

# 胰腺导管单克隆上皮样干细胞的多分化潜能

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**摘要** 体外诱导源于成年大鼠的胰腺导管单克隆上皮样干细胞分化形成胰岛、神经、脂肪及成骨细胞, 探讨干细胞的多分化潜能。扩增培养源于成年大鼠的胰腺导管单克隆上皮样干细胞, 采用不同的诱导液体外诱导其向胰岛、神经、脂肪及成骨细胞分化, 并通过DTZ染色、糖刺激试验、免疫荧光反应、油红O染色、茜素红染色或Vonkossa染色的方法对分化细胞进行检测。结果显示, 体外诱导培养干细胞分化形成类胰岛, DTZ染色阳性, 糖刺激分泌胰岛素、C-肽; 分化形成类神经细胞, 表达神经元特异性烯醇化酶; 分化形成类脂肪细胞, 油红O染色阳性; 分化形成类成骨细胞, 其分泌物呈岛状矿化结节, 茜素红和Vonkossa染色阳性。这表明, 该源于成年大鼠的胰腺导管上皮样干细胞系具有多分化潜能。

**关键词** 胰腺导管干细胞; 分化; 胰岛; 神经细胞; 脂肪细胞; 成骨细胞; 大鼠

## Multipotent of Monoclonal Epithelial Stem Cells Derived from Pancreatic Duct

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**Abstract** Monoclonal epithelial stem cells derived from pancreatic duct of an adult rat were *in vitro* induced to differentiate into pancreatic islets, nerve cells, adipose cells and osteoblasts. And pluripotency of these stem cells was studied. Monoclonal epithelia stem cells derived from pancreatic duct of an adult rat were proliferated. With different inducement medium, epithelial stem cells were respectively induced to differentiate into pancreatic islets, nerve cells, adipose cells and osteoblasts. Characteristics of differentiated cells were respectively detected with DTZ staining, glucose stimulation experiment, immune-fluorescence reaction, oil red staining, alizarin red staining or Vonkossa staining. The results showed that in inducement culture conditions, monoclonal epithelial stem cells derived from pancreatic duct of an adult rat differentiated into functional pancreatic islet-like tissue that was positive for DTZ staining, secreted insulin and C-peptide using glucose stimulation. Monoclonal epithelial stem cells were induced to differentiate into nerve-like cells which expressed neuron specific enolase, and were induced to differentiated into adipose-like cells that displayed accumulation of lipid vacuoles as detected by oil red O. Also stem cells were induced to differentiate into osteoblast-like cells that excretion formed mineralized nodules. Alizarin red or Vonkossa staining was positive. This indicated that the monoclonal epithelial stem cells derived from pancreatic duct of an adult rat were

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pluripotent stem cells that could differentiate into cells from three germ layers.

**Keywords** pancreatic duct stem cell; differentiation; pancreatic islet; nerve cell; adipose cell; osteoblast; rat

胰腺导管干细胞是成体干细胞,具有较强的扩增能力和多分化潜能。但是,由于定向诱导胰腺导管干细胞分化形成功能性胰岛移植治疗糖尿病的巨大应用潜力,目前国际上有关该干细胞分化特性的研究主要集中在定向诱导其分化形成功能性胰岛上<sup>[1-6]</sup>,而对其分化形成其他细胞的多分化潜能的研究报道甚少。本研究体外定向诱导大鼠胰腺导管上皮样干细胞分化形成胰岛、神经细胞、脂肪细胞和成骨细胞,对其多分化潜能进行探讨,将为进一步确定胰腺导管干细胞的生物学特性积累资料,也为成年胰腺组织存在胰腺导管干细胞的理论提供有力的支持。

## 1 材料与方法

### 1.1 材料

为本实验室分离培养的1例源于成年大鼠的胰腺导管单克隆上皮样干细胞系。部分细胞保存在中国典型培养物保藏中心。

### 1.2 主要试剂

RPMI-1640、无糖-DMEM及H-DMEM购自Gibco公司;NBS购自Hyclone公司;表皮生长因子(epidermal growth factor, EGF)、骨形态发生蛋白7(bone morphogenetic protein 7, BMP7)、葡萄糖、尼克酰胺、双硫脲(dithizon, DTZ)、地塞米松(dexamethasone, DXM)、胰岛素(insulin)、3-异丁基-1-甲基黄嘌呤(3-1-methyl isobutyl-xanthin, IBMX)、油红O及茜素红购自Sigma公司;Hepes购自Solabio公司;BSA、 $\beta$ -磷酸甘油(sodium glycerophosphate,  $\beta$ -Sod Gly)及维生素C(vitamin C, VitC)购自Amerisco公司;大鼠胰岛素和C-肽(C-peptide)的ELISA试剂盒购自上海科兴商贸有限公司;兔抗大鼠神经元特异性烯醇酶(neuron specific enolase, NSE)一抗购自北京中杉试剂公司;山羊抗兔荧光二抗购自Epitomics公司;Triton X-100、多聚甲醛、硫代硫酸钠及硝酸银购自广东光华科技股份有限公司。

### 1.3 诱导干细胞分化形成胰岛 $\beta$ 细胞及鉴定

1.3.1 诱导方法 采用含有10% NBS、10 ng/mL EGF和2.2 g/L NaHCO<sub>3</sub>的RPMI-1640培养液,稀释干细胞至 $1 \times 10^5$ /mL,24孔板每孔加入0.5 mL细胞悬液,扩增培养3 d,细胞生长至单层。诱导组换加含有

10% NBS、10  $\mu$ g/mL BMP7和25 mmol/L葡萄糖配制的无糖-DMEM培养液诱导培养,阴性对照组换加含有10% NBS的无糖-DMEM继续扩增培养,隔天换液。诱导试验共进行28 d,每7 d进行DTZ染色,第28 d进行糖刺激试验。

1.3.2 DTZ染色 参照Shiroi等<sup>[7]</sup>的方法。弃诱导液,PBS缓冲液清洗细胞,加DTZ染色液,染色30 min,PBS缓冲液清洗多余染料,拍照。换加诱导液继续培养,5 h后,着色细胞红色消失,细胞仍正常生长。DTZ能与胰岛 $\beta$ 细胞富含的Zn<sup>2+</sup>特异性络合,阳性细胞呈棕红色。源于成年大鼠的胰岛作DTZ染色阳性对照。

1.3.3 糖刺激试验 弃诱导液,PBS缓冲液清洗细胞,其中,6孔中分别加入1 mL含5 mmol/L葡萄糖的无糖-DMEM刺激液(低糖刺激液),另外6孔分别加入1 mL含25 mmol/L葡萄糖的无糖-DMEM刺激液(高糖刺激液),培养2 h。分别收集每孔细胞培养液,2 000 r/min离心10 min,收集上清液。采用ELISA法测定胰岛素和C-肽分泌量。

### 1.4 诱导干细胞分化形成神经细胞及鉴定

1.4.1 诱导方法 扩增干细胞的方法同1.3.1。当干细胞生长至单层时,诱导组换加含有10% NBS、5 mg/mL大鼠脑组织提取液(本实验室制备)、4 g/L HEPES和3.7 g/L NaHCO<sub>3</sub>的H-DMEM诱导液,诱导培养8 d。荧光免疫染色。阴性对照为无糖-DMEM、10% NBS扩增培养的单层上皮样干细胞。

1.4.2 制备大鼠脑组织提取液 取健康的SD成年大鼠,腹腔注射戊巴比妥钠,麻醉致死。解剖大鼠,无菌取全脑,称重。PBS缓冲液清洗,匀浆器研磨,加入10 mL PBS缓冲液稀释,3 000 r/min离心10 min。收集上清液,再10 000 r/min离心10 min。重复2次。收集上清液,0.22  $\mu$ m滤膜过滤,制备大鼠脑组织提取液。分装,-20  $^{\circ}$ C冷冻保存。动物实验在广东省医学实验动物中心完成并经广东海洋大学伦理委员会批准。

1.4.3 免疫荧光反应 诱导培养第8 d,当观察到典型的神经样细胞时,弃诱导液,PBS缓冲液清洗,4%多聚甲醛固定细胞15 min。PBS缓冲液清洗,1% Triton X-100穿透30 min,1% BSA封闭45 min。PBS

缓冲液清洗, 加含1% BSA的PBS缓冲液稀释的兔抗大鼠NSE(1:100稀释), 4 °C过夜。PBS缓冲液清洗, 加含1% BSA的PBS缓冲液稀释的山羊抗兔荧光二抗(1:1 000稀释), 室温避光孵育1 h, PBS缓冲液清洗, 观察并拍照。

## 1.5 诱导干细胞分化形成脂肪细胞及鉴定

**1.5.1 诱导方法** 扩增干细胞的方法同1.3.1。当干细胞生长至单层, 换加含有10% NBS、1  $\mu\text{mol/L}$  DXM、1  $\mu\text{g/L}$  胰岛素、0.5  $\text{mmol/L}$  IBMX、4  $\text{g/L}$  HEPES和3.7  $\text{g/L}$   $\text{NaHCO}_3$ 的H-DMEM诱导液, 诱导培养28 d。每7 d进行油红O染色。阴性对照为无糖-DMEM、10% NBS扩增培养的单层上皮样干细胞。

**1.5.2 油红O染色** 弃去诱导培养皿中的诱导液, PBS缓冲液清洗, 10%中性甲醛固定细胞10 min, 油红O浸染10 min。60%异丙醇洗去多余染液, PBS缓冲液清洗细胞10 min, 显微镜下观察并拍照。

## 1.6 诱导干细胞分化形成成骨细胞及鉴定

**1.6.1 诱导方法** 扩增干细胞的方法同1.3.1。当干细胞生长至单层, 换加含有10% NBS、0.1  $\mu\text{mol/L}$  DXM、10  $\text{mmol/L}$   $\beta$ -Sod Gly、50  $\mu\text{g/mL}$  VitC和2.2  $\text{g/L}$   $\text{NaHCO}_3$ 的RPMI-1640诱导液, 诱导培养28 d。每7 d分别进行茜素红染色和Vonkossa染色。阴性对照为无糖-DMEM、10% NBS扩增培养的单层上皮样干细胞。

**1.6.2 茜素红染色** 弃诱导液, PBS缓冲液清洗细胞, 95%乙醇室温下固定细胞30 min。弃固定液, 0.1%茜素红染色液染色10 min, PBS缓冲液清洗, 显微镜下观察并拍照。

**1.6.3 Vonkossa染色** 弃诱导液, PBS缓冲液清洗细胞, 4%多聚甲醛室温下固定细胞15 min。弃固定液, 5%硫代硫酸钠室温下作用30 min。PBS缓冲液清洗, 加1%硝酸银, 在紫外线照射下室温反应1 h, PBS缓冲液清洗, 显微镜下观察并拍照。

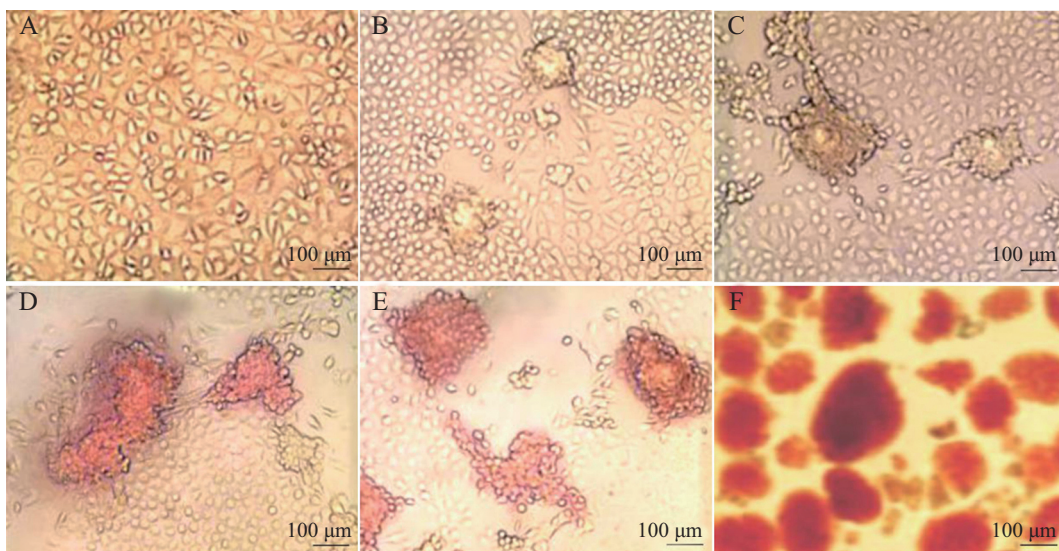
## 1.7 数据统计

胰岛素和C-肽分泌量数据先用Excel初步整理, 再采用SPSS 19.0统计软件进行单因素方差分析(One-Way ANOVA), 处理后的数据以平均值 $\pm$ 标准差表示。各平均值间的比较采用LSD进行差异显著性分析,  $P < 0.05$ 表示差异显著。

## 2 结果

### 2.1 干细胞分化形成类胰岛

经扩增培养, 干细胞贴壁生长, 呈多角形上皮样, 生长至单层时, 呈“铺路石”样(图1A)。采用该方法, 诱导培养7 d时, 一些干细胞由多角形上皮样分化形成球形细胞, 并开始聚集成团(图1B)。14 d时, 更多的干细胞分化形成球形细胞, 聚集成团(图1C)。21 d时, 一些较大的细胞团分化形成包含 $\beta$ 细胞的



A: 干细胞增殖至单层; B~E: 干细胞诱导培养7、14、21、28 d; D、E: 分化形成的类胰岛包含 $\beta$ 细胞, DTZ染色阳性; F: 大鼠胰岛DTZ染色阳性对照。  
A: stem cells proliferated a layer; B-E: stem cells were induced for 7, 14, 21 and 28 days; D,E: differentiated pancreatic islets-like containing  $\beta$  cells that DTZ staining was positive; F: DTZ staining positive control of pancreatic islets from rat.

图1 胰腺导管单克隆上皮样干细胞分化形成类胰岛

Fig.1 Monoclonal epithelia stem cells from pancreatic duct differentiated into pancreatic islets-like

类胰岛, DTZ染色阳性(图1D)。28 d时, 大部分干细胞分化形成包含 $\beta$ 细胞的类胰岛, DTZ染色阳性(图1E)。图1F是大鼠胰岛DTZ染色阳性对照。

分别采用低糖、高糖刺激液进行刺激,  $1 \times 10^5$  干细胞诱导分化形成的类胰岛其胰岛素和C-肽释放量如表1所示。与对照组相比, 差异极显著( $P < 0.01$ )。这表明, 体外诱导培养源于成年大鼠的胰腺导管单克隆上皮样干细胞分化形成包含 $\beta$ 细胞的功能性类胰岛。

## 2.2 干细胞分化形成类神经细胞

采用该方法诱导培养第4 d, 约30%的多角形上皮

样干细胞伸出突起, 形成类神经细胞(图2A); 第8 d, 约60%以上的干细胞分化形成类神经细胞(图2B); 免疫荧光反应显示, 分化形成的类神经细胞表达神经元特异性烯醇化酶(图2C)。

## 2.3 分化形成类脂肪细胞

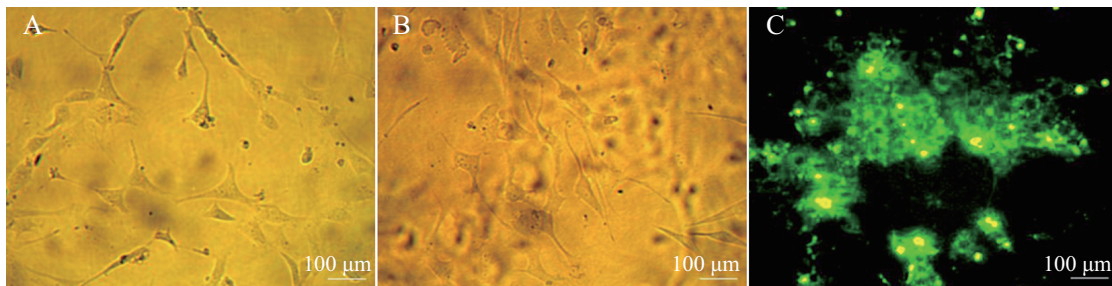
体外定向诱导大鼠胰腺导管上皮样干细胞分化形成脂肪细胞, 在诱导培养14 d时, 一些多角形上皮样干细胞分化形成圆形类脂肪细胞, 细胞内出现脂滴, 脂滴小而分散(图3A); 21 d时, 约有40%的干细胞分化形成类脂肪细胞, 细胞内脂滴增大、增多(图

表1 不同浓度葡萄糖刺激诱导胰岛胰岛素和C-肽释放量

Table 1 Insulin and C-peptide release of induced pancreatic islets using different concentration of glucose			
组别	糖刺激浓度(mmol/L)	胰岛素(mU/L)	C-肽(pg/mL)
Groups	Concentration of glucose (mmol/L)	Insulin (mU/L)	C-peptide (pg/mL)
Control group	5	3.82±0.15	0.05±0.05
	25	4.56±0.12	0.05±0.02
Inducement group	5	26.59±0.33**	0.33±0.07**
	25	37.16±0.25**	0.57±0.03**

数值用平均数±标准差表示, 样本数=6, \*\* $P < 0.01$ , 分别与对照组相比较。

Data are presented as mean±S.D.,  $n=6$ , \*\* $P < 0.01$ , compared with the control group respectively.

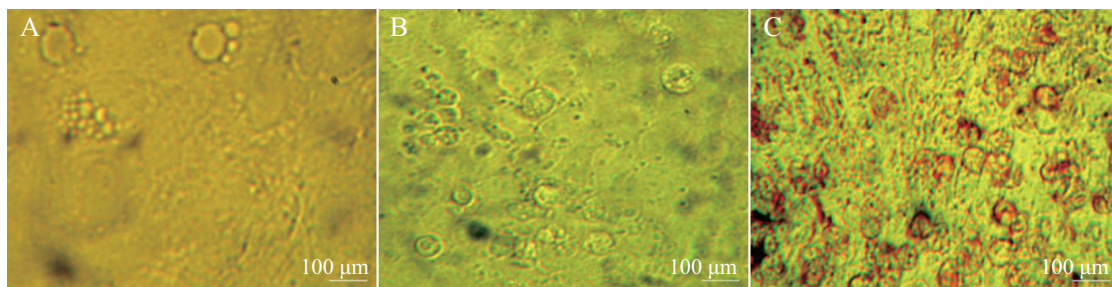


A、B: 干细胞诱导培养4、8 d; C: 分化形成的类神经细胞表达神经元特异性烯醇化酶。

A,B: stem cells were induced for 4, 8 days; C: differentiated nerve-like cells expressed neuron-specific enolase.

图2 胰腺导管单克隆上皮样干细胞分化形成类神经细胞

Fig.2 The monoclonal epithelia stem cells from pancreatic duct differentiated into nerve-like cells

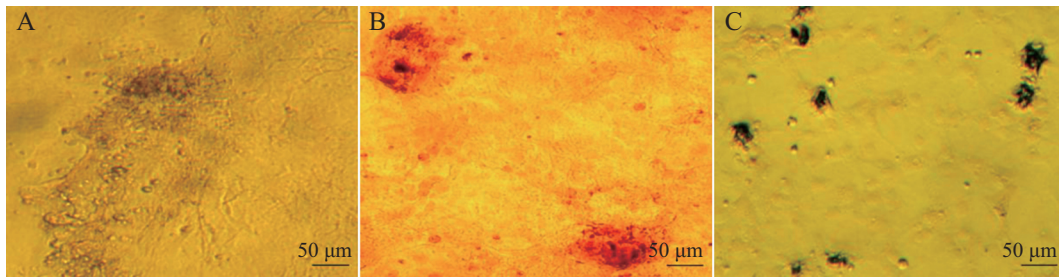


A~C: 干细胞诱导培养14、21、28 d; C: 分化形成的类脂肪细胞内脂滴明显, 油红O染色阳性。

A-C: stem cells were induced for 14, 21 and 28 days; C: differentiated adipose-like cells displayed accumulation of lipid vacuoles that detected by oil red O.

图3 胰腺导管单克隆上皮样干细胞分化形成类脂肪细胞

Fig.3 The monoclonal epithelia stem cells from pancreatic duct differentiated into adipose-like cells



A: 干细胞诱导培养21 d; B、C: 诱导培养28 d, 分化的类成骨细胞分泌物形成明显的矿化结节, 茜素红染色阳性, Vonkossa染色呈现黑色。

A: stem cells were induced for 21 days; B,C: induced for 28 days, mineralized nodules derived from differentiated osteoblasts-like that alizarin red and Vonkossa staining were positive.

图4 胰腺导管单克隆上皮样干细胞分化形成类成骨细胞

Fig.4 The monoclonal epithelia stem cells from pancreatic duct differentiated into osteoblasts-like

3B); 28 d时, 分化形成的类脂肪细胞内脂滴明显, 油红O染色阳性(图3C)。

#### 2.4 分化形成类成骨细胞

采用该方法诱导培养大鼠胰腺导管干细胞7 d时, 少数多角形上皮样干细胞开始分化形成类成骨细胞, 细胞分泌功能增强; 14 d时, 分化形成的类成骨细胞数量增加, 分泌物增多, 并出现成骨细胞分泌物特有的基质样结节; 21 d时, 分泌物形成明显的岛状矿化结节(图4A); 28 d时, 分化形成的类成骨细胞分泌更加旺盛, 分泌物形成的岛状矿化结节显著, 茜素红染色阳性(图4B), Vonkossa染色呈现黑色(图4C)。

### 3 讨论

近年来, 分离克隆胰腺导管干细胞并诱导其分化形成功能性胰岛移植治疗糖尿病的巨大应用潜力已吸引众多的研究者投入其中, 该研究进展迅速。例如, Peck<sup>[8]</sup>领导的研究小组最早报道采用低糖低血清培养液诱导培养源于糖尿病成年小鼠的胰腺导管上皮样干细胞, 可快速分化形成小而圆的胰岛祖细胞; 进一步诱导, 可分化形成类胰岛细胞团, 糖刺激分泌胰岛素。将诱导胰岛移植给糖尿病小鼠, 小鼠血糖浓度恢复至正常水平<sup>[8-10]</sup>。Jin等<sup>[11]</sup>用含层黏连蛋白的半固体培养基悬浮诱导培养源于成年小鼠的胰腺导管干细胞, 干细胞可分化形成胰岛和腺泡细胞, 糖刺激分泌胰岛素/C-肽。Rovira等<sup>[12]</sup>采用含有20 ng/mL EGF、20 ng/mL FGF、100 μmol/L β-巯基乙醇、10 ng/mL LIF、3% FBS的DMEM/F-12诱导液, 诱导培养源于成年小鼠胰腺导管末端/泡心干细胞, 干细胞可分化形成胰岛细胞, 糖刺激分泌胰岛素; 也

可分化形成腺泡细胞, 产生淀粉酶。Huch等<sup>[13]</sup>将源于小鼠的Lgr5阳性胰腺导管干细胞移植在裸鼠肾囊内, 干细胞分化形成胰腺导管细胞和胰岛细胞。Noguchi等<sup>[14]</sup>报道, 定向诱导源于成年小鼠的胰腺导管干细胞可分化形成表达胰岛素的胰岛细胞。Bonner-Weir等<sup>[15]</sup>采用含有1 g/L ITS、2 g/L BSA、10 ng/mL KGF和10 mmol/L尼克酰胺的无血清DMEM/F12诱导液, 诱导培养上皮样干细胞分化形成胰岛细胞团, DTZ染色阳性, 糖刺激分泌胰岛素。Hoesli等<sup>[16]</sup>体外定向诱导源于成人的胰腺导管上皮样干细胞分化形成胰岛。将诱导胰岛移植在NOD成年小鼠肾囊内, 糖刺激分泌人胰岛素。Lee等<sup>[3,17]</sup>构建Neurog3腺病毒载体, 转染, 诱导人胰腺导管干细胞分化形成胰岛细胞, 分泌胰岛素。效梅等<sup>[2,18-20]</sup>分离培养1例源于流产胎儿的胰腺导管干细胞系, 体外定向诱导, 干细胞分化形成分泌胰岛素和C-肽的功能性类胰岛; 分化形成神经细胞, 表达神经纤维蛋白。将诱导胰岛移植给糖尿病模型大鼠, 大鼠血糖水平降低。本研究结果显示, 分别采用不同的诱导液体外定向诱导, 该源于成年大鼠的胰腺导管单克隆上皮样干细胞分化形成功能性类胰岛细胞团, DTZ染色阳性, 糖刺激分泌胰岛素和C-肽; 分化形成具有突触的类神经细胞, 荧光免疫化学反应表达神经元特异性烯醇化酶; 分化形成具有脂滴的类脂肪细胞, 油红O染色阳性; 分化形成类成骨细胞, 其分泌物形成典型的岛状矿化结节, 茜素红染色阳性, Vonkossa染色呈现黑色。与其他研究相比, 本研究较全面地评价了该源于成年大鼠的胰腺导管上皮样单克隆干细胞系的多分化潜能。神经细胞来源于外胚层, 胰腺来源于内胚层, 脂肪细胞和成骨细胞均来源于中胚层, 这表明该干细胞具有

能分化形成三胚层细胞的分化潜能。该研究为成年胰腺导管组织存在干细胞提供了有力的支持。

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