iPSC derived building blocks for the fat liver axis

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Thank you all for your support!

Oslo, February 2023

Saphira Felicitas Baumgarten
Summary

This thesis is submitted in fulfilment with the requirements for the degree of Philosophy Doctor at the University of Oslo. It contains four independent research papers, describing the differentiation approach for liver organoids through the definitive endoderm governed by autophagy, the application of the organoids in a setting with parenteral nutrition, and the differentiation of adipocytes with the goal of supplying building blocks to establish the fat-liver axis in vitro.

The number of obese adults has increased to over 300 million since 2000, affecting general health in light of the COVID pandemic and putting an immense strain on health services to cover the costs. To mitigate this will require access to effective models that allow us to understand disease aetiology, and develop novel therapeutic interventions, particularly concerning the fat-liver axis in metabolic diseases. Metabolic syndrome is complex and multifactorial and therefore not restricted to just one tissue or cell. Accordingly, we encounter the necessity of multiple cell types for in vitro modelling, since animal models are not up to the task, e.g. due to differences in cytochrome P450 enzymes of mice and humans. Thus, we are proposing minimal requirements to build an in vitro fat-liver axis by utilising induced pluripotent stem cells and their differentiation to liver organoids and adipocytes for the described application.

Autophagy has been shown to play a crucial role in both development and differentiation, however early events during development including the exit from the pluripotent state and commitment toward a specific germ layer still require more research. The first paper of this study aimed to elucidate the role of autophagy in the early events of lineage commitment, to that end, human embryonic and induced pluripotent stem cells have been applied for the differentiation of definitive endoderm, mesoderm and ectoderm. The results indicate that a dip in autophagy facilitates the exit from pluripotency. The induction of autophagy is promoted by sex determining region y-box 2 degradation and thereby formation of mesendoderm and its accumulation by inhibition of autophagy caused neuroectoderm formation. These results highlight the importance of autophagy for lineage commitment and together could potentially improve differentiation approaches of pluripotent stem cells in future applications.

The collected knowledge on endoderm formation was further applied for the second paper. The generation of an organoid model, containing parenchymal (foregut endodermal origin) and non-parenchymal (mesodermal origin) cells of the liver, recapitulating a developmental relevant pathway for liver development. The developed protocol is extracellular matrix independent, in suspension and growth factor free, decreasing production costs in comparison to growth factor based differentiation cocktails, labour time and plastic ware. Demonstrated by single-cell ribonucleic acid sequencing and immunofluorescence, a liver like cellular repertoire was proven, including vascular luminal structures, hepatic stellate cells and a population of resident macrophages – the kupffer cells. Key liver functions including drug metabolism, serum protein production, urea synthesis and coagulation factor production, which preserved post-translational modifications such as N-glycosylation and functionality, were demonstrated. Additionally de novo vascularization and albumin secretion were validated, offering several application possibilities of the organoids. Based on the validated characteristics of the organoids, they appeared to be a suitable model to investigate parenteral nutrition associated disease. Parenteral nutrition (PN) is used for patients of varying ages with intestinal failure to supplement calories, but unfortunately, premature new-borns with low birth weight are at a high risk for developing parenteral nutrition associated liver disease (PNALD) including steatosis, cholestasis, and gallbladder sludge/stones. There are no human model systems available for
testing PN; therefore, paper III investigated the potential of our liver organoids as a testing platform for PNALD, since they have early postnatal maturity making them a suitable model for premature newborns. To mimic PN treatment we used medium supplemented with either clinoleic (80% olive oil and 20% soybean oil) or intralipid (100% soybean oil) for 6 days and the treatment did not directly affect organoid viability. Both PN treatments caused a significant increase of *de novo* lipogenesis, further analysis for Cytochrome P450 3A4 activity under different culture conditions revealed a significantly decreased activity in organoids, but no effect on Cytochrome P450 1A2 activity. We therefore concluded that the organoids could be utilised as a pre-screening platform for the development of new, less hepatotoxic PN solutions.

Finally yet importantly, paper IV was dedicated to the differentiation of adipocytes from induced pluripotent stem cells, because currently available adipocyte differentiation protocols are inefficient, and lack many features of their primary equivalents. A robust protocol that guides human induced pluripotent stem cells to adipocytes over a 53-day period through a neural crest stem cell population, requiring single cell adaptation of induced pluripotent stem cells, followed by differentiation of these cells to an expandable neural crest stem cell population. This step was followed by the conversion of neural crest stem cells to mesenchymal like stem cells, and finally the generation of adipocytes under 3-dimensional culture conditions in suspension culture, a method transfer from paper II. The derived adipocytes have similar gene expression, lipid storage and adipokine secretion profiles as compared to adipocytes differentiated from stem cells derived from adipose tissue. The induced pluripotent stem cells derived adipocytes can be potentially exploited in co-culture application with the liver organoids, since both models are cultured in suspension are transferable to any other culture vessel.

Taken together, the results of this thesis deliver the two major building blocks to establish the fat liver axis *in vitro*. This could potentially facilitate the study of metabolic diseases like non-alcoholic fatty liver disease *in vitro*, delivering an alternative to animal models in the future.

**Sammendrag**

Denne oppgaven leveres i samsvar med kravene til graden doctor philosophiae (dr.philos.) ved Universitetet i Oslo. Den inneholder fire uavhengige forskningsartikler, som beskriver differensiering av leverorganoider gjennom den definitive endodermen styrt av autofagi, anvendelsen av organoidene for å undersøke effekten av parenteralt ernæring og differensiering av stamceller for å lage et modellsystem for å undersøke interaksjon mellom lever og fettvev *in vitro*.


Autofagi har vist seg å spille en avgjørende rolle i både utvikling og differensiering, men tidlige hendelser under utviklingen inkludert utgang fra den pluripotente tilstanden og dannelse av spesifike
kimlag krever fortsatt mer forskning. Det første arbeidet i denne studien hadde som mål å belyse rollen til autofagi i de tidlige hendelsene i dannelsen av kimlag. Vi har brukt humane embryonale og induserte pluripotente stamceller for differensiering av definitiv endoderm, mesoderm og ektoderm. Resultatene indikerer at redusert autofagi fremmer utgangen fra pluripotens. Indusjonen av autofagi øker SOX2-nedbrytning og derved dannelse av mesendoderm. Akkumulering av SOX2 ved inhibering av autofagi forårsaker nevroektodermdannelse. Disse resultatene fremhever viktigheten av autofagi for utvikling av kimlag og kan potensielt forbedre prosessene for differensiering av pluripotente stamceller.


Begge PN-behandlingene forårsaket en signifikant økning av de novo lipogene. Analyse av CYP3A4-aktivitet under ulike betingelser viste en signifikant påvirket CYP3A4-aktivitet i organoider, men ingen effekt på CYP1A2-aktivitet. Vi konkluderte med at organoidene kan brukes som en screeningsplattform for utvikling av nye, mindre hepatotoksiske parenterale ernæringsløsninger.

I det fjerde arbeidet undersøkte vi differensiering av adipocytter fra iPSCs, fordi tilgjengelige differensieringsprotokoller for adipocytter er ineffektive og mangler mange funksjoner i deres primære ekvivalenter. En robust protokoll som utvikler hiPSC-er til adipocytter over en 53-dagers periode ble etablert. Denne krever enkeltcelletilpasning av hiPSC-er, etterfulgt av differensiering av hiPSC-er til en ekspanderbar nevral-cellepopulation. Dette trinnet ble fulgt av konvertering av nevrals kamstamceller til mesenkymallignende stamceller (iMSC), og til slutt generering av adipocytter under 3-dimensjonale kulturbetingelser i suspensjon på en cellenkulturshaker (en metodeoverføring fra papir 2). Disse adipocyttenes har lignende genuttrykk, lipidlagring og adipokinsekresjonsprofiler sammenlignet med adipocytter differensiert fra stamceller fra fettvevet. De hiPSC-avledede adipocyttenes kan potensielt utnyttes i samkultursystem med leverorganoidene, siden begge modellene dyrkes i suspensjon og kan overføres til andre cellenkulturer.
Samlet gir resultatene av denne oppgaven av grunnsteinene for å etablere et modellsystem for å studere fett-leveraksen in vitro. Dette kan potensielt lette studiet av metaboliske syndrom inkludert ikke-alkoholisk fettleversykdom in vitro, og være et alternativ til dyremodeller i fremtiden.

List of publications


III. Saphira F. Baumgarten, Sean P. Harrison, Maria E. Chollet, Benedicte Stavik, Anindita Bhattacharya, Runar Almaas, Gareth J. Sullivan. Parenteral nutrition emulsion inhibits CYP3A4 in an iPSC derived liver organoids testing platform. Manuscript is with the reviewers of The Journal of Pediatric Gastroenterology and Nutrition.

SI Units

**Concentration**

- nM  Nanomolar, $1 \times 10^{-9}$ M
- µM  Micromolar, $1 \times 10^{-6}$ M
- mM  Millimolar, $1 \times 10^{-3}$ M
- M   Molar, $1 \times 10^0$ M

**Distance**

- nm  Nanometre, $1 \times 10^{-9}$ m
- µm  Micrometre, $1 \times 10^{-6}$ m
- m   Metre, $1 \times 10^0$ m

**Mass**

- ng  Nanogram, $1 \times 10^{-9}$ g
- µg  Microgram, $1 \times 10^{-6}$ g
- mg  Milligram, $1 \times 10^{-3}$ g
- g   Gram, $1 \times 10^0$ g

**Volume**

- µl  Microlitre, $1 \times 10^{-9}$ l
- ml  Millilitre, $1 \times 10^{-6}$ l
- l   Litre, $1 \times 10^0$ l

**Temperature**

- °C  Degrees Celsius
Abbreviations

2D  2-dimensional
3D  3-dimensional
3Rs Replacement, reduction and refinement
A1AT Alpha 1-antitrypsin
ACLY ATP citrate lyase
ADIPOQ Adiponectin
ADIPOR1 Adiponectin receptor 1
ADIPOR2 Adiponectin receptor 2
AdrRβ Beta-adrenergic receptor
AFP Alpha-fetoprotein
AKT AKT serine/threonine kinase
ALGS Alagille syndrome
ALT Alanine aminotransferase
AMPK AMP activated protein kinase
APAP Acetaminophen
APPL1 Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
ASCs Adipose tissue derived stem cell/progenitors
AST Aspartate aminotransferase
ATP Adenosine triphosphate
BAPX1 Bagpipe homeobox protein homolog 1
BAT Brown adipose tissue
BDNF Brain derived neurotrophic factor
BIO 6-Bromoindirubin-3'-oxime, GSK3 inhibitor
BM-MSCs Bone marrow mesenchymal stem cells
BMP4 Bone morphogenetic protein-4
BMP7 Bone morphogenetic protein 7
BMP8b Bone morphogenetic protein 8b
BPEL Bovine serum albumin (BSA) polyvinylalcohol essential lipids medium
C/EBPα/β/γ/δ/ CCAAT/enhancer binding protein alpha, beta, delta, gamma
cAMP  Cyclic adenosine monophosphate
CD105  Endoglin
CD106  CD106 antigen
CD13  CD13 Antigen
CD146  Melanoma cell adhesion molecule
CD24  CD24 molecule
CD29  Integrin beta-1
CD31  Platelet endothelial cell adhesion molecule (PECAM-1)
CD34  CD34 Molecule
CD36  Scavenger receptor class b, member 3
CD44  Hematopoietic cell e- and l-selectin ligand
CD45  Protein tyrosine phosphatase receptor type C
CD49d  CD49D antigen
CD54  CD54 antigen
CD73  Ecto-5'-nucleotidase
CD90  Thy-1 cell surface antigen
CK18  Cytokeratin 18
CK7  Cytokeratin 7
CK8  Cytokeratin 8
cMYC  Myc avian myelocytomatosis viral oncogene homolog
CO₂  Carbon dioxide
COVID-19  Coronavirus disease of 2019
CXCL11  C-X-C motif chemokine 11
CYP2D6  Cytochrome P450 2D6
CYP2E1  Cytochrome P450 2E1
CYP3A4  Cytochrome P450 3A4
CYP450  Cytochromes P450 enzymes
Dex  Dexamethasone
DIHEXA  N-hexanoic-Tyr-Ile-(6) aminohexanoic amide
DIO2  Iodothyronine deiodinase 2
DLK1  Delta like non-canonical notch ligand 1
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNL</td>
<td>De novo lipogenesis</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>E8</td>
<td>Essential E8</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>EPCAM+</td>
<td>Epithelial cell adhesion molecule positive</td>
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<td>ESC</td>
<td>Embryonic stem cells</td>
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<td>F-12</td>
<td>Ham’s F-12 Nutrient Mixture</td>
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<td>F7</td>
<td>Coagulation factor VII</td>
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<td>FABP4</td>
<td>Fatty acid binding protein 4</td>
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<td>FASN</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBG</td>
<td>Plasma fibrinogen</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>fg</td>
<td>Fore-gut</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGF21</td>
<td>Fibroblast growth factor 21</td>
</tr>
<tr>
<td>FLT3LG</td>
<td>FMS-related tyrosine kinase 3 ligand</td>
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<tr>
<td>FOXF1</td>
<td>Forkhead box f1</td>
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<td>GBP4</td>
<td>Guanylate binding protein 4</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma-Glutamyltransferase</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
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</table>
GSC  Goosecoid homeobox
GSK3β  Glycogen synthase kinase 3 beta
HC  Haemopoietin cocktail
HC21  Hydrocortisone 21 hemisuccinate sodium salt
HepaRG  Hepatoma cell line
hg  Hind-gut
hiPSCs  Human induced pluripotent stem cells
HMW  High-molecular weight
HNF4α  Hepatocyte nuclear factor 4 alpha
HO  Hepatic organoids
HO1  Primary hepatic organoid
HO2s  Secondary hepatic organoids
HOX11  Homeo box 11
HOXA5  Homeobox protein hox-a5
HOXC8  Homeobox protein hox-c8
HOXC9  Homeobox protein hox-c9
HPC  Haematopoietic stem cells and progenitor cells
HSC  Hepatic stellate cell
HUVECs  Human umbilical vein endothelial cells
IBMX  3-isobutyl-1-methylxanthine
ICM  Inner cell mass
IDO1  Indoleamine 2,3-dioxygenase 1
IFNG  Interferon gamma
IGF-2  Insulin-like growth factor 2
IL-6  Interleukin 6
IMDM  Iscove's modified dulbecco's medium
iMSCs  iPSC derived mesenchymal stem cells
Indo  Indomethacin
iPSCs  Induced pluripotent stem cells
ITS  Insulin transferrin selenium
KITLG  KIT ligand/ stem cell factor
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<td>KLF4</td>
<td>Kruppel like factor 4 a</td>
</tr>
<tr>
<td>KSR</td>
<td>Knockout serum replacement</td>
</tr>
<tr>
<td>L-15</td>
<td>Leibovitz 15 medium</td>
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<td>LC3 I</td>
<td>Microtubule-associated proteins 1A/1B light chain 3-I</td>
</tr>
<tr>
<td>LC3 II</td>
<td>Microtubule-associated proteins 1A/1B light chain 3-II</td>
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<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
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<td>LSECs</td>
<td>Liver sinusoidal endothelial cells</td>
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<td>LY6E</td>
<td>Lymphocyte antigen 6 family member e</td>
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<td>MCHS</td>
<td>Myocerebrohepatopathy spectrum</td>
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<td>MESP-1</td>
<td>Mesoderm posterior bhlh transcription factor 1</td>
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<td>MESP1</td>
<td>Mesoderm posterior protein 1</td>
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<td>mg</td>
<td>Mid-gut</td>
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<tr>
<td>MLXIPL</td>
<td>MLX-interacting protein-like</td>
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<td>CHREBP</td>
<td>Carbohydrate-responsive element-binding protein</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>Myf5</td>
<td>Myogenic factor 5</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
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<td>NANOG</td>
<td>Homeobox transcription factor nanog</td>
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<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone imine</td>
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<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NCCs</td>
<td>Neural crest cells</td>
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<td>NG2</td>
<td>Neuron-glial antigen 2</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NODAL</td>
<td>Nodal growth differentiation factor</td>
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<td>NOTCH2</td>
<td>Neurogenic locus notch homolog protein 2</td>
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<tr>
<td>NRG4</td>
<td>Neuregulin 4</td>
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<td>NT-3</td>
<td>Neurotrophin-3</td>
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12
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>OB-R</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4, also known as POU5F1</td>
</tr>
<tr>
<td>PAX3</td>
<td>Paired Box 3</td>
</tr>
<tr>
<td>PAX7</td>
<td>Paired box 7</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCSK9</td>
<td>Proprotein convertase subtilisin/kexin type 9</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet-derived growth factor receptor beta</td>
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<tr>
<td>PN</td>
<td>Parenteral nutrition</td>
</tr>
<tr>
<td>PNALD</td>
<td>Parenteral nutrition associated liver disease</td>
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<tr>
<td>POLG</td>
<td>DNA polymerase gamma</td>
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<tr>
<td>POU5F1</td>
<td>POU domain, class 5, transcription factor 1, also known as OCT4</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator activator receptor gamma</td>
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<tr>
<td>PRDM16</td>
<td>PR/SET domain 16</td>
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<tr>
<td>PREF1</td>
<td>Preadipocyte factor 1</td>
</tr>
<tr>
<td>PS</td>
<td>Primitive streak</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative real-time polymerase chain reaction</td>
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<tr>
<td>SCD</td>
<td>Stearoyl-coa desaturase</td>
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<tr>
<td>SMAD2/3</td>
<td>Suppressor of mothers against decapentaplegic 2/3</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SOX2</td>
<td>Sex determining region y-box 2</td>
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<tr>
<td>STEMPPro34</td>
<td>Hematopoietic stem cell medium</td>
</tr>
<tr>
<td>STRO-1</td>
<td>Protein marker of mesenchymal stem cells</td>
</tr>
<tr>
<td>SVCs</td>
<td>Stromal vascular cells</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>sWAT</td>
<td>Subcutaneous white adipose tissue</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodothyronine</td>
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</table>
**Introduction**

Metabolic syndrome and co-morbidities are a global burden on healthcare services, and leave people susceptible to diseases such as diabetes and non-alcoholic fatty liver disease (NAFLD), making patients vulnerable to viral infections, like the coronavirus disease of 2019 (COVID-19). The metabolic syndromes are complex, multifactorial, and not restricted to just one tissue or cell type, but involve organ crosstalk and whole body homeostasis. Therefore, there is a necessity to utilise *in vitro* models that contain multiple cell types, thus representing different tissues. It should be noted that animal models are a potential solution but they have a number of limitations, such as physiological differences between rodents and humans (Maloney et al., 2014; Masopust et al., 2017), not amenable to scaling, costly, and there is a concerted effort to drastically reduce animal usage in compliance with the 3Rs (Replacement, Reduction and Refinement). It was our vision to develop surrogate organs to provide scalable human organotypic *in vitro* models for research. We cannot rebuild an entire body, but propose to build an *in vitro* fat-liver axis equivalent by utilising induced pluripotent stem cells (iPSCs) to produce both liver organoids and spheroids of adipocytes.

**iPSCs – potential and drawbacks**

Embryonic stem cells (ESC) have held great promise in the study of disease mechanism and the development of potential new treatment strategies. This is due to the ability to proliferate indefinitely while maintaining their capacity to differentiate into the three germ layers (Thomson et al., 1998). ESCs are derived from the inner cell mass (ICM) of mammalian blastocysts, but they also come with ethical controversies with respect to the use of human embryos (Evans and Kaufman, 1981). In 2007, human somatic cells were reprogrammed to iPSCs by Yamanaka and his lab (Takahashi et al., 2007). This reprogramming was achieved by transduction of several key transcription factors, octamer-binding transcription factor 4 (Oct4 or Pou5f1), sex determining region Y-box 2 (Sox2), Klf4 (Kruppel like factor 4), and myc avian myelocytomatosis viral oncogene homolog (c-Myc). These human iPSCs were similar...
to human ESC in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and their telomerase activity. Additionally, these cells could be differentiated into cell types of the three germ layers both in vitro and via teratomas. The transcription factors POU5F1, SOX2, and homeobox transcription factor nanog (NANOG) are known master regulators of self-renewal and pluripotency and it has been shown that changes in the levels of these factors can lead to exit from pluripotency and ultimately differentiation (Mizushima and Komatsu, 2011; Yang and Klionsky, 2010). Also, studies have indicated that SOX2 is a lineage specifier toward neuroectoderm, but how SOX2 levels are regulated is still under investigation (Sharma et al., 2022).

For the reprogramming of somatic cells to pluripotent stem cells, autophagy is required. Autophagy is also relevant for the maintenance of the pluripotent state (Cho et al., 2014; He et al., 2012) and furthermore cell fate determination during embryonic development (Mizushima and Levine, 2010). Autophagy and its relevance has been reviewed in detail in other publications (Chang, 2020; Chen et al., 2021), and will only be summarised in the following section.

Extracellular stress such as starvation, can induce autophagy (He et al., 2012). By the process of autophagy, molecules and subcellular elements, including nucleic acids, proteins, lipids and organelles, are degraded via lysosome-mediated degradation to promote homeostasis, differentiation, development and/or survival (Cho et al., 2014). When autophagy is induced, the pre-autophagosomal structure is formed, the phagophore. The phagophore is elongated and matures, leading to the formation of the autophagosome with double membranes. LC3 I (microtubule-associated proteins 1A/1B light chain 3-I) is conjugated with phosphatidylethanolamine to become LC3 II (microtubule-associated proteins 1A/1B light chain 3-II), which associates with both the outer and inner membranes of the autophagosome. The autophagosome fuses with the lysosome to create the autolysosome, leading to degradation of the contained cargo (Abounit et al., 2012).

It is of note, autophagy has a central role during iPSC reprogramming, clearing the mitochondria, during reprogramming, at which point a metabolic switch from mitochondrial oxidative phosphorylation to glycolysis occurs (Chen et al., 2021; Ma et al., 2015; Xu et al., 2013). The emergence of human iPSCs (hiPSC) offers a number of possibilities, including access to cell types with limited availability such as hepatocyte-like cells, since primary human hepatocytes are usually destined for transplantation. A potential downside of these cells is the presence of genetic and epigenetic variations, which will have potential impact on basic, translational and clinical applications (Liang and Zhang, 2013; Pera, 2011). Liang and Zhang list 3 possible sources for the observed variation, firstly genetic variations stemming from heterogeneous genetic makeup of source cell population, secondly the reprogramming process itself may introduce de novo variations, and lastly, the culture of iPSCs may introduce / select genetic alterations favouring cell proliferation. Additionally, other routes of variation have been investigated, like chromosomal abnormalities by Laurent and colleagues (Laurent et al., 2011), “de novo” copy number variations (Hussein et al., 2011) and point mutations in protein coding regions (Gore et al., 2011; Huch et al., 2015b). Incomplete reprogramming of somatic cells into iPSCs might contribute to the heterogeneous in differentiation capacity observed amongst iPSC lines and some evidence suggests that epigenetic memory predisposes iPSCs to enhanced differentiation into the parental cell type (Efrat, 2021). None of the possible reasons for the presence of genetic and epigenetic variations have been proven incorrect at the moment and it is a topic of ongoing research efforts.

While genomic instability is a potential issue when considering the use of iPSCs in regenerative medicine/cellular therapy, they still have versatile application possibilities, such as the differentiation to different cell types from the same source. Especially when looking at disease mechanisms, e.g. NAFLD, where multiple cell types are involved and one cell type alone cannot deliver the complexity
of a tissue. To generate these different types of cells, one has to look at the different stages involved in development. iPSCs potentially deliver a plethora of applications, such as disease modelling, tissue engineering, development, and regenerative medicine, however a detailed overview is out of the scope of this thesis, but these applications are discussed in the following reviews (del Carmen Ortuño-Costela et al., 2019; Singh et al., 2015; Wiegand and Banerjee, 2019).

Endoderm development and hepatic specification

Embryonic development starts with a set of pluripotent stem cells within the ICM, which differentiate to form the epiblast and then during gastrulation (Figure 1) the three germ layers (ectoderm, endoderm and mesoderm) are formed (Kiecker et al., 2016; Sharma et al., 2022). The definitive endoderm arises through an epithelial to mesenchymal transition (EMT) and is regulated by the nodal growth differentiation factor (NODAL) and wingless-type mmmtv integration site family (WNT) signalling pathways. The definitive endoderm folds to create the gut tube which can be separated into fore-, mid- and hind-gut (Sheaffer and Kaestner, 2012) with the ventral foregut being the main site of hepatogenesis (Zorn and Wells, 2009). The septum transversum mesenchyme, together with the cardiac mesoderm, signals to promote the initial phase of hepatic induction, leading to the formation of hepatoblasts, giving rise to the parenchymal cells (hepatocytes and cholangiocytes) of the liver (Harrison et al., 2021; Rossi et al., 2001).
Primary hepatocytes and 3D models

Access to human hepatocytes is restricted because high quality livers are destined for transplantation. This is further compounded with an inability to culture human primary hepatocytes long term, even with numerous attempts to improve culture conditions (Hewitt et al., 2007) to compensate for loss of metabolic function and longevity (Bell et al., 2016; Giwa et al., 2017). For example, a sandwich approach that utilises two layers of gelled extracellular matrix protein (hydrated rat tail tendon collagen matrix) has had some success prolonging the culture life span, together with new media compositions (Dunn et al., 1991; Hewitt et al., 2007; Pascussi et al., 2000a; Pascussi et al., 2000b). While the expansion of human primary hepatocytes in vitro (Michailidis et al., 2020; Zhang et al., 2018) through their amplification as liver progenitors is possible, it does not deliver the complexity of the
liver, because it is only one cell type (Garnier et al., 2018). An alternative model is the cell line HepG2, isolated from a hepatocellular carcinoma (Knowles et al., 1980), and has been used to study oncogenesis, and cytotoxicity testing of substances (Arzumanian et al., 2021). However when compared to normal hepatocytes, HepG2 utility is limited by its weak expression of the cytochrome P450 enzymes (CYP450) superfamily, hampering the assessment of phase 1 xenobiotic oxidation in the liver after drug exposure (Gerets et al., 2012; Westerink and Schoonen, 2007). Another possible replacement for primary hepatocytes is the cell line HepaRG (Gripon et al., 2002), established from a liver tumour associated with chronic Hepatitis C, it is a human bipotential progenitor cell line, which can give rise to both hepatocytes and cholangiocytes (Marion et al., 2010). CYP450 activity can be induced in this line, and it has been proven to be a better screening substitute for primary hepatocytes than HepG2 (Gerets et al., 2012). On the other hand, this cell line does have karyotypic abnormalities, due to being isolated from a liver tumour. In addition, transcripts and basal activity of the non-inducible cytochrome P450 2D6 (CYP2D6) are at the limit of detection suggesting that HepaRG cells are derived from a CYP2D6 poor metabolizer patient (Guillouzo et al., 2007).

2-dimensional (2D) systems, such as monolcultures of hepatocytes or hepatic cell lines, do not reflect the complexity of the liver, such as an oxygen gradients, nutrient distribution, and cell to cell signalling. Hepatocytes have a fixed polarity, apical and basal. Polarity is crucial for hepatocytes to perform many diverse functions, such as canalicular bile secretion and sinusoidal secretion of serum proteins into blood, a loss of hepatocyte polarity is a characteristic of many diseases including cholestasis (Wang and Boyer, 2004). The polarization of hepatocytes is initiated by cell–cell or cell–extracellular matrix contacts and the widely used monolayer culture (2D) of hepatocytes, does not provide the extracellular matrix (ECM), which contributes to the three-dimensional setup of the hepatocytes (Huang et al., 2020; Wang and Boyer, 2004). It should further be mentioned that a monolayer cultures allow hepatocytes to migrate with no constraints. Additionally, cell adhesion molecules, which support binding to the ECM, would usually act as biosensors, enabling the hepatocyte to change behaviour based on their environment, but in monolayer, adhesions are fixed (Hintermann and Christen, 2019). Substrate stiffness is sensed by the adhesion molecules, and has an impact on the liver, e.g. it was shown that the stiffness of a cirrhotic liver was higher than that of a normal liver, indicating that a high substrate stiffness is not an ideal culture setting for hepatocytes, underscored by the findings that matrix stiffness and shear stresses can modulate hepatocyte functions in a fibrotic liver sinusoidal model (Li et al., 2021). Modulating substrate stiffness has been proposed to improve hepatocyte culture (Cozzolino et al., 2016), but for investigating disrupted wound healing in the liver and fibrogenesis (a continuous scarring of the liver tissue) with extensive deposition of ECM, not only the hepatocytes are implicated, but other cell types resident in the liver (Hintermann and Christen, 2019). This underscores the need for more complex models.

To address the shortcomings of the above mentioned models / approaches, different 3-dimensional (3D) systems have been developed to attempt to mimic organ characteristics. These include 3D aggregates containing a single cell type or a mixture of cells, termed spheroids, which are either derived from terminally differentiated cell(s) or stem cells undergoing the differentiation process in a sphere, and so called organoids. Harrison et. al. defined an organoid as something that undergoes a developmentally relevant process, while changing over time in an autonomous manner resulting in self-organized heterogeneity in a 3D structure from stem cells (Harrison et al., 2021) and different examples of available organoid models will be given in the next sections.

Comparison of different liver organoid models
A thorough overview of different types of liver organoids is presented in a review from Harrison et. al, 2021, but in order to assess the liver organoid model developed in this thesis, an overview of the current organoid landscape will be given, in the context of their make-up, cell types and complexity, and their limitations.

Currently available 3D hepatic culture models can be categorized in four groups illustrated in Figure 2. These include aggregates of cells containing only a single cell type such as hepatocytes (Ogawa et al., 2013); aggregates/condensates of different cell types (Coll et al., 2018), for example cells representing endoderm, mesenchymal stem cells (MSCs) and human umbilical vein endothelial cells (HUVECs) (Takebe et al., 2015; Takebe et al., 2013); hepatobiliary organoids containing cholangiocytes, bile canaliculi and hepatocytes but have no mesenchyme (Wu et al., 2019a); and epithelium organised in a sphere (cyst) with only one layer of cells, consisting of hepatic stellate cells, kupffer cells, and hepatocytes surrounding a lumen on the inside, but are not organised in a liver like architecture i.e. lack of sinusoid, no space of Disse, no vasculature or evidence of zonation (Ouchi et al., 2019). The current organoid landscape will be described in detail below.

An example of hepatocyte aggregates (single cell type aggregate) is the work of Ogawa and colleagues, they combined 3D aggregation and cyclic adenosine monophosphate (cAMP) signalling to enhance the maturation of hiPSC-derived hepatoblasts to a hepatocyte-like population (Ogawa et al., 2013). They were able to show that their aggregates had expression profiles and metabolic enzyme levels...
comparable to primary human hepatocytes and that the activation of the cAMP pathway within the 3D aggregates changed the gene expression profile, indicative of maturation of the hepatic lineage (Ogawa et al., 2013).

There have been a number of publications describing mixed cell aggregates, a good example is the work of Coll and colleagues, where they mix hiPSC derived hepatic stellate cells (HSCs) with the immortal bipotential hepatoma cell line HepaRG (Gripon et al., 2002) to create spheroids containing HSCs along with hepatocytes and cholangiocytes (Coll et al., 2018). The resulting aggregates contained HSCs, which could store retinol, and be activated leading to deposition of ECM, indicative of mature HSC function (Coll et al., 2018). The functionality of the HSCs was underscored by increased gene expression of fibrogenesis markers and pro-collagen type 1 secretion upon acetaminophen (APAP) treatment. APAP is metabolized by the hepatocytes through cytochrome P450 2E1 (CYP2E1) and Cytochrome P450 3A4 (CYP3A4) to the toxic intermediate N-acetyl-p-benzoquinoneimine (NAPQI), demonstrating the application of the model for both fibrotic response and toxicity testing. The downsides to this model was the use of the HepaRG cancer cell line and the lack of organisational structure, such as the space of Disse in which the HSCs would normally reside, along with the absence of immune cells which contribute to inflammation due to their secretion profile (Coll et al., 2018; Harrison et al., 2021).

A different approach developed by Takebe and colleagues, leveraged off organ bud formation, by combining iPSCs derived progenitors or primary samples with endothelial and MSCs (Takebe et al., 2015). This combination of cells when mixed together condensed to form liver buds, driven by the inclusion of the MSCs. In a further refinement of the protocol, Takebe’s laboratory was able to develop an early hepatic endoderm stage with broad hepatocyte function, including albumin secretion, metabolism, and functional vasculature. Additionally, this approach was scalable and could be further matured by transplantation into mice (Takebe et al., 2017). However the requirement for transplantation for maturation and a lack of immune cells, such as kupffer cells and HSCs, limits its in vitro applications.

Wu and colleagues generated a hepatobiliary organoid model (Wu et al., 2019a), by simultaneously inducing endoderm and mesoderm in the dish. This was achieved by delaying early hepatic differentiation and driving biliary specification by activation of neurogenic locus notch homolog protein 2 (NOTCH2) and transforming growth factor beta (TGFβ) pathways. They were able to produce hepatocyte and cholangiocyte populations leading to the formation of hepatobiliary organoids through the addition of cholesterol and a cocktail of small molecules. This model contained biliary epithelial cells that had bile acid accumulation in cystic structures. A limitation was the functional level of hepatocytes, which were comparable to fetal liver. However, there was no requirement for exogenous supportive cells or ECM, making this model more cost efficient.

Another hepatobiliary organoid protocol was developed by Guan and colleagues, starting from iPSCs they recapitulate embryonic development, deriving hepatic organoids (HO) which contained hepatocytes and cholangiocytes (Guan et al., 2017). Furthermore, they were organized into epithelia surrounding lumen of bile duct–like structures. This was achieved by first growing them in suspension with ECM, and a cocktail of small molecules and growth factors to pattern foregut endoderm into hepatic and gallbladder cells during organogenesis, followed by maturation until day 20 and the resulting organoid structures were named HO1 (Guan et al., 2017). Organoid size is in general limited by oxygen supply, therefore Guan and colleagues decided to dissociate their organoids, and reseed them in matrigel for 6 days in proliferation medium, followed by 6 days of differentiation. This unorthodox approach yielded secondary HOs (HO2s) with luminal structures expressing hepatic markers such as hepatocyte nuclear factor 4 alpha (HNF4α) and cytokeratin 18 (CK18). This model is
of particular interest because of the ability to generate secondary organoids, thus allowing both expansion and long-term maintenance due to the presence of a progenitor population. They also displayed key hepatocyte functions, such as bile acid and albumin secretion, and CYP450 metabolism, being further supported by transcriptome data showing similarity to both primary human hepatocytes and liver tissue.

Ouchi and colleagues succeeded in developing liver organoids containing both parenchymal and non-parenchymal cell types, creating multi-cellular human liver organoids, containing hepatocyte-, HSCs, cholangiocyte- and kupffer-like cells that exhibited a transcriptional resemblance to in vivo-derived liver tissue (Ouchi et al., 2019). They further demonstrated CYP3A4 expression, vitamin A storage in the HSCs and their activation. Activation was achieved by treatment with lipopolysaccharide, which acts through Toll-like receptor 4 (TLR4), by enhancing the effect of TGFB (Harrison et al., 2021; Pradere et al., 2010) and exemplifying a capacity for an inflammatory response. Of particular interest was treatment with oleic acid, leading to lipid accumulation and the production of triglycerides, causing hepatic ballooning, considered a pathologic hallmark of steatohepatitis activity. A downside of this model was the fetal-like hepatocyte functional activity, even though the transcriptome data indicated a resemblance to liver tissue and also a questionable low number of kupffer cells.

Another source of organoids is primary tissue, Clevers and colleagues designed a protocol for the generation of adult hepatic organoids, consisting of hepatocytes and cholangiocytes. Their starting material was an epithelial cell adhesion molecule positive (EPCAM+) biliary epithelial cell population (Huch et al., 2015b). These organoids were clonally derived, expandable and could be maintained for several months in culture while maintaining genomic stability which is an advantage over iPSC derived organoids (Bayart and Cohen-Haguenauer, 2013; Huch et al., 2015a). Unfortunately this approach requires access to donated liver tissue, cultivation with matrigel and the use of growth factors is far from being cost efficient. The model application is further limited by the absence of non-parenchymal cells. The thorough overview of applications of primary material is outside the scope of this thesis, but for an in depth description, the reviews of Lewis et. al. and Peng et. al. are recommended (Lewis et al., 2021; Peng et al., 2021).

**General thoughts on liver model selection**

It is clear from this multitude of presented approaches, there are different possibilities and drawbacks depending on how 3D structures are generated, as well as the cell types present and input cell type origin. Coll and colleagues approach delivers a spheroid model containing iPSC derived HSCs and HepaRG derived hepatocytes with no discernible tissue-like organization (Coll et al., 2018). These spheroids can be used for toxicity tests and assessing fibrosis response due to the presence of HSCs that can be activated and produce ECM. Unfortunately the use of the HepaRG line is a limitation, while other iPSC lines can be used to differentiate the HSCs, the HepaRG will remain the same, limiting the investigation of the role of single nucleotide polymorphisms (SNPs) with regards to phase 1 and 2 metabolism or the overall genetic background.

The organoid model developed by Huch and colleagues utilised an EPCAM+ biliary epithelial cell population, which in turn could be differentiated to cystic organoids containing only the parenchymal cells of the liver, hepatocytes or cholangiocytes, but importantly no non parenchymal cell types (Huch et al., 2015a). This was also the case in the study of Wu and colleagues who produced a hepatobiliary model without any non-parenchymal cell types (Wu et al., 2019a). A general difficulty in the generation of organoids containing HSCs, is their activation in routine culture, as described by Guan...
and Ouchi (Guan et al., 2017; Ouchi et al., 2019). Takebe’s liver bud approach on the other hand contains no biliary or kupffer cells (Takebe et al., 2015). But it is important to take a step back and appreciate that all these protocols were developed for a specific application, highlighting the importance of model choice for the research question to be answered.

The scope of this thesis was to provide the building blocks to investigate the fat-liver axis in the context of the metabolic syndrome and NAFLD, and the following section will give an overview of this disease area but also explain the necessity for the presence of both parenchymal and non-parenchymal cell types, and explain the contribution of HSCs to the disease state.

**Non-alcoholic liver disease**

Metabolic syndrome is an accumulation of several disorders, together raising the risk of an individual developing atherosclerotic cardiovascular disease, insulin resistance, and diabetes mellitus (Swarup et al., 2021) and even though NAFLD is not one of the defining criteria for metabolic syndrome, it is a common hepatic manifestation (Williams, 2015). NAFLD is defined as the presence of ≥5% hepatic steatosis, in the absence of other competing liver diseases (Younossi et al., 2016). Among those disease etiologies are viral hepatitis and chronic liver diseases, such as autoimmune hepatitis, hemochromatosis, and Wilson’s disease. NAFLD has become the most common chronic liver disease globally, it is associated with a high risk of progression to non-alcoholic steatohepatitis (NASH), liver cirrhosis and hepatocarcinoma (Rojano et al., 2022). A characteristic of NAFLD is triglyceride accumulation in the cytoplasm of hepatocytes as a result of an imbalance between lipid input and output, initiated by increased free fatty acid uptake from the circulation, due to increased lipolysis from adipose tissue and/or from the diet in the form of chylomicrons (Berlanga et al., 2014). Also increased glucose and insulin levels in response to carbohydrate intake, promotes de novo lipogenesis (DNL), leading to further lipid accumulation. Another driver is decreased fatty acid mitochondrial oxidation along with decreased triglyceride hepatic secretion (Berlanga et al., 2014).

The accumulation of lipids, together with fatty acid oxidation increases reactive oxygen species (ROS) and cytotoxic lipid metabolites, causing cellular damage and inflammation (Zisser et al., 2021). The liver resident immune cells, the kupffer cells, secrete pro-inflammatory cytokines, which increase inflammation and further add to hepatic stress. HSCs account for 5%–8% of all liver cells and reside in the space of Disse. Upon inflammation the HSCs become activated, causing them to differentiate to a myofibroblast like phenotype, with associated loss of lipid droplets. In addition, they express contractile fibres, and have increased cell proliferation and deposition of fibrous ECM (Zisser et al., 2021). It has been accepted in the field that HSCs are activated as a reaction to pro-inflammatory signals such as TGFβ, but the exact mechanism is not fully understood (Fan et al., 2019). However, HSCs also secrete TGFβ and express the receptor, indicating autocrine signalling contributes to their activation (Kamm and McCommis, 2022). HSCs can also activate T cells by acting like an antigen-presenting cell and take part in the inflammation process in the liver together with hepatic macrophages, T- and B-lymphocytes, Natural killer cells, driving the progression of NAFLD (Kamm and McCommis, 2022; Wang et al., 2022; Winau et al., 2007). Based on the interplay of these different cell types, a model recapitulating NAFLD would need to contain both parenchymal and non-parenchymal cell types and be responsive to lipid treatment. Since NAFLD is usually connected to obesity, it is also closely connected to adipose tissue function. Obesity changes the secretion profile of the adipose tissue to a pro-inflammatory profile, with decreased adiponectin, and increased leptin secretion.

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Tumour necrosis factor alpha (TNFα) secretion increases and macrophage infiltration into the adipose tissue, leads to increased local inflammation, which in turn reduces insulin sensitivity both in adipose tissue and systemically (Parker, 2018; Surmi and Hasty, 2008), discussed in more detail in a later paragraph.

Parenteral nutrition associated liver disease

Parenteral nutrition associated liver disease (PNALD) shares characteristics with NAFLD, and in light of the fact that it takes up to two years for a human liver to mature (Beath, 2003), an organoid model expressing glutamine synthetase (GS), which is absent from fetal hepatocytes, indicating that the organoids are at a developmental stage past in utero, delivers a unique application for the investigation of PNALD. The following paragraph will explain the occurrence of PNALD and its parallels with NAFLD, highlighting why testing how organoids respond to lipids in the setting of PNALD is an important checkpoint, before giving the green light to increase the experimental complexity by adding adipose tissue /spheroids to the culture system.

Parenteral nutrition (PN) is used as nutrient source for infants suffering gastrointestinal failure, providing nutrition intravenously (Kapoor et al., 2019; Mundi et al., 2016), but some infants receiving PN can suffer from PNALD. The three main types of PNALD are steatosis, cholestasis, and gallbladder sludge/ stones (Nowak, 2020). It is proposed that the lipid emulsion composition has an effect on the outcome of the PN treatment, especially soybean oil based lipid emulsion, which contain high levels of ω-6 fatty acids, a polyunsaturated fatty acid (PUFA), and phytosterols. The rationale is that ω-6 fatty acids are thought to be pro-inflammatory, thus a potential driver of the liver injury (Nowak, 2020). Additionally, high levels of PUFAs and a high ω-6/ω-3 ratio may cause an increased incidence of cholestasis, steatosis, and sepsis (Madnawat et al., 2020). There are some similarities between PNALD and NAFLD, e.g. NAFLD is initiated through fat infiltration of the liver (steatosis), which can progress to NASH, presenting with inflammation, hepatocyte ballooning and/or fibrosis (Lambert et al., 2014). Since PNALD also begins with steatosis, which can manifest in fibrosis, cirrhosis, and biliary changes such as cholestasis (Murillo et al., 2015), some mechanistic similarities would be expected. Currently, there are no physiologically relevant in vitro cell models available to determine potential PN effects on the new born liver, making PN treatment a good starting point for the investigation of organoid response to different lipids, as discussed in detail in the discussion section of paper III.

The adipose tissue and its secretory properties

Since NAFLD is connected to the presence of obesity, it is also closely connected to adipose tissue function. The following section will summarize adipose tissue characteristics and its implication for the disease state.

Adipose tissue acts as a complex organ that can comprise more than 40% of the total body composition (Ikeda et al., 2018). It is not only an energy depot for lipids, it also serves as a crucial integrator of glucose homeostasis. With the global rise in obesity, this area has gained more attention (Rosen and Spiegelman, 2006). The potential to store lipids is conserved among unicellular and multicellular organisms alike, prokaryotes store lipids in intracellular organelles, the lipid droplet (Driskell et al., 2014; Ottaviani et al., 2011; Zwick et al., 2018). While multicellular organisms have developed specialized cells to store lipids, the adipocytes. In the whole organism the anatomical location of fat
tissues varies between species, but mammals, birds, amphibians, and reptiles all have adipocytes (Rosen and MacDougal, 2006).

Even though adipocytes are the main component of the adipose tissue, providing crucial functions like energy storage and endocrine activity, the adipose tissue also houses pre-adipocytes, fibroblasts, vascular cells, nerves, and immune cells, which belong to the stromal vascular fraction (SVF). The blood vessels (endothelium) are surrounded by stromal and/or vascular smooth muscle cells. This includes the adipose stem/progenitor cells (ASCs), and larger blood vessels associated with vascular smooth muscle cells. Blood vessels in the adipose tissue distribute nutrients, oxygen, and adipokines, while vascular cells are responsive to these factors (Gil et al., 2011; Kershaw and Flier, 2004).

Sources of mature primary adipocytes include direct harvesting from donors or differentiated from primary ASCs harvested from the SVF from individuals undergoing surgery or liposuction. However, these depots might be accessible once during surgery, thus providing only a limited amount of material. Also it has been shown that marker expression of isolated ASCs changes during the culture period (Peng et al., 2020) making the selection of primary material as a control for iPSC derived cells more complex in regard to their surface marker expression profile. Therefore stable marker expression might require further refinement of the established culture conditions.

Bone marrow mesenchymal stem cells (BM-MSCs) and ASCs share many characteristics, such as the ability to differentiate to bone and cartilage in vivo (Cawthorn et al., 2012) as well as expression of key surface markers. An issue is that different marker profiles have been suggested for subpopulations of ASCs, Yoshimura et al. designated ASCs as a CD34 molecule (CD34)/ platelet endothelial cell adhesion molecule (CD31)/ protein tyrosine phosphatase receptor type C (CD45)/ Thy-1 cell surface antigen (CD90)/ endoglin (CD105)/ melanoma cell adhesion molecule (CD146) subpopulation of stromal vascular cells (SVCs) (Yoshimura et al., 2006), while recent publications show that CD34 expression is lost with passaging (Scherberich et al., 2013). The BM-MSC marker profile has been discussed in the field for some time (Camilleri et al., 2016; Lv et al., 2014; Mabuchi et al., 2021) and their subpopulations have been studied for different applications, such as tissue engineering, and especially orthopedic applications (Pérez-Silos et al., 2016). It has been demonstrated by flow cytometry analyses of BM-MSC and ASCs, that both cell types express CD13 antigen (CD13), CD29, CD44, CD90, CD105, CD73, and protein marker of mesenchymal stem cells (STRO-1). But differences in the expression of antigen CD49D (CD49d), CD54 antigen (CD54), CD34 and CD106 antigen (CD106) between the two cell types (De Ugarte et al., 2003; Hass et al., 2011) were reported. This highlights the importance of surface marker expression profiling for cell characterization. Another compounding issue for the observed differences between BM-MSCs and ASCs could be due to differences in isolation process, the region of isolation in the human body, and culture protocols (Strioga et al., 2012). The number of stem cells which can be obtained from adipose tissue is much higher than from bone marrow, one gram of adipose tissue harvested by liposuction yields approximately 5000 putative stem cells, which is 500 times greater than the number of stem cells isolated from 1 g of bone marrow (Brown and Katz, 2019; Fraser et al., 2006). This makes the adipose tissue an ideal source for stem cells, since fat depots are more readily available than bone marrow and the stem cell yield is higher.

An important feature of the adipose tissue is that it can react to hormone signals and the central nervous system, by secreting adipokines, such as leptin, visfatin, and adiponectin, that can act locally or systemically (Kershaw and Flier, 2004; Khan and Joseph, 2014). Identified in 1994 by Dr. Friedman, leptin is an important adipokine (Mechanick et al., 2018; Vegiopoulos et al., 2017). It regulates lipid metabolism, energy expenditure, and even though leptin levels increase in patients with obesity, most patients develop leptin resistance (Vegiopoulos et al., 2017). For visfatin it has been shown that it can cause inflammation in adipocytes, and induce insulin resistance (Kumari and Yadav, 2018). While
adiponectin, discovered in 1995, is a key regulator of insulin sensitivity and inflammation (Whitehead et al., 2006), circulating at high concentrations in the blood (Scherer et al., 1995) (Kershaw and Flier, 2004). Different molecular weight forms of adiponectin exist due to multimerisation, low-, middle-, high-molecular forms and high-molecular weight (HMW) complexes are thought to be of special importance for the liver (Gamberi et al., 2018). Adiponectin increases insulin sensitivity and previous studies have shown that serum adiponectin is inversely related to body fat mass in people, while diabetic (Type II) patients have even lower levels of adiponectin (Gavrila et al., 2003).

Different types of adipose tissue exist, the white adipose tissue (WAT) and the brown adipose tissue (BAT) (Cinti, 2005; Gil et al., 2011). They differ both at the morphological and functional levels, WAT accumulates excess energy mainly in the form of triacylglycerols, while BAT dissipates energy as heat, using uncoupling protein-1 (UCP-1) to uncouple electron flux and adenosine triphosphate (ATP) synthesis in the mitochondrial respiratory chain (Gil et al., 2011). For many years, BAT was thought to be only present in human newborns and rodents, but fluorine-18 fluorodeoxyglucose positron emission tomography/computed tomography revealed the presence of BAT in adults. This might be because functional BAT in adult humans is only located in a few areas of the body, namely the neck, supraclavicular, mediastinal and interscapular areas (Gil et al., 2011; Zwick et al., 2018). Brown adipocytes contain multilocular lipid droplets and high numbers of mitochondria (Zwick et al., 2018). There is a third group of adipocytes that resides in the WAT depots, the beige / brite adipocytes, they are an inducible form of the thermogenic adipocytes (Min et al., 2016b). Since thermogenic activity of adipose tissue inversely correlates with obesity and obesity related diseases, the activation of brown and beige adipocytes has gained more attention in obesity research (Guénantin et al., 2017).

BAT secretes multiple peptides and protein factors, however it is not clear if the brown fat has a similar extensive secretome as WAT (Carobbio et al., 2019), but leptin and interleukin 6 (IL-6) are known to be secreted by both (Almudena Gómez-Hernández and Sabela Díaz-Castroverde, 2016). The secreted factors can enhance or inhibit thermogenic activity, among the enhancers are bone morphogenetic protein 8b (BMP8b), endothelin-1, fibroblast growth factor 21 (FGF21), and IL-6.

There are two major types of WAT, the subcutaneous (s) and the visceral (v) WAT (intra-abdominal WAT) (Ouchi et al., 2011). Interestingly, it has been shown that obese patients and diabetics who have large amounts of vWAT, also suffer from insulin resistance and other health issues, like hypertension and dyslipidemia, which contribute to high rates of mortality. However, sWAT seems to have health promoting effects, despite large amounts in obese patients (Bluher, 2012; Ouchi et al., 2011; Tran et al., 2008). One explanation for the different health outcomes for sWAT and vWAT could be that visceral fat produces /releases substances that cause metabolic abnormalities, such as cardiovascular disease systemic inflammation and diabetes, as visceral adipocytes cannot store as much triglycerides as subcutaneous adipocytes (Jensen, 2008). It has further been shown that vWAT has more innervations and contains larger numbers of inflammatory and immune cells. sWAT also has a better differentiation efficiency, while vWAT is more metabolically active and more sensitive to lipolysis, thus more insulin resistant (Ibrahim, 2010). The fact that sWAT adipocytes can accumulate more triglycerides, and grow bigger because of decreased lipolysis, compared to vWAT, adds to the health detrimental profile of the vWAT, making it a predictor for greater mortality than sWAT (Fang et al., 2015). It is acknowledged that the size of an adipocyte determines its secretory status (Stenkula and Erlanson-Albertsson, 2018). During the onset of obesity, not only does the cellular composition change, but also adipokine secretion changes, affecting not only the function of the adipocyte, but also the cross-talk with other organs, such as the liver and muscles (Ouchi et al., 2011; Vegiopoulos et al., 2017).
Developmental origins of adipocytes

Adipocytes developmentally originate from different sources. It is generally accepted, that WAT originates from the mesoderm (Berry et al., 2013; Duan et al., 2007), which is organised in different segments, the paraxial, somatic and splanchnic mesoderm (Figure 3) (Sebo and Rodeheffer, 2019). These different segments have been investigated for their ability to give rise to adipocytes, but more specifically if the derived adipocytes are white, brown or beige/brite.

The paraxial mesoderm has been pinpointed as a source of brown adipocytes in mice, interestingly interscapular BAT is derived from paraxial mesoderm precursors which are positive for myogenic factor 5 (Myf5), Pax7 (paired box 7), and Pax3 (paired box 3) (Altshuler-Keylin et al., 2016; Brown, 2020; Roh et al., 2018). However the paraxial mesoderm is not exclusively a source of brown adipocytes, (Sanchez-Gurmaches et al., 2012; Shan et al., 2013), David Guertin and colleagues showed through lineage tracing experiments, that mesenchymal precursors expressing Myf5 also gave rise to a subset of white adipocytes, further supported by Tizhong Shan’s findings in 2013 (Shan et al., 2013).

The lateral plate mesoderm, more specifically the somatic mesoderm gives rise to white adipocytes, stemming from mural cells (smooth muscle cells and pericytes of the vasculature)(Lin et al., 2021). These cells reside in the adipose vasculature, and are derived from Myf5 negative precursors of the lateral plate mesoderm (Hassan et al., 2012; Tang et al., 2008). Lineage tracing studies also revealed that a Wilms tumour 1 (Wt1⁺) precursor population present within the lateral plate mesoderm generated a significant portion of all visceral WAT depots, whereas there was no Wt1⁺ precursor contribution to either BAT or sWAT (Brown, 2020; Chau et al., 2014).

Figure 3: Embryonic origins of adipocytes, modified from Aron Brown, 2020. Lineage tracing studies in mice demonstrate that white and brown adipocytes arise from different mesodermal layers during development, including the lateral plate (visceral white) and paraxial mesoderm (brown) and from the neural crest.
Aron Brown and colleagues developed a protocol to derive beige adipocytes from hiPSCs (Su et al., 2018). They utilised a commercially available serum-free mesoderm induction medium (StemDiff™), which is highly specific for generating forkhead box f1 (FOXF1)⁺ precursors. FOXF1⁺ precursors are common to the splanchnic layer within lateral plate mesoderm during embryonic development. The splanchnic mesoderm gives rise to different cell types, including the pericytes and vascular smooth muscle cells which are accepted as precursors of beige adipocytes (Brown, 2020; Mahlapuu et al., 2001; Su et al., 2018; Vishvanath et al., 2017; Wang and Seale, 2016). FOXF1⁺ cells were cultured in serum-free commercial MSC medium (MesenCult™-ACF), the exact medium composition is not known, but highly specific for the generation of beige adipocyte precursors (Brown, 2020), since it couldn’t be substituted with other MSC media without losing the beige phenotype.

Several studies using chick-quail chimeras and CRE-dependent cell marking studies in mice have shown that adipocytes can also originate from the neural crest (Berry et al., 2013; Billon et al., 2007; Le Lièvre and Le Douarin, 1975). It has been speculated that since PAX3 plays a role in brown versus white adipocyte fate determination, the possibility exists that in addition to white adipocytes from the neural crest, brown adipocytes could be derived from PAX3⁺ neural crest (Brown, 2020). A good review on lineage tracking experiments in mice was published by Sebo and Rodeheffer in 2019 for more detailed descriptions on adipocyte specification please see (Sebo and Rodeheffer, 2019).

Existing protocols and strategies for the adipocyte differentiation

A variety of protocols to generate adipocytes have been published, often as proof of concept to validate iPSC derived MSCs (iMSCs). The characterization of the derived adipocytes was mainly by gene expression levels of peroxisome proliferator activator receptor gamma (PPARγ), the master regulator of adipogenesis, fatty acid binding protein 4 (FABP4) which maintains glucose homeostasis (Prentice et al., 2019) and the accumulation of fat droplets validated by oil red O or bodipy staining. There are several possibilities to differentiate adipocytes, either from the mesoderm or the neural crest cell (NCCs) population as discussed above (Billon and Dani, 2012). The majority of protocols to differentiate iPSCs to are based on protocols to generate iMSCs because of the adipocyte`s developmental origin, the mesoderm. The following paragraph will therefore give an overview of currently available differentiation approaches to differentiate iPSCs to iMSCs, to provide an understanding of the complexity and effort of these protocols and how these protocols were tailored to adipocyte differentiation.

Protocols for iPSC derived MSCs

MSCs are of great interest for a variety of reasons, including its potential to differentiate to osteocytes, chondrocytes and adipocytes. Many protocols focus on the differentiation of MSCs from iPSCs and perform adipogenesis to confirm their tri-lineage potential. It is of importance to note, that these protocols deliver a variety of cells with different characteristics, often referred to as “pre-MSCs” or MSC-like cells, as will be shown with different examples below. The caveat is that these differentiated MSCs are supposed to serve as an intermediate towards adipocytes.

Original differentiation strategies were based on spontaneous differentiation of pluripotent stem cells. This entailed cells being seeded as single cells, and maintained in MSC medium, deprived of pluripotent signals, with serial passaging until they expressed MSC markers (Hynes et al., 2016). Hynes and colleagues observed a high similarity to MSCs with regard to both morphology and marker expression, but exhibited low differentiation potential, particularly toward the adipogenic lineage. Another
strategy utilised embryoid body (EB) formation, for this approach cells were seeded and spontaneously formed 3D structures that committed to the three germ layers. Outgrowing cells were cultured in MSC culture medium, containing 10% fetal bovine serum (FBS) (Hafner and Dani, 2014). Solvig Diederichs and Rocky S. Tuan compared different protocols to produce iMSCs, comparing four different approaches side by side: EB formation, spontaneous differentiation, indirect bone BM-MSC co-culture, and BM-MSC growth medium. The iPSCs used were derived from human BM-MSCs, allowing direct comparison with the original source BM-MSCs (Diederichs and Tuan, 2014). The study revealed that the derived iMSCs exhibited typical MSC morphology, surface marker profiles, and they were capable of differentiation in vitro along the osteogenic, chondrogenic, and adipogenic lineages, but in comparison to BM-MSCs, they were less responsive to traditional BM-MSC differentiation protocols.

Devito, Klontzas and colleagues compared two protocols for their ability to generate MSC and importantly their differentiation potential (Devito et al., 2019). The first protocol (called ARG) was a modification of the spontaneous MSC differentiation, taking 30 days to generate iMSCs. This involved single cell iPSC culture on vitronectin coated plates, in minimum essential medium (MEM) supplemented with 10% human platelet lysate. After 14 days the cells were replated on vitronectin coated plates in aMEM medium supplemented with ROCK inhibitor. This protocol was selected as it had been used successfully by a number of groups (Frobel et al., 2014; Luzzani et al., 2015; Shao et al., 2013). The second protocol employed (named TEX) included the inhibitor of suppressor of mothers against decapentaplegic 2/3 (SMAD2/3), SB-431542, to direct the differentiation. The iPSCs were passaged for 25 days in media supplemented with SB-431542 on vitronectin coated plates, followed by culture on uncoated plastic using standard MSC medium (aMEM supplemented with 10% human platelet lysate). After the differentiation, the cells were sorted for the MSC markers CD73, CD90 and CD105 and only triple positive cells were used. Devito and Klontzas reported that iMSC lines differentiated with TEX had an attenuated response to interferon gamma (IFNG) stimulation, assessed by messenger ribonucleic acid (mRNA) level quantification of indoleamine 2,3-dioxygenase 1 (IDO1), guanylate binding protein 4 (GBP4), and C-X-C motif chemokine 11 (CXCL11), which have been used extensively as markers of IFNG response. The iMSCs obtained by the TEX protocol retained a more stem cell like phenotype, meaning an improved differentiation potential in comparison to the ARG protocol, especially TEX differentiated adipocytes had higher expression of adipogenic markers in comparison to the ARG protocol. However, only a minimal characterization was performed, FABP4 expression and lipid accumulation measured by lipidTOX staining were analyzed, there is no further information about other adipogenic markers or the secretion profile of the differentiated adipocytes. Since the TGFβ signaling family is implicated in the regulation of stemness, the authors speculate that the small molecule SB-431542, which is a TGFβ signaling inhibitor, acts to preserve the stemness (Devito et al., 2019; Sakaki-Yumoto et al., 2013). This work is of special interest because they used two different MSC differentiation protocols (TEX versus ARG), which resulted in iMSCs similar to their parental native MSCs but had entirely different properties when it came to differentiation potential and proliferation.

Protocols for iPSC derived adipocytes

The above protocols aim was to deliver a reliable source of iMSCs for different applications and the differentiation to adipocytes was only a by-product to show proof of tri-lineage capability of the iMSCs.

Nonetheless, the protocols with a focus to specifically produce iPSC derived adipocytes vary in their approaches, mostly because the field is developing in vitro models for different applications, e.g. to differentiate beige or brown adipocytes because of their thermogenic potential and possible
applicability in obesity research. More detailed information on brown adipocyte protocols is discussed in Brown, 2020 (Brown, 2020). However white adipocytes are the majority in the body and therefore the desired type for co-cultivation experiments. It is of note to mention that protocols for white/beige/brown adipocytes vary in how detailed the intermediate cell types were characterized in regard to their differentiation capabilities. The following section will give some examples of the protocols developed to deliver iPSC derived adipocytes as their main goal and their characterization.

<table>
<thead>
<tr>
<th>Study</th>
<th>Technique</th>
<th>Mesoderm induction</th>
<th>Mesoderm markers</th>
<th>MSC phenotype</th>
<th>Adipocyte</th>
<th>Adipocyte induction</th>
<th>Adipokine secretion</th>
<th>Transferable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taura et al. 2009</td>
<td>EB outgrowth</td>
<td>ES cell culture medium w/out FGF2</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Insulin, IBBX, DEX, Indo and PPARgamma</td>
<td>not tested</td>
<td>adherent</td>
</tr>
<tr>
<td>Ahfeldt et al. 2012</td>
<td>EB outgrowth and transgene</td>
<td>DMEM, 15% FBS</td>
<td>GSC/TBX3</td>
<td>CD73 and CD105</td>
<td>white</td>
<td>Insulin, DEX, Rosiglitzone, and PPARgamma</td>
<td>adiponectin and leptin secretion, ELISA of cell medium</td>
<td>adherent</td>
</tr>
<tr>
<td>Ahfeldt et al. 2012</td>
<td>EB outgrowth and transgene</td>
<td>DMEM, 15% FBS</td>
<td>GSC/TBX3</td>
<td>CD73 and CD105</td>
<td>brown</td>
<td>Insulin, DEX, Rosiglitzone, and CEBPB transgene</td>
<td>not tested</td>
<td>adherent</td>
</tr>
<tr>
<td>Nishio et al. 2012</td>
<td>EB outgrowth</td>
<td>B3G, L6 and IGF-II, BPEL medium BMN, VEGFA, KITLG, F8L, FLT3LG</td>
<td>CD90, CD29, integrin beta 1</td>
<td>CD90, CD29, integrin beta 1</td>
<td>beige</td>
<td>Insulin, DEX, th1-1 cell surface antigen; DMEUM, dubecco’s modified eagle medium; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; F-12, Ham's F-12 Nutrient Mixture; FGF21, fibroblast growth factor 21; FLT3L, Fms-related tyrosine kinase 3 ligand; FOXF1, forkhead box f1; GSC, Goosocoid homeobox; HOX11, homeo box 11; IGF-2, Insulin-like growth factor 2; IL-6, interleukin 6; Indo, indomethacin; KITLG, KIT ligand; KSR, knockout serum replacement; LY6E, lymphocyte antigen 6 family member e; MESP-1, mesoderm posterior blh transcription factor 1; Myf5, myogenic factor 5; NG2, neuron-glial antigen 2; NRG4, neuregulin 4; PAX3, paired box 3; PAX7, paired box 7; PDGFRa, platelet derived growth factor receptor alpha; PDGFRB platelet-derived growth factor receptor beta; STEMPro34 HSC, STEMPro34 hematopoietic stem cell medium; T3, triiiodothyronine; TBOX, t-brachyury homolog; VEGFA, vascular endothelial growth factor A.</td>
<td>not tested</td>
<td>adherent</td>
</tr>
<tr>
<td>Guénanin et al. 2017</td>
<td>Monolayer</td>
<td>STEMPro34 HSC medium + BMP4, Activin A</td>
<td>TBOX and MESP-1</td>
<td>PDGFRa, CD44, CD29, and CD40</td>
<td>beige</td>
<td>Insulin, IBBX, DEX, Rosiglitzone, SB431542, ascorbic acid, hydrocortisone, and FGF</td>
<td>not tested</td>
<td>adherent</td>
</tr>
<tr>
<td>Su et al. 2018</td>
<td>Monolayer</td>
<td>STEMDiff Mesoderm induction medium</td>
<td>FOXF1, BAP1, and HOX11</td>
<td>PPARgamma, CD20, CD29, and CD40</td>
<td>beige</td>
<td>Insulin, IBBX, DEX, Indo, T3, Rosi, SB431542, ascorbic acid, hydrocortisone, and FGF</td>
<td>not tested</td>
<td>adherent</td>
</tr>
</tbody>
</table>

Table 1: Overview modified from Aron Brown: Adipokine secretion was added to demonstrate the lack of characterisation and relevance of the differentiation protocol. Abbreviations: ADIPOQ, adiponectin; BAPX1, bagpipe homebox protein homolog 1; BMP4, Bone morphogenetic protein-4; BPEL, bovine serum albumin (BSA) polyvinylalcohol essential lipids medium; CD105, endoglin; CD146, melanoma cell adhesion molecule; CD29, integrin beta-1; CD44, hematopoietic cell e- and l-selectin ligand; CD73, ecto-5'-nucleotidase; CD90, thy-1 cell surface antigen; DMEUM, dubecco’s modified eagle medium; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; F-12, Ham's F-12 Nutrient Mixture; FGF21, fibroblast growth factor 21; FLT3L, Fms-related tyrosine kinase 3 ligand; FOXF1, forkhead box f1; GSC, Goosocoid homeobox; HOX11, homeo box 11; IGF-2, Insulin-like growth factor 2; IL-6, interleukin 6; Indo, indomethacin; KITLG, KIT ligand; KSR, knockout serum replacement; LY6E, lymphocyte antigen 6 family member e; MESP-1, mesoderm posterior blh transcription factor 1; Myf5, myogenic factor 5; NG2, neuron-glial antigen 2; NRG4, neuregulin 4; PAX3, paired box 3; PAX7, paired box 7; PDGFRa, platelet derived growth factor receptor alpha; PDGFRB platelet-derived growth factor receptor beta; STEMPro34 HSC, STEMPro34 hematopoietic stem cell medium; T3, triiiodothyronine; TBOX, t-brachyury homolog; VEGFA, vascular endothelial growth factor A.

In 2009 the Nakao group described the generation of adipocytes from iPSCs (Brown, 2020; Taura et al., 2009). They utilized EBs in suspension with transient retinoic acid (RA) treatment to generate adherent
Nishio and colleagues published a protocol for brown adipocytes derived from iPSCs using a specific haemopoietin cocktail (HC) without exogenous gene transfer in a base medium composed of 1:1 ratio of Iscove’s modified dulbecco’s medium (IMDM) and Ham's F12, supplemented with bovine serum albumin, synthetic lipids, α-monomioglycerol, insulin-transferrin-selenium, Glutamax, protein-free hybridoma mix, and ascorbic acid-2-phosphate. The HC was composed of KIT ligand/ stem cell factor (KITLG), FMS-related tyrosine kinase 3 ligand (FLT3LG), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) and bone morphogenetic protein 7 (BMP7) and removing the HC components from the base medium favoured an increase in white adipocyte markers. Functionally the brown adipocytes showed respiratory and thermogenic activation by β-adrenergic receptor (AdrRβ) stimulus and augmented lipid and glucose tolerance (Nishio et al., 2012).

Mohsen-Kanson et al looked into the generation of an expandable adipogenic precursor that could generate either brown or white adipocytes, developing a more specified differentiation method (Mohsen-Kanson et al., 2014). EBs were formed from iPSCs and either treated or not with RA between days 3 and 6 of the differentiation. At day 10, the EBs were plated and outgrowths were maintained in proliferative medium until day 20. An adipogenic cocktail was added from day 20 to 30 and after which gene expression profiles were analysed. Cells that were treated with RA showed a white adipocyte gene expression profile; bone morphogenetic protein-4 (BMP4), homeobox protein hox-c9 (HOXC9), homeobox protein hox-c9 (HOXC9), and homeobox protein hox-a5 (HOXA5) while untreated cells expressed a brown adipocyte profile (PR/SET domain 16 (PRDM16), iodothyronine deiodinase 2 (DIO2, and PAX3). They also discovered in functional experiments that ectopic expression of PAX3 converted white adipocyte progenitors to brown adipocyte progenitors, speculating that PAX3 was a new marker for brown adipocyte progenitors.

Guènantin and colleagues developed a protocol to differentiate beige adipocytes from iPSCs with medium supporting hematopoietic differentiation. The prediction was that this would enhance subsequent adipocyte differentiation, since adipogenesis and angiogenesis are interdependent during development (Tang et al., 2008), together with the knowledge that human beige adipocyte progenitor proliferation is connected to capillary networks (Guènantin et al., 2017; Min et al., 2016a). Their protocol started with induction of iPSCs to mesoderm, the culture medium was composed of STEMPro34 (hematopoietic stem cell medium) supplemented with GlutaMAX, ascorbic acid, BMP4, and activin A. After culturing the iPSCs in the above medium for 4 days they expressed platelet derived growth factor receptor alpha (PDGFRα), hematopoietic cell e- and l-selectin ligand (CD44), integrin beta-1 (CD29), and lymphocyte antigen 6 family member e (LY6E). Next they were induced with an adipogenic cocktail (insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and indomethacin) for a further 6 days. The medium was changed on day 10 to Dulbecco’s Modified Eagle Medium / Ham’s F-12 Nutrient Mixture (DMEM/F12) supplemented with 10% fetal calf serum and insulin, after a total culture time of 20 days, the resulting adipocytes were analysed. This protocol was performed
in monolayer (2D), without an embryoid body intermediate, shortening the derivation of adipocytes to 20 days. The adipocytes had a beige phenotype and respiratory function. Unfortunately the authors did not comment on the secretory phenotype e.g. if adiponectin was secreted.

The secretion of adipokines has not been the main focus of any of the aforementioned protocols (Table 1), while they all provide differentiated adipocytes, the focus of this thesis, was to produce adipocytes with an adipokine secretion profile, to provide the tools to address the fat liver-axis and organ cross-talk, which is choreographed by secreted factors. Another important caveat for this thesis was the transferability of the differentiated adipocytes for co-culture applications, which has not gained any attention in any of the other protocols described (Table 1).

The examples described above were picked to give an overview of adipocyte differentiation landscape from iPSCs. While most of the differentiation protocols for iPSCs use similar cocktails as discussed above, they first needed to be translated to the iPSC field from protocols developed for the differentiation of primary ASCs. There are a number of state of the art protocols for differentiation of ASCs to adipocytes available (Ahmad et al., 2020; Rosen and MacDougald, 2006), and an in depth summary by Michelle Scott and colleagues is available for information on the differentiation cocktail compositions (Scott et al., 2011).

How is adiponectin implicated in metabolic syndrome

The following section will outline why adipose tissue is of relevance for the investigation of metabolic syndrome. Obesity changes the secretion profile of the adipose tissue to an inflammatory state, with decreased adiponectin, but increased leptin secretion. Increased TNFα secretion and macrophage infiltration into adipose tissue, leads to elevated local inflammation, which in turn reduces insulin sensitivity both in the adipose tissue and importantly systemically (Parker, 2018; Surmi and Hasty, 2008). As mentioned before, adiponectin does not only increase insulin sensitivity, but previous studies have shown that serum adiponectin is inversely connected with body fat mass in humans, and type 2 diabetic patients having even lower levels of adiponectin (Gavrila et al., 2003), which makes adiponectin a factor of interest in the context of organ crosstalk in disease state. Accordingly it is important to understand, what controls adiponectin secretion/concentration and how it exerts its effect on the liver. It has been acknowledged, that adiponectin`s plasma concentration decreases upon accumulation of visceral fat (Shehzad et al., 2012). Serum levels are also decreased in obese, insulin-resistant, type 2 diabetic rodents, and humans. Furthermore, overexpression of adiponectin has a protective effect on mice fed with high fat diet, preventing hepatic lipid accumulation in rodent models of obesity (Kim et al., 2007; Scherer, 2006). All these findings highlight that adiponectin secretion is vital for metabolic health.

Adiponectin signalling in the liver

Adiponectin signalling in the liver is a complex process and I will share a few examples that are relevant to this thesis. The adiponectin signalling pathway in the liver is regulated by adiponectin receptor 1 (ADIPOR1) and 2 (ADIPOR2), T-cadherin, AMP activated protein kinase (AMPK), and adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) (Combs and Marliss, 2014). Adiponectin itself has two receptors, AdipoR1 and AdipoR2. These receptors have seven-transmembrane domains, but are distinct from the topology of G-protein-coupled receptors, whereas the ADIPOR1 gene encodes for a 375 amino acid protein with an estimated molecular mass of 42.4
kDa, and ADIPOR2 encodes for a 311 amino acid protein of 35.4 kDa. It has been shown that AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for full-length adiponectin. While ADIPOR2 has an intermediate affinity for both (Hug et al., 2004; Shehzad et al., 2012; Yamauchi et al., 2003). Tomita and colleagues were able to demonstrate in a mouse model that inhibition of AdipoR2 signalling increased lipid peroxidation and thereby up-regulated hepatic production of TGFβ at all stages of NASH, while an enhancement of AdipoR2 expression in the liver improved NASH. This underscores the protective role of AdipoR2 signalling in the progression of NASH in mice (Tomita et al., 2008) and demonstrates why the presence of adiponectin secretion is essential for the establishment of co-culture model of liver organoids and adipocytes.

With all this in mind the study of metabolic syndrome requires protocols that give both organotypic and functional organoids/spheroids to allow the study of organ crosstalk. This requires an understanding of the development process of these tissues, and how these developmental stages can be integrated in a differentiation protocol. It is also of importance to have well characterised cells/organoids in the context of their function (drug metabolism, cell composition) and a well characterised secretion profile.

**Key methodologies**

**Cell culture conditions**

All cells were maintained in different sized flasks, dishes or multi-wells at 37°C with 5% carbon dioxide (CO₂) and 100% relative humidity. The cells were kept under these conditions until harvesting. All cell work was carried out under a class 2 laminar flow hood. All media were supplemented with 1% pen-strep (Gibco, 15140122).

**Pluripotent Stem Cell Culture**

The human pluripotent stem cell lines utilised in this study were as follows: the hiPSC line AG27 (reprogrammed using Sendai virus from AG05836B fibroblasts, obtained from Coriell Cell Repositories), Detroit8, CRLs20, CRLR8 (Siller et al., 2015; Siller et al., 2016). hiPSCs were maintained under feeder free conditions on Geltrex (Gibco, A1413201) coated tissue culture plates using Essential 8 (E8) medium made in house as described previously (Siller and Sullivan, 2017) or in commercial E8 (Gibco, Essential 8™ A1517001).

**Cardiomyocyte differentiation**

The protocol for cardiomyocyte differentiation was based on Lian et al., single cells were seeded in E8 supplemented with Y-27632 (Tocris, 1254) at 10µM with a seeding density of 4 x10^4 cells per cm² on Geltrex (Gibco, A1413201) coated plates, the medium volume were 2ml per well for 6-well plates. 24 hours later the cells were washed with Dulbecco’s phosphate buffered saline (DPBS) (Gibco, 14040133) and fed with E8. The next day cells should have reached a confluence of 70-80%, the differentiation was initiated by switching the medium to RPBMI/B27 (2%) without insulin (Gibco, A1895601,) and 8µM CHIR99021 (Tocris, 4423). After 24 hours, the medium was replaced by RPMI/B27 without insulin, but with double the medium volume, 4ml per well in a 6-well plate, 48 hours later the medium was replaced with RPMI/B27 (Gibco, 21875034) without insulin (Gibco, A1895601, 2%), supplemented with 5µM IWP2 (Tocris, 3533) (day 3). Cells were left for 48 hours and then changed to RPMI/B27 without insulin for another 48 hours. From day 7 onwards, the medium was replaced with RPMI/B27 with
insulin (Gibco, 17504044), the cells were expected to beat after 9 days of differentiation. Note that the CHIR99021 concentrations may need to be adjusted depending on cell line.

**Definitive endoderm**

For definitive endoderm differentiation (Mathapati et al., 2016; Siller et al., 2015), hiPSCs were seeded as single cells at a density of 4.75 x 10^4 cells per cm^2 in E8 supplemented with 10μM Y-27632 (Tocris, 1254). When cells reached 30% confluency, the differentiation was initiated by adding RPMI/B27 (Gibco, 21875034) without insulin (Gibco, A1895601, 2%) and 4μM CHIR99021 (Tocris, 4423) for 24 hours, after 24 hours the medium was exchanged to RPMI/B27 alone. Depending on cell line, the CHIR99021 concentrations will need to be optimised and some lines may require insulin supplement.

**Liver organoid differentiation**

For the liver organoid differentiation (Harrison et al., 2020), hiPSCs were harvested by treating with Accutase (Gibco, A1110501) for 5 minutes to obtain single cells. Single cells were resuspended in E8 supplemented with 10μM Y-27632 (Tocris, 1254) and transferred to an Erlenmyer flask on a flat bed shaker in a regular cell incubator at 80 rpm. The next day the differentiation was initiated by changing the medium to RPMI/B27 (Gibco, 21875034) without insulin (Gibco, A1895601, 2%), supplemented with 4μM CHIR99021 (Tocris, 4423) (day 0), after 24 hours of incubation, the medium was changed to un-supplemented RPMI/B27 without insulin (day 1). From day 2 to 7, the culture medium was switched to serum replacement medium/ dimethyl sulphoxide (SR-DMSO) for hepatic specification this medium contained KnockOut™ DMEM (Gibco, 10829018), Knockout Serum Replacement (Gibco, 10828028), Dimethylsulfoxide (DMSO) (Sigma-Aldrich, 472301), GlutaMAX™ Supplement (Gibco, 35050061), MEM Non-Essential Amino Acids Solution (100X) (Gibco, 11140050), 50mM 2-Mercaptoethanol (Life Technologies, 31350-010). The medium was changed every 48 hours. From day 7 till day 21 the organoids were cultured in L-15 Leibovitz base medium (L-15) supplemented with 100nM Dihexa (N-hexanoic-Tyr-Ile-(6) aminohexanoic amide)(Active Peptide, Cat#AP201016; CAS#1401708-83-5) and 100nM Dexamethasone (Tocris, 1126). The L-15 base (Sigma-Aldrich, L5520), contained FBS (Biowest, 51800-500), Trypsese Phosphate Broth Solution (Sigma-Aldrich, T8159), GlutaMAX™ Supplement (Gibco, 35050061), Insulin-Transferrin-Selenium (ITS) (100X) (Gibco, 41400045), (±) Sodium L-ascorbate (Ascorbic Acid) (Sigma-Aldrich, A7631), and Hydrocortisone 21 Hemisuccinate Sodium Salt (HC21) (Sigma-Aldrich, H4881).

**Neural epithelium differentiation**

The neural epithelium differentiation was based on Chambers (Chambers et al., 2009) and Maroof’s work (Maroof et al., 2013). hiPSCs were split with EDTA in a ratio of 1:3 on Geltrex (Gibco, A1413201) coated plates in E8, the next day differentiation was induced (at 20-30% confluency) by neural phase I medium (advanced DMEM/F12 (Gibco, 12634010), with Glutamax (Gibco, 35050061, 1%), and N2 supplement (Gibco, 17502048, 1%) supplemented with 10μM SB431542 (Tocris, 1614), 100nM LDN 193189 (Selleckchem, S2618) and 2μM XAV-939 (Tocris, 3748) with 4ml per well in a 6-well plate. Medium was changed every 2 days until day 8, when neural rosette formation was visible.

**Neural crest differentiation**

The neural crest differentiation protocol was modified from Menendez et al, 2013. hiPSCs were adapted to single cell passaging with Accutase (Gibco, A1110501) for two weeks (approximately 4 passages). Cells were plated at a density of 6 x 10^3 cells per cm^2 or a 1:4 ratio on Geltrex (Gibco, A1413201) coated plates and incubated at 37°C in 5% CO2 in E7 (E8 without TGFB) medium. For neural
crested induction, cells were passaged with Accutase (Gibco, A1110501) and seeded at a density of 9.2 x10^3 cells per cm^2 on Geltrex (Gibco, A1413201) coated plates in E7. The next day, the medium was replaced with neural crest medium, containing 0.5µM BIO (Tocris, 3194) and 20µM SB431542 (Tocris, 1614) for the inhibition of GSK3 and TGFβ. Several different concentrations of BIO were tested and 0.5µM was chosen for all experiments.

**Neuronal differentiation**

For the production of peripheral neurons the protocol from Menendez et al 2013 was followed, it is summarized as follows (Menendez et al., 2013). NCCs were treated with Accutase (Gibco, A1110501) and plated at a density of 1 x10^4 cells per cm^2 in NCC medium on Geltrex (Gibco A1413201) coated plates. The next day the medium was changed to peripheral neuron medium. The peripheral medium was changed every 2 days and contained DMEM/F12 (Sigma, #D8437), 1% of N2 supplement (Gibco, 17502048), 10ng/ml brain derived neurotrophic factor (BDNF) (Tocris, 2837), 10ng/ml nerve growth factor (NGF) (Sigma-Aldrich, H9666), 10ng/ml glial cell line-derived neurotrophic factor (GDNF) (R&D, 212-GD-010/CF), 10ng/ml Neurotrophin-3 (NT-3) (R&D, 267-N3), 200µM ascorbic acid (Sigma-Aldrich, A8960) and 0.5mM cAMP (Sigma-Aldrich, A9501). After 14-18 days peripheral neurons were stained for β-tubulin III and peripherin.

**Cell culture medium for mesenchymal differentiation and adipose tissue derived stem cells**

The basis for the maintenance medium was DMEM/F12 (Sigma, #D8437) for both ASCs and iMSCs. The DMEM/F12 was supplemented with 33µM biotin (Sigma-Aldrich, B4501), 17µM pantothenic acid (Sigma-Aldrich, 21210-5G-F), and 10% FBS (BioWest, S1810-100), the serum was inactivated before use by incubating at 56°C for 45 minutes, or alternatively using heat inactivated bovine serum (BioWest, S181H).

**Differentiation to iMSCs**

NCCs were detached with Accutase (Gibco, A1110501) and plated onto non-coated tissue culture dish at a density of 6.5 x10^3 cells per cm^2 in maintenance medium (see above for recipe). Media was changed every second day. Cells were passaged after 4-6 days with trypsin-EDTA (Sigma-Aldrich, T4049-500ML) and seeded for further experiments. Cell identity was confirmed by flow cytometry analysis of the following markers: CD73+, CD44+, CD105+ and CD90+ and furthermore by osteogenic (Gibco, A1007201) and chondrogenic potential (Gibco, A1007101) using StemPro™ differentiation kits. Adipose tissue derived stem cells were grown in medium supplemented with 0.25ng/ml FGF2 (Peprotech, 100-18B).

**Differentiation of ASCs to adipocytes expressing UCP-1**

For the differentiation, 2.5 x10^4 cells per cm^2 were seeded in maintenance medium with 0.25ng/ml FGF2 (Peprotech, 100-18B). When the cells reached confluency, the medium was changed to maintenance medium without FGF2 and cultivated for 48 hours. Then cells were induced with the beige differentiation medium, consisting of maintenance medium, supplemented with 10µg/ml insulin (Sigma-Aldrich, I9278-5ML), 1µM dexamethasone (Tocris, 1126), 0.5µM IBMX (Sigma-Aldrich, I5879-100MG), 200µM indomethacin (Sigma-Aldrich, I7378), 1µM rosiglitazone (Sigma-Aldrich, R2408-10MG), and changed every 3 days. Adipocytes were harvested for RNA isolation after 12 days of differentiation.

**Adipogenesis in monolayer**

The iMSCs were plated onto Geltrex (Gibco A1413201) coated plates at a density of 6.25 x10^4 cells per cm^2 and allowed to attach overnight. 24 hours later the iMSCs were induced with either an “in
house” adipogenic cocktail or the commercially available StemPro™ adipogenesis differentiation media (Gibco, A1007001). The “in house” adipogenic cocktail contained DMEM/F12 (Sigma, #D8437) supplemented with 0.2μM insulin (Sigma-Aldrich, I9278-5ML), 1μM dexamethasone (Tocris, 1126), 60μM indomethacin (Sigma-Aldrich, I7378), 10μg/ml transferrin, and 10% FBS (Biowest, S1810-100). Only for the initial induction 0.5mM IBMX (Sigma-Aldrich, I5879-100MG) was added up to day 3 and then removed. The medium was changed every 3 days. The resulting putative adipocytes were characterised 20 to 26 days post initiation of the differentiation.

**Adipogenesis in spheroids**

NCCs were seeded as 8.5 x10^3 cells per cm² in maintenance medium, containing DMEM/F12 (Sigma, #D8437), supplemented with 33μM biotin (Sigma-Aldrich, B4501), 17μM Pantothenic Acid (Sigma-Aldrich, 21210-5G-F) and 10% FBS (Biowest, S1810-100) on non-coated plastic wells. Medium was refreshed every 2 days. After 4-6 days the cells were harvested with trypsin-EDTA (Sigma-Aldrich, T4049-500ML) and resuspended in maintenance medium at 1 million cells per millilitre. Using a multichannel pipette 20µl drops containing 20,000 cells were seeded onto a petri dish lid. The lid was inverted and placed on a petri dish bottom containing DPBS (Gibco, 14040133). After 2-3 days, the aggregated spheroids were collected in DPBS (Gibco, 14040133) and transferred to 6-well plates or cell culture flasks with “in house” adipogenic cocktail, only for the initial induction. 0.5mM IBMX (Sigma-Aldrich, I5879-100MG) was added. The medium was changed every 3-4 days, but one fourth of the medium was left behind (conditioning). Adipocyte spheroids were analysed after 10-21 days in culture.

**Osteogenesis and chondrogenesis**

For osteogenesis and chondrogenesis, iMSCs were generated from neural crest stem cells, which were seeded as, 8.5 x10^3 cells per cm² in maintenance medium (DMEM/F12 (Sigma, #D8437) supplemented with 33μM biotin (Sigma-Aldrich, B4501), 17μM pantothenic acid (Sigma-Aldrich, 21210-5G-F), and 10% FBS (Biowest, S1810-100)) to differentiate the iMSCs. The medium was changed every second or third day. After 6 days, the cells were treated with trypsin-EDTA (Sigma-Aldrich, T4049-500ML) for 5 minutes and seeded for osteogenesis and chondrogenesis as follows: For the monolayer differentiation, cells were seeded on Geltrex (Gibco A1413201) coated 6-well plates 6.4 x10^4 per cm². The next day the osteogenesis cocktail (StemPro™, A1007201) or chondrogenesis cocktail (StemPro™, A1007101) was added to induce the differentiation and cells were cultured until day 20 with a medium change every 3-4 days.

For standing drops, iMSCs were collected as described for the monolayer and resuspended in the same maintenance medium (DMEM/F12 (Sigma, #D8437) supplemented with 33μM biotin (Sigma-Aldrich, B4501), 17μM pantothenic acid (Sigma-Aldrich, 21210-5G-F), and 10% FBS (Biowest, S1810-100)). 20,000 iMSCs per drop were seeded in a 6-well plate as standing drops (10 drops in total per well). The next day induction medium was added (osteogenesis cocktail (StemPro™, A1007201) or chondrogenesis cocktail (StemPro™, A1007101)), medium was changed every 3-4 days until day 20. For hanging drops, again 20,000 cells per drop (iMSCs resuspended in the described maintenance medium) were seeded on a petri dish lid, but the lid was inverted. After 2-3 days, the drops were collected in DPBS (Gibco, 14040133) and resuspended at a ratio of 1 million iMSCs per ml (calculated from cell number before seeding the hanging drops) in osteogenesis cocktail (StemPro™, A1007201) or chondrogenesis cocktail (StemPro™, A1007101). Approximately 5 million iMSC aggregated as spheroids were transferred to a Geltrex (Gibco A1413201) coated 6-well plate and cultured for 20 days with medium changes every 3-4 days. For the suspension culture, hanging drops in osteogenesis cocktail (StemPro™, A1007201) or chondrogenesis cocktail (StemPro™, A1007101) were transferred to a suspension plate and cultivated on a shaker for 20 days.
Immunofluorescence

Samples were fixed at room temperature for 30 minutes with 4% PFA (Sigma-Aldrich, P6148-500G), 10% of goat serum (Sigma-Aldrich, G9023) for blocking in DPBS (Gibco, 14040133) supplemented with 0.1% Triton-X100 (Sigma-Aldrich, 93443) was performed for 45 minutes, followed by incubation with primary antibody in 5% goat serum (Sigma-Aldrich, G9023) in DPBS (Gibco, 14040133) with 0.1% Triton-X100 (Sigma-Aldrich, 93443) over night at 4°C. After washing the samples three times for 10 minutes in DPBS (Gibco, 14040133) with Triton 0.1%, the secondary antibody in 1% goat serum in DPBS (Gibco, 14040133) with Triton 0.1% was added for 1 hour at 37°C. For a list of antibodies, see material section of paper II.

Flow cyt fluorimetry

The Human MSC Analysis Kit (BD Bioscience, 562245) was used according to the manufacturer’s instructions. Plates were analysed using the Attune NxT Flow Cytometer (Thermo Fisher Scientific). The lasers were calibrated using OneComp eBeads™ Compensation Beads (Thermo Fisher Scientific, 01-1111-42) for surface staining markers, and ArC™ Amine Reactive Compensation Beads (Thermo Fisher Scientific, A10628) for the live-dead stain. Both were used as stated by the manufacturer.

The data was then analysed using the FlowJo™ v10.7 software (BD Bioscience). Briefly, analysis first removed doublets before discriminating between live and dead cells. Isotype controls and positive controls were used to evaluate cell line expression of CD73, CD90, and CD105. Data was normalised to mode and presented as histograms.

Flow panel adipokines

The LEGENDplex™ Human Adipokine Panel (13-plex) w/FP (BioLegend, 740196) for simultaneous analyses of 13 different cytokines was performed for 2 cell lines according to the manufacturer’s instructions and normalized detection levels to cell number. Analysis was performed with the LEGENDplex™ software according to the manufacturer’s instructions.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Samples were collected in TRI Reagent® (T9424, Sigma-Aldrich) and ribonucleic acid (RNA) was isolated according to the manufacturer’s instructions. 500ng of RNA were converted to cDNA using the High capacity cDNA Reverse Transcription kit (Applied Biosystems™, 4368814), validated TaqMan™ gene expression assays were used with the SsoAdvanced Universal Probes Supermix (BioRad 1725280). All reactions were performed according to the manufacturer’s instructions, with 5ng of cDNA template, in a final volume of 15µl.

Aim of the study

The main aim of this study was to develop differentiation protocols for the separate building blocks that make up the fat-liver axis, in order to provide a model to investigate metabolic syndrome, in the context of NAFLD and PNALD, in a multi-tissue model system. This was an ambitious goal, with several tasks. (1) Investigation of developmental processes during definitive endoderm specification (paper I), as a base to create/optimise a liver model. (2) The establishment of a characterised/validated scalable liver organoid model (paper II), and (3) testing the liver organoid model for its response to lipids in a
parenteral nutrition setting (paper III). (4) Finally the establishment of an adipocyte model, representing the secretory properties of adipose tissue (paper IV).

**Paper I: Autophagy modulates cell fate decisions during lineage commitment**

**Aim of paper I**

Paper I explores the role of autophagy during the formation of the three germ layers, by utilising hiPSCs and it demonstrates that down regulation of autophagy is crucial for iPSCs to exit pluripotency. Subsequent upregulation of autophagy activates mesoderm and endoderm formation. SOX2 has a unique role because it is selectively degraded by autophagy in contrast to NANOG and OCT4 and it was concluded that SOX2 degradation is a determining factor for lineage specification.

**mTOR inhibition has similar effect as autophagy activation by rapamycin**

Autophagy is the major cellular digestion process that removes damaged macromolecules and organelles (Kim and Guan, 2015). Nutrient and growth factor deprivation together with low cellular energy levels, which are known to activate autophagy, have also been proven to inhibit mammalian target of rapamycin complex 1 (mTORC1) activity. mTORC1 promotes cell growth when energy is sufficient and catabolism when energy levels are low, it regulates cell growth and metabolism, while mammalian target of rapamycin complex 2 (mTORC2) mainly controls cell proliferation and survival (Unni and Arteaga, 2019; Zou et al., 2020). The inhibition of mTORC1 improves proteostasis, increases autophagy and enhances stem cell function (Fontana and Partridge, 2015). This inverse coupling of autophagy induction and mTORC1 activation is especially interesting in the context of cell commitment. Our findings, that autophagy is upregulated during definitive endoderm differentiation is underscored by Emanuel Nazareth’s report (Nazareth et al., 2016). Nazareth and colleagues patterned human iPSCs in engineered microenvironments and screened the response to 400 small-molecule kinase inhibitors, measuring yield and purity outputs of undifferentiated, neuroectoderm, mesendoderm, and extra-embryonic populations. They observed that mTOR inhibition enhances mesendoderm, hemogenic endothelium, and blood progenitors, which is in alignment with our observations.

**Autophagy and WNT signalling**

In the paper, it was hinted at the possibility that the WNT pathway together with autophagy could guide lineage commitment. WNT signalling plays a central part in early embryonic development, morphogenesis and cell division. It is further implicated in adult tissue homeostasis, embryonic and adult stem cell maintenance (Logan and Nusse, 2004; Lorzadeh et al., 2021; Nusse and Clevers, 2017; Steinhart and Angers, 2018). It was shown that GSK3β inhibition rapidly directed iPSCs into a primitive streak-like cell type, a 24 hour pulse of CHRI99021 results in primitive streak (mesendoderm) and the withdrawal of CHIR99021 allowed the transition to definitive endoderm. Since the formation of ectoderm does not pass through the mesendoderm stage, it is interesting that the dip in autophagy induced by bafilomycin A1 and chloroquine elevated markers of all three lineages. Together with the finding that enhanced autophagy facilitates the processing of SOX2 and thereby definitive endoderm formation, it could be interesting to look into other key players of differentiation and see if they are also a special target of autophagy, since the crosstalk of WNT and autophagy might facilitate the
commitment together (Lorzadeh et al., 2021). It can be speculated that autophagy activators could be utilised to improve protocols and their definitive endoderm efficiency, as it has been shown that PI3K inhibition promotes mesendoderm formation (McLean et al., 2007).

**Autophagy versus mTOR in mature hepatocytes**

Because autophagy directs between the three lineages, it would be interesting to further dissect the hepatic maturation after the definitive endoderm commitment of liver organoids during the entire differentiation process, to assess if autophagy plays a part there as well. This hypothesis is supported by the knowledge, that activation of mTORC1 is important for liver regeneration and hepatocyte maturation in vivo (Boon et al., 2020; Fournaschen et al., 2013; Sugiyama et al., 2017). It has been demonstrated that mTORC1 signalling is linked to the induction of CYP3A4, as addition of the mTORC1 inhibitor rapamycin blocked the induction of CYP3A4, in both HepG2 and hepatocyte-like cells (Boon et al., 2020).

Therefore, hepatocyte maturation depends on mTORC1 activation, which means inhibition of autophagy. It was also shown in mice that autophagy drives HSC activation but generated fatty acids from cleaving retinyl esters, underlined by the observation that treatment with autophagy inhibitor bafilomycin A1 decreased proliferation and the expression of HSC activation markers (Tsuchida and Friedman, 2017). This was further supported by the work of Xiu and colleagues; they were able to demonstrate that doxazosin inhibited HSC proliferation and migration, while PI3K/Akt/mTOR pathway was activated by doxazosin. HSC activation was attenuated by doxazosin in a concentration-dependent manner in vivo and in vitro (Xiu et al., 2021).

**Autophagy versus mTOR in other differentiations**

Autophagy’s involvement in cell differentiation has been established for different cell types. Adipogenic differentiation of 3T3-L1 pre-adipocytes is associated with activation of autophagy and increased PPARγ2 protein levels (Zhang et al., 2013). Likewise, mTORC1 inhibition promotes adipogenesis (Magdalon and Festuccia, 2017), and autophagy was shown to be part of the mechanism that promotes morphological and structural changes, cell differentiation into an adipocyte, and accumulation of triglycerides which fuse into a single large cytoplasmic lipid droplet (Lafontan, 2008). Cells treated with 3-methyl-adenine, a pharmacological inhibitor of autophagy, displayed less triglyceride accumulation, and decreased markers of adipocyte differentiation (Vessoni et al., 2012). Zhang and colleagues investigated the role of autophagy in a mouse model with a targeted deletion of the autophagy-related gene 7 (Atg7), in adipose tissue, Atg7 drives the fundamental stages of degradative autophagy and its importance is reviewed elsewhere (Collier et al., 2021). Mice with this deletion had a lower fed plasma concentrations of fatty acids, the mutant mice displayed increased insulin sensitivity with a decrease in leptin secretion and an overall increase in β-oxidation, and infertility (Zhang et al., 2009). Another example of mTOR versus autophagy regulation in cell commitment is exemplified in psoriasis. mTOR signalling is inactive when keratinocytes switch from proliferation to a terminally differentiated state, but in the disease state (psoriasis), mTOR signalling is hyper-activated and keratinocyte proliferate excessively. Already in 2011, it had been discovered that autophagy abnormalities cause inflammatory cytokine production and cell proliferation in keratinocytes (Klapan et al., 2022; Lee et al., 2011).

**Outlook**

Taken together, paper I delivers relevant information on early stage lineage commitment, and accordingly autophagy should be considered when establishing a differentiation protocol, because it is at the very heart of decision-making.
Paper II: Scalable production of tissue-like vascularised liver organoids from human PSCs

Aim of Paper II

The aim of this paper was to provide a physiological hiPSC derived liver organoid model, to provide a tool for toxicology, drug metabolism, development and disease modelling, namely to supply one part of the fat-liver axis for the investigation of metabolic syndrome and disease.

Scalable production of tissue-like vascularised liver organoids from human PSCs

Current liver organoid models do not have the cellular diversity of the organ, containing only a subset of representative cells such as hepatocytes and/or cholangiocytes. To address this we developed a cocktail of small molecule growth factor mimetics, to direct differentiation in a developmentally relevant sequence, mimicking in vivo liver development. This protocol was performed in suspension, without the requirement for 2D patterning, ECM or growth factors. This approach decreased production costs by orders of magnitude, allowing large quantities of organoids to be generated. As shown by our analysis, the organoids have a liver like cellular complexity with vascular luminal structures and a resident macrophage population. They produce serum proteins, synthesise urea, coagulation factors, and can be transplanted and maintained in mice producing albumin. Additionally they have documented drug metabolism, shown by long-term CYP450 activity for 80 days and non-CYP450 mediated metabolism demonstrated with the opioid heroin. The organoids produce and secrete the coagulation machinery including factors FII, AT, plasma fibrinogen (FBG), Protein C, Protein S, FVII, and FVIII (synthesised by endothelial cells).

Application of the model for FVII deficiency

Due to the secretion of coagulation factors by our organoid, we can propose our model for the investigations of the coagulation machinery and potential treatment strategies for coagulation deficiencies. Congenital FVII deficiency is the most frequent among the recessively inherited bleeding disorders (Kavlie et al., 1998). It is caused by heterogeneous mutations in the F7 (coagulation factor VII) gene with varying severity of symptoms from intracranial haemorrhage to mild bleeding (Bernardi and Mariani, 2021). The current treatment is the replacement of the deficient or defective factor, using plasma-derived or recombinant products (Peyvandi and Menegatti, 2016). Another potential course of treatment in the future, is potentially gene editing, and a recent success has been reported that exploits CRISPR technology to gene edit a patient’s hematopoietic stem and progenitor cells, causing an increase in fetal haemoglobin, resulting in a significant reduction in blood transfusions in patients with hemoglobinopathies as a treatment for sickle cell disease and β-thalassemia (Frangoul et al., 2021). A similar approach to correct the F7 gene in lines generated from patients could open the door to an alternative treatment and is currently under development in our collaborator’s lab. This is a multifaceted project, because the correction of the F7 gene with CRISPR also requires screening for off-target effects via whole genome sequencing. A safety concern with the use of iPSCs is their ability to create tumours after transplantation, exemplified by the teratoma formation assay, used to demonstrate pluripotency of generated lines in immune-deficient mice (Buta et al., 2013; Nelakanti et al., 2015; Wesselschmidt, 2011). It is therefore crucial to exclude the presence of iPSCs in the differentiated organoid before transplantation, this is especially important, as potential transplantation will be autologous. It should be noted that to date we have not observed the formation of tumours in our transplantation studies, however the period was only 5 weeks and a long-term study will be required to provide absolute certainty. Additionally we could incorporate additional fail-safes
into our differentiation protocol such as the use of small molecules to target residual iPSCs (Martin et al., 2020; Wu et al., 2019b). Martin and colleagues genome edited iPSC lines, which contained two small molecule inducible safeguards, one to deplete undifferentiated iPSCs (targeting **NANOG**), and the second to kill all iPSC derived cell types, creating the possibility to remove the transplanted cells **in vivo**, in case of side effects (Martin et al., 2020). Another possibility is to culture the cells in medium without methionine, it has been demonstrated that a short methionine deprivation poises human ESCs/iPSCs for differentiation, but prolonged methionine deprivation induced apoptosis (Shiraki et al., 2014) and furthermore, zinc depletion altered methionine metabolism. Zinc depletion of iPSCs mimicked methionine depletion and is presented with impaired pluripotency (Sim et al., 2022), so changing the medium constitution could be a possibility to exclude/remove any remaining iPSCs.

To date a number of clinical trials involving iPSCs have been conducted. Japan is leading in therapeutic trials and several companies are utilising iPSC technology worldwide, but only 13 are developing iPSC-based therapeutic products (Kim et al., 2022). Already now, there are completed clinical trials, e.g. for the transplantation of allogeneic iPSC-derived retinal pigment epithelium (RPE) cells, for the treatment of neovascular age-related macular degeneration (AMD), a phase I/II autologous trial, giving hope for future applications of iPSC for patients suffering from genetic disease.

**Genetic disease models**

The most immediate and extensive use of iPSCs to date has been in modelling genetic disease and this is a possibility with our system for liver specific diseases. Models for monogenic disorders such as Alagille syndrome (ALGS) and α1-antitrypsin (A1AT) deficiency have already been demonstrated (Guan et al., 2017; Huch et al., 2015a). A1AT is produced and secreted from the liver to protect the lungs against proteolytic damage from neutrophil elastase. An inherited mutation in the corresponding gene *SERPINA1* predisposes to chronic obstructive pulmonary disease and chronic liver disease (Huch et al., 2015a; Stoller and Aboussouan, 2005). Huch and colleagues developed a model for A1AT deficiency by aggregating EPCAM⁺ biliary epithelial cells from A1AT deficient patients to model A1AT. In A1AT deficiency, one characteristic of the disease state, is the aggregation of the protein within the endoplasmic reticulum of hepatocytes, which was observed within the cells of the differentiated organoids, as well as a reduced protein secretion of A1AT and reduced ability to block elastase activity (Huch et al., 2015a).

Another study Guan and colleagues developed a model of ALGS, which is a genetic disorder where NOTCH signalling pathway mutations impair bile duct formation, and presents with substantial variability in associated clinical features in patients (Guan et al., 2017). Examples for severe genetic disorders are mutations in the DNA polymerase gamma (*POLG*) gene, which encodes the catalytic subunit of DNA polymerase gamma, the enzyme responsible for mitochondrial DNA replication and repair (Copeland and Longley, 2003). *POLG* related conditions, which are prominent in children, include Alpers-Huttenlocher syndrome and childhood myocerebrohepatopathy spectrum (MCHS) (Hikmat et al., 2017; Nguyen et al., 2006). The children experience epilepsy, and if the *POLG* mutation is not detected, they have a high risk of acute liver failure when treated with sodium valproate (Stewart et al., 2010). This organoid platform could provide a testing platform for the mechanism of toxicity in these patients and provide guidance for new drug development.

**Infectious disease models**

Furthermore, infectious diseases could be investigated with this model, there are several viruses, like Epstein-Barr virus, Yellow fever virus, Hepatitis (A, B, C, D and E) (Cao et al., 2021) and Dengue virus, which cause liver damage (Talwani et al., 2011). It has been reported for dengue that the liver injury is
caused by direct infection of the kupffer cells and the hepatocytes (Ling et al., 2007), both present in our model.

Another interesting application of our model would be malaria. Malaria is caused by different Plasmodium species, the sporozoite infects and resides in the liver before moving to the blood stage of the life cycle (Valenciano et al., 2022). Different species of Plasmodium exist, Plasmodium falciparum is the most prevalent and deadliest cause of malaria globally. Elimination efforts for P. falciparum have shown that, the emptied niche is being taken over by less prevalent and less studied species of Plasmodium (Valenciano et al., 2022). To make matters worse, Chloroquine resistance in P. vivax has emerged and compromises clinical efficacy (Price et al., 2020; Price et al., 2014; Valenciano et al., 2022), highlighting the need to develop new and improved treatments of malaria is of importance.

It is furthermore of concern that 8-aminoquinoline which is the only treatment against the P. vivax hypnozoite (parasite stage that remains quiescent through the primary liver infection; can result in a relapse weeks or years later), can cause haemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals (Baird, 2015). Additionally, a patient’s genotype in regard to cytochrome P450 2D6 polymorphism status, can correspond with a varying metabolizer phenotypes and low activity (Baird, 2015; Valenciano et al., 2022). CYP2D6 is responsible for metabolising several anti-malaria drugs of the 8-aminoquinolines, such a primaquine (Pybus et al., 2013) and tafenoquine (St Jean et al., 2016). Investigating, how SNP are contributing to altered drug metabolism, could be achieved with our model.

It is of note that CYP2D6 expression varies throughout the life, being very low at birth and gradually increasing with age (Darney et al., 2021). It has furthermore been shown that CYP2D6 expression in liver samples from neonates less than 7 days of age was higher than that observed in first and second trimester samples, but not significantly higher than third trimester fetal samples (Stevens et al., 2008). The organoids could serve as a “housing” for the hypnozoite stage for in vitro experiments. The presence of CYP2D6 in our organoids should be investigated to evaluate if the y could be used for CYP450 polymorphism studies and their drug response in malaria.

**Hepatic stellate cells**

Having a liver like cellular complexity in our organoid model opens the door to an array of applications. Of special interest, is the HSCs population within the organoids. The presence of HSCs was initially investigated by smooth muscle actin (αSMA) immunofluorescence, revealing cells with a star-like morphology, and others resembling myofibroblasts, indicating a potential quiescent and activated population. These populations were further validated using single cell RNAseq, confirming the presence of both quiescent and activated populations of HSCs. To pin down HSCs origins, has been a difficult task, because HSCs express gene markers of all three germ layers (Kamm and McCommis, 2022). WT1 expressing and mesoderm posterior 1 (MESP1) expressing cells develop into HSCs, this cell population stems from the mesoderm, originating from the septum transversum (Asahina et al., 2009; Asahina et al., 2011; Kamm and McCommis, 2022). Yet, HSCs also express desmin, cytokeratin 7 /8 (CK7/8), CD34, which hints at an endodermal origin and glial fibrillary acidic protein (GFAP), nestin, and neurotrophins indicating ectodermal origin (Friedman, 2008). This issue has been reviewed in other literature (Asahina, 2012; Asahina et al., 2011; Miyata et al., 2008), but the consensus drawn from mice lineage tracing is, that the mesoderm is the most likely origin of HSCs and that αSMA expression is considered the defining marker of activated HSCs (Asahina, 2012; Kamm and McCommis, 2022). Hepatic lineage tracing has been discussed in the field for its pitfalls (Lemaigre, 2015), and tracing HSCs is still an ongoing project (Chen et al., 2022). Since our model recapitulates a developmental relevant
pathway for liver development, the liver organoids could prove to be a useful tool to track the HSCs origins by organoid based lineage.

**Stellate cells and inflammation**

Currently supplies of quality primary human HSCs are limited and this is compounded with batch differences in terms of genotype etc. Several protocols for the isolation of stellate cells from the mouse liver exist (Chang et al., 2014; Mederacke et al., 2015; Modak and Zaiss, 2019). To have an unrestricted source of HSCs, we have adapted a mouse protocol for isolation of HSCs from liver biopsies to our human organoids, where we can isolate a regular supply of genetically defined HSCs, which are positive after 4 days in culture (personal communication). This needs further refinement, but shows the utility of our organoid model as a source of HSCs for examining organoid response to drug treatments for inflammation and in parallel the response of the corresponding HSCs and their activation.

Similarities between adipocytes and quiescent HSCs have been proposed concerning fatty acid storage and gene expression. Comparison of quiescent and activated HSCs in culture revealed higher expression of adipogenic transcription factors in quiescent HSCs. Among these were C/EBPα, C/EBPβ, C/EBPδ, PPARγ, in quiescent HSCs (She et al., 2005). Also the expression of leptin (Potter et al., 1998) and its receptors (Ikejima et al., 2004; Ikejima et al., 2002; Otte et al., 2004a; Saxena et al., 2002) mediating inflammation (Friedman, 2008; Han, 2006) have been characterised in that regard. HSC activation causes progressive accumulation of excess ECM (collagen), resulting in progressive fibrosis and cirrhosis (Friedman et al., 2018), illustrated by elevated leptin mRNA levels in fibrotic and cirrhotic liver samples, correlating with the degree of fibrosis (Otte et al., 2004b). Our preliminary results, demonstrate that our organoids do express the leptin receptor (OB-R), but the expression of leptin was variable (unpublished data). We also performed RT-qPCR analysis and observed the expression of ADIPOR1 and ADIPOR2 (personal communication). The presence of these receptors is crucial for adiponectin signalling between the liver and the adipose tissue as discussed above. These are very exciting preliminary results, which warrants further investigation, especially in co-culture applications such as the liver-fat axis and investigating the effect of adiponectin in this system with regards to inflammation.

**Activated stellate cells in Cachexia**

Another application possibility is in the context of cancer cachexia. Cachexia is a wasting syndrome, which induces metabolic disruption, causing both, loss of fat and muscle mass. An impaired hepatic lipid metabolism in cancer cachexia, leads to worsening symptoms (Gonçalves et al., 2019). During the progression of cachexia, deposition of liver collagen has been reported in connection with changes in mitochondrial quality control (Rosa-Caldwell et al., 2020). As activated HSCs are well known to produce the majority of the abnormal ECM, including fibrillar collagens and basement membrane proteins, and thereby contributing to pathological inflammation and fibrosis observed in the liver, they could be a possible target for cachexia development (Sherman, 2018). Likewise, cachexia induced changes in bile acid metabolism seems to be a contributing factor to hepatic inflammation (Thibaut et al., 2021). All these findings underscore the utility of our organoids, due to the presence of hepatocytes, cholangiocytes and HSC populations.

**Fat accumulation in liver organoids and its implications**

Metabolic diseases/syndromes, like diabetes and NAFLD, are systemically connected, having implications for the liver (being over loaded with lipids, increased DNL) and the adipose tissue. A hallmark of NAFLD is increased liver fat content, which is tightly connected to insulin resistance, limited expandability and the dysfunctional adipose tissue (Godoy-Matos et al., 2020) which can in some cases
progress to NASH, characterised by hepatocyte ballooning (Hirsova and Gores, 2015). We investigated if we could develop a model with these characteristics, by exploring whether the organoids can take up and accumulate fatty acids. We demonstrated this by exposing the liver organoids to fatty acids (300μM Sodium Oleate/BSA complex) supplemented in the culture media and observed massive accumulation of lipids and evidence of hepatic ballooning. This is of relevance, because it has been shown in primary human hepatocytes, that lipid droplets are intracellular mechanical stressors, which can affect tissue stiffness (Loneker et al., 2022). Additionally, tissue stiffness in the context of liver disease, is treated as a risk factor for hepatocellular carcinoma development (Loneker et al., 2022). Therefore future characterisation of lipid treated organoids should also include a thorough analysis of DNL, since this is considered an important area/target for the treatment of NASH (Finck, 2018; Mashek, 2021) and is only touched upon in paper IV.

**Potential improvements for the future**

The protocol could be further refined/optimised by replacing FBS. It has been shown for various systems, that by switching from bovine to human serum, cell performance improves. Especially proliferation and differentiation of human MSCs, cultivation of human corneal stromal keratocyte and fibroblasts (Aladhamsh et al., 2011; Guiotto et al., 2020; Seidelmann et al., 2021) were improved. For example, HepG2 cells cultured in human serum, resulted in increased bile acid and proprotein convertase subtilisin/kexin type 9 (PCSK9) secretion, with an overall improved functionality of the HepG2 cells (Pramfalk et al., 2016). This was further supported by Steenbergen and colleagues, who replaced FBS with human adult serum, they noted key morphological and metabolic features, amongst was the restoration of xenobiotic degradation in human hepatoma cells (Steenbergen et al., 2018). In light of these findings, replacing FBS with human serum could potentially benefit our liver organoid model.

Another approach that could be considered for future applications is that FBS is not compliant with current good manufacturing practice (GMP) for cellular therapy. This will be an absolute requirement for the utilisation of our organoids in replacement therapies for bleeding disorders caused by FVII defects. The translation of organoid technology to the clinic would require following the guidelines on GMP specific to advanced therapy medicinal products (ATMPs). Conformity of our protocol and thus further development of the differentiation cocktail would need to be established/refined. A possible FBS replacement would be Knockout Serum Replacement (Gibco) or B27 supplements (Gibco), which are xeno-free and available at GMP grade and have already been tested in our preliminary experiments.

We are aware that no model fits all purposes, there are applications for mouse models, or cancer lines, but the main advantage of our human organoid model, is the presence of different cell types in the organoids, their interactions, no prescribed polarity, etc., which monoculture cell systems do not provide. It has been established in the above examples, and especially in the setting of NAFLD, that there are multiple drivers of liver disease, resulting in a complex set of questions, which can be potentially addressed using our model.

**Paper III: Parenteral nutrition decreases CYP3A4 activity in iPSC derived liver organoids**

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Aim of Paper III

The aim of this paper was to assess if liver organoids, which have a proven CYP1A2 and CYP3A4 activity (Harrison 2023), could be utilised to investigate the effect of parental nutrition (PN) in the development of PNALD.

Parenteral nutrition emulsion inhibits CYP3A4 in an iPSC derived liver organoid testing platform

PN is used for patients of varying ages with intestinal failure to supplement calories. Premature newborns with low birth weight are at a high risk for developing PNALD including steatosis, cholestasis, and gallbladder sludge/stones (Kapoor et al., 2019). To optimize nutrition regimens, a new model system needs to be established.

The hypothesis was that liver organoids, which were developed in paper II, would be an ideal model to investigate the effects of PN due to their following characteristics; hepatic cellular diversity, expression of GS which is absent from fetal hepatocytes, indicating a developmental stage past the fetal liver), and basal CYP1A2 and CYP3A4 activity (Guengerich, 1999; Jamwal et al., 2018; Zanger and Schwab, 2013).

Some of the DNL markers including MLX-interacting protein-like /Carbohydrate-responsive element-binding protein (MLXIPL/CHREBP), Stearoyl-CoA Desaturase (SCD), and ATP citrate lyase (ACLY) were increased after PN treatment, but only clinoleic treatment increased FASN (fatty acid synthase) significantly. Additionally, the mRNA levels of scavenger receptor class b, member 3 (CD36) were analysed, but only clinoleic treated organoids showed significant elevation. After 6 days of PN treatment, none of the emulsions affected CYP1A2 levels or activity, but intralipid treatment decreased CYP3A4 activity significantly in treated organoids. In conclusion, our liver organoids can be utilised as a potential pre-screening platform for the development of new parenteral nutrition solutions concerning their influence on CYP450 activity.

Alternative models

While our organoid model has a number of advantages as already pointed out, it is also important to discuss if alternative models could have added more information to our line of questioning. This should be evaluated in the context of the cell types present and model performance, in regard to cholangiocytes, and in the context of PNALD. The cholangiocyte are derived from the endoderm, which stems from the hepatoblast, which line the biliary ducts. Our organoid model has, shown by immunostaining, a cell population surrounding luminal spaces, which stain positive for the cholangiocyte marker (CK7), and is expressed in vivo from 16-20 weeks post conception. Alcian blue staining also revealed the presence of mucins secreted from these epithelial cells, which along with RNaseq data, supports the presence of cholangiocytes. However, we have not yet investigated the production of bile acids in our cell medium.

Cholestasis is defined as the impairment of bile flow and is characterised by the accumulation of bile acids in the liver and serum (Chen et al., 1995; Chen et al., 2014) and the PN supplement intralipid has been reported to have several pro-inflammatory properties, contributing to cholestasis and hepatic inflammation (Casson et al., 2020; Nandivada et al., 2016). Hepatobiliary organoids (HOs) could provide an alternative to investigate PNs effect on the liver, because they contain both hepatocytes and biliary epithelial cells (cholangiocytes) with cyst like structures, and have demonstrated bile acids accumulated within these ductal structures, using liquid chromatography–mass spectrometry (LC-MS)
This protocol requires matrigel embedding and a cocktail of growth factors for the differentiation, it is also labour intensive. However, since it can support the production of secondary organoids, it could deliver the obvious advantage of studying effects on bile acid secretion in contrast to our model.

A model that produces bile acids is of special interest, as lowered CYP3A4 activity (reported in PNALD patients and our model) is part of this vicious circle. CYP3A4 is implicit in the biotransformation of bile acids lowering toxicity by hydroxylation (Chen et al., 2014). However, lower CYP3A4 leads to higher levels of bile acid, which in turn further inhibits CYP3A4 activity. By using a HO model, it might be possible to observe changes in bile acid secretion due to PN treatment, giving more insight into the mechanism of our PN mediated CYP3A4 activity decrease and the mechanism of cholestasis development. It is worth noting that (Casson et al., 2020) the prophylactic use of SMOFlipid did not decrease the incidence or degree of cholestasis compared to intralipid in infants, but when cholestasis did develop there was a more rapid resolution of hyper-bilirubinemia, demonstrating that cholestasis is part of the PN dilemma and can be addressed.

Another avenue is the use of animal models such as mouse and piglet. In the case of mice, it is accepted that there are physiological differences. As highlighted in our paper, humans and mice have a different number of CYP450, making it difficult to translate observations made in mice to patients. Another consideration is the costs of using mice and the time taken to breed different mouse strains. Another animal model is the piglet, for example different types of PNs have been studied to assess the risk to induce PNALD in pre-term pigs (Vlaardingerbroek et al., 2014). The pig has been widely accepted as a model for physiological studies in human for nutrient sensing, appetite regulation, gut barrier function, intestinal microbiota and nutritional neuroscience (Roura et al., 2016). Piglet models used for PN treatment studies show symptoms after 14 days (Guthrie et al., 2020; Ng et al., 2016; Vlaardingerbroek et al., 2014). Our study was 7 day exposure to PNs and changes were observed within this timeframe i.e. CYP3A4 activity, but there is no reason why an extended exposure could not be performed in future experiments. While the piglet model delivers a whole organ set, variation is observed in terms of overall health and condition of the piglets during the treatment, with piglets presenting with diarrhoea, impacting the study outcome. Ultimately it is a about prioritizing a models complexity versus its cost and the scope of the study. One other caveat that should be considered when using animal models is the importance of the 3Rs in modern research practices.

Limitations of our model
The concentrations for PN treatment are calculated daily based on the patient’s weight, the desired amount of calories, and enteral feeding. This approach is difficult to mimic in vitro since babies should gain weight during the treatment period and the amount of PN administered will vary throughout the treatment course. Another complication is the infusion rate with which patients receive PN. The rate is adjusted during the first hour of administration, and without lipid concentration measurements and data on the half-life of the PN in the patient it is difficult to accurately extrapolate the lipid concentration to the petri dish. A possibility to compensate for this would be a microfluidic chip with adjustable flow rate to replicate the infusion rate of PNs administered to the patient. Many different microfluidic devices are already on the market. Our collaborator has developed a 3D printed device with in situ printed vessels that houses 1000s of organoids in parallel. Additionally, the device has both oxygen and nutrient gradient across the device creating an artificially vascularized tissue like observed in the acinar (Paper in review, Biomaterials). Another problem with PN testing in vitro is possible
interference of the PN (lipids) with downstream assays. To address this organoids were washed in DPBS and resuspended in fresh medium before analysis of CYP450 activity or harvesting in general. Unfortunately, the analysis of factors which are secreted such as liver enzymes gamma-glutamyl transferase (GGT), alanine and aspartate aminotransferase (ALT, AST) commonly analysed for liver health assessment (van Beek et al., 2013) would be very difficult. This is an age-old problem as in the clinic lipemic specimens are a source of significant analytical errors.

**Cytochrome P450 possibilities**

CYP450 are involved in the metabolism of between 70-80% of all clinically used drugs (Hammer et al., 2021; Sutton et al., 2010), which makes them a highly relevant field of study when it comes to PN. As preterm babies not only receive PN to compensate for the shortage of calories, but also suffer a range of other issues including necrotizing enterocolitis and short bowel syndrome which require different drug treatment regimes. As a consequence, drug metabolism could be directly affected by PN treatment, leading to a decrease in CYP450 activity, leaving these children vulnerable to undesired side effects. Our organoid platform delivers a system, which can be used to assess drug metabolism pre and post PN treatment to potentially identify CYP450 mediated issues.

An advantage of our system is it can utilise iPSC lines from different genetic backgrounds, thus taking into account SNPs. For example CYP3A5, which is detected in the livers of 10–30% of adult Caucasians (Aoyama et al., 1989; Quaranta et al., 2006; Schuetz et al., 1994; van Schaik et al., 2002; Wrighton et al., 1990) and can be compensated for with CYP3A4 activity. In our publication (paper III), it is highlighted that when CYP3A4 activity is compromised, this can potentially lead to complications, but also highlights why mouse models cannot deliver the same genetic relevance as human cell lines. From cancer research studies it is appreciated that race/ethnicity has a great impact on cancer incidence and survival, but when it comes to patient-derived oncological models, 8.3% have no record of the donor’s race/ethnicity (Guerrero et al., 2018). To further underline this, Popejoy and Fullerton analysed 2,511 genome-wide association studies (GWAS) (35 million samples) and observed that 81% of these samples were isolated from European descents (Guerrero et al., 2018; Popejoy and Fullerton, 2016). This raises the question: Could we build a testing platform for PN utilising different iPSC lines, providing representative cytochrome P450 family SNPs that account for different ethnic backgrounds to provide an inclusive screen for PN associated drug metabolism effects? This is also important in light of gender differences. It has been reported that CYP3A4 expression in females at the mRNA and protein levels were higher, as well as its activity (Waxman and Holloway, 2009; Wolbold et al., 2003; Yang et al., 2012), highlighting the opportunities of using cell lines representing both genders from a variety of genetic backgrounds.

**Outlook**

Among the experiments that were not performed, but are of interest for validating this model, is to assess different concentrations of PNs. Namely, clinoleic at 6.25% versus intralipid 6.25% and 0.93% clinoleic and 0.93% intralipid to investigate whether clinoleic at higher concentrations also decreases CYP3A4 activity. We did not include this in the first round of experiments, because we only assessed physiologically relevant concentrations, but from a mechanistic standpoint, it should be followed up. Also of significance is the contribution of ROS on liver organoids, since this has been implicated in the pathogenesis of PN in the mouse (Ferrucci-Da Silva et al., 2020). Investigating effects for different treatment periods of PN could also be carried out to see if treatment length has an effect in respect to
the piglet studies carried out over 14 days. Omegaven was originally part of paper III, but due to the culture conditions, the lipids accumulated as a layer on top of the medium, resulting in organoid death (data not shown). Omegaven has relevance for the treatment of patients with cholestasis (Park et al., 2011) and optimising the cell culture conditions, e.g. with a chip platform, would provide the possibility to investigate a broader spectrum of PNs, even in combination with prescribed drug regiments used to address bowel issues to elucidate the disease mechanism behind PNALD.

Paper IV: iPSC derived adipocyte spheroids

Aim of Paper IV

The aim of study IV was to establish a protocol for the differentiation of iPSC derived adipocytes, which should fulfil the general characteristics of an adipocyte. Adipocytes do not only take up energy in the form of glucose and fatty acids, and convert them to triglycerides (Galton, 1971), but also secrete a variety of adipokines which circulate in the blood stream to facilitate communication with other organs (Kwon and Pessin, 2013), including the liver, high-lighting their role in organ-cross talk.

iPSC derived adipocytes

This established protocol guides hiPSC to adipocytes via an expandable NCC population to iPSC derived MSCs (iMSCs). We then we turned to a 3D hanging drop intermediate, to generate adipocyte spheroids, 21 days post induction through an adipogenic cocktail. This protocol is cost efficient, based on an in-house E8 medium for culturing the iPSCs, with NCC differentiation medium based on E7 (E7 supplemented with BIO and SB431542), followed by MSC differentiation medium, which does not contain growth factors. The NCCs are bankable, therefore saving time, and seeding of the hanging drops only requires a multichannel pipette, making the preparation of the hanging drops easy and cost efficient.

To the best of our knowledge, there is no reported 3D model of hiPSC derived adipocytes, with only monolayer approaches being reported. Since it is known in the field (Guénantin et al., 2017), that the adipogenic capacity of iMSCs is low, our approach to improve the process employed 3D differentiation, based on primary cell approaches with ASCs. While adipose tissue is a relatively accessible tissue in comparison to liver or kidney, it is still an invasive process, requiring continuous access to the clinic and patients. To overcome this barrier, and importantly to generate organotypic models from the same cell line, we developed our protocol using hiPSCs as source material.

For the generation of hiPSC-derived adipocytes we employed an approach described by Klingelhutz and colleagues, to generate 3D spheroids from our iMSCs, since the 2D differentiation of iMSCs did not results in measurable mRNA levels of adiponectin (ADIPOQ). Klingelhutz and colleagues used immortal human pre-adipocytes and a SVF to form adipocyte spheroids which showed improved adiponectin secretion and lipid droplet accumulation in comparison to 2D differentiations (Klingelhutz et al., 2018).

Validating the primary control and pre-selected markers

In order to have a positive control for our hiPSC-derived adipocytes, we subjected primary human ASCs to the same protocol, which we optimised for our iMSCs. When differentiating iPSCs, it is of special importance to select a proper positive control and to narrow down a set of markers for benchmarking of the differentiated cells. Another protocol inspired by Klingelhutz and colleagues, was published in 2022 by Mandl and colleagues, they used an uncharacterised primary SVF to generate WAT organoids.
In the following paragraph Mandl’s WAT model will be used as a reference, to demonstrate that the markers we selected for analysis are relevant in the field. While the starting material was different, both approaches were initiated with hanging drop culture, but one striking difference is the composition of the medium. SVF cells were resuspended and seeded as hanging drops, in medium with growth factors (pidermal growth factor (EGF) and FGF) plus serum, and collected after 5 days. While our iMSCs were seeded without FBS for serum starvation, contained no growth factors and were collected after 2-3 days.

The resulting WAT organoids from the Mandl approach (Mandl et al., 2022) were transferred to agar coated plates in medium without FBS for serum starvation for 2 days, then transferred to an adipogenic differentiation cocktail. While our spheroids were transferred to a suspension culture plate in adipogenic differentiation media. After a further 18 days of differentiation (Mandl protocol), WAT organoids were processed for lipid accumulation and marker expression, while our spheroids were collected after 21 days in suspension. Our control spheroids derived from ASCs, stained positive for bodipy and lipitox (Paper IV, Supplementary Figure S6), comparable to the WAT organoids from Mandl and colleagues and also displayed significant gene expression marker increase in comparison to the undifferentiated control. This not only justifies our approach and validates our control, but it also underscores the time, required to effectively differentiate adipocytes loaded with lipid droplets.

Delta like non-canonical notch ligand 1 (DLK1)/ preadipocyte Factor 1 (PREF1) inhibits adipogenesis in mice (Smas and Sul, 1993) and human (Mitterberger et al., 2012) adipocyte progenitors, while knockdown of DLK1 in ASC causes an increase of PPARγ2 in differentiated adipocytes and is used as a pre-adipocyte marker (Rodeheffer et al., 2008; Tseng et al., 2005). It should be noted, that DLK1 was elevated in our hanging drops, before decreasing over the differentiation. This is in alignment with in vitro studies of ASCs, unfortunately Mandl and colleagues did not analyse their hanging drops before they induced them with their adipogenic cocktail. It is of note that the authors did not sort or characterize their patient derived SVFs. Since the SVF consists of different subpopulations as assessed by marker expression, it would have been interesting to see if different subpopulations would lead to different outcomes of the differentiations. It has been shown that stemness is higher and adipogenic capacity lower in DLK−/CD34+/CD24− relative to DLK+/CD34+/CD24− subpopulations, making the composition of the SVF noteworthy (Hatzmann et al., 2021).
Another important characteristic of adipocytes for differentiation success, is the secretion of adipokines. WAT produces a myriad of adipokines that play a role in energy/metabolic homeostasis. This includes adiponectin, an important adipokine involved in the regulation of glucose levels, lipid metabolism, and insulin sensitivity. The cell culture medium was assessed for secreted adiponectin and was detected in our study and also by Mandl and colleagues. It is worth pointing out, that WAT organoids had no detectable leptin secretion (Mandl et al., 2022) and the mRNA levels did not change significantly during the differentiation process. This is in alignment with our findings and the literature, where leptin secretion in vitro, is very challenging to reproduce (Church et al., 2012).

One aspect that was assessed in the Mandl paper was a colony formation assay; this was to determine the presence of a potential undifferentiated ASC population in the final organoids at the end of differentiation. We did not include a proliferation assay in our study, but it would have been interesting to investigate whether there were still a proliferative cell population present in our spheroids. However, this was not the scope of our article. The comparison is concluded by stating, that the protocol which we developed for our positive control ASC differentiated spheroid, is valid in regard to differentiation approach and choice of markers analysed, as demonstrated by comparison with the published work of Mandl et al. from 2022. Therefore serving as a good comparison for our iMSC derived adipocyte spheroids (Mandl et al., 2022).

Improving the organoid model via oxygen supply

The size of our adipocyte spheroids was determined by the number of cells seeded as hanging drops. Our measurements were comparable to the adipose organoid from the Mandl study (Mandl et al., 2022), which had a radius between 150 and 200 µm, which is very close to our measurements (Paper IV, Figure 5c) and seems to be in line with the reported oxygen diffusion minimum for cell survival in tumours. Cell survival is always connected to cell metabolism and oxygen consumption which is affected by culture, e.g. growing spheroids in gel or suspension (Al Tameemi et al., 2019; Mandl et al., 2022). A possible approach to overcome the size limit, could be the introduction of a vasculature network using endothelial cells or using a microfluidic chip with synthetic vasculature for oxygen supply. We have embarked on the latter, through a collaboration with the Danish Technical University (paper in review); we applied their chip technology, originally developed to house our liver organoids, to adipocyte spheroids. Due to time constraints only a preliminary test was performed, which included loading a chip with the adipocyte spheroids and maintaining them for 4 days. This application needs further work, but could open the door for new possibilities, e.g. providing the adipocyte spheroids with an artificial vasculature. This chip format would also potentially prevent adipocytes from floating off the spheroid due to shear stress in the suspension culture, and would enable investigations of adipocyte size and secretion potential on chip, as an adipocyte size increase can shift the secretion profile to pro-inflammatory cytokines (Skurk et al., 2007).

Solving the issue of inefficient adipogenesis

It has been accepted by the field that current protocols of hiPSC differentiation to adipocytes are relatively inefficient (Guénantin et al., 2017) and a study from Diederichs and Tuan’s indicated the adipogenic capacity is not down to the derivation methods, such as via EB formation or other approaches tested in the study (Diederichs and Tuan, 2014). This led to the speculation that a different developmental approach is required, this is in part why the NCCs were chosen, as adipocyte generation has previously been described from both quail and mouse embryonic stem cells via neural crest (Billon et al., 2007). Another problem is the lack of comparability, since different labs use different adipogenic
induction cocktails. Our approach of using neural crest, as a cell intermediate alone did not result in adipocytes, only transitioning from 2D to 3D delivered differentiated adipocytes. It can only be speculated if the protocols discussed in the section above would benefit from a 3D environment for differentiation or if only the combination of NCCs and the modification of the adipogenic differentiation accomplished iPSC derived adipocytes.

In addition, not all reports validate their tri-lineage potential (osteogenesis, chondrogenesis, adipogenesis) of the iMSCs beyond staining (Menendez et al., 2013) or a few expression markers (Bloor et al., 2020). For example, protocols for chondrogenesis from iPSCs are still under investigation, Prado and colleagues compared four approaches to generate iPSC derived chondrocyte like cells (Castro-ViñUelas et al., 2020) to formulate general recommendations for chondrocyte differentiation, they conclude that formation of EBs and the sequential addition of growth factors enabled the best differentiation of the iPSC into chondrogenic-like cells.

The push to produce a progenitor (hiPSC derived MSC), that can effectively give rise to all the lineages may be hampered by the lack of optimised protocols for further downstream differentiation, rather than the cells` lack of multipotency. Thus, our focus was a protocol for the differentiation to adipocytes. Another compounding issue is cell line specific differentiation propensities which has been described for pancreatic differentiations (Siehler et al., 2021), as well in a study which assessed 17 human ESC lines and showed some lines exhibited a marked propensity to differentiate into specific lineages (Osafune et al., 2008). One could therefore in the future try to optimize differentiation protocols by modifying differentiation cocktails, or pay more attention to the niche and be selective in hiPSC clones used.

Outlook and other possible applications

Another advantage that our protocol offers is the possibility to use the different cell types for other applications. It has been demonstrated that transplanting NCCs with murine pancreatic islets, improved insulin release after transplantation in mice (Olerud et al., 2009). It has further been shown that human intestinal organoids can be combined with vagal NCCs derived from iPSCs to generate an innervated organoid (Loffet et al., 2020). We could exploit iMSCs for their secretion properties, for example a study demonstrated that co-culture of human islets with human ASCs improved islet secretory function in vitro, assessed by glucose-stimulated insulin secretion (Arzouni et al., 2017). MSCs have been used in multiple co-culture applications, for example the in vitro expansion of haematopoietic stem cells and progenitor cells (HPC) from umbilical cord blood where the MSCs potentially provide a suitable cellular environment (Walenda et al., 2010). Other examples include co-culture with SY5Y-differentiated neuronal cells to rescue the neurons after they were subjected to in vitro cerebral ischemia-like stress (Alhazzani et al., 2018) and another was investigating the regenerative potential of hypoxic human cardiomyocytes in vitro with either direct co-culture or conditioned medium (Kastner et al., 2020). Even though there are different subtypes of MSCs as discussed before, we envisage that iMSC co-culture applications would be of great interest due to their secretion profile (Paper IV, Supplementary Figure S3B) and importantly the ability to generate a limitless supply of genetically defined material.

In summary we have developed a protocol for the differentiation of hiPSCs to adipocytes, which can be potentially utilised for organ crosstalk, microfluidic application, and due to their proven secretory profiles, deliver one of the building blocks for future fat-liver axis studies in a dish.
Outlook

This thesis provides protocols for the differentiation of iPSCs to liver organoids, through a developmentally relevant pathway. In addition, the differentiation of iPSC derived adipocytes has also been established. Due to the secretion of adiponectin by the adipocytes, and the receptor expression of ADIPOR1 and ADIPOR2 by the liver organoids, the main building blocks to investigate adiponectin signalling are now in place. It has been demonstrated in the organoid paper that the organoids can take up oleic acid (Harrison et al., 2020), and that PN treatment of the liver organoids has an effect on CYP450 metabolism. This could potentially facilitate the investigation of DNL and lipolysis, and the effect of circulating free fatty acids on the liver organoids in a connected culture system. Also the liver organoids and the adipocyte spheroids are cultured in suspension, they can be transferred to a new culture vessel at any differentiation time point. The microfluidic chip developed by our collaborationpartner at the DTU could be utilised to house both of the models, by providing an artificial vasculature to connect the spheroids with the organoids. The examination of changes in their secretion profiles, inflammation state and possible response to drug treatment could be established in the near future. In conclusion, the thesis provides new tools for the investigation of liver-fat organ cross talk without the ethical concerns of animal models.

References


Original publications
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Autophagy modulates cell fate decisions during lineage commitment

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ABSTRACT

Early events during development leading to exit from a pluripotent state and commitment toward a specific germ layer still need in-depth understanding. Autophagy has been shown to play a crucial role in both development and differentiation. This study employs human embryonic and induced pluripotent stem cells to understand the early events of lineage commitment with respect to the role of autophagy in this process. Our data indicate that a dip in autophagy facilitates exit from pluripotency. Upon exit, we demonstrate that the modulation of autophagy affects SOX2 levels and lineage commitment, with induction of autophagy promoting SOX2 degradation and mesendoderm formation, whereas inhibition of autophagy causes SOX2 accumulation and neuroectoderm formation. Thus, our results indicate that autophagy-mediated SOX2 turnover is a determining factor for lineage commitment. These findings will deepen our understanding of development and lead to improved methods to derive different lineages and cell types.

Abbreviations: ACTB: Actin, beta; ATG: Autophagy-related; BafA1: Bafilomycin A1; CAS9: CRISPR-associated protein 9; CQ: Chloroquine; DE: Definitive endoderm; hESCs: Human Embryonic Stem Cells; hiPSCs: Human Induced Pluripotent Stem Cells; LAMP1: Lysosomal Associated Membrane Protein 1; MAP1LC3: Microtubule-Associated Protein 1 Light Chain 3; MTOR: Mechanistic Target Of Rapamycin Kinase; NANOG: Nanog Homeobox; PAX6: Paired Box 6; PE: Phosphatidylethanolamine; POU5F1: POU class 5 Homeobox 1; PRKAA2: Protein Kinase AMP-Activated Catalytic Subunit Alpha 2; SQX2: SRY-box Transcription Factor 2; SQSTM1: Sequestosome 1; ULK1: unc-51 like Autophagy Activating Kinase 1; WDFY3: WD Repeat and FYVE Domain Containing 3.

Introduction

Macroautophagy (hereafter autophagy) is a conserved cellular catabolic pathway involving lysosomal degradation of cytoplasmic material. Autophagy is important for cellular homeostasis, but is also activated under various intrinsic and extrinsic conditions leading to cellular stress to facilitate cell survival [1,2]. Animal models lacking essential autophagy genes are characterized by increased tumorigenesis, neurodegeneration and developmental defects, indicating that autophagy plays an important role in development [2,3]. Additionally, studies have shown an important role for autophagy in embryo development, as well as during erythropoiesis, lymphopoiesis and adipogenesis [4]. The exact roles of autophagy in the early events of development are however not clear.

Induction of autophagy is tightly regulated by several autophagy related (ATG) proteins, including the ULK1 (unc-51 like autophagy activating kinase 1) complex, the class III phosphatidylinositol 3-kinase (PtdIns3K) complex and the ATG12–ATG5–ATG16L1 complex [5,6]. The activity of the ULK1 kinase is tightly regulated by the MTOR (mechanistic target of rapamycin kinase) and PRKAA2 (protein kinase AMP-activated catalytic subunit alpha 2)/ AMP-activated protein kinase (AMPK). The activated ULK1 complex facilitates production of PtdIns3P at early autophagic membranes, leading to recruitment of the ATG12–ATG5–ATG16L1 complex through its interaction with the PtdIns3P binding protein WIP12 (WD repeat domain, phosphoinositide interacting 2) [7] and direct membrane interactions [8,9]. The ATG12–ATG5–ATG16L1 complex further facilitates the conjugation of Atg8 homolog proteins of the MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) and GABARAP (GABA type A receptor-associated protein) subfamilies to phosphatidylethanolamine (PE) in the autophagic membrane to allow cargo selection and autophagic flux [10,11]. During selective

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autophagy, cargo to be degraded is recognized by specific autophagy receptors that can bind to cargo (often ubiquitinated) and Atg8 proteins in the autophagic membrane [12,13]. One such autophagy receptor is the ubiquitin-binding protein SQSTM1/p62 (sequestosome 1) that also facilitates recruitment of the ULK1 complex and the Ptdlns3P binding protein WDFY3/ALFY (WD repeat and FYVE domain containing 3) to stimulate formation of the autophagosomal membrane around the cargo to be degraded [14–16]. After its completion, the autophagosome fuses with acidic lysosomes to generate an autolysosome where the sequestered cargo becomes degraded. The latter can be effectively blocked by lysosomal inhibitors, such as BafilomycinA1 (BafA1) and chloroquine (CQ), thus inhibiting downstream cargo degradation and recycling of sugars, amino acids or nucleic acids [17,18].

During embryonic development, cells undergo a number of changes including the initiation of organogenesis and differentiation of functional tissues. The development of the early embryo involves a pluripotent cell population within the inner cell mass differentiating to the epiblast and then to the three germ layers, ectoderm, mesoderm and endoderm, being choreographed through both nodal growth differentiation factor (NODAL) and WNT signaling pathways [19]. There is considerable insight into the role of autophagy during the differentiation into erythrocytes, lymphocytes, adipocytes, keratinocytes, osteoclast, etc., and also in human embryonic stem cells (hESCs) [4,20–22]. However, our knowledge about the role of autophagy in germ layer specification is sparse. hESCs and hiPSCs serve as a potentially limitless source of cells to facilitate studies related to cellular reprogramming [23]. These cells offer a unique window to explore the earliest events of differentiation during early human development [24]. To date, researchers have developed differentiation procedures that mimic the developmental trajectory, coaxing human pluripotent stem cells (hPSCs) to cell types that are representative of the three germ layers, using either growth factors, a combination of growth factors and small molecules or solely small molecules [25–30].

POUS1/FCT4 (POU class 5 homeobox 1), SOX2 (SRY-box transcription factor 2) and NANOG (Nanog homeobox) are intrinsic factors that regulate pluripotency and the modulation of their levels is associated with lineage commitment [31,32]. For example, SOX2 has been shown to be important for neuroepithelial formation, while POUS1/F1 acts as a lineage specifier for mesendoderm [4,33]. Interestingly, both POUS1/F1 and SOX2 counteract the formation of definitive endoderm (DE), while NANOG activates the transcriptional network required for DE formation [34]. A recent study utilizing cancer stem cells reported that SOX2, after post-translational modification, is exported from the nucleus and targeted for degradation by autophagy [35,36]. The impact of autophagy on the levels of SOX2 during lineage commitment has however yet to be explored.

In this study, we elucidate the role of autophagy during the formation of the three germ layers, using hiPSCs as our model system. We show that downregulation of autophagy is important for hiPSCs to exit pluripotency. A further upregulation of autophagy facilitates mesoderm and endoderm formation, while continuous downregulation of autophagy takes cells toward neuroectoderm. Importantly, we find that SOX2, but not the other pluripotency factors, is selectively degraded by autophagy during endoderm differentiation. In conclusion, we show that autophagy-mediated degradation of SOX2 determines lineage specificity during hiPSC differentiation.

**Results**

**Autophagy is induced during DE differentiation from hiPSCs**

To investigate the kinetics and role of autophagy during early events of germ layer specification, we used well established procedures that have been shown to effectively differentiate human iPSCs to cell types representative of the three lineages, definitive endoderm (DE), mesoderm (cardiomyocytes) and ectoderm (neuroepithelial stem cells) [28,29,37].

DE is one of the three primary germ layers (innermost), contributing to the formation of endodermal-related organs, including the liver, thyroid, stomach, lungs, pancreas and intestine [38,39]. To efficiently differentiate hiPSCs toward DE, we took advantage of a growth-factor-free protocol that employs the GSK3 (glycogen synthase kinase 3) inhibitor CHIR99021 (WNT agonist) [28,40,41]. In short, the hiPSC line AG27 [42] was treated with CHIR99021 for 24 h, which drives the cells toward primitive streak (PS/mesendoderm) (Fig. S1A). Upon the withdrawal of CHIR99021, the cells transit to DE over the next 24 h. The cells were confirmed for pluripotency using immunofluorescence before setting up differentiations (Fig. S1B). The various stages of differentiation toward DE were confirmed morphologically over the 48 h (Fig. S1C). This was further verified by immunofluorescence microscopy, showing high expression levels of the DE markers FOXA2 (forkhead box A2) and SOX17 (SRY-box transcription factor 17) (Fig. S1D), in line with previous reports describing an average DE differentiation efficiency of 80% or greater using this protocol [28,40,41,43]. The observed changes in morphology were supported by expected changes in gene expression, as assessed by expression analysis of stage specific markers by real-time quantitative PCR (RTqPCR).

Increased mRNA levels of MIXL1 (Mix paired-like homeobox) at 24 h indicated the progression through a PS/mesendoderm stage [44,45], while elevated expression levels of FOXA2 and SOX17 were observed at 48 h (Fig. S1E) [46–48].

To examine the role of autophagy in DE formation, we analyzed transcript and protein levels of various autophagy-related genes involved in bulk and selective autophagy in AG27 cells. The transcript levels of LC3B and SQSTM1 remained stable throughout, whereas WDFY3, ATG5 and ATG7 showed an increase in transcript levels by the 48 h differentiation of AG27 cells to endoderm (Fig. S1F–J). Similarly, the levels of several autophagy related proteins were modulated during DE formation of AG27 cells as analyzed by Western blotting. While the total level of the ULK1 kinase remained stable during DE differentiation, the levels of AMPK- mediated ULK1 phosphorylation at Ser555 fluctuated during DE differentiation, dipping at 4 h and 24 h (Figure 1A, B). Also, the levels of WDFY3, involved in selective
Figure 1. Autophagy is induced during DE differentiation. (A) Cell lysates from AG27 hiPSCs were collected at the indicated time points until DE formation (confirmed by RTqPCR and fluorescence microscopy) and analyzed by Western blotting using the indicated antibodies. The blots are representative of three independent experiments (n = 3). ACTB/beta actin was used as a loading control. (B–D) Quantification of the indicated protein levels during DE formation (0 and 48 h) in AG27 cells. The relative expression levels were normalized to that at 0 h and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001; (B) WDFY3 levels (relative to ACTB) and phospho-ULK1 (Ser-555):total ULK1 levels, (C) SQSTM1 levels (relative to ACTB), (D) LC3B-I and LC3B-II levels (relative to ACTB). (E) The human ESC line, H1, was differentiated to DE and lysed in RIPA buffer at 0 and 48 h, followed by Western blotting using the indicated antibodies. The blots are representative of three independent experiments. ACTB was used as a loading control. * represents RPS6KB p85 and ** represents phosphorylated RPS6KB p85 that are both recognized by the anti-RPS6KB kinase antibody. (F) Quantification of p-ULK1:ULK1 and p-RPS6KB:RPS6KB levels (relative to ACTB) during DE formation from H1 cells (0–48 h). The relative expression levels were normalized to that at 0 h and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (G) Quantification of SQSTM1 and LC3B-II levels (relative to ACTB) during DE formation from H1 cells (0–48 h). The relative expression levels were normalized to that at 0 h and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001.
autophagy, showed a transient dip at 4 h and increased again from 8 h, while SQSTM1 levels decreased at both 24 and 48 h of differentiation, indicating an upregulation of autophagy (Figure 1A,C). In line with this, protein levels of the PIK3C3/VPS34 kinase increased while the levels of phosphorylated RPS6KB1/p70S6K (ribosomal protein S6 kinase B1; a direct target of MTOR) decreased during DE formation (Figure S1K). There were no significant differences in the protein levels of LC3B and GABARAP during DE differentiation (Figure 1A,D and S1K), but it is difficult to conclude about the autophagic flux as these blots represent steady state levels of these autophagy markers.

An induction of autophagy upon DE differentiation was also seen in the human ESCs line H1. DE differentiation was induced by treating the cells with CHIR99021 for 24 h, followed by 24 h incubation in the absence of CHIR99021 (Fig. S1L). Increased autophagy levels at 48 h was demonstrated by increased ULK1 phosphorylation, reduced MTOR activity and reduced SQSTM1 levels (Figure 1E–G; Fig. S1M).

As autophagy is a dynamic process where cargo and membrane-bound LC3-II become rapidly degraded in the autolysosome, it is difficult to evaluate levels of autophagic flux at steady state. We therefore generated a stable iPSCs line containing a doxycycline (Dox) inducible mCherry-EGFP-tagged LC3B reporter (hereafter referred to as double-tagged LC3B, dt-LC3B), which can be visualized as yellow puncta in early autophagic structures and as red-only puncta upon autophagosome fusion with a lysosome, due to quenching of the EGFP signal in the low pH environment of the autolysosome (Figure 1H). Both yellow and red puncta were seen upon induction of dt-LC3B expression in iPSCs, indicating basal levels of autophagy for pluri potency maintenance (Figure 1I, left panel). The dt-LC3B line was validated for the expression of the pluripotency factors POU5F1, SOX2 and NANOG using immunofluorescence microscopy (Fig. S2A). To demonstrate that the dt-LC3B iPSC cell line responded to known autophagy modulators, the cells were starved for 1 h (EBSS media) to induce autophagy in the absence or presence of an inhibitor of ULK1 activation (MRT68921 dihydrochloride) [49]. As expected, starvation caused an increase in the total number of both yellow and red puncta (Figure 1I, middle panel), while MRT68921 drastically reduced the number of red puncta (Figure 1I, right panel), demonstrating that the dt-LC3B iPSC line responded to autophagy modulation.

To study the kinetics of autophagic flux during DE formation, cells were fixed, and analyzed by confocal microscopy at 0, 12, 24 or 48 h during DE differentiation and the relative number of red-only puncta was quantified. In line with our Western blot data, an increased autophagic flux was observed at 12 h with increasing numbers of red-only puncta present by the end of DE differentiation at 48 h (Figure 1J,K). This was further confirmed by an increased number of endogenous LC3B puncta at 48 h after induction of DE differentiation (Figure 1L,M). Taken together, our data indicate that autophagy is upregulated during differentiation of hiPSCs and hESCs toward DE formation.

**Opposite roles of autophagy during differentiation of hiPSCs to cardiomyocytes and neuroectoderm**

To investigate the kinetics of autophagy in mesoderm specification, AG27 iPSCs were treated with a protocol that leads to generation of beating cardiomyocytes after 12–17 days [29] (Video SV1 and SV2) that were positive for the sarcomere marker ACTN1 (actin alpha 1) (Fig. S2B). This was further confirmed by qRT-PCR data from beating cardiomyocytes showing a strong increase in the levels of cardiomyocyte-specific markers, NKX2.5 (NK2 homeobox) and TNNT2 (troponin T2, cardiac type) (Fig. S2C).

The levels of WDFY3, p-ULK1 (Ser555), SQSTM1 and LC3B proteins were determined by immunoblotting of cell lysates at different time points throughout cardiomyocyte formation until beating was observed. Enhanced protein levels of WDFY3 and the phosphorylated form of ULK1 (Ser555), as well as reduced levels of SQSTM1 and increased LC3B-II levels (Figure 2A–C) indicated an induction of autophagy during cardiomyocyte differentiation. To further corroborate our findings, the mCherry-EGFP-LC3B iPSC line was stimulated toward cardiomyocyte differentiation and the level of yellow and red-only puncta was analyzed by confocal imaging at different days during differentiation. Indeed, the total number of LC3B puncta, as well as red-only puncta increased upon mesoderm differentiation (Figure 2D,E), in line with previous findings showing that both endoderm and mesoderm follow a common path until the formation of mesendoderm where there is a bifurcation to either endoderm or mesoderm [50].

In contrast to DE and mesoderm differentiation, the formation of the ectoderm does not involve the PS/mesendoderm stage [51]. We therefore assessed whether the kinetics of autophagy during ectoderm formation would differ from that seen during endoderm and mesoderm formation. Ectoderm formation was initiated using a well-established procedure of

(n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (H) Schematic diagram showing the principle of the mCherry-EGFP double-tag (dt) approach to monitor autophagic flux. mCherry-EGFP-tagged LC3B is visualized as yellow puncta in both phagophore and autophagosome structures, while after lysosomal fusion only red puncta are observed due to quenching of the EGFP signal in the acidic lysosome. (I) AG27 iPSCs with stable doxycycline-inducible expression of mCherry-EGFP-LC3B were grown in full media or starved (EBSS) for 1 h in the absence or presence of the ULK1 inhibitor MRT68921 (EBSS+MRT) to study autophagosome formation and autophagic flux. Arrowheads indicate autolysosomes (red only structures). Image is representative of three independent experiments. Scale bar: 10 µm. (J) AG27 iPSCs expressing mCherry-EGFP-LC3B were seeded as single cells and differentiated to DE. The cells were fixed at different time points after induction of differentiation and processed for microscopy. Images are representative of three independent experiments. Scale bar: 10 µm. (K) Quantification of the number of red-only puncta formed during DE differentiation from images as shown in (J). A minimum of 20 images at each time point (n = 20) were used for quantification with ImageJ analysis. Error bars represent SD. *P < 0.1. (L) AG27 iPSCs were fixed before (0 h) and after (48 h) DE formation and processed for immunofluorescence microscopy using an anti-LC3B antibody. Representative images with endogenous LC3B spots (green, arrowheads) are shown. Nuclei were counterstained with DAPI. Images are representative of three independent experiments. Scale bar: 10 µm. (M) The number of LC3B puncta formed during DE formation were quantified from a minimum of 20 images from each time point as shown in (L) using ImageJ analysis. Error bars represent SD. **P < 0.01.
Figure 2. Kinetics of autophagy during differentiation to mesoderm and neuroectoderm. (A) Autophagy kinetics during cardiac differentiation. AG27 human iPSCs were seeded and treated with CHIR on day 3 (8 μM). On day 6, cells were treated with IWP2 (5 μM). Cells started beating between day 8 and 17. Cell lysates were collected at different time points and processed for Western blotting using the indicated antibodies. The blots are representative of three independent experiments (n = 3); ACTB was used as a loading control. (B) Quantification of SQSTM1 and LC3B-II levels (relative to ACTB) during mesoderm (cardiomyocyte) formation from AG27 cells. The relative expression levels were normalized to that at day 0 and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (C) Quantification of WDFY3 and p-ULK1 (Ser-555):total ULK1 levels (relative to ACTB) during mesoderm (cardiomyocyte) formation from AG27 cells. The relative expression levels were normalized to that at day 0 and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (D) AG27 hiPSCs with stable Dox-inducible expression of mCherry-EGFP-LC3B were seeded as single cells and differentiated to cardiomyocytes. The cells were fixed at different days after induction of cardiomyocytes differentiation until day 7 and processed for microscopy. Images are representative of three independent experiments. Scale bar: 10 μm (day 0–5), 100 μm (day 7). (E) The number of LC3B puncta...
dual SMAD inhibition, leading to the generation of neuroectoderm over a 6-day period [37]. The successful formation of the neuroectoderm was confirmed at day 6 by assessing both the protein and mRNA levels of the ectodermal lineage markers NES (nestin) and PAX6 (paired box 6) (Fig. S2D-F). Importantly, the protein levels of WDFY3 and active p-ULK1 (Ser555), were reduced over the 6-day period (Figure 2F,G), while LC3B lipidation and SQSTM1 levels remained rather stable during ectoderm formation. To further characterize the autophagic flux during ectoderm differentiation, the mCherry-EGFP-LC3B iPSC line was treated to induce the formation of ectoderm. In contrast to what was observed during endoderm and mesoderm formation, no significant increase in red-only puncta was observed during the 6 days treatment protocol (Figure 2H,I), indicating that autophagic flux is kept at a low level during neuroectoderm formation.

**Short-term inhibition of autophagy facilitates exit from pluripotency**

Autophagy has been shown to be essential for both attaining and maintaining a pluripotent state [52,53]. In line with this, our data show a transient dip in the protein levels of several proteins involved in autophagy (p-ULK1[Ser555], PIK3C3 and WDFY3) at 4 h during DE formation compared to the iPSC state (Figure 1A; Fig. S1K), indicating that a transient inhibition of autophagy brings iPSCs out of pluripotency. This was confirmed by experiments showing that hiPSCs treated with the lysosomal inhibitors bafilomycin A1 (BafA1) and chloroquine (CQ) [17,18] for 4 h displayed a change in cell morphology commencing as early as 8 h (data not shown) and within 24 h the cells had lost their typical compact pluripotent morphology, becoming looser and in some cases flatter, with increased cytoplasmic volume and were more elongated (Figure 3A). Corroborating these morphological findings, we found that mRNA levels of the pluripotency factors SOX2, NANOG and KLF4 (Kruppel like factor 4) were significantly reduced in both BafA1 and CQ treated hiPSCs (Figure 3B), indicating that inhibition of autophagy was sufficient to drive cells out of pluripotency.

To further understand how inhibition of autophagy affected the state of the hiPSCs, we performed RTqPCR against a panel of various pluripotency and lineage-specific markers in an array card format (Fig. S3A) [42]. Concomitant with the observed morphological changes, we found elevated transcript levels of markers of all the three germ layers in iPSCs treated with autophagy inhibitors, including GATA4 (GATA binding protein 4), HAND1 (heart and neural crest derivatives expressed 1) and GATA2 as well as PAX6 (Figure 3C), which are markers for endoderm, mesoderm and ectoderm, respectively [54–57]. These results indicate that a short 4 h inhibition of autophagy in pluripotent stem cells results in their exit from a pluripotent state, allowing commitment toward the three germ layers.

**Prolonged inhibition of autophagy drives hiPSC cells toward Neuroectoderm**

We next investigated how prolonged inhibition of autophagy could influence the fate of hiPSCs. In brief, hiPSCs were incubated with BafA1 or CQ for 24 h, followed by RTqPCR analysis of the resulting population of cells against our panel of markers as described above (Fig. S3A). Interestingly, the levels of PAX6 (neuroectoderm) were greatly elevated (up to 500-fold as compared to control) after 24 h following treatment with either BafA1 or CQ (Figure 3D), indicating that extended inhibition of autophagy may drive hiPSCs to a neuroectodermal fate. This is supported by our protein kinetics data, showing that autophagy is downregulated during neuroectoderm formation (Figure 2F–I).

To further assess the effect of inhibition of autophagy on lineage commitment, we used clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing to construct an ATG7 hiPSC knockout line. ATG7 functions as an E1-like enzyme in the lipidation of LC3 and GABARAP proteins and its disruption will thus inhibit autophagic flux [58,59]. For the depletion of ATG7 protein production, we targeted the region close to the start codon of the ATG7 sequence. The CAS9-induced cleavage led to a 32 bp deletion resulting in a premature stop codon (Fig. S3B–C). Interestingly, while a number of heterozygous (ATG7<sup>+/−</sup>) clones were obtained, no homozygotes (ATG7<sup>−/−</sup>) clones were found, suggesting that ATG7 is essential for survival of hiPSCs. Importantly, LC3B lipidation was substantially reduced in the heterozygous ATG7<sup>+/−</sup> hiPSC lines as compared to the isotype control (Figure 3E), indicating a reduction of ATG7 activity in the heterozygous clones. When ATG7<sup>−/−</sup> hiPSCs was subjected to neuroectoderm formation, an early induction (by day 4) of neuroectoderm marker SOX2 was observed as compared to wild-type AG27 cells (Figure 3F, Fig. S3D). The levels of pluripotency markers in the ATG7<sup>−/−</sup> hiPSC were reduced as compared to the parental AG27 cells, indicating relatively less stability of these cells in pluripotency state (Fig. S3E). Moreover, when taken toward the endoderm, the level of the DE marker SOX17 was reduced in ATG7<sup>−/−</sup> hiPSC as compared to wild-type AG27 cells at 48 h, indicating a possible delay toward endoderm differentiation (Figure S3F).

Intriguingly, when the hiPSCs were grown under standard culture conditions, we observed that a distinct cell population formed during DE formation were quantified from a minimum of 100 cells from each time point (except day 7) as shown in (D) using Imagej analysis. Error bars represent SD. ***P < 0.001. (F) Autophagy kinetics during neuroectoderm differentiation. AG27 cells were collected at the indicated time points during neuroectoderm differentiation and processed for Western blotting using the indicated antibodies (n = 3). ACTB was used as a loading control. (G) Quantification of WDFY, p-ULK1[ULK1] and LC3B-II levels (relative to ACTB) during neuroectoderm formation (day 0-day 6) from AG27 cells. The relative expression levels were normalized to that at day 0 and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (H) AG27 hiPSCs with stable Dox-inducible expression of mCherry-EGFP-LC3B were seeded as colonies (EDTA splitting) and differentiated to neuroectoderm. The cells were fixed at different days after induction of neuroectoderm differentiation and processed for microscopy. Images are representative of three independent experiments. Scale bar: 10 µm. (I) The number of red puncta were quantified from a minimum of 100 cells from each time point as shown in (H) using Imagej analysis. Error bars represent SD.
Figure 3. Inhibition of autophagy facilitates exit from pluripotency and drives hiPSC cells toward neuroectoderm. (A) AG27 iPSCs were treated or not with the autophagy inhibitors bafilomycin A1 (BafA1) or chloroquine (CQ) for 4 h before replacing with E8 medium. Representative images of the cell morphology at the 24 h time point are shown (n = 3). Scale bar: 50 µm. (B) AG27 iPSCs were treated as in (A) and processed for RTqPCR analysis at 24 h using TaqMan probes for major pluripotency genes (KLF4, SOX2, NANOG). Untreated iPSCs served as control. The graph shows the average of three independent experiments (n = 3). Error bars represent SD. **P < 0.01, ***P < 0.001. (C) AG27 iPSCs were treated as in (A) and processed for RTqPCR analysis at 24 h using an array card (Fig. 5S3A) to analyze the transcript levels of genes of various germ layers. The most significant changes in transcript levels are shown and graphs represent an average of three independent experiments (n = 3). Error bars represent SD. *P < 0.05, **P < 0.01, ***P < 0.001. (D) AG27 iPSCs were treated or not with BafA1 or CQ for 24 h and then processed for RTqPCR analysis using an array card (Fig. 5S3A) to analyze the transcript levels of genes of various germ layers. The graph shows PAX6 expression levels and represents an average of three independent experiments (n = 3). Error bars represent SD. **P < 0.01. (E) AG27 iPSCs cells were electroporated with guide RNA for ATG7 (Table 1). The tracr-RNA was fluorescently labeled with ATTO dye. Clones were screened using restriction fragment length polymorphism (RFLP). Cell lysates from wild
began to migrate from the ATG7- hiPSC colonies and not from the isotype controls, when the cells became more confluent (6–7 days in culture) (Figure 3G). In line with this, we noted a loss of typical pluripotent stem cell morphology by day 5 and the appearance of neural “rosette-like” structures in the ATG7- hiPSCs by phase contrast microscopy. By day 8, the number of “rosette-like” structures had further increased (Figure 3G, upper right panel). A similar morphology was observed in many other ATG7- hiPSC clones, confirming that this was not an artifact (Fig. S3G). By day 24, we noted the formation of several neurosphere-like structures with putative neuron-like cells emerging (Figure 3G, lower right panel) in contrast to wildtype control (Figure 3G, lower left panel). As long-term chemical inhibition of autophagy was found to drive iPSCs toward neuroectoderm (Figure 3D), we speculated that the cells migrating from the ATG7- hiPSC colonies might be neuroectodermal in nature. Indeed, PAX6 positive cells were detected as early as 3 days post plating and these cells corresponded to the cells that migrated from the colony or close to the leading edge of the colony (Figure 3H). Moreover, strong upregulation of PAX6 was detected in the ATG7- hiPSCs (day 5) by RTqPCR (Figure 3I). The neuron-like cells derived from the ATG7- hiPSCs were positive for the neuronal marker TUBB3 (tubulin beta 3 class III) [60], as analyzed by immunofluorescence microscopy at day 15 (Figure 3J). As expected, the formation of endogenous LC3B puncta was strongly reduced in ATG7- hiPSCs compared to WT cells (Figure 3J, zoom). Taken together, our results indicate that a brief inhibition of autophagy facilitates exit from pluripotency, extended inhibition of autophagy appears to drive the cells toward an ectodermal fate.

**SOX2 is degraded by autophagy during DE differentiation**

Having confirmed that autophagy is upregulated during DE differentiation, we next asked whether induction of autophagy would facilitate differentiation of iPSCs to DE. To investigate this, iPSCs were treated with the autophagy inducer rapamycin for the first 24 h of DE differentiation. Interestingly, rapamycin-treated cells showed typical DE morphology by 48 h, with reduced areas of undifferentiated cells as compared to the standard protocol (Fig. S4A). Moreover, both the mRNA and protein levels of SOX17 increased upon rapamycin treatment as compared to control (Figure 4A,B), suggesting that induction of autophagy may improve DE formation.

To further elucidate the role of autophagy during DE formation, we asked whether autophagy might facilitate degradation of a pluripotency factor (POUSF1, SOX2 or NANOG) during DE formation. As expected, we observed a strong reduction in the levels of SOX2, NANOG and POU5F1 by 48 h of DE differentiation in control cells (Figure 4C). Remarkably, SOX2 degradation was delayed (up to 12 h) under DE differentiation in cells treated with the ULK1 inhibitor MRT68921 as compared to control cells (Figure 4C,D), indicating that SOX2 might be a potential cargo for autophagy. This was further confirmed in cells treated with Bafilomycin A1 (BafA1) for 2 h before collection at 12 or 48 h during DE formation, showing increased levels of SOX2 at both time points as compared to control cells (Figure 4E,F), as well as decreased level of SOX17 at 12 h, indicating a possible delay in DE formation upon blocking autophagy. Importantly, SOX2 was also stabilized at 12 h of DE differentiation in ATG7- hiPSCs compared to WT cells (Figure 4G,H; Fig. S4B), further indicating that SOX2 is processed through autophagy. ATG7- hiPSCs were able to make DE, but the level of SOX17 was reduced at 48 h compared to WT cells (Fig. S3F). Further supporting the role of autophagy in SOX2 degradation, we found a reduction in the levels of SOX2 at 12 h in cells treated with the autophagy inducer rapamycin (MTORC1 inhibitor) (Figure 4E,F). As rapamycin treatment also enhanced mRNA and protein levels of SOX17 (Figure 4A,B), our data indicate that enhanced autophagy facilitates SOX2 processing and DE formation.

To further confirm these findings, we generated stable hiPSC lines carrying inducible double-tagged (Cherry-EGFP) pluripotency factors (dt-POUSF1, dt-SOX2 or dt-NANOG). As expected, all cell lines showed nuclear localization of the double-tagged proteins under pluripotent conditions (Fig. S4C). Next, the double-tagged lines were subjected to DE differentiation to assess if any of the pluripotency factors were substrates for autophagy. Indeed, the autophagic degradation of dt-SOX2 was indicated by the clear formation of red-only puncta outside the nucleus within 24 h (mesendoderm), which increased further by 48 h (Figure 4I). The number of red-only puncta were significantly reduced upon treatment with the ULK1 inhibitor MRT68921 at both time points, indicating that SOX2 is a cargo for autophagy during DE differentiation (Figure 4I). By 48 h, the nuclear expression of SOX2 was undetectable (as compared to both 24 h and control), indicating that the majority of SOX2 was targeted to lysosomes. In contrast, we observed no significant formation of red puncta for dt-NANOG or dt-POUSF1 during DE formation (data not shown). Lysosomal degradation of SOX2 was further supported by demonstrating that endogenous SOX2 colocalized extensively with LAMP1 (lysosomal associated membrane protein 1) at the perinuclear region, at both 24 and 48 h in control cells, which also was prevented by inhibition of ULK1, majorly at 24 h (Figure 4K). Furthermore, some colocalization between SOX2 and SQSTM1 was detected.
Table 1. Cells, antibodies and other reagents used in the study are indicated below.

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at 24 h, suggesting that the selective autophagy machinery might be involved in degradation of SOX2 (Figure 4L). Taken together, these data indicate that SOX2 is degraded through the process of autophagy during DE formation.

To investigate whether the degradation of SOX2 by autophagy is specific for DE formation, dt-SOX2 iPSCs were differentiated to neuroectoderm. According to the literature, SOX2 is required for neuroectoderm formation and should therefore not be degraded [61]. As expected, the nuclear expression of dt-SOX2 was maintained upon neuroectoderm formation and no red dt-SOX2 puncta were observed over the 6 days differentiation protocol (Fig. S4D). Interestingly, a continuous overexpression of dt-SOX2 hiPSCs resulted into a morphology similar to ATG7+ hiPSCs, indicating that increased SOX2 levels drive iPSCs toward neuroectoderm (Fig. S5).

**Discussion**

It is well established that autophagy is required for the reprogramming of somatic cells to a pluripotent state and for maintenance of pluripotency [53,62,63]. However, little is known about the molecular programs, including autophagy, that regulate exit from pluripotency and entry to germ layer commitment. As expected, we found that a transient inhibition of autophagy stimulate exit from pluripotency, toward

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**Software and Algorithms**

| GraphPad prism | Graph Pad | https://www.graphpad.com/scientific-software/prism/ |
| SnapGene | SnapGene | https://www.snapgene.com/ |
| Fiji | ImageJ | https://fiji.sc/ |
Figure 4. Autophagy modulation affects DE differentiation through SOX2 degradation. (A) AG27 iPSCs were treated with CHIR to induce DE formation in the absence or presence of the MTOR inhibitor rapamycin. After 48 h, SOX17 levels were assessed by RTqPCR. The graph is representative of three independent experiments (n = 3). Error bars represent SD. ***P < 0.001. (B) AG27 iPSCs were treated as in (A) and processed for Western blotting using a SOX17 antibody (n = 3). ACTB was used as a loading control. (C) AG27 cells were treated with CHIR to induce DE differentiation in the absence or presence of the ULK1 inhibitor MRT68921. Cell lysates were collected at different timepoints of DE differentiation and analyzed by Western blotting using antibodies against the pluripotency factors POU5F1, SOX2 and NANOG (n = 3). ACTB was used as a loading control. (D) Quantification of SOX2 levels (relative to ACTB) in lysates from cells treated as in (C) at 0, 12 and 48 h during DE differentiation. The relative expression levels were normalized to that at day 0 and are presented as the mean of three independent experiments (n = 3); error bars represent SD. *P < 0.1, **P < 0.01, ***P < 0.001. (E) Cell lysates from AG27 cells treated or not with autophagy inhibitor, BafA1 or autophagy inducer, rapamycin and were collected at different timepoints (0, 12 and 48 h) of DE differentiation, followed by immunoblotting with the indicated antibodies (n = 3). ACTB was used as a loading control. (F) Quantification of SQSTM1, SOX2 and SOX17 levels (relative to ACTB) in cell lysates from BafA1 and rapamycin treated cells during DE
a differentiated state as evident by the expression of markers of all the three germ layers. Remarkably, upon extended inhibition of autophagy, or when reducing the levels of the autophagy-specific gene ATG7, cells differentiated toward the neuronal lineage. In line with this, the autophagy levels were reduced during neuronal differentiation. In contrast, an increase in autophagy was seen when pluripotent stem cells transit toward mesoderm and endoderm, both derived from a common mesendoderm population [64,65]. The role of autophagy in mesendoderm differentiation was further supported by improved DE differentiation in cells treated with Rapamycin or Torin, drugs known to induce autophagy by inhibition of mTOR activity.

The transcription factors POU5F1, SOX2, and NANOG are known master regulators of self-renewal and pluripotency and skewing of the levels of these factors can lead to exit from pluripotency and ultimately differentiation [31,32]. Previous studies have indicated that SOX2 is a lineage specifier toward neuroectoderm differentiation [61,66], but the factors that regulate the level of SOX2 as well as the other master transcription factors remain largely unknown. We here show that SOX2 is a target for autophagy during DE formation, but is stabilized during neuroectoderm differentiation when autophagy levels are low. In line with this, SOX2 levels were increased at an earlier timepoint in ATG7− hiPSC compared to WT cells when differentiated into neuroectoderm. Thus, we propose that autophagy-mediated SOX2 turnover may contribute to lineage commitment (Figure 5).

Our data indicate that autophagy-specific turnover of SOX2 facilitates DE differentiation, as inhibition of the ULK1 kinase or lysosomal degradation (by BatA1 or chloroquine) delayed SOX2 turnover and SOX17 expression, a marker for endoderm. Furthermore, the levels of SOX2 were also stabilized at 12 h as compared to the AG27 parental cells when ATG7− hiPSCs were differentiated into DE. The mechanisms involved in autophagy of SOX2 remains elusive, but we could detect SOX2 colocalization with the ubiquitin-binding autophagy receptor SQSTM1 and LAMP1 positive lysosomes found in close proximity to the nucleus. Interestingly, only red puncta (no yellow) were observed outside the nucleus in the mCherry-EGFP-SOX2 hiPSC line, with the majority of red-only structures observed in the juxtanuclear region. A similar phenomenon was observed for the degradation of nuclear lamina in human senescent cells [68]. In yeast, a unique micro-autophagic process termed piecemeal micro-autophagy of the nucleus has been reported, which involves the pinching-off and degradation of nonessential portions of the nucleus [69,70].

During this process, a nuclear bleb is formed that instead of being initially packaged into an autophagosome becomes directly engulfed by the vacuole, the yeast equivalent of the lysosome [68]. Whether a similar process is involved in the autolysosomal degradation of SOX2 is worthy of future investigation. Also, it will be interesting to explore the contribution of the proteasomal machinery in SOX2 processing during DE formation, if any. A recent study suggested that SOX2 serves as a target for autophagy in cancer stem cells [36]. It was found that MAPK3 (mitogen-activated protein kinase 3)/ERK1 kinase promoted autophagic degradation of SOX2 via phosphorylation of SOX2 at Ser251, but the underlying mechanisms are unclear. Interestingly, the activation of ERK signaling during DE differentiation has been observed [71] and it will be interesting to explore whether ERK-mediated phosphorylation of SOX2 facilitates its autophagic degradation during DE specification. It has also been reported that p300-mediated acetylation of SOX2 induces its nuclear export in ESCs [35], but a possible link to autophagy is unknown.

Interestingly, we did not observe any evidence for autophagy-mediated degradation of SOX2 during neuroectoderm formation. Further supporting this, we found that hiPSCs overexpressing the mCherry-EGFP-SOX2 for a week lost pluripotency and showed a neuronal morphology similar to hiPSCs depleted of ATG7 (ATG7− hiPSCs). In contrast, POU5F1 has been proposed as a lineage specifier for mesendoderm [4,33], which is supported by our data showing the presence of POU5F1 protein until DE formation with no evidence of autophagy-mediated degradation of dt-POU5F1. Although we cannot rule out that the modulation of autophagy is a result of the small molecules being used to induce differentiation rather than a reflection of the cells progressing through differentiation, we demonstrate the importance of autophagy for lineage commitment using different inducers and inhibitors as well as by genetic downregulation of autophagy. One could speculate that a crosstalk of the WNT pathway and autophagy may facilitate these lineage commitment decisions, but this would require more work.

The main findings of our study can be compared to a junction on a highway, one leading uphill (mesendoderm) the other downhill (ectoderm) (Figure 5). The vehicle at the junction (a pluripotent state) comes to a transient stop (mimicked by the early dip in autophagy) at which point, it is free to go in any direction, either up or downhill. While autophagy is required to drive uphill, thus directing pluripotent stem cells to a mesendoderm fate, an inhibition or
downregulation of autophagy will cause the cells to travel downhill (to a neuroectodermal fate). Autophagic degradation of SOX2 is one of the determining factors of these events, where its turnover is the driver of a mesendoderm fate, while its accumulation drives cells toward a neuronal fate.

Taken together, our results show for the first time the importance of autophagy in cell fate decisions during differentiation of hiPSCs. These findings will not only pave the way for a deeper understanding of the molecular mechanism and cargo involved in cell fate decisions, but could also be relevant to understand the tight regulation of maintenance and exit from pluripotency.

Materials and methods

Cell culture

H1 hESCs (WiCell) [72] and the Sendai virus-derived hiPSC line AG05836B [42] clone #27 (AG27) were cultured under feeder-free conditions in Essential 8 Medium (E8) on Gelatin-coated plates at 37°C/5% CO₂. The cultures were routinely passaged 1:3 ratio every 4–5 days.

Electroporation of human iPSCs

For optimal results, human pluripotent stem cells at 75–80% confluency and no evidence of differentiation were used. Note that 1 million per reaction is required. The cells were pre-treated with Y27632 (final concentration 10 µM) for 2–3 h. For the electroporation, 82 µl of Human stem cell Nucleofector solution 2 was combined with 18 µl of Supplement 1 (reaction A hereafter) and incubated at 37 degrees for at least 5 min. While reaction A was incubating, the cells were washed with 4 ml of PBS, followed by 1 ml of accutase, when the cells detached easily on gentle mixing the accutase was neutralized in E8 supplemented with Y27632. The cells were then passed through a cell strainer (a small aliquot was removed prior to pelleting to assess the cell number), followed by centrifugation at 300 g for 3 min. After centrifugation the cell pellet was resuspended in E8 supplemented with Y27632 to give 1 million cells/ml. Next, 1 ml of cells were transferred to a sterile 1.5 ml microcentrifuge tube and pelleted, the supernatant was aspirated and the resulting pellet was in resuspended in 100 µl of reaction A. DNA or RNA was added directly to the cell suspension and the cells were transferred to a cuvette and electroporated using A-023 program on the Amaxa 2D machine. The electroporated cells were carefully resuspended in E8 medium supplemented with Y27632 and seeded at the required density.

Generation of human iPSC lines harboring a double tag reporter

AG27 hiPSCs were processed for single-cell seeding as described above. For each electroporation, 1 million cells were combined with 5 µg of the respective PiggyBac reporter construct. The cells were seeded at low density to aid downstream picking of colonies and were selected under puromycin. Colonies formed were picked, cultured and then replica plated. The replica plate was supplemented with doxycycline and colonies exhibiting the expected fluorescence (EGFP and mCherry) were further expanded and validated.

Generation of ATG7+ human iPSC cells

CRISPOR (http://crispor.tefor.net) was used to identify potential ATG7 guides (Table 1). In a PCR tube, 5 µl of guide crRNA (100 µM, IDT) was mixed with 5 µl of tracr RNA (100 µM, IDT). The complex was annealed using a PCR block with the following conditions: 95°C, 5 min, 70°C 10 min, 45°C, 5 min and 4°C hold (gradual cooling, ramp rate 0.1°C per sec). For RNP formation, 2 µl of the annealed crRNA-tracr complex was mixed with 4 µg of CAS9 protein (stock 10 µg/µl). The mixture was incubated for 1 h at room temp. The cells were prepared when the RNP complexes were forming, the hiPSC line AG27 was processed for single-cell seeding and electroporated as described above. When colonies appeared, they were picked, expanded and screened. Screening was performed as follows, genomic DNA was
isolated with the Phire animal tissue direct PCR kit and the resulting genomic was subjected to PCR with ATG7 specific primers (Table 1). The amplified DNA was then digested with BamHI enzyme and resolved on an agarose gel. The colonies that generated the expected restriction pattern were validated by sequencing. Positive clones were then expanded, validated, banked and used for subsequent experiments.

**Endoderm differentiation**

hiPSCs or HsESCs were plated as single cells at a density of 47,500/cm² in E8 supplemented with Y27632, the cells were culture overnight and differentiated [28,40,41]. In brief, the differentiation was initiated when the cells reach approximately 30% confluent (approximately 18–20 h post plating). Differentiation to definitive endoderm was achieved by a 24 h treatment with RPMI-B27 ± insulin supplemented with CHIR99021, after 24 h the medium was exchanged to RPMI-B27 ± insulin alone.

**Mesoderm differentiation**

hiPSC cells were plated as single cells at a density of 40,000/cm² in E8 supplemented with Y27632. A slightly modified protocol described by Lian and colleagues [29] was used. Briefly, the cells were cultured in E8 for 2 days. On day 3 post seeding, (approximately 75–80% confluence), cells were cultured in RPMI/B27-minus insulin supplemented with 8 μM CHIR99021 (WNT agonist) for 24 h. The next day, cells medium was exchanged to RPMI/B27-minus insulin and cultured for 48 h. The cells were then treated with RPMI/B27 (minus insulin) supplemented with IWP2 (5 μM) (WNT antagonist working through the inactivation of PORCN (porcupine O-acetyltransferase) [29]), for another 48 h. After this, the cells were cultured in RPMI/B27-minus insulin for another 48 h. Finally, the medium was exchanged RPMI/B27-with insulin until beating was observed in the culture.

**Ectoderm differentiation**

The protocol described by Maroof and colleagues was followed [37]. In brief, high-quality hiPSCs at a confluency of 75–80% were passaged 1:3 as described above. The next day, the cells were treated every alternate day with Neural Phase 1 Base Medium (Advanced DMEM/F12 with 1% Glutamax, 1% penicillin-streptomycin and 1% N2 base) supplemented with 10 μM SB431542, 100 nM LDN 193189, and 2 μM XAV-939 until day 6.

**Immunofluorescence staining**

Cells were fixed with 4% formaldehyde for 15 min, permeabilized with 0.05% saponin in PBS, and blocked with 10% goat serum. The cells were incubated with the appropriate dilution of primary antibody for 1 h at room temperature. After 1 h the antibody was removed and cells were washed three times with 0.05% saponin in PBS. The respective secondary antibodies (1:500 dilution) were applied and incubated for 45 min at room temperature. To visualize the nucleus, samples were either incubated with Hoechst (1 μg/ml) and then mounted in ProLong Diamond Antifade or mounted directly with Fluoroshield with DAPI. Antibodies used are listed in Table 1. Cells were imaged using a confocal microscope (Zeiss LSM 710) with a 63x objective lens or with a high throughput fluorescent microscope (Zeiss Axiovert Observer Z1).

**Western blot analysis**

Cell extracts were generated by manually scraping the cells from the culture well and homogenized in RIPA buffer (50 mM Tris-Cl, pH 7.4, 120 mM NaCl, 1 mM EDTA pH 8.0, 1% NP-40, 0.25% Triton X-100). After protein quantification by Pierce BCA Protein Assay Kit, 20 μg of protein was applied to a SDS gel (4–20%, Bio-Rad Criterion) and separated by electrophoresis. Then the resulting gel was blotted onto a PVDF Immobilon FL membrane, 0.45 μm pore size. The membrane was blocked with casein buffer for 1 h and then incubated in the appropriate dilution of primary antibody overnight at 4°C. After three washes in PBST (PBS +0.01% Tween 20), each of 5 min, the membrane was incubated with the appropriate dilution of the secondary antibody for 1 h at room temperature. Protein bands were visualized using the Odyssey imager (LI-COR). The antibodies used are listed in Table 1.

**RNA isolation and RTqPCR**

RNA was isolated using TRIzol as per the manufacturer’s recommendations and the resulting RNA quantified using a Nanodrop spectrophotometer. cDNA was generated using the High Capacity Complementary DNA Reverse-Transcription kit following the manufacturer’s recommendations. Gene expression was determined by quantitative real-time PCR (RTqPCR) on the Taqman Via7 either using optimized array cards with SYBR green master mix (Thermo Fisher Scientific) or with validated Taqman Gene expression assays along with Taqman Gene expression Master Mix (Life Technologies). Individual gene expression was normalized using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as endogenous housekeeping gene. The expression level of each gene was determined by using the comparative Ct method (2−ΔΔCt). Samples were normalized to undifferentiated pluripotent control samples or untreated pluripotent control samples in case of drug treatment experiments. The primers used are detailed in Table 1. For array card based RTqPCR, cDNA was added to the wells containing lyophilized SYBR green linked primers against various genes (detailed in Fig. S3A). GAPDH was used as a control. Statistical analyses and graphics were carried out with GraphPad Prism 5 software and Microsoft Excel.

**Characterization of the fluorescent reporter human iPSC cell lines**

The fluorescent double tag reporter lines were characterized for pluripotency by immunofluorescence analysis of stemness cell markers NANOG, SOX2 and POUSF1. The cells were
processed for immunofluorescence staining as described above. In addition, we assessed each line by RTqPCR for the above markers and their ability to differentiate the three germ layers (data not shown). Only lines that passed these criteria were used for subsequent experiments.

**Autophagy flux analysis**

The AG27 mCherry-EGFP reporter lines were induced with doxycycline and processed for various treatments. The cells were fixed and mounted. Confocal microscopy was used to acquire images and cells were scored for red puncta, readout of autophagic flux. A minimum of 20 fields were used for the quantification. Quantification of red puncta per cell was acquired using a pipeline based on ImageJ.

**Cloning details**

The double tag (dt) constructs were generated by fusing the following genes, LC3B, POU5F1, SOX2, and NANOG with a dt fluorescent protein reporter based on mCherry and EGFP. Each of the above genes were synthesized as gBlock Gene Fragments. The sequences for MAP1LC3, POU5F1, SOX2, and NANOG were based on the original RefSeq sequences. A codon optimized dt-mCherry-EGFP gBlock Gene Fragment was synthesized, containing restriction sites to allow the in-frame cloning with the recipient gene as either a C-terminal fusion with respect to dt-mCherry-EGFP for MAP1LC3 or as N-terminal fusions for the pluripotency factors. In all cases, the gBlock Gene Fragments were codon optimized and had engineered restriction sites introduced to allow seamless cloning downstream into a PiggyBac vector backbone (gift from Gang Wang, Harvard). The PiggyBac vector was based upon the vector described in Wang et al., 2017 [73]. In short, the gBlock Gene Fragments were cloned into pCRBluntII-TOPO vector and validated by sequencing. Sequence verified clones were digested with NcoI and AgeI and subcloned onto the inducible PiggyBac vector restricted with the same enzymes. Positive clones were validated by restriction with multiple enzymes.

**Quantification and statistical analysis**

Results are expressed as the arithmetic mean ± standard deviation of the mean (SD). All the experiments were performed with n ≥ 3, which means three independent experiments, each performed with replicates, from at least three independent differentiations. Statistical analysis comparisons between groups were determined by unpaired Student’s t test. A P value of <0.05 was considered significant and was represented by *. Very significant and extremely significant values were represented by ** and *** with P values of <0.01 and <0.001, respectively. Densitometric quantification of Western blots was performed using ImageJ software (https://imagej.nih.gov/ij/). Analyses were carried out using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**References**


Title: Scalable production of tissue-like vascularised liver organoids from human PSCs.

Running Title: Scalable liver organoid production.

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ABSTRACT

Organoid models have been developed for a number of tissues including the liver. Current hepatic organoid models are generally simplistic, composed of hepatocytes or cholangiocytes, rendering them less physiologically relevant when compared to native tissue. To address this we have developed an approach that does not require 2D patterning, which is ECM independent and growth factor free, to mimic embryonic liver development that produces massive quantities of liver like organoids. Using single-cell RNA sequencing and immunofluorescence we demonstrate a liver-like cellular repertoire, presenting with vascular luminal structures, and a population of resident macrophage – the Kupffer cells. The organoids exhibit key liver functions including drug metabolism, serum protein production, urea synthesis and coagulation factor production, which preserved post-translational modifications such as N-glycosylation and functionality. The organoids can be transplanted and maintained in mice producing human albumin long term. The organoids exhibit a complex cellular repertoire reflective of the organ, have \textit{de novo} vascularization, and liver like function. This is a pre-requisite for a myriad of applications from cellular therapy, tissue engineering, drug toxicity assessment, disease modeling, to basic developmental biology.

Keywords: Human Pluripotent Stem Cells, Organoids, Liver, Hepatocytes, Scalable, Cellular Diversity, Single Cell RNAseq, Proteomics, Coagulation, Vascularised, Stellate Cells, Kupffer Cells, Cholangiocytes, ECM Independent.
INTRODUCTION

The liver serves as the primary site of xenobiotic metabolism, production of coagulation factors, and removal of ammonia as well as a multitude of other essential functions. In the setting of liver failure, no other curative treatment approach besides liver transplantation (LTX) exists. Additionally, the gold standard for the evaluation of hepatic metabolism and drug toxicity, amongst other things, are primary human hepatocytes (PHHs). However, these are extremely limited in supply and rapidly lose function in vitro. This highlights the necessity to identify potential surrogates to fill this void, providing a stable, scalable and genetically defined alternative.

Human pluripotent stem cells (hPSCs) have the ability to self-organise into organotypic structures called organoids. However, organoid models do not fully recapitulate the cellular diversity and architectural features of the organ in question. This is combined with current approaches being labour intensive, highly dependent on expensive growth factors and not amenable to scaling. This lack of such organotypic in vitro models has lead us to develop an approach that utilises a suspension culture system that leverages off the ability of hPSCs to self aggregate on seeding as single cells. In addition, we have overcome the requirement to pattern the hPSCs in a 2D format to produce definitive endoderm by directly patterning the 3D pluripotent aggregates through the addition of small molecules, in an extracellular matrix (ECM) independent manner, that is scalable to generate organoids which contain vasculature while recapitulating the complexity of cell types associated with the liver. The resulting organoids also replicate key functional features of the liver, including cytochrome p450 activity that is long lived and other phase 1 metabolising enzymes such as carboxyl esterases, which are involved in xenobiotic metabolism. We demonstrate that the organoids have a scavenger function via uptake of labelled substrates. We also demonstrate the utility of this platform to allow modelling of biological processes and potentially disease. One area of particular interest is the coagulation machinery. The organoids produce and secrete a myriad of functional liver specific proteins including albumin, serpins (alpha-1 antitrypsin (A1AT) and antithrombin (AT) among others) and the coagulation factors, which are post-translational modified and produced at comparable levels to primary hepatocytes. The coagulation machinery is functional with respect to Factor VII and will provide an important model to study coagulation, characterize coagulation factor deficiencies and set the basis for the development of cell-based therapy.

MATERIALS AND METHODS

hPSC Culture

The stem cell lines utilised in this study were: the human embryonic stem cell line H1 (WiCell) and the previously described hPSC lines AG27 (reprogrammed using retrovirus from AG05836B fibroblasts, obtained from Coriell Cell Repositories), and Detroit RA (reprogrammed using Sendai virus from Detroit 551 fibroblasts, obtained from ATCC). hPSCs were maintained under feeder free conditions on Geltrex (Life Technologies)
coated tissue culture plates using Essential 8 medium made in house as described previously.  

**Culture of primary human hepatocytes**

Human plateable hepatocytes (primary hepatocytes (PH)) were purchased from Thermo Fisher Scientific and cultured in Williams’ Medium E (1x, no phenol red) (Thermo Fisher Scientific) following the manufacturer’s instructions.

**2D hepatocyte-like-cell differentiation**

Cells were differentiated to hepatocyte-like-cells (HLCs) in 2D as described previously.

**Suspension culture and differentiation to liver organoids**

To differentiate hPSCs to organoids the cells were harvested by incubating with Accutase (Life Technologies) for 10 minutes at 37°C until all cells had detached. The cells were pelleted by centrifugation at 300 x g for 5 minutes at room temperature. After counting, the cells were seeded into 125 ml Erlenmeyer flasks (Corning) at a bulk cell density of 3.5 - 4 ml of media/million cells, (see 66) in Essential 8 supplemented with 10 μM Y-27632 (BOC Sciences). The cells were allowed to self-organise into aggregates for 24 hours on an orbital shaker at 70 RPM in a humidified 37°C, 5% CO₂ incubator. The conditions for suspension culture (orbital shaker at 70 RPM in a humidified 37°C, 5% CO₂ incubator) were utilised for all further steps of the differentiation. To initiate differentiation, the hPSC aggregates were collected from the flask and transferred to a 50 ml conical tube and pelleted by centrifugation for 5 minutes at 300 x g at room temperature. After removal of the supernatant, the aggregates were resuspended in 3.5 ml/million cells of stage 1 medium (see supplementary information) and 3 or 4 μM CHIR99021 (BOC Sciences). Optimal conditions need to be established for each line based on our previously established protocol. The aggregates were then transferred back to the Erlenmeyer flask and the incubator. After 24 hours, the aggregates were collected as described and the cell pellet was gently resuspended in the same volume of stage 1 medium, without any small molecules, and transferred back to the Erlenmeyer flask. After 24 hours the cells were directed towards hepatic endoderm. The aggregates were collected as above and resuspended at 3.5 ml/million cells in stage 2 medium and incubated for 5 days, with medium changes every 48 hours. On day 7, the resulting organoids were switched to stage 3 medium for maturation to liver organoids. The organoids were cultured from day 7 to day 20, with a medium exchange every 48 hours. Organoids were collected and analysed or maintained in long-term culture as indicated.

**Fixation of organoids**

For TEM the organoids were fixed in 1% glutaraldehyde / 1% paraformaldehyde (PFA) in 0.12 M phosphate buffer and 0.02 mM CaCl₂, pH 7.2 – 7.5; Sigma (buffer 1) for 4 hours at room temperature, washed in 8 % glucose in buffer 1 and post-fixed in 2% OsO₄ in buffer 1 for 90 minutes at room temperature. For histology and immunohistochemical detection the organoids were briefly rinsed in 0.1 M Sörensen buffer (pH 7.4) and...
immersed in 3% PFA and 0.05% glutaraldehyde in 0.1 M Sörensen buffer (pH 7.4) for 2 hours at room temperature followed by 30 minutes at 4°C. After a thorough washing in 0.1 M Sörensen buffer (pH 7.4), the organoids were dehydrated and embedded in paraffin. 6 µm thick serial sections were cut from paraffin blocks using a microtome and every tenth slide was stained with hematoxylin-eosin for histological examination.

For whole-mount immunofluorescence and confocal imaging organoids were fixed in 4% PFA for 45 minutes, then pelleted (300 x g for 3 minutes) and washed in PBS for 20 minutes, three times. The fixed organoids were blocked for 30 minutes in PBS supplemented with 0.1% Triton X-100 (Sigma Aldrich) x 100 (PBS- Triton X-100) and 10% goat serum (Life Technologies) in 1ml total volume in a 1.5ml Eppendorf tube. The primary antibody was then diluted at the appropriate dilution in PBS- Triton X-100 containing 10% goat serum and incubated overnight at 4°C. The primary antibody was removed and the sample washed for 3 x 20 minutes in PBS-Triton X-100. All Alexa-Fluor secondary antibodies (Life Technologies) were diluted 1:1500 with PBS-Triton X-100 and added to the samples for 4 hours at 4°C in the dark and subsequently washed 3 times for 20 minutes in PBS-Triton X-100. To dual label the samples, the above steps (block, primary, secondary) were repeated. Nuclei were counterstained with DRAQ5 at 1:1500 in PBS, for a minimum of 15 minutes before imaging.

Immunohistochemical detection was performed by indirect two-step method in paraffin-embedded sections. After deparaffinization and rehydration of sections, antigen retrieval was performed in HistoStation (Milestone, Sorisole, Italy). Endogenous peroxidase was blocked in 5% H2O2 (3 x 10 minutes) and then, sections were incubated with primary mouse anti-cytokeratin 19, clone BA17 (DAKO, Glostrup, Denmark; 1:50) antibody for 1 hour at room temperature. After washing in PBS, the sections were exposed to anti-mouse DAKO EnVision+ System-HRP Labeled Polymer (DAKO, Glostrup, Denmark) for 35 minutes at room temperature. Then the reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were dehydrated, counterstained with hematoxylin and mounted in DPX (Sigma-Aldrich).

**Microscopy**

Phase-contrast images were obtained using an Axio Primovert upright light microscope (Zeiss). Images were captured using Zen Software (Zeiss). Tissue sections were examined in Olympus BX51 microscope equipped with DP71 camera. Confocal images were obtained using an Olympus BX61 confocal microscope with submersion lenses, images were captured using Olympus Fluoview and compiled using Image J software. Scale bars represent 100 µm unless otherwise stated in the figure legends.

**Spinning disk confocal microscopy.**

An ultrafast confocal system IXplore SpinSR Olympus (Olympus, Tokyo, Japan) was used. We utilized our previously published imaging settings 67 and apparatus 68.
Fluorescence confocal images were acquired using software cellSens (Olympus, Tokyo, Japan). Icy open source software was used for image processing and 3D reconstruction.

Cryosectioning

PFA fixed organoids were transferred to a cryomold and embedded in OCT compound (Thermo Fisher Scientific) and cooled to -80°C on a bath of isopropanol on dry ice. The cryo-embedded organoids were then sectioned at 50 μm on a cryotome and transferred to slides. Slides were stored at -80°C until immunostained and imaged as described above.

Transmission electron microscopy

The fixed organoids (described above) were rinsed and incubated overnight in 10% sucrose (in water) at 4°C, the organoids were dehydrated in graded alcohols (50%, 75%, 96%, 100%), cleared in propylene oxide and embedded in a mixture of Epon 812 and Durcupan (Sigma; polymerization for 3 days at 60°C). Firstly, semithin sections were cut on Ultrotome Nova (LKB, Sweden) and stained with toluidine blue. Subsequently, ultrathin sections were cut on the same ultramicrotome, collected onto formvar carbon-coated copper grids, counterstained with uranyl acetate and lead citrate and examined under JEOL JEM-1400Plus transmission electron microscope (at 120 kV, JEOL, Japan).

LSEC functionality testing

Day 21 organoids were transferred to a 6-well suspension plate, 3ml/well. To separate wells were added either Alexa Fluor™ 488 AcLDL (Thermofisher, L23380) or fitc-FSA (Gift from Karen Sørensen) both at 2ug/ml, in the culture medium. These were incubated at 37°C for both 15 and 75 minutes before being washed two times in culture medium and then fixed as described previously. Organoids were then immunostained with endothelial markers as described above.

RNA

Two methods were employed to isolate RNA (i) Cells were collected for RNA isolation from 2D controls by washing the cells once with DPBS⁻/⁻, followed by scraping the cells into DPBS⁺/⁺. The resulting cell suspension was pelleted by centrifugation at 300 x g for 1 minute at room temperature. The supernatant was carefully removed and Trizol (Life Technologies) was added to lyse the cells. For organoids, RNA isolation was performed by removing 2 ml of suspension culture medium from the Erlenmeyer flasks and collecting the organoids by centrifugation at 300 x g for 5 minutes at room temperature. The supernatant was carefully removed and the cells were washed with 5 ml of DPBS⁻/⁻ and repelleted as previously. The DPBS⁻/⁻ was gently removed and Trizol added to lyse the cells. The Trizol samples were then either processed immediately for RNA isolation according to the manufacturer’s instructions or stored at -80°C for subsequent processing. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop). (ii) For the vitamin K dependent enzyme analysis total RNA was isolated using the MagMAX™-96 Total RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor as described by the manufacturer (both from Thermo Fisher Scientific, Waltham, MA, USA).
cDNA synthesis

500 ng of RNA was used as a template for reverse transcription to cDNA. cDNA synthesis was performed using the High Capacity Reverse Transcriptase Kit (Life Technologies) with random primers, following the manufacturer’s instructions for reactions without RNase inhibitor.

Gene expression analysis with RT-qPCR

Gene expression was analysed via reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using TaqMan probes (Life Technologies) and SSO Universal Probes Master Mix (Bio-Rad). For a complete list of probes used in this study, please see Supplementary Table 1. All samples were analysed in triplicate. Data is presented as the average of three independent experiments +/- the standard deviation.

CYP450 activity and induction

Analysis of Cytochrome P450 (CYP) basal activity and inducibility was performed as previously described with several modifications for organoid cultures. 72 hours post induction the cells were assayed for CYP1A2 and CYP3A4 activity using the P450-Glo CYP3A4 (Luciferin-PFBE) Cell-based/Biochemical Assay kit (Promega, Cat. no. V8902) and the P450-Glo CYP1A2 Induction/Inhibition Assay kit (Promega, Cat. no. V8422) according to the manufacturer’s instructions. Data was normalised to 1 million hepatocytes and is presented as the average of three independent experiments +/- the standard deviation.

Heroin metabolism

After 21 days differentiation, 50 organoids per well were loaded in triplicate into a 96-well plate and treated with culture media supplemented with 10 µM heroin for 1, 3, 6, and 24 hours. For controls, we used culture media without organoids; these were performed in parallel to measure heroin degradation throughout the experiment. To stop metabolism at each time point the samples were transferred to a new 96-well plate prefilled with formic acid (final conc. 0.1 M) along with internal standards. The samples were centrifuged for 10 minutes at 1000 x g at 4°C. The supernatants were transferred to auto-sampler vials and analysed for heroin, morphine, and M3G using an Acquity UPLC system (Waters, Milford, MA) coupled to a Xevo-TQS triple quadrupole mass spectrometer with an electrospray ionization interface (Waters) based on a method previously described. Data acquisition, peak integration, and quantification of samples were performed using MassLynx 4.0 SCN509 software (Waters Corp., Milford, MA, USA).

Coagulation factor studies

For coagulation studies hepatic organoids were cultured in L15 medium supplemented with Vit K (Konakion Roche) 5 ug/ml for at least 48 hours before harvesting.

FVII activity

Factor VII (FVII) activity in the cell medium was determined using the Human FVII Chromogenic Activity Kit (Nordic BioSite AB, Täby, Sweden) according to manufacturer’s
instructions. Primary hepatocytes (Thermo Fisher Scientific) were used as control. Functional activity of FVII from supernatants of iPSC-HO was assessed by two methods: a) by western blot after activation with Tissue Factor (TF, STA Neoplastin) and CaCl₂, and incubating with Antithrombin (AT), purified from human plasma, (Grifols®, Spain) and unfractionated heparin (Hospira®) to measure the ability of FVII to become active and form a complex with AT; and b) by thrombin generation (CAT, Stago®, Valencia, Spain) of supernatants derived from organoids with and without FVII-depleted plasma. Briefly, 40 µl of organoid supernatants was added to 40 µl of FVII-depleted plasma and activated with tissue factor 5 pM. Fluorescence measurements started just after the addition of Fluka® reagent diluted in a commercial buffer containing calcium. As controls we used only FVII-depleted plasma 80 µl, and organoid medium (SFM L15) 40 µl with FVII-depleted plasma 40 µl, activating also with tissue factor 5 pM. Fluorescence measurements were recorded during 60 min in the fluorimeter (Stago ®, Valencia, Spain).

**Western analysis of FII, AT, A1AT and FVII**

Intracellular levels of FII were determined by western blot (WB). Hepatic organoids were lysed in T-PER™ buffer (Thermo Fisher Scientific), and anti-FII (Novus biologicals, Centennial, CO, USA) was used following the previously published method. Primary hepatocytes were used as control.

AT, alfa-1-antitrypsin (A1AT) and FVII were evaluated by western blot on 8% polyacrylamide gel electrophoresis performed under 10% SDS denaturing conditions in the presence 0.05 M reducing agent. AT, A1AT and FVII were immunostained with rabbit anti-human AT (A9522, Sigma-Aldrich), anti-human A1AT (Dako Diagnostics, Denmark) and anti-human FVII polyclonal antibodies, respectively.

**Evaluation of N-glycosylation of secreted proteins**

N-glycosylation was evaluated by comparative Western blots in basal conditions and after digestion with neuraminidase (α(2→3,6,8,9) neuraminidase from *Arthrobacter ureafaciens*, Sigma-Aldrich), following manufacturer indications, and with N-glycosylase F (PNGase F, Sigma-Aldrich, Madrid, Spain). Cell supernatants (up to 200 µg of glycoprotein in a volume of 35 µl) were denatured with 10 µl of 250 mM phosphate buffer and 2.5 µl of 2% SDS with 1 M 2-mercaptoethanol, heated at 100 ºC for 5 minutes and cooled. TRITON x-100 (2.5 µl of 15%) (v/v) was added. Then, 2.0 µl of PNGase F (≥5,000 units/ml) were added and incubated overnight at 37 ºC. Samples were run in SDS-PAGE and detected as described above.

**Serum protein analysis via ELISA**

ELISAs for human Albumin (Bethyl Laboratories Inc cat# E80-129), human A1AT (Abcam cat# ab108799) and hepatocyte growth factor (Antibodies-online cat# ABIN624992) were used as described by the supplier. Colorimetric readings were taken at the specified wavelength on a SPECTRAMax PLUS 384. Values were derived from standards using appropriate lines of best fit generated on Softmax pro software. For clotting factors organoids were collected by centrifugation for 10 minutes at 300 x g and the cell medium
was collected. Hepatic organoids were lysed in T-PER™ buffer (ThermoFisher) containing Halt protease and phosphatase inhibitor cocktail 1X (Thermo Fisher cat# 78440). FVII and FX antigen (FVIIAg and FXAg) were measured in the cell medium using FVII ELISA kit and FX ELISA kit (Abcam, cat# ab168545 and ab108832) respectively. FII, AT, Protein C, Protein S, were measured using Procarta-plex coagulation 6 plex panel 1 and coagulation 4 plex panel 3 (Thermo Fisher Scientific). Primary hepatocytes were used as control. The FVIIAg and FXAg levels (ng/ml) were normalized to 1x10⁶ cells and the ratio organoid/primary hepatocytes was calculated.

**Oleic acid accumulation assay**

1 M Oleic acid (Sigma Aldrich) was diluted with NaOH (Sigma Aldrich) and heated at 70°C for 30 minutes to form a 20 mM Sodium Oleate solution. This was then diluted with a 5% BSA/PBS solution at 37°C to form a 5 mM Sodium Oleate/BSA complex. This was further diluted to 300 μM in culture media and incubated with organoids for 5 days, changing the media each day. After the fatty acid treatment, the organoids were then incubated with 3.8 μM BODIPY 493/503 (Life Technologies) for 30 minutes in culture media at 37°C, then washed two times with PBS before replacing with fresh culture media containing DRAQ5 (Thermofisher) at 1:1500. Organoids were then imaged on a confocal as described above.

**Sample preparation, processing and data processing of proteomics data**

**Patient liver samples**

Five patient samples were collected from liver explants from patients undergoing LTX at Oslo University Hospital. The samples were stored in liquid nitrogen. The regional ethics committee approved the use of the patient material (REK 2012-286) in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Samples were processed as follows, the proteins were precipitated with acetone/TCA (Sigma Aldrich). The pellets were resuspended in 8 M Urea in 50 mM NH₄HCO₃, and the proteins were reduced, alkylated and digested into peptides with trypsin (Promega). The resulting peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a C18 resin disk (3M Empore). Each peptide mixture was analyzed by a nEASY-LC coupled to QExactive Plus (ThermoElectron, Bremen, Germany) with EASY Spray PepMap®RSLC column (C18, 2 μl, 100 Å, 75 μm x 25 cm) using a 120 minute LC separation gradient.

The resulting MS raw files were submitted to the MaxQuant software version 1.6.1.0 for protein identification. Carbamidomethyl (C) was set as a fixed modification and acetyl (protein N-term), carbamyl (N-term) and oxidation (M) were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed mis-cleavages. The minimal unique+razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. The Uniprot database with ‘human’ entries (October 2017) was used for the database searches.

Proteins with log2(intensity) > 10 average intensity value were defined as “expressed proteins”. Pearson correlation coefficient between liver and organoid was
calculated from log2(intensity) with cor.test function in R. Differential expression of proteins between liver and organoid was defined with more than 2 fold change and p<0.05 by two-sided T test. Gene Ontology analysis was conducted with GOstats Bioconductor package. Multiple test correction was performed by Benjamin-Hochberg method with p.adjust function in R.

**Library preparation and data processing of scRNAseq**

scRNA-seq libraries were prepared from the liver organoids at day 48 with Chromium Single Cell 3’ Reagent Kits (version 2 - 10x Genomics) as described previously. Conversion to fastq format, mapping/UMI counting in human genome (hg19) and data aggregation were implemented by mkfastq, count and aggr functions with default parameters in CellRanger software (v2.1.0). Subsequent data processing, such as batch effect normalization, was performed by Seurat software (v3.1.0). In each replicate, the feature UMI count was normalized to the total count and multiplied by 10,000. Top 2,000 Highly-Variable Features (HVFs) were then identified by variance stabilizing transformation. Anchor cells across different scRNAseq libraries were identified with HVFs under 20 dimensional spaces from canonical correlation analysis and used for the transformation of multiple scRNAseq datasets into a shared space. Gene expression values were scaled for each gene across all integrated cells and used for principal component analysis (PCA). 20 PCs were further assigned into two dimensional space using Uniform Manifold Approximation and Projection (UMAP) and also used to identify cell clusters. Differentially-expressed genes in each cluster were identified with more than 1.25 fold change and p<0.05 by two-sided T test. Overrepresented GO terms were identified by GOstats (v2.24.0). Multiple test correction was performed by Benjamin-Hochberg method with p.adjust function in R.

The cluster labels were assigned by cell type specific markers and GO terms (Supplementary Fig. 2c,e). Nine out of 22 clusters were first separated by the overrepresentation of “extracellular matrix (GO:0031012)”, which is a feature of stellate and endothelial cells. Active (AST) and resting stellate cells (RST) were defined by genes involved in “mitotic nuclear division (GO:0140014)” and its markers (MGP and ELN). Non-stellate clusters were labeled as endothelial cell (EC) and further divided into liver sinusoidal (LSEC) and macrovacular endothelial cells (MVEC) by the absence and presence of vasculogenesis markers (KDR and HAND1). Seven out of 13 other clusters were assigned hepatocyte (HEP), cholangiocyte (CHO), Kupffer cell (KPC) and Kupffer precursors (KPP) using the enrichment of GO terms “Cholesterol homeostasis (GO:0042632)”, “Keratinization (GO:0031424)”, “Phagocytosis (GO:0006909)” and “hematopoietic stem cell differentiation (GO:0060218)”, respectively. Five clusters were assigned as peripheral nervous system with the expression of neuronal lineage markers (SOX2 and PAX6) and further divided into neuron (Neu), glia (Glia), neuro progenitor (NPC) and cilia-bearing cell (CBC) with “axon development (GO:0061564)”, “glial cell development (GO:0010001)”, “mitotic nuclear division (GO:0140014)” and “cilium assembly (GO:0060271)”, respectively. We could not identify any unique marker and relevant GO terms in one cluster and
labeled it as unknown (UN). The cluster labelling strategy was schematically represented in Supplementary Fig. 2b.

Public transcriptome profiles were downloaded from NCBI Gene Expression Omnibus database. Single-cell transcriptome of the liver organoid from Ouchi et al. (GSE130073) and human liver atlas (GSE124395) were merged with our scRNAseq data and plotted into the share UMAP space by Seurat as described above. Clusters, which are mainly composed of CD45+ cells and unique to human liver atlas, were labeled as “other immune cell”. Genes were sorted by the difference of average expression of all cells between our and Ouchi et al. liver organoid and used for GSEA (v2.2.2) of REACTOME genes without collapsing gene set. Cell-type specific gene signatures were constructed from bulk RNA-seq in primary hepatocyte (GSE98710, GSE112330 and GSE135619), biliary tree stem (GSE73114), stellate (GSE119606) and endothelial cells (GSE114607). The RNA-seq read was aligned to hg19 human genome by Tophat (v2.2.1) with default parameters. The mapped reads were counted in each gene by HTSeq software (v0.9.0) with options “-s no -f bam”.

The factors of technical variations across multiple transcriptome datasets were minimized by RUVs function in RUVSeq (v1.8.0). Subsequently, differentially expressed genes in each cell type were identified by DESeq2 (v1.14.1). To evaluate the enrichment of the cell-type specific genes, genes were sorted in individual cells by relative expression level to average of all cells and used for GSEAPY software (v0.9.3) with options “--max-size 50000 --min-size 0 -n 1000”. Hepatic zone-specific genes were obtained from transcriptome profiles of hepatocyte from laser-microdissected human livers (GSE105127). After processing the bulk RNA-seq, the zone-specific genes were defined with more than 1.5-fold change and p<0.05 by two-sided T test. The enrichment was evaluated by GSEAPY software with pre-ranked genes in individual cells relative to all cells in all hepatocyte clusters.

To investigate transcriptional bias between LSEC and MVEC, cells from EC clusters were ordered in pseudotemporal spaces by Monocle (v2.99.3). Briefly, the monocle object was first constructed from the UMI count matrix for cells in EC clusters and preprocessed according to the instruction. We then replaced data in “normalized_data_projection” and “reducedDimW” with non-transposed and transposed PCA dimensional matrix. In addition, “reducedDimS”, “reducedDimA” and “reducedDimK” slot were replaced with transposed UMAP dimensional matrix. The principal graph was learned by learnGraph function with “RGE_method = 'DDRTree', close_loop=T, prune_graph=F, euclidean_distance_ratio=5”. Subsequently, cells are ordered according to the trajectory by orderCells function using MVEC1 as a root cluster. Differentially-expressed genes were identified by differential GeneTest function with the model “~sm.ns(Pseudotime)”. Finally, Genes with q<1e-50 were selected as EC ordering-dependent genes and used for GO analysis by GOstats as described above.

Animal work
Male and female NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NOD scid gamma, NSG) mice (purchased from The Jackson Laboratory, Bar Harbor, ME, USA) were housed in a Minimal Disease Unit at the animal facility at Oslo University Hospital Rikshospitalet, Oslo, Norway, with a 12 hour light–dark cycle and <i>ad libitum</i> access to water and standard rodent diet. All experiments were performed with co-housed age-matched mice. Mice undergoing surgery were not fasted and were 15 weeks of age at the time of surgery. All animals received human care and the animal experiments were approved the Norwegian National Animal Research Authority (project license no FOTS 19470) and performed according to the European Directive 2010/63/EU, the Animal Research: Reporting of In Vivo Experiments guidelines and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press).

**Implantation of human liver organoids under the rodent kidney capsule**

Male and female immunodeficient NSG mice were used in this study. The transplantation of the organoids under the kidney capsule or the sham laparotomy was performed as described in 80, 81. A 1 ml syringe with a 25G needle containing either the organoid suspension or pure Matrigel (Thermo Fisher Scientific) (sham surgery) was gently pushed under the capsule towards the inferior pole of the kidney in order to avoid perforation and damage to the blood vessels. 50-80 µl organoid suspension or Matrigel matrix was very slowly discharged under the kidney capsule and the needle simultaneously slowly pulled out of the capsule to avoid backflow. Next, the kidney was returned to the body cavity, the abdominal wall closed with suture and the skin incision closed. Once every week, blood was sampled from the saphena vein for serum markers measurement.

**RESULTS**

**Differentiation of human pluripotent stem cells to liver organoids**

We employed a cocktail of small molecule mimetics in a developmentally relevant sequence to mimic <i>in vivo</i> liver development (Fig. 1A)4-6. On initiating organoid formation, we observed aggregation of the hPSCs post single cell seeding, which maintained the pluripotency markers, OCT4, SOX2 and NANOG over a 24 hour period (Fig. 1B). To initiate the developmental programme, aggregates were treated with CHIR99021 (CHIR) for 24hours. By day 2 the aggregates exhibited markers of both mesoderm and definitive endoderm (DE); T, GSC, FOXA2, SOX17, HHEX and CER1 etc (Fig. 1C and D). The mesendodermal aggregates were then subjected to hepatic specification until day 7, the resulting organoids were characterised for the presence of hepatic markers. We observed increased expression of AFP, CEBPa, HNF4α and TTR coinciding with decreased expression of DE markers such as HHEX, SOX17 and GATA4 (Fig. 1F). Expression of the T-box family protein TBX3 was observed (Fig. 1F), which is a factor involved in hepatic endoderm delamination and subsequent invasion into the adjacent septum transversum mesenchyme (STM). Using immunostaining we revealed further characteristics of a mixed lineage liver bud stage. The outer epithelial layer of cells expressed ECAD (CDH1), FOXA2, and CK8, as well as being HNF4α and AFP positive, indicative of an early hepatic phenotype (Fig. 1G and
Supplementary Fig. 1) while these epithelial markers were absent from the core of the organoids (Supplementary Fig. 1).

We characterised the non-epithelial cells of day 7 organoids, where we observed a population of MESP1 positive cells, indicative of mesodermal STM (Fig. 1H). During development a mesodermal MESP1+ population gives rise to mesothelial/sub-mesothelial populations marked by ALCAM and Wilms Tumour (WT1), which in turn give rise to the hepatic stellate cells (HSCs). We also identified ALCAM and Wilms Tumour (WT1) positive populations within the mesenchyme of the organoids (Fig. 1H). We then directed these early hepatic organoids to a mature liver-like stage over a 14-day period resulting in the formation of organotypic 3D structures (Figure 1I) with an average size of 250 μm (Fig. 1J). Analysis by RT-qPCR clearly demonstrated the expression of hepatic markers such as ALB, A1AT, ASGPR1 as well as genes enriched in the liver such as APOA2, TDO2 and TTR and those involved in xenobiotic metabolism cytochrome P450 (CYP) 3A4 and CYP3A7 (Fig. 1K). Immunofluorescence staining demonstrated that the hepatocytes were located on the surface of the organoids visualised via HNF4α and ALB staining (Fig. 1L). We assessed the production of HGF during the course of the differentiation and noted secretion of HGF increased throughout organoid differentiation (Fig. 1M).

**Single cell transcriptome analysis of liver organoids**

To further dissect the cellular diversity within the liver organoids, we profiled the single-cell transcriptomes of a total of 21,412 cells by scRNAseq. A total of 22 clusters were detected and systematically assigned into liver cell types by unique markers, Gene Ontology (GO) functions and reference transcriptome profiles (Fig. 2A and Supplementary Fig. 2A-E). We identified three hepatocyte-like clusters (HEP1-3) with substantial expression of glycerolipid or cholesterol metabolic genes. To evaluate the spatial heterogeneity of hepatocytes within the liver organoid, we performed Gene Set Enrichment Analysis (GSEA) of gene signatures for human perportal hepatocytes to individual cells in the hepatocyte clusters (Fig. 2B). The enrichment of perportal gene signatures was significantly different across the three hepatocyte clusters. In particular, the HEP3 cluster was closest to the perportal zone, while HEP1 was more distant suggesting that gene expression zonation of hepatocytes was present in the liver organoids. We plotted normalized enrichment scores of perportal genes and showed a significant difference across three HEP clusters (Fig. 2C and D).

Next, the endothelial cell (EC)-like clusters were characterized by ECM genes and reference transcriptome annotation (Fig 2E and 5E) and divided into liver sinusoidal (LSEC) and macrovascular endothelial cells (MVEC) by the expression pattern of vascularization markers (Fig. 2E). To infer continuous EC heterogeneity, we ordered cells from EC clusters along with their gene expression patterns (Fig. 5E). Six co-expression modules that are transiently expressed with the EC ordering were involved in different cellular events. The modules biased to LSEC included genes related to oxidative reaction and anti-inflammatory response, which are characteristics of the liver sinusoid. In contrast, genes involved in smooth muscle and calcium transport were significantly enriched in MVEC-biased gene modules. We next explored LSEC zonation, we mapped gene signatures of LSEC in central
and periportal zones and observed no significant differential expression of LSEC markers and gene signatures in the EC clusters.

In addition to the above liver cell types, we also identified clusters expressing genes for the development of peripheral nervous system (SOX2, PAX6, STMN2) (Fig. 2A, G and H and Supplementary Fig. 2B and E).

Our scRNAseq data were compared against scRNAseq of FACS-sorted human adult liver cell populations, liver organoids derived from another protocol and data from all sources in the shared UMAP space (Fig. 2F and Supplementary Fig. 2F) \(^3,28\). The liver cell types (hepatocytes, HSCs, Kupffer and endothelial cells) in our liver organoid are close to cells derived from the human liver, indicating similar gene expression profiles of these cells between our organoid and primary human liver. The liver organoids from the two different protocols exhibited similar cell composition, but displayed apparent differences in the amount of non-endodermal cell types (Fig. 2G). In addition, peripheral nervous system-like cells were also detectable in the aforementioned study. Comparative analysis revealed that our liver organoids displayed the generation and maturation of Kupffer cells (Fig. 6C). Cells expressing the cell surface marker (MARCO, CD45, CD163, CD16A, LY86 and CD86), complement components (C1QA, C1QB and C1QC) and macrophage regulators (VSIG4, TREM2 and PU.1) were clearly enriched in our protocol. For further comparison we analyzed the preferential pathways between the two protocols. GSEA for REACTOME database revealed that collagen and polyamine metabolic genes are significantly enriched in our protocol (Fig. 2H). In contrast, cholesterol biosynthetic genes were enriched in Ouchi and colleagues protocol.

**Proteomic analysis of liver organoids**

We subjected the organoids to global proteomic analysis. This revealed that 1,842 out of 2,461 detected proteins were expressed in biopsies from human primary liver and *in vitro* generated liver organoids, GO analysis indicated that these enriched proteins were involved in a variety of metabolic pathways and functions including blood coagulation (F2, SERPINC1, SERPING1, FGG, FGA etc.) and glutamine family amino acid metabolic processes (ARG1, ASS1, FAH, GLUL, GFPT1, GOT1; GOT2 etc.) (Fig. 3A). We used iBAQ plot to illustrate key liver markers in each intensity range (Fig. 3B). Liver markers such as SOD1 and ALB showed highest expression in both organoid and liver. Other liver markers such as KRT18 (CK18), GSTA2 and SERPINA1 were also expressed in both organoid and liver, but their intensity varied between organoid and liver. GO analysis indicated that similar protein sets were enriched in organoid and liver samples. For example, proteins related to “response to toxic substitution” and “detoxification” were enriched in the highest expression range (25~) (Fig. 3C). Overall, these results show that key liver proteins are commonly expressed in both organoid and liver samples. Next, we used unbiased clustering of the proteomic data, clustering was performed to log2(intensity) value of all-expressed genes (log2(intensity) > 10) (Fig. 3D). We noted a separation of organoid and liver that was robustly observed by various cut-off values (e.g. 10, 15, 20) of the expressed genes. A heatmap shows differentially expressed proteins between organoids and liver, identifying approximately 200-400 proteins that were differentially expressed (Fig. 3E).
These differences were proteins related to blood cells (e.g. immune response and heme-binding), which were significantly enriched in primary liver (Fig. 3E). While in the organoids, cell cycle and early developmental genes were enriched.

**Organoids contain hepatic parenchymal cells**

Immunofluorescence was performed on the organoids (day 20 to 30) to corroborate the findings of liver-like cellular complexity. We verified the presence of hepatocytes by the cytosolic expression of GS and CPS1 along with nuclear expression of hepatic transcription factor HNF4α, which were located on the outer surface of the organoids (Fig. 4A). The enzyme GS is specifically absent from foetal liver hepatocytes \(^{20}\) and only detectable in the liver parenchyma after day 2 *post partum* in humans \(^{20}\). The expression of GS in our organoids is thus indicative of a developmental stage surpassing that of foetal liver. This is further reinforced by the expression of the xenobiotic metabolising enzyme CYP2A6 (Fig. 4A), a *bona fide* marker distinguishing adult from foetal hepatocytes \(^{30}\) and the expression of ASGP1, a marker of maturity used to purify mature hepatocytes (Fig. 4A) \(^{31}\). To further validate our findings we assessed a panel of highly enriched adult liver genes and observed enrichment of this panel in our organoids (Supplementary Fig. 3).

Another feature of hepatocytes is polarisation, this was first confirmed by the expression of the tight junction protein ZO-1 and the apical export protein MRP2, which are both enriched in bile canaliculi (Fig. 4A) \(^{32, 33}\). Polarisation was also assessed at the ultrastructural level, revealing features of primary liver tissue, including epithelial cells lining luminal structures arranged in a layer and connected by tight junctions, a feature of the hematobiliary barrier (Fig. 4B). Along with a polarised epithelial cell morphology with the lumen facing surfaces presenting with numerous microvilli, while the abluminal surface facing the ECM was devoid of microvilli and appeared to be attached to an underlying basal lamina (Fig. 4B). The other endoderm-derived cell type is the cholangiocyte. We confirmed the presence of cholangiocytes, in cells surrounding lumen, through immunohistochemical staining of CK19 which is present in cholangiocytes and hepatoblasts but is lost in hepatocytes (Fig. 4D) and CK7 which is expressed *in vivo* from 16-20 weeks post-conception (wpc) in humans (Fig. 4D) \(^{34}\). *In vivo* the cholangiocytes produce mucins to form a mucus layer. Using Alcian blue staining we observed epithelial cells lining the lumen stained blue indicating the presence of mucopolysaccharides i.e. mucin secreting cells (Fig. 4C).

**Organoids are de novo vascularized**

The human liver is also contains non-parenchymal cell types. We investigated the endothelial populations identified by scRNAseq analysis using antibodies to delineate the different endothelial populations. First we investigated the macrovasculature and observed branched chains and lumen surrounding structures positive for CD31 throughout the organoids, often forming vascular networks (Fig. 5A and Supplementary Fig. 4A B and C). On investigating volume rendered 3D reconstructions in cross-section, we observed clear lumen with a diameter of \(8\mu\text{M}\), which is in the range of capillary lumens (5-10\(\mu\text{m}\)) (Fig. 5B) \(^{36}\).
We also observed small clusters of CD34+ endothelial structures suggesting continued neo-vascularization in the end stage organoids (Fig. 5C). The microvasculature in the liver bud is acquired from the endothelial cells of the STM. These begin as CD34+/CD31+ continuous vasculature gradually acquiring liver sinusoidal endothelial cell (LSEC) specific features. In the adult liver LSECs exhibit distinct zonal markers 37, this is recreated in the organoids where the endothelial structures acquire increasing CD54+ expression in proximity to the hepatocyte layer, which suggests that endothelial cell specialisation is potentially a product of the niche (Fig. 5A and Supplementary Fig. 4C). We also investigated the distribution of LYVE1, another marker of LSECs 38, this too revealed luminal structures within the organoids (Fig. 5D).

scRNAseq data identified a peripheral neuron population (Fig. 2A, E and F and Supplementary Fig. 2B and E). In order to corroborate these findings we performed immunohistochemistry against TUBB3 (Fig. 6E), we detected TUBB3 positive neurons throughout the organoids. Interestingly the neural crest lineage arises from the pluripotent epiblast, prior to definitive germ layer formation 26, 27. We investigated early points in the differentiation (Day 2 and 7) for the emergence of a neural crest like population. Using RT-qPCR we observed the neural plate border markers PAX3, PAX7, ZIC1 and the neural crest specifiers AP2 and SOX10. At day 7 also we observed the neural crest stem cell marker p75 (Fig. 6F).

**A resident macrophage and hepatic stellate population**

scRNAseq data identified a discrete population of Kupffer cells, which are the resident macrophage population of the liver and are derived in situ during the hematopoetic phase of liver development in vivo 39, 40. We investigated the presence of Kupffer cells within the organoids using the marker CD68 41, and observed a population exhibiting typical cytoplasmic granule staining (Fig. 6A). The endothelial and hematopoetic cells share a common precursor early in development called the hemangioblast. We speculated that the endothelial and Kupffer cell types potentially arise from a common mesodermal population at an early stage of differentiation. To that end we studied early time points in organoid differentiation using RT-qPCR for orchestrators of hematopoietic commitment and observed expression of RUNX1 on day 2, which is essential for hematopoietic commitment. By day 7 we observed the expression of both RUNX1 and GATA2 both involved in the early hemangioblast core circuit (Fig. 6B) 42. The scRNAseq analysis identified a putative hepatic stellate cell (HSC) population via canonical markers such as BGN, CTGF, TPM2, SPARC, IGFBP, TAGLN, DCN, CCL2, COL1A1, etc. In vivo HSCs originate from the undifferentiated mesenchyme 8. Above ALCAM+, MESP1+ and WT1+ populations were observed in the organoids (potentially a mesothelial/submesothelial equivalent) (Fig. 1H). We speculate that the WT1+ population potentially gives rise to a HSC population. We first assessed αSMA, revealing positive cells seen in close proximity to the hepatocyte population, marked by HNF4α (Fig. 6D). On closer inspection both HSCs with a star-like morphology, that had long cytoplasmic processes with fine branches, and cells resembling myofibroblasts were observed suggesting quiescent and activated populations. This is corroborated by the scRNAseq data, where both activated and resting populations...
were detected in UMAP space (Fig. 2A). One function of HSCs is the production of ECMs including the laminins. Immunohistochemistry using a pan-laminin antibody along with αSMA revealed a close relationship between laminin and HSCs (Fig. 6D).

Organoids display liver like function

We assessed the basal and induced levels of CYP1A2 and CYP3A4, which play an important role in metabolism of a range of drugs including caffeine and acetaminophen.\(^4^4\) The organoids were benchmarked against primary human hepatocytes and a 2D protocol for generating hiPSC derived hepatocytes\(^5\), \(^6\). The organoids presented with greatly elevated levels of activity for both basal and inducible metabolism compared with the equivalent time-point in 2D (Fig. 7A). When we compared organoids to primary human hepatocytes we observed robust CYP activity in both basal and induced conditions. We observed comparable basal levels, while after induction we observed a 4 fold difference in activity for CYP3A4 and approximately a 15 fold for CYP1A2, between organoids and primary hepatocytes. These levels of activity were maintained over a 40-day period in the organoids, while activity rapidly declined to almost undetectable levels in 2D culture (Fig. 7A). We then assessed long-term activity, observing maintenance of basal and inducible activity for 80 days (Fig. 7B). The signal started to decline from day 50 for CYP3A4 and day 60 for CYP1A2; we speculate that this may be a feature of suboptimal culture conditions for long-term maintenance (Fig. 7B).

Non-CYP450-mediated metabolism has attracted increasing attention as an important player in absorption, distribution, metabolism, and excretion – (ADME)\(^4^5\). We investigated liver carboxyl esterases (CES),\(^4^6\) which metabolize a wide range of xenobiotic substrates including heroin, metabolized by sequential deacetylation (phase I reaction) to 6-monoacetylmorphine (6-MAM) and morphine. Morphine is then glucuronidated, by a phase II reaction via UDP-glucuronosyltransferase (UGT) to morphine-3-glucuronide (M3G) (Fig. 7C). We tested organoids by exposure to 10 µM heroin and quantification by UPLC-MS/MS. We also compared organoid metabolism to human liver microsomes and the human S9 fraction (unfractionated microsomes and cytosol). The kinetics of metabolism was slower in the organoids, where we observed phase I metabolism of heroin by CES to morphine in approximately 6 hours, while the controls produced morphine in approximately 12 minutes. However, phase II metabolism (UGT) to morphine glucuronides was detectable in the organoids and absent in the controls (Fig. 7C).

Another essential liver function explored was hepatic urea synthesis, which is required for the removal of excess nitrogen. Above we show the expression of CPS1 and GS enzymes, which are involved in the urea cycle (Fig. 4A). The organoids on testing against a primary human hepatocyte control, produced and secreted urea into the medium (Fig. 7E)\(^4^8\).

A study from Ouchi et al. (2019) demonstrated the accumulation of lipids in a liver organoid model, which displayed a steatohepatitis-like phenotype.\(^2^5\) Using the neutral lipid dye Bodipy we established the steady-state level of lipids in untreated organoids, which
was low (Fig. 7D). We next treated organoids with free fatty acid (Oleic acid), which lead to massive lipid accumulation, in enlarged droplets (Fig. 7D).

We next investigated endothelial cell functionality, we first looked at Factor VIII, an essential component of the hemostatic system, which is expressed in the sinusoidal endothelial cells of liver in vivo. We performed immunofluorescence, confirming the expression of FVIII protein in CD31 positive cells (Fig. 5F). The LSEC population are also equipped with high-affinity receptors (scavenger) enabling the removal of large molecules and nanoparticles from the blood to maintain blood and tissue homeostasis. The proteomic data also indicated the presence of scavenger receptors (e.g. LYVE, LRP1, CD36 and SCARB1) in the organoids. It is also established that LSECs can bind and uptake acetylated low-density lipoprotein (AcLDL) and formaldehyde-treated serum albumin (FSA), which is linked to the scavenger receptor function. On treatment of the organoids with either AcLDL or FSA combined with immunofluorescence staining against the LSEC marker CD54, we observed an association of both AcLDL and FSA with the CD54 LSEC population indicating the binding and uptake of these molecules (Fig. 5G).

Our ECM independent system combined with small molecules is capable of producing 300-500 organoids per ml of culture media, where we routinely produce 10’s to 100’s of thousands of organoids (see Supplementary Fig. 5a). Importantly, the cost of production has been reduced by nearly 3 orders, as compared to conventional 2D approaches (Supplementary Fig. 5b). With access to such numbers of organoids we assessed if these could be transplanted and maintained in the kidney capsule of mice. The organoids were introduced into the kidney capsule with or without ECM. Using a human specific albumin assay as readout, we assessed the blood of mice over a 5 week period. No albumin was detectable in the first 96 hours, however we observed secretion of human albumin into the bloodstream of the recipient mice from weeks 1 to 2 post transplantation. This was maintained until the mice were sacrificed at week 5 post-transplantation, while no human albumin was detectable in the control sham mice (Fig. 7F). We then performed immunofluorescence staining on sections from the graft/host material to assess the presence and maintenance of hepatic cell types after transplantation. We demonstrate the retention of hepatic populations and structures that were observed in the in vitro cultures. We observed CK7 positive cells surrounding small and large luminal spaces within the transplanted material (Fig. 7G) and branching networks of human CD31 positive cells within the transplanted organoid that enter and extend throughout large areas of the mouse kidney parenchyma. We also see the maintenance of both layers and luminal structures positive for HNF4α within the transplanted organoids (Fig. 7G). We show cells highly positive for injected dextran within the mesenchyme area of the transplanted organoid, away from the kidney parenchyma, which are positive for human albumin (Fig. 7G). The presence of CD31 (human specific antibody) vascular structures that appear to have anastomosed into the mouse kidney parenchyma further supports the presence of vascularisation within our organoids and ongoing de novo vascularisation.

Liver organoids secrete plasma proteins and have functional coagulation machinery.
An important feature of the liver is the production of serum proteins. We have demonstrated the production of HGF from our organoids; on inspecting the proteomic datasets numerous serum proteins were identified, including the apolipoproteins (APOA1, APOA4, APOC3 and APOD), hormones (the IGFs) and serine protease inhibitors (A1AT, AT, C1-inhibitor etc). We first assessed the production/secretion of albumin and A1AT by ELISA, previously shown to be transcribed and expressed above (albumin) in hepatocytes. Both these proteins are secreted from hepatocytes into the circulation in vivo and their secretion into the culture medium was also verified. We then assessed A1AT in both the supernatants and lysates of organoids by western analysis. Notably, the A1AT electrophoretic mobility was very similar to that of those forms detected in both human plasma and primary hepatocytes (PH). A1AT contains a number of post-translational modifications (PTMs) that increase protein stability, for example by protecting against proteolysis and being degraded, amongst other things. We therefore analysed the N-glycosylation status of A1AT, which has four known N-glycosylation sites. N-glycosylation status was investigated by treatment of A1AT derived from iPSC-HO with PNGase F. We clearly showed a higher electrophoretic mobility, compatible with the removal of 4-glycans on the A1AT. These results were similar to those observed in the control primary human hepatocytes, where a similar shift in mobility was observed.

Another key function of the liver is the production and secretion of coagulation factors and inhibitors to maintain balanced hemostasis. We investigated if we could detect the expression of vitamin K-dependent coagulation factors, endogenous anticoagulants, as well as a number of other coagulation factors by RT-qPCR. The expression of vitamin K-dependent coagulation factors (with the exception of F9 which exhibited very low expression), the two main natural hepatic anticoagulants (protein C (PC) and AT), F8 (expressed in endothelial cells), and plasma fibrinogen (FBG) were all observed. We are particularly interested in FVII and its associated machinery, and the development of cell based models that are currently lacking in this space. We first assessed FVII by immunofluorescence and we observed that this procoagulant molecule was localized to the outer epithelial cells, corresponding to the hepatocytes. We next assessed the production and secretion of FVII into the media by ELISA. We observed robust production of FVII at comparable levels to primary human hepatocytes. As FVII has a key role in the initiation of blood coagulation, the activity of FVII was assessed using a chromogenic assay, where again we observed levels comparable to primary human hepatocytes. We then assessed intracellular and secreted FVII production by organoids using western analysis under SDS reducing conditions, where we observed similar electrophoretic mobility in both the lysate and secreted compartments. FVII also contains PTMs, notably it undergoes N-glycosylation, which is important for its secretion. Therefore we investigated N-glycosylation of secreted FVII by PNGase F digestion. After digestion the secreted FVII showed a higher electrophoretic mobility compatible with the loss of 2 N-glycans. We then evaluated the ability of the
secreted FVII (and the other coagulation factors) to generate thrombin using a thrombin generation assay. We found that the cell medium from the hepatic organoids shortened the lag time of a FVII deficient plasma when mixed at equal volume (Fig. 8L).

We assessed the production of other components of the coagulation machinery including AT, which is one of the most potent anticoagulant proteins that inhibits multiple coagulation serine-proteases, mainly thrombin (FIIa) and activated factor X (FXa), but also FVIIa, FIXa, FXIa and FXIIa. Western analysis showed that intracellular and secreted AT from organoids had similar electrophoretic mobility to primary hepatocytes and human plasma AT (Fig. 8N). We then investigated PTMs of AT using PNGase F and neuraminidase based digestion. We observed that after treatment with PNGase, the organoid-derived AT showed a higher electrophoretic mobility, which was similar to the plasma pool control, and compatible with 4-glycan removal (Fig. 8O). Evident mobility changes were also appreciated after neuraminidase digestion, consistent with the loss of 8 terminal sialic acids associated with the N-glycans, (Fig. 8O) and importantly similar to those observed with human plasma pool AT (Fig. 8O). The functionality of AT produced by organoids was tested by the detection of covalently-bound thrombin-AT complexes, which were generated by addition of thromboplastin (TF+) to iPSC-HO supernatants. These analysis also detected FVII-AT complexes (Fig. 8N). Additionally, we also evaluated the expression of procoagulant proteases that are targets of AT. Secretion of FX, FII, PC, PS and AT into cell medium was quantified using ELISA and procarta-plex assays and production was observed (Fig. 8M and Supplementary Fig. 6A and 6B). Intracellular levels of prothrombin (FII), is shown by western blot (Supplementary Fig. 6C).

**DISCUSSION**

The production of scalable liver-like tissue that exhibits functionally long lived human liver characteristics has remained elusive. Here we describe an approach that can generate massive amounts of functionally mature hPSC derived liver-like organoids. The protocol is efficient and reproducible and organoids can be produced in just 20 days. It follows a liver-like developmental route, producing organoids that contain a cellular repertoire including the parenchymal and non-parenchymal cells.

The HGF secretion is further support for the developmental accuracy of the organoid differentiation, as in vivo it is secreted early in development by the mesenchymal population and later by their derivative, the hepatic stellate cells (HSCs). Where it drives the expansion and maturation of the liver bud suggesting it potentially fulfils a similar role here in our organoids. The detected hemangioblast markers may belong to the population that will go on to form hematopoietic stem cells, but are not yet specified to a myeloid lineage, from which Kupffer cells would ultimately emerge. Together these data suggest that the mesoderm by virtue of undergoing differentiation adjacent to the endodermal derived cells, facilitate the same role as the mesoderm in liver development in vivo.
Further analysis by scRNAseq indicated the establishment of early hepatocyte zonation. However, at the individual gene level, these showed low expression and only a few genes were differentially expressed across HEP clusters. Therefore we demonstrate partial zonation is present via markers differentially expressed across the hepatocyte clusters. For example several zonation markers such as CYP2E1 and LGR5 were lowly expressed in the liver organoid. We speculate this is because our organoids mimic the developing liver and are not yet fully mature i.e. at an early postnatal stage, where full zonation of the acinus architecture is not established at birth and takes many years to fully organise. In a study from Liang et al., they demonstrated that in mice hepatocyte zonation only became defined after day 56 post birth.

In the case of LSEC zonation, we did not detect significant differential expression of LSEC markers and gene signatures in the EC clusters, indicating the LSEC zonation is not yet established in our organoid model. Again this is not unexpected as in the Liang et al. study they only observe pericentral and periportal LSEC populations after day 56 in rodents, equivalent to many months to years in humans, while in neonates they observed heterogeneity in the pre-mature liver.

Proteomic analysis also clearly demonstrated a liver like phenotype. scRNAseq data was further confirmed using immunostaining. Interestingly, we observed parenchymal cell polarity, which was further supported by electron microscopy. We also confirmed the presence of the non-parenchymal cell types including the stellate and Kupffer cells. Remarkably we also observed de novo vascularization within our organoids, along with the presence of neuronal markers. This is not surprising as the human liver is highly innervated, it has also been demonstrated that neural crest cells appear in the epiblast prior to emergence of definitive ectoderm and mesoderm.

The organoids showed the presence of continuous luminal structures, reconstructed in 3D, with a diameter of 8μm. The organoids present with liver like functional features, which included the production of serum proteins, urea and the coagulation factors. They are proficient in drug metabolism exhibiting long-term CYP450 activity, 80 days. They also exhibited non-CYP mediated metabolism using heroin as an exemplar. The organoids have the ability to accumulate fatty acids, presenting with a steatotic like phenotype, potentially providing a useful model for NAFLD. Finally the organoids can be successfully transplanted into mice where they stably produce human albumin. The resulting engrafted organoids clearly show human CD31 positive endothelial structures throughout large areas of the transplanted organoid, extending into the mouse kidney parenchyma. This could only occur through vascularisation from the organoids into the surrounding mouse tissue. Thus supporting our claims of de novo vascularization.

With respect to disease modeling and indeed normal cellular processes, we show the utility of our liver organoids to model the coagulation machinery. There are a dearth of suitable cellular models that can recapitulate the key features of this process and indeed produce physiological and correctly PTMed proteins. Here we clearly demonstrate that
organoids are able to produce AT, A1AT, high levels of FVII as well as other vitamin K specific factors including FII and FX. Moreover, the electrophoretic mobility of the intracellular and extracellular forms of the organoid derived proteins, were very similar to their plasma equivalents of AT, A1AT and FVII. The organoid derived proteins AT, A1AT and FVII were N-glycosylated, compatible with 4, 4 and 2 mature N-glycans, respectively, and again similar to those found in the plasma of healthy adults. Finally the above approach to evaluate organoid derived FVII function suggests that, although these experiments are not specific for FVII activity per se, supernatants (supplemented with vitamin K) show haemostatic potential due to the secretion of serine-proteases (probably gamma-carboxylated) into the conditioned medium that mimic FVII effect. Interestingly the mRNA levels of F10 showed elevated expression compared to the other coagulation factors. We speculate that the elevated levels maybe a feature of the activated HSC population which (indicative of fibrosis) can lead to elevated F10 and worthy of further investigation. We also cannot rule out that the HSCs are fetal in nature, as undifferentiated fetal HSCs also express αSMA.

Organoids can provide both a unique and powerful model to interrogate disease, understand regeneration and potentially provide the building blocks for bridging therapies for patients waiting for an organ or ultimately provide a replacement organ. A key limitation is scaling, for example in children 10^9 hepatocytes are required to correct specific metabolic liver function, however researchers are currently producing organoids in the 10’s to 100’s in combination with Matrigel (ECM) and recombinant growth factors, making scaling both a financial and technical bottleneck. Our approach currently produces around 500 organoids per ml of medium used (Supplementary Fig. 5a) in Erlenmeyer flasks. Therefore based on our approach we would require culture volumes of 1-3 litres to achieve these numbers. It is also compatible with the controlled production in stirred tank bioreactors as recently demonstrated for cardiac and hematopoietic lineages and will provide the field with a game-changing resource to allow the development of clinical as well as screening platforms where the requirement will be in the millions of organoids rather than today’s laboratory scale.

We envisage these organoids will provide a powerful tool to address developmental biology in the dish, where we could utilise lineage tracing to investigate the emergence of for example the HSCs and the tissue-resident hematopoietic cells i.e. Kupffer population. In addition the organoids will provide a powerful tool to allow the investigation of cellular interactions i.e. the mesoderm and endoderm and how they contribute to induce or co-develop these lineages. Organoid production is scalable at a cost-effective level, but will require standardization in order to provide a platform for drug screening, toxicology, disease modeling, to act as the building blocks to produce liver micro-tissues and potentially scaling to larger tissue units.

References:


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Author contributions: GJS, SPH, RS and IHP designed and executed the development of the study. GJS, TY, IHP, YX and BP prepared libraries, ran single cell RNA seq. TY, IHP, FSS, TAN and GJS performed single cell RNA sequencing bioinformatical analysis and proteomic analysis. Proteomic data acquisition was performed by TAN, SRW, RA and FSS. Establishment of suspension system SPH, RS, GJS, RZ, and HK. Metabolism data acquisition RS, SPH, SRW, FSS, ICB and ILB. Serum protein and coagulation factor data acquisition GJS, SPH, RS, MEC, PMS, EA, MEM-B, CB and JC. Modeling organoid growth JJW. Electron microscopy and immunohistochemistry data acquisition GJS, SPH, AB, DC and JM. Transplantation work and data acquisition EM, KSÅ, EA and SPH. Other data acquisition was performed by SPH, RS, SFB, SM, KS, FB, DK, RA. GJS, SPH and RS wrote the manuscript, final manuscript was edited by GJS, SPH, RS, MEC, SFB and JC. All authors critically evaluated the manuscript. Competing Interests statement: FB is partially funded by Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 812616.
Figures

Figure 1. Differentiation of multicellular liver organoids from human PSCs, mimics stages of in vivo development.

(A) Schematic overview of organoid differentiation from PSCs. (B) Representative images of Day 0 (D0) pluripotent spheroids, left panel brightfield. Remaining panels show whole-mount immunostaining of the pluripotency markers OCT4, SOX2 and NANOG. (C) Whole-mount immunostaining of the definitive endoderm (DE) marker FOXA2 at D2 of the differentiation. (D) RT-qPCR analysis of pluripotency and DE associated genes at D1 and D2 of the differentiation relative to D0 spheroids on a log10 scale. Results from three independent experiments are presented as mean ±SD. (E) Graph showing shift in size of organoids from D0 to D2 (Black bar D0, Hatched bar D1 and Grey bar D2). The size is expressed as a mean diameter (µm). The range from 100 to >200 µm was sub-divided into five size distributions. The x-axis represents the percentage of each mass in each category. The y-axis is the organoid size distribution, expressed as a mean diameter (µm). The percentages were based upon the amount in each category (representing a total number of 570 organoids). (F) RT-qPCR analysis of DE and early liver development genes at D7 of the differentiation relative to D2, on a log10 scale. Results from three independent experiments are presented as mean ±SD. (G) Whole-mount immunostaining of D7 organoids showing epithelial (ECAD) and early hepatocyte markers (FOXA2, CK8, HNF4α, AFP) on the outer surface of the organoids. (H) Whole-mount immunostaining of D7 organoids showing heterogeneous expression of mesoderm (MESP1) and mesenchymal (ALCAM, WT1) associated markers. (I) Brightfield image of D20 organoids scale bar is 500 µm. (J) Violin-plot of D20-D27 organoid diameter from three representative experiments, solid line represents the mean while dotted lines represent the quartiles. (K) RT-qPCR analysis of both early and later (developmentally) hepatocyte genes at D20 of the differentiation relative to D2, on a log10 scale. Results from three independent experiments are presented as mean ±SD. (L) Whole-mount immunostaining of D20 organoids showing expression of the hepatocyte markers HNF4α and Albumin on the outer surface of the organoids. (M) Demonstration of increasing secretion of HGF into the culture medium by the organoids throughout the differentiation as measured by ELISA, results from two independent experiments are presented as mean ±SD. All above experiments were performed with the hiPSC line AG27. All Scale bars are 100 µm unless stated otherwise.
**Figure 2. Single-cell RNA sequence analysis of liver organoids.** (A) UMAP plot of single cells distinguished by cell types. (B) GSEA of gene signatures of periportal hepatocyte on hepatocyte clusters. The enrichment was compared across three hepatocyte clusters. *p*<0.05 by two-sided student T test. (C) Differential expression of SOX9 and APOE across hepatocyte clusters. (D) Pseudotemporal ordering of hepatocyte clusters. SOX9 and APOE expression level are positively and negatively correlated with pseudotime. (E) Heatmap showing expression of genes related to oxidative stress, collagen, vascularization, early hepatocyte development, keratin and Kupffer cell development. (F) UMAP plot of single cells derived from our and Ouchi et al. liver organoids and human liver. (G) Pie charts representing cell composition in our and Ouchi et al. liver organoid. (H) GSEA of pathway-related genes between our and Ouchi et al. liver organoid. All above experiments were performed with the hiPSC line AG27.

**Figure 3. Proteomic analysis of liver organoids.**

(A) Venn diagram representing proteins shared between in vivo liver and in vitro organoid. The correlation is also shown with the scatterplot and the overrepresented GO terms of the shared proteins are shown by bar graph. (B) Ranked plot of log2(intensity) of proteomics data in organoid (blue) and liver (red). Proteins are categorized by four intensity range (25 to 25, 20-25, 15-20 and 10-15). Representative liver markers in each intensity range are shown in the right panel. (C) Bar plot showing overrepresented Gene Ontology Terms in each intensity range. (D) Unbiased clustering of proteomic data. Clustering was performed to log2(intensity) value of all-expressed genes (log2(intensity)>10). (E) Differentially-expressed proteins and their overrepresented GO terms. All above experiments were performed with the hiPSC line AG27.

**Figure 4. Liver organoids contain the parenchymal cell types of the liver.**
(A) Immunostaining showing expression of various maturity and polarity associated hepatocyte markers in the outer most layer of the organoids (whole-mount except for ASGP/HNF4α which are from 50 μm cryosections). (B) Electron micrograph revealing ultrastructural features associated with hepatocytes including epithelial cells lining a luminal structure arranged in one layer, connected with tight junctions (circles) indicative of polarization. The surface facing the lumen contains numerous microvilli whereas the abluminal surface facing the extracellular matrix remains smooth and attached to underlying basal lamina (arrowheads). Scale bar 2 μm. (C) Paraffin embedded section of organoid stained with alcian blue and counterstained with nuclear red. The lumen of this organoid shows cells containing pale blue cytoplasm indicating the presence of mucopolysaccharides. Scale bar 50 μm. (D) Immunostaining of an organoid cryosection showing a later developmental cholangiocyte marker (CK7) positive population of cells separate to the hepatocyte population. These form smaller ring like structures as well as lining large luminal or cyst-like spaces within the organoids. Immunohistochemical staining of AG27 derived organoids showing CK19 positive cells, arrow denotes cholangiocytes surrounding lumen. All above experiments were performed with the hiPSC line AG27.

**Figure 5. Liver organoids are de novo vascularised and contain neuronal cell types**

(A) Max projection and cross section of whole-mount immunostained organoids showing overlapping populations expressing one or both of the endothelial markers CD54 and CD31. Cross-section reveals stronger CD54 expression towards the outer surface contrasted with stronger CD31 expression toward the centre of the organoid. Also visible are multiple conjoined luminal spaces bounded by the positive cells. (B) Immunofluorescence of CD31-expressing endothelial cells (green) under high magnification. Plane image and 3D volume rendering are shown. Hoechst 33342 (blue) dye was used to counterstain nuclei. Right panels show 3D volume rendering and plane image of selected region. Insert visualize vessel cross-section. (C) Immunostaining of a 50 μm cryosection showing adjacent and overlapping expression of CD34 and CD31 in a small structure indicative of neo-vascularization. (D) Whole-mount staining of LYVE1 a sinusoidal endothelial marker showing positive cells demarking a diversity of lumen shapes and sizes. (E) Endothelial cell ordering from LSEC to MVEC. Heatmap represents expression pattern of genes, which are dependent on the EC ordering and categorized into six groups. Significant GO terms in each gene group are also shown. (F) Immunostaining of two 50 μm cryosection showing co-localisation of liver endothelial associated FVIII and endothelial cell marker CD31 and luminal spaces in the organoids. (G) Immunofluorescence analysis of AcLDL-488 and FSA-FITC binding and uptake in CD54+ endothelial cells within the organoids. All above experiments were performed with the hiPSC line AG27. Nuclei in blue, scale bars are 100 μm.
Figure 6. Liver organoids contain a resident macrophage and hepatic stellate population.

(A) Immunostaining of 50 μm cryosection showing CD68 in a granular pattern in the cytoplasm of the Kupffer-like cells. (B) RT-qPCR analysis of hemangioblast (RUNX1) and hematopoietic (GATA2) associated genes involved in the development of macrophages, at D2 and D7 of the differentiation relative to D0 spheroids. Results from three independent experiments are presented as mean ±SD. (C) Ratio of cells expressing Kupffer cell markers. (D) Immunostaining of cryosection showing expression of endodermal and stellate cell associated proteins HNF4α and αSMA (left and middle panel) and (right panel) whole-mount showing expression of mesenchymal and stellate cell associated proteins (αSMA, Laminin) beneath the hepatocyte layer of organoids. (E) Immunostaining for neuronal population in an organoid (TUBB3). (F) RT-qPCR analysis of neural crest stem cells markers, at D2 and D7 of the differentiation relative to D0 spheroids. All above experiments were performed with the hiPSC line AG27. Results from three independent experiments are presented as mean ±SD. All Scale bars are 100 μm unless stated otherwise.

Figure 7. Assessment of liver organoid function, maintenance and transplantability.

(A) Assay demonstrating inducibility and increasing activity of CYP1A2 (top row) and CYP3A4 (bottom row) drug metabolizing enzymes at D20, D30 and D40 in the hESC line H1 organoids. To induce CYP1A2 the cells were pretreated with omeprazole and for CYP3A4 rifampicin. Graphs show comparison of suspension cultures, 2D differentiation and cryo-preserved primary human hepatocytes. Results from three independent experiments are presented as mean ±SD. (B) Assay demonstrating long-term maintenance of activity and inducibility of CYP1A2 (top row) and CYP3A4 (bottom row) proteins in suspension up to D80. Results from three independent experiments are presented as mean ±SD. (C) D20 AG27 derived organoids show Phase I and Phase II metabolism of heroin dosed at 10 μM. Left panel represents the metabolic pathway of heroin. Heroin is metabolized by sequential decacetylation (phase I reaction) to 6-monoacetylmorphine and morphine by esterase enzymes. Morphine is further glucuronidated (phase II reaction) by UDP-
glucuronosyltransferases (UGTs) to morphine-3-glucuronide (M6G) and morphine-6-glucuronide (M3G). Center panel; Ion chroma tograms of an extracted organoid sample analysed by LC-MSMS. Right panel; Metabolism of heroin (10 µM) in organoids, primary human liver microsomes (HLM) and human S9 fraction (S9). (D) Whole-mount live imaging of Oleic acid treated (right column) and untreated (left column) AG27 derived organoids showing accumulation of non-polar fats after treatment (BODIPY in green). Oleic acid was used at 300 µM for 5 days. (E) Demonstrating the production and secretion of urea into the culture medium from AG27 derived organoids (ipSC-HO) and primary human hepatocytes after 48 hours and normalized to mass per million hepatocytes (n=3, mean ±SD, no urea was detectable in cell-free medium). (F) Transplanted AG27 derived organoids can be maintained in mice. Human albumin was consistently detected over a 5 week period in mouse blood samples. D8 organoids were transplanted with either Matrigel / FGF2 supplementation (MG+FGF) or Matrigel / FGF2 free (ORG) (mean ±SD; MG+FGF n=3, ORG n=2, sham n=4). All Scale bars are 100 μm unless stated otherwise. (G) Transplanted AG27 derived organoids can be maintained in mice. Human albumin was consistently detected over a 5 week period in mouse blood samples. D8 organoids were transplanted with either Matrigel / FGF2 supplementation (MG+FGF) or Matrigel / FGF2 free (ORG) (mean ±SD; MG+FGF n=3, ORG n=2, sham n=4). All Scale bars are 100 μm unless stated otherwise. (J) Immunostaining of mouse kidney/organoid transplant cryosections, demonstrate the retention of human hepatic populations (CK7, CD31, HNF4α and ALB) and structures seen in vitro. They also demonstrate clear endothelial engraftment via CD31 staining. Texas red dextran is strongly localised in the kidney parenchyma and albumin (ALB) positive clusters of the transplanted organoids, whilst being detectable at lower levels in other areas of the transplanted material. The boundary between kidney parenchyma (marked with *) and organoid is delineated by a white dotted line while a yellow dotted line marks the external surface. Nuclei in blue, scale bars are 100 μm.

**Figure 8. Assessment of liver organoid coagulation machinery.**

(A) Demonstrating the production and secretion of A1AT and albumin into the culture medium by D21-24 AG27 derived organoids and primary human hepatocytes over 48 hours as measured by ELISA (n=3, mean ±SD). (B) Alpha-1-antitrypsin (A1AT) is present in cell lysates (lane 2 and 3) and supernatants (lane 5 and 6) derived from liver organoids assessed by SDS-PAGE under reducing conditions. Plasma pool (lane 7) and primary hepatocytes (lane 1) were used as a reference of plasma A1AT; SFM L15 medium was used as a negative control (lane 4). (C) Assessment of the N-glycosylation content of A1AT from the supernatant of organoids by PNGase F, compared to supernatants from primary human hepatocytes (PHH). (D) Levels of coagulation factors and inhibitors in iPSC derived organoids (gray bars) and primary human hepatocytes (black bars). mRNA levels of coagulation factors II, VII, VIII, IX, X, fibrinogen (F2, F7, F8, F9, F10, FBG), coagulation inhibitors protein C and antithrombin (PC, AT) and hepatic markers alpha-1 antitrypsin and hepatocyte nuclear factor 4 alpha (A1AT, HNF4α) were determined using quantitative RT-qPCR with 18S as endogenous control. The results are
presented as mean of the fold-change expression of the respective gene. Results from three independent experiments are presented as mean ±SD. (E) FX protein (Ag) levels (ng/ml) in culture medium from iPSC-HO and PH as measured by ELISA. The total concentration of FX was adjusted to 1x10^6 cells and the results are expressed as the ratio iPSC-HO/PH. Results from three independent experiments are presented as mean ±SEM. (F) Lysates were obtained from iPSC-HO (lanes 1-3) and PH (lane 4). Equal amounts of proteins were separated by SDS-PAGE under reducing conditions, blotted onto a PVDF membrane and incubated with antibody against FII. β-actin was used as loading control. Results from three independent experiments are presented. (G) Demonstrating the production and secretion of FVII protein in culture medium of AG27 derived liver organoids (iPSC-HO) and primary human hepatocytes (PH) as determined using ELISA. The total concentration of FVII was adjusted to 1x10^6 cells and the results are expressed as the ratio iPSC-HO/PH. Results from three independent experiments are presented as mean ±SEM. (H) Demonstrating FVII activity (IU/ml), culture medium from AG27 derived iPSC-HO and PH was determined using a FVII chromogenic assay. Results were adjusted to 1x10^6 cells and expressed as the ratio iPSC-HO/PH. Results from three independent experiments are presented as mean ±SEM. (I) FVII is present in cell lysates and supernatants derived from liver organoids assessed by SDS-PAGE under reducing conditions. SFM L15 medium was used as a negative control (C). (J) Assessment of the N-glycosylation content of secreted FVII of organoids by PNGase F treatment. (K) Immunostaining of two 50 μm cryosections showing localization of FVII (Green) to the outer hepatocyte layer of the AG27 derived organoids, co-stained with phalloidin (Red). Nuclei in blue, scale bars are 100 μm. (L) Assessment of thrombin generation in FVII-depleted plasma (Black) complemented with either Serum Free medium (SFM) (Red); organoid supernatant (Green) or fetal bovine serum (FBS) supplemented serum free medium (SFM) L15 (Red). (K) Demonstrating the production and secretion of AT protein in culture medium of AG27 derived liver organoids (iPSC-HO) and primary human hepatocytes (PH) as determined using procarta-plex assay. The total concentration of AT was adjusted to 1x10^6 cells and the results are expressed as the percentage of plasma calibrator control. Results from three independent experiments are presented as mean ±SEM. (L) AT is present in cell lysates and supernatants derived from liver organoids assessed by SDS-PAGE under reducing conditions. Plasma pool and primary hepatocytes were used as a reference of plasma AT; SFM L15 medium was used as a negative control (C). (M) Assessment of the N-glycosylation content of secreted AT from the supernatant of organoids by PNGase F and Neuraminidase treatment, compared to human plasma pool. (N) Western analysis of AT after activation of organoid derived supernatants by Tissue Factor (TF) and CaCl_2 and incubated with AT and unfractioned heparin. Activated FVII-AT (FVIIa-AT) complexes are indicated by an arrow and bracket.