·基础研究·

与羊膜上皮细胞共培养对羊膜间充质干细胞生物学特性的影响

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[摘　要]　目的：研究与羊膜上皮细胞(amniotic epithelial cells，AEC)共培养对羊膜间充质干细胞(amniotic mesenchymal stem cells，AMSC)生物学特性的影响，并探讨基质细胞衍生因子1(stromal cell derived factor-1, SDF-1)/CXCR4轴在AMSC归巢、迁移过程中的作用。方法：从人羊膜中分别分离、培养AMSC和AEC，进行扩增及鉴定。实验设AMSC与AEC共培养组、无血清AMSC培养组以及血清AMSC培养组，培养24、48、72 h后，通过CCK-8及锥虫蓝实验检测AMSC细胞增殖活性，免疫荧光流式细胞术和Real-time PCR检测CXCR4 mRNA的表达水平，细胞迁移实验检测AMSC细胞体外迁移能力。结果：48、72 h共培养组及血清培养组细胞增殖活性及增殖率均高于无血清培养组(P<0.05)；共培养组与无血清培养组AMSC细胞的CXCR4 mRNA和蛋白表达明显高于血清培养组(P<0.05)，两组AMSC细胞依赖迁移能力明显高于血清培养组(P<0.05)。结论：与AEC共培养的AMSC细胞仍具备间充质干细胞的基本生物学特性，且能保持良好的增殖活性，共培养能上调AMSC表面CXCR4，并在体外增强其迁移能力。

[关键词]　羊膜间充质干细胞；羊膜上皮细胞；CXCR4；归巢；迁移

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Effect of co-culture with amniotic epithelial cells on biological characteristics of amniotic mesenchymal stem cells

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[Abstract]　Objective: The aim of this study was to investigate the effect of co-culture with AEC (amniotic epithelial cell) on the biological characteristics of AMSC (amniotic mesenchymal stem cell), and to investigate the roles of SDF-1/CXCR4 axis in the homing and migration of AMSC. Methods: AMSC and AEC were isolated from human amnion, and then cultured, amplified and identified, respectively. The AMSC were divided into three groups: AEC co-cultured group, serum-free cultured group and serum cultured group. After culture for 24 h, 48 h, and 72 h, the proliferation viability of AMSC was measured by CCK-8 assay and trypan blue staining; the expression of CXCR4 mRNA was analyzed by flow cytometry and Real-time RT-PCR, and the migration ability of AMSC in vitro was observed by migration assay. Results: Cell viability (48 h and 72 h) and survival rate in the co-culture and serum groups were higher than those in the serum-free group (all P<0.05). The mRNA and protein expressions of CXCR4 in AMSC of the co-culture and serum-free groups were significantly higher than those of the serum group (P<0.05). The migration ability of AMSC in the co-culture and serum-free groups, which increase with the SDF-1 (stromal cell derived factor-1) concentration gradient, were higher than that in the serum group (P<0.05). Conclusion: AMSC co-cultured with AEC still have the basic biological characteristics of MSC, and showed good

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growth activity. Co-culture with AEC can up-regulate CXCR4 on AMSC surfaces and enhance the migration ability of AMSC *in vitro*.

[Key words] amniotic mesenchymal stem cell (AMSC); amniotic epithelial cell (AEC); CXCR4; homing; migration

间充质干细胞（mesenchymal stem cell，MSC）是来源于中胚层的一类多能干细胞，在组织修复、抗炎、预防及治疗移植物抗宿主病（graft-versus-hostdis-ease，GVHD）中发挥重要作用[1-2]。骨髓间充质干细胞（bone marrow MSC，BMSC）的获取需通过有创途径获得，且其分化能力随年龄增长而减弱[3]，而羊膜间充质干细胞（amniotic mesenchymal stem cell，AM-SC）在体外与BMSC具有相似的支持造血功能及多向分化潜能，同时免疫原性低，能显著抑制淋巴细胞增殖，成为一种更加理想的MSC临床研究及应用的来源[4-5]。临床联合移植BMSC和造血干细胞（hema-topoietic stem cell，HSC）是提高造血重建能力、减少GVHD发生的有效手段[1,6]；切实有效地提高MSC归巢和植入才能使MSC移植获得良好的治疗效果[7]。有研究[8-10]表明，细胞因子刺激可增加BMSC表面CXCR4表达，SDF-1/CXCR4轴是促进BMSC归巢、加快受体造血恢复重要的生物轴。这种方法尽管有效，但这些细胞因子并不适于临床，因此，寻找更加简便、安全、有效的促进MSC归巢方法仍是有待解决的问题。羊膜上皮细胞（amniotic epithelial cell，AEC）源自胚胎外胚层，可以合成和分泌多种细胞因子，在无血清的条件下仍能生长繁殖[11-12]。因此推测与AEC共培养能不但能维持AMSC的活性，同时在细胞因子刺激下，AMSC表面CXCR4表达亦可增加，提高其迁移和归巢能力，从而为获得更好的AMSC移植疗效提供理论支持。本课题采用与AEC共培养方式，探讨AMSC细胞的生物学特性，为AMSC应用于临床提供理论依据。

1　材料与方法

1.1　组织标本、主要试剂和仪器

人羊膜由昆明医科大学第一附属医院产科提供（肝炎全套、HIV、梅毒等血清学反应均显示阴性）。本研究经昆明医科大学第一附属医院伦理委员会批准，并经产妇知情同意。优等澳大利亚胎牛血清、DMEM/F12液体培养基购自北京海克隆公司，青-链霉素溶液、胰蛋白酶溶液购自以色列Bioind公司，Cy-tofix/Cytoperm、Perm/Wash购自美国BD公司，Pan-Cytokeratin Antibody (C11) PE购自美国Santa Cruz公司。小鼠抗人的单克隆抗体CD11a、CD11b、CD29、CD31、CD34、CD44、CD45、CD105、HLA-DR、Pan-CK购自美国BioLegend公司，Goat anti-mouse IgG(H+L)、Human ads-FITC购自美国Southern Biotech公司，

Anti-Human CD184(CXCR4) PE以及Anti-Human CD184(CXCR4) Purified购自美国Ebioscience公司。Human SDF-1 alpha购自美国Peprotech公司，Human Plasma Fibronrctin Purified Protein、Millicell悬挂式培养皿购自美国Millipore公司，TRIzol Reagent购自美国Invitrogen公司，反转录、PCR试剂盒、SYBR Green Real-time PCR试剂盒、DL2000 DNA Marker以及引物均购自日本TaKaRa公司。流式细胞仪购自美国Becton Dickinson公司，荧光定量PCR仪购自美国ABI StepOnePlus公司。

1.2　AMSC的分离、培养与鉴定

无菌条件下分离羊膜并反复漂洗，去除血凝块后剪碎，分散接种到培养瓶底上，加入完全DMEM/F12培养基（含10%胎牛血清、1%青-链霉素）并置于37 ℃、5% CO_2饱和湿度培养箱中静置培养。细胞达到80%~90%汇合时进行传代。采用间接免疫荧光法，一抗为小鼠抗人的单克隆抗体CD11a、CD11b、CD29、CD31、CD34、CD44、CD45、CD90、CD105、HLA-DR、Pan-CK，4 ℃孵育30 min，加入FITC标记的羊抗鼠二抗，采用同型抗体作为对照，4 ℃孵育30 min。流式细胞仪检测，结果采用WinMDI 2.9软件分析。AMSC以$3×10^4$/cm接种于培养瓶中，在成脂肪细胞诱导分化液（$1×10^{-6}$ mol/L地塞米松+0.5 mol/L 1-甲基-3-异丁基-黄嘌呤+0.1mol/L维生素C+1%青-链霉素+10% FBS的IMDM）中诱导3周，10%甲醛固定，油红O染色。成骨诱导采用含10% FBS、$1×10^{-7}$ mol/L地塞米松、10 mol/L β-磷酸甘油、0.05 mol/L维生素C、1%青-链霉素的IMDM培养基，诱导3周后Von Kossa染色。

1.3　AEC的分离、培养与鉴定

以0.125%胰酶在37 ℃中消化羊膜组织3次，每次30~40 min。收集消化的液体，200目筛网过滤，离心后收集细胞，静置培养。细胞彼此汇合达80%~90%时传代。将AEC制成细胞爬片，以4%中性甲醛固定、苏木精浸染后，依次以1%盐酸乙醇分化0.5~1 min、1%氨水返蓝1 min、伊红水溶液浸染30 s，最后乙醇脱水，透明5~10 min后吹干后封片。显微镜下观察。将AEC细胞爬片以4%中性甲醛及冰丙酮固定、3% H_2O_2灭活、动物血清封闭后，滴加适当一抗(Pan-CK)孵育45 min。冲洗后二抗孵育30 min，最后抗生物素蛋白-过氧化物酶(SABC)溶液作用30 min，DAB溶液显色，显微镜下观察。采用直接免疫荧光法，Fix/Perm破膜、固定后，加入PE标记的Pan-CK抗

体,室温,暗室,孵育40 min。重悬细胞后上流式细胞仪行检测。结果用WinMDI 2.9软件分析。

1.4 AEC共培养体系的建立

消化收集细胞,以无血清培养基重悬,AMSC以 1×10^5 个/孔接种至6孔板,放入Millicell小室(孔径 0.4 μm),AEC以 1×10^4 个/孔接种至小室内,标记为共培养组。取同批次AMSC,消化后分别以无血清培养基和完全培养基重悬,按上述方法和浓度接种至6孔板,不加入AEC,作为对照组,分别标记为无血清培养组、血清培养组。于培养箱内培养24、48、72 h后,消化收集AMSC待用。

1.5 CCK-8和锥虫蓝法检测共培养后AMSC增殖活力

将各组各时间位点的AMSC悬液接种到96孔板,培养过夜。每孔加适量CCK-8溶液孵育3~4 h,酶标仪测定各孔光密度值(D)值。在各组各时间位点的AMSC悬液中加入0.4%锥虫蓝染液,吹打均匀后吸取细胞悬液于显微镜下计数蓝染细胞。计算公式:细胞增殖率(%)=(细胞总数-蓝染的细胞数)/细胞总数×100%。

1.6 直接免疫荧光法分析AMSC的CXCR4蛋白的表达

应用PE标记的小鼠抗人CXCR4单抗对细胞表面进行标记,对胞内CXCR4的检测,则先用未作标记的CXCR4单抗(clone 12G5)孵育来封闭细胞表面蛋白,经Fix/Perm破膜、固定后,用PE标记的CXCR4单抗染色。流式细胞仪检测,结果采用WinMDI 2.9软件分析。

1.7 Real-time PCR检测 *CXCR4* mRNA的表达

提取总RNA并反转录为cDNA,采用SYBR Green染料法在荧光定量PCR仪上扩增并作图。目的基因 *CXCR4* 序列为:5' - ACTTCAGTTTGTTGG CTGCGGC - 3',5'-ACCGCTGGTTCTCCAGATGCG- 3'。内参基因 *GAPDH* 序列为5'-GAAGGTGAAGGT CGGAGTC - 3',5' - GAAGATGGTGATGGGATTTC - 3',反应条件:95 ℃预变性10 s;95 ℃变性5 s,60 ℃退火40 s,循环40次。实验重复3次。分析结果以 $2^{-\Delta\Delta Ct}$ 值表示,公式如下:Δ Δ Ct= Δ Ct(共培养组或无血清培养组)- Δ Ct(血清培养组),经共培养或无血清培养的细胞与血清培养组的细胞之间的差异就是 $2^{-\Delta\Delta Ct}$ 倍。

1.8 体外迁移实验观察AMSC体外迁移能力

实验在Millicell小室中进行,上室膜(孔径12 μm)用纤维连接蛋白FN包被,下室中加入不同质量浓度的SDF-1(0、100、200、300 ng/ml),将AMSC悬液加入上室,培养24 h后,取出滤膜,经结晶紫染色后,显微镜下计数迁移至滤膜外表面的细胞数。每张滤膜随机取5个视野计数。实验重复3次。

1.9 统计学处理

采用SPSS17.0统计学软件,计量资料以 $\bar{x} \pm s$ 表示,多组间比较采用单因素方差分析,两组间比较采用LSD-*t*检验,以 $P < 0.05$ 或 $P < 0.01$ 表示差异有统计学意义。

2 结 果

2.1 成功培养AMSC

培养后AMSC形态呈长梭形或多角形,细胞均质、明亮,透明度大、折光度强(图1A、B)。流式免疫分析结果((图1C-L)显示,AMSC表达CD29、CD44、CD90、CD105,不表达或低表达CD11a、CD11b、CD31、CD34、CD45、HLA-DR、pan-CK,在体外可成功向脂肪和成骨细胞诱导分化(图1M-P)。

2.2 成功培养AEC

AEC呈多角形或卵圆形,轮廓清晰,胞质丰富,生长成片后呈铺路石样外观。H-E染色可见胞质丰富,胞核蓝染(图2A)。免疫组化(图2B、C)及流式免疫分析(图2D、E、F)均可观察到Pan-CK在AEC中表达。

2.3 共培养后AMSC的基本生物学特征

AMSC在培养24、48、72 h后,在形态学上未见明显变化,仍保持稳定的免疫表型特征:高表达基质和间质细胞抗原,而造血干祖细胞标志、主要组织相容性抗原复合物II类分子、上皮以及内皮标志均显示阴性表达(图3)。

### 2.4 共培养后AMSC增殖活力明显提高

CCK-8法检测结果(图4A)显示,在共培养24 h后3组细胞增殖率无明显差别($P > 0.05$);48、72 h后,共培养组及血清培养组的 D 值显著高于无血清培养组($P < 0.05$),提示细胞增殖活性较好。锥虫蓝法检测结果(图4B)显示,3个时间位点均显示共培养组及血清组AMSC的增殖率明显高于无血清培养组($P < 0.05$)。

2.5 共培养后AMSC中CXCR4的表达情况

直接免疫荧光法检测结果(图5A、B)显示,在3个时间位点,共培养组和无血清培养组细胞表面CX-CR4的表达量均明显高于血清培养组($P < 0.05$);对于胞内CXCR4,在24 h共培养组的表达明显高于无血清培养及血清培养组($P < 0.05$);在48、72 h 3组细胞胞浆内CXCR4无明显差别($P > 0.05$)。RT-PCR检测结果(图5C)显示,*CXCR4* mRNA的表达:在24 h共培养组及无血清培养组AMSC中 *CXCR4* mRNA的表达量分别为血清培养组的(1.664 ± 0.288)倍、(1.227 ± 0.289)倍;在48 h该差异倍数值分别为

（2.875±0.260）倍和（2.842±0.413）倍；在 72 h 该差异倍数值分别为（3.241±0.511）倍和（2.998±0.632）倍。
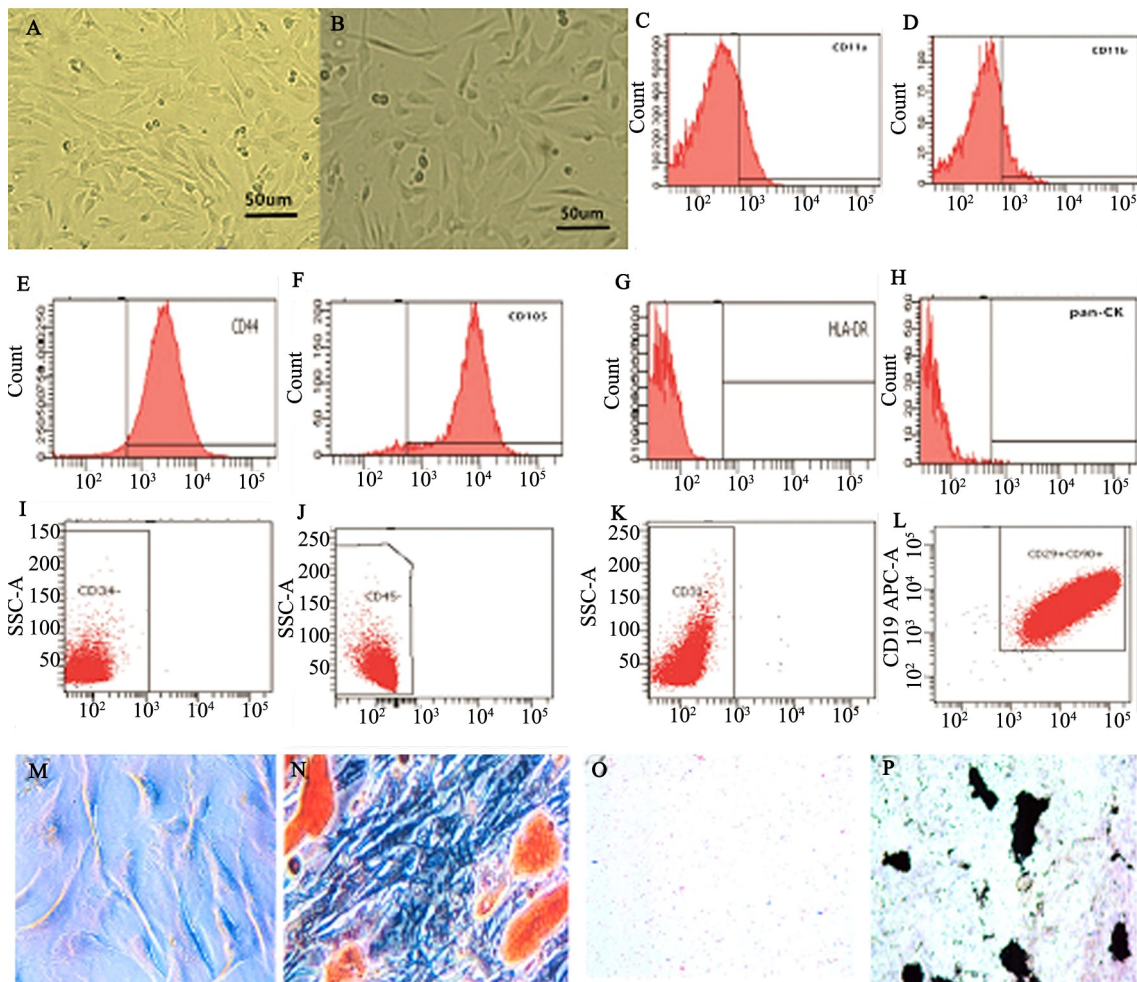
A-B：The morphology of AMSC（magnification，×40）；C-L：Phenotype analysis of AMSC，AMSC highly express matrix and stromal cell antigen, but not express the hematopoietic stem and progenitor cell markers, MHC II molecules, epithelial and endothelial markers；M-N: Adipogenic differentiation of AMSC(×200). M：Negative control for oil red O staining of AMSC；N：AMSC exhibited positive oil red O staining, indicating successful adipogenic differentiation；O-P: Osteogenic differentiation of AMSCs(×200). O: Negative control for Von Kossa staining of AMSC; P: Von Kossa staining demonstrated that AMSC exhibited positive staining for calcium accumulation following osteogenic induction

图1　AMSC 的鉴定

Fig.1　Identification of AMSC

2.6　共培养后 AMSC 体外迁移能力明显增强

体外迁移实验结果（图6）显示，3组的细胞均能向 SDF-1 迁移，并呈剂量依赖性。与血清培养组比较，共培养组与无血清培养组 AMSC 均呈现明显迁移优势（$P<0.05$），而共培养组与无血清培养组迁移能力无明显差异（$P>0.05$），且用中和抗体预先与 AMSC 共孵育能阻止这种迁移，证实了该迁移的特异性。

3　讨　论

MSC因其多向分化潜能及免疫调控等特性被应用于干细胞移植等科研领域[13]。MSC 归巢的数量与疗效紧密相连，但是其归巢的数量与移植的数量却不呈正相关[14]。因此，MSC 特异性归巢和植入靶组织的能力和效率，是 MSC 治疗疾病的关键。有研究[15-16]显示，SDF-1/CXCR4 轴在 MSC 归巢中发挥重要作用增加 CXCR4 的表达有助于 MSC 向靶器官迁移，若阻断受体，其迁移能力降低。

目前细胞共培养体系主要靠两种方法建立：直接共培养体系和间接共培养体系，前者不同种类的细胞之间相互接触，后者不同种类的细胞共用同一个培养环境而不直接接触。已有实验[17]表明，共培养

体系中的辅助细胞可以维持目的细胞的基本生物学功能。有研究[13-15]显示，培养后AMSC形态呈长梭形或多角形，细胞均质、明亮，透明度大、折光度强，流式免疫分析显示其上表达CD29、CD44、CD90、CD105，不表达或低表达CD11a、CD11b、CD31、CD34、CD45、HLA-DR、Pan-CK，在体外可成功向脂肪和成骨细胞诱导分化。本研究利用AEC能分泌多种细胞因子这一特点，在无血清的条件下与AMSC共培养，以期能维持AMSC良好的生长活性并上调CXCR4表达，提高归巢及迁移能力。通过Millicell小室（孔径0.4 μm）将AMSC与AEC在无血清条件下进行非接触共培养，仅靠细胞分泌的各种活性物质维持营养需要，并排除了细胞接触的影响因素。结果显示，共培养的AMSC在形态、免疫表型和多向分化能力等基本生物学特性上与共培养前的细胞相比无明显差异。在细胞增殖活性及存活率方面，共培养的细胞能达到与血清培养组无差异的生长能力，并优于无血清培养。这提示，AEC所产生的细胞因子至少在72 h内，足以维持AMSC的生物学活性。
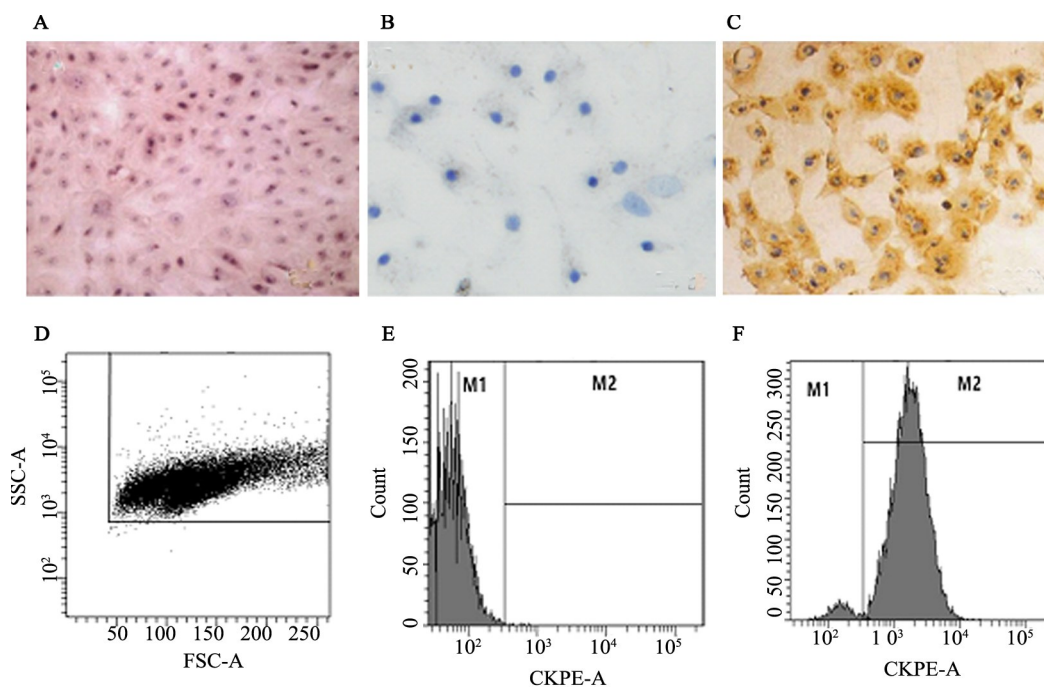
A：The morphology of AEC by H-E staining (magnification, ×100); B: Negative control for Pan-CK immunohistochemical staining
（magnification, ×200）; C: Immunohistochemical staining for Pan-CK indicated positive expression in AEC (magnification,×200);
D: The scatter plot of flow cytometry; E: Negative control for Pan-CK immunofluorescence and flow cytometry experiment; F: Flow
cytometry following immunofluorescence staining indicated a high expression of Pan-CK in AECs; M1: Indicates negative cells；
M2：Indicates positive cells

图2　AEC的鉴定

Fig.2　Identification of AEC

CXCR4大部分存在于胞质内，细胞通过内摄作用调节CXCR4在细胞膜表面的表达[21]。正常情况下，MSC表面只有极少部分的CXCR4表达（1.0%~3.9%），经过破膜后，胞内抗原暴露，CXCR4才大量表达[21]，LI等[22]的研究亦表明存在于MSC表面的CXCR4微乎其微，这与本实验用免疫荧光法测得的结果一致，无论哪种培养方式，CXCR4胞内的表达一直处于高水平（95%以上），但表面表达量却相对较少（平均不到5%）。研究表明细胞表面CXCR4的表达可以通过外化和内吞来调节[21, 23-24]。由此推测，AEC与AMSC在无血清的环境下，可能通过自分泌或旁分泌，产生能促进AMSC表达CXCR4的细胞因子，这些细胞因子甚至有可能促进胞内CXCR4向表面外化。此外，有趣的是在无血清条件下，细胞表面CXCR4表达亦有增高，这可能是在缺乏营养的条件下，AMSC为维持自身生长需要，自分泌部分细胞因子，这些细胞因子增加了表面CXCR4的表达，但是这些有限的细胞因子满足不了细胞生长增殖的需要，这就解释了无血清培养组表面CXCR4表达相对较高，而细胞活力却不如共培养组及血清培养组的原因。同样，虽然血清培养组在细胞活力方面未呈现不足，但表面CXCR4的表达却明显低于其他两组。因此，与AEC共培养的AMSC兼顾了CXCR4表达的上调和良好的细胞活力两个方面，显示出该培养的优势。而无血清培养的细胞即便在迁移实验中与共培养组不相上下，

但综合考虑细胞活性等方面,却略逊一筹。
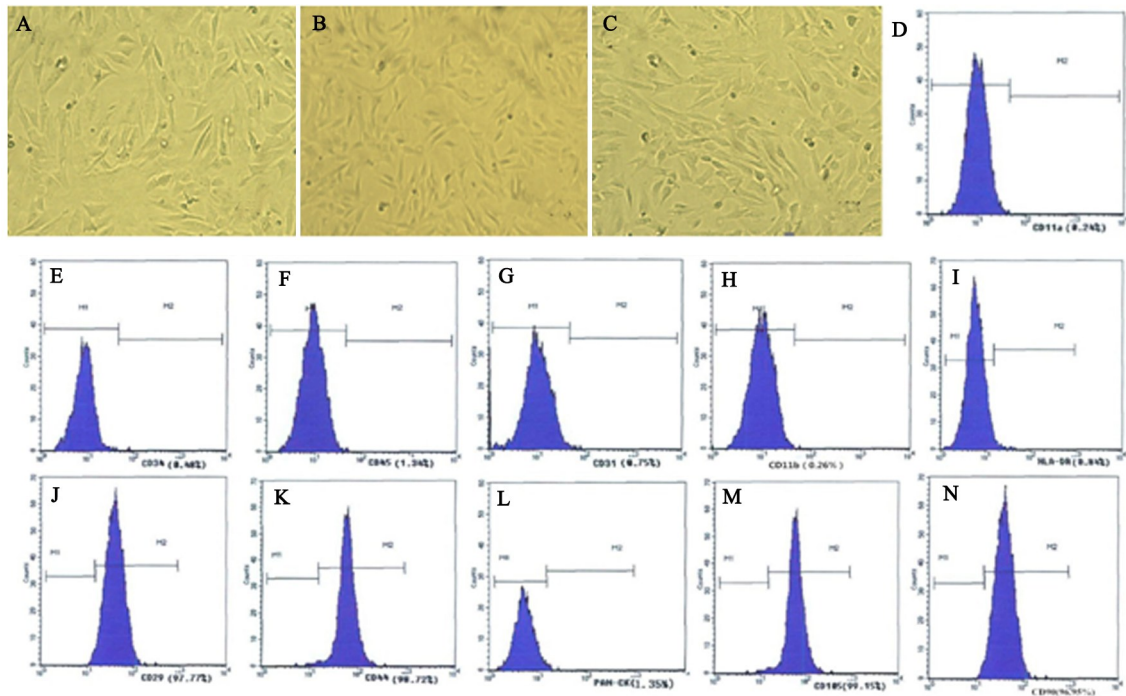
A:The morphology of AMSC in co-cultured group (magnification,×40); B: The morphology of AMSC in serum-free cultured group (magnification,×40);C:The morphology of AMSC in serum cultured group (magnification,×40). Phenotype analysis of AMSC after co-culture (D-N): the graph outlined the region of fluorescent intensity for cells labeled with control antibodies: AMSC highly expresses of matrix and stromal cell antigen, but negative for the hematopoietic stem and progenitor cell markers, MHC II molecules, epithelial and endothelial markers (M1: The expression rate of negative cells; M2: The expression rate of positive cells)

图3 共培养 72 h后 AMSC 的生物学特征

Fig. 3 Biological characteristics of AMSC after co-culture for 72 h
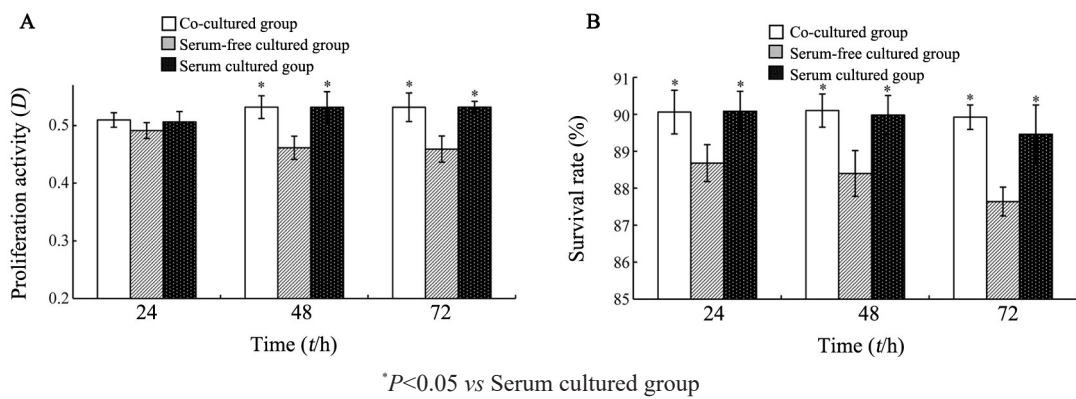

$^*P<0.05$ *vs* Serum cultured group

图4 CCK-8 法(A)和锥虫蓝染色法(B)检测共培养后 AMSC 细胞的增殖活力

Fig. 4 AMSC proliferation after co-culture were detected by CCK-8 assay (A) and trypan blue staining (B)

事实上,趋化因子受体发挥其生物学作用还是依赖于细胞表面表达的量。本课题组前期研究[8]显示,用5种细胞因子的联合处理BMSC后,使CXCR4在细胞表面及内部的表达上调,增强了向SDF-1的迁移的能力,并促进其向辐照后的NOD/SCID小鼠的骨髓归巢和植入。亦有研究[22, 25]表明,表达CXCR4的MSC能沿SDF-1的浓度梯度趋化到靶器官或组织,

参与组织修复。本研究中迁移实验结果显示,共培养组由于表面CXCR4表达的增加,其体外沿SDF-1浓度梯度迁移的能力亦有所增加,这一现象更进一步证实了迁移中发挥作用的是细胞表面趋化受体的表达,而非胞内。而预先加入CXCR4中和性抗体能使该迁移过程受阻,证实了这种迁移的特异性。

*P<0.05 *vs* Serum cultured group
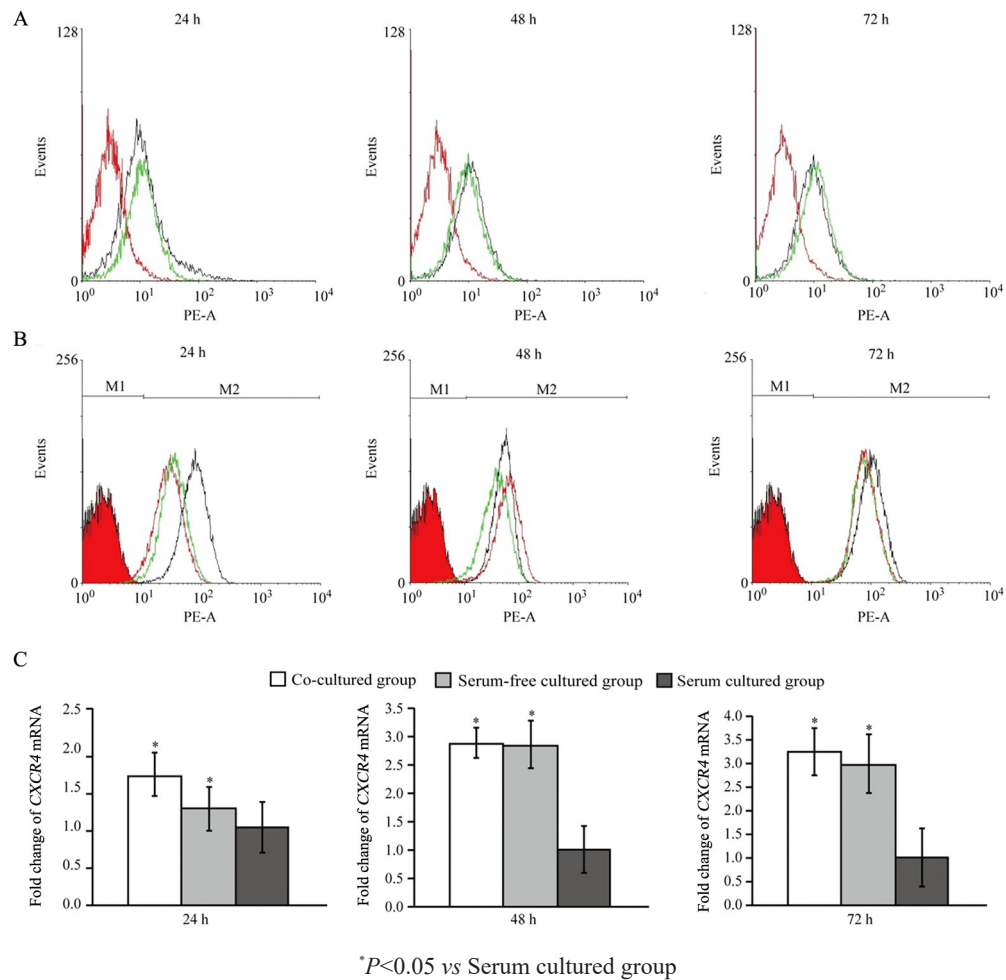
A：The surface CXCR4 mRNA expression of AMSC in co-cultured group (black curve) and serum-free cultured group (green curve) were higher than that in serum cultured group (red curve)；B：The intracellular CXCR4 mRNA expression of co-cultured group (black curve) was significantly higher than the serum-free cultured (green curve) and serum cultured group (red curve) at 24 h, while it was no significant difference in the three groups at 48，72 h；C：Detection of *CXCR4* mRNA expression in three groups at different time by RT-PCR

图5 共培养后 AMSC 中 *CXCR4* 的表达

Fig. 5 Expression of *CXCR4* in AMSC after co-culture
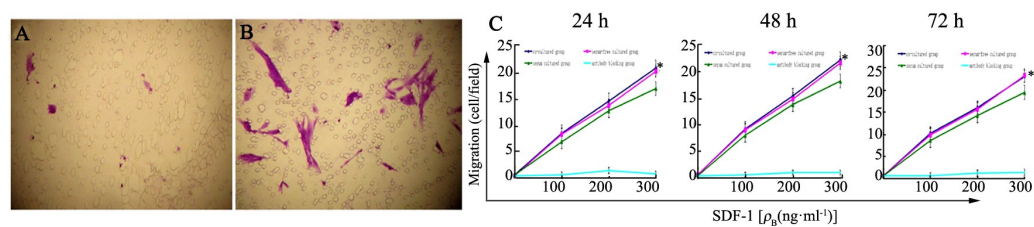
*P<0.05 *vs* Serum culture group

A: 0 ng/ml SDF-1 gradient after 48 h (magnification, ×200); B: 200 ng/ml SDF-1 gradient (magnification, ×200); C: The chemotactic activity of AMSC towards various doses of SDF-1 at the three time sites

图6 共培养后 AMSC 体外迁移能力

Fig.6 The *in vitro* migration ability of AMSCs after co-culture

综上所述，本研究证实了与 AEC 共培养能在保持良好细胞活性的基础上，上调 AMSC 表面 CXCR4 的表达，提高其迁移能力，探索增加 AMSC 定向迁移及归巢的方法，为 AMSC 体内归巢能力的研究奠定基础。这为更好地将 AMSC 应用于临床提供了可靠的理论依据，并希望为临床提高 AMSC 移植疗效和

造血干细胞移植成功率提供新策略。

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