

SIMPLE and RELIABLE ISOLATION of PRIMARY HEPATOCYTES

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Isolation of hepatocytes from various liver tissues by a novel, semi-automated perfusion technology

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1 Introduction and objectives

Primary liver cells represent a major tool in biomedical research. Presently, different protocols for the isolation of liver cell subsets have been introduced, which require trained staff to avoid considerable fluctuations in quality and yield.

We aimed at establishing an easy workflow for the generation of hepatocytes.

2 Methods

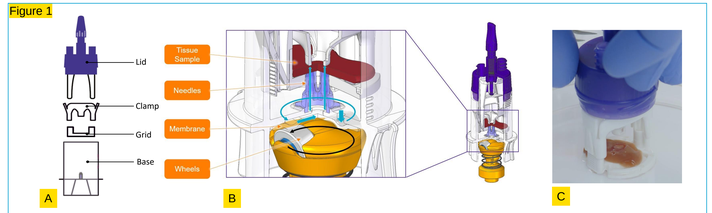
A new semi-automated perfusion technology was developed, which is suitable for the gentle, rapid, and efficient generation of single-cell suspensions from rodent livers. Tissue is clamped into a newly designed disposable (Perfuser) and enzymatically digested using optimized components. Afterwards, single-cells are liberated from the tissue by a short mechanical disruption of the perfused tissue and the sample is applied to a strainer to remove any remaining larger particles from the single cell suspension. Yield, viability and purity of cells were determined as well as immunofluorescence microscopy was performed. Cells were stimulated with different cytokines and expression of target genes was analyzed by Western blot analysis.

3 Results

Isolation of Primary Hepatocytes with the newly designed Perfusion Technology

We have constructed a new tube format (fig. 1A) which is run in combination with optimized reagents and newly designed sleeves (fig. 1B) on the gentleMACS instrument for optimal isolation of hepatocytes. After perfusion of the liver lobule (fig. 1C), the perfused tissue is disrupted by another tube format (C Tube) and hepatocytes are collected after filtration and a low-spin centrifugation step. The yield isolated from one liver lobule is ~1,4 x 10^7 hepatocytes with ~83% viability.

Figure 1

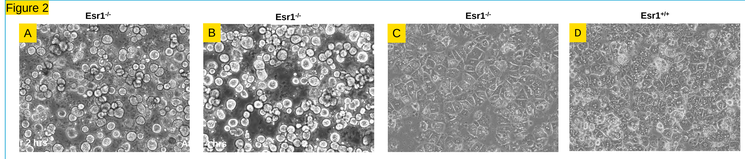


The Perfuser can be disassembled into four parts: Lid, Clamp, Grid, and Base. The Base contains an in-built peristaltic pump which delivers liquids via a needle array into the penetrated tissue sample. To aspirate buffer from the inside of the Perfuser, a Pasteur pipette with elongated tip is introduced though one of the luer lock connectors to the base bottom. The Adjuster on top of the lid can be used to adapt the penetration depth of the needle array into the sample material.

Cultivation of Primary Hepatocytes

In culture, primary hepatocytes take on the characteristic flattened, hexagonal appearance and can be grown for several days (fig. 2A-D).

Figure 2

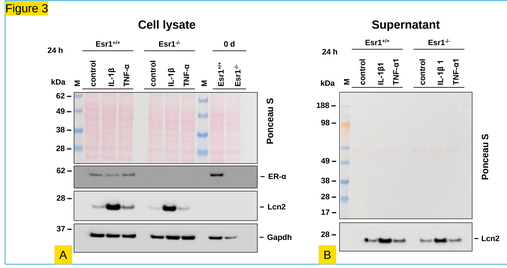


Primary hepatocytes isolated from 3-months-old male Esr1$^{+/+}$ (A-C) or Esr1$^{-/-}$ (D) mice and were seeded in HepatoZyme on collagen coated six-well plates (400,000 – 500,000 cells per well). After 2 hrs medium was changed to DMEM (containing supplements and 10% FBS). Images were taken after 2 hrs (A), 4hrs (B) or on the next day (C-D) at 100x magnification.

Stimulation of Primary Hepatocytes with pro-inflammatory cytokines

After stimulation with IL-1β, the cells express and secrete large quantities of LCN2, previously shown to be a target of IL-1β.

Figure 3

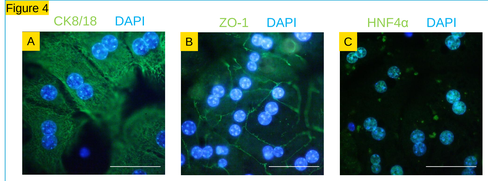


Primary hepatocytes were isolated from 3-months-old male (Esr1$^{+/+}$ or Esr1$^{-/-}$) mice and were seeded in HepatoZyme. A small amount of the hepatocytes were directly lysed in RIPA buffer and used for protein analysis (0 d). After 2 hrs, the medium was changed to DMEM (containing supplements and 10% FBS). On the next day, primary hepatocytes were stimulated, after starvation (2-3 hrs in DMEM containing 0.5% FBS), with indicated cytokines (in DMEM containing supplements and 0.2% FCS) or left untreated for 24 h. IL-1β = 2.5 ng/ml; TNF-α = 10 ng/ml. Cells were harvested in RIPA buffer for protein analysis. Equal amounts of protein (40 µg for cell lysate, A, or 21 µl supernatant, B) were used for Western blot analysis.

Immunofluorescence Microscopy of isolated Primary Hepatocytes

Primary hepatocytes show the expected staining of typical hepatocyte markers.

Figure 4



Primary hepatocytes were characterized by immunofluorescence staining of hepatocellular markers cytokeratin 8/18 (CK8/18), zona occludens-1 (ZO-1), and hepatocyte nuclear factor 4 alpha (HNF4α) with nuclear DAPI staining (markers appear in green, DAPI in blue). Magnification 1000x, bars 50 µm.

4 Conclusions

- Perfuser and appropriate reagents were optimized for the generation of parenchymal rodent hepatocytes but can be adapted to non-parenchymal cells.
- The semi-automated workflow enabling liver perfusion in a closed system is easy to apply and helps to implement the 3R principle because only one liver lobule is perfused, while others can be used for further experimentation. Nested processing allows simultaneous handling of up to 8 samples.
- The protocol does not require inconvenient perfusion in living animals avoiding the need of animal ethics approval.

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