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Regenerative medicine: from new insights to new applications

ORGANTRANS

Controlled Organoids transplantation as enabler for regenerative medicine translation

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= Deliverable D1.1 =

Six established and fully characterized individual hepatic organoid lines

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RE	Restricted to a group specified by the consortium (including the Commission Services)	
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Executive Summary

In order to obtain standardized liver spheroids with hepatic function and tissue architecture (deliverable 1.3), we need to isolate and fully characterize LGR5-positive stem cells in the form of organoids that are required throughout the project. We have received liver tissue (pseudo-anonymous) from the transplant centre in Rotterdam, the Netherlands. From this tissue we isolated LGR5-positive stem cells and cultured them as organoids. Six lines were established, three male and three female, and kept into culture for at least 10 passages. Characterisation of the cells was performed on early passage (P3-5) and late passage (P10), for both expanding as differentiating conditions. Results indicated that all cell lines proliferated normally (average split rate of 1:4 per week), and during expanding conditions, high levels of *LGR5*, *SOX9* and *AXIN2* were expressed. Successful differentiation into hepatocyte like cells was achieved at passage 5 and 10, indicated by an upregulation of the hepatocyte markers *HNF4α*, *CYP3A4* and *MRP2*. The deliverable 1.1 has yielded six organoid lines that can be successfully differentiated into hepatocyte-like cells needed for the generation of spheroids.

Table of Contents

1. Introduction

This deliverable, 1.1 “six established and fully characterized individual hepatic organoid lines”, part of work package 1 (WP1), reports the establishment of six hepatic cell lines. The overall aim of WP1, Stem cell source and requirements, is to create standardized liver spheroids with hepatic function and tissue architecture. In order to achieve this, we have isolated six individual adult stem cell cultures from liver tissue (male and female) and differentiated them towards the hepatocyte-lineage. In task 1.3, hepatocytes that are derived from these organoid lines will be combined with supporting cells including bone-marrow derived Multipotent Stromal Cells (BM-MSCs) and endothelial cells, in order to create standardized spheroids using the Sphericalplates 5D from KUG, and liver function and tissue architecture of the multicellular spheroids will be assessed. Task 1.2, scale-up the culture of adult stem cells from the liver cultured as organoids in spinner flasks, has already been completed and described in the deliverable 1.2.

2. Results and Discussion

To establish six lines of liver organoids, we received fresh liver tissue from 3 male and 3 female donors. Bile ducts were isolated from all donors, and organoid cultures were established according to previously published protocols.[1] Organoids were maintained in expansion medium (EM) for at least 10 passages, with weekly passaging at a 1:4 ratio. EM samples were analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunofluorescence (IF) at P3 (early passage) and P9 (late passage). Additionally, at P5 (early passage) and P10 (late passage), all donors were differentiated towards hepatocyte-like cells for 9 days in differentiation medium (DM) and subsequently analysed by qRT-PCR and IF.

qRT-PCR analysis confirmed a high expression of stem cell markers *LGR5*, *SOX9* and *AXIN2* in organoids cultured in EM, which was 5-40 fold higher compared to freshly isolated liver tissue. Levels of expression were comparable at early and late organoid passages in EM, and were downregulated in differentiation conditions (DM) (Fig. 1a). Conversely, early and late hepatocyte markers *HNF4α*, *CYP3A4* and *MRP2* were upregulated in DM conditions compared to EM conditions and reached levels of 5-100% compared to fresh liver tissue (Fig. 1b).

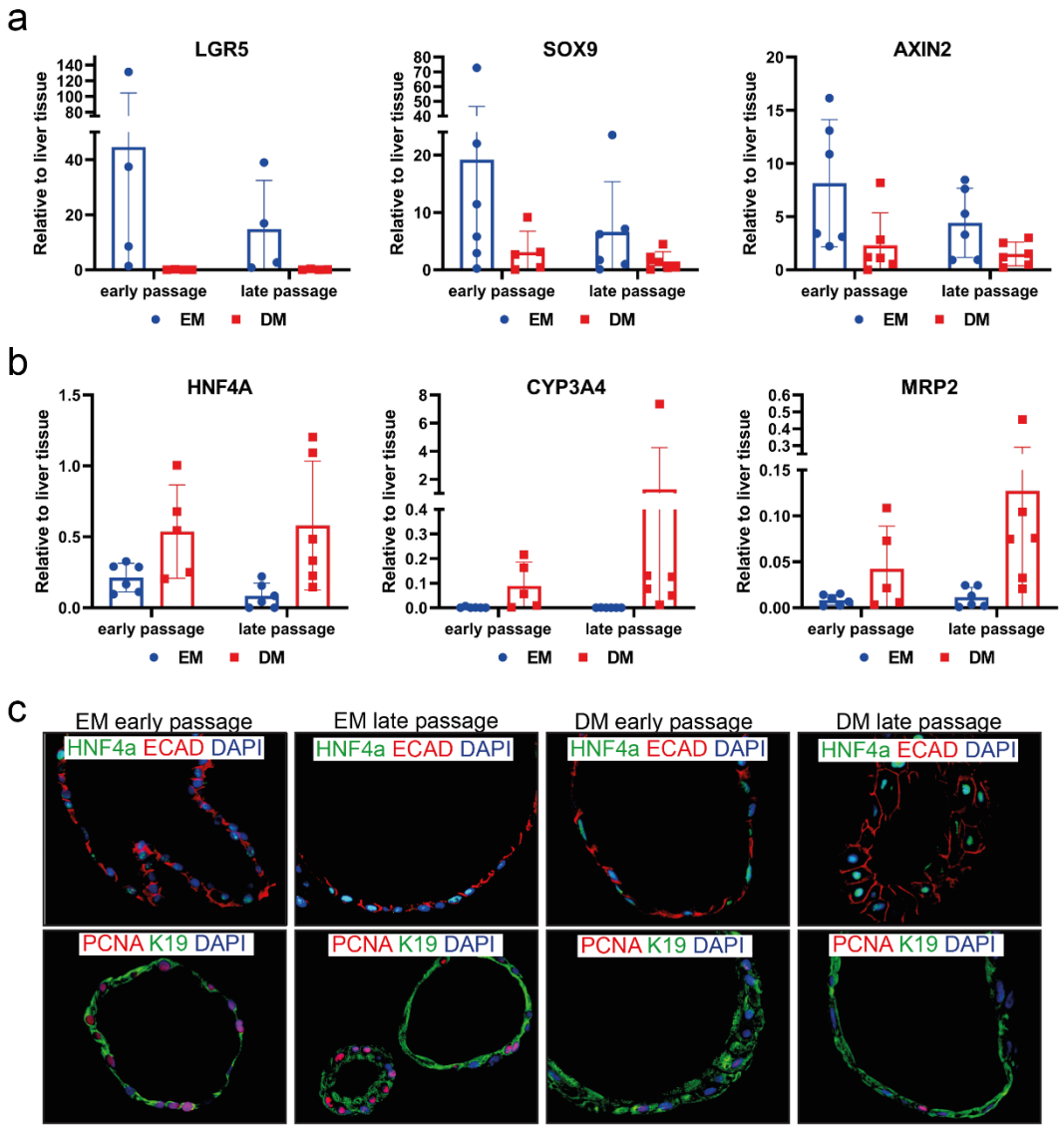

FIG 1. Characterization of human liver organoids in expansion medium (EM) and differentiation medium (DM) at early and late passages. Liver organoids from six donors were maintained in EM for ten weeks with weekly passaging. (A) Expression levels of stem cell markers in organoids from six donors in EM and DM. Stem cell markers *LGR5*, *SOX9* and *AXIN2* are highly expressed in EM conditions, and expression levels are comparable at early and late passages. In DM conditions, stem cell markers are downregulated. (B) Expression levels of differentiation markers in organoids from six donors in EM and DM. Early (*HNF4α*) and late (*CYP3A4, MRP2*) hepatocyte markers are highly upregulated in DM conditions compared to EM conditions at both, early and late passage. (C) Immunofluorescent analysis confirms an epithelial (ECAD) liver (K19) phenotype in all samples. The early hepatocyte marker HNF4a is highly expressed in DM organoids and shows only low expression in EM organoids (top panel). PCNA staining confirms that almost all cells are proliferative in EM conditions, whereas only very few cells proliferate after differentiation (DM conditions).

Immunofluorescent stainings confirmed those observations on a protein level. E-cadherin (ECAD) stainings confirmed that organoids in both conditions (EM and DM) at early and late passages displayed an epithelial phenotype. The early hepatocyte marker HNF4α was lowly expressed in some EM organoid cells, whereas high expression was observed in DM conditions. Conversely, the proliferation marker PCNA was highly expressed in EM conditions, in accordance with our observed high organoid proliferation. In DM conditions, when organoids adapt a hepatocyte-like phenotype, only very few cells showed a positive PCNA staining. Keratin 19 (K19) stainings confirmed a liver cell phenotype in all conditions (Fig. 1c).

In this report, we established and characterised six lines of human LGR5-positive adult stem cell–derived liver organoids. These cells were maintained in culture for at least 10 passages and retained a stable stem cell phenotype at early and late passages. Upon differentiation towards the hepatocyte lineage, stem cell markers were downregulated and hepatocyte markers were upregulated at comparable levels at early and late passages.

Reference:

1. Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MMA, et al. Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver. Cell. 2015 Jan 15;160(1):299–312.

3. Conclusions

We fully characterized six organoid lines in expansion and differentiation conditions. All organoid lines could successfully be differentiated into hepatocyte-like cells. The organoids are now ready to be used in co-culture experiments, where we will establish standardized multicellular spheroids (task 1.3).

4. Degree of Progress

This deliverable is part of Task 1.1: Products and regulatory requirements and specifications, and more specifically the part 'Isolation and differentiation capacity'. This deliverable D1.1 "Six established and fully characterized individual hepatic organoid lines" has been 100% fulfilled.

5. Dissemination Level

The deliverable is provided as a report, parts of the characterisation of the organoid lines are planned to be published in a research journal in due course. Therefore, the deliverable is considered as public.