获得诱导性肝细胞样细胞的策略研究

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摘要 原位肝移植是目前治疗终末期肝衰竭等疾病最有效的办法, 但供体来源少、手术费用昂贵等问题使得每年能够接受肝移植的病人非常少。肝细胞移植弥补了整个肝脏移植的不足, 成为治疗肝病的最佳方案, 但实验证明不论是在二维还是三维培养体系下原代肝细胞均无法在体外大量扩增, 这极大地限制了其在临床上的广泛应用。多能干细胞以及肝干细胞可以在体外大量扩增且具有肝向分化潜能, 因此近年来研究者致力于研究如何获得大量的具备成熟肝细胞功能的肝细胞样细胞。该文概述了目前获得诱导性肝细胞样细胞的策略及其潜在临床应用价值, 以期为今后临床上终末期肝病肝细胞治疗的应用提供有效思路。

关键词 终末期肝病; 诱导性肝细胞样细胞; 细胞移植; 干细胞分化

The Strategy for Acquiring Induced Hepatocyte-Like Cells

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Abstract Orthotopic liver transplantation is the most effective method for the treatment of end-stage liver diseases including liver failure, but only a few patients achieve liver transplantation because of the limited organ donor and expensive surgery. Hepatocyte transplantation has been considered as the best alternative to orthotopic liver transplantation in the treatment of liver diseases. However, evidences have reported that primary hepatocytes can not be massively expanded *in vitro* under either 2D or 3D culture system, greatly limiting its clinical application. Pluripotent stem cells or liver stem cells have the advantage of *in vitro* expansion for several passages and the potential capacity of differentiation. In recent years, researchers focused on how to acquire a large number of mature hepatocyte-like cells. This review summarized the current strategies and potential clinical value for acquiring induced hepatocyte-like cells, for developing clinical approaches of cell therapy in end-stage liver diseases.

Keywords end-stage liver disease; induced hepatocyte-like cells; cell transplantation; stem cell differentiation

原位肝移植是治疗终末期肝脏疾病最为有效的方式, 但由于供体肝脏数量不足, 每年仅有2 000~3 000人可接受肝移植。原代肝细胞作为肝移植的替代方式, 移植成本低, 手术较简单, 且获得的肝细胞再殖效率是所有细胞中最高的[1]。近20年来原代肝细胞移植已经逐步实现从实验室到临床的转变, 但成熟肝细胞在体外无法大量扩增, 移植后易发生免疫排斥反应, 从供体直接获得的肝细胞质量不佳等问题极大地限制了其

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在临床上的广泛应用。

干细胞因其具有体外增殖和分化能力在细胞治疗研究领域展现了强大的优势。有研究显示肝干细胞具有治疗肝损伤疾病的潜能[2], 但干细胞不表达成熟肝细胞相关的功能基因, 且与成熟肝细胞相比其在动物肝脏内的再殖效率低下。因此, 如何提高干细胞的诱导效率以获得成熟肝细胞是目前发展终末期肝病细胞治疗技术的关键问题。近十几年来研究者已在二维和三维培养体系下建立了许多切实可行的获得诱导性成熟肝细胞样细胞的研究方案。

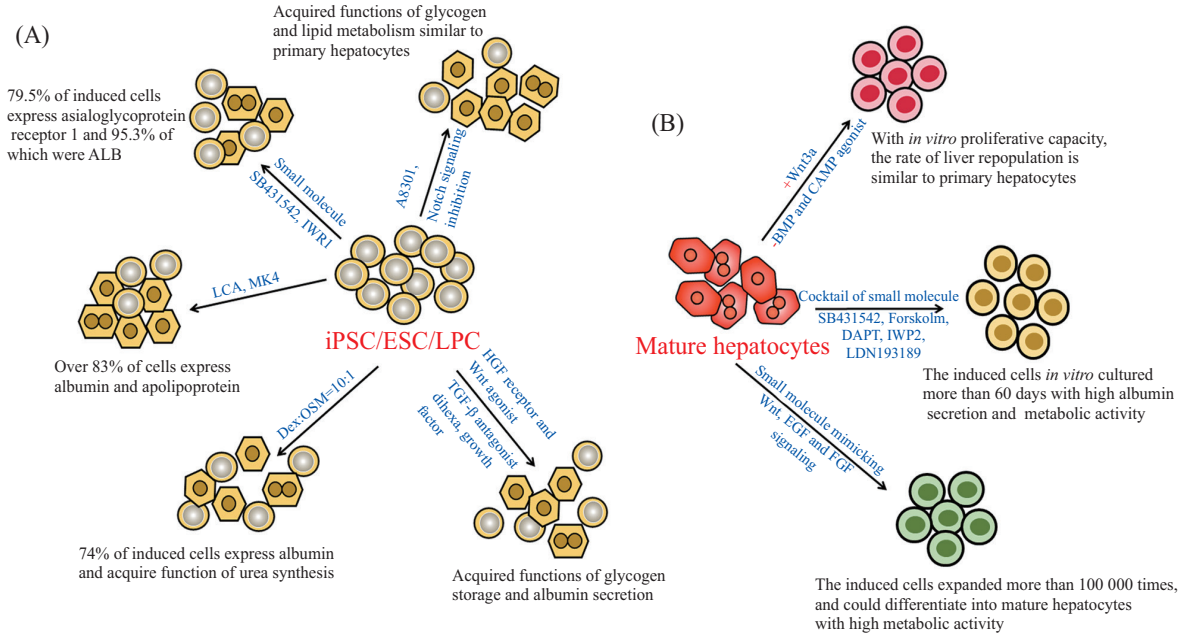
1 二维培养体系下干细胞向肝细胞分化的研究策略

1.1 基因水平调控

研究显示, 过表达或者抑制特异性基因的表达水平都可以调控干细胞的分化过程, 比如过表达*HNF4A*基因可促进胚胎干细胞向肝细胞分化[3]。特异性敲除*TLL1*基因, 可降低TGF-β及其靶基因的表达水平, 从而促进肝细胞分化[4]。过表达*FOXA2/HNF1A*基因可促进人诱导多能干细胞(human induced pluripotent stem cell, hiPSC)向肝细胞样细胞分化, 所得肝细胞样细胞中α-1抗胰蛋白酶、细胞色素P450相关蛋白表达水平与原代肝细胞相同。所获得诱导性肝细胞样细胞中的白蛋白阳性细胞比例高达80%[5]。miR-122和miR-375可促进诱导多能干细胞在培养基未添加生长因子的情况下向成熟肝细胞分化[6]。miR-199a-5p抑制剂处理也会促进胚胎干细胞向肝细胞样细胞分化。分化产生的肝细胞样细胞移植到*FAH*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$小鼠后, 其血清中白蛋白含量提高了4倍[7]。在OCT4、SOX2、KLF4和cMYC 4个转录因子的作用下, 人包皮成纤维细胞(human foreskin fibroblasts, HFFs)可被直接重编程为肝细胞样细胞, 表达成熟肝细胞特异性标志物如白蛋白、TAT和TTR等[8]。不仅如此, 最新研究显示, FOXA3、HNF1A和HNF6 3个转录因子即可将人脐带静脉内皮细胞直接重编程为人肝脏前体细胞(human hepatic progenitor cells, hHepPCs), hHepPCs不仅可以长期培养, 而且在体外可分化为更成熟的肝细胞和胆管细胞, 实现损伤肝脏再生修复[9]。

1.2 小分子化合物诱导调控

相较于基因水平上进行修饰可能会诱使细胞发生突变, 小分子诱导获得功能性肝细胞效率较高且可有效规避基因水平调控的问题(图1)。研



A: 从干/前体细胞诱导产生肝细胞样细胞。B: 小分子化合物将成熟肝细胞诱导成具有增殖能力的肝细胞。iPSC: 诱导多能干细胞; ESC: 胚胎干细胞; LPC: 前体肝细胞。

A: hepatocyte-like cells are induced from stem/precursor cells. B: mature hepatocytes can be induced to proliferative hepatocytes with small molecules. iPSC: induced pluripotent stem cell; ESC: embryonic stem cell; LPC: liver precursor cell.

图1 小分子诱导产生肝细胞样细胞

Fig.1 Hepatocyte-like cells induced by small molecules

究报道小分子化合物HGF受体抑制剂、Wnt激动剂、GSK-3β抑制剂、TGF-β抑制剂、抑瘤素M(oncostatin M, OSM)以及一些生长因子如EGF和HGF等是促进人多能干细胞向肝细胞分化的关键, 获得的诱导性肝细胞可以表达肝细胞特异质标志物, 具有白蛋白分泌能力和糖原合成能力[10-12]。OSM、Dex等的联合处理可显著促进人诱导多能干细胞和人胚胎干细胞分化, 所得细胞具有糖原储存、白蛋白分泌和尿素合成能力以及CYP450代谢活性[13]。维生素K2和石胆酸可提高诱导性肝细胞样细胞与成熟肝细胞的相似度, 诱导性细胞中有超过83%的细胞可以分泌白蛋白和脂蛋白, 且其中白蛋白和载脂蛋白B100的表达水平与原代人肝细胞的一致[14]。而将小分子化合物GSK-3β抑制剂、DLPC(dilauroyl phosphatidylcholine)、丁酸钠(NaB)和转录因子结合可以使人成纤维细胞直接重编程为诱导多潜能细胞(induced multipotent progenitor cell, iMPC), 并能够快速使iMPC分化为与成熟肝细胞功能十分相似的细胞(iMPC-Heps)[15]。另外, 小分子SB431542、化合物E可通过抑制TGF和Notch通路来促进胚胎干细胞的肝向分化, 并促进肝细胞样细胞白蛋白的表达[15]。而SB431542和端锚聚合酶抑制剂IWR1共同作用也可促进诱导多能干细胞向肝细胞分化, 诱导所得的细胞中肝细胞特异性基因的表达水平仅为原代肝细胞的50%, 但其中ALB阳性细胞可高达95.3%[16]。此外, Wnt、EGF和FGF信号通路相关小分子化合物共同作用可将成熟肝细胞去分化为具有增殖能力的肝前体细胞, 其体外增殖速率增加了近1 000倍, 而且保留了原肝细胞中较强的药物代谢能力和HBV病毒感染能力[17]。而在成熟肝细胞培养体系中添加Wnt3a并去除BMP和CAMP信号激动剂也可促进成熟肝细胞向具有更强增殖能力的前体细胞去分化, 所产生的多潜能样肝细胞移植后仍可再殖并损伤肝脏, 具有与原代肝细胞相似的再殖效率[18]。小分子KOSR、制瘤素等可促进人胚胎干细胞分化为肝细胞样细胞, 表达CYP3A4等细胞色素相关蛋白并具有白蛋白分泌功能[19]。SB431542、Forskolin、DAPT、IWP2和LDN193189这5个小分子可以使成熟肝细胞处于长期稳定增殖状态, 且该细胞体外培养达60多天仍能保留白蛋白分泌能力和CYP等代谢功能[20]。

1.3 细胞共培养的诱导分化调控作用

研究显示胚胎干细胞与肝非实质细胞系TWNT-1等共培养, 诱导产生的细胞表达肝脏特异性基因如*ASGPR*、*ALB*、*AFP*、*SOX17*、*FOXA2*, 并且可以合成尿素和代谢氨等[21]。人诱导多能干细胞和人脐静脉内皮细胞共培养可促进hiPSCs向肝细胞分化, 诱导分化的细胞从第7天开始其ALB和AFP蛋白表达量明显增加, 且细胞色素CYP相关酶活性明显增强[22]。

1.4 细胞外基质的调控作用

针对传统的利用多能干细胞实现的二维分化无法模拟细胞与胞外基质间的相互作用, 从而导致肝细胞的生理特性和功能缺陷的问题, 很多研究团队开发了能模拟细胞周围环境中的物质如纳米纤维和水凝胶等[23]。如半乳糖凝集素-3(galectin-3)可通过激活Wnt通路促进大鼠骨髓间充质干细胞向成熟肝细胞分化, 所得肝细胞特异性基因的表达水平与成熟肝细胞高度一致[24]。水凝胶可一次性持续释放多种生长因子, 能够促进脂肪干细胞分化为肝细胞样细胞, 所得细胞中肝特异性基因如*HNF4A*、*AAT*、*CYP3A4*表达量增加, 且糖原储存能力和白蛋白分泌能力显著提高[25]。此外, 人重组层黏连蛋白111、层黏连蛋白511和IV型胶原蛋白也可促进诱导多能干细胞向肝细胞样细胞分化[26], 所得细胞具有储存糖原和尿素合成能力, 并具有细胞色素P450相关蛋白介导的药物代谢能力。

1.5 微流体生物芯片技术

早在2011年, DAUJAT-CHAVANIEU等[27]就利用一种多室模块化生物反应器以促进人肝细胞分化。结果表明所得肝细胞样细胞中的*CYP1A1*、*CYP1A2*、*CYP2B6*等多种肝特异性基因表达水平均有特异性上调, 表达水平接近或高于成熟肝细胞。2017年, 有课题组开发了一种生物芯片培养技术, 此技术主要利用透明、疏水和生物相容的材料如聚二甲基硅氧烷预处理生物芯片表面, 并在此芯片表面接种并培养细胞[28]。通过微流控系统模拟体内细胞环境, 能够促进人诱导多能干细胞向肝细胞样细胞分化, 而分化而来的细胞高表达stabilin-1和白蛋白[29]。更有意思的是, 将多能干细胞诱导产生的肝窦状内皮细胞和肝细胞样细胞在微流控系统中的生物芯片上共培养, 能够促进细胞分泌白蛋白并表达细胞色素CYP450蛋白, 使得白蛋白表达水平相比于未共培养组提升了约68%[30]。

2 三维培养体系下干细胞向肝细胞分化的研究策略

2.1 三维空间结构的诱导调控

NAGATA等[31]建立了一种以微流体纤维封装技术为基础的“细胞纤维技术”三维培养系统，以提高细胞与细胞外基质的相互作用，这种方式促进了肝细胞特异性特异性基因*ALB*、*CYP3A4*、*HNF4A*、*G6P*的表达。HUI团队[18,32]开发了一种三维大规模悬浮培养体系，通过低氧条件及激活cAMP相关通路诱导肝细胞样细胞的大量产生，且使得分化的肝细胞样细胞白蛋白分泌水平比原代人肝细胞高10倍。所得肝细胞样细胞的胆汁酸和尿素分泌以及氨代谢水平与人原代肝细胞水平一致。DENG团队[33]将二维培养条件下产生的肝前体细胞转移至低吸附培养皿后，利用悬浮培养方式产生成熟的肝细胞，并诱导细胞进行受体肝脏体内移植以救治急性肝衰竭猪模型。将诱导产生的肝细胞样细胞包裹在海藻酸盐聚l-赖氨酸珠中移植入小鼠体内，可促进人白蛋白的分泌，并提高细胞色素CYP2C9和CYP3A4的蛋白水平[34]。同样，在生物人工肝体系中用海藻酸盐珠包裹培养的细胞也可以促进细胞稳定保持白蛋白合成能力[35-36]。在人多能干细胞来源的定向内胚层细胞诱导为肝细胞的过程中，将细胞转移到铺有胶原的转移小室内，可明显促进肝细胞极性结构的产生，并提高肝细胞分泌白蛋白和尿素的能力[37]。

2.2 生物打印

生物打印技术是使用计算机控制的打印技术，以细胞或细胞团为原料逐层或逐点地构建组织和器官。与二维培养系统相比，诱导性肝细胞样细胞可在海藻酸盐水凝胶或明胶中进行生物打印，此方法可以改善白蛋白分泌和尿素生产并能促进细胞色素相关蛋白如CYPP450的高表达，尤其在促进细胞色素相关蛋白表达方面，不同于一般培养方式下相关酶只在培养最初几天表达，生物打印技术组的酶表达水平是随天数递增的，并且是在与正常肝细胞表达量相近的情况下。生物打印方法也应用于构建球状体组织[38]，或同时利用不同类型的细胞同时打印，如来自人诱导多能干细胞的肝球状体与非实质细胞[39]的生物打印。

2.3 类器官

类器官是指在三维培养条件下产生的具有器官特异性功能的细胞群(图2)。CLEVERS团队[40]最早用*Lgr5*$^{+}$的干细胞建立了体外稳定增殖且具有分化潜能的肝脏类器官，所建立的类器官表达多个肝细胞蛋白标记物和胆管标记物，其中*Wnt6*、*Lgr5*的表达水平是原来的两倍多。将类器官移植到*FAH*$^{-/-}$小鼠后其再殖肝脏的效率为30%且能够100%的促进肝脏功能恢复。在此基础上，他们又进一步将*Lgr5*$^{+}$干细胞类器官诱导分化为成熟肝细胞，将所产生的肝细胞类器官移植到千里光碱结合四氯化碳诱导的急性肝损伤的小鼠中可再生小鼠肝脏，而且在宿主血清中也可检测到白

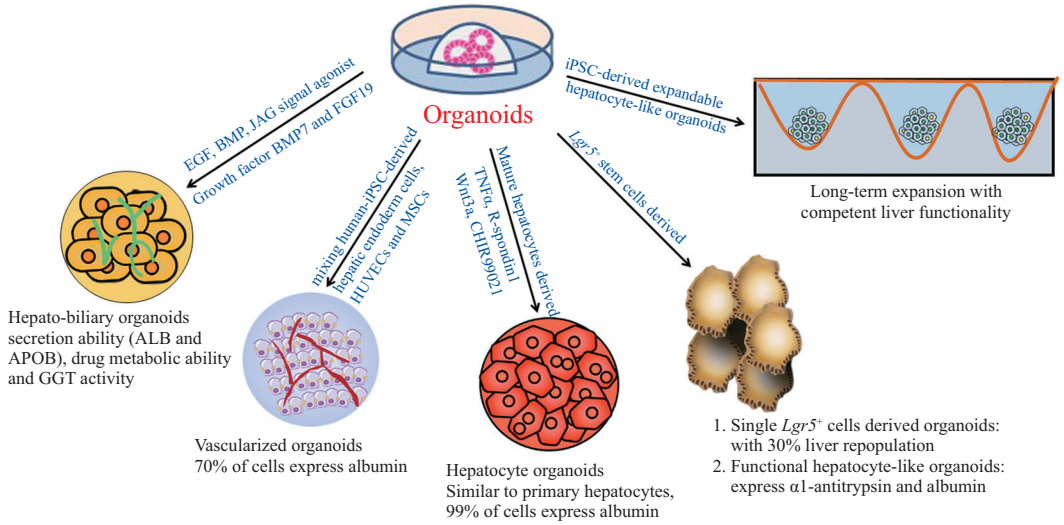


图2 类器官培养条件下诱导性肝细胞的获得

Fig.2 Hepatocyte-like cells are acquired under organoids culture system

蛋白和a1-抗胰蛋白酶的活性[41]。2018年, CLEVERS团队[42]又进一步优化培养条件, 建立了人胎肝细胞和成熟肝细胞来源的肝脏类器官, 它们在基因表达和肝细胞功能上都更接近原代肝细胞, 如约99%的细胞中都有白蛋白的表达。TAKEBE团队[43]发现, 将hiPSCs来源的内胚层细胞、人脐带血内皮细胞(human umbilicalvein endothelial cells, HUVECs)和人骨髓间充质干细胞(human mesenchymal stem cells, hMSCs)混和培养可产生血管化肝芽。单纯由hiPSCs诱导产生内胚层细胞、血管内皮细胞和间质细胞混合培养后也可产生肝芽结构, 且肝芽内肝细胞样细胞中有约70%表达白蛋白并且具有强大的药物代谢解毒能力[44]。此后该团队又建立了血管化类器官, 进一步模拟了体内微环境以促进肝细胞功能的成熟[45-46]。小肠组织去细胞后留下的胞外基质可以促进内胚层细胞直接产生肝脏类器官, 所得类器官移植到动物体内后可与基质胶来源的肝脏类器官产生类似的功能[47]。WANG团队[48]利用基质胶包被培养体系, 将人胚胎干细胞诱导产生了具有增殖和肝细胞功能特性的肝脏类器官。诱导产生的肝脏类器官表达肝细胞特异性基因且具有肝脏药物代谢能力, 并能够体外模拟酒精性肝损伤模型。此外, 利用小分子化合物激活EGF、BMP、JAG通路, 抑制TGF和Notch通路可促进人多能干细胞分化为具有肝细胞和胆管细胞特征的类器官, 在该类器官中添加脂肪酸可模拟非酒精性脂肪肝模型[49-50]。将二维条件下诱导产生的肝细胞样细胞转移至类器官培养体系后, 可获得功能与成熟肝细胞更为相似的具有药物代谢和解毒功能的细胞。类器官培养体系下的肝细胞能维持较高的增殖能力, 体外传代次数可高达24代, 能长期培养5个月之久[51]。除了肝内胆管系统可产生肝脏类器官外, 近年来研究显示肝外系统的胆管也可以产生肝脏类器官, 然而通过两种来源类器官的基因表达分析以及体内移植实验, 发现肝外来源的肝脏类器官更偏向胆管细胞特性, 能够促进胆管损伤的修复而对肝细胞功能损伤的修复作用却微乎其微[52-53]。

3 获得性肝细胞样细胞的应用潜能

为了进一步检验诱导性肝细胞样细胞的功能以为后期临床试验打下基础, 不同课题组设计了不同肝脏损伤动物模型如*FAH*$^{-/-}$小鼠、肝切小鼠、APAP急性肝衰竭小鼠模型等以更好地模拟临床终末期肝病, 通过将细胞移植入受损的肝脏检测移植细胞的再殖效率以判断获得的肝细胞样细胞是否成熟且具备成熟肝细胞相关的代谢解毒功能(表1)。*FAH*$^{-/-}$小鼠是延胡索酰乙酰乙酸水解酶(Fuarylacetoacetatehydrolase, *FAH*)基因缺失小鼠, 该模型模拟了临床上的II型酪氨酸血症。研究显示诱导性肝细胞样细胞移植入*FAH*$^{-/-}$/*Rag2*$^{-/-}$/*IL2rg*$^{-/-}$小鼠后, 不仅能够延长小鼠的生存时间, 而且能够在宿主肝脏实现再殖, 再殖效率高达60%[18]。经类器官诱导分化的肝细胞同样可以实现*FAH*$^{-/-}$小鼠肝脏的再殖[48]。小分子诱导产生的肝细胞样细胞移植入*FAH*$^{-/-}$/*Rag2*$^{-/-}$/*IL2rg*$^{-/-}$小鼠后也能缓解肝损伤, 并提高血清人白蛋白的含量。在uPA动物模型中, 肝细胞过表达uPA会导致肝脏凝血功能障碍从而引发肝损伤。研究报道经成纤维细胞诱导获得的人肝细胞样细胞移植入*Tet-uPA*/*Rag2*$^{-/-}$/*γc*$^{-/-}$小鼠后能够成功再殖小鼠肝脏并改善小鼠肝功能[54], 且移植后约50%的外源细胞表达人白蛋白。对于急性肝损伤小鼠模型, 人诱导多能干细胞来源的肝脏类器官通过肾包膜或者腹腔移植后都可以提高小鼠生存率, 降低血清AST、ALT、胆红素水平[55]。在二维培养条件下, 利用小分子或者胞外基质诱导获得的肝细胞样细胞移植后也可促进急慢性肝损伤功能的修复, 提高动物生存率。多数研究也证明了诱导性肝细胞样细胞可以用于救治急性肝衰竭的大鼠甚至猪模型。如HUI团队[18]通过激活cAMP通路联合低氧(5%)培养促进肝细胞样细胞产生, 肝细胞样细胞移植到急性肝衰竭大鼠后使得约50%的大鼠存活了至少7天以上。LI团队[56]用骨髓干细胞成功治愈了D-gal诱导肝损伤的猪, 与对照组存活了2.6天相比, 实验组动物存活了4.6天, 存活率有了显著提高。DENG团队[57]于2020年将肝球状体移植到急性肝衰竭猪模型中, 移植后的猪血清ALT、AST、氨、总胆红素(total bilirubin, TBIL)水平显著下调, 肝脏结构恢复。治疗2个月后猪上述所有指标均恢复到健康水平且未有复发。

4 展望

原代成熟肝细胞具备蛋白合成、解毒和代谢活性能力, 对急性肝衰竭或代谢性肝病患者具有重要的意义。但人原代肝细胞在体外无法大量扩增, 近年来科研人员应用了很多方法如改善细胞外基质等成功解决了此问题, 但肝细胞短缺仍然是限制肝

表1 诱导性肝细胞样细胞移植治疗肝损伤的情况

Table 1 Treatment of liver injury with transplantation of induced hepatocyte-like cells

细胞 Cells	诱导策略 Induced strategy	动物模型 Animal model	移植方法 Transplantation methods	结果 Results	参考文献 References
hiPSC induced hepatocyte-like cells	OSM, Dex, etc.	Chronic liver injury mice	Intravenous	Increased survival rate, serum total LDH and total bilirubin reduced	[12]
	Inhibition of EGF signaling	Hemophilia B mice	Kidney capsule	Bleeding time shortened; the human FIX level increased	[16]
	FOXA2/HNF1A overexpression	Acute liver injury mice	Cell sheet	Survival rate increased, and serum AST and ALT reduced	[4]
	Human recombinant laminin 111, laminin 511, or type IV collagen	(1) Acute liver injury mice (2) Hepatic fibrosis model	Intrasplenic	(1) Survival rate increased up to 50% (2) Decreased liver fibrosis associated genes level; serum hALB increased to 350 ng/mL	[25]
hESC induced hepatocyte-like cells	(1) Activating HGF, EGF, VEGF signaling (2) MicroRNA-199a-5p inhibitor	*FAH*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice	(1) Subcutaneous (2) Intrasplenic	(1) Decreased liver injury gene level (2) Serum AST increased to 320 ng/mL after 3 weeks transplantation	[6,19]
Reprogrammed hepatocyte-like cells	Small molecules mimicking cAMP signaling, inhibiting TGF-β signaling	*uPA*/*Rag2*$^{-/-}$/*γc*$^{-/-}$ mice	Intrasplenic	Improved liver function, 50% hepatocyte-like cells expressed hALB	[54]
hiPSC-derived hepatic spheroids	Small molecules FSK, FB431542, CHIR99021, and EGF	Acute liver injury pigs	Portal vein	Increased survival rate, improved liver function	[57]
hiPSC-derived liver buds	Cocultivation of MSCs, HUVECs and endodermal cells	TK-NOG mice	Mesentery	Formed functional vessel, secreted human serum ALB	[42]
hiPSC-derived liver organoids	(1) Cocktail of growth factors including EGF, VEGF, bFGF (2) HGF, EGF, VEGF, KOSR, oncostatin, etc.	Acute liver injury mice	(1) Kidney capsule (2) Intraperitoneal	(1) Increased survival rate, human serum ALB was 2 000 ng/mL (2) Increased survival rate, serum AST and ALT reduced	[6,55]
(1) ESCs-derived liver organoids (2) Hepatic progenitor cells organoids	(1) DAPT, FGF19, DEX, etc. (2) Activating Wnt, CAMP signaling, inhibiting BMP signaling	*FAH*$^{-/-}$/*Rag2*$^{-/-}$/*IL2rg*$^{-/-}$ mice	(1) Intrahepatic (2) Intrasplenic	(1) (20.0±5.6)% liver repopulation (2) Up to 60% liver repopulation	[52-53]
Lgr5^{+} cells derived liver organoids	(1) Inhibiting Notch and TGF-β signaling (2) DAPT, FGF19, Dex, BMP7, etc.	(1) *FAH*$^{-/-}$ mice (2) Acute liver injury mice	Intrasplenic	(1) 30% liver repopulation (2) 2/5 of mice survived to 120 d; serum human ALB, α-1 antitrypsin increased	[39-40]

细胞移植治疗广泛应用的主要问题。近年来三维培养模式也被广泛应用于诱导性肝细胞样细胞的分化研究中, 其中类器官培养模式展示出了广阔的前景。利用肝外胆管来源类器官可以解决肝脏内成体肝干细胞获取途径难以及数量少的问题。但实验证明目前仍无法通过肝外来源类器官获得成熟肝细胞。若能综合本文所述手段, 通过研究得到一种方案, 获得体外可扩增、具备成熟肝细胞功能, 并能实现受体肝脏内再殖的诱导性成熟肝细胞, 不仅有助于理解肝脏干细胞分化成熟的分子调控机制, 而且可为临床救治肝衰竭等终末期肝病病患者提供福音。希望随着科技的不断进步, 人类在未来可以真正解决肝衰竭疾病的治疗问题。

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