人脐带间充质干细胞提取物对人肺癌细胞A549作用的研究

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摘要 该研究探讨了人脐带间充质干细胞(human umbilical cord-derived mesenchymal stem cells, hUC-MSCs)提取物对人肺腺癌细胞A549增殖和迁移能力的影响及其作用机制。利用超声破碎法制备hUC-MSCs提取物, 并处理经链球菌溶血素O(streptolysin O, SLO)通透的A549细胞。采用MTT法、平板克隆形成实验检测hUC-MSCs提取物对A549细胞增殖的影响, Transwell实验检测细胞迁移能力, 实时荧光定量PCR检测促凋亡基因胱冬肽酶-3(*caspase-3*)、胱冬肽酶-9(*caspase-9*)、抑癌基因*RUNX3*(runt-related transcription factor 3)和存活蛋白基因(*survivin*)的mRNA水平, 亚硫酸氢盐测序检测*RUNX3*启动子区CpG岛甲基化水平。结果发现, 经hUC-MSCs提取物处理后, A549细胞的增殖、迁移能力均显著降低, *caspase-3*、*caspase-9*和*RUNX3* mRNA水平显著升高, *survivin* mRNA的表达量显著降低, *RUNX3*启动子区CpG岛显著去甲基化。该研究结果表明, hUC-MSCs提取物可能通过逆转*RUNX3*启动子区甲基化状态恢复其表达, 并通过提高caspase-3、caspase-9表达及降低survivin的表达来抑制A549的增殖及迁移能力。

关键词 肺癌; 人脐带间充质干细胞; 提取物

Effects of Human Umbilical Cord-derived Mesenchymal Stem Cells Extracts on Human Lung Cancer Cells A549

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Abstract In this study, the effects of cell extracts from human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) on the proliferation and migration of human lung cancer A549 cells were studied. After permeated by streptolysin O (SLO), A549 cells were cultured in medium containing hUC-MSCs extracts. Then, the proliferation of A549 cells were assessed by the MTT and colony formation assay. The invasion of A549 cells were examined by the transwell chambers assay. Meanwhile, the mRNA expression of pro-apoptotic gene *caspase-3* and *caspase-9*, tumor suppressor gene *RUNX3* (runt-related transcription factor 3) and anti-apoptotic gene *survivin* were detected by real-time quantitative PCR. The DNA methylation status in the promoter region of *RUNX3* gene were detected by bisulfite sequencing. The results showed that the proliferation and migration of A549 cells were significantly decreased after hUC-MSCs extracts treatment and the mRNA levels of *caspase-3*, *caspase-9* and *RUNX3*

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were significantly increased, while the mRNA level of *survivin* was significantly decreased and the CpG islands on the promoter of *RUNX3* was significantly demethylated. These results demonstrated that hUC-MSCs extracts inhibited the proliferation and migration of A549 cells probably through reversing *RUNX3* expression by demethylated its promoter region, activating the expression of caspase-3, caspase-9 and inhibiting the expression of survivin.

Keywords lung cancer; human umbilical cord-derived mesenchymal stem cells; extracts

肺腺癌是严重危害人类健康的恶性肿瘤, 其起病隐匿、进展迅速、恶性程度高, 很多患者被确诊时已处于中晚期, 传统肿瘤治疗是通过手术切除、放疗和化疗的手段来杀灭癌细胞。但由于手术和放疗不可能将所有癌细胞杀灭, 且化疗在杀死癌细胞的同时会对正常的细胞带来极大的损伤[1]。所以研发新的肺腺癌治疗药物和治疗手段迫在眉睫。

近年来, 国内外一些研究显示, 某些特殊细胞的提取物作用于人恶性肿瘤细胞, 可发挥重编程效应并有效抑制其恶性表型。例如, 蝾螈卵母细胞提取物会逆转沉默的肿瘤抑制基因并抑制乳腺癌细胞在小鼠肿瘤模型中的致瘤性[2]; 斑马鱼卵母细胞提取物通过pRb/E2F1(retinoblastoma protein/adenovirus E2 gene involves the activation of the cellular transcription factor 1)细胞凋亡通路呈现抗肿瘤特性[3]; 鸡胚提取物会通过对抑癌基因的去甲基化来上调其表达并逆转骨肉瘤高转移特性[4]等。牛卵母细胞提取物在不上调多能性基因*OCT4*(POU class 5 homeobox 1)、*NANOG*(Nanog homebox)、*KLF4*(Kruppel like factor 4)和*Myc*(v-myc avian myelocytomatosis viral oncogene homolog)表达的情况下, 通过对抑癌基因*RUNX3*(runt-related transcription factor 3)和*CDH1*(cadherin 1)去甲基化来上调其表达, 有效抑制肺癌细胞的增殖、锚定非依赖性生长、迁移和侵袭能力[5]。这表明, 细胞提取物可能会为癌症治疗提供一种新的药物来源。

人脐带间充质干细胞(human umbilical cord-derived mesenchymal stem cells, hUC-MSCs)是间充质干细胞家族的新生代表, 具有组织来源原始、生物性能稳定、增殖分化能力强、免疫原性低、无病毒感染风险、对供者无侵害、收集不涉及社会伦理问题等优点[6]。许多研究表明, hUC-MSCs可以与癌细胞相互作用并抑制它们的生长[7-10]。Yang等[7]研究证明, 人脂肪组织来源的间充质干细胞(adipose derived mesenchymal stem cells, ASCs)和hUC-MSCs都会诱导人脑胶质瘤细胞系U251的凋亡和分化, 然而hUC-MSCs诱导细胞凋亡能力更强, 两者诱导分化能力不相上下。Han等[8]证明了hUC-MSCs是通过激活JNK和下调PI3K/AKT信号通路来发挥对前列腺癌细胞PC-3的抑制作用。Leng等[9]用分子成像技术证明了hUC-MSCs可以通过诱导细胞凋亡和抑制血管生成来抑制乳腺癌细胞的生长。Lee等[10]证明, hUC-MSCs有向共培养的胶质瘤细胞以及胶质瘤干细胞传递外源合成miR-124和miR-145的能力, 且被传递了miR-124和miR-145模拟物的癌细胞各自的报告靶基因*SCP1*(CTD small phosphatase 1)和*SOX2*(SRY-box 2)荧光活性显著减少, 且胶质瘤细胞的迁移和自我更新能力显著降低。Chao等[7]的研究显示, hUC-MSCs条件培养基可通过促进胱冬肽酶-3基因、胱冬肽酶-9基因、*RUNX3*和*CDH1*并抑制存活蛋白(*survivin*)基因和*XIAP*(X-linked inhibitor of apoptosis)的表达来抑制癌细胞的增殖、迁移和锚定非依赖性生长能力。

在肿瘤的发生和发展过程中, 会引起大量癌基因和抑癌基因表达的失调。抑癌基因和原癌基因表达的长期失衡, 促使正常细胞向肿瘤细胞转化以及肿瘤由低级向高级演进。这启发我们去制备hUC-MSCs提取物, 研究其是否能抑制肺腺癌A549细胞的恶性表型并对癌基因及抑癌基因产生的影响, 通过探讨hUC-MSCs提取物对肿瘤细胞的作用及明确可能的作用作用分子及作用途径, 为癌症治疗提供新思路。

1 材料与方法

1.1 材料

A549细胞株、hUC-MSCs为本实验室留存。

1.2 试剂及仪器

$MgCl_2$、ATP、GTP、NaCl、磷酸肌酸(phosphocreatine)、肌酸激酶(creatine kinase)、链球菌溶血素O(streptolysin O, SLO)和EpiJET Bisulfite Conversion Kit购自Sigma公司; 蛋白酶抑制剂混合液(protease inhibitor cocktail)购自Active Motif公司; MTT购自Amresco公司; PCR引物、逆转录试剂盒和实时定量

PCR试剂盒购自TaKaRa公司; 8 μm孔径Transwell小室购自Costar公司; 酶标仪购自Bio-Rad公司; 7500 Real-Time PCR Systems购自Applied Biosystem公司。

1.3 细胞培养

A549细胞培养于含10% FBS的RPMI 1640中, hUC-MSCs培养于含10% FBS的DMEM/F12中, 培养条件为37 ℃、5% CO_2饱和湿度, 常规换液和传代。

1.4 细胞提取物制备

收集细胞后用PBS和细胞裂解液各洗1次, 去上清后加1体积细胞裂解液重悬(一般而言, 1 μL提取物从10^5细胞中获得), 冰上裂解30~45 min。超声破碎仪破碎细胞, 直到细胞核完全破碎, 4 ℃、15 000 ×*g*离心15 min, 收集上清, 分装存于–80 ℃。细胞裂解液配方: 0.1 mol/L HEPES pH8.2、0.05 mol/L NaCl、5 μmol/L $MgCl_2$、1 μmol/L DTT、蛋白酶抑制剂(100×)、0.1 μmol/L PMSF、H_2O, 每250 μL裂解液中加入5 μL 0.5 mol/L EDTA(清洗过程使用不添加EDTA的裂解液)。

1.5 提取物处理通透细胞

1.5.1 细胞通透 A549细胞用无钙、镁离子的DPBS清洗, 以去除钙、镁离子, 用1 mL DPBS重悬约1×10^5细胞分装到预冷的1.5 mL离心管中, 于4 ℃、300 ×*g*离心5 min, 去上清, 用488 μL DPBS重悬细胞, 37 ℃水浴中孵育2 min, 然后加入12 μL链球菌溶血素O(0.5 μg/mL), 37 ℃水浴中孵育50 min。迅速将离心管置于冰中, 加入500 μL预冷的DPBS, 4 ℃、300 ×*g*离心5 min, 弃上清。

1.5.2 细胞处理 5 μL ATP再生系统与100 μL hUC-MSCs提取物混合, 加入到已通透A549细胞中, 对照组加入相同体积的DPBS。细胞于37 ℃水浴中孵育1 h。孵育结束后, 加入500 μL含有2 μmol/L $CaCl_2$的完全培养液, 修复通透细胞膜。将修复后的细胞移到24孔板中培养4 h后, 换新鲜的无$CaCl_2$培养液继续培养。ATP能量再生系统: ATP(0.1 mol/L)、磷酸肌酸(1 mol/L)、肌酸激酶(2.5 mg/mL)、GTP(0.01 mol/L)用1:1:1:1的体积混合。

1.6 MTT法检测细胞增殖

制备细胞悬液1×10^5/mL, 100 μL/孔接种于96孔培养板, 设6个平行孔, 分别培养24、48、72、96、120 h后加入MTT(5 mg/mL), 20 μL/孔, 4 h后弃上清, 每孔加入150 μL DMSO, 振荡10 min, 490 nm波长处测定各孔的吸光度。

1.7 平板克隆形成实验

收集细胞并计数, 100/孔接种于6孔板, 设3个平行孔, 37 ℃、5% CO_2培养2周。出现肉眼可见克隆时终止培养, PBS漂洗, 空气干燥, 甲醇固定15 min, 空气干燥; 4%结晶紫染色15 min, 空气干燥, 流水缓慢洗去染液, 空气干燥。在显微镜下对形成的克隆计数(≥50个细胞为一个克隆), 平板克隆形成率=形成克隆数/接种细胞数×100%。

1.8 Transwell小室检测细胞迁移能力

取3组细胞悬于无血清培养液中, 1×10^5/200 μL加到上室, 下室加600 μL含20%胎牛血清培养液。37 ℃、5% CO_2培养48 h, 用PBS洗3次, 用棉签擦去上室未迁移细胞。用95%乙醇固定10 min, 苏木素染色5 min, 流水冲洗, 盐酸酒精分化20 s, 流水冲洗, 0.5%氨水返蓝, 流水冲洗。随机选取5个不同视野计迁移细胞数。

1.9 实时定量PCR检测基因表达

提取细胞总RNA。取1 μg RNA反转录得到cDNA,

表1 实时定量PCR引物

Table 1 Real-time PCR primers

引物名称 Primer name	引物序列(5′→3′) Primers sequence (5′→3′)	基因序列号 GeneBank accession
Caspase-3	F: CAG TGG AGG CCG ACT TCT TG R: TGG CAC AAA GCG ACT GGA T	NM_004 346.3
Caspase-9	F: TGT CCT ACT CTA CTT TCC CAG GTT TT R: GTG AGC CCA CTG CTC AAA GAT	NM_001 229.4
Survivin	F: ACC ACC GCA TCT CTA C R: TCC TCT ATG GGG TCG T	NM_001 012 270.1
RUNX3	F: CAG CAC CAC AAG CCA CTT CA R: GGT CGG AGA ATG GGT TCA GTT	NM_004 350
GAPDH	F: TGT CCC CAC TGC CAA CGT GTC A R: GCG TCA AAG GTG GAG GAG TGG GT	NM_002 046

进行实时定量PCR反应, 反应体系为20 μL, 扩增反应程序为: 95 ℃预变性30 s; 95 ℃变性5 s, 60 ℃退火31 s, 72 ℃延伸15 s, 40个循环; 以*GAPDH*为内参, 采用$2^{-\Delta\Delta Ct}$法计算每个基因的相对mRNA表达量。各基因引物见表1。

1.10 亚硫酸氢盐测序

提基因组DNA。亚硫酸氢盐转换使用EpiJET Bisulfite Conversion Kit, 操作按试剂盒说明进行, 亚硫酸氢盐转换所用引物*RUNX3*-F: TAA TAA TGG TGG TGG ATA ATG GTA G(5′→3′); *RUNX3*-R: ACC CAA AAA ACA AAA CTA AAA AC(5′→3′)。PCR产物连入pEASY-T1载体, 每组最少选10个样本进行测序。

1.11 统计方法

每个实验设置3个平行组(n=3), 实验所得结果采用SPSS 23.0软件进行单因子方差分析。数据统计以mean±S.D.表示, P<0.05为具有显著性差异, P<0.01为具有极显著性差异。

2 结果

2.1 hUC-MSCs提取物处理对细胞增殖能力的影响

为检测细胞增殖能力, 利用MTT实验, 对hUC-MSCs提取物处理后A549进行了观察。1×10^5 hUC-MSCs制备成1 μL提取物, 取100 μL hUC-MSCs提取物处理经0.5 μg/mL SLO通透的1×10^{5}个A549细胞, 经测定蛋白浓度在2 500~3 000 ng/mL。结果表明, 与未处理组及缓冲液处理组相比, 从培养72 h开始, 实验组细胞增殖明显受到抑制; 培养96 h后, 实验组细胞增殖能力降低了34%; 培养120 h后, 实验组细胞增殖降低了45.97%(图1)。

2.2 hUC-MSCs提取物处理对细胞克隆形成能力的影响

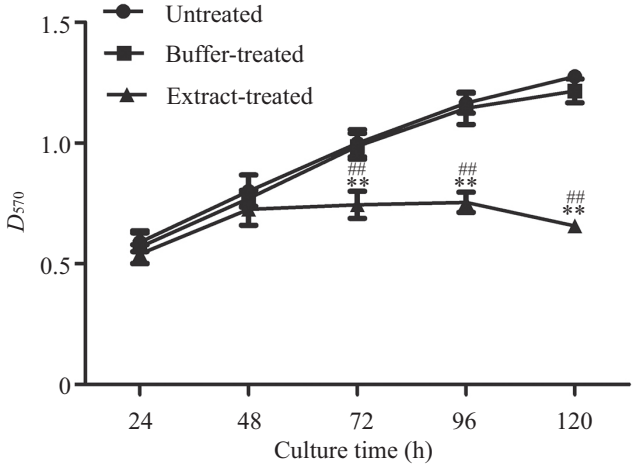
为检测细胞克隆形成能力, 利用平板克隆形成实验, 对提取物处理后的A549细胞进行了观察(观察方法同结果2.1)。结果表明, 2周后, 与未处理组及缓冲液处理组相比, 实验组克隆数减少了53.18%(图2)。

2.3 hUC-MSCs提取物处理对细胞迁移能力的影响

为检测细胞迁移能力, 利用Transwell方法对hUC-MSCs提取物处理后A549进行了观察(观察方法同结果2.1)。结果表明, 与未处理组及缓冲液处理组相比, 实验组穿膜细胞数减少了75.47%(图3)。

2.4 hUC-MSCs提取物处理对促凋亡、存活蛋白、抑癌相关基因的影响

为探究细胞增殖和迁移能力变化机制, 利用荧光定量PCR检测mRNA表达量的方法对hUC-MSCs提取物处理A549后进行了检测(观察方法同结果2.1)。结果表明, 24 h后, 与未处理组及缓冲液处理组相比, 实验组的促凋亡基因胱冬肽酶-3的相对表达量上升了36.37%, 胱冬肽酶-9相对表达量升高了2.44倍, 存活蛋白基因相对表达量降低了39.17%, 抑癌基因*RUNX3*相对表达量升高了3.17倍(图4)。

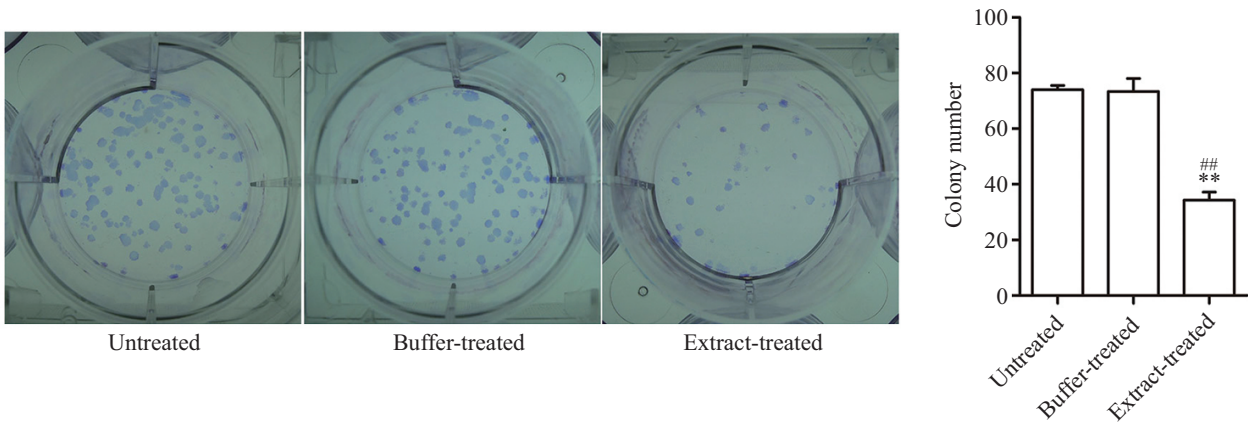


hUC-MSCs提取物处理后抑制了A549细胞的增殖。**P<0.01, 与未处理组比较; ##P<0.01, 与空白对照组比较。

Treatment with hUC-MSCs extracts inhibited proliferation of A549 cells. **P<0.01 compared with untreated group; ##P<0.01 compared with buffer-treated group.

图1 hUC-MSCs提取物处理A549后细胞增殖能力

Fig.1 Proliferation ability of A549 cells treated with hUC-MSCs extracts

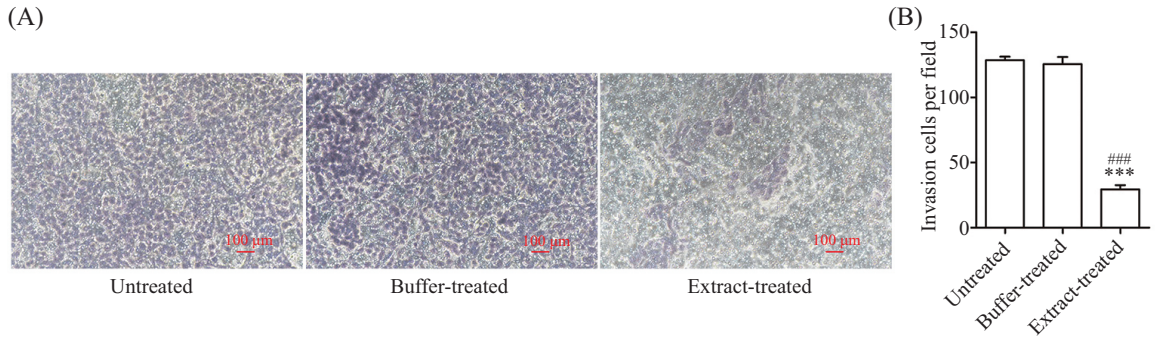


A: 克隆经结晶紫染色, hUC-MSCs提取物处理后抑制了A549克隆形成能力; B: 克隆计数, hUC-MSCs提取物处理后抑制了A549克隆形成数。**P<0.01, 与未处理组比较; ##P<0.01, 与空白对照组比较。

A: clonies are stained by crystal violet, treatment with hUC-MSCs extracts inhibited clony forming ability of A549 cells; B: counting the number of the clonies, treatment with hUC-MSCs extracts reduced the number of clones of A549 cells. **P<0.01 compared with untreated group; ##P<0.01 compared with buffer-treated group.

图2 hUC-MSCs提取物处理A549后细胞克隆的形成能力

Fig.2 Clone forming ability of A549 cells treated with hUC-MSCs extracts



A: A549细胞经苏木素染色, hUC-MSCs提取物处理后抑制了A549细胞的迁移能力; B: 迁移细胞计数, hUC-MSCs提取物处理后抑制了A549细胞迁移的数量。***P<0.001, 与未处理组比较; ###P<0.001, 与空白对照组比较。

A: A549 cells are stained by hematoxylin, treatment with hUC-MSCs extracts inhibited migration ability of A549 cells; B: counting the migration number of the A549 cells, treatment with hUC-MSCs extracts reduced the migration rate of A549 cells. ***P<0.001 compared with untreated group; ###P<0.001 compared with buffer-treated group.

图3 hUC-MSCs提取物处理A549后细胞迁移能力变化

Fig.3 Changes of migration ability of A549 cells treated with hUC-MSCs extracts

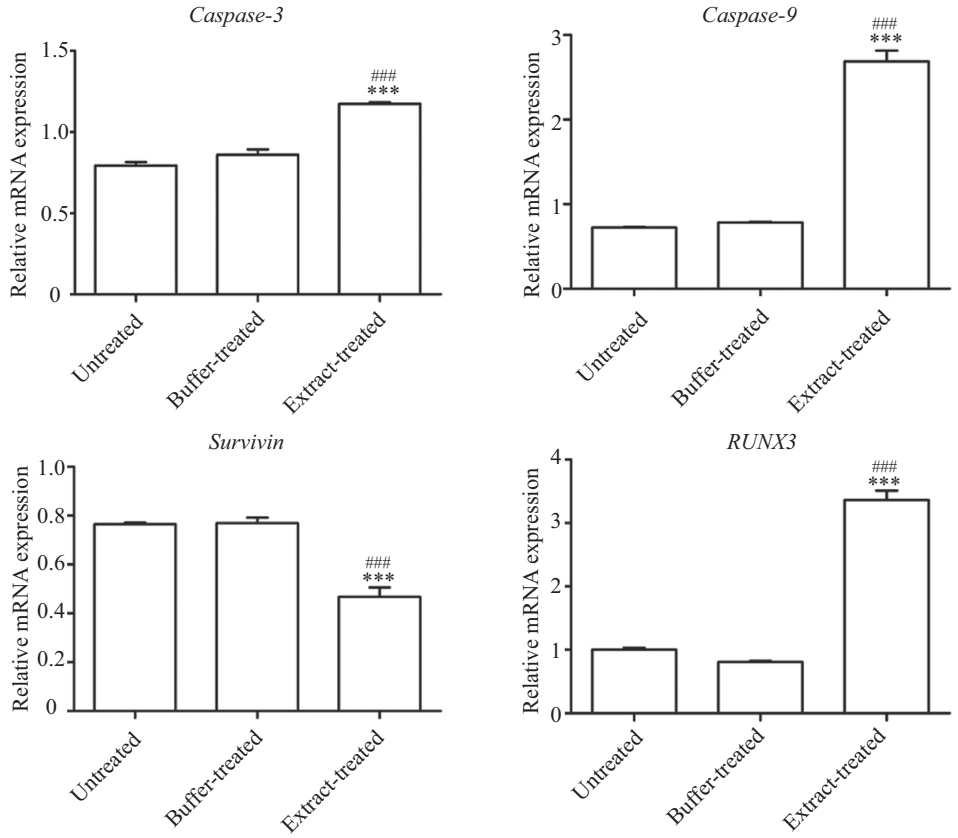
2.5 hUC-MSCs提取物处理对抑癌基因*RUNX3*启动子区CpG岛甲基化水平的影响

为探究细胞增殖和迁移能力变化机制, 利用亚硫酸氢盐测序的方法, 对hUC-MSCs提取物处理A549后进行了检测(观察方法同结果2.1)。结果表明, 24 h后, 与未处理组及缓冲液处理组相比, 实验组A549细胞中*RUNX3*启动子区CpG岛甲基化水平降低18.18%(图5)。

3 讨论

细胞提取物介导的重编程是近年新发展起来的一种安全、高效的重编程方法。此法无需转基因操作, 无诱发插入突变的危险。本研究使用hUC-MSCs提取物处理A549细胞后, 经MTT、平板克隆、Transwell等实验检测, 结果表明, hUC-MSCs提取物处理后, A549细胞的增殖能力下降了53.18%, 迁移能力降低了75.47%, 证明hUC-MSCs对A549的生长和迁移有抑制作用。近年来研究表明, hUC-MSCs可通过分泌物来激活各种信号通路, 还可在癌症治疗中作为病毒、纳米颗粒负载基因及药物的运载工具[11-13], 在癌症治疗中展现了广阔的应用前景。

荧光定量PCR结果表明, 经hUC-MSCs提取物处理后, 影响了促凋亡和抗凋亡基因的表达。凋亡信号通路主要分为外源性和内源性凋亡通路。在内

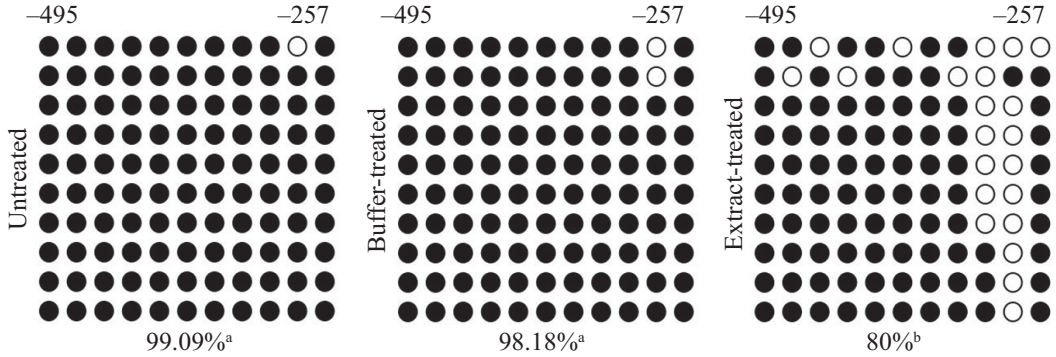


hUC-MSCs提取物处理A549后激活了胱冬肽酶-3、胱冬肽酶-9和*RUNX3* mRNA的表达, 抑制了存活蛋白mRNA的表达。***P<0.001, 与未处理组比较; ###P<0.001, 与空白对照组比较。

A549 cells treated with hUC-MSCs extracts activated the expression of *caspase-3*, *caspase-9* and *RUNX3*, and repressed the expression of *survivin* at mRNA level. ***P<0.001 compared with untreated group; ###P<0.001 compared with buffer-treated group.

图4 hUC-MSCs提取物处理A549后基因表达变化

Fig.4 Changes of gene expression of A549 cells treated with hUC-MSCs extracts



hUC-MSCs提取物处理后降低A549细胞中*RUNX3*启动子区CpG岛甲基化水平。图上数字表示*RUNX3*第1外显子上游CpG岛位置, 空心圈表示未甲基化的CpG位点, 实心圈表示甲基化的CpG位点; 每个CpG岛的甲基化CpG位点数/总CpG位点数作为该区域的甲基化率; 图下数字中, 右上标相同字母表示无显著差异(P>0.05), 不同字母表示有显著差异(P<0.05)。

A549 cells treated with hUC-MSCs extracts leads to demethylation of the promoter CpG islands of *RUNX3*. Number above the figure represents *RUNX3* CpG island from the first exon location. The open circles represent unmethylated CpG sites, and closed circles represent methylated CpG sites. The methylation level of each region is presented as the number of methylated CpG sites as a percentage of the total number of unmethylated and methylated CpG sites. Means that share the same superscript letter are not significantly different from each other (P>0.05); means with different superscript letters are significantly different (P<0.05).

图5 hUC-MSCs提取物处理A549后*RUNX3*启动子区CpG岛甲基化水平变化

Fig.5 Changes of methylation in CpG island of *RUNX3* promoter region in A549 cells treated with hUC-MSCs extracts

源性线粒体途径中, 凋亡信号刺激细胞后, 线粒体内的细胞色素c就会释放到胞质中, 当dATP存在时, 存在于胞质内的细胞色素c就会与凋亡蛋白酶激活因子-1(apoptotic protease activating factor-1, APAF-1)结合, 募集并激活胱冬肽酶-9, 随后胱冬肽酶-3活化, 胱冬肽酶级联反应启动, 造成细胞凋亡[14]。存活蛋白(survivin)是在肿瘤中表达一个重要抗凋亡蛋白, 而在正常组织中表达量很低, 被认为是重要的癌基因[15]。这种蛋白的表达与肿瘤的侵袭性生物学特性、抗辐射和化疗的耐药性及临床疗效差有关。本实验中, hUC-MSCs提取物处理A549后, 胱冬肽酶-3和胱冬肽酶-9的表达量均显著升高, 存活蛋白基因的表达量显著降低, 表明hUC-MSCs提取物可以有效增加A549的凋亡率。有研究表明, 存活蛋白主要通过抑制胱冬肽酶-3、胱冬肽酶-9等酶的活性而发挥阻断凋亡的作用[16]。细胞提取物介导的重编程可能通过抑制存活蛋白的表达来激活胱冬肽酶-3、胱冬肽酶-9等酶的活性来促使细胞凋亡。目前, 某些存活蛋白小分子抑制剂已经进入到肿瘤临床研究阶段[17], 对细胞提取物成分的分析可能为开发具有选择性的新型存活蛋白抑制剂提供思路。

本研究结果表明, 经hUC-MSCs提取物处理后, 影响了抑癌基因*RUNX3*的表达和启动子区CpG岛甲基化水平。*RUNX3*是重要的抑癌基因[18], 通过与TGF-β-catenin形成复合物和阻止其与靶启动子(如*c-Myc*和*cyclinD1*启动子)的结合来抑制肿瘤起始Wnt信号通路[19]。*RUNX3*表达下调参与了肺癌的发生、发展并与肺癌的浸润、分化和转移相关, 其失活的机制主要为基因启动子区CpG岛的高甲基化[20]。本研究中, hUC-MSCs提取物处理A549细胞后, *RUNX3*启动子区CpG岛显著去甲基化且其表达量显著上升。此外, Ruibal等[21]发现, 脑胶质细胞瘤*RUNX3*基因高甲基化与肿瘤分级成正相关。Kang等[22]发现, 前列腺癌中*RUNX3*基因甲基化是其癌变过程中的早期事件。*RUNX3*基因甲基化情况的检测可能为癌症早期诊断、判断预后和判断肺癌恶性程度提供一种新手段。

综上所述, hUC-MSCs提取物可能通过逆转*RUNX3*启动子区甲基化态恢复其表达, 激活胱冬肽酶-3、胱冬肽酶-9并抑制存活蛋白基因的表达来抑制A549的增殖及迁移能力。本研究为hUC-MSCs提取物对癌细胞的作用机制研究奠定基础, 为癌症治疗提供新的药物资源和新的方法。然而, hUC-MSCs提取物中具体的活性作用分子值得进一步深入研究。

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