From Department of Cell and Molecular Biology
Karolinska Institutet, Stockholm, Sweden

LINEAGE TRACING OF EARLY ORGANOGENESIS AND LIVER MESENCHYMAL CELLS WITH ULTRASOUND-GUIDED IN UTERO NANO-INJECTION

Jingyan He

何婧妍

Karolinska Institutet

Stockholm 2023

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2023

ISBN 978-91-8017-155-7
Cover illustration: E9.5 mouse embryos collected from amniotic cavity (left) and exocoelomic cavity (right) injections with H2B-GFP lentivirus at E7.5 and immunofluorescent staining showing the different targeting patterns. GFP in green, DAPI in blue.

Lineage tracing of early organogenesis and liver mesenchymal cells with ultrasound-guided in utero nano-injection

Thesis for Doctoral Degree (Ph.D.)

By

Jingyan He

The thesis will be defended in public at Lecture hall Peter Reichard, Biomedicum, Solnavägen 9, 171 65 Solna , December 15th, 14:00

Principal Supervisor:
Associate Professor Emma R Andersson
Karolinska Institutet
Department of Cell and Molecular Biology

Co-supervisor(s):
Professor Katja Petzold
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Uppsala University
Dept. of Medical Biochemistry and Microbiology

Associate Professor Niklas Björkström
Karolinska Institutet
Department of Medicine
Center for Infectious Medicine

Associate Professor Martin Ivarsson
Karolinska Institutet
Department of Medicine
Center for Infectious Medicine

Opponent:
Professor Anna Mae Diehl
Duke Unversity
Department of Medicine

Examination Board:
Associate Professor Claudio Cantù
Linköping University
Department of Molecular and Developmental Biology
Division of Molecular Medicine and Virology (MMV)

Dr. Fredrik Lanner
Karolinska Institutet
Department of Clinical Science, Intervention and Technology

Professor Goncalo Castelo-Branco
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Molecular Neurobiology

To my family and friends who always love me and believe in me

谨以此书献给永远爱我 相信我的家人和朋友们

道阻且长 行则将至，行而不辍 未来可期

《诗经·蒹葭》《荀子·修身》

Abstract

Current methods used to investigate embryo development and alter gene expression in mouse embryos are often time consuming and require large numbers of mice. To circumvent these limitations, we were aiming to develop a flexible and efficient method to investigate embryo development and manipulate gene expression *in utero*. Two tissues of interest to target in vivo are the ectodermal/ neural compartment, and mesoderm. Neurectoderm gives rise to the brain, spinal cord and peripheral nervous system, among others, while mesoderm gives rise to blood, muscle, and mesenchymal cells, among other cell types.

Mesenchymal cells in liver, including peri-portal fibroblasts, mesothelial cells and hepatic stellate cells (HSCs), play multiple crucial roles in normal liver development, liver regeneration or liver fibrosis when liver is injured. Mesenchymal cells express both neural and mesenchymal markers, therefore both a neural crest (NC) and mesoderm origin have been proposed. The embryonic origin of HSCs is a long-debated topic in this field due to the lack of specific marker genes and potential convergent differentiation from different origins.

To investigate nervous system development, we first hypothesized that by injecting lentivirus into the amniotic cavity (AC) prior to the neural plate closure, the open neural plate should be labeled and therefore label the future brain and spinal cord. In Paper I and II, we developed NEPTUNE (NEural Plate Targeting by *in Utero* NanoinjEction) to transduce either nervous system with up to 99% efficacy or selectively achieve the expression in specific cell types by using cell-specific MiniPromoters.

In Paper III, the first aim was to develop a method to target mesoderm, and then to apply this technology to investigate liver mesenchymal cells. We hypothesized that exclusive labeling of the mesoderm and its progeny could be achieved by injecting into the exocoelomic cavity (ExC) during gastrulation after the segregation of three germ layers and full establishment of two cavities (AC and ExC), since mesoderm is in contact with the ExC. Therefore, we further adapted ultrasound-guided *in utero* nano-injection to lineage trace mesoderm descendants by injecting a diverse lentivirus barcode library into the ExC at embryonic day (E)7.5. In parallel, we used NEPTUNE, to target ectoderm/neural crest as well as primitive streak (PS) by injecting into AC at E7.5. Embryos were collected at E9.5 and E10.5 to address early organogenesis and the origin of septum transversum mesenchyme (STM), a transit tissue believed to contribute to the mesenchymal compartments of diverse internal organs. E16.5 livers were collected to resolve the clonal relations between different mesenchymal cells in the liver.

In summary, during my doctoral studies, we developed two new approaches to target embryonic tissues during development. Ultrasound-guided *in utero* nano-injection is a

flexible and efficient tool to elucidate clonal relations among tissues in early mouse embryo development, as well as for gene manipulation. It significantly minimizes both the financial cost and ethical burdens associated with animal research, in the meantime accelerating the progression from hypothesis to *in vivo* results.

List of scientific papers

I. Mangold, K., Mašek, J.*, **He, J.***, Lendahl, U., Fuchs, E., & Andersson, E. R. (2021). Highly efficient manipulation of nervous system gene expression with NEPTUNE. Cell Reports Methods, 0(0), 100043.

II. Mangold, K., **He, J.**, Stokman, S., & Andersson, E. R. (2022). Murine Neural Plate Targeting by *In Utero* Nano-injection (NEPTUNE) at Embryonic Day 7.5. JoVE (Journal of Visualized Experiments), 2022(180), e63148.

III. **Jingyan He**, Sandra De Haan, Lenka Belicova, Agustin Corbat, Noémi Van Hul, Stefaan Verhulst, Michael Ratz, Leo Van Grunsven, Jonas Frisén, Emma R Andersson. Single cell lineage tracing of ectodermal and mesodermal compartments contributing to organogenesis and liver mesenchymal cells **(Manuscript)**

Scientific papers not included in the thesis

I. Emma Patey*, **Jingyan He***, Andrea Ponzetta, Emma R Andersson#, Niklas K. Björkström#. Tracing ontogeny of group 1 ILCs in mouse peripheral tissues (**Manuscript**)

II. Mašek J#, Filipovic I*, Hankeová S*, Van Hul N*, **He J*** , Belicová L, Jiroušková M, Maria Frontino A, Oliveira DV, Turetti F, Iqbal A, Červenka I,Sarnová L, Verboven E,Brabec T, Björkström NK, Gregor M, Dobeš J, Andersson ER#. Jag1 modifies hepatic fibrosis via immune cells in a mouse model of Alagille syndrome (**Submitted**)

III. Mašek J#, Belicová L; Iqbal A; Červenka I; Van Hul N; Hankeova S; **He J**; Jiroušková M; Maria Frontino A; Oliveira DV; Turetti F; Sarnová L; Verboven E; Filipovic I; Brabec T; Björkström NK; Gregor M; Andersson ER#. A scRNAseq atlas of Jag1Ndr/Ndr mice reveals disrupted hepatocyte differentiation in Alagille syndrome (**Submitted**)

* These authors have equal contribution

CONTENTS

List of abbreviations

AAV	Adeno-associated virus
AER	Apical ectodermal ridge
Ad	Adenovirus
AGM	Aorta-gonad-mesonephros
A-P	Anterior-posterior
BM	Bone Marrow
BMP	Bone Morphogenetic Proteins
CAR	Coxsackie adenovirus receptor
CLE	Caudal lateral epiblast
CRISPR	Clustered regularly interspaced short palindromic repeats
ECM	Extracellular matrix
EHBDs	Extrahepatic bile ducts
EHF	Early head fold
EMT	Epithelial-mesenchymal transition
ENS	Enteric nervous system
ER	Estrogen receptor
ExE	Extra-embryonic ectoderm
ExM	Extra-embryonic mesoderm
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast Growth Factors
FL	Fetal liver
HNF	Hepatocyte nuclear factors
HSCs	Hepatic stellate cells
HSV	Herpes simplex virus
IGF	insulin-like growth factor
IHBDs	Intrahepatic bile ducts
LDLR	Low-density lipoprotein-receptor
LPM	Lateral plate mesoderm

NEPTUNE	NEural Plate Targeting by *in Utero* NanoinjEction
NC	Neural crest
NCCs	Neural crest cells
NGF	Nerve growth factor
NMPs	Neuromesodermal progenitors
NSB	Node-streak border
PAC	Proamniotic cavity
P/Sp	Para-aortic splanchnopleure
PGC	Primordial germ cells
PNS	Peripheral nervous system
PS	Primitve streak
PSM	Presomitic mesoderm
RA	Retinoic acid
RTK	Receptor Tyrosine Kinases
SHH	Sonic Hedgehog
sm-LPM	somatic lateral plate mesoderm
sp-LPM	somatic lateral plate mesoderm
TS	Theiler stage
VE	Visceral endoderm
YS	Yolk sac
4-OHT	4-hydroxy-tamoxifen

Introduction

The core aim of my doctoral studies is to innovate technologies for manipulating mouse embryos, enabling novel experiments like lineage tracing and conditional perturbations etc. in developing mouse embryos. In order to develop these techniques and comprehend the potential and limitations of these technologies, a thorough understanding of mouse embryo development is essential. Consequently, in this review, I will provide an overview of early mouse embryo development, focusing on gastrulation, early organogenesis, and liver development. In addition, I will also summarize advances in lineage tracing approaches, including studies on hepatic stellate cells (HSCs) and other mesenchymal cells in liver. Lastly, I will introduce different viral vectors for gene manipulation and gene therapy and ultrasound-guided *in utero* injections as a new tool for lineage tracing and gene manipulations.

1 Early embryo development

During early embryo development, embryos start from a single-cell zygote and undergo several rounds of division, progress to a blastocyst, subsequently progressing to gastrulation[1,2]. Early embryo development is a precisely controlled process, regulated by key factors and signaling pathways. In mice, implantation takes place on embryonic day (E)4.5, at which point the embryo consists of three distinguishable lineages: epiblast, trophectoderm (TE) and primitive endoderm (PrE). Shortly following implantation, the epiblast rapidly undergoes epithelialization and subsequently cavitation, ultimately giving rise to the proamniotic cavity (PAC)[1–4]. At E5.5, the cup-shape embryo, known as the egg cylinder, comprises three distinct populations: pluripotent epiblast cells, enveloped by a layer of visceral endoderm (VE), and the extra-embryonic ectoderm (ExE)[1–3]. From E6.25 to E9.5, during gastrulation, the pluripotent epiblast gives rise to the three multipotent germ layers of the fetus, including mesoderm, definitive endoderm and ectoderm. In the meantime, the extraembryonic tissue assumes the role of developing supportive structures, including the placenta[1–3]. Following gastrulation, organogenesis initiates around E8.0, refining the previously established pattern into intricate organ systems, and subsequently forming nearly all major organ systems in the following days from E9.5 to E13.5[5](Fig1).

During mouse embryonic development, pluripotent cells undergo rapid division and differentiation, resulting in the speedy dynamic shift of morphogenic process. This process is regulated by a complex, successive series of interactions between molecules and tissues [1–4].

1.1 Gastrulation and organogenesis

Gastrulation represents a key developmental process during early mouse embryo development from E6.25 to E9.5 during which pluripotent epiblast cells give rise to definitive ectoderm, mesoderm and endoderm germ layers that will contribute to diverse organisms[1–3,5,6]. At ~E6.5, the primitive streak (PS) first appears at the posterior end of the embryo, at the boundary of extraembryonic/ embryonic compartment, which indicates the initiation of gastrulation. Pluripotent epiblast cells ingress through the PS, go through the epithelial-mesenchymal transition (EMT) and contribute to mesoderm and endoderm fate[1–3,5]. Pluripotent epiblast cells that do not ingress remain in the outermost layer and differentiate into ectoderm.

Genetic studies have identified several key transcriptional networks, which are highly conserved across vertebrates, governing the specification of three germ layers, including Nodal pathway, BMP, Wnt, FGF, SMAD, FOXH1, FOXA2, Brachyury/T, T-box gene eomesodermin (Eomes)[2]. The EMT process in PS is highly orchestrated and controlled through the signaling pathways of Wnt, Bone Morphogenetic Proteins (BMPs) and Fibroblast Growth Factors (FGFs)[1–3,5,6]. The current model suggests that the determination of diverse cell destinies depends on temporal and spatial aspects of cell ingression through the PS, indicating distinct regulatory signaling contexts[7].

Additionally, in mammalian embryos, early pluripotent epiblast cells are capable of giving rise to both somatic and germ cell fates. During gastrulation, the specification of primordial germ cells (PGCs) is triggered by extrinsic signaling concurrent with the patterning of the embryonic axis[8,9].

1.2 Organogenesis

Following gastrulation, organogenesis initiates by forming the neural plate and heart tube around E8.0-E8.5 and subsequently forming nearly all major organ systems in the following days from E9.5 to E13.5[5,6,10]. Early organogenesis constitutes a pivotal stage in animal development, during which cells originating from the three primary germ layers undergo rapid division and differentiation to initiate the establishment of all major organs [5,10].

Due to the extremely restricted cell numbers in early embryos and the lack of distinguishing marker proteins for the isolation of individual cell types, the comprehensive characterization of cellular identities during early organogenesis has been challenging. The proper establishment of all major organs requires the spatiotemporal orchestration of diverse gene expression that controls the cell type specification, migration and localization[11,12]. The emergence of single-cell profiling techniques brought the breakthrough and improved our understanding of the cell diversity and increased the resolution of early regulatory dynamics in early mouse embryos[5,13–17].

1.3 Three germ layers

Gastrulation represents a key developmental process from E6.25 to E9.5 during which pluripotent epiblast cells give rise to the definitive ectoderm, mesoderm and endoderm germ layers that will give rise to diverse organs.

1.3.1 Ectoderm

The anterior PS cells do not undergo EMT and instead form multipotent ectoderm, which differentiates into surface ectoderm or neurectoderm[1,5]. The part of definitive ectoderm nearest to the node undergoes differentiation into neurectoderm or neural plate. The neural plate is a pseudostratified columnar sheet of neuroepithelium symmetrically positioned along the anterior midline of the embryo. Neural tube formation is a complicated process that requires the orchestration of various cell signaling pathways, such as Shh, BMP signaling[18,19]. Primary neurulation begins with an open neural plate and undergoes progressive closure in a zipper-shape manner and eventually form a closed neural tube. The initial/ primary closure point is located at the hindbrain/cervical boundary (closure 1) around E8.5 (~6 somites stage), following by the second closure at forebrain/midbrain boundary (closure 2), and achieved a full closure at the third closure point the rostral end of the forebrain (closure 3) around E10.5 (~30 somites stage). The closure can also vary in different mouse strains[18,19]. Subsequently, both the neurectoderm and non-neuronal ectoderm will undergo remodeling, culminating in the formation of a closed tube, covered by a single layer of non-neuronal ectoderm. Only the brain and anterior spinal cord are generated during primary neurulation. The secondary neurulation occurs around E11-E12 after the primary neurulation, encompasses the creation of a medullary cord from cells in the tail bud region and subsequently transformed into a neural tube through a cavitation process[18–20]. The lumen from the secondary neurulation is continuous with primary neural tube lumen.

Neurulation is regulated by multiple factors, including sonic hedgehog (Shh) from the node and floor plate, which triggers the elevation of neural folds and facilitates the formation of the neural groove and floor plate and as well as acts as an essential ventral patterning factor[18,19,21,22]. Wnt6, produced by the epidermal ectoderm adjacent to the neural plate, along with BMPs, induces the expression of slug in the forthcoming neural crest cells (NCCs). Furthermore, BMPs appear to sustain the dorsal expression of Pax transcription factors[18,19].

The full commitment of definitive ectoderm to either surface ectoderm or neurectoderm is established around E7.5. Subsequently, patterning of the brain and spinal cord is regulated by the morphogen activity gradients in a dorsoventral pattern[21]. Some of the cells at the border of neural and non-neural ectoderm will delineate and migrate to form the NC.

1.3.2 Neural crest (NC)

In addition to the formation of the three germ layers during gastrulation, a fourth highly multipotent cell population is the NC, which is normally considered as the fourth germ layer[23–26]. Neural crest cells (NCCs) are first identified as distinct, migratory cells occurs when they undergo EMT and delaminate from the neuroepithelial cells[23–27].

The NC is composed of four subpopulations: cranial, trunk (including cardiac), vagal and sacral NC[28,29]. These subpopulations emerge successively as the embryo extends along the anterior-posterior axis, marking the progression in both temporal and spatial dimensions. NCCs go through EMT and migrate ventrally along the anterior-posterior axis, after delaminating from the dorsal neural tube, and give rise to diverse cell types including neurons, glial cells that contribute to the peripheral nervous system (PNS) and enteric nervous system (ENS), as well as non-neural cell populations[25].

Cranial NCCs contribute to most connective tissues, smooth muscle, bone and cartilage of the head and face, as well as nerve ganglia and pigment cells[30–32]. Cardiac NCCs contribute to the aorticopulmonary septum formation of the outflow tract uring heart development[33–35]. The trunk NC gives rise to neurons, glia and PNS, as well as the endocrine system and pigment-synthesizing melanocytes. Vagal NCCs contribute to diverse organs, including thymus, lung, heart, and the ENS that colonizes the entire gastrointestinal tract[16,36]. Sacral NCCs contribute to the ENS that populates the post-umbilical gut[16,37].

Signaling molecules such as BMPs, FGFs, Wnt proteins, retinoic acid (RA), Notch and Receptor Tyrosine Kinases (RTKs), play an active role in inducing, specifying, guiding cell migration, and promoting neural differentiation of the NC. These highly coordinated signals among different tissue types, including ectodermal, mesodermal cells, the neural and non-neural ectoderm are working in a tightly orchestrated fashion[24,30,38].

1.3.3 Mesoderm

The formation of nascent mesoderm occurs when epiblast cells ingress through the PS, undergo EMT, and establish a new layer between the epiblast and visceral endoderm[39,40]. The EMT process includes loosening of the epithelial adherent junctions, basement membrane disruption and cytoskeletal rearrangement. Several transcriptional factors play important roles in this process, including BMP signaling from the ExE, and nodal-SMAD signaling, as well as canonical Wnt signaling from the epiblast, FGF signaling, including FGF8, FGFR1 and Snail; MAPK signaling, EOMES, as well as transcription factors mesoderm posterior 1 (MESP1) and MESP2 [39,40].

Fate mapping studies have revealed that the ultimate identities of mesodermal cells are regulated by both the timing and location of cell ingression along the anterior-posterior (A-P) axis of the PS [39,40]. The segregation initiates around E6.25, when the posterior streak first generates extraembryonic mesoderm (ExM), and the middle section produces lateral

mesoderm, while the anterior region predominantly gives rise to paraxial mesoderm, gut, and notochord [39,40].

1.3.3.1 Extra-embryonic mesoderm (ExM)

Extra-embryonic mesoderm (ExM) first arises at the posterior PS and forms the mesodermal compartment of the visceral yolk sac, which further gives rise to the amnion, chorion and allantois, facilitating nutritional and respirational support of the fetus through the umbilical connection[4,39]. Additionally, the first two waves of hematopoiesis occur in the yolk sac: the first wave occurs in the blood islands at E7.5 producing primitive erythroid cells, macrophages, and megakaryocytes, and the second wave occurs when hemangioblasts generate erythromyeloid progenitors (EMP) that will contribute to erythroid cells and myeloid cells[41].

1.3.3.2 Paraxial mesoderm

The establishment of a segmented body plan represents a fundamental and highly conserved characteristic shared by all vertebrate species [42]. It bestows both the stability and flexibility to the body. Segmentation is established through the process of somite formation, also known as somitogenesis[42–44]. In vertebrates, the process of segmentation is governed by the segmentation clock driven by Wnt and Notch signaling pathway, which operates within the PSM[4543,44].

Somites manifest as epithelial blocks of paraxial mesodermal cells originating from the presomitic mesoderm (PSM), forming on both sides of the neural tube synchronously[42–44]. Following their formation, the epithelial somites undergo a course of maturation and differentiation. The surrounding tissues play a crucial role by providing signals that steer the specification into sclerotome, the dermatome, and the myotome[46–48]. Cells in the ventral portion of the somite undergo de-epithelialization and ultimately give rise to the sclerotome, which is responsible for shaping the vertebral elements and syndetome, the precursor of tendons. This process is regulated by Shh signals from the notochord. These signals exhibit highest concentrations in the ventral part of somite and induce the expression of paired box 1 (Pax1) and Pax9 in sclerotome cells[46–48]. Conversely, dermomyotome originates from the dorsal somite and retains its epithelial characteristics. Wnt1/3a from the neural tube and Wnt8c from the ectoderm induce the expression of Pax3 and Pax7 in the dermomyotome[46]. Subsequently, cells from the tips of the dermomyotome develop to myotome, initiating the myogenic factors Myf5 and MyoD expression, ultimately leading to the development of the muscles in the back and body wall, as well as a portion of the dermal tissue of the back. The specification of dermatome, which is the precursor of dermal tissue, is influenced by neurotrophin 3 (NTF3) from the neural tube[47].

1.3.3.3 Intermediate mesoderm (IM)

Fate mapping studies have shown that the paraxial mesoderm originates from anterior PS, and lateral plate mesoderm (LPM) originates from the posterior PS. However, it is challenging to define intermediate mesoderm (IM) at early post-gastrula stage, since very few specific marker genes have been identified for the IM region. IM contributes to the development of urogenital development, that ultimately give rise to the gonads, adrenal cortex, kidney, and reproductive tract[49–51].

Genetic studies in mice indicate that Osr1, Lhx1 and Pax2/Pax8 play crucial roles in early specification of the IM[49,50]. This activation seems to rely on BMP signals originating from either the LPM or the overlying ectoderm, in conjunction with opposing signals from the somite. Low concentrations of BMPs induce IM-specific genes, while higher concentrations trigger the expression of lateral plate markers[49,51]. Consequently, it appears that the source of BMPs is situated more laterally, likely in a dorsal position. Additionally, introducing BMPs in ectopic locations can alter the IM's spatial position and even induce paraxial mesoderm to adopt an IM-like phenotype[40].

1.3.3.4 Lateral plate mesoderm (LPM)

Initially, the lateral plate mesoderm (LPM) is a homogeneous mesenchymal structure lateral to the somitic mesoderm. The specification of LPM is governed by BMP4 activity. Subsequently the LPM splits into somatic LPM and splanchnic LPM and the distinctive striped architecture becomes evident during segmentation[52].

The limb is a complex organ originating from the LPM, whose development is coordinated along three different major axes: proximal-distal, dorsal-ventral and anterior-posterior[53]. Forelimb initiation starts at approximately E9.5 in the form of a forelimb bud consisting of undifferentiated mesenchymal cells, enveloped by an ectoderm layer. The apical ectodermal ridge (AER) governs the proximal-distal axis, while the zone of polarizing activity and Shh regulates the anterior-posterior axis. WNT7A from the ectoderm influences the dorsal-ventral axis.

The mesothelium is a layer of simple squamous epithelial cell lining all coelomic organs and coelomic cavities, originating from the splanchnic LPM[54–56]. It constitutes the largest epithelial organ in the mammalian body. Increasing evidence indicates that mesothelium gives rise to wide range of cell types within developing organs through EMT. This includes smooth muscle cells in the vascular system, gastrointestinal, and respiratory tracts as well as fibroblast cells, and stellate cells[55,56]. Early gene markers such as *Wt1*, *Gata4* and *Msln* are used for lineage studies[57–59]. In addition, mesothelium contributes to the spleen, visceral white adipose tissue and gonads.

The anterior LPM contributes to the heart formation during early somitogenesis[40,60]. In addition, progenitors from the anterior LPM also contribute to the craniofacial muscle lineages alongside the contributions of paraxial mesoderm and NCCs [40,60].

1.3.4 Neuromesodermal progenitors (NMPs)

Biopotent neuromesodermal progenitors (NMPs) were identified in 2009, which challenged the conventional understanding of the three-germ layer paradigm[61–64]. This discovery has introduced the prospect that certain posterior neural tissues may be produced independent of the mechanisms responsible for inducing the anterior neural plate.

Fate mapping studies have shown that the NMPs are located at the caudal lateral epiblast (CLE) and neighboring node-streak border (NSB). Currently, there are no unique molecular markers exclusively identified for NMPs. Instead, NMPs are identified by co-expression of Sox2, the neural progenitor marker and brachyury (T/Bra), an early mesodermal marker.

Once established, NMPs continue to generate new neural tissue and the adjacent paraxial mesoderm for the elongation of the body axis [62–65]. This simultaneous generation of neural and mesodermal tissues from a common precursor plays a vital role in the differentiation and patterning of trunk tissues. This dynamic process is essential for the proper development and organization of various organs and systems in the body. Subsequently, RA signals from the mesoderm suppress FGF and Wnt signaling, which play a pivotal role in the induction and maintenance of NMPs and promote neural differentiation. Meanwhile, Hox genes are crucial for establishing the anterior to posterior identity, by regulating the Wnt, FGF and RA signaling pathways[62,65].

1.3.5 Endoderm

Definitive endoderm originates from the anterior PS during gastrulation, and gradually incorporates into the overlying VE. Endoderm cells are first identified in the VE around E6-6.5. After the establishment of the primary germ layers, the endoderm undergoes a sequence of transformations to form the gut tube, ultimately developing into the epithelial lining of the digestive and respiratory systems[66,67].

In general, the anterior endoderm contributes to the formation of the anterior intestinal portal, while the posterior endoderm cells give rise to the caudal (posterior) intestinal portal[66]. Interactions among different germ layer derivatives play a crucial role in the morphogenesis and organogenesis of the gut tube[5,40,68]. The establishment of A-P patterning is regulated by multiple signaling pathways, including FGF, Wnt/ β-catenin, BMP, RA and Nodal signalings[66,69–71].

Following the establishment of the A-P pattern, the foregut develops into the esophagus, thyroid, lungs, liver, pancreas, as well as the biliary system. The midgut gives rise to the small intestine and stomach. The hindgut differentiates into the large intestines, as well as the cells enveloping the genitourinary system[66,67,69].

Increasing evidence has shown that a common mesendoderm progenitor is responsible for generating both mesoderm and endoderm[72]. However, it is still uncertain whether all endoderm cells originate from a common mesendoderm progenitor.

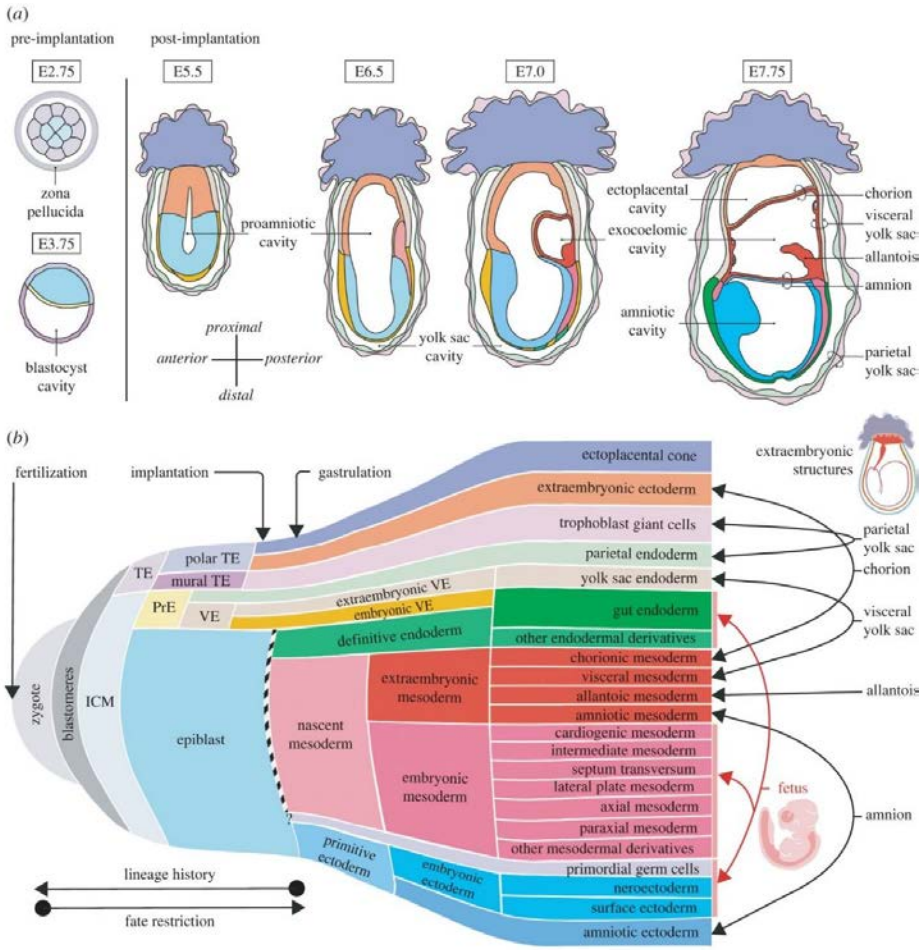
1.4 Amniotic cavity & Exocoelomic cavity

During early embryo development, the PAC becomes divided into a small ectoplacental cavity, an amniotic cavity (AC) and an exocoelomic cavity (ExC) by amnion and chorion membranes, which are formed completely around E7.5[4]. Starting from early gastrulation, the amnion is initiated with the formation of a posterior amniotic fold (PAF, also known as amniochorionic fold (ACF)) resulting from the aggregation of ExM. Subsequently, folds expand laterally along the sides of the egg cylinder and converge at the anterior midline. In addition, the small anterior fold is formed by the accumulation of ExM at the anterior portion. Ultimately, the ExC forms by the accumulation and merging of these small cavities.

During development, the coelomic cavity develops inside the body of all vertebrates. This cavity is a confined space within the body trunk, featuring two distinct layers of cells: one that lines the inner surface of the body wall, and another encases the digestive tract[55,73,74]. The formation of the coelomic cavity represents a pivotal event giving rise to a tube-within-a-tube configuration of the embryo[73,74]. The coelomic fluid acts as a hydroskeleton and allows the growth and free movement of internal organs, and is essential for the transport of gases, nutrients and wastes around the body.

The formation of coelomic cavity is the result of LPM splitting. Originally, the LPM is a homogeneous mesenchymal structure that resides alongside the somitic mesoderm. Subsequently, the LPM splits into two layers along the dorsoventral axis: somatic LPM (sm-LPM) and splanchnic LPM (sp-LPM). sm-LPM is located dorsally underneath the ectoderm and sp-LPM is located ventrally and establishes the inner cavity to support the organ development and placement. The combination of sp-LPM and endoderm is commonly known as splanchnopleure, while sm-LPM, along with ectoderm, is denoted as somatopleure[55,73,74].

Fig1. Schematic diagram of early mouse embryo development. (a) Mouse embryo development. The process from pre-implantation of zygote at E2.75 and blastocyst at E3.75 to post-implantation and the gastrulation stage. (b) The sequential emergence of embryonic and extraembryonic compartments from a single zygote. Extraembryonic structures are shown in black arrows and embryonic structures are shown in red bars and arrows. (TE, trophectoderm; ICM, inner cell mass; PrE, primitive endoderm; VE, visceral endoderm.) **Reprint from Shifaan Thowfeequ and Shankar Srinivas. Phil. Trans. R. Soc. 2022, with open access.**



(Fig1, legend on last page)

2 Liver development and liver fibrosis

The liver is one of the most crucial organs in our body, and is necessary for digestion, detoxification and essential metabolic functions, which include bile production, glucose regulation, and blood homeostasis modulated by clotting factors and serum proteins[75,76]. The liver gradually and delicately transforms from a transient hematopoietic site in embryos to a metabolic organ, but also harbors the immune system for a while after birth[75–77].

The fetal liver (FL) consists of different cell types, including endoderm-derived hepatoblasts and mesoderm-derived hepatic mesenchyme, including resident macrophages, hepatic stellate cells (HSCs), fibroblasts, thus creating a specific microenvironment[75,76]. Regarding these diverse cell types and the embryonic origins of the liver, it is not surprising that numerous signaling pathways are involved in the delicate regulation of liver development[75,76]. At the initiation stage, FGF secreted by adjacent cardiogenic mesoderm and BMP from STM induces the hepatic fate in foregut endoderm, and promotes liver bud growth. In addition, Wnt/β-catenin and TGF-β signaling are essential for liver induction[75,76]. Several hepatocyte nuclear factors (HNFs), such as HNF-4α, HNF-1β, have abundant functions in different steps of liver development t[75,76].

Liver fibrosis is a dynamic process characterized by the progressive accumulation of extracellular matrix (ECM) due to chronic liver injury resulting from various liver diseases. HSCs or portal fibroblast are activated and transdifferentiated into myofibroblasts, which play pivotal roles in the fibrotic response following liver injury[77–80].

After injury, the liver has a remarkable capability for regeneration. Experimental models of liver injury underscore the involvement of sympathetic and parasympathetic nerves in the process of liver regeneration, although the mechanism is less understood[81].

2.1 Endoderm contribution to liver development

The development of the liver begins as early as E7.0[2,5,69]. During gastrulation, the endoderm is established and patterns into a primitive gut tube, consisting of the midgut, hindgut and foregut, from which the embryonic liver originates[5,40,69,75,76]. At E8.75, the anterior portion of the hepatic diverticulum is formed, giving rise to the liver and intrahepatic bile ducts (IHBDs), while the posterior portion develops into the gall bladder and extrahepatic bile ducts (EHBDs). By E9.5, hepatoblasts derived from the foregut invade the septum transversum mesenchyme (STM) and form hepatic cords, which are divided by sinusoids filled with blood cells[75,76]. Starting from E13.5, hepatoblasts begin to differentiate into cholangiocytes and hepatocytes. The final maturation is gradual and continues until the postnatal period. The liver lobule, the basic functional unit of the liver, is fully established 2 to 3 weeks after birth (Fig2)[75,76].

2.2 Mesoderm contribution to liver development

Three germ layers arise during gastrulation and contribute to liver formation when septum transversum mesenchyme (STM, thought to be derived from mesoderm) interacts with endoderm to induce liver formation[75,76,82]. Furthermore, STM differentiates into intrahepatic mesenchymal cell types, including hepatic stellate cells (HSCs) and portal fibroblasts[57,82,83]. HSCs are crucial mesenchymal cells in the liver that control fibrosis and regeneration in response to injury in adult liver[80,84,85], and act as a niche for hematopoietic cells in the fetal liver[86].

HSCs express both mesodermal markers, including Wt1, Desmin, and neural markers, such as p75 neurotrophin receptor (p75NTR), glial fibrillary acidic protein (GFAP) etc. Therefore, the embryonic origin of HSCs was long debated[87,88]. Several lineage tracing studies supported a mesoderm and STM origin HSCs[89–92], *Tg (hand2: EGFP)* lineage tracing in zebrafish supported a LPM origin for HSCs[93]. *MesP1-Cre* suggested a mesoderm origin of HSCs, submesothelial cells, and perivascular mesenchymal cells[57,82]. *Wt1-Cre* suggested Wt1+ STM cells contribute to HSCs and other liver mesenchymal cells[57,82]. Similarly, *Gata4-Cre* with G2 enhancer also labelled STM and the liver mesenchymal compartment including HSCs[58]. Mesothelin *(MSLN)-Cre* labeled fibroblast and smooth muscle cells of the trunk[59]. *αSMA-CreERT2* labeled myofibroblast which acts as the progenitor of hepatic epithelial cells, contributing to liver regeneration after partial hepatectomy[94]. *Reelin-CreERT2* labeled a new subset of HSCs, which showed different properties from Desmin+ HSCs in cholestatic liver injury[95]. However, the developmental trajectory and relation to other mesenchymal populations in liver is not fully understood. Additionally, it is still less clear if different STM compartments/subgroups make different mesenchymal lineages. It remains to be clarified if all HSCs go through an intermediate mesothelium stage.

On the other hand, fate mapping studies with neural marker genes result in different conclusions. *Wnt1-cre* lineage tracing does not support a NC origin for HSCs[91,96]. Interestingly, it has also been reported that some stromal-like cells are labeled in embryonic liver with *Sox10-CreERT2* (Tamoxifen injection at E9.5) and *Wnt1-Cre* mice[96]. human GFAP *(hGFAP) -Cre* labels HSCs but also cholangiocytes. hGFAP-labeled HSCs acts as the progenitors for liver epithelial cells after liver injury[97]. Intriguingly, in another study, both *hGFAP* and mouse GFAP *(mGFAP)- Cre* model did not label HSCs. Additionally, *hGFAP-Cre* model also labeled the vascular smooth muscle cells (VSMCs) and endothelial cells[98]. *Reelin-Cre* labeled a subset of HSCs representing different properties from Desmin+ HSCs for cholestatic liver injury[95].

Interestingly, transplantation of hematopoietic stem cells isolated from bone marrow into adult liver also showed a potential to transdifferentiate into HSCs after liver injury[99].

2.3 Ectoderm contribution to liver development

In contrast to the endoderm-originated hepatic epithelial cells and the mesoderm contribution to liver mesenchymal cells, the ectoderm contribution to liver development and the innervation of liver are less studied and remains unclear.

The liver is innervated by autonomic fibers and sensory fibers belonging to the sympathetic and parasympathetic nervous system[100]. Recent findings have shed light on the importance of the hepatic nervous system in liver homeostasis, regeneration and facilitating its recovery. However, there are still substantial gaps in understanding the precise mechanisms by which hepatic nerves influence liver diseases and vice versa[100].

In mice, the initial presence of innervation is detected at the extrahepatic bile ducts around E17.5, and over the first few postnatal weeks[101]. Developmental studies have shown that the nerve fibers first emerge at the center before birth and gradually extend towards the periphery. In murine models, the nerve fibers are extended from the periportal regions to the pericentral area, which is stimulated by nerve growth factor (NGF) from the biliary epithelial cells and the adjacent mesenchymal cells and NGF signals from hepatocytes[101].

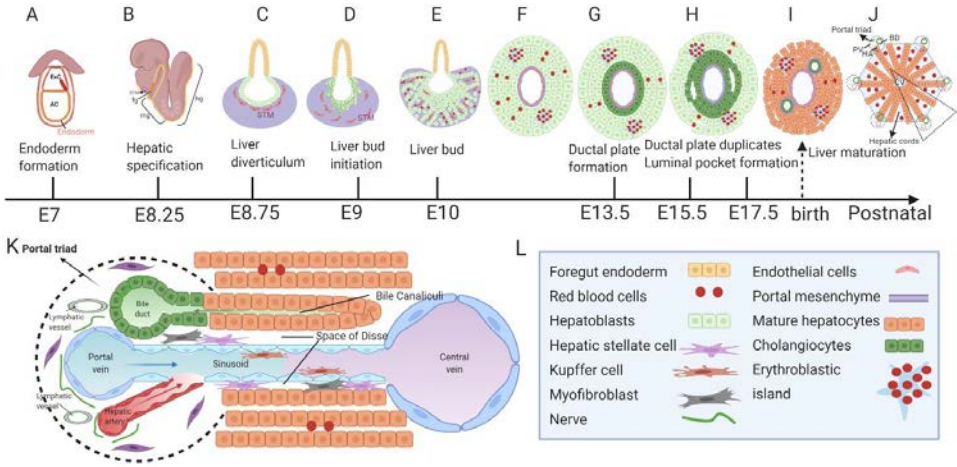


Figure2. Schematic diagram of liver development and liver morphology in mouse. (A) Endoderm is established by gastrulation and liver development is initiated around E7.0. (B) At E8.25, the endoderm patterns into a primitive gut tube (foregut, midgut and hindgut). The embryonic liver originates from foregut. (C-E) Transverse sections to show the mouse liver bud growth process. (C) Around E8.75, foregut endoderm differentiates into hepatoblasts, which then invade the septum transversum mesenchyme (STM). (D) Hepatoblasts transition from columnar to pseudostratified morphology. (E) Invading hepatoblasts form hepatic cords, divided by sinusoids filled with blood cells. (F) Prior to bile duct development, hepatoblast is the only epithelial cell types in the fetal liver. (G) E13.5, bile duct development initiates with formation of the ductal plate, which is composed of a single layer of cholangiocyte precusors in contact with the portal mesenchyme. (H) Between E15.5-E17.5, a second layer of cholangiocytes emerges at discrete points of the ductal plate. Luminal pockets are formed between the two layers of cholangiocyte precursors. (I) In the perinatal period, some luminal pockets form bile ducts. The remaining hepatoblasts differentiate into mature hepatocytes. Erythroblastic islands (EI) are also present in the fetal liver, until 2weeks after birth. (I, J) The final liver maturation is gradual and continues until the postnatal stage. (J) Mature liver contains a repeating hexagonal unit known as a Liver lobule (K), consisting of hepatic cords, which radiate from a central vein towards portal triads at six corners (composed of a bile duct, hepatic artery and portal vein). (K) Schematization of triangular region in J. Different cell types are present in mature liver. (L) Legend for structure of different cell types. ExC= exocoelomic cavity, AC= amniotic cavity, fg= foregut, mg= midgut, hg= hindgut, CV= central vein, BD= bile duct, HA= hepatic artery. **The figure is created with Biorender.com. Some parts are adapted from**[76].

Tabel 1. Summary of Cre mouse models for lineage studies of different liver mesenchymal cells.

Mouse model	Marker expression	Cells labeled
MesP1-Cre[57,82]	Mesoderm	STM, MC, subMC, PMCs, HSCs
Wt1-CreERT2[57]	STM, Mesothelium lining the coelomic cavity	STM, MC, subMC, PMCs, HSCs
WT1-Cre[57,102]	STM Mesothelium lining the coelomic cavity	STM, MC, subMC, PMCs, HSCs Endothelium in hepatic sinusoids
G2-Gata4-Cre[58]	Epicardium Mesothelium	Epicardium and broad mesenchymal cells in liver, esophagus, gonad, lung
MSLN-Cre[59]	Mesothelium	Fibroblast and smooth muscle cells of trunk
αSMA-CreERT2[94]	Smooth muscle cells	aHSCs/ Myofibroblast
Pdgfrβ-Cre[103,104]	HSCs	HSCs, VSMCs
Vimentin-CreER[105]	Mesenchymal	Minimal HSCs. aHSCs/ Myofibroblast
Tg(hand2: EGFP)	NC & LPM	Intestinal smooth muscle cells,[106] HSCs[93]
Col1a1-Cre & *Col1a2-Cre*[107]	aHSCs/ Myofibroblast	aHSCs/ Myofibroblast
LRAT-Cre[93]	HSCs	Majority: HSCs
Reelin-CreERT2[95]	Brain	Subset of HSCs
Wnt1-Cre[96,108,109]	Neural cells	**Yes**: CNS, NC decendents[108], Peripheral glia[96] SOX10+ cells and P75+ cells in liver[109] **No**: HSCs
Wnt1-Cre *GLAST-CreERT2*[96]	GLAST: radial glia, myelinating Schwann cells, pericytes	Pericytes in bone marrow contribute to endothelial and hepacotye-like cells after liver injury
hGFAP-Cre & *mGFAP-Cre*[110]	Astrocytes Enteric glial cells	**Yes**: Cholangiocytes (Extra- and intra-hepatic bile duct)[110] **No**: HSCs
hGFAP-Cre[111]	Astrocytes Enteric glial cells	HSCs, Cholangiocytes

HSCs: hepatic stellate cells, **aHSCs**: activated HSCs, **LRAT**: Lecithin-retinol acyltransferase, **MC**: mesothelial cells, **PMC**: peri-vascular mesenchymal cells, **STM**: septum transversum mesenchyme, **subMC**: sub-mesothelial cells, **VSMCs**: vascular smooth muscle cells

2.4 Embryonic liver is a hematopoietic site

Hematopoiesis is a highly conserved process during embryogenesis that involves multiple anatomical sites. In mice, hematopoiesis occurs in three distinct waves: the first wave is termed primitive hematopoiesis, and occurs in the blood islands formed in the extraembryonic mesoderm of the yolk sac (YS) as early as E7.0-E7.5. Primitive hematopoiesis produces primitive erythroid cells for oxygenation, embryonic macrophages for tissue remodeling and defense, and primitive megakaryocytes required for vascular maintenance[41,112]. However, lymphoid cells or hematopoietic stem cells are not generated during primitive hematopoiesis[112]. Shortly thereafter, the second wave of definitive (adult-type) hematopoiesis occurs in the YS, characterized by definitive erythromyeloid progenitor cell production (around E8.25)[113]. Definitive hematopoiesis continues with the production of definitive hematopoietic stem cells, definitive hematopoietic progenitor cells and lymphoid progenitors[113]. It remains largely unknown which signals activate definitive hepatopoiesis[112]. In parallel, in another important hematopoietic niche, the placenta, clusters of hematopoietic stem and progenitor cells are present in the vasculature, attached to endothelial cells[114]. Dramatic expansion continues until E12.5-13.5, and declines by E15.5 in the placenta. Prior to the establishment of circulation, para-aortic splanchnopleure (P-Sp), which develops from the intraembryonic lateral plate mesoderm, starts to produce multilineage progenitors at E8. At E9.5, P-Sp gives rise to the aorta-gonad-mesonephros (AGM) region. The third wave is characterized by hematopoietic stem cell production in hemogenic endothelium of the AGM region around E9.5-10.5. At E10.5-11, hematopoietic progenitor cells derived from YS, AGM region and placenta migrate to the FL. After that, the FL becomes the essential transitional site where hematopoietic cells undergo dramatic expansion and proliferation, until they colonize the bone marrow (BM) in late fetal life[115]. However, the FL itself does not directly produce hematopoietic cells. BM maintains hematopoiesis throughout adulthood.

The FL is a critical niche for hematopoietic cell expansion, proliferation and maturation. It consists of multiple cell types which originate from different germ layers, which makes it a unique microenvironment. Hepatic epithelial, mesenchymal and hematopoietic system development have been extensively investigated, but typically independently. The crosstalk between these cell types is beginning to be investigated. Some studies suggest that several cytokines, chemokines and growth factors, such as angiopoietin-3, insulin-like growth factor (IGF), Wnt family growth factors, CSF1, EPO, and CXC chemokine ligand (CXCL2), secreted by different cell types, such as stromal cells, endothelial cells, mesenchymal progenitors and hepatoblasts, are essential for hematopoietic stem cell colonization, retention, proliferation and survival[116]. Angiopoietin-like factors, β-integrin, and SOX17 are also essential for HSC expansion in FL[117]. However, the mechanisms by which the FL maintains its self-renewal capacity despite exposure to high levels of cytokines, which can induce HSC differentiation, are still not fully understood.

2.5 Liver fibrosis and HSCs

Liver fibrosis is a dynamic process marked by accumulation of ECM due to chronic liver injury, caused by various liver diseases, toxins, infections, or metabolic diseases. HSCs maintain a non-proliferative and quiescent state in normal livers. When liver is injured, HSCs are activated and transdifferentiate into myofibroblasts which play crucial roles in the fibrotic process[78–80].

Fate mapping studies have revealed that in experimental liver fibrosis models, more than majority of collagen-producing cells are composed of activated HSCs and activated portal fibroblasts. This observation strongly indicates that these cells serve as the primary contributors to myofibroblast populations[78,79].

Previously, liver fibrosis was believed to not be reversible. However, sequential liver biopsies have shown that early liver fibrosis is reversible by removing the underlying etiological agent[118–121].

Over the recent years, numerous essential strategies have arisen to prevent and slow down the onset and progression of liver fibrosis. These strategies include liver protective approaches, anti-inflammatory agents, restraining the activation and proliferation of HSCs, reducing the excessive production of ECM, and promoting the degradation of ECM. Furthermore, gene therapy has shown promise as a potential treatment of fibrosis[122–124]. However, most anti-fibrosis drugs are still in the preclinical stage, and there is currently a lack of effective treatments for liver fibrosis[122–124].

3 Lineage tracing

Cells serve as the fundamental units of all living organisms. One of the core and challenging topics in developmental biology is to decipher the origin of cells and lineage trace cells from the progenitor stage to its progeny[125,126]. The golden standard for deciphering the relationship between progenitors and progeny is lineage tracing, a method to identify all descendants originating from a single progenitor cell[125,126]. Better understanding of early embryo development and the underlying mechanism is of remarkable importance for developmental biology, as well as for regenerative medicine and treatment for related diseases.

The history of lineage tracing can be traced back to the developmental biology studies of invertebrate embryos under light microscope in the 19th-century[127]. Lineage tracing offers a potent approach to unravel the intricacies of tissue development and homeostasis. It can be broadly classified into prospective tracing, which involves labeling progenitor cells and tracking their offspring with the same label and retrospective tracing, which relies on inherited genetic markers in the descendant cells after multiple divisions, allowing the inference of their lineage connections based on shared markers.

By combining lineage tracing and gene manipulation, we can pinpoint the essential roles of individual genes in determining cell fates. In recent times, the synergistic use of inducible recombinases, multi-color reporter constructs, and live-cell imaging has provided novel insights and significantly advanced our understanding of developmental biology[125]. Peturb-seq method by combining sc-RNA seq and clustered regularly interspaced short palindromic repeats (CRISPR) based perturbations has been developed to elucidate the functions of multiple genes in parallel[128,129].

In this section, I will discuss the various experimental techniques for lineage tracing, as well as associated advantages and limitations.

3.1 Conventional lineage tracing

3.1.1 Direct observation & Vital dye labeling

As early as the 19th century, lineage tracing was already pioneered by Conklin and colleagues with directly observing the early cleavages in invertebrate embryos with light microscopy[127]. Lineage tracing through direct observation is quick and noninvasive but can be only used for transparent embryos with limited number of cells. Therefore, it is unsuitable for post-implantation mammalian embryo studies unless cells or embryos are cultured, which may not fully recapitulate the *in vivo* behavior.

When direct observation is not possible for non-transparent embryos and *in vivo* development, vital dyes and radioactive tracer can be applied to label cells in embryos without killing them, allowing for following of the fate of their progeny during development[130]. However, these dyes are diluted with each division and potentially leak to neighboring cells, thus perturbing the interpretation[125].

3.1.2 Genetic labeling

Genetic markers include fluorescent proteins and Escherichia coli β-galactosidase, can be introduced by direct injection, transfection, viral transduction and electroporation[131,132]. By labeling the progenitors, a set of labeled clones express the same marker. One can subsequently investigate their locations, marker expressions, as well as the lineage separation during this timeframe[125]. Additionally, a more comprehensive lineage structure can be established by consistently marking the progenitor cells at defined developmental stages[125].

In contrast to vital dyes, genetic markers offer distinct advantages, notably the stable inheritance to the progeny and no leakage to neighboring cells. Nevertheless, one potential challenge associated with genetic labeling is the efficacy of gene introduction[125].

3.1.3 Genetic recombination

Genetic recombination is one of the most widely used lineage tracing approaches in development biology. The Cre-loxP system is commonly employed in mouse models for genetic lineage tracing studies. The system involves the use of two mouse lines: one expressing Cre recombinase under a tissue- or cell-specific promoter, and another containing a reporter gene flanked by a loxP-STOP-loxP ("floxed" loxP) sequence at the Rosa26 locus. When both constructs are present, Cre recombinase can remove the STOP cassette and activate the expression of the reporter gene, permanently labeling cells and their progeny. Reporters such as β-galactosidase[133] and fluorescent reporters[134,135] are widely used in this approach. Cre-loxP has significant advantages, including higher specificity, longevity and tunability of time and location.

To achieve spatial and temporal control of Cre activity, inducible recombination can be achieved by fusing Cre recombinase with human estrogen receptor (ER). In the absence of ligands, the Cre recombinase-ER fusion protein (CreER) is sequestered in the cytoplasm. Upon application of the ligands, such as tamoxifen, or 4-hydroxy-tamoxifen (4-OHT), the CreER translocates to the nucleus, enabling inducible recombination[125].

To prevent the CreER activation by endogenous 17β-oestradiol, two different mutants of CreER, including mouse ER ($CreER^{TAM}$)[136] and human ER ($CreER^{T}$) has been developed [137]. Second generation $CreET^{T2}$ is established[138], which is sensitive to low levels of tamoxifen.

Furthermore, it can be problematic when the reporter is expressed in more than one cell type within the same tissue. In order to overcome this problem, a "split-Cre" system has been developed[139], in which inactive Cre fragments are under the control of distinct promoters, and the recombination occurs only when their expression patterns overlap.

Some *promoter-Cre* mice may experience leakage issues, where the cre activity is observed when it is not expected. This can occur due to genetic editing disturbances or unforeseen expression patterns[140,141].

Multicolor labeling systems such as Brainbow and Confetti have been developed to lineage trace individual recombined cells and their clonal progeny simultaneously using imaging[142,143]. By combining various flox sites and fluorescent reporters, multiple colors can be generated upon activation to distinguish different cells. Another method inducible, fluorescent, and functional genetic mosaic (ifgMosaic) has been developed to enable the investigation of multiple gene combination with a higher cellular and temporal resolution[144]. However, achieving single-cell resolution with multicolor lineage tracing remains challenging, and co-staining with antibodies is also technically difficult[125,126,142,143].

Despite the advancements in lineage tracing techniques, conventional methods still have inevitable limitations, such as a low resolution and limited number of clones to follow[125]. In

addition, the establishment of Cre mouse models can be very time-consuming and labor-intensive, requiring large number of mice. Furthermore, the administration of tamoxifen can be toxic and "off-targeting" poses challenges to data interpretation[125].

3.2 Single-cell lineage tracing and clonal analysis

Compared with the low throughput of conventional lineage tracing approaches, recent advances have led to the development of high throughput methods with single-cell resolution. Single cell lineage tracing provides the possibility to derive cell identities and perform lineage tracing studies[125,126,145].

Single cell lineage tracing can be combined with a barcoding approach[126]. Barcodes are random nucleic acid fragments contain highly variable sequences, providing a new method for labeling individual cells[146]. Genomic integration of barcodes can be achieved with transposons or lentiviruses, and inclusion of the barcode in the 3' untranslated region (3'UTR) after a fluorescent protein reporter serves several purposes. The fluorophore allows for straightforward retrieval of labelled cells using fluorescence-activated cell sorting (FACS)[15,126], and sequencing can detect the fluorophore and barcode in tandem. However, one limitation is that the integration of barcodes into the host genome is random and may not be easily adapted to certain *in vivo* settings. To overcome this challenge, polylox and CRISPR barcodes have been developed[126]. By combining the clonal analysis, single cell or spatial transcriptomic lineage tracing provides the possibility to simultaneously derive cell identities and clonal information[15,125,126,145].

In summary, significant improvements of the techniques, ranging from the microscopic time-lapse imaging to sc-RNA and spatiotemporal transcriptomic analysis, lineage tracing and multiomic studies have significantly enhanced our ability to map the cell lineage atlas and improved our understanding of early mouse embryo development with higher resolution[5,13–15,126].

4 Viral vectors

Viral vectors play a crucial role in genetic manipulation in developmental biology and gene therapy for human diseases[147]. Nowadays, three main vector strategies based on adenoviruses (Ads), adeno-associated virus (AAV) and lentivirus have facilitated significant advances in both preclinical and clinical settings. Nevertheless, despite these achievements, there are still obstacles that hinder their full potential. In this section, I will discuss these three widely used vectors, describing their mechanisms of action and their contributions to gene manipulation studies.

4.1 Adenovirus (Ad)

Adenovirus (Ad) is a non-enveloped virus that has a protein capsid with an icosahedral structure. It contains a linear, double-stranded DNA genome ranging from 26 to 45 kb in

size. Adenoviruses (Ads) enter host cells by interacting with receptors on the cell surface. The efficiency of binding and entry depends on the distribution of these receptors and the affinity of different receptor subgroups[148,149]. The most common cell surface receptor is the Coxsackie adenovirus receptor (CAR)[150], but recent research has identified additional receptors for various serotypes, such as CD46, CD86, proteoglycans, and sialic acid[149]. Upon entering the host cell, the viral protease gradually breaks down the viral capsid by cleaving viral structural proteins[149]. The viral particle is then transported through the nuclear pore into the nucleus. Within the nucleus, viral-encoded proteins interact with the cellular nuclear matrix, facilitating primary transcription initiation. Ads exhibit a high degree of transduction efficiency in both quiescent and dividing cells. They also have a wide range of tissue targets due to their broad tropism.

It is worth noting that, under normal circumstances, the viral DNA does not integrate into the host cell genome, instead, it persists as an episome within the nucleus. The adenovirus genome is transcribed and replicated at discrete replication centers within the nucleus of infected cells.

4.2 Adeno-associated virus (AAV)

Adeno-associated virus (AAV) is a parvovirus requiring co-infection with another helper virus (such as Ads or Herpes simplex virus (HSV)) to facilitate its replication. Unlike adenovirus, AAV lacks the essential genes required for replicating and expressing its own genome. AAV virions are compact, non-enveloped, and have an icosahedral structure with diameters ranging from 18 to 26nm. They encapsulate a single-stranded DNA molecule containing inverted terminal repeats (ITR). The ITRs facilitate site-specific integration of wild-type AAV into chromosome 19 and promote the formation of secondary structures within the viral genome, aiding viral DNA replication with the host cell polymerase[149,151]. AAV consists of different serotypes, including AAV serotype2 (AAV2), AAV1, AAV5, AAV8, AAV9 which have high tropism for the neural system, muscle, retina, liver and heart, respectively[149]. Tissue- or cell- specific promoters are widely used to drive the specific expression of AAVs but the promoters can be leaky.

Furthermore, tissue- or cell-specific targeting can be fine-tuned through pseudotyping, which involves modifying the viral surface or envelope proteins to resemble those of a different viral subtype[152,153]. Directed evolution has been employed to engineer novel AAV capsids for efficient gene delivery. In addition, machine learning has opened up new possibilities for AAV library engineering and precise next-generation gene delivery, offering high potential in this field[154,155].

4.3 Lentivirus

Lentiviral vectors are a subclass of retrovirus family. Retroviruses are approximately 100nm in size and have a spherical shape. They are enveloped, with a single-stranded RNA genome. Lentiviral vectors allow for stable long-term transduction in both dividing and non-dividing cells, by integrating into the host genome. Lentivirus exhibit a broad tropism for diverse cell types. Firstly, lentivirus binds to the membrane receptor, low-density lipoprotein-receptor (LDLR). After binding to the receptor, viruses enter the cell by endocytosis. Subsequently, viral RNA is transcribed by reverse transcriptase, resulting in the production of double-stranded (ds) DNA. Then dsDNA enters the nucleus, and the transgene is integrated into host genome with the assistance of lentiviral integrase enzymes. The integration is not random and shows a preference for transcriptionally active sites[156,157].

Nevertheless, developing an effective and universally applicable system for cell entry remains challenge. The efficiency of lentiviral transduction is limited by many factors, including restriction of the cell entry step[147,158]. Quiescent lymphocytes and NK cells represent limited transduction efficiency due to a lack of LDLR expression[147,158]. Lentiviral vectors have been modified by lentiviral pseudotyping, incorporating the glycoproteins derived from other enveloped viruses, thus to achieve the tropism of the virus the glycoproteins derived from. The most widely used glycoprotein for pseudotyping is the vesicular stomatitis virus glycoprotein (VSV-G) in order to achieve broad tropism and high stability. Pseudotyping improves the transduction efficiency and reduce toxicity.

To achieve cell type-specific expression, tissue- or cell-specific promoters can be employed. For instance, temporal regulation can be achieved using a tetracycline-inducible promoter[159]; while post-transcriptional regulation of transgene expression can be achieved with endogenous miRNAs, thus reducing the immune response[160]. In Paper I, we achieved cell-type specific expression by using cell-type specific MiniPromoters with lentiviral vectors[161].

5 Ultrasound-guided *in utero* nano-injection as a new tool for lineage tracing and gene manipulation

High throughput investigation of how different genes control developmental processes in mammals has previously been challenging, though a wide variety of approaches are available. Conventional gene knockout technology is a powerful technique in which genes are inactivated in all tissues at the same time. However, it also has some limitations: about 15% of gene knockouts are developmentally lethal[162]. It can be very time-consuming to create traditional mouse strains. Conditional mouse models are more advanced and widely used nowadays, involving manipulation of gene expression only in specific tissues

or organs at specific timepoints. As discussed above, Cre-loxP mouse models are widely used but also time-consuming, labor intensive and can have "off-target" issues. As an alternative often employed in neuroscience, electroporation is widely used to facilitate the transfer of different molecules across the membrane to cellular cytoplasm[163]. Electroporation achieves efficient delivery of a wide range of substances in different tissues. It can also be applied to all cell types at all cell cycle stages. However, this method has several disadvantages: it sometimes leads to cell damage, permanent membrane permeabilization and nonspecific transportation. The delivery efficiency is limited by the electrical properties of different cell types. Our lab is developing ultrasound-guided *in utero* nano-injection to target different organ systems, with the ultimate aim of investigating organogenesis and and the embryonic origin of HSCs, as well as the clonal relations of different mesenchymal cells.

Elaine Fuchs's laboratory established ultrasound-guided *in utero* nano-injection to manipulate gene expression in the skin by injecting embryos with lentiviruses carrying RNA interference (RNAi) or Cre recombinase at E9.5[164,165]. Targeting of embryonic rat lung interstitium with Ads and lentiviral vectors using ultrasound biomicroscopy has also been reported [166]. Our lab has adapted ultrasound-guided *in utero* nano-injection to target the nervous system, a technique we called "NEPTUNE". Rapid and flexible transduction of the neural plate and future adult nervous system was achieved by injecting the amniotic cavity (in contact with ectoderm) with gene expression-modifying viruses prior to neurulation. Conditional transduction was achieved with virus encoding tissue-specific Mini-Promoters driving expression of the transgene of interest. We can recapitulate existing mouse knockouts (the Olig2 knockdown phenotype) and investigate gene function (Sptbn2 shRNA). **(Paper I & II)**. Based on our knowledge of mesoderm development, we hypothesized that injection into the exocoelomic cavity would exclusively label mesoderm-derived cells, allowing us to lineage trace their progeny and resolve the clonal relations of liver mesenchymal cells **(Paper III).**

Ultrasound-guided *in utero* nano-injection will open the door to enable gene manipulation and investigation of gene function in a faster and more flexible way. This approach requires fewer mice and allows for faster data acquisition. It emerged as a feasible, cost-effective and powerful approach to investigate organogenesis and development of liver mesenchymal cells.

6 TREX pipeline

In Paper III, we used a published custom-written computational TREX pipeline to analyze the barcode reads to infer the clonal information[15] (pipeline available at https://github.com/frisen-lab/TREX with several further ongoing optimizations by our

group). The TREX pipeline can be used to extract genetic barcodes/ CloneIDs from single-cell or spatial transcriptomes and to reconstruct related cells or spots.

Before TREX analysis, a custom reference must be generated by adding the custom library marker genes to the reference mouse genome (cellranger mkref pipeline). After establishing the reference genome, the TREX workflow starts with the detection of the CloneID region in the reference by specifying the starting and ending site of the extra "chromosome" in which the CloneID is located. In this extraction step, all reads must contain a cellular identifier (cellID) and a unique molecular identifier (UMI). Additionally, the cellID of retrieved reads must be included in the cellID list generated from the "clean" single cell object, the output of Seurat and DoubletFinder packages.

After the retrieval of usable **reads**: The next step is to group reads to **molecules** based on the cellID and UMI identifier and most sequencing errors can be removed in this step. Subsequently, molecules with common cellID were compared with each other and group into **cells** if the positional differences of CloneIDs are minor. The metric for measuring the differences is Hamming distance, and is defined simply as the number of positions that differ between two sequences. The Hamming distance was calculated for 1000 randomly selected barcodes. In our barcode library, the random nucleotides are 30bp and the average Hamming distance between different sequences is 22, with value >=15 representing a highly diverse of the barcode library (Supp Fig2 in Paper III).

To increase the accuracy of the CloneID retrieval and clone assignment, rare sequences (single read barcodes) are removed as they are likely contaminations from other cells. Finally, cells are grouped into **clones** with the "clone calling" module. The CloneID overlap between cells was calculated with the Jaccard index, an index measuring the similarity of two sets of CloneIDs. Jaccard Index is calculated as the number of shared CloneIDs between Cell A and Cell B, divided by the total numbers of CloneIDs in Cell A and Cell B. Jaccard index was calculated for all pairs of cells and a threshold is set as 0.7, meaning two cells are merged into one clone if they have more than 70% of CloneIDs in common. All pairs or groups of cells above the threshold are considered clones.

Ultimately, the clonal output from TREX pipeline can be integrated with the sc-RNA analysis to simultaneously perform the transcriptomic and clonal analysis.

7 Concluding remarks

Early mouse development is a precisely controlled process orchestrated by key factors and signaling pathways. Hepatic stellate cells (HSCs) express diverse cell markers from different germ layers, therefore making its origin long-debated. Significant methodological improvements, ranging from microscopic time-lapse imaging to sc-RNA sequencing, spatiotemporal transcriptomic analysis, and lineage tracing, have played pivotal roles in enhancing the mapping of the cell lineage atlas and has improved our understanding of early mouse embryogenesis with higher resolution. By combining ultrasound-guided *in utero* nano-injection, with single cell barcode lineage tracing, we achieved a powerful tool to decipher mouse embryo development and lineage trace liver mesenchymal cells.

8 Research aims

The overall aims of my PhD project are to establish a new flexible and efficient tool to elucidate early organogenesis, including ectodermal and mesodermal contribution to the liver in mouse.

- To develop an alternative method to manipulate gene expression in the mouse nervous system. (NEPTUNE, **Paper I & II**)
- To trace the diverse derivatives of neural crest with NEPTUNE and investigate the potential contribution to liver mesenchymal cells (**Paper III**)
- To flexibly and efficiently trace the mesoderm derivatives (**Paper III**)
- To lineage trace the hepatic stellate cells (HSCs) and other mesenchymal cells in mouse liver (**Paper III**)
- To resolve the clonal relations between HSCs and other mesenchymal cells in moue liver (**Paper III**)

9 Ethical consideration

All experiments were performed according to the ethical approval granted by the Swedish Board of Agriculture (Jordbruksverket, N59/14, 8188-2017 and 2987-2020).

The purpose of my Ph.D. study is to investigate the organogenesis during early mouse embryo development, as well as the embryonic origin of hepatic stellate cells and other mesenchymal cells during liver. The technique we are using/optimizing is ultrasound-guided *in utero* nano-injection. Science workhorse, mice are used for our research.

The principles of scientific ethics demand honesty and integrity at every stage of scientific practice, spanning from data collection through publication and beyond. For personal ethics, morality is the first place. For professional ethics, standards and expectations are needed. For societal ethics, law is used for regularizing behaviors. Methodological standards were employed throughout the entire scientific process, encompassing the design, experimental procedures, data analysis, data interpretation and data publication. For my research, the whole project plan should be based on honesty. All the experiments design and process should have an ethical permit and follow the Swedish law.

Another crucial topic in scientific ethics is the difference between mistake and misconduct. Everyone makes mistakes. Actually, the line between mistakes and misconduct is not very clear. Serious career pressure potentially motivates desperate scientists to fabricate results. There are three categories of research misconduct: fabrication, falsification, and plagiarism. My Ph.D. project is based on a new and promising technique, ultrasound-guided *in utero* nano-injection. During my doctoral studies, we developed two new approaches to target embryonic tissues during development. Ultrasound-guided in utero nano-injection is a flexible and efficient tool to elucidate clonal relations among tissues in early mouse embryo development, as well as for gene manipulation. It's difficult to develop and optimize a new technique. All the injection stage, injection volumes and virus titers should be optimized. All these tests and optimization should base on the real data from each embryo and repeat multiple times to assess reproducibility and have robust numbers for statistical testing.

Authorship is becoming more and more complicated problems in scientific work since the cooperation is becoming broader and more popular. Lots of journals are adopting DORA (Declaration of Research Assessment) and implementing rules related to it: the authors should be significantly involved in the study design, data collection, analysis and interpretation. They should contribute to the manuscript drafting and revising. The author orders represent the magnitude of contributions. Normally, the first author contributes more to the experiment and analysis and the last author is the most senior one who plays

the predominantly supervisory role. The Committee on Publication Ethics recommends that that researchers should have discussions about authorship order from the inception of a project to the drafting of the manuscript, its submission, revisions, and record each decision in written form. For my Ph.D. projects, Paper I and II are collaborative ones, I contributed mostly to some experimental work, scientific discussion and revision work. Paper III is my main project, and I will be the first author. For papers not included in this thesis, Emma P. and Jingyan He et al is also one of my main projects, which I have been working since the beginning of my PhD. I have optimized the targeting of three waves of hematopoiesis and paving the way for this project. I was also doing the embryo injections, sample collections etc. Emma P and I will be co-first authors.

In scientific research, animal model, or animal experiments are very important. Regarding the *in vitro* and *in vivo* environment are quite different, normally the conclusions from the in vitro cell experiments should be tested and verified in animals (*in vivo*). Research involving animals is for human benefit, either for medical reasons or for improving understanding of bodily systems. In conclusion, ethical issues are closely related to animals. In Swedish legislation, laboratory animals are animals that are used in, or bred for, animal experiments. Embryos from the last third of their development are included. Therefore, for my research project, the mum and embryos are all laboratory animals.

For researchers who use animals should be consider the welfare of animals and familiar with the law (Sweden's current Animal Welfare Act includes legislation for using animals in research). Ethics in laboratory animal research can be shortly described as the study of theories of human responsibilities on animals used in research. The starting ethical problem we should consider is "doing good" (beneficence), at least, "not doing bad" (non-maleficence).

From the beginning of the research, the responsibility should be considered. Each action should be aiming at maximizing good outcome, at least minimizing the bad consequences. In scientific research, animal welfare should be implemented as a scientific concept. Another important principle one should consider is "3R's", which are replacement, reduction and refinement. Replacement refers to methods that include no experimental animals, which have substituted any method that previously included experimental animals. Reduction means that the number of experimental animals used should be reduced to the absolute minimum to obtain satisfactory results. Refinement means that the experimental animals should suffer as little distress as possible.

My Ph.D. project is closely related with mice models. One of the advantages of our ultrasound-guided *in utero* nano-injection is fewer mice are needed, which means we follow the reduction principle very well. After injection, the mice are closely observed and checked every day. If the mice were found to be suffering, they would be sacrificed out of humanity. This means we also follow the "not doing bad" principle.

10 Results and Discussion

10.1 Paper I & II

In these two studies, we developed NEPTUNE (NEural Plate Targeting by *in Utero* NanoinjEction): a rapid, adaptable and cost-effective technique to target the neural plate with virus before neurulation, and thus manipulate gene expression during neural development and label the future brain, spinal cord (**Paper I**) and peripheral nervous system (PNS), neural retina (NR), retinal pigment epithelium (RPE) (**Paper II**). Additionally, surface ectoderm can be labeled as well, and thus manipulate gene expression in future lens, corneal epithelium and salivary glands (**Paper II**).

In order to achieve maximum survival and transduction efficiency, we needed to optimize different parameters. First, we tested different timepoints and observed that E8.25-E8.5 injection results in high transduction of forebrain and low transduction in hindbrain, which might be due to hindbrain/ cervical boundary closure occurring first, while the forebrain/ midbrain closes second, and the rostral end of forebrain closes last. We identified ~E7.5 as the optimal stage prior to the neural tube closure for targeting the neural tube exposed to the amniotic fluid. This stage was accessible to injected virus, allowing the labeling of the future nervous system. Furthermore, we also observed that the Theiler stage (TS) 11b is the optimal injection stage, to target the neural tube before closure.

Next, we tested a range of different volumes and titers for lentivirus. By comparing the relative increase in amniotic volume after injections, we observed that a volume increase of >90% resulted in embryo resorption. Injections of different titers of lentivirus showed a dose-dependent transduction efficiency. In conclusion, optimal volume 207nL and optimal titer 2 x 10^{10} IFU/mL resulted with reproducible maximum transduction ~95%. By using IF staining, we observed even GFP distributions across CNS regions and different cell types, including in SOX2+ neural progenitors, and NeuN+ mature neurons.

After achieving stable integration in more than 95% of cells and long-term expression in the brain, we developed conditional expression by utilizing cell-type-specific MiniPromoters. We cloned MiniPromoter sequences driving expression in neuronal progenitors (Doublecortin, DCX miniP), astrocytes (Glial Fibrillary Acidic Protein, GFAP miniP), and oligodendrocytes (Oligodendrocyte Transcription Factor 1, OLIG1 miniP). These Minipromoters were then used to replace the hPGK promoter in *hPGK-H2B-GFP*, resulting in the generation of *DCX-H2B-GFP*, *GFAP-H2B-GFP*, and *OLIG1-H2B-GFP* lentivirus.

Ultimately, we recapitulated the phenotype of *Olig2-/-* embryos by using NEPTUNE to knockdown the expression of *Olig2* using shRNA. We also revealed the neurodevelopmental defects by knocking down the expression of Spectrin Beta, Non-

Erythrocytic 2 (*Sptbn2*) with *Sptbn2* shRNA lentivirus, using NEPTUNE. This resulted in a strong downregulation of *Sptbn2* mRNA and dose-dependent defects in the neural tube, embryonic turning, and abdominal wall closure. The knockdown of *Sptbn2* with NEPTUNE is quite variable. One potential reason for this variability could be the variable efficiency of lentiviral integration. In addition, *Sptbn2* has essential roles in multiple cell types and the early embryo is highly dynamic and potentially different germ layer compartments are accessible to the virus when injecting different embryos. This may contribute to the observed differences in efficiency.

One of the key advantages of NEPTUNE is achieving cell-type-specific labeling without the need of dedicated Cre mice. This not only simplifies experimental procedures but also significantly reduces the number of mice required for studies, thereby promoting ethical and more efficient research practices.

There are some caveats to consider when using NEPTUNE with shRNA knockdown, including the risk of variable knockdown and potential off-target effects due to the variable lentivirus integration. Another limitation to consider is the packaging capacity of lentiviral vectors. With the high-titer lentivirus production technique, we were able to achieve a titer up to $2x10^{10}$ IFU/mL with *hPGF-H2B-GFP* plasmid but a 50% reduction in virus titer when using MiniPromoters due to the limitation of lentiviral packaging capacity.

10.2 Paper III

The first aim of this study was to develop a method to target mesoderm, and then to apply this technology to investigate liver mesenchymal cells. We hypothesized that exclusive labeling of the mesoderm and its progeny could be achieved by injecting into the exocoelomic cavity (ExC) during gastrulation after the segregation of three germ layers and full establishment of two cavities (AC and ExC), since mesoderm is in contact with the ExC. In parallel, we wanted to map the progeny of NEPTUNE-targeted cells to understand better what cells are accessible at E7.5. Therefore, we further adapted ultrasound-guided *in utero* nano-injection by injecting a diverse lentivirus barcode library[15] into the AC and ExC at embryonic day (E)7.5 to lineage trace the progeny. Our results showed that we were able to precisely label and track the progeny of ectodermal and mesodermal compartments. This allowed us to map the lineage trajectories and clonal relationships within developing organs during organogenesis as well as in embryonic liver.

Firstly, to address the contribution of ectodermal and mesodermal cells to organogenesis, we first collected embryos at early developmental stages E9.5 and E10.5, after AC or ExC injections at E7.5. By combining immunofluorescent (IF) staining and sc-RNA sequencing, we observed clear different targeting patterns between the two injection approaches. Neural derivatives are extensively targeted by AC injection and mesoderm-derived cells were predominantly labeled by ExC injection. These results indicated that amniotic

injection at E7.5 targeted the open neural tube, which subsequently proliferates and undergoes neurogenesis. Conversely, exocoelomic injection at E7.5 exclusively labeled the mesoderm.

Interestingly, we observed that both mesodermal and endodermal cells were labeled by AC injection, which suggested that pluripotent epiblast cells were accessible to the virus prior to their ingression through the PS and undergoing EMT. The conventional method to stage the mouse embryos during development includes the Theiler stage criteria described in 1989[167], and supplemented by cell number, somite number etc[168]. Theiler Stages (TS)9- TS10 are defined as primitive streak stage and the neural plate stage started at TS11a, initiated by the development of the head process. We meticulously chose embryos at appropriate developmental stages, considering their morphology and the optimal size of cavities for injections. Specifically, we selected embryos around TS11a-TS11c for our injections, when three germ layers and two cavities are fully established. Strikingly, we observed that pluripotent epiblast cells could still be labeled by AC injection at TS11a, the neural plate stage, to TS11c, the early head fold (EHF) stage.

Next, to address the targeting patterns in E16.5 livers with different injection approaches, we repeated the injections described above and determined that liver mesothelial and mesenchymal cells are highly enriched with ExC injection.

Regarding the clonal analysis, we observed that, two days after injections at E9.5, AC-injected embryos have a slightly larger clone size than ExC-injected embryos, but three days after injections at E10.5, AC-injected embryos have much larger clone sizes than ExC embryos, which might relate to the proliferation rate of different progenitors during early embryo development.

Additionally, we observed variation in the number of barcodes per cell, which may be attributed to the exposure time of progenitor cells to amniotic or exocoelomic liquid, in which the virus is present. Generally, AC-labeled cells tended to have more barcodes per cell compared to ExC-labeled cells, suggesting that pluripotent epiblast cells on the surface have a longer contact time with the amniotic fluid before their ingression, while surface mesoderm cells in contact with the coelomic/ exocoelomic fluid may spend a relatively shorter time in contact with the fluid.

Our findings are in line with previous findings using lineage tracing studies using Cre mouse models, such as *Mesp1-cre*, *Wt1-cre*, and *Gata4-cre*, suggesting the predominant contribution of the mesoderm to the septum transversum mesenchyme (STM) and intrahepatic mesenchymal cells[57,82].

In addition, it has been proposed that HSCs may have an ectodermal/neural crest (NC) origin, as they express neural markers such as *GFAP* and *p75NTR*[169,170]. However, Cre mice

studies yielded both positive and negative results. *Wnt1-Cre*, *hGFAP-Cre*, and *mGFAP-Cre* were reported to not successfully label HSCs [96,108,109], but another study demonstrated HSC labeling with *hGFAP-Cre*[111]. In our study, we observed a clonal correlation between NC and mesothelium/ STM populations at E9.5 and E10.5, supporting the possibility of a shared origin between STM and some mesothelial cells.

Together, our clonal analysis of E9.5 and E10.5 embryos injected with AC and ExC at E7.5 suggested a heterogeneous contribution to the transient STM during development.

11 Conclusions and future perspectives

11.1 Paper I & II

In conclusion, NEPTUNE is a powerful and versatile technique for modulating gene expression during embryonic development. It offers the possibility to achieve widespread, stable, and conditional expression in the brain and spinal cord, providing insights into the roles of genes in crucial nervous system development.

Moreover, NEPTUNE can be further enhanced by combining with other genetic tools such as shRNA, Cre, or Cas9 mice. This integration allows for improved conditionality and the ability to modify specific genes, thereby enabling researchers to unravel the complex mechanisms underlying nervous system development and diseases.

Furthermore, NEPTUNE provides an opportunity for lineage tracing studies in the nervous system. By injecting a barcode lentivirus library with NEPTUNE, lineage studies can be performed in embryonic or adult brain, spinal cord or peripheral nervous system (PNS).

shRNA knockdown strategies in combination with NEPTUNE can be somewhat variable. It is potentially resulted by the variable efficiency of lentiviral integration or different germ layer compartments labeled in individual embryos due to the high dynamics in early mouse embryo. On the other hand, strong knockdown of *Sptbn2* is normally lethal and embryos are died at early stages. Therefore, the variable knockdown of shRNA with NEPTUNE can be used as an advantage for future work to decipher gene roles. We can compare the traditional shRNA knockdown and CRISPR editing of corresponding genes with NEPTUNE-mediated CRISPR gene editing and shRNA knockdown, to compare phenotypes upon knockdown and genetic perturbation.

In summary, NEPTUNE represents a cutting-edge approach that empowers researchers to manipulate gene expression with precision and specificity during embryonic development. The application in combination with other genetic tools holds immense promise for uncovering the mechanisms as well as reconstructing the lineage trees during nervous system development.

11.2 Paper III

In summary, the findings from this study provided additional support for the mesodermal origin of hepatic stellate cells (HSCs) and other mesenchymal cells in the liver. Our results also indicate a contribution of the neural crest (NC) to septum transversum mesenchyme (STM), which is believed to be a transient tissue contributing to mesenchymal cells of multiple internal organs. This finding suggests a potential dual origin for HSCs and other mesenchymal cells in the liver. However, it is important to note that due to a lack of direct

continuous lineage information from NC to STM and liver mesenchymal cells in the current study, the continuous lineage trajectories cannot be fully studied. To address this gap in knowledge, future investigations could focus on stages between E10.5 and E16.5, encompassing tissues composed of both liver mesenchymal cells and NC populations.

To further validate our findings, orthogonal validation using conventional lineage tracing methods, such as Cre mouse models, based on markers identified in our datasets can be used to confirm the mesodermal and neural crest contributions to liver mesenchymal cells.

Paper III is still an ongoing project. We are aiming to optimize the TREX pipeline to improve the retrieval of CloneID information and enhance clone identification. To avoid the potential misreading and misalignment, we are implementing a per cell correction module that will replace the previous method of correcting partial CloneIDs to Clone IDs found throughout the dataset. This new approach will help to prevent accidental bridging and improve the accuracy of clone identification.

Additionally, we are cross-checking all datasets generated from the same barcode library and implementing an extra filtering process to exclude the overrepresented and less complex barcodes. This step is crucial in ensuring the quality and reliability of the clonal information obtained from the TREX pipeline. In the end, by removing potential doublets, "single cells" expressing CloneIDs specific to two distinct clones, we can minimize the error bridging of two likely separate clones and improve the accuracy of the clone retrieval and lineage tree construction.

With these updates and improvements of TREX pipeline, we anticipate extracting more reliable clonal information and constructing a more precise lineage tree for organogenesis and liver mesenchymal cells. This will provide valuable insights into developmental biology and contribute to the potential new therapeutic strategies for developmental disorders and liver fibrosis diseases.

12 Acknowledgements

Time files. My Ph.D. journey is now coming to an end, even though it takes much longer than I expected. It is the time to thank all people who have accompany and support me during the long journey. I could not have done this without you. I am truly grateful to all the colleagues, friends and family who are always there to love me, support me and believe in me.

First and foremost, I would like to thank all my supervisors. My main supervisor, **Emma**. Thanks so much for giving me the opportunity to start my PhD study in your lab. You are always so kind and supportive and give me the opportunity/ freedom to explore different possibilities and directions. Thanks for all the encouragement and compliment which indeed helped me to be more and more confident. Your unwavering diligence and boundless enthusiasm consistently motivate me through my PhD journey. You guided me towards becoming a progressively more independent scientist. Furthermore, I highly appreciate your help and guidance with revising my writings. Each time I have learned a lot and greatly benefited from your expertise. My co-supervisors, **Katja**, thank you so much for always encouraging me on miR meetings. Your endless passion on work also motivated me. All the best wishes to your new career journey in Uppsala. **Niklas**, thanks for your guidance and support on the hematopoiesis project. I really love this project, looking forward to working more on this project after my defense. **Martin**, thank you for your guidance and support and prompt response during the preparation of my defense.

To my mentor, **Xiaojun**, thank you for your unwavering support and keep encouraging me since the beginning when I was not confident and didn't see the future of my PhD journey. Thank you for caring for me when I was not doing well.

Also, hug thanks go to all former and current members in **ERA lab**. **Simo**, you are the first person who communicated with me through email to help me smoothly transit to Sweden and picked me up on my first day at KI. Thanks for always being so nice to me/everyone. Thanks for always being so supportive and helpful. I was inspired and motivated a lot by your scientific passion with your "wondering" moments during all scientific sessions. **Bettina**, thanks for being so patiently and carefully teaching me all the animal techniques and always taking care of our mice and virus room. You always have so many interesting stories to share. I laughed so much by your funny stories and small talks. **Jia**, thanks for not only being a great colleague but also the best friend, thank you for always listening to me no matter how many times I have being complaining about the same things. I cannot survive my PhD without you. Thank you for all your company, your understanding, your encouragement. **Katrin**, thank you for showing me a lot of techniques and inspired me a lot with your many years' hardworking on NEPTUNE. **Janek**, thank you for your unwavering support and encouragement. Thanks a lot for including me in your project and gave me

so many invaluable guidance and suggestions. Thank you for always being so helpful and cheerful with your unlimited energy and jokes. **Noemi**, you are always so thoughtful, so heartwarming and care all the people around you. Thank you so much for your kindness and help both on work and in personal life. I enjoyed so much talking to you. You always have the magic techniques to make every small thing in your life so interesting and super fun. I never feel bored listening/talking to you.

To current PhD buddies, **Afshan**, thanks for your kindness and the delicious food and snacks. It is your turn soon. All the best wishes and best luck to you. **David**, my desk mate, you don' talk much but we do laugh a lot with your sudden jokes. You are so supportive and always joins all the events in the lab. You are always there. **Sandra**, thanks for working together on the injection and TREX papers. We are an excellent team when we do the library experiment together. I still remember very clearly how stressful it was when we did library prep independently the very first time. Thanks for being a good colleague and a good friend. I really like talking to you. I will miss all our talks, gossips and of course our teamwork in the lab. Lastly, all the best wishes to you three, you are all almost there.

To all postdocs in ERA lab. **Paulina**, thank you for taking over the liver miRNA project from my shoulder. Thank you for your kindness and support. I really enjoyed a lot when we share the cats' stories. Cats are the best!!! **Eli**, thanks for always being so kind, cheerful, and energetic. I like your sweet smiles so much. You'd never imagine that such a sweet girl would rock the rugby match in Sweden. Keep smiling, champ! **Lenka**, thanks so much for all your help on the projects and all the helpful scientific discussions. **Agus**, thank you so much for all your help and support on the bioinformatics part.

To my former colleagues, **Ile**, **Ezel**, thanks for helping me so much on the miRNA projects. Thank you for your consistent kindness and support.

Additionally, to my first and only student, **Anne-lin**, such a sweet girl, I was so happy and proud seeing you growing from a baby bachelor student to an independent junior researcher. I am glad that you found a job that you genuinely enjoy, especially one that contributes to helping patients. Beyond the work, I am also deeply touched by your warmth and thoughtfulness. Thank you for your unwavering support and belief. Good luck with our new journey.

Lastly, I would like to specially express my sincere gratitude to **all ERA members** for the presentation practices. This is the part/ tradition I like most. All the suggestions and input from all members were super helpful for me to become better and better to present my data/ project.

To all the collaborators, I have learnt so much from all of you. **Michael,** thank you for kindly sharing your experience, knowledge, and the reagents. All the best wishes to your next journey as a PI. **Leonie**, thanks for all the discussions and collaborative efforts. Despite not

formally establishing the project, we both gained a lot of valuable insights. Beyond the work, also big thanks for being so thoughtful and heartwarming in daily life. **Alek**, thanks for bringing so much fun. I enjoyed a lot when listening to your fun stories. Also, super grateful that you showed me how to do the library prep when I initially learning. **Iva**, thank you so much for working together at the initial stage of the hematopoietic project. A huge thanks for sharing me your expertise on flow cytometry when I knew so little about it. You have been the motivation for my efforts to learn how to run multi-color FACS. **Emma P**, I appreciated a lot the opportunity to work together with you on the hematopoiesis project. Thanks for all your support and help. **Andrea**, thank you for your guidance and support on the collaborative project. **Stefaan**, I am incredibly grateful for your participation in my project and for the invaluable help, guidance, and input you have provided. I was so impressed by your bioinformatics techniques and your boundless enthusiasm in exploring new and interesting things in this beautiful world.

To all the colleagues at KI, **Hongyan**, **Shaobo**, **Anna, Sarah, Lara**, **Joanna**, **David G**, **Frank**, thanks for all your help and support. Thanks for being part of my good memories of KI.

To all my friends at KI. My thanks start with **Yanan** and **Hui**, the Zhengzhou University "Three Musketeers". **Yanan**, you know you are the motivation behind my pursuit of a PhD position at KI? You came first and encouraged me to apply for PhD positions during my last year of my master's studies and gave me a lot of good suggestions. We three have so much in common in our life journeys. It feels so great that we can both talk about life here in Sweden and classmates, colleagues from the past. We were having so much fun chatting/ gossiping together in our local dialect. I am so grateful having you two here during my PhD. Next, special thanks to **Taotao** for showing me how to take the public transport to work on my very first day. You even do not remember that you helped a new girl on her first day. But see? the destiny makes us best friends after six years. Thank you, **Alex**, for taking care of Taotao and being a good and warm friend. I am so happy for you **Can** for finding your Mr. Right, **Niclas (Xiaoni)**, in Sweden and settle down here after your PhD. As I said, I am always impressed by your perseverance and hard working. All the best wishes to your Swedish medical license journey. **Taotao** and **Can**, I love our trips all around Europe so much. I know, I know, I still owe you two a tour as the tour guide. Let's plan a new trip next year. Then, to our hotpot guys, **Lipeng**, **Yanhua**, **Zeyu** and **Danyang**, thank you all for being such good friends and all your company. We had so much fun and nice food together to celebrate special moments and the festivals. **Shanshan** and **Rui**, thank you **Shanshan** for organizing a lot of activities and invited me when I first came without knowing anyone. Thank you for always supporting me and all the warmhearted conversations. I am genuinely happy for you two. Over the years, witnessing you two from knowing each other, being together, navigating through ups and downs, and finally coming to a happy ending. Thank you for choosing me as the witness of your wedding. I feel so

honored. Thank you for your consistent invitation to your home and warm hospitality, especially the delightful food. **Shan** and **Bowen**, thank you for your company and unwavering support. I really have the most fun and relaxing time in our Wechat group. Thank you for all whimsical and fantastical, intriguing conversations. Thank you for being open-minded and non-judgmental. It feels great that we can all be "the true selves". **Jielu**, **Yuanzhe** and **Yiyi**, thank you for inviting me to your home and taking me to pick up mushrooms. I enjoyed so much when we were making nice food together. You showed me the best example of a happy family who consistently support one another. **Yanan** and **Shuangyang**, I'm delighted to have witnessed your marriage and the joy of becoming parents. Thank you for consistently inviting me to your home and treating me with nice food. We also had so much fun on our movie nights. Looking forward to your wedding ceremony in China. **Lin** and **Jiangwei**, thank you for inviting me to your home and teaching me how to play "Legend of Three Kingdoms". A heartfelt thanks to **Aunt** for preparing such delicious food, which brought the flavors of our hometown to life for me. I am so happy for the expansion of your family. It is heartening to see **Qiqi** growing into a big boy. He's sure to be a wonderful big brother. **Ruining** and **Hanxiong**, thank you so much for all the fun fika time. Thank you for inspiring me with your smile and "happy life" and easygoing attitude, which really helped me becoming more and more relaxed and casual with daily life and find life more relaxing and enjoyable. **Fan** and **Fan**, thank you for inviting me to your Kitty house and enjoy the happiness surrounded by a full house of cats. Thank you for always treating me with delicious food and sharing so many fun stories. **Lei Li**, thank you for inviting me to your dissertation party, which was the very first one in my PhD journey. I am so glad for you that you settled down in Sweden and are living a cozy, happy life with **Xiaofan**. **Yuanyuan**, the best baker, the pastry master, thank you for your unlimited delicious cakes and pastries. Thank you for always so thoughtful and heartwarming. **Yiran**, thank you for your company and encouragement. We share the happiness and worries. We share the best and the worst. After so many years' hard working, we finally come to the end of our PhD journeys. Hope both of us will find a dream job and live a happy life. **Feifei** and **Long**, thank you for inviting me to your house and treat me with the best BBQ. You two showed me the best example of a couple who accompany and support one another. **Ting Huang**, thank you for not only being a great colleague but also a good friend. Don't forget to take good care of yourself while working hard on your PhD. **Qing Liu**, I am so happy for you that you are finally standing at the end point. You already went through the darkest period. Your life will only get better from here. **Ziwei**, I am very glad that we are working on the collaborative project together. Thanks so much for lending me the reagents and resolving the emergency. Thank you for asking me out to explore different brunch, bakery stores in Stockholm. **Chenhong**, thanks for inviting me to your fancy apartment and showed me all the fancy equipment. It was very fun. Thank you for treating me with nice food and delicious ice cream and the enjoyable conversations. **KK** and **Tong**, I am very happy for you two on the best way to the brightest future. **KK**, I

am so happy for you that you found the perfect person and place to settle down after your diverse experiences in different countries over the years. I sincerely hope your dream comes true, and I am ready for your good news.

Thank you to all the friends I have met at KI, **Chenggui, Keyi, Chenfei & Chikai, Xueqi, Yue Li, Mingzhi, Yangjun, Yaxuan, Jiarui, Junjie, Mu Nie, Shengduo, Dandan, Yawen, Yunbing, Le Tong, Dongmei, Ting Wang, Yang Wang, Dang Wei, Yujiao, Xu Wen, Huazhen & Meng Yu, Xiangling, Rong Yu, Le Zhang, Yujie, Leilei, Baoyi & Yujie, Zhengbing,** without your support, I cannot stand where I am now and achieve what I have done.

To my **family**- My parents, grandparents, thank you for being my pillar of strength. You always support my decisions even though you didn't fully grasp my thought sometimes. But love always wins, your unwavering support means the whole world to me. Fueled by your love, I consistently move forward with courage. To my sister and brother, thank you for your continuous encouragement, trust, and support. Thank you for taking care of our parents and grandparents while I am away. My mother and aunt, your profound love for each other as siblings and your genuine care for our parents serve as a guiding example for my future life. I love my family forever!

致**家人**，我的爸爸妈妈，姥姥姥爷，我永远最坚实的支柱，谢谢你们永远都无条件支持我所有的人生决定，尽管很多时候你们并不是很理解我的内心世界。爱永远战胜一切，你们的爱就是我的全世界，你们无条件的支持就是我人生前进的动力。我的姐姐和弟弟，谢谢你们永远的信任，永远的支持和鼓励。也谢谢你们在我不能在长辈身边的时候对父母和姥姥姥爷的照顾。妈妈，姨，你们之间深厚的姐妹情谊以及对长辈的真心爱护和照料是我的人生榜样。我永远爱我的家人！

Next, my special thanks go to my cat, **Timmy (甜茶)**, who has been a constant source of comfort and joy. You came into my life during my most depressing days, the darkest time in my life. You cured me and warmed my heart. I cannot imagine a happy life without you. I love you so much.

In the end, I would like to express the gratitude myself. **Jingyan (婧妍)**, thank you for your perseverance. Thank you for studying hard and working hard on your PhD projects. Thank you for keeping pursuing hobbies to make your life more fulfilling and more fun. I see the significant growth in you, both on work and in personal life. Keep embracing happiness and continue living a fun life!

13 References

1. Tam, P. P. L. & Behringer, R. R. Mouse gastrulation: the formation of a mammalian body plan. *Mech Dev* **68**, 3–25 (1997).

2. Bardot, E. S. & Hadjantonakis, A. K. Mouse gastrulation: Coordination of tissue patterning, specification and diversification of cell fate. *Mech Dev* **163**, 103617 (2020).

3. Rivera-Pérez, J. A. & Hadjantonakis, A. K. The Dynamics of Morphogenesis in the Early Mouse Embryo. *Cold Spring Harb Perspect Biol* **7**, a015867 (2015).

4. Pereira, P. N. *et al.* Amnion formation in the mouse embryo: The single amniochorionic fold model. *BMC Dev Biol* **11**, 1–13 (2011).

5. Pijuan-Sala, B. *et al.* A single-cell molecular map of mouse gastrulation and early organogenesis. *Nature 2019 566:7745* **566**, 490–495 (2019).

6. Thowfeequ, S. & Srinivas, S. Embryonic and extraembryonic tissues during mammalian development: shifting boundaries in time and space. *Philosophical Transactions of the Royal Society B* **377**, (2022).

7. Qu, F. *et al.* Three-dimensional molecular architecture of mouse organogenesis. *Nature Communications 2023 14:1* **14**, 1–18 (2023).

8. Barton, L. J., LeBlanc, M. G. & Lehmann, R. Finding their way: themes in germ cell migration. *Current Opinion in Cell Biology* vol. 42 128–137 Preprint at https://doi.org/10.1016/j.ceb.2016.07.007 (2016).

9. Grimaldi, C. & Raz, E. Germ cell migration—Evolutionary issues and current understanding. *Seminars in Cell and Developmental Biology* vol. 100 152–159 Preprint at https://doi.org/10.1016/j.semcdb.2019.11.015 (2020).

10. Sampath Kumar, A. *et al.* Spatiotemporal transcriptomic maps of whole mouse embryos at the onset of organogenesis. *Nature Genetics 2023 55:7* **55**, 1176–1185 (2023).

11. Tam, P. P. L. & Loebel, D. A. F. Gene function in mouse embryogenesis: get set for gastrulation. *Nature Reviews Genetics 2007 8:5* **8**, 368–381 (2007).

12. Arnold, S. J. & Robertson, E. J. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nature Reviews Molecular Cell Biology 2009 10:2* **10**, 91–103 (2009).

13. Cao, J. *et al.* The single-cell transcriptional landscape of mammalian organogenesis. doi:10.1038/s41586-019-0969-x.

14. Lohoff, T. *et al.* Integration of spatial and single-cell transcriptomic data elucidates mouse organogenesis. *Nature Biotechnology 2021 40:1* **40**, 74–85 (2021).

15. Ratz, M. *et al.* Clonal relations in the mouse brain revealed by single-cell and spatial transcriptomics. doi:10.1038/s41593-022-01011-x.

16. Jacobs-Li, J., Tang, W., Li, C. & Bronner, M. E. Single-cell profiling coupled with lineage analysis reveals vagal and sacral neural crest contributions to the developing enteric nervous system. *Elife* **12**, (2023).

17. Han, L. *et al.* Single cell transcriptomics identifies a signaling network coordinating endoderm and mesoderm diversification during foregut organogenesis. *Nature Communications 2020 11:1* **11**, 1–16 (2020).

18. Nikolopoulou, E., Galea, G. L., Rolo, A., Greene, N. D. E. & Copp, A. J. Neural tube closure: cellular, molecular and biomechanical mechanisms. *Development* **144**, 552–566 (2017).

19. Singh, R. & Munakomi, S. Embryology, Neural Tube. *StatPearls* (2023).

20. Schoenwolf, G. C. Histological and ultrastructural studies of secondary neurulation in mouse embryos. *Am J Anat* **169**, 361–376 (1984).

21. Dessaud, E., McMahon, A. P. & Briscoe, J. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* **135**, 2489–2503 (2008).

22. Copp, A. J., Greene, N. D. E. & Murdoch, J. N. The genetic basis of mammalian neurulation. *Nature Reviews Genetics 2003 4:10* **4**, 784–793 (2003).

23. Hall, B. K. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic. *Evol Dev* **2**, 3–5 (2000).

24. Rocha, M., Singh, N., Ahsan, K., Beiriger, A. & Prince, V. E. Neural crest development: insights from the zebrafish. *Developmental Dynamics* **249**, 88–111 (2020).

25. Achilleos, A. & Trainor, P. A. Neural crest stem cells: discovery, properties and potential for therapy. *Cell Research 2012 22:2* **22**, 288–304 (2012).

26. Achilleos, A. & Trainor, P. A. Neural crest stem cells: discovery, properties and potential for therapy. *Cell Research 2012 22:2* **22**, 288–304 (2012).

27. Zhao, R. & Trainor, P. A. Epithelial to mesenchymal transition during mammalian neural crest cell delamination. *Semin Cell Dev Biol* (2022) doi:10.1016/J.SEMCDB.2022.02.018.

28. Rocha, M. *et al.* From head to tail: Regionalization of the neural crest. *Development (Cambridge)* **147**, (2021).

29. Soldatov, R. *et al.* Spatiotemporal structure of cell fate decisions in murine neural crest. *Science (1979)* **364**, (2019).

30. Méndez-Maldonado, K., Vega-López, G. A., Aybar, M. J. & Velasco, I. Neurogenesis From Neural Crest Cells: Molecular Mechanisms in the Formation of Cranial Nerves and Ganglia. *Front Cell Dev Biol* **8**, 549609 (2020).

31. Magaletta, M. E. *et al.* Integration of single-cell transcriptomes and chromatin landscapes reveals regulatory programs driving pharyngeal organ development. *Nature Communications 2022 13:1* **13**, 1–16 (2022).

32. Fabio Santagati and Filippo M. Rijli. CRANIAL NEURAL CREST AND THE BUILDING OF THE VERTEBRATE HEAD. *Nature reviews* (2023) doi:10.1038/nrn1221.

33. Chen, W. *et al.* Single-cell transcriptomic landscape of cardiac neural crest cell derivatives during development. *EMBO Rep* **22**, e52389 (2021).

34. George, R. M., Maldonado-Velez, G. & Firulli, A. B. The heart of the neural crest: cardiac neural crest cells in development and regeneration. (2020) doi:10.1242/dev.188706.

35. Chen, W. *et al.* Single-cell transcriptomic landscape of cardiac neural crest cell derivatives during development. *EMBO Rep* **22**, e52389 (2021).

36. Hutchins, E. J. *et al.* Migration and diversification of the vagal neural crest. *Dev Biol* **444**, S98–S109 (2018).

37. Burns, A. J. & Le Douarin, N. M. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. *Development* **125**, 4335–4347 (1998).

38. Martik, M. L. & Bronner, M. E. Riding the crest to get a head: neural crest evolution in vertebrates. *Nature Reviews Neuroscience 2021 22:10* **22**, 616–626 (2021).

39. Ferretti, E. & Hadjantonakis, A. K. Mesoderm specification and diversification: from single cells to emergent tissues. *Curr Opin Cell Biol* **61**, 110–116 (2019).

40. Probst, S. *et al.* Spatiotemporal sequence of mesoderm and endoderm lineage segregation during mouse gastrulation. *Development (Cambridge)* **148**, (2021).

41. Al-Drees, M. A. *et al.* Making Blood: The Haematopoietic Niche throughout Ontogeny. *Stem Cells Int* **2015**, (2015).

42. Maroto, M., Bone, R. A. & Kim Dale, J. Somitogenesis. *Development* **139**, 2453–2456 (2012).

43. Weldon, S. A. & Münsterberg, A. E. Somite development and regionalisation of the vertebral axial skeleton. *Semin Cell Dev Biol* **127**, 10–16 (2022).

44. Ibarra-Soria, X. *et al.* A transcriptional and regulatory map of mouse somitogenesis. *bioRxiv* 2023.01.24.525253 (2023) doi:10.1101/2023.01.24.525253.

45. Pourquié, O. The segmentation clock: Converting embryonic time into spatial pattern. *Science (1979)* **301**, 328–330 (2003).

46. Christ, B., Huang, R. & Scaal, M. Amniote somite derivatives. *Developmental Dynamics* **236**, 2382–2396 (2007).

47. Yusuf, F. & Brand-Saberi, B. The eventful somite: Patterning, fate determination and cell division in the somite. *Anat Embryol (Berl)* **211**, 21–30 (2006).

48. Guibentif, C. *et al*. Diverse Routes toward Early Somites in the Mouse Embryo. *Dev Cell* **56**, 141-153.e6 (2021).

49. Ranghini, E. & Dressler, G. R. Early Specification and Patterning of the Intermediate Mesoderm: Genetics and Epigenetics. *Kidney Development, Disease, Repair and Regeneration* 17–26 (2016) doi:10.1016/B978-0-12-800102-8.00002-3.

50. Dressler, G. R. Advances in early kidney specification, development and patterning. *Development* **136**, 3863–3874 (2009).

51. James, R. G. & Schultheiss, T. M. Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. *Dev Biol* **288**, 113–125 (2005).

52. Prummel, K. D., Nieuwenhuize, S. & Mosimann, C. The lateral plate mesoderm. (2020) doi:10.1242/dev.175059.

53. Petit, F., Sears, K. E. & Ahituv, N. Limb development: a paradigm of gene regulation. *Nature Publishing Group* (2017) doi:10.1038/nrg.2016.167.

54. Ariza, L., Carmona, R., Cañete, A., Cano, E. & Muñoz-Chápuli, R. Coelomic epithelium-derived cells in visceral morphogenesis. *Developmental Dynamics* **245**, 307–322 (2016).

55. Winters, N. I. & Bader, D. M. Development of the Serosal Mesothelium. *Journal of Developmental Biology 2013, Vol. 1, Pages 64-81* **1**, 64–81 (2013).

56. Koopmans, T. & Rinkevich, Y. Mesothelial to mesenchyme transition as a major developmental and pathological player in trunk organs and their cavities. *Communications Biology 2018 1:1* **1**, 1–14 (2018).

57. Asahina, K., Zhou, B., Pu, W. T. & Tsukamoto, H. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* **53**, 983–995 (2011).

58. Ariza, L., Carmona, R., Ca~, A., Cano, E. & On Mu~ Noz-Ch Apuli, R. Coelomic Epithelium-Derived Cells in Visceral Morphogenesis. doi:10.1002/dvdy.

59. Rinkevich, Y. *et al*. Identification and prospective isolation of a mesothelial precursor lineage giving rise to smooth muscle cells and fibroblasts for mammalian internal organs, and their vasculature. *Nature Cell Biology 2012 14:12* **14**, 1251–1260 (2012).

60. Ferretti, E. & Hadjantonakis, A. K. Mesoderm specification and diversification: from single cells to emergent tissues. *Curr Opin Cell Biol* **61**, 110–116 (2019).

61. Tzouanacou, E., Wegener, A., Wymeersch, F. J., Wilson, V. & Nicolas, J. F. Redefining the Progression of Lineage Segregations during Mammalian Embryogenesis by Clonal Analysis. *Dev Cell* **17**, 365–376 (2009).

62. Henrique, D., Abranches, E., Verrier, L. & Storey, K. G. Neuromesodermal progenitors and the making of the spinal cord. (2015) doi:10.1242/dev.119768.

63. Attardi, A. *et al.* Neuromesodermal progenitors are a conserved source of spinal cord with divergent growth dynamics. *Development (Cambridge)* **145**, (2018).

64. Shaker, M. R. *et al.* Spatiotemporal contribution of neuromesodermal progenitor-derived neural cells in the elongation of developing mouse spinal cord. *Life Sci* **282**, 119393 (2021).

65. Martin, B. L. Mesoderm induction and patterning: Insights from neuromesodermal progenitors. *Semin Cell Dev Biol* **127**, 37–45 (2022).

66. Spence, J. R., Lauf, R. & Shroyer, N. F. Vertebrate intestinal endoderm development. *Developmental Dynamics* **240**, 501–520 (2011).

67. Franklin, V. *et al.* Regionalisation of the endoderm progenitors and morphogenesis of the gut portals of the mouse embryo. *Mech Dev* **125**, 587–600 (2008).

68. Mitiku, N. & Baker, J. C. Developmental Cell Resource Genomic Analysis of Gastrulation and Organogenesis in the Mouse. doi:10.1016/j.devcel.2007.10.004.

69. Tam, P. P. L. *et al.* Sequential allocation and global pattern of movement of the definitive endoderm in the mouse embryo during gastrulation. *Development* **134**, 251–260 (2007).

70. Zorn, A. M. & Wells, J. M. Molecular Basis of Vertebrate Endoderm Development. *Int Rev Cytol* **259**, 49–111 (2007).

71. Tremblay, K. D. & Zaret, K. S. Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Dev Biol* **280**, 87–99 (2005).

72. Lewis, S. L. & Tam, P. P. L. Definitive endoderm of the mouse embryo: Formation, cell fates, and morphogenetic function. *Developmental Dynamics* **235**, 2315–2329 (2006).

73. Funayama, N., Sato, Y., Matsumoto, K., Ogura, T. & Takahashi, Y. Coelom formation: binary decision of the lateral plate mesoderm is controlled by the ectoderm. *Development* **126**, 4129–4138 (1999).

74. Schlueter, J. & Mikawa, T. Body Cavity Development Is Guided by Morphogen Transfer between Germ Layers. *Cell Rep* **24**, 1456–1463 (2018).

75. Zorn. Liver Development. *Stembook* 1–26 (2008) doi:10.3824/stembook.1.25.1.

76. Gordillo, M., Evans, T. & Gouon-Evans, V. Orchestrating liver development. *Development* **142**, 2094 LP – 2108 (2015).

77. Lotto, J., Stephan, T. L. & Hoodless, P. A. Fetal liver development and implications for liver disease pathogenesis. *Nature Reviews Gastroenterology & Hepatology 2023 20:9* **20**, 561–581 (2023).

78. Kisseleva, T. & Brenner, D. Molecular and cellular mechanisms of liver fibrosis and its regression. *Nature Reviews Gastroenterology & Hepatology 2020 18:3* **18**, 151–166 (2020).

79. Iwaisako, K. *et al.* Origin of myofibroblasts in the fibrotic liver in mice. *Proc Natl Acad Sci U S A* **111**, E3297–E3305 (2014).

80. Yin, C., Evason, K. J., Asahina, K. & Stainier, D. Y. R. Hepatic stellate cells in liver development, regeneration, and cancer. *J Clin Invest* **123**, 1902–1910 (2013).

81. Miller, B. M., Oderberg, I. M. & Goessling, W. The Hepatic Nervous System in Development, Regeneration, and Disease. *Hepatology* **74**, 3513 (2021).

82. Asahina, K. *et al.* Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology* **49**, 998–1011 (2009).

83. Asahina, K. Hepatic stellate cell progenitor cells. *J Gastroenterol Hepatol* **27**, 80–84 (2012).

84. Kisseleva, T. & Brenner, D. Molecular and cellular mechanisms of liver fibrosis and its regression. *Nat Rev Gastroenterol Hepatol* **18**, 151–166 (2021).

85. Kitto, L. J. & Henderson, N. C. Hepatic Stellate Cell Regulation of Liver Regeneration and Repair. *Hepatol Commun* **5**, 358–370 (2021).

86. Lee, Y., Leslie, J., Yang, Y. & Ding, L. Hepatic stellate and endothelial cells maintain hematopoietic stem cells in the developing liver. *J Exp Med* **218**, (2021).

87. Geerts, A. On the origin of stellate cells: mesodermal, endodermal or neuro-ectodermal? *J Hepatol* **40**, 331–334 (2004).

88. Kordes, C., Bock, H. H., Reichert, D., May, P. & Häussinger, D. Hepatic stellate cells: Current state and open questions. *Biol Chem* **402**, 1021–1032 (2021).

89. Asahina, K., Zhou, B., Pu, W. T. & Tsukamoto, H. Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* **53**, 983–95 (2011).

90. Yin, C., Evason, K. J., Maher, J. J. & Stainier, D. Y. R. The basic helix-loop-helix transcription factor, heart and neural crest derivatives expressed transcript 2, marks hepatic stellate cells in zebrafish: analysis of stellate cell entry into the developing liver. *Hepatology* **56**, 1958–70 (2012).

91. Cassiman, D., Barlow, A., Vander Borght, S., Libbrecht, L. & Pachnis, V. Hepatic stellate cells do not derive from the neural crest. *J Hepatol* **44**, 1098–1104 (2006).

92. Asahina, K. *et al.* Mesenchymal origin of hepatic stellate cells, submesothelial cells, and perivascular mesenchymal cells during mouse liver development. *Hepatology* **49**, 998–1011 (2009).

93. Yin, C., Evason, K. J., Maher, J. J. & Stainier, D. Y. R. The basic helix-loop-helix transcription factor, heart and neural crest derivatives expressed transcript 2, marks hepatic stellate cells in zebrafish: Analysis of stellate cell entry into the developing liver. *Hepatology* **56**, 1958–1970 (2012).

94. Swiderska-Syn, M. *et al.* Myofibroblastic cells function as progenitors to regenerate murine livers after partial hepatectomy. *Gut* **63**, 1333–1344 (2014).

95. Chen, N. *et al.* Fate tracking reveals differences between Reelin+ hepatic stellate cells (HSCs) and Desmin+ HSCs in activation, migration and proliferation. *Cell Prolif* e13500 (2023) doi:10.1111/CPR.13500.

96. Sierra, R. *et al.* Contribution of neural crest and GLAST+ Wnt1+ bone marrow pericytes with liver fibrogenesis and/or regeneration. *Liver International* **40**, 977–987 (2020).

97. Yang, L. *et al.* Fate-Mapping Evidence That Hepatic Stellate Cells Are Epithelial Progenitors in Adult Mouse Livers. *Cells* **26**, 2104–2113 (2008).

98. Osman, I., Wang, L., Hu, G., Zheng, Z. & Zhou, J. GFAP (Glial Fibrillary Acidic Protein)-Positive Progenitor Cells Contribute to the Development of Vascular Smooth Muscle Cells and Endothelial Cells - Brief Report. *Arterioscler Thromb Vasc Biol* **40**, 1231–1238 (2020).

99. Miyata, E. *et al.* Hematopoietic origin of hepatic stellate cells in the adult liver. *STEM CELLS IN HEMATOLOGY* (2008) doi:10.1182/blood-2007-07.

100. Miller, B. M., Oderberg, I. M. & Goessling, W. Hepatic Nervous System in Development, Regeneration, and Disease. *Hepatology* **74**, 3513–3522 (2021).

101. Tanimizu, N., Ichinohe, N. & Mitaka, T. Intrahepatic bile ducts guide establishment of the intrahepatic nerve network in developing and regenerating mouse liver. *Development (Cambridge)* **145**, (2018).

102. Li, Y., Wang, J., Asahina, K. & Hogan, B. L. M. Mesothelial cells give rise to hepatic stellate cells and myofibroblasts via mesothelial-mesenchymal transition in liver injury. doi:10.1073/pnas.1214136110.

103. Arnold, T. D. *et al.* Targeting of αv integrin identifies a core molecular pathway that regulates fibrosis in several organs. (2013) doi:10.1038/nm.3282.

104. Hamberger, F., Mederacke, Y. S. & Mederacke, I. An inducible model for genetic manipulation and fate-tracing of PDGFRβ-expressing fibrogenic cells in the liver. *Scientific Reports 2023 13:1* **13**, 1–7 (2023).

105. Troeger, J. S. *et al.* Deactivation of Hepatic Stellate Cells During Liver Fibrosis Resolution in Mice. *Gastroenterology* **143**, 1073–1083.e22 (2012).

106. Gays, D. *et al.* An exclusive cellular and molecular network governs intestinal smooth muscle cell differentiation in vertebrates. (2017) doi:10.1242/dev.133926.

107. Kisseleva, T. *et al.* Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proc Natl Acad Sci U S A* **109**, 9448–9453 (2012).

108. Cassiman, D., Barlow, A., Vander Borght, S., Libbrecht, L. & Pachnis, V. Hepatic stellate cells do not derive from the neural crest. *J Hepatol* **44**, 1098–1104 (2006).

109. Nagoshi, N. *et al.* Ontogeny and Multipotency of Neural Crest-Derived Stem Cells in Mouse Bone Marrow, Dorsal Root Ganglia, and Whisker Pad. *Cell Stem Cell* **2**, 392–403 (2008).

110. Mederacke, I. *et al.* Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nature Communications 2013 4:1* **4**, 1–11 (2013).

111. Yang, L. *et al.* Fate-mapping evidence that hepatic stellate cells are epithelial progenitors in adult mouse livers. *Stem Cells* **26**, 2104–2113 (2008).

112. Gao, X., Xu, C., Asada, N. & Frenette, P. S. The hematopoietic stem cell niche: From embryo to adult. *Development (Cambridge)* **145**, (2018).

113. Fraser, S. T. *et al.* Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin+ population. *Exp Hematol* (2002) doi:10.1016/S0301-472X(02)00887-1.

114. Sasaki, T. *et al.* Regulation of hematopoietic cell clusters in the placental niche through SCF/Kit signaling in embryonic mouse. *Development* **137**, 3941–3952 (2010).

115. Mikkola, H. K. A. & Orkin, S. H. The journey of developing hematopoietic stem cells. *Development* **133**, 3733–3744 (2006).

116. Mahony, C. B. & Bertrand, J. Y. How HSCs colonize and expand in the fetal niche of the vertebrate embryo: An evolutionary perspective. *Front Cell Dev Biol* **7**, 438520 (2019).

117. Medvinsky, A., Rybtsov, S. & Taoudi, S. Embryonic origin of the adult hematopoietic system: advances and questions. *Development* **138**, 1017–1031 (2011).

118. Dixon, J. B., Bhathal, P. S., Hughes, N. R. & O'Brien, P. E. Nonalcoholic fatty liver disease: Improvement in liver histological analysis with weight loss. *Hepatology* **39**, 1647–1654 (2004).

119. Kweon, Y. O. *et al.* Decreasing fibrogenesis: An immunohistochemical study of paired liver biopsies following lamivudine therapy for chronic hepatitis B. *J Hepatol* **35**, 749–755 (2001).

120. Arthur, M. J. P. Reversibility of liver fibrosis and cirrhosis following treatment for hepatitis C. *Gastroenterology* **122**, 1525–1528 (2002).

121. Hammel, P. *et al.* Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* **344**, 418–423 (2001).

122. Tan, Z. *et al.* Liver Fibrosis: Therapeutic Targets and Advances in Drug Therapy. *Front Cell Dev Biol* **9**, 730176 (2021).

123. Zhang, C. Y., Liu, S. & Yang, M. Treatment of liver fibrosis: Past, current, and future. *http://www.wjgnet.com/* **15**, 755–774 (2023).

124. Nathwani, R. *et al.* A Review of Liver Fibrosis and Emerging Therapies. *EUROPEAN MEDICAL JOURNAL* (2019).

125. Kretzschmar, K. & Watt, F. M. Leading Edge Primer Lineage Tracing. doi:10.1016/j.cell.2012.01.002.

126. Chen, C., Liao, Y. & Peng, G. Connecting past and present: single-cell lineage tracing. *Protein Cell* **13**, 790–807 (2022).

127. Parsons, C. L., By, A. E. & Conklin, E. G. The Organization and Cell-lineage of the Ascidian Egg. By Professor E. G. Conklin, Journal of the Academy of Natural Sciences of Philadelphia, Second Series, Volume XIII., Part I., 1905. *Science (1979)* **23**, 340–344 (1906).

128. Dixit, A. *et al.* Perturb-seq: Dissecting molecular circuits with scalable single cell RNA profiling of pooled genetic screens. *Cell* **167**, 1853 (2016).

129. Yao, D. *et al.* Scalable genetic screening for regulatory circuits using compressed Perturb-seq. *Nature Biotechnology 2023* **10**, 1–14 (2023).

130. Vogt, W. Gestaltungsanalyse am Amphibienkeim mit Örtlicher Vitalfärbung - II. Teil. Gastrulation und Mesodermbildung bei Urodelen und Anuren. *Wilhelm Roux Arch Entwickl Mech Org* **120**, 384–706 (1929).

131. Itasaki, N., Bel-Vialar, S. & Krumlauf, R. 'Shocking' developments in chick embryology: Electroporation and in ovo gene expression. *Nat Cell Biol* **1**, (1999).

132. Kumar, P., Nagarajan, A. & Uchil, P. D. Electroporation. *Cold Spring Harbor Protocols* vol. 2019 519–525 Preprint at https://doi.org/10.1101/pdb.top096271 (2019).

133. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature Genetics 1999 21:1* **21**, 70–71 (1999).

134. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1**, 1–8 (2001).

135. Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H. & Orkin, S. H. Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* **97**, 324–326 (2001).

136. Littlewood, T. D., Hancock, D. C., Danielian, P. S., Parker, M. G. & Evan, G. I. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**, 1686–1690 (1995).

137. Feil, R. *et al.* Ligand-activated site-specific recombination in mice. *Proceedings of the National Academy of Sciences* **93**, 10887–10890 (1996).

138. Feil, R., Wagner, J., Metzger, D. & Chambon, P. Regulation of Cre Recombinase Activity by Mutated Estrogen Receptor Ligand-Binding Domains. *Biochem Biophys Res Commun* **237**, 752–757 (1997).

139. Hirrlinger, J. *et al.* Split-cre complementation indicates coincident activity of different genes in vivo. *PLoS One* **4**, (2009).

140. Song, A. J. & Palmiter, R. D. Detecting and Avoiding Problems When Using the Cre/lox System. *Trends Genet* **34**, 333 (2018).

141. Stifter, S. A. & Greter, M. STOP floxing around: Specificity and leakiness of inducible Cre/loxP systems. *Eur J Immunol* **50**, 338–341 (2020).

142. Weissman, T. A. & Pan, Y. A. Brainbow: new resources and emerging biological applications for multicolor genetic labeling and analysis. *Genetics* **199**, 293–306 (2015).

143. Livet, J. *et al.* Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature 2007 450:7166* **450**, 56–62 (2007).

144. Pontes-Quero, S. *et al.* Dual ifgMosaic: A Versatile Method for Multispectral and Combinatorial Mosaic Gene-Function Analysis. *Cell* **170**, 800-814.e18 (2017).

145. VanHorn, S. & Morris, S. A. Next-Generation Lineage Tracing and Fate Mapping to Interrogate Development. *Dev Cell* **56**, 7–21 (2021).

146. Weinreb, C. & Klein, A. M. Lineage reconstruction from clonal correlations. *Proc Natl Acad Sci U S A* **117**, 17041–17048 (2020).

147. Bulcha, J. T., Wang, Y., Ma, H., Tai, P. W. L. & Gao, G. Viral vector platforms within the gene therapy landscape. doi:10.1038/s41392-021-00487-6.

148. Russell, W. C. Update on adenovirus and its vectors. *J Gen Virol* **81**, 2573–2604 (2000).

149. Howarth, J. L., Lee, Y. B. & Uney, J. B. Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). *Cell Biology and Toxicology 2009 26:1* **26**, 1–20 (2009).

150. Roelvink, P. W. *et al.* The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol* **72**, 7909–7915 (1998).

151. Ni, T.-H. *et al.* Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. *J Virol* **72**, 2777–2787 (1998).

152. Havenga, M. J. E. *et al.* Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. *J Virol* **76**, 4612–4620 (2002).

153. Bangari, D. S. & Mittal, S. K. Development of nonhuman adenoviruses as vaccine vectors. *Vaccine* **24**, 849–862 (2006).

154. Fantastic AAV Gene, D., Rommelaere, J., Becker, J., Fakhiri, J. & Grimm, D. Fantastic AAV Gene Therapy Vectors and How to Find Them—Random Diversification, Rational Design and Machine Learning. *Pathogens 2022, Vol. 11, Page 756* **11**, 756 (2022).

155. Cronin, J., Zhang, X.-Y. & Reiser, J. Altering the Tropism of Lentiviral Vectors through Pseudotyping. *Curr Gene Ther* **5**, 387 (2005).

156. Wei Qian, A., Yong Wang, A., Rui-fu Li BCF Xin Zhou BCF Jing Liu ABCDEFG Dai-zhi Peng, B. & Peng, D. Prolonged Integration Site Selection of a Lentiviral Vector in the Genome of Human Keratinocytes. **23**, 1116–1122 (2017).

157. Ciuffi, A. Mechanisms Governing Lentivirus Integration Site Selection. *Curr Gene Ther* **8**, 419–429 (2008).

158. Bouard, D., Alazard-Dany, N. & Cosset, F. L. Viral vectors: from virology to transgene expression. *Br J Pharmacol* **157**, 153–165 (2009).

159. Vigna, E. *et al.* Robust and Efficient Regulation of Transgene Expression in Vivo by Improved Tetracycline-Dependent Lentiviral Vectors. *Molecular Therapy* **5**, 252–261 (2002).

160. Brown, B. D., Venneri, M. A., Zingale, A., Sergi, L. S. & Naldini, L. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. (2006) doi:10.1038/nm1398.

161. Mangold, K. *et al.* Highly efficient manipulation of nervous system gene expression with NEPTUNE. *Cell Reports Methods* **0**, 100043 (2021).

162. Hall, B., Limaye, A. & Kulkarni, A. B. Overview: Generation of Gene Knockout Mice. *Current Protocols in Cell Biology* Preprint at https://doi.org/10.1002/0471143030.cb1912s44 (2009).

163. Wang, C. & Mei, L. In Utero Electroporation in Mice. in *Methods in Molecular Biology* vol. 1018 151–163 (Humana Press, Totowa, NJ, 2013).

164. Beronja, S., Livshits, G., Williams, S. & Fuchs, E. Rapid functional dissection of genetic networks via tissue-specific transduction and RNAi in mouse embryos. *Nat Med* **16**, 821–827 (2010).

165. Beronja, S. *et al.* RNAi screens in mice identify physiological regulators of oncogenic growth. *Nature* **501**, 185–190 (2013).

166. Henriques-Coelho, T. *et al.* Targeted gene transfer to fetal rat lung interstitium by ultrasound-guided intrapulmonary injection. *Molecular Therapy* **15**, 340–347 (2007).

167. Davidson, D. The House Mouse: Atlas of Embryonic Development. By K. Theiler. Second printing, 1989. New York: Springer-Verlag. 178 pages. DM 168.00. ISBN 3 540059407. doi:10.1017/S001667230002872X.

168. Downs, K. M. & Davies, T. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255–1266 (1993).

169. Suzuki, K. *et al.* p75 Neurotrophin Receptor Is a Marker for Precursors of Stellate Cells and Portal Fibroblasts in Mouse Fetal Liver. *Gastroenterology* **135**, 270-281.e3 (2008).

170. Morini, S. *et al.* GFAP expression in the liver as an early marker of stellate cells activation. *Italian Journal of Anatomy and Embryology* **110**, (2005).