激活素 A 促进人胚胎干细胞定向分化为视网膜色素上皮细胞

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摘要 视网膜色素上皮细胞(retinal pigment epithelium, RPE)的功能紊乱、退化和丢失导致的感光细胞的损伤是造成老年性黄斑变性(age-related macular degeneration, AMD)和视网膜色素变性(retinitis pigmentosa, RP)的主要原因。目前临床常规治疗只能延缓患者视力减退的进程, 无法根治该类疾病。近期的临床研究表明, 视网膜下腔移植RPE能够改善患者视力, 这为RPE细胞用于临床治疗提供了很好的依据。由于原代RPE不能再生且资源有限, 所以可以通过人胚胎干细胞(human embryonic stem cells, hESCs)分化获得RPE。为解决hESCs自发分化获得RPE的效率很低、分化时程较长的问题, 在常规分化RPE的前7天加入激活素A(Activin A), 结果发现Activin A能够促进hESCs向RPE的分化。鉴定结果表明, 分化获得的细胞表达RPE相关蛋白: OTX2, MITF和BEST1, 以及RPE特异标记物: MITF和RPE65, 而且具有正常的形态特征和吞噬功能。上述结果表明分化得到的细胞具有RPE的基本特性。该分化方法能够为后期的研究和临床治疗提供大量的细胞。

关键词 激活素 A, 人胚胎干细胞, 视网膜色素上皮细胞, 定向分化

视网膜色素上皮细胞(retinal pigment epithelium, RPE)是一种位于神经视网膜和脉络膜之间的单层细胞, 在维持视网膜及其光感受器的功能中扮演很重要的角色。其功能包括吸收杂散光, 给神经视网膜提供营养物质, 吞噬感光细胞脱落的外节和视色素的再生等^[1]。RPE移植被认为是治疗视网膜变性疾病的具有广阔前景的治疗方法之一^[2]。在视网膜变性疾病模型大鼠的视网膜下注射分化得到的RPE^[3-5], 视觉功能和光感受器都得到明显的改善, 在患者眼部注射自体的RPE, 也能使视力得到改善^[2]。

人胚胎干细胞(human embryonic stem cells, hESCs)是定向分化RPE的理想种子细胞^[6]。目前, hESCs来源的RPE已应用于老年性黄斑变性(age-related macular degeneration, AMD)和Stargardt黄斑营养不良(Stargardt macular dystrophy, SMD)临床试验, 中期结果显示患者接受RPE移植后无不良反应, 部分患者视力得到提升^[7,8]。hESCs自发分化获得RPE的效率很低^[9], 而且所需要的分化周期也较长^[10], 为了保证临床实验的需要, 提高RPE的分化效率尤为重要。

本实验在hESCs定向分化RPE体系的前7天加入

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Activin A, 通过统计黑色素灶数量证明加入Activin A之后能够促进RPE分化. 分化得到的细胞具有正常RPE的形态特征, 并且表达正常RPE的特异性标记物, 具有吞噬能力, 说明分化得到的细胞具有RPE应有的特性. 该分化方法大大提高了RPE的分化效率, 为未来临床应用时所需大量的细胞资源提供了更好的保障.

1 材料和方法

(i) 细胞培养. 本研究使用的hESCs(Q-CTS-hESCs-2)来自中国科学院动物研究所北京干细胞库. hESCs接种在Vitronectin (0.5 $\mu\text{g}/\text{cm}^2$, Gibco)基质包被的培养板上, Essential 8TM(Gibco)培养液每天换液培养, 每隔3~5 d用1 mg/mL dispase(Gibco)消化传代.

hESCs培养至8~10 d, 出现90%克隆融合时开始分化. 分化液成分为KnockoutTM-DMEM, 20% KOSR, 1% NEAA, 1% Penicillin Streptomycin, 1% L-Glutamine以及0.1% β -mercaptoethanol(Gibco). hESCs定向分化RPE的前7天加入50 ng/mL Activin A(R&D), 第8天开始只加分化液, 在第50天统计黑色素灶数量.

(ii) 流式细胞分析. Tryple(Gibco)消化hESCs定向分化得到的RPE, 悬浮在PBS中. 分别加入一抗MITF(1:200, Santa)和RPE65(1:200, Abcam)室温孵育1 h, PBS洗3次后加入二抗(1:200)室温孵育1 h, 对照的细胞只用二抗进行孵育, 洗3次之后使用流式细胞分析仪进行结果分析.

(iii) 免疫荧光染色. 4%多聚甲醛在室温固定细胞30 min, PBS洗3次后0.5% Triton X-100透膜1 h. PBS洗3次, 2% BSA室温封闭1 h. 一抗孵育: 用2% BSA稀释OTX2抗体(1:200, Millipore), MITF抗体(1:200, Santa), BEST1抗体(1:200, Norus Biologicals), 放入4℃冰箱内孵育过夜. 次日, 用PBS洗3次, 加入2% BSA稀释的二抗, 室温避光孵育细胞1 h. 最后用PBS洗3次后, 使用1 mg/mL Hoechst 33342(Thermo)室温5 min染细胞核, 激光共聚焦显微镜获取图像.

(iv) 透射电子显微镜法. 单细胞消化hESCs定向分化得到的RPE, 收集不少于 1×10^7 个细胞, 1200 r/min离心3 min后弃上清, 沿离心管壁缓慢加入2.5%戊二醛溶液固定, 在中国科学院生物物理研究所进行后续的切片和透射电子显微镜观察实验.

(v) 吞噬作用实验. 在37℃下荧光微球(Thermo)

与hESCs定向分化得到的RPE共培养16 h, PBS洗3次后4%多聚甲醛固定30 min, 0.05% Triton X-100通透10 min, ZO-1抗体(1:200, Thermo)标记细胞, Hoechst 33342进行细胞核染色. 最后使用共聚焦显微镜获得图像.

(vi) 核型分析. hESCs定向分化得到的RPE送样至北京协和医院进行核型分析检测.

2 结果与讨论

2.1 RPE的定向分化

RPE分化实验模仿在胚胎时期RPE发展的流程, 通过找到一些在眼发育过程中对形成RPE起到关键作用的因子, 猜想这些因子很可能在体外培养过程中也能促进hESCs向RPE的分化. 之前的报道认为Activin A或者其他来自TGF- β 超家族的因子能够促进视泡向RPE的分化^[11]. 所以, 本实验在hESCs定向分化RPE的过程中添加Activin A (图1(a)), 当分化到第50天的时候可以观察到明显的黑色素灶生成(图1(b)), 对形成的黑色素灶数量进行统计(图1(c))后发现添加Activin A组产生的黑色素灶明显多于对照组, 说明Activin A能促进hESCs向RPE的分化.

2.2 RPE的鉴定

RPE是神经视网膜和脉络膜血管层之间的一种单层细胞, 具有六边形的形态, 细胞质内有黑色素颗粒存在, 且能够吞噬光感受器外段以维持光感细胞的新陈代谢. 基于RPE本身的这些特点, 我们对分化获得的细胞进行了一系列鉴定. 首先, 在光学显微镜下可以观察到分化得到的细胞具有明显的六边形结构(图2(a)), 且通过流式分析来检测RPE的特异性标记物, 结果显示hESCs定向分化的RPE能够表达MITF和RPE65(图2(b)和(c)), 另外, 免疫荧光染色检测结果显示hESCs定向分化得到的RPE能表达RPE早期发育的OTX2蛋白以及RPE特有的MITF和BEST1蛋白(图2(d)~(f)), 这些蛋白是RPE特有的早期和成熟时期的蛋白. 为了确定分化得到的RPE具有RPE的超微结构, 我们通过透射电子显微镜进行观察, 结果发现hESCs定向分化的RPE具有原代RPE特征, 包括细胞表面微绒毛、细胞质内含黑色素颗粒(图2(g)). 进一步地通过吞噬荧光微球的实验检测分化得到的RPE是否具有吞噬作用, 经过荧光微球与分化得到

图1 (网络版彩色)RPE分化的流程和结果图。(a) 细胞分化流程图; (b) 分化50 d时黑色素灶形成的结果图片; (c) 黑色素灶数量统计。**, $P < 0.01$

Figure 1 (Color online) The process and results of RPE differentiation. (a) The flow chart of cell differentiation; (b) the pigments clusters can be observed on day 50; (c) number counting of pigments clusters. **, $P < 0.01$

的RPE共同孵育后, 固定, 染色, 经共聚焦显微镜观察, 结果显示RPE细胞质内有荧光微球, 说明分化所得的RPE具有吞噬作用(图2(h)). 最后经核型分析发现hESCs定向分化得到的RPE具有与hESCs相同的正常的46条染色体(图2(i)), 说明经过分化和Activin A因子处理后不影响细胞核型的稳定。

2.3 讨论

近期的临床研究表明注射RPE可以使AMD和SMD患者的视力得到改善, 这为RPE细胞疗法替代常规疗法提供了很好的依据。RPE可以通过hESCs分化得到, 但是分化效率普遍不高, 如何提高分化效率, 确保足够的细胞数量, 这些在临床治疗中至关重要。

Activin A可以调节RPE自分泌和旁分泌, 控制眼睛发育过程中的细胞增殖与分化^[12]。TGF- β 超家族因子可以促进胚胎期视泡向RPE的分化^[11], 也能够通过特定的信号通路调节RPE迁移^[13]。

本实验在分化的1~7 d加入Activin A, 结果显示该因子可显著提高hESCs向RPE分化的效率。hESCs定向分化所得的RPE表达RPE特定的标记物和蛋白, 具有RPE的形态特征和吞噬作用功能。本实验中得到的结论与Idelson等人^[14]的相符, 但是他们是在分化的最后2周加入Activin A, 而且是在加入烟酰胺(Nico-

tinamide, NIC)的基础之上促进hESCs向RPE分化, 黑色素灶比率从17.7%提高到50.7%, 本实验在加入的时间点和时程与他们不同, 只在开始分化的1周单独加入Activin A, 可以提高近3倍的分化效率, 分化得到的细胞均具有正常的形态和功能。相对于Idelson等人^[14]的RPE分化方案, 我们的因子在分化的开始阶段加入, 且添加时程大大缩短, 更加明确了Activin A的作用时间。本分化方法大大提高了RPE的分化效率, 同时也降低了因子添加的成本, 为后期进行RPE分化相关信号通路的探索提供了依据。

本实验中所使用的hESCs是国内首株经过验证的临床级别人胚胎干细胞株, 其分化得到的RPE在临床应用中有很大前景, 随着眼类疾病的逐渐增多, 对可用于治疗部分眼类疾病的RPE需求也逐渐增多, 希望后期可以通过本实验得到的分化方法获得更多可应用于临床的RPE, 早日恢复更多病人的视觉功能, 使更多的病人受益。

3 结论

本实验中得到的结论是在分化的前7天加入Activin A能够促进RPE分化。在未来实验中, 我们还将尝试其他相关的因子, 并在添加时间、作用时长等方面进行系统性研究, 为获得临床上使用的RPE提供参考。

图 2 hESCs分化得到的RPE的鉴定结果. (a) 在光学显微镜下观察到RPE的细胞形态; (b)~(c) 流式分析分化得到的RPE能够高表达MITF和RPE65; (d)~(f) 免疫荧光染色能够表达OTX2, MITF和BEST1; (g) 通过透视电子显微镜可以看出RPE细胞的结构特征; (h) 分化得到的RPE具有吞噬作用; (i) 核型分析RPE具有正常的46条染色体. 图片标尺: (a) 200 μ m, (d) 200 μ m, (e) 200 μ m, (f) 200 μ m, (g) 2 μ m, (h) 50 μ m

Figure 2 The identification results of hESC-derived RPE. (a) RPE cell morphology can be observed under the phase-contrast microscope; (b)~(c) flow cytometry analysis show that RPE highly express MITF and RPE65; (d)~(f) immunofluorescence staining show that the RPE express markers of OTX2, MITF and BEST1; (g) electron microscopic analysis of RPE show feature characteristics of RPE; (h) RPE have the function of phagocytosis; (i) G-banding analysis of RPE. Scale bars: (a) 200 μ m, (d) 200 μ m, (e) 200 μ m, (f) 200 μ m, (g) 2 μ m, (h) 50 μ m

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Activin A promotes human embryonic stem cells to directly differentiate into retinal pigment epithelium cells

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Retinal pigment epithelium (RPE) is a kind of monolayer cells situated between neural retina and choroid, and plays an important role in maintaining the functions of retina and photoreceptor. Its functions mainly include absorbing stray light, providing nutrients for neural retina and phagocytizing outer section of photoreceptor. Photoreceptor damage caused by dysfunctions, degeneration and loss of RPE is the main reason that leads to age-related macular degeneration (AMD) and retinitis pigmentosa (RP).

After subretinal transplantation of differentiated RPE into the rat model with retinal degeneration disease, visual functions and light sensors have been improved. At present, the conventional clinical treatments can only delay the process of patient's visual deterioration, and cannot cure the disease. Recent clinical studies showed that cell therapy based on subretinal transplantation RPE could promote patient's vision, which provide a good basis for the RPE replacement therapy. Because primary RPE is not renewable and its resource is limited, so human embryonic stem cells (hESCs) were used to be differentiated into RPE. However spontaneous differentiation of hESCs into RPE is low efficient, and the process is long, so improving differentiation efficiency of RPE is of large urgency.

Transforming growth factor- β (TGF- β) superfamily adjusts RPE formation during the period of embryonic development, adding TGF- β into differentiation system can promote the RPE differentiation. Activin A is a member of the TGF- β superfamily. In this study, Activin A was added in the first 7 days of conventional differentiation of RPE, and according the results of number counting of pigment cluster Activin A could promote the differentiation of hESCs into RPE. Next, identification of the hESCs derived RPE was performed. Under the optical microscope, the cells showed a primary RPE-like hexagonal morphology. Furthermore, the hESCs derived RPE expressed PRE specific markers: MITF, RPE65, OTX2 and BEST1, which were confirmed using the method of flow cytometry and immunofluorescence staining.

In order to detect the ultrastructure of RPE derived from hESCs, transmission electron microscopy was used, and the results showed that the hESCs derived RPE had the primary RPE-like features, including cell surface microvilli, cytoplasmic melanin granules. Next, the hESCs derived RPE was co-cultured with fluorescent microspheres, and then fixed, stained and observed using the confocal microscopy, the results showed that the obtained RPE had phagocytosis functions. Finally, the karyotype analysis revealed that the hESCs derived RPE had the same normal 46 chromosomes as hESCs, which indicated that Activin A treatments had not effect on karyotype stability during the PRE differentiation. The above results indicate that our obtained hESCs derived RPE have the basic features of RPE, and the differentiation method can provide a large number of cells for the future research and clinical applications.

Activin A, human embryonic stem cells, retinal pigment epithelium, directional differentiation

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