

# 博士論文 (要約)

## Systematic Integration of a Biomimetic Hepatic Microenvironment *in vitro* for Improved Physiological Drug Toxicity Assays

(薬物評価のための生理的肝細胞培養系の構築を目指した肝微小環境の統合的再現)

シャイデカ ベネディクト

# Systematic Integration of a Biomimetic Hepatic Microenvironment *in vitro* for Improved Physiological Drug Toxicity Assays

薬物評価のための生理的肝細胞培養系の構築を目指した肝微小環境の統合的再現

Benedikt Scheidecker (37-177284)

**Sakai-Nishikawa Laboratory**

## **Abstract:**

Pre-clinical drug screening is an important step in assessing the metabolic effects and hepatic toxicity of new pharmaceutical compounds. However, due to the complexity of the liver microarchitecture, simplified *in vitro* models do not adequately reflect *in vivo* situations. Especially spatial heterogeneity, known as metabolic zonation, is often lost due to limitations introduced by typical homogenous culture conditions. Restoring this phenotypic functionality in hepatocellular culture models is a crucial factor to improve clinical relevance in drug toxicity assessments. Thus, identifying critical parameters of the hepatic microarchitecture and translating them into *in vitro* systems with gradually increasing complexity will improve currently available toxicity models in a physiological manner. In this work, supplying hepatocytes with biomimetic oxygen flux was shown to enable the formation of zone-specific phenotypes through the variation of oxygen tension but could not overcome physiological hormonal and paracrine signals. Similar induction in reduced oxygen flux, mimicking currently used technology, instead resulted in phenotypes resembling hepatocellular carcinoma with impaired drug metabolism. Further, implementation of endothelial, paracrine signaling enabled zone-specific activation of morphogenic pathways largely responsible for cellular drug metabolism. Lastly, by developing an oxygen-controlled perfusion mechanism, fluid dynamic shear stress was shown to increase angiogenic signaling and  $\beta$ -catenin dependent drug metabolism, but not the functional increase as reported in perfusion systems with additional oxygen influx. In combination, this work elucidates the influence of various biomimetic culture parameters and their need in hepatic cell-based models. Reducing animal experimentation in pre-clinical studies inherently requires the availability of such physiologically accurate models. By providing a roadmap of relevant parameters this work will ultimately enable a better understanding of the hepatic drug metabolism *in vitro*.

## **1. General Introduction:**

The development of pharmaceuticals requires strict compliance with regulatory requirements. Especially efficacy and toxicity of drugs are essential parameters that need to be considered before further testing in clinical trials. In these pre-clinical studies, the safety of pharmaceutical compounds is assessed through a myriad of *in vivo* and *in vitro* assays regarding their absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox). Even though these functions require the interaction of multiple organs, the focus of research often centers on the liver as the main site of metabolism. While *in vivo* models do represent the metabolism of an organism adequately, utilization of animal models faces multiple issues ranging from the ethical questions regarding animal use to the applicability of results due to interspecies differences<sup>1</sup>. These problems, however, can be circumvented by simplified models of human hepatocytes cultured *in vitro*. Nevertheless, these *in vitro* models generally do not deliver the required level of accuracy due to the complexity of the hepatic metabolism and microarchitecture. For these assays to be more reliable, it is therefore imperative to develop and use highly functional and representative

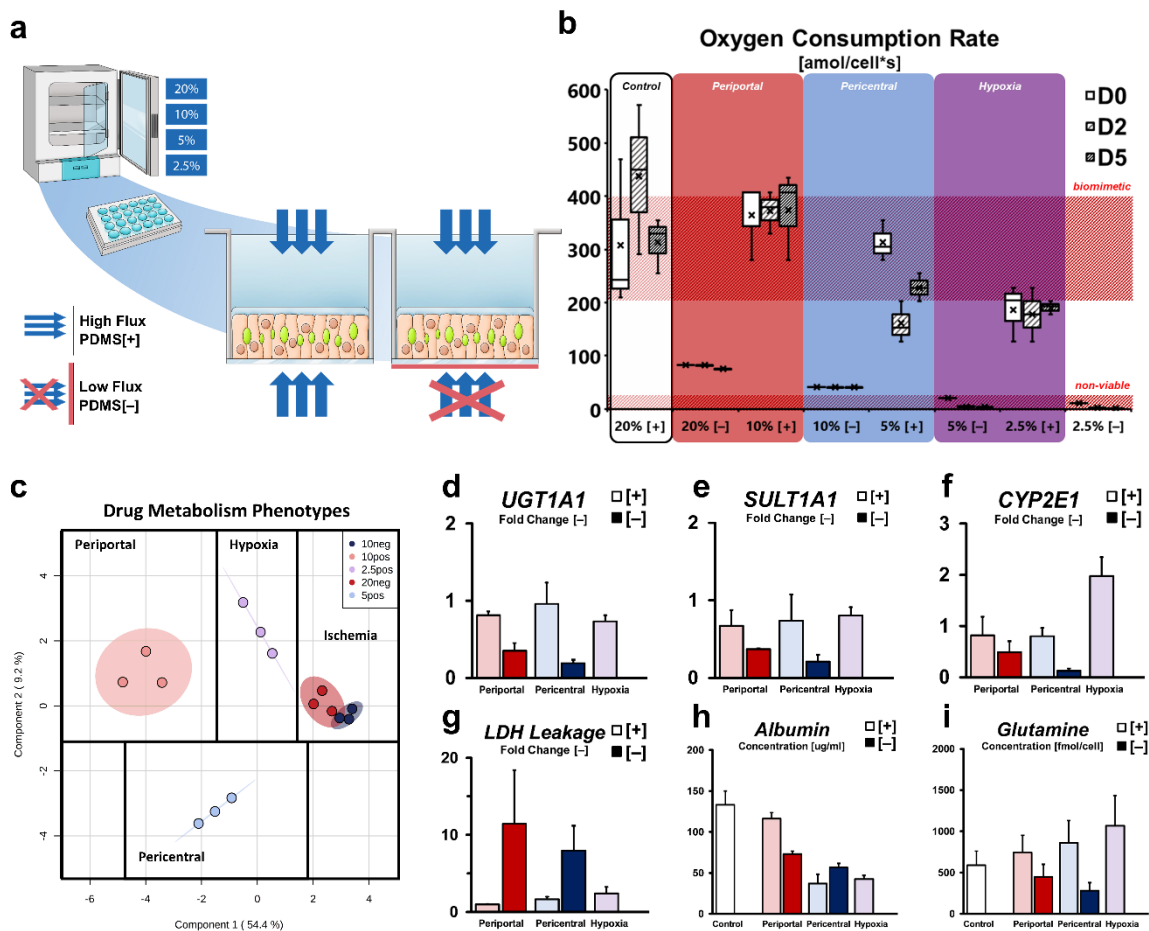
models of liver metabolism. A vertebrates' metabolism consists of a multitude of different pathways, which fundamentally rely on the liver as the central organ for metabolic functions. Blood glucose homeostasis, metabolism of xenobiotics and endogenous byproducts, and synthesis of bile acids are just a few of this organ's core functions<sup>2</sup>. Managing these pathways requires distinct patterning of enzymes in order to be efficient since certain pathways are inherently opposing each other. This compartmentalization is referred to as metabolic zonation, which describes the spatial difference of cellular functions along the sinusoids of the liver's structural units - the lobule. Accordingly, periportal hepatocytes, named after their location near the portal triad at the perimeter of the lobule, can be phenotypically distinguished in terms of their metabolic functions from pericentral hepatocytes located close to the central vein. This is especially relevant in terms of drug-metabolizing enzymes, which are often expressed with pericentral bias<sup>3</sup>. Although this systematic patterning is clearly described through extensive research<sup>4-6</sup>, translation of this phenomenon into cell-based *in vitro* models is still lacking, in part due to the complex molecular interactions involved. Spatial heterogeneity of hepatocytes is inherently associated with the respiration-based oxygen gradient along the sinusoid – ranging from 65 mmHg (8.5%) at the portal triad to roughly 30 mmHg (4%) near the central vein<sup>2</sup>. As a result of oxygen's signaling function, and in conjunction with hormonal gradients, cells assume distinct spatial functions. Additionally, morphogenic Wnt/ $\beta$ -catenin has been implied as a key regulating component through paracrine Wnt protein secretion and oxygen-dependent degradation<sup>2</sup>.

In order to successfully translate this metabolic patterning into routine cell-based systems, relevant physiological parameters have to be evaluated in terms of their contribution to the formation of heterogeneous phenotypes, while maintaining the simplicity of the model to facilitate their use. This work, therefore, aims to induce stable, zonation-like hepatocytes *in vitro* by including biomimetic parameters into hepatocellular culture systems with gradually increasing physiological relevance and complexity. First, current culture practices and regulatory rationales are reviewed in order to identify relevant biomimetic parameter for inclusion. Based on the apparent importance of cellular oxygen levels, hepatocytes cultures are then screened regarding their metabolic response to different forms of oxygen tension and supply. Next, this model is expanded to include non-parenchymal sinusoidal endothelial cells in order to investigate oxygen-dependent signaling functions caused by the hepatic microarchitecture. Lastly, further complexity is added with auxostatic fluid shear stress to investigate the importance of interstitial mechanical stimuli on hepatocytes without interfering effects from perfusion-related oxygenation. Elucidating the metabolic plasticity of hepatocytes caused by oxygen and the surrounding microarchitecture will ultimately lead to improved hepatocellular *in vitro* assays which mimic the *in vivo* metabolism more accurately. This will allow for a physiologically relevant assessment of drug interactions and toxicity while enabling reductions in current animal experimentation.

## **2. Induction of *in vitro* Metabolic Zonation in Primary Hepatocytes Requires Both Near Physiological Oxygen Concentration and Flux**

**Methods:** Zonation-like phenotypes of primary rat hepatocytes were induced by modulation of oxygen concentration and availability. In order to modulate oxygen availability, PDMS membranes of the culture vessels were either left as-is for oxygenated cultures (denominated as [+]) or sealed with an optical adhesion polyester film to only allow oxygen supply by diffusion through the medium (denominated as [-]). Oxygen concentration was further varied between experimental groups by transferring cultures into incubators with different ambient oxygen levels (Fig. 1a). To allow for the formation of stable phenotypes, hepatocytes were cultured for 5 days after induction, before being subjected to 20 mM acetaminophen and collected for analysis.

**Results:** Cellular respiration of hepatocyte cultures was found to heavily depend on the oxygenation method, with high flux cultures exhibiting self-regulated, phenotype-specific oxygen consumption rates (OCR) within biomimetic ranges (Fig.1b). In contrast, conventional low flux setups resulted in limited, ischemic OCR values due to pericellular oxygen depletion regardless of ambient oxygen tension. Principal component analysis (Fig. 1c) regarding drug metabolism-related gene expression generated distinct phenotypes according to previously defined groups in high flux situations, while low flux groups were overlapping as one ischemic phenotype. Detailed analysis of genes involved in acetaminophen metabolism revealed stark downregulation in ischemic conditions (Fig. 1d-f), resulting in increased cellular injury (Fig. 1g) due to dysfunctional, energy-dependent drug metabolism. High flux conditions, in contrast, exhibited homeostatic drug metabolism (Fig. 1d, e), but relevant zone-specific gene expression (Fig. 1f) was found to occur only in hypoxic groups below physiological oxygen tension. In general, cultures were assuming zonation-like phenotypes in high flux conditions based on biomarker content (Fig. 1h, i), while low flux conditions instead exhibited reduced biomarker concentration.



**Fig. 1:** Oxygen-dependent induction of metabolic zonation *in vitro*. (a) Experimental setup comprising high [+] and low [-] oxygen diffusion exposed to different oxygen tension. Cultures exhibit differential (b) OCR based on their environment and assume (c) distinct drug metabolic phenotypes. Zone-specific expression of (d-f) drug metabolic genes leads to varying (g) cellular drug toxicity, with zonation-like functionality confirmed by gradual (h) periportal and (i) pericentral biomarker content.

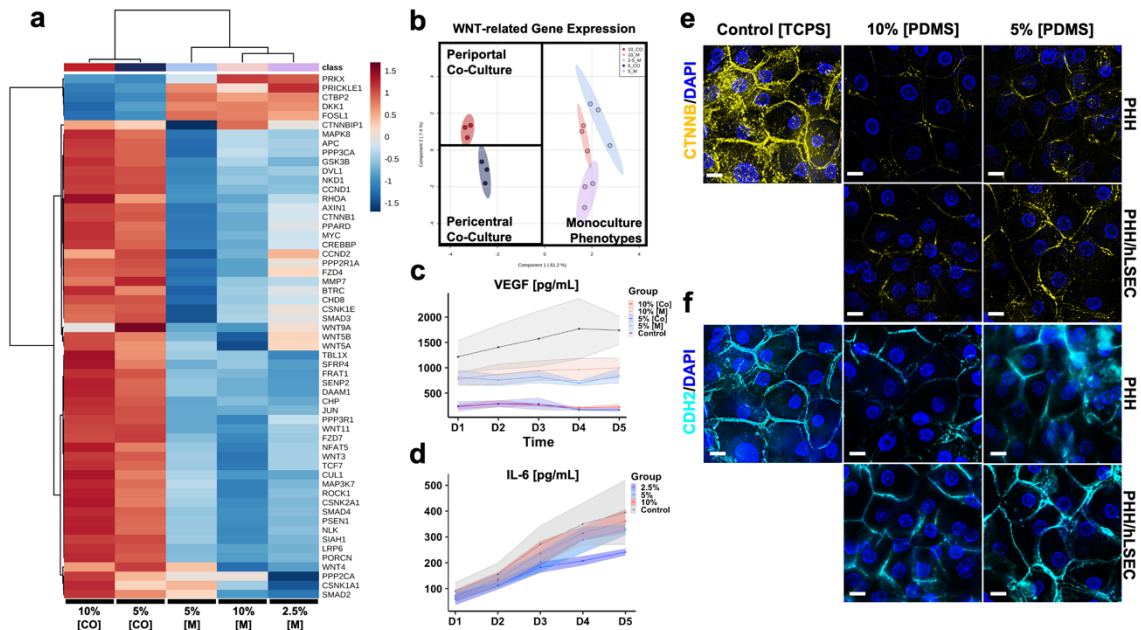
**Conclusion:** Modulation of ambient oxygen tension enables the induction of distinct zonation-like phenotypes in high flux conditions but cannot overcome hormonal or morphogenic signaling

within physiological oxygen ranges. Similar oxygen modulation in setups mimicking conventional culture technology instead induces one ischemic phenotype closely resembling hepatocellular carcinoma and exhibiting increased drug-induced cell toxicity. Conclusively, this shows the importance of biomimetic oxygen flux in physiological hepatocytes cultures, while also highlighting the need for increased complexity of such systems to include zone-specific signaling.

### 3. Endothelial Signaling *in vitro* Enables Oxygen-dependent Induction of Metabolic Zonation in Primary Hepatocellular Co-Cultures

**Methods:** Human liver sinusoidal endothelial cells (hLSECs, expanded to P4) and primary human hepatocytes (PHH) were cultured in varied ambient oxygen conditions either in monolayer or co-culture configuration on PDMS culture plates to investigate oxygen-dependent phenotypes and paracrine effects. Hepatocytes were maintained in confluent cultures, while hLSECs were seeded sub-confluent and formed monolayers over 5 days of culture in differential physiological ambient oxygen before being collected for analysis. Drug metabolic functions were additionally assessed by exposure to pharmaceutical compounds with known cytochrome P450 (CYP450) interactions and subsequent targeted metabolite measurement by LC-MS quantification.

**Results:** Exposing hLSECs to differential oxygen tension during monolayer formation induced small changes in their transcript profiles (Fig. 2a), indicating a slight hypoxic influence in their morphogenic gene expression. Contrastingly, co-cultured hLSECs exhibited strongly increased transcript levels of the same genes (Fig. 2a, b), highlighting the importance of hepatocyte signaling effects towards hLSEC functionality. This is most likely caused by an increase in hepatocyte-derived VEGF concentration (Fig. 2c), with periportal hLSECs consuming this growth factor at higher rates compared with pericentral groups.



**Fig. 2:** (a) Differential gene expression relating to the Wnt signaling pathway in hLSECs show a distinct difference between (b) mono- and co-cultured groups with slight oxygen-dependency based on sPLS-DA. Co-culture effect is likely caused by (c) increased, hepatocyte-derived VEGF concentration, leading to higher growth factor consumption in periportal groups. (d) hLSECs are shown to secrete IL-6 in an oxygen-dependent manner, with low oxygen tension significantly reducing cytokine concentration. As a result, co-cultured hepatocytes express (e) cytosolic active  $\beta$ -catenin (CTNNB) in pericentral conditions, confirmed by (f) CDH2 staining signifying the cell membrane. (Bands represent 95% CI; scale bars = 10  $\mu$ m)

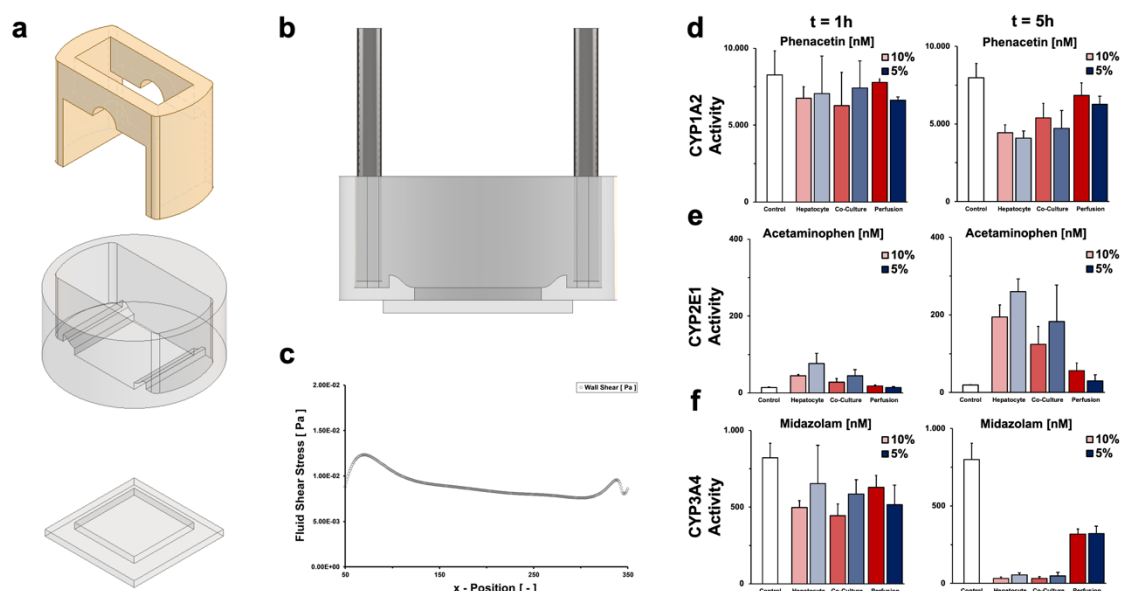
Further, normoxic hLSECs have been shown to secrete interleukin 6 (IL-6), a typical periportal upstream signal known to inhibit Wnt signaling (Fig. 2d), while hypoxic cells exhibited significantly reduced levels of this cytokine. As a result, and in combination with previous gene expression profiles, zone-specific  $\beta$ -catenin activation (Fig. 2e, f) was observed in pericentral hepatocytes in co-cultures, but not in monocultures.

**Conclusion:** Sinusoidal endothelial cells are known to be a major factor in physiological Wnt/ $\beta$ -catenin signaling. Exposure to different oxygen tension weakly induced zonation-like phenotypes, with normoxic hLSECs expressing Wnt-inhibiting factors, and hypoxic hLSECs exhibiting elevated Wnt gene expression. Consequently, co-cultured hepatocytes in pericentral ambient oxygen tension were found to show zone-specific active  $\beta$ -catenin signaling, which could not be achieved in monocultures. Conclusively, these hepatocellular co-cultures are mutually beneficial for both cell types and further add physiological accuracy to the system.

#### 4. Physiological Fluid Shear Stress in Open-Well Perfusion Improves Hepatocellular Functionality in Induced Zonation-like Phenotypes

**Methods:** Hepatocellular co-cultures comprising hLSECs and PHH were cultured on custom PDMS sheets fit into perfusion inserts (Fig. 3a). Oxygen-controlled perfusion was achieved by constant aspiration of culture medium with a peristaltic pump in the absence of an external medium reservoir within multi-gas incubators. After 5 days, cellular drug metabolism was assessed by LC-MS quantification of CYP450 metabolites and compared with control cultures.

**Results:** While perfusion systems *in vitro* have been reported to generally enhance hepatic functionality, a distinction of this effect between fluid shear stress and additional oxygen influx is often not clear. In order to elucidate the effect of interstitial shear stress *in vitro*, an oxygen-controlled perfusion system (Fig. 3a, b) was developed to deliver a biomimetic mechanical stimulus (Fig. 3c) to the hepatocyte layer in laminar open-well configuration for simplicity of use.



**Fig. 3:** Assembly of the developed oxygen-controlled perfusion insert comprising (a) PDMS bottom sheet, polycarbonate insert structure and temporary seeding geometry. After initial cell attachment the yellow seeding geometry is removed and the (b) perfusion mechanism is inserted. Numerical shear stress computation confirms nearly homogeneous shear rate along the culture area. Comparison of drug metabolic activity of (d) CYP1A2, (e) CYP2E1, and (f) CYP3A4 shows differences in drug metabolic rates between mono-, co-, and perfusion cultures after 5 days.

With the cellular environment being kept identical to static cultures, pericellular oxygen tension was comparable between mono-, co-, and perfusion culture, contributing differential cellular functions to the applied shear stress. Interestingly, commonly stated perfusion enhancements like albumin secretion were not significantly increased but instead improved zonation-like distinction between oxygen tension groups. Further, angiogenic secretion of VEGF was increased in perfused cultures. Drug metabolic activity (Fig. 3d-f) of perfused cultures was comparable to static groups but resulted in increased *CYP2E1* and decreased *CYP3A4* activities in prolonged drug exposure.

**Conclusion:** By developing a perfusion system with constant oxygen levels, the direct effect of interstitial shear stress was shown to enhance angiogenic signaling, as well as increase  $\beta$ -catenin dependent drug metabolism. Contrasting previous reports, generally increased hepatocyte function was not observed, indicating that this effect stems largely from improved oxygen supply in other microfluidic perfusion systems.

## 5. General Conclusion and Future Perspectives

In order to replace current pre-clinical animal experimentation with cell-based *in vitro* assays, such models need to accurately replicate an organism's metabolism. With model relevance being a trade-off between physiological accuracy and model simplicity, required parameters have to be evaluated in terms of their benefit to the system performance. In this work, relevant biomimetic factors have been systematically included in hepatic *in vitro* models to improve their physiological functions and replicate spatial heterogeneity of the hepatocellular metabolism. Especially oxygen tension and physiological supply rates have been shown to critically affect hepatocyte functionality and their drug metabolism. Recapitulating spatial heterogeneity within physiological oxygen tension required the addition of sinusoidal endothelial cells, which can mediate specific paracrine signaling. In summary, this work has shown the functional plasticity of hepatic cells, allowing for a directed reprogramming through physiological oxygen levels and ultimately improving tissue functionality for *in vitro* assays.

While sinusoidal endothelial cells have shown to assume differential phenotypes in this work, their propagation before utilization might inadvertently mask aspects regarding their possible functional reprogramming in comparison to freshly isolated hLSECs. Such cell sources might further improve zonation-like heterogeneity of oxygen-induced phenotypes *in vitro*, as well as allow the formation of mid-lobular phenotypes and therefore increase spatial resolution. Similarly, further inclusion of other liver-resident cell types can introduce important metabolic and signaling functions to the *in vitro* system, generating a more accurate representation of the hepatic tissue model. Lastly, an important consideration for the broad application of such models remains the availability of utilized cells. With primary cell sources being extremely limited, proliferating cells or reprogrammed stem cells can be an alternative to explore. The evaluation of relevant biomimetic factors outlined in this work should similarly allow these cell types to assume physiologically relevant functionality *in vitro*.

## 6. References

1. Begley, C. & Ellis, L. Raise standards for preclinical cancer research. *Nature* **483**, 531–533 (2012).
2. Kietzmann, T. Metabolic zonation of the liver: The oxygen gradient revisited. *Redox Biol.* **11**, 622–630 (2017).
3. Braeuning, A. *et al.* Differential gene expression in periportal and perivenous mouse hepatocytes. *FEBS J.* **273**, 5051–5061 (2006).
4. Halpern, K. B. *et al.* Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. *Nature* **542**, 1–5 (2017).
5. Halpern, K. B. *et al.* Paired-cell sequencing enables spatial gene expression mapping of liver endothelial cells. *Nat. Biotechnol.* **36**, 962 (2018).
6. Ben-Moshe, S. *et al.* Spatial sorting enables comprehensive characterization of liver zonation. *Nat. Metab.* **1**, 899–911 (2019).