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Generation of 3d organoids of human fetal biliary tree stem cells (hbtscs) as innovative tool for the regenerative medicine of liver and pancreas

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3D organoids represent an advanced culture technology in the field of stem cells and regenerative medicine, recapitulating embryonic organ development. Adult or fetal biliary tree represent ideal cell sources of stem/progenitor cells to be used for the regenerative medicine of liver and pancreas. The aim of our study was to generate 3D organoid cultures of hBTSCs and differentiate them toward hepatocyte cells which are suitable for cell therapy and regenerative medicine of liver. The fetal biliary tree (N=3, obtained from elective pregnancy termination) was digested, mechanically and enzymatically, to isolate *EpCAM/LGR5*-enriched hBTSCs, we also used the fragments of undigested bile duct to cultivate the organoids. Cells and bile duct fragments were then embedded in Matrigel and cultured in an expansion organoid medium containing soluble factors typical of the stem cell niche (e.g. EGF, FGF, Noggin, R-Spondin1) that represent LGR5 ligands and Wnt agonists and favor the expansion of stem cells and maintenance of stemness. Culture medium was also supplemented with Forskolin, a cAMP activator and with a TGFβR inhibitor to induce cell proliferation and arrest of differentiation. After 7 days the medium was changed to differentiation medium for a period of 10 days. We analyzed colony formation efficiency, organoid size and morphology, cell proliferation and gene expression by RT-qPCR. An average of 85 ± 7 million (N=3) *EpCAM/LGR5* enriched fetal hBTSCs were obtained. The cells isolated from fetal biliary tree showed a high tendency to generate organoids with high colony formation efficiency (> 60%). After 5 days in culture, the organoids were microscopically detected as spherical structures and after 7 days, they reached a macroscopically visible size. Cell proliferation and population doubling in organoids was significantly higher compared to 2D conditions ($p < 0.05$). Fetal biliary tree organoids were composed of single layered cuboidal epithelium and inner cell masses. RT-qPCR analysis demonstrated that organoids in expansion condition expressed multipotency stem cell markers (SOX2, NANOG, OCT4), endodermal stem/progenitor cell markers (LGR5, EpCAM, PDX1, SOX17), hepatic progenitors and ductal markers (CK19, CK7) and stem/progenitor surface genes (NCAM, CD133, CD44), recapitulating major processes of self-organization during embryonic development, whereas the differentiated organoids expressed high level of mature hepatocyte marker like CYP3A and ALB. Interestingly, LGR5 Expression reduced notably in organoids in differentiation condition compared to expansion condition ($p < 0.01$). Moreover, differentiated organoids acquired a hepatocyte morphology, including polygonal cell shape and secreted significant high level of albumin into medium respect to the same cells in 2D culture. We have demonstrated that organoids expand clonogenically stable *in vitro* for at least two months, maintaining a stable phenotype of multipotent stem cells and they can differentiate toward mature functional hepatocyte. This system has potential applications in regenerative medicine of liver and pancreas and in disease modelling.

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