A NATURE-INSPIRED PROTOCOL TO GENERATE MATURE hiPSC-DERIVED HEPATOCYTES: UNVEILING THE ROLE OF HUMAN INTESTINAL MICROBIOME

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The production of hepatocytes derived from human induced pluripotent stem cells (hiPSC-HLC) holds great promise for multiple cell therapies and tissue engineering applications. Nonetheless, the current protocols to generate HLC *in vitro* are not yet successfully established resulting in low yields of mainly immature cells when compared to the adult counterparts. The major hurdle in recapitulating *in vitro* the physiological liver maturation process is due to its complexity as it takes approximately 2 years after birth and involves a wide range of biological events (1). Recent findings have been suggesting that liver maturation, that naturally occur during the early postnatal period, can be strongly associated with human intestinal microbiome (2). For example, lithocholic acid and vitamin K2, two intestinal postbiotics, were shown to induce the expression of CYP450 enzymes in HLC and fetal hepatocytes (3). Additionally, studies on germ-free animals reported dissimilar xenobiotic enzyme profiles (4) and an impaired liver regeneration (5) compared to wild type animals. Considering these evidences, we developed a nature-inspired bioprocess to produce relevant numbers of highly functional and mature HLC for application in regenerative medicine.

In this study, hiPSC-HLC were generated as 3D cell aggregates in stirred-tank bioreactors according to the integrated bioprocess developed previously by our group (6), and matured with a novel strategy based on human intestinal microbiota's secretome. The maturation profile of hiPSC-HLC was evaluated at transcriptional and functional levels, and the composition of microbial secretome formulation was also characterized by UPLC-MS/MS, GC-MS and LC–MS/MS technologies.

Our results showed an efficient hiPSC differentiation into hepatic lineage with a production of $2.8x10^6$ HLC/mL (~370 million cells in a 200mL ST-BR), displaying a mixture of adult (~80%ALB^+ cells) and fetal traits (~30%AFP^+ cells and *CYP3A7*$^+$ *cells*). Noteworthy, HLC treated with bacterial secretome showed higher ALB expression (87%ALB^+ cells), ALB and A1AT secretion, urea synthesis, and basal and inducible CYP3A4 metabolism, when compared to untreated HLC that were cultured in standard hepatocyte maintenance medium. Detailed analytical characterization of the microbial secretome revealed some of the potential biologically active molecules, such as bile acids, short-chain fatty acids and vitamins that could be responsible for HLC *in vitro* maturation.

In conclusion, the protocol developed herein presents high technological relevance due to its efficiency, scalability, and reproducibility, but also unveils the potential role of human intestinal microbiome in hepatic cell maturation. Noteworthy, we also demonstrated that the 3D aggregates of mature hiPSC-HLC were able to adhere and migrate in human hepatic extracellular matrix scaffolds, while maintained their viability and functional features, showing to hold great promise to be used as cell therapy products or as cell ingredients for liver bioengineering applications.

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