation within the ductal compartment. On the contrary, SCA1 expression does segregate the sorted H/E and Msc populations. The portal fibroblast identity of the Msc SCA1+ cells is supported by enrichment of classical mesenchymal markers (*Pdgfra, Pdgfrb, Col1a1, Col1a2*) but also portal-specific ones (*Eln, Il6*), and by the diminished expression of markers of HSCs (*Des*) and smooth muscle cells (*Acta2*), which instead cluster in the Msc SCA1- fraction. Also noteworthy is the expression of *Cd34* in the Msc SCA1+ cells; a marker that shares progenitor-specific links with SCA1 across diverse lineages (Brown, Greaves and Molgaard, 1991; Torrente et al., 2001), including mesenchymal cells (Hittinger et al., 2013). In the liver, CD34 expression is most commonly linked with abnormal angiogenesis in hepatocellular carcinoma (Cui et al., 1996; Kimura et al., 1998), but has also been detected on homeostatic portal blood vessels and biliary epithelium, as well as in damage-induced ductular reactions (Omori et al., 1997). Lineage tracing experiments in chick embryos have shown a
shared mesothelial ancestor between endothelial and stellate cells of adult hepatic sinusoids (Perez-Pomares et al., 2004). Given that portal fibroblasts may also originate from mesothelial cells (Asahina et al., 2011), it would be interesting to investigate whether the ontogeny of portal SCA1+ endothelial and mesenchymal cells can be traced back to a shared mesothelial progenitor expressing SCA1.

6.4 SCA1+ mesenchymal cells can be expanded in vitro

When grown in vitro on standard tissue culture plastic, HSCs undergo progressive cellular activation towards a myofibroblast-like phenotype – as characterised by loss of vitamin A, increased collagen synthesis and αSMA (Acta2) expression (Geerts et al., 1989; Bachem et al., 1992); whilst they retain features of quiescence in a basement membrane matrix like Matrigel (Friedman et al., 1989). Conversely, the myofibroblast fate can be partially reversed when cells are re-inserted into Matrigel (Sohara et al., 2002). Portal fibroblasts undergo similar, if not more pronounced, phenotypic activation when cultured in vitro (Lepreux and Desmoulière, 2015). In line with this, we demonstrated that primary SCA1+Msc cells can be serially passaged (up to p5) on plastic but not Matrigel, where they arrest growth much sooner; and expanded cells upregulate various myofibroblast markers including Acta2, Col1a1 and Col1a2. Given that a proliferative myofibroblastic signature is characteristic of wound healing across many tissues (Gabbiani, 2003), in vitro expanded SCA1+ Msc cells may model more accurately the hepatic periportal niche in the context of regeneration than in homeostasis. Differentially up and down-regulated genes from the t=0 to d15 transition of SCA1+ Msc cells should then be studied more thoroughly in order to understand the paracrine signalling capacity of these cells after activation as well as the underlying molecular identity that drives it.

Returning to the contested idea of a biological role for SCA1, it was interesting that SCA1+PDGFRα+ Msc cells expanded more readily than SCA1·PDGFRα+ Msc in our in vitro system. Although this could simply reflect differing proliferative capacities between two distinct mesenchymal lineages, Bonyadi et al. have shown that loss of SCA1 in bone marrow mesenchymal progenitor cells severely hampers their ability to be serially passaged (Bonyadi et al., 2003). The knock-down of Ly6a in our cultures
could then ascertain if any proliferative, as well as niche signalling, defects may develop as a consequence of SCA1 loss.

6.5 SCA1+ mesenchyme supports organoid formation *in vitro* via soluble factors

Making use of distinct methods for co-culturing cells, mainly cell-contact independent ones, we confirmed that primary SCA1+ Msc cells support the transition of single EpCAM+ ductal epithelial cells into proliferative, self-organising 3D organoids that morphologically and molecularly resemble the cultures grown in full growth factor medium. Both freshly sorted and expanded SCA1+ Msc cells were capable of this, albeit with the confounding variable of culturing on matrigel vs plastic. This implies that it is the SCA1+ Msc signature retained after *in vitro* culturing that promotes organoid growth. SCA1+ Msc cells indeed express various mitogenic factors (*Hgf*, *Fgf7*, *Rspo1* and *Rspo3*) normally supplemented in EM and implicated in liver regeneration (Ishikawa *et al.*, 2012; Takase *et al.*, 2013; Planas-Paz *et al.*, 2016). Whilst *Egf* expression was not detected, both HGF and EGF signal through receptor tyrosine kinases and are functionally redundant in the lung (Engelman *et al.*, 2007) and in PHx-induced liver regeneration (Paranjpe *et al.*, 2016). Even if the SCA1+ Msc ‘secretome’ does encompass most EM growth factors, their blockade via monoclonal antibodies and/or small molecule inhibitors is still required to confirm contribution towards organoid sustenance, particularly since SCA1+ Msc cells express multiple other cytokines that have not been directly associated with liver regeneration yet.

An ‘ideal’ combination and concentration of signalling factors must exist to sustain hepatic regeneration. Michalopoulos *et al.* reported early on that HGF, EGF and the corticoid dexamethasone are essential factors for culturing adult primary hepatic cells in a way that retains histological organisation, although these conditions do not support ductal progenitor expansion (Michalopoulos *et al.*, 2001). *Ex vivo* studies with E10 embryonic livers have on the other hand shown that the WNT + HGF combination is sufficient for maintaining hepatocytes, cholangiocytes and progenitor cells (Hussain *et al.*, 2004). In the case of standard adult organoids, removal of either FGF10, EGF, HGF or RSPO1 from the complete culture medium prevents their long-term passaging (Huch *et al.*, 2013). Organoids sustained by soluble factors from SCA1+
Msc cells do grow *in vitro* but have limited expansion potential, and their molecular signature shows skewing towards a more differentiated ductal state. This could be due to insufficient secretion of mitogens by the mesenchyme, or contrary, a supply of pro-differentiation signals. In the muscle, FAPs that co-expand with myofibroblast progenitors after damage promote their differentiation in *ex vivo* co-cultures (Joe *et al.*, 2010). Periportal SCA1+ mesenchyme could then entertain a similar relationship with the ductal progenitors following injury, so that their ductal de-differentiated state is only maintained transiently. Extrapolating from this, and from the concept of local cell-fate choice (Boulter, Lu and Forbes, 2013), SCA1+ mesenchymal signals may act as molecular ‘reminders’ of homeostatic cell identity for ductal cells in the portal area. Such a mechanism is observed in skin, where the site-specific HOX transcriptional program of fibroblasts regulates epidermal fate and thus acts as positional memory to pattern the epithelia (Rinn *et al.*, 2008).

### 6.6 Liver organoids support the growth of SCA1+ mesenchyme

In the mid-to-late phases of epidermal wound healing, fibroblasts receive keratinocyte-derived stimuli such as PDGF, IL-1 and TGFβ that in a positive feedback loop potentiate myofibroblast differentiation for the continuous support of keratinocyte-driven regeneration (Werner, Krieg and Smola, 2007). This bidirectionality of epithelial-to-mesenchymal communication holds true for many other wounded adult tissues (Holgate *et al.*, 2004; Chapman, 2011; Ding *et al.*, 2012), possibly including the liver. In rat models of biliary fibrosis, ductal cells have been shown to express PDGF-B, connective tissue growth factor (CTGF) and TGFβ2 (Milani *et al.*, 1991; Grappone *et al.*, 1999; Siedlaczek *et al.*, 2001); although these are well-established mesenchymal mitogens and/or activators (of both portal fibroblasts and hepatic stellate cells) (Li *et al.*, 2007; Friedman, 2008), most of these studies have been correlational and the mesenchymal response to purported biliary-derived stimuli requires further characterisation. In our system, conditioned medium from liver, but apparently not small intestinal, organoids enhances the growth of SCA1+ Msc cells after splitting, suggesting that there could indeed be a signalling crosstalk between ductal epithelium and SCA1+ Msc during liver regeneration. At present, the
6.6 Liver organoids support the growth of SCA1+ mesenchyme

identity and mode of action (pro-proliferation or pro-survival) of this ductal derived signal(s) remains unknown, but we have RNAseq data of both DC and SCA1+ Msc cells cultured alone or in a transwell co-culture which could shed some light on key signalling pathways for mesenchymal growth. The power of our in vitro co-culture system is that can we can directly probe the effect of previously reported mesenchymal mitogens/activators (PDGF-B, CTGF and TGFβ2) but also novel ones via gene knock-down and/or small molecule inhibitors. Given that SCA1+ Msc cells grow well in Basal + WNT CM medium, and that addition of the GSK-β inhibitor, CHIRON, enhances mesenchymal cell survival in vitro (data not shown), we could hypothesise that one of the ductal-derived signals is a WNT ligand such as Wnt7, which is expressed by DC and organoids, but not SCA1+ Msc, in our RNAseq. In breast cancer, epithelial cells secrete WNT7A to recruit and activate cancer associated fibroblasts in a TGFβ-like mechanism, which subsequently feeds back on tumour aggressiveness (Avgustinova et al., 2016). The exclusive growth response of SCA1+ Msc cells to liver organoid CM tantalisingly suggests tissue specificity in signalling. Organ-specific gene signatures have been identified in stellate cells of the adult liver and the pancreas despite their suspected shared developmental origin (Buchholz et al., 2005), whilst in the embryo, Sneddon and colleagues have shown that co-culturing ESC-derived pancreatic progenitors with organ-matched mesenchyme is crucial for the self-renewal and proliferation of the latter. Extrapolating from this, we could envision liver-specific interactions between hepatic ductal cells and SCA1+ Msc. Future experiments should include pancreatic cells as an additional control given that the liver shares more features with the pancreas (both in development and in adulthood) than with the rapidly self-renewing small intestine.

6.7 Strategies for the in vivo ablation of SCA1+ mesenchymal cells

Judging from the contested biological function of SCA1 in other tissues (see section 6.2), knocking out this gene in the periportal mesenchyme is unlikely to reveal any niche-related phenotype. Instead, we strove to ablate the population as a whole, on the basis of a CreloxP, iDTR system. The lack of temporal control of the Ly6-Cre transgenic line renders it unsuitable for this purpose, considering that Ly6a
expression is reported as early as E9 on the endothelial layer of the dorsal aorta, concurrent with the emergence of hematopoietic progenitors (De Bruijn et al., 2002). The multi-organ pattern of Ly6 tracing we observed in adulthood could reflect a variety of HSC-derived blood lineages, although at the macroscopic level, reporter fluorescence did not appear to localise within vessels. Specifically for the liver, SCA1 expression has also been reported in putative foetal liver hepatoblasts (Nierhoff et al., 2005), which could explain a more widespread epithelial staining in the adult organ. Closer analysis of the tissue would be required to properly assess the extent and types of cell lineages traced, but it is clear that Ly6-Cre expression would not be restricted to hepatic periportal cells.

To circumvent the hurdle of constitutive transgenics, we turned to postnatal gene transfer with viral vectors. Lentiviruses are capable of infecting quiescent and/or non-dividing cells (Naldini et al., 1996), which makes them advantageous for targeting tissues with slow turnover like the liver. Transduction efficiency may nonetheless be higher in cells that do cycle, as evidenced after partially hepatectomy (Park et al., 2000), but other reports have shown no such bias towards cell cycle state, and discrepancies may be due to vector design (Pfeifer et al., 2001). In homeostasis, our experiments showed preferential viral infection of the H/E fraction, particularly in adults, where there were signs of tissue damage and/or inflammation. Pfeifer and colleagues have similarly demonstrated that the bulk of viral load (78.7%) in transduced adult livers is found within non-parenchymal cells (Pfeifer et al., 2001). This is caused by immune recognition and sequestration of the virus, predominantly by professional scavengers like Kupffer cells (van Til et al., 2005), although both endothelial cells and HSCs are competent for antigen uptake and presentation (Knolle and Gerken, 2000; Viñas et al., 2003). In line with this, inhibition of the innate immune response results in increased parenchymal cell targeting (van Til et al., 2005; Brown et al., 2007). Lentiviral infection was attempted in pups because their immune system is relatively immature compared to adults (Simon, Hollander and McMichael, 2015); however, despite the feasibility of targeting of SCA1+ Msc cells in vivo, efficiency remains too low for the purpose of cell ablation. In the future, infection can be enhanced by screening naturally occurring viruses with tropism for mesenchymal cells or via pseudotyping (Cronin, Zhang and Reiser, 2005). This has been shown for
6.7 Strategies for the in vivo ablation of SCA1+ mesenchymal cells

adenoviral-associated viruses (AAVs) (Di Pasquale et al., 2003; Rezvani et al., 2016), of which AAV5 is of particular interest because it is recognised by PDGFR (Di Pasquale et al., 2003). In its current form, the strategy of lentiviral infection could be used for lineage tracing of SCA1+ Msc cells at the clonal level in order to address if they indeed expand in situ following damage.

6.8 Contact between SCA1+ Msc and ductal cells inhibits organoid growth in a ratio-dependent manner

The cellular proximity between ductal epithelium and SCA1+ Msc cells in vivo made us reassess the nature of the signalling interactions between these two cell types. Taking Le Douarin’s work as an example again, close contact between hepatic mesenchyme and endodermal cells is required for stimulating the proliferation and differentiation of the latter population during embryogenesis (Le Douarin, 1975). We thus generated, via two different methods, chimeric organoids that incorporate adult ductal and SCA1+ Msc cells. Interestingly, the arrangement of these two populations within the organoid structure appears to respect the in vivo rules of cellular organisation: mesenchyme surrounding the ductal epithelium from the ‘outside’, with no apparent disruption of inter-epithelial cell junctions. Our method of promoting cell-cell to aggregation relies on confining the two cell populations in small spaces to increase their likelihood of meeting, after which the cells assemble spontaneously, perhaps due to combined cell-autonomous and neighbour-to-neighbour cues. This is conceptually different from conventional tissue engineering methods which rely on artificial scaffolds to force complex cellular structures, as opposed to allowing 3D self-organisation (Woodford and Zandstra, 2012; Sasai, 2013). The ‘self-renewal’ of our DC/SCA1+ Msc chimeras in vitro remains uncertain; once assembled, ductal cells proliferate depending on the ratio of mesenchymal cells (see below) but SCA1+ Msc cells rarely do so within the chimera. Although potentially unsuitable for in vitro expansion, mesenchyme-contacted organoids may better recapitulate the physiology of healthy liver precisely because of their low proliferative nature, given that hepatic parenchymal cells divide seldomly (MacDonald, 1961); standard EM-grown liver organoids on the other hand mimic the transient proliferative state of the ductal epithelium following tissue damage.
Using both the microfluidic-based and 2D Matrigel method of cell aggregation, we found that the ratios of mesenchyme-to-epithelial cells contacting each other determines whether SCA1+ Msc promotes or inhibits organoid growth and proliferation. A ratio of 0.1:1 (SCA1+ Msc : DC) in growth-factor reduced medium enhances organoid formation compared to controls lacking mesenchyme, whilst higher ratios gradually suppress organoid growth until completely impairing it. Consistent with our data from cell-contact independent co-cultures, the mitogenic effect of the SCA1+ Msc at 0.1:1 is likely due to the soluble factors that these cells express (e.g. Hgf, Rspo1, Fgf7), and indeed, the phenotype is abolished when culturing in growth factor complete medium. In the microfluidic co-cultures, because all chimeric and non-chimeric organoids grow within the same well, paracrine signalling from neighbouring SCA1+ Msc cells likely affects non-chimeric organoids (which would otherwise grow poorly in Basal + WNT CM medium), thus confounding the analyses of growth dynamics and justifying the lack of increased organoid growth at 0.1:1.

What is so special about the 0.1:1 ratio? We entertain two possibilities: either a single mesenchymal cell is unable to physically contact all organoid cells and inhibit their proliferation, or it cannot synthesise sufficient amounts of a short-range cytostatic factor. In both cases, after the threshold is surpassed (>0.1:1), the cytostatic effect of the mesenchyme outcompetes its mitogenic capacity. To discern if actual cell-to-cell contact is required for the block of proliferation, we will make use of SCA1+ Msc cells expressing membrane reporters (mTmG or mGFP) to delineate all cytoplasmic projections that may be contacting the ductal cells. Likewise, we can make use of Lgr5-Cre, R26-stop-tdTomato reporter ductal cells to see if mesenchymal contact somehow targets Lgr5+ progenitors within the organoid.

Beyond arrest of proliferation, the final fate of the mesenchyme-contacted ductal cells remains unclear. Some cells stained positive for cleaved caspase 3, a marker of apoptosis, and there was variable upregulation of differentiated cell markers accompanied of non-cystic morphology. The mechanism through which SCA1+ Msc cells hinder organoid growth is as yet undetermined; we disfavour
6.8 Contact between SCA1+ Msc and ductal cells inhibits organoid growth in a ratio-dependent manner

nutrient competition as a hypothesis given that transwell co-cultures of a 10:1 (SCA1+ Msc : DC) ratio do support robust organoid formation (Figure 4.14). Unlike their mitogenic 'secretome', the cytostatic signal of SCA1+ Msc cells must be shared with the SCA1- Msc population given that they both induce contact-dependent organoid growth arrest. On that note, Le Douarin’s studies in the embryo had hinted at the non-specificity of mesenchymal cells (e.g. hepatic mesenchyme, lateral plate mesoderm) in regulating hepatic endodermal cell behaviour via contact-dependent mechanisms (Le Douarin, 1975). Suitable candidate molecules must either be membrane-bound or known to act within a short range; meeting these requirements are ligands of the Notch, TGFβ and Hippo pathway, which according to our RNAseq data are expressed by the mesenchyme and their corresponding receptors by the ductal cells (data not shown). In the liver, the TGFβ and Hippo pathway in particular have been proposed to terminate regeneration and/or control organ size (Michalopoulos, 1990; Lee et al., 2010; Lu et al., 2010). Although TGFβ is not membrane bound, it is secreted in a latent form and must be processed extracellularly (Lyons et al., 1990; Shi et al., 2011). Efficient TGFβ activation in dermal fibroblasts requires contact-dependent cocultures with keratinocytes (Shephard et al., 2004), an observation that holds true for co-cultures of other cell types (Sato and Rifkin, 1989; Sato et al., 1990). Inhibitor studies will be performed in the future to identify the signal that rescues organoid growth arrest upon mesenchymal contact.

A fraction of the SCA1+ Msc : DC ratios that we 'engineered' in vitro, and that modulate organoid growth, do exist in homeostatic and DDC-damaged mouse livers. In the absence of damage, we detect a ratio of 0.3:1, which in vitro falls within a range that would allow organoid growth though not to its maximum. Given that homeostatic livers proliferate very rarely, the 0.3:1 ratio could represent a ‘poised’ state for expansion in the case of damage. At the late stage of regeneration (d12), the shift towards a higher (0.5:1) SCA1+ Msc : DC ratio is expected to limit ductal cell expansion even further compared to homeostasis, and could thus be a mechanism to terminate regeneration. That being the case, for ductal expansion to occur between d0 and d12, our in vitro data suggests that the SCA1+ Msc : DC ratio should drop transiently. Although results are still preliminary, we have indeed detected a pro-proliferative
ratio close to 0.1:1 around d4 after liver damage (data not shown). Tuchweber et al. had measured the kinetics of portal fibroblast and ductal cell expansion in the early stages of cholestatic fibrosis in rats (Tuchweber et al., 1996), showing that 24h post BDL ductal cells and periductular fibroblasts exhibit proliferative indices of 36.8% and 16.7% respectively, whilst they switch to 29.5% and 31% at 48h and to 12.0% and 11.6% at 7 days. These data suggest delayed expansion of the periportal mesenchyme compared to ductal cells immediately after damage, which would fit well with the drop in SCA1+ Msc : DC ratio at d4 that we predict permits ductal cell proliferation. At the later stages, the converse situation (increased SCA1+Msc cell numbers) may be necessary for cessation of epithelial growth. Regenerative processes must indeed be self-limiting, because if left unchecked, they may progress into pathologies like cancer (Tang et al., 2008).

In our in vitro system, SCA1+ Msc : DC ratios higher than 1:1 nearly abolish organoid growth; despite failing to detect such ratios in the mouse liver so far, it would be interesting to re-assess tissues in the context of chronic damage. As introduced in Chapter 1, the activation and proliferation of ECM-depositing myofibroblasts is a normal process of regeneration, but when overstimulated due to repeated injury, it leads to scarring of the tissue (fibrosis) and impaired epithelial replacement. We could thus hypothesise that ratios of >1:1 (SCA1+ Msc : DC) may be relevant for chronically damaged livers. Particularly interesting would be if an increased ratio of SCA1+Msc per se, and not their ECM depositing capacity, proves to be inhibitory for ductal cell proliferation. This scenario would fit well with the apparently low contribution (compared to HSCs) of periportal fibroblasts to myofibroblast generation and ECM deposition in fibrotic livers (Mederacke et al., 2013). To verify this is the case, we will probe the ECM depositing capacity of the SCA1+ Msc cells across different SCA1+Msc : DC ratios in vitro and in vivo, and considering that fibrosis is reversible, we will make use of the iDTR lines we have generated to inducibly ablate SCA1+ Msc cells and assess the effect on organoid growth.
6.9 Conclusion and working hypothesis

In this dissertation, we have made use of co-culture assays to highlight the mesenchymal (Msc) cell compartment of the liver as a niche cell population that supports organoid growth in vitro, and which may consequently be relevant for ductal-driven liver regeneration. Mesenchymal cells expressing the cell-surface antigen SCA1 localise periportally, in the immediate vicinity of biliary ducts (Figure 6.1, in vivo), whilst SCA1+ cells are interspersed throughout the hepatic parenchyma. Judging from their anatomical coordinates and the expression of markers like Elastin, SCA1+ Msc cells are likely to overlap – at least partially – with the previously described portal fibroblast lineage of the liver. When tissues are challenged with damage models that induce ductal-driven regeneration (such as DDC), the in situ expansion of DC is seemingly mirrored by the periportal SCA1+ Msc population; yet with distinct dynamics, given that the ratio of SCA1+ Msc : DC changes with time: we observed an increase from 0.3:1 to 0.5:1 (SCA1+ Msc : DC) between homeostasis and the late phase of the regenerative response (DDC d12), respectively; and according to preliminary data, there could be a transient drop (~0.1:1) soon after damage (DDC d4) (Figure 6.1, in vivo). Primary and in vitro expanded SCA1+ Msc cells are enriched in a battery of pro-regenerative growth factors (Rspo1/3, Hgf, Fgf7) and robustly support, in a contact-independent manner, the transition of differentiated EpCAM+ DC into highly proliferative organoid structures. Organoid-secreted factor(s), yet to be identified, also support SCA1+ Msc growth in a positive feedback loop (Figure 6.1, in vitro). Although this would seem to suggest that the SCA1+ Msc-to-DC crosstalk is designed to promote DC expansion, our in vitro data also suggests that physical contact and/or proximity from the SCA1+ Msc cells inhibits ductal cell proliferation in a ratio-dependent manner. At levels of 0.1:1 SCA1+ Msc : DC, the mitogens secreted by the SCA1+ Msc cells override their contact-dependent cytostatic action on the DC; in contrast, progressively higher ratios tilt the balance towards impaired DC proliferation until nearly abolishing organoid growth (Figure 6.1, in vitro).
Conclusions of the *in vivo* and *in vitro* cross-talk between SCA1⁺ Msc cells and DC. In the liver lobule, SCA1⁺ Msc cells reside in very close proximity to the ductal cell (DC) epithelium. Hepatotoxic damage through a DDC diet induces expansion of both DC and SCA1⁺ Msc cells, so that the ratio between these two populations (SCA1⁺ Msc : DC) changes from 0.3:1 at DDC d0, to 0.1:1 at DDC d4 (preliminary) and to 0.5:1 at DDC d12. SCA1⁺ Msc cells express pro-regenerative growth factors like Rspo1/3, Hgf and Fgf, and *in vitro* co-culturing of DC with SCA1⁺ Msc in the absence of cell-cell contact enhances DC proliferation and thus organoid formation. The proliferating DC in turn support SCA1⁺ Msc growth. However, when SCA1⁺ Msc and DC are in physical proximity, only a mesenchymal-to-epithelial ratio of 0.1:1 supports DC proliferation, whilst higher ratios arrest it.

By merging our *in vitro* and *in vivo* data, we can conceive a working model as follows (Figure 6.2): in the homeostatic liver, DC and SCA1⁺ Msc physically contact each other in a ratio (0.3:1) where the cytostatic factors of the mesenchyme limit DC proliferation, yet still allow DC to remain responsive to pro-proliferative inputs; damage and inflammation may provide such stimuli in the early phases of the regenerative cascade, so that DC begin to expand with respect to the SCA1⁺ Msc cells until reaching a 0.1:1 ratio where the mitogens of the mesenchymal cells override their contact-dependent block of proliferation. Given that the proliferating DC secrete mitogenic and/or pro-survival factors that help the SCA1⁺ Msc cells expand, the process of regeneration may be self-limiting, as the increased numbers of SCA1⁺ Msc cells will re-instate a higher SCA1⁺ Msc : DC ratio (0.5:1) that will consequently
6.9 Conclusion and working hypothesis

terminate proliferation through cell-cell contact interactions. To prove this model, a careful temporal regulation of the SCA1⁺ Msc numbers will be required, either via iDTR-based strategies or conversely, via transplantation. In the meantime, we will make use of our in vitro tools to dissect the molecular mechanisms of the mesenchymal-to-epithelial interplay, particularly with respect to the SCA1⁺ Msc-derived cytostatic factor.

In conclusion, our study has shown that periportal SCA1⁺ Msc cells behave as a niche population for DC with antagonistic properties: mitogenic vs cytostatic; a duality that may be extremely pertinent for the activation and termination phases of liver regeneration, respectively.

**Figure 6.2 Working model of the role of SCA1⁺ Msc cell in regulating ductal-driven liver regeneration.** We propose that in homeostasis, SCA1⁺ Msc and DC exist at a 0.3:1 ratio that predominantly limits DC proliferation through contact-dependent cytostatic signals from the mesenchyme. Damage stimuli, still-to-be characterised, induce DC proliferation and change the SCA1⁺ Msc : DC ratio towards 0.1:1, which in turn exacerbates DC expansion due to fewer DC being physically contacted by the mesenchyme yet receiving the mesenchyme-secreted growth factors. In the later phases of regeneration, the proliferating DC secrete a mitogen that promotes SCA1⁺ Msc expansion, which establishes a new ratio of 0.5:1 whereby increased contact with the mesenchyme shuts down DC proliferation.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>αSMA</td>
<td>α Smooth muscle actin</td>
</tr>
<tr>
<td>2-AAF</td>
<td>2-acetylaminofluorene</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>A1AT</td>
<td>α1-antitrypsin</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AFP</td>
<td>α-Fetoprotein</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BD</td>
<td>Biliary duct</td>
</tr>
<tr>
<td>BDL</td>
<td>Bile duct ligation</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone marrow mesenchymal stem cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone morphogenetic protein 2</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCl₃⁺</td>
<td>Trichloromethyl radical</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CDE</td>
<td>Choline-deficient ethionine-supplemented</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CPT</td>
<td>Camptothecin</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CV</td>
<td>Central vein</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Cytochrome P450 2E1</td>
</tr>
<tr>
<td>DC</td>
<td>Ductal cell</td>
</tr>
<tr>
<td>DDC</td>
<td>Diethoxycarbonyl-1,4-dihydrocollidine</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EdU</td>
<td>Ethynyldeoxyuridine</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EM</td>
<td>Organoid expansion medium</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
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<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
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<tr>
<td>Fab</td>
<td>Fumarylacetoacetate hydrolase</td>
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<tr>
<td>FAP</td>
<td>Fibro-adipogenic progenitors</td>
</tr>
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<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FFD</td>
<td>Flow focusing device</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FGF7</td>
<td>Fibroblast growth factor 7</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Foxf1</td>
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<td>Foxo1</td>
<td>Forkhead box L1</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GPI-AP</td>
<td>Glycosyl phosphatidylinositol-anchored cell surface protein</td>
</tr>
<tr>
<td>H/E</td>
<td>Hematopoietic/endothelial</td>
</tr>
<tr>
<td>HA</td>
<td>Hepatic artery</td>
</tr>
<tr>
<td>HC</td>
<td>Hepatocyte</td>
</tr>
<tr>
<td>Hes1</td>
<td>Hes family bhlh transcription factor 1</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Hlx</td>
<td>H2.0-like homeobox</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>HNF-6</td>
<td>Hepatocyte nuclear factor-6</td>
</tr>
<tr>
<td>Hnf4a</td>
<td>Hepatocyte nuclear factor 4-alpha</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic stellate cell</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical cord endothelial cells</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>Itgb1</td>
<td>Integrin β1</td>
</tr>
<tr>
<td>Krt19</td>
<td>Keratin 19</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine rich repeat containing G protein coupled receptor 5</td>
</tr>
<tr>
<td>LPM</td>
<td>Lateral plate mesoderm</td>
</tr>
<tr>
<td>Lrat</td>
<td>Lecithin-retinol acyltransferase</td>
</tr>
<tr>
<td>LSECs</td>
<td>Liver sinusoidal endothelial cells</td>
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<tr>
<td>LV</td>
<td>Lentiviral vector</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<tr>
<td>Mdm2</td>
<td>Transformed mouse 3T3 cell double minute 2</td>
</tr>
<tr>
<td>MEFs</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MF</td>
<td>Myofibroblasts</td>
</tr>
<tr>
<td>mGFP</td>
<td>Nuclear-localised green fluorescent protein</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MP</td>
<td>Myogenic progenitors</td>
</tr>
<tr>
<td>Msc</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>mtdTomato</td>
<td>Membrane-localised tdTomato protein</td>
</tr>
<tr>
<td>nGFP</td>
<td>Nuclear-localised green fluorescent protein</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NPC</td>
<td>Non-parenchymal cell</td>
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<tr>
<td>ntdTomato</td>
<td>Nuclear-localised tdTomato protein</td>
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<td>One-cut-2</td>
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<td>OPN</td>
<td>Osteopontin</td>
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<td>OSM</td>
<td>Oncostatin m</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<td>PCK</td>
<td>Pancytokeratin</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet derived growth factor α</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet derived growth factor β</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
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<td>PHx</td>
<td>Partial hepatectomy</td>
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<tr>
<td>PV</td>
<td>Portal vein</td>
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<tr>
<td>RPKM</td>
<td>Reads Per Kilobase of transcript per Million mapped reads</td>
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<tr>
<td>Ri</td>
<td>ROCK kinase inhibitor</td>
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<tr>
<td>Rspon1</td>
<td>Rspondin 1</td>
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<tr>
<td>Rspon3</td>
<td>Rspondin 3</td>
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<tr>
<td>S.I.</td>
<td>Small intestine</td>
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<td>SCA1</td>
<td>Stem cell antigen 1</td>
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<td>Sfrp5</td>
<td>Secreted frizzled-related protein 5</td>
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<tr>
<td>Sox9</td>
<td>SRY-related HMG box transcription factor 9</td>
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<td>STM</td>
<td>Septum transversum mesenchyme</td>
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<tr>
<td>Tert</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Thy1</td>
<td>Thymus cell antigen 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase-1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>Ttr</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
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<tr>
<td>VEGFR3</td>
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