

基础研究

大鼠脂肪干细胞和骨髓间充质干细胞向内皮分化的能力比较

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摘要:目的 研究脂肪干细胞(ASCs)向内皮分化的能力,并与骨髓间充质干细胞(BMSCs)对比。方法 从SD大鼠中分离培养ASCs、BMSCs,选择生长良好的一定代数的细胞,流式细胞仪鉴定细胞表面标志物;用CCK-8法绘制细胞生长曲线;进行标准内皮诱导3周,用qPCR检测内皮细胞特异性标志物CD31、KDR、vWF mRNA的表达;免疫荧光染色观察表面抗原CD31的表达;用Dil标记的乙酰化低密度脂蛋白(Dil-Ac-LDL)检测诱导组细胞的摄取功能;在Matrigel上验证诱导组细胞的成管能力。结果 成功分离培养大鼠ASCs和BMSCs,形态上,ASCs与BMSCs类似,均呈长梭形、纺锤状,类成纤维细胞样形态;流式检测结果显示BMSCs与ASCs均高表达间充质干细胞表面标志物CD29(100%与96.9%)、CD90(96.5%与99.2%),低表达造血系细胞表面标志物CD45(3.9%与0.8%),证实所提取的是间充质干细胞;CCK-8增殖实验结果表明,ASCs的增殖速度比BMSCs的增殖速度快($P<0.05$)。标准内皮诱导后,qPCR显示诱导组BMSCs和ASCs表达CD31、KDR、vWF mRNA水平均高于未诱导组($P<0.05$);免疫荧光染色显示CD31抗原表达在诱导组的细胞膜表面;荧光显微镜下诱导组细胞摄取Dil-Ac-LDL(未诱导组不摄取);诱导组细胞在Matrigel上均具备成管能力,证实诱导后的细胞为内皮细胞。结论 表明BMSCs和ASCs均可诱导向内皮细胞分化,ASCs广泛分化为内皮细胞的时间更短且增殖能力更强,提示ASCs比BMSCs更具有应用前景。

关键词:脂肪干细胞;骨髓间充质干细胞;内皮分化;内皮细胞;糖尿病足

Comparison of endothelial differentiation capacity of adipose-derived stem cells and bone marrow mesenchymal stem cells from rats

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Abstract: Objective To compared the differentiation capacity of rat adipose-derived stem cells (ASCs) and bone marrow mesenchymal stem cells (BMSCs) into endothelial cells. **Methods** Rat BMSCs and ASCs were isolated, cultured and identified for cell surface markers using flow cytometry. The cell growth curves were drawn by CCK-8 assay, and the cells in active growth were induced for endothelial differentiation following standard protocols. On day 21 of induction, the cells were examined for mRNA expressions of endothelial cell specific markers CD31, KDR, and vWF using qPCR. Immunostaining was performed to observe the expression of CD31 on the cells. The induced cells were also tested for Dil-labeled acetylated low-density lipoprotein (ac-LDL) uptake ability. The tube-forming ability of the induced cells was verified on Matrigel. **Results** We successfully isolated rat ASCs and BMSCs. Morphologically, ASCs were similar with BMSCs, both having long spindle-shaped and fibroblast-like morphology. Flow cytometry showed that both BMSCs and ASCs had high expressions of mesenchymal markers CD29 and CD90 and a low expression of hematopoietic cell surface markers CD45. CCK-8 assay showed that ASCs proliferated more quickly than BMSCs. The cells with induced endothelial differentiation exhibited increased levels of CD31, KDR, and vWF mRNA expressions and immunofluorescent staining identified CD31 antigen expression on the cell membrane. Fluorescence microscopy revealed red fluorescence in the induced cells suggesting uptake of Dil-Ac-LDL by the cells. The induced cells were capable of forming tube on Matrigel, confirming their identity of endothelial cells. **Conclusion** Both rat BMSCs and ASCs can be induced to differentiate into endothelial cells, but ASCs differentiate more quickly into endothelial cells and possess a stronger proliferation ability, suggesting its greater potential than BMSCs in future applications.

Key words: adipose-derived stem cells; bone marrow mesenchymal stem cells; endothelial differentiation; endothelial cells; diabetic foot

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糖尿病足是糖尿病患者最严重的慢性并发症之一,糖尿病患者发生足部溃疡的终生风险高达25%^[1]。目前针对糖尿病足缺乏有效的临床治疗手段,间充质干细胞(MSCs)能促进新生血管形成、恢复缺血肢体的血流

灌注,是治疗糖尿病足的新选择^[2-3]。缺血性疾病的主要病因是内皮功能障碍,治疗性血管新生的传统理念是通过注射内皮细胞和生长因子来促进组织修复,然而,由于免疫原性和细胞来源的限制,大多数情况下内皮细胞用于血管治疗并不可行^[4]。间充质干细胞具备低免疫原性、多分化潜能特征,在组织工程的血管新生中发挥重要作用。研究最早、最广泛的是骨髓间充质干细胞(BMSCs),但抽取骨髓是高度侵入性操作,且BMSCs只占骨髓细胞的0.01%~0.001%,抽取骨髓量大,对病人造成创伤较大^[5]。而脂肪干细胞(ASCs)从脂肪组织中分离出来,具有来源广泛、容易获得的特征,ASCs占脂肪抽吸物有核细胞的2%^[6],且研究显示ASCs的增殖能力比BMSCs更强^[7]。两者均具备多向分化潜能,但对于分化为血管治疗最有价值的内皮细胞方面,尚无研究比较两者的强弱。因此,本研究拟诱导ASCs分化为内皮细胞,并与“金标准”BMSCs对比,有助于选出有利于组织工程血管新生的间充质干细胞来源,有利于血管疾病细胞疗法的发展。

1 材料和方法

1.1 材料和试剂

SPF级200~250 g SD大鼠10只,购于南方医科大学实验动物中心,动物合格证编号:SCXK(粤)2011-0015。主要试剂:胎牛血清购自德国PAN,DMEM/F12培养基购自Gibco,VEGF₁₆₅和bFGF购自PeproTech, Anti-Rat CD29 FITC、Anti-Rat CD90 PE、Anti-Rat CD45 APC购自eBioscience, PECAM-1(M-20)购自Santa Cruz, Trizol购自Invitrogen, CCK-8法细胞增殖检测试剂盒、DAPI染液购自南京凯基生物, Dil-乙酰化低密度脂蛋白购自广州奕源生物。

1.2 实验方法

1.2.1 大鼠脂肪干细胞的分离、培养 SD大鼠用3%戊巴比妥钠腹腔注射麻醉,取双侧腹股沟部脂肪组织, PBS缓冲液清洗,眼科剪剪碎,加入2倍体积的0.1% I型胶原酶混匀,于37℃振荡消化60 min。消化结束后,加入全培(10%FBS, DMEM/F12, 1%青链霉素混合液)终止消化,吹打混匀, 800 g离心5 min,沉淀用全培重悬,相继用100 μm、70 μm的细胞筛网过滤,收集滤液,细胞接种于培养皿中,置于37℃, 5% CO₂, 饱和湿度的培养箱中培养。24 h后首次换液,以后每2~3 d换液,细胞汇合达80%~90%时进行传代培养。

1.2.2 大鼠骨髓间充质干细胞的分离、培养 SD大鼠用3%戊巴比妥钠麻醉后断颈处死, 75%酒精浸泡5 min,取双侧胫骨、股骨,去除表面肌肉, PBS液反复冲洗,剪断股骨、胫骨两端,用全培反复冲洗骨髓腔至变白,收集冲洗液, 800 g离心5 min,沉淀用全培重悬,接种于培养

皿中,置于37℃, 5% CO₂, 饱和湿度的细胞培养箱中培养。24 h后首次换液,以后每2~3 d换液,细胞汇合达80%~90%时进行传代培养。

1.2.3 流式细胞仪鉴定 BMSCs 和 ASCs 细胞表面标志物 取生长旺盛的第3代BMSCs和ASCs,制成1×10⁵/mL的细胞悬液, PBS洗涤2次,加入100 μL含0.1%BSA的PBS液,每管相继加入FITC-CD29, PE-CD90, APC-CD45抗大鼠抗体,充分混匀后冰上避光孵育30 min,用含0.1%BSA的PBS液洗涤2次, 250 g离心5 min,加200 μL 0.1%BSA的PBS液重悬,混匀,流式细胞分析仪上样检测。

1.2.4 CCK-8法绘制细胞生长曲线 取第3代BMSCs和ASCs,制成5×10⁴/mL的单细胞悬液,在96孔板加入100 μL/孔,置于细胞培养箱培养24 h,于第1~7天每天统一时间点行CCK-8法检测细胞增殖,每次取3个复孔,加3个单纯完全培养基复孔,每孔加入CCK-8 10 μL,培养箱孵育3 h,酶标仪在450 nm波长处检测每孔的A值,其余孔每2 d换液1次。以时间为横轴, A值为纵轴绘制生长曲线。

1.2.5 诱导 BMSCs 和 ASCs 向内皮细胞分化 制备BMSCs和ASCs单细胞悬液,以2.5×10⁵/孔密度接种在6孔板培养,待细胞生长密度达到80%后,加入内皮细胞诱导液(含DMEM, 10% FBS, 50 μg/L VEGF₁₆₅, 10 μg/L bFGF), 3 d换液1次,诱导21 d,或延长诱导,定期于倒置相差显微镜下观察细胞的形态变化。

1.2.6 内皮细胞的鉴定

1.2.6.1 细胞形态观察 诱导期间用倒置相差显微镜观察细胞的形态变化,拍照记录。

1.2.6.2 qPCR检测内皮细胞特异性标志物 CD31、KDR、vWF mRNA的表达情况 经内皮诱导21 d后,提取细胞RNA,进行qPCR检测,每组细胞设有3个平行复孔。Primer5.0设计引物,以GAPDH作为内参基因,设计的引物见表1。

表1 荧光定量PCR引物序列

Tab.1 Primer sequence for real-time PCR

Gene	Primer sequence	Primer length (bp)
CD31	F: AGCAGCCGACTCCTGTATTG	99
	R: AATTGGATGGCTTGGCCTGA	
KDR	F: GTGGGCTATGAGCCAAGTGT	128
	R: AGGCAACATGCTCACTTCCA	
vWF	F: CAAAGCCCCTGGACAACCTCG	73
	R: TAAGCCATGTCCCTGAGCAAA	
GAPDH	F: AGACAGCCGCATCTTCTTGT	142
	R: TGATGGCAACAATGTCCACT	

1.2.6.3 免疫荧光染色及激光共聚焦显微镜观察 将诱导后的细胞均匀铺于玻底培养皿中,培养箱孵育 24 h,去全培,4%多聚甲醛固定 15 min,PBS 清洗 3 次,加入 500 μ L 3% BSA 封闭非特异性抗原,30 min 后去掉封闭液,加入 1:50 的 CD31 抗大鼠抗体,4 $^{\circ}$ C 孵育过夜。第 2 天取培养皿,吸走一抗,PBS 清洗 3 次,加入 1:50 的二抗,避光孵育 1 h。去掉二抗,PBS 液清洗 3 次,加入 500 μ L DAPI 工作液染色 15 min,弃去染色液,PBS 清洗,滴加 300 μ L 甘油,在激光共聚焦显微镜下观察、拍照。

1.2.6.4 荧光显微镜检测内皮样细胞摄取 Dil-Ac-LDL 的能力 将诱导的 BMSCs 和 ASCs 以 5×10^4 /孔的密度均匀铺于 24 孔板,至细胞铺满孔 80% 以上,更换含 Dil 标记的乙酰化低密度脂蛋白 20 mg/L 的完全培养基,5% CO₂,37 $^{\circ}$ C 培养箱孵育 4 h,去掉培养基,4%多聚甲醛固定 15 min,PBS 液清洗 3 次,加入 DAPI 工作液染色 10 min,PBS 液清洗后于倒置荧光显微镜下观察拍照,红色荧光表示摄取了 Dil-Ac-LDL 的细胞,蓝色荧光表示细胞核。

1.2.6.5 Matrigel 成管实验 取出 Matrigel 于 4 $^{\circ}$ C 过夜融

化,96 孔板、枪头盒均 4 $^{\circ}$ C 过夜预冷;次日在冰盒上进行铺胶,96 孔板每孔加入 50 μ L Matrigel,诱导组和未诱导组各 3 个复孔,于 4 $^{\circ}$ C 平衡 10 min 后,置于 37 $^{\circ}$ C 孵育 60 min;每孔加入细胞约 2×10^4 个,置于 37 $^{\circ}$ C 孵育 6 h 后,于倒置显微镜下观察拍照。

1.2.7 统计方法 采用 SPSS 21.0 统计软件分析数据,数据以均数 $\pm$ 标准差表示,进行正态性、方差齐性检验,两样本均数比较采用独立样本 *t* 检验,以 *P*<0.05 为差异有统计学意义。

2 结果

2.1 大鼠骨髓间充质干细胞的分离培养

全骨髓贴壁法培养原代细胞,接种后镜下可见大量大小不一呈圆形或类圆形的混合细胞;24 h 首次换液后可见部分细胞贴壁,第 3 天可见细胞克隆团块形成;约 10 d,细胞密度汇合达 80%。第 3 代细胞呈均一的长梭形,类成纤维细胞样形态,流式细胞仪检测间充质干细胞表面标志物 CD29、CD90 均为阳性(100%、96.5%),造血系细胞表面标志物 CD45 阴性(3.9%)(图 1)。

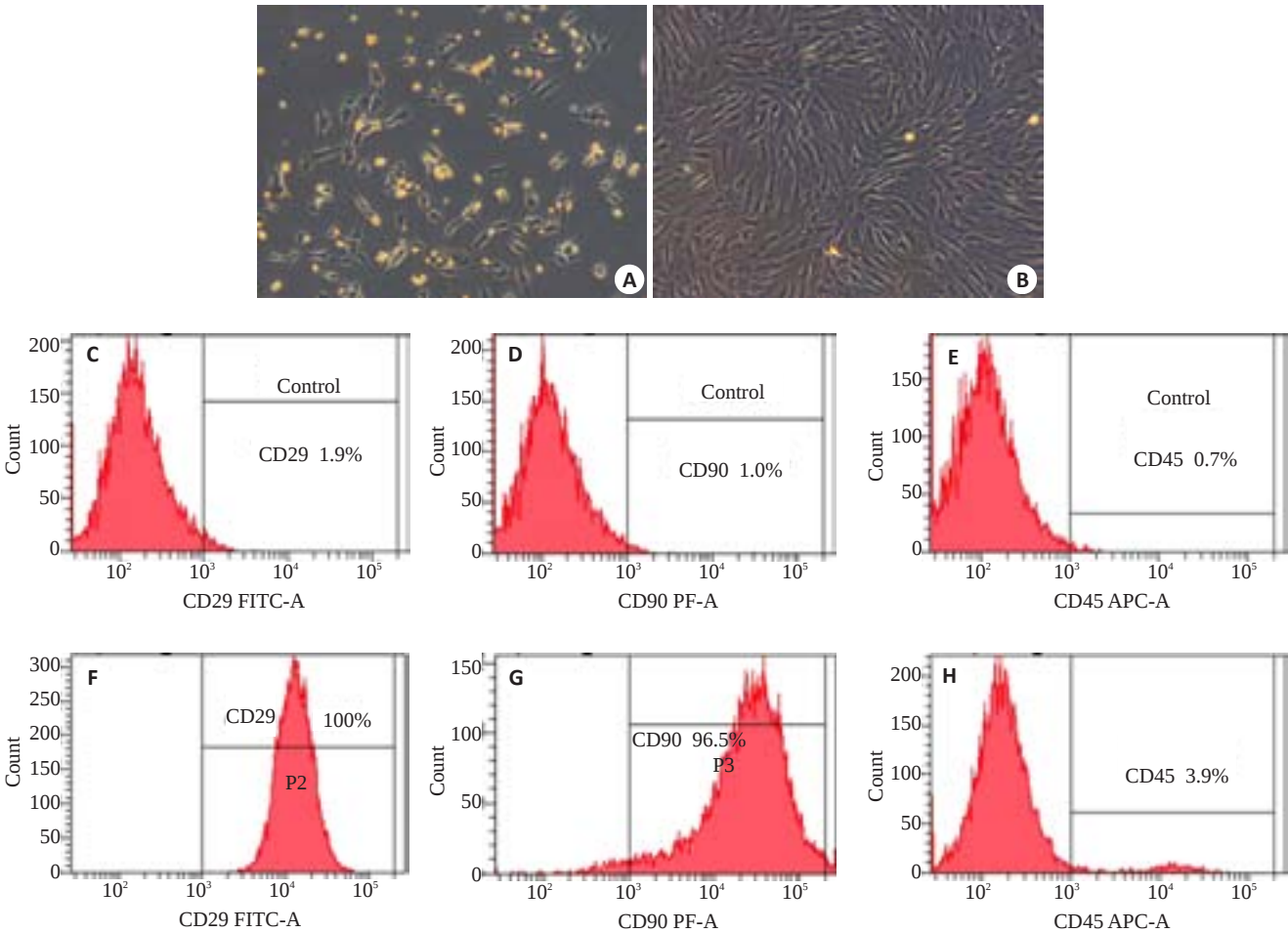


图 1 骨髓间充质干细胞的鉴定

Fig.1 Identification of bone marrow mesenchymal stem cells. A: Microscopic images of BMSCs on day 3 (Original magnification: $\times 100$); B: Passage 3 of BMSCs ($\times 100$); C-H: Flow cytometry for identification of the phenotype of BMSCs.

2.2 大鼠脂肪干细胞的分离培养

贴壁法培养原代细胞,接种后镜下可见大量大小不一呈圆形或类圆形的混合细胞;24 h首次换液后可见部分细胞贴壁,第3天可见较多长梭形样的贴壁细胞;约

7 d,细胞密度汇合即达80%。第3代细胞呈均一的长梭形,类成纤维细胞样形态,流式细胞仪检测间充质干细胞表面标志物CD29、CD90均为阳性(96.9%和99.2%),造血系细胞表面标志物CD45阴性(0.8%)(图2)。

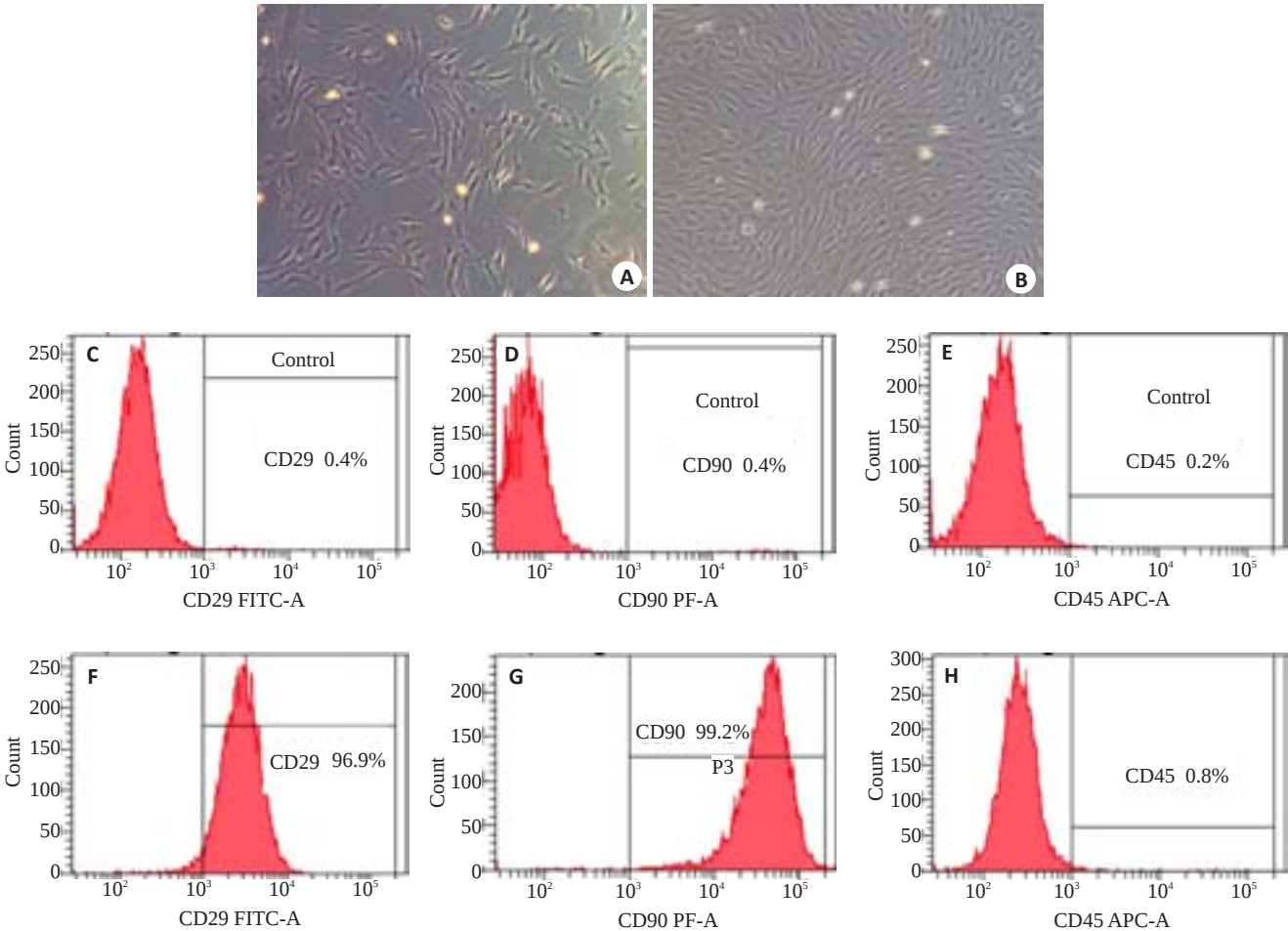


图2 脂肪干细胞的鉴定
Fig.2 Identification of adipose-derived stem cells. A: Microscopic images of ASCs on day 3 (×100); B: Passage 3 of ASCs (×100); C-H: Flow cytometry for identification of the phenotype of ASCs.

2.3 CCK-8法绘制细胞生长曲线

采用CCK-8法检测1~7 d ASCs和BMSCs的A值和单纯完全培养基的A值,两者相减得出最后的A值。经独立样本t检验分析数据,结果如图3所示,除第1、2、3天和第7天外,第4、5、6天两组细胞的A值均有统计学差异($P<0.05$),说明ASCs增殖能力强于BMSCs。

2.4 诱导BMSCs和ASCs向内皮样细胞分化

2.4.1 细胞形态观察 诱导4 d后,BMSCs和ASCs出现多角形,11 d后,细胞开始变圆,21 d后,BMSCs和ASCs出现“岛状的”典型内皮细胞“铺路石”样排列,继续培养35 d后,可见细胞广泛由长梭形变成类圆形样的细胞,整体成“铺路石”状,镜下观察ASCs分化成类圆形的细胞面积比BMSCs更大(图4)。

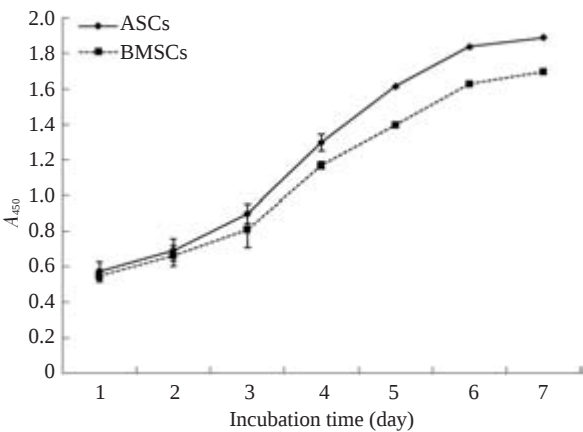


图3 ASCs和BMSCs的生长曲线
Fig.3 Cell growth curve of ASCs and BMSCs. CCK-8 proliferation experiment showed that ASCs proliferated more quickly than BMSCs ($P<0.05$).

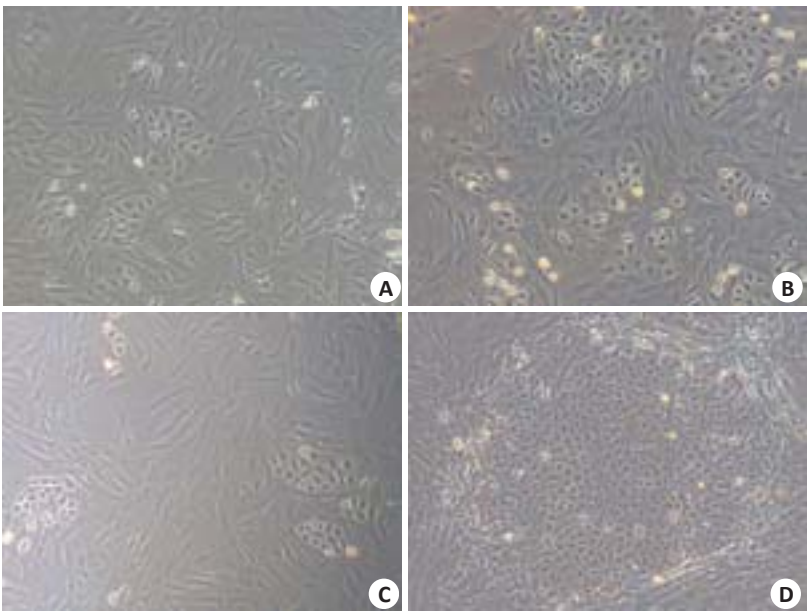


图4 向内皮诱导后的BMSCs和ASCs

Fig.4 Endothelial differentiation of BMSCs and ASCs. A: Microscopic images showing endothelial differentiation of BMSCs at 21 days ($\times 100$); B: Endothelial differentiation of BMSCs at 35 days ($\times 100$); C: Endothelial differentiation of ASCs at 21 days ($\times 100$); D: Endothelial differentiation of ASCs at 35 days ($\times 100$).

2.4.2 qPCR检测内皮细胞特异性标志物CD31、KDR、vWF mRNA相对表达量 本研究采用 $\Delta\Delta CT$ 法,以未诱导组的表达量为参照,分别测定两个诱导组的MSCs相对未诱导组的CD31、KDR、vWF mRNA表达水平。采

用独立样本 t 检验对数据进行统计分析,结果如图5所示,BMSCs、ASCs经内皮诱导后,表达CD31、KDR、vWF mRNA的水平比未诱导组增高,差异有统计学意义($P<0.05$)。

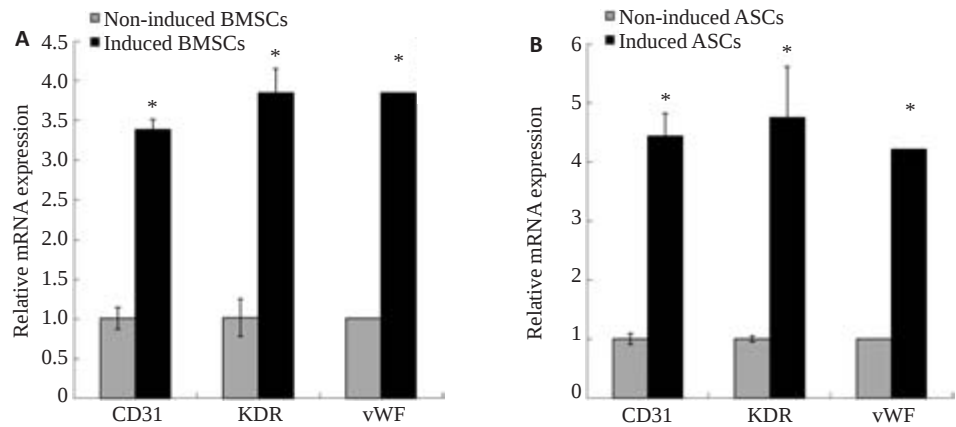


图5 Real-time PCR分析BMSCs和ASCs内皮相关因子表达水平

Fig.5 Real-time PCR analysis of expressions of endothelial-related factors in BMSCs (A) and ASCs (B). The results are expressed as fold increase compared with the expression in non-induced cells. Values are expressed as $Mean\pm SD$ ($n=3$). $*P<0.05$.

2.4.3 免疫荧光染色及激光共聚焦显微镜观察 免疫荧光染色 CD31 抗原,激光共聚焦显微镜下观察可见 CD31 抗原(FITC,绿色荧光)表达在MSCs的细胞膜表面,诱导组ASCs和BMSCs均表达CD31抗原,进一步

证实ASCs和BMSCs均可诱导为内皮样细胞(图6)。

2.4.4 荧光显微镜检测内皮样细胞摄取Dil-Ac-LDL的能力 荧光显微镜下观察,蓝色荧光代表细胞核,红色荧光代表摄取了Dil-Ac-LDL的细胞,结果显示,未诱导

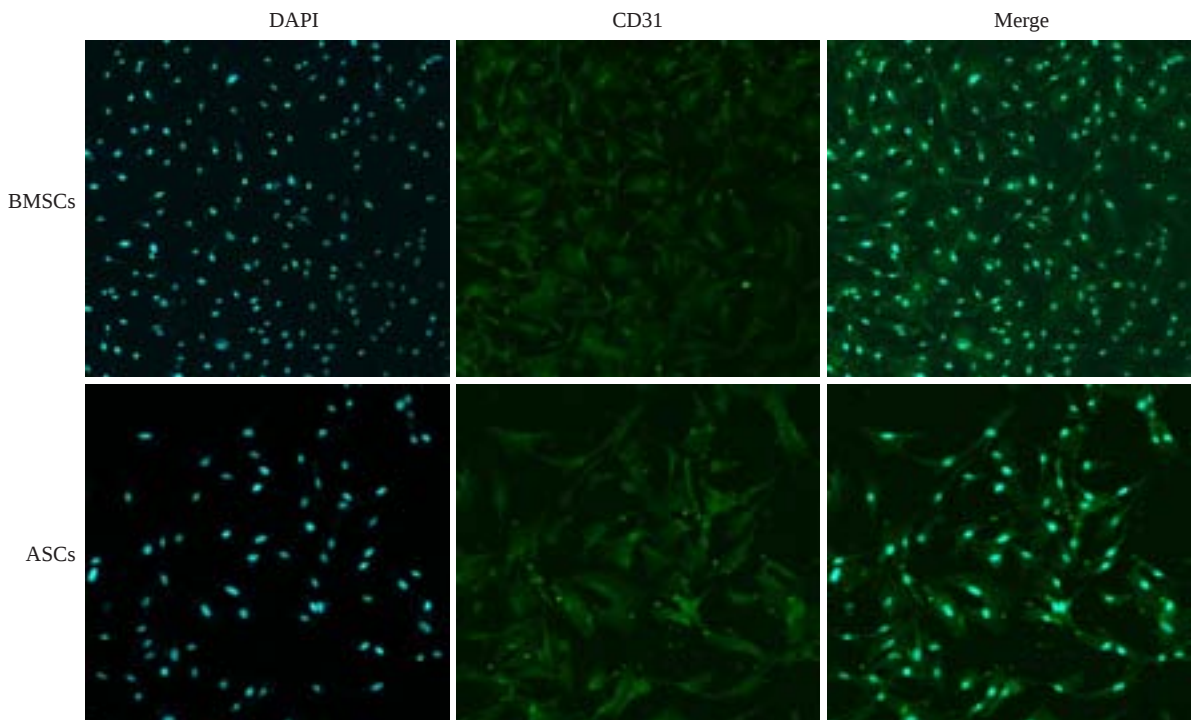


图6 诱导组BMSCs和ASCs表达CD31抗原
Fig.6 Confocal images of CD31 (green) expressed on BMSCs and ASCs after endothelial differentiation (×100). Cell nuclei are counterstained with DAPI (blue).

组的MSCs均不具备摄取Dil-Ac-LDL的能力,而诱导组的BMSCs和ASCs均能够摄取Dil-Ac-LDL,进一步证实诱导后的细胞为内皮样细胞(图7)。

2.4.5 Matrigel 成管实验 细胞在Matrigel上孵育6 h

后,倒置显微镜下观察可见未诱导组细胞呈球状聚拢,而诱导组ASCs和BMSCs均成管样结构,进一步证实ASCs和BMSCs均可诱导为内皮细胞(图8)。

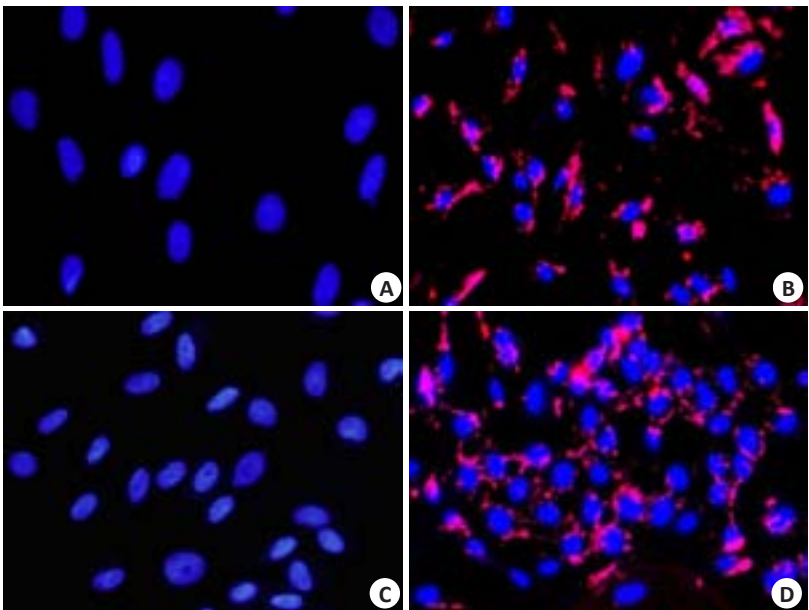


图7 诱导组BMSCs和ASC摄取Ac-LDL的能力
Fig.7 Immunocytochemical staining of BMSCs and ASCs (×100). Cell nuclei are counterstained with DAPI (blue), and red fluorescence in the cells indicates Ac-LDL uptake. A: Control BMSCs; B: BMSCs after endothelial differentiation; C: Control ASCs; D: ASCs after endothelial differentiation.

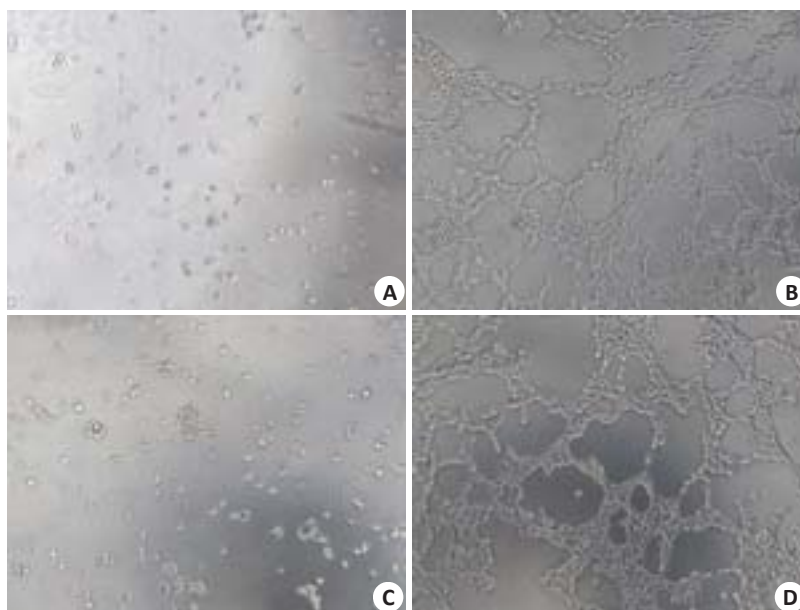


图8 Matrigel成管试验

Fig.8 Ability of BMSCs and ASCs after endothelial differentiation for tube formation on Matrigel (Original magnification: $\times 50$). A: Control BMSCs; B: BMSCs after endothelial differentiation; C: Control ASCs; D: ASCs after endothelial differentiation.

3 讨论

骨髓间充质干细胞和脂肪干细胞作为研究最广泛的成体干细胞^[8],两者均具有间充质干细胞的典型特征,能进行自我更新、多向分化潜能^[9]。本研究从SD大鼠中原代提取BMSCs和ASCs,通过流式鉴定膜蛋白的表达证实获得的细胞为间充质干细胞,提示全骨髓贴壁法和0.1%I型胶原酶消化法提取的BMSCs和ASCs质量较佳,能获得比较纯的间充质干细胞,呈经典的成纤维细胞样形态,可用于后续MSCs的研究。通过CCK-8法绘制BMSCs和ASCs的生长曲线,结果表明,ASCs的增殖能力更强,这与大多数学者的研究结果一致^[7, 10],再加上ASCs更容易获得,来源丰富等特征,提示ASCs比BMSCs更具有应用前景。

为解决糖尿病足溃疡以及外周动脉闭塞性疾病等下肢循环差、血管供应不足的问题,需要新生的血管内皮细胞恢复缺血组织的血管化^[11],本研究诱导SD大鼠骨髓间充质干细胞和脂肪干细胞向内皮细胞分化,使用最经典的、研究最深入的VEGF和bFGF作为内皮细胞诱导液^[12]。结果证实来源于中胚层的BMSCs和ASCs不仅能分化为文献报道的成骨、脂肪、软骨等中胚层细胞^[13],还能分化成内皮细胞,通过qPCR和免疫荧光检测证实其具备成熟内皮细胞的特定标志物,进一步的验证也表明诱导后的细胞具备摄取Ac-LDL的能力以及成血管的能力,证实BMSCs和ASCs可以分化成功能型的内皮细胞。功能型的内皮细胞能快速形成新生血管^[14],是组织工程成功的前提^[15],因此间充质干细胞

是治疗血管性疾病具有前景的干细胞来源。而与BMSCs相比较,相同培养条件下ASCs的增殖能力更强,细胞状态更好,相同诱导条件下ASCs广泛分化为内皮细胞的时间更短,提示其能力更强,更适合用于治疗糖尿病足溃疡这种全身基础条件差、血运重建难的情况^[16]。而且抽取骨髓对于患者是较大的创伤,BMSCs的增殖潜能、多向分化潜能会随着年龄的增长而下降^[17]。脂肪组织从抽脂术中废弃的脂肪组织中获得,研究显示年龄、性别、肥胖、肾功能衰竭和血管疾病不影响分离的有效性^[18],本文创新性地比较BMSCs和ASCs向内皮分化的能力,有利于选出对于组织工程血管新生更优的间充质干细胞来源,结果表明ASCs是干细胞移植治疗糖尿病足更合适的来源。

目前也有大量的临床前研究探索脂肪干细胞治疗糖尿病伤口模型的有效性,证实其可通过分泌多种生长因子和细胞因子,改善慢性炎症,整合到微血管循环中,从而加快伤口愈合^[19-21],而利用脂肪干细胞及相关细胞产品注册的临床试验也日益增多^[22-24]。与骨髓单个核细胞对比,脂肪组织干细胞的治疗性血管新生的作用还体现在促进局部抗炎因子IL-10的表达、释放M2型巨噬细胞等^[25]。随着研究的更广泛和深入,更充分证实ASCs在组织工程应用中具有广阔的前景。

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