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·基础研究·

低氧诱导因子-1α对骨髓间充质干细胞成骨分化与血管生成相关因子的影响

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【摘要】　目的　探讨低氧诱导因子-1α(hypoxia-inducible factor-1α，HIF-1α)在SD大鼠骨髓间充质干细胞(bone marrow mesenchymal stem cells，BMSCs)中对成骨分化和血管生成相关细胞因子的影响。**方法**　分离并培养BMSCs，采用流式细胞术进行鉴定；分别构建上调和下调HIF-1α基因的质粒载体及对照组质粒，使用Lipofectamine®LTX转染试剂将各组质粒分别转染至BMSCs，将细胞分为过表达实验组、过表达对照组、低表达实验组和低表达对照组。各组成骨诱导3、7 d后分别进行茜素红染色；RT-PCR检测目的基因HIF-1α，成骨分化特异标志物Runt相关转录因子2(Runt-related transcription factor 2，Runx2)，以及血管生成特异标志物血小板衍生生长因子-BB(platelet derived growth factor-BB，PDGF-BB)、转化生长因子-β(transforming growth factor-β，TGF-β)的mRNA表达水平；用Western blot检测目的蛋白HIF-1α及Runx2、PDGF-BB的蛋白表达量。**结果**　流式鉴定BMSCs表面标记物CD29、CD45阳性表达率分别为98.2%和4.2%；RT-PCR结果显示：过表达实验组BMSCs中HIF-1α、Runx2、TGF-β和PDGF-BB的mRNA表达显著升高($P < 0.001$)；低表达组中HIF-1α、Runx2、TGF-β和PDGF-BB的mRNA表达显著降低($P < 0.001$)；Western blot结果显示：过表达实验组BMSCs中HIF-1α、Runx2和PDGF-BB的蛋白表达量均增高($P < 0.001$)，低表达实验组中HIF-1α、Runx2和PDGF-BB蛋白表达量降低($P < 0.001$)。茜素红染色结果显示，低表达实验组钙结节面积小于其对照组，过表达实验组红色钙结节面积显著大于其对照组，随着成骨诱导时间的增加，各组钙化面积也增大。**结论**　上调和下调HIF-1α后可调控BMSCs的成骨分化及血管生成相关因子表达。

【关键词】　低氧诱导因子-1α；　骨髓间充质干细胞；　成骨分化；　血管生成；　Runt相关转录因子2；　血小板衍生生长因子-BB；　转化生长因子-β；　磷脂酰肌醇3-激酶；　蛋白激酶B；　骨组织工程

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Effects of hypoxia inducible factor-1α on osteogenic differentiation and angiogenesis related factors of bone marrow mesenchymal stem cells

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【Abstract】　Objective　To investigate the level of hypoxia inducible factor-1α (HIF-1α) on osteoblasts and angiogenesis-associated cytokines in bone marrow mesenchymal stem cells (BMSCs) from SD rats. **Methods**　BMSCs were isolated and cultured and identified by flow cytometry. Plasmid vectors containing upregulated and downregulated HIF-1α gene and a control vector were constructed. The plasmids were transfected into BMSCs by Lipofectamine®LTX trans-

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fection reagent, and the cells were divided into an overexpression experimental group, an overexpression control group, a low expression experimental group and a low expression control group. All components were stained with a lizarin red 3 d and 7 d after osteogenesis induction. The mRNA expression levels of the target gene HIF-1α, osteogenic differentiation-specific markers, including Runt-related transcription factor 2 (Runx2) and angiogenic markers, including platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β (TGF-β), were detected by RT-PCR. Western blot was used to detect the protein expression of the target proteins HIF-1α, Runx2, and PDGF-BB. **Results** The CD29- and CD45-positivity rates of BMSC surface markers identified by flow cytometry were 98.2% and 4.2%, respectively. RT-PCR results showed that the mRNA expression of HIF-1α, Runx2, TGF-β and PDGF-BB was observably increased (P < 0.001). The mRNA expression levels of HIF-1α, Runx2, TGF-β and PDGF-BB in BMSCs from the low expression experimental group were significantly reduced (P < 0.001). Western blot results showed that the expression levels of HIF-1α, Runx2 and PDGF-BB in BMSCs from the overexpression experimental group were all increased (P < 0.001). The expression levels of HIF-1α, Runx2 and PDGF-BB in BMSCs from the low expression experimental group were reduced (P < 0.001). Alizarin red staining results showed that the area of calcium nodules in the low expression experimental group was smaller than that in low expression control group, the area of red calcium nodules in the over expression experimental group was larger than that in over expression control group, and with the increase of osteogenic induction time, the calcification area of each group also increased. **Conclusion** Upregulation and downregulation of HIF-1α can regulate the osteogenic differentiation and the expression of angiogenesis related factors of BMSCs.

【Key words】 hypoxia inducible factor-1α; bone marrow mesenchymal stem cells; osteogenic differentiation; angiogenesis; Runt-related transcription factor 2; platelet derived growth factor-BB; transforming growth factor-β; phosphatidylinositol 3-kinase; protein kinase B; bone tissue engineering

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组织工程骨为解决骨组织的缺损和修复提供了新的方向，其中种子细胞、细胞因子与支架材料是构成组织工程骨的三大要素。骨髓间充质干细胞（bone marrow mesenchymal stem cells，BMSCs）具有多向分化和自我更新的功能，可作为理想的种子细胞[1-2]，成熟的基因治疗技术也为细胞构建更加稳定的生长环境，延缓其老化过程，促使细胞增殖能力和分化能力等提供了可靠的方法[3]。低氧诱导因子-1α（hypoxia-inducible factor-1α，HIF-1α）通过调控其编码相对应的生长因子产物，可以直接或者间接参与血管发生的整个过程。研究发现在骨缺损修复过程中HIF-1α也发挥着调控作用，其通过不同途径诱导成骨因子的生成，进而促进缺损部位血管生成[4]。利用RNA干扰（RNAi）技术使目的基因表达沉默，具有高特异性[5]。

本研究通过体外实验上调和下调BMSCs中的HIF-1α的表达，观察各组BMSCs中的成骨和血管生成相关因子的变化，探究HIF-1α对BMSCs成骨分化和血管生成相关因子表达的影响，为HIF-1α在骨组织工程中的应用提供实验依据。

1 材料与方法

1.1 主要材料和仪器

HIF-1α过表达质粒和低表达质粒（由上海吉凯基因科技有限公司提供）；清洁级SD大鼠，雌雄不限，体重（100 ± 10）g，由新疆医科大学动物实验中心提供，许可证号：SCXK（新）2016-0003。

主要试剂：MEM basic（1 ×）培养基（Gibco，美国）；胎牛血清（BI，美国）；0.25%胰蛋白酶（HyClone，美国）；质粒提试剂盒（BIOMIGA，美国）；限制性内切酶（Thermo，美国）；Lip LTX转染试剂盒（Invitrogen，美国）；茜素红染色液（北京索莱宝公司，中国）；引物序列（上海生物工程公司，中国）；QuantiNova®SYBR®Green PCR Kit试剂盒（QIAGEN，德国）；BCA蛋白定量试剂盒（Thermo，美国）；Rabbit-Anti-HIF-1α-antibody（Abcam，美国），Rabbit-Anti-Runx2-antibody（Abcam，美国），Rabbit - Anti-PDGF-BB-antibody（Abcam，美国），Rabbit-Anti-GAPDH-antibody（Abcam，美国）。主要仪器：CO_2恒温培养箱（371型，Thermo，美国）；荧光定量PCR仪（CFX96，Bio-RAD，美国）；高速离心机（5810R，Ep-

pendorf，德国）；western-blot电泳装置（PowerPacHC，Bio-RAD，美国）；酶标仪（Multiskan Go，Thermo，美国）；激光共聚焦显微镜（TCS-SP8 SR，Leica，德国）。

1.2　实验方法

1.2.1　质粒提取　根据GenBank中HIF-1α的基因序列，参照shRNA设计原则进行在线分析设计。后为获取更多质粒，将HIF-1α过表达质粒和筛选出的低表达质粒转化入大肠杆菌感受态细胞中，将菌落分别接种于含有卡那霉素和氨苄霉素的SOC培养基中。依据质粒小体试剂盒的说明书提取质粒，并检测浓度，用BamHI内切酶将质粒单切后进行电泳实验，测试目的条带，送往吉凯公司进行测序。

1.2.2　细胞分离培养与鉴定　将SD大鼠采用颈椎脱臼处死法，处死后放入75%（mL/mL）乙醇中浸泡5 min后分离出股骨与肱骨，剪开骨骺两端冲出骨髓，充分吹打混匀至悬液后离心。弃上清，管内加入含15%胎牛血清、链霉素和青霉素的MEM完全培养基，分装至细胞培养瓶内放入培养箱中培养。以后每2～3 d更换培养基，待细胞汇合度至85%时，用0.25%胰蛋白酶消化1∶2传代，细胞传至第3代加入荧光抗体上流式细胞仪进行鉴定。

1.2.3　质粒转染至BMSCs　取第3代细胞，以密度为5×10^5个/孔接种于6孔板内，待细胞汇合度达90%左右时，按课题组前期已证实安全有效的转染剂量，使用Lipofectamine®LTX转染试剂将各组质粒分别转染至BMSCs。转染后48 h，使用激光共聚焦显微镜观察荧光表达量，计算转染率（转染率 = 镜下荧光细胞数/总细胞数 × 100%）。按照转染质粒的不同将细胞分为过表达实验组、过表达对照组、低表达实验组和低表达对照组。

1.2.4　茜素红染色　BMSCs转染48 h后，换液为成骨诱导液（α-MEM+10% FBS+10 nmol/L β甘油酸钠+100 nmol/L地塞米松+50 μmol/L VitaminC），每3 d换液一次，成骨诱导3 d和7 d后分别进行染色。PBS洗涤2遍，4%多聚甲醛固定30 min，ddH_2O洗3遍，1%茜素红染液染色20～30 min，ddH_2O洗3遍后显微镜下观察钙结节并拍照。

1.2.5　RT-PCR检测相关基因mRNA　用Trizol法裂解转染后的各组细胞，提取总RNA，以2 μg反转20 μL体系反转录为cDNA，后用RT-PCR试剂盒检测成骨分化特异标志物Runt相关转录因子2（Runt-related transcription factor 2，Runx2），以及血管生成特异标志物血小板衍生生长因子-BB（platelet derived growth factor-BB，PDGF-BB）、转化生长因子-β（transforming growth factor-β，TGF-β）的mRNA表达量。所用引物序列见表1。检测结果通过公式$X = 2^{-\Delta\Delta Ct}$计算各实验组目的基因较对照组的相对表达水平。

表1　RT-PCR引物序列

Table 1　Synthetic primers used for RT-PCR

Primers	Sequence
GAPDH	F:5'-GGCACAGTCAAGGCTGAGAATG-3' R:5'-ATGGTGGTGAAGACGCCAGTA-3'
HIF-1α	F:5'-CACCGCAACTGCCACCACTG-3' R:5'-TGAGGCTGTCCGACTGTGAGTAC-3'
Runx2	F:5'-CCATAACGGTCTTCACAAATCCT-3' R:5'-TCTGTCTGTGCCTTCTTGGTTC-3'
PDGF-BB	F:5'-TCTCTGCTGCTACCTGCGTCTG-3' R:5'-AAGGAGCGGATGGAGTGGTCAC-3'
TGF-β	F:5'-GGCACCATCCATGACATGAACCG-3' R:5'-GCCGTACACAGCAGTTCTTCTCTG-3'

HIF-1α: hypoxia inducible factor-1α; Runx2: runt-related transcription factor 2; PDGF-BB: platelet derived growth factor-BB; TGF-β: transforming growth factor-β

1.2.6　Western blot检测相关蛋白　在转染48 h后分别提取实验组和对照组的总蛋白，胰蛋白酶消化后至离心管内，用PBS洗涤三遍，视细胞量的多少加入RIPA裂解液，在冰上将细胞充分裂解30 min。后离心（4 ℃，12 000 r，5 min），取上清，加入5 × Loading Buffer在100 ℃下煮沸10 min。电泳后，转膜，脱脂奶粉封闭各2 h，一抗稀释后（GAPDH稀释比例为1∶10 000，HIF-1α、Runx2和PDGF-BB稀释比例为1∶2 000）分别加入，放置摇床，4 ℃孵育过夜。1 × TBST清洗3遍，加入二抗室温下孵育1 h，洗后加入AP显色液，使用Image lab version分析上述蛋白结果。

1.3　统计学分析

运用SPSS 24.0统计软件分析所得实验数据，计量资料符合正态性，两组间比较采用独立样本t检验，$P < 0.05$为差异具有统计学意义，使用GraphPad Prism软件绘制统计图。

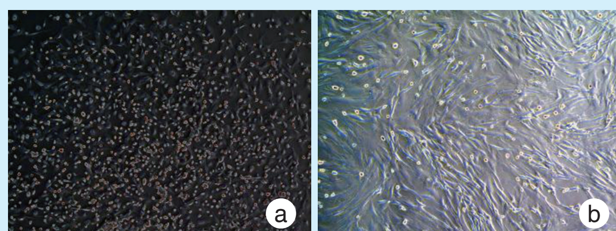
2　结　果

2.1　质粒提取

分别将过表达和低表达组质粒用BamHI酶切后，过表达实验组、低表达实验组的条带大小分别为：7.8 kb、6.4 kb。与吉凯公司设计结果一致。

2.2 BMSCs培养和鉴定

原代细胞首次换液后镜下观察呈圆形，传至P3代可见细胞呈贴壁生长的长梭形、旋涡状，见图1。流式细胞术鉴定：间充质干细胞抗原CD29阳性率为98.2%；表面阴性标记物：造血干细胞抗原CD45阳性率为4.2%。以上结果均符合间充质来源的干细胞特征。



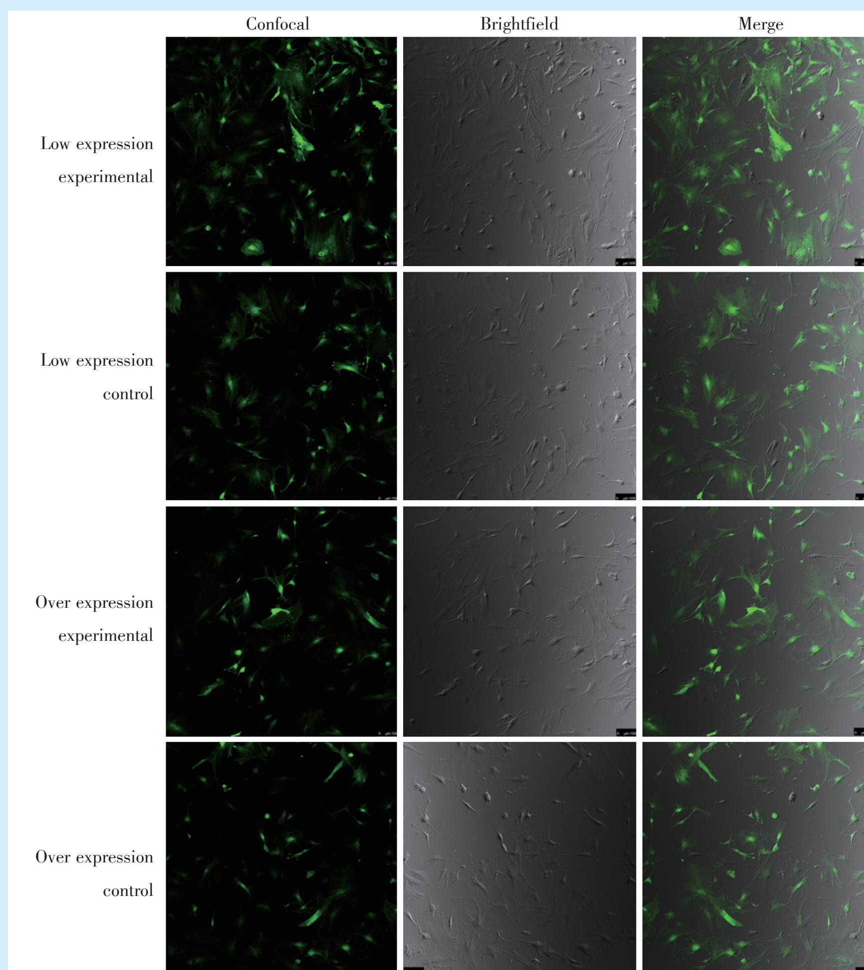
a: the primary cells were round; b: P3 cells, the cells adhered to the wall and grew in long fusiform or whirlpool shape; BMSCs: bone marrow mesenchymal stem cells

Figure 1 Cell culture of BMSCs(× 50)

图1 BMSCs的培养(× 50)

2.3 细胞转染率

转染48 h后激光共聚焦显微镜下可见，成功转染质粒的BMSCs呈绿色荧光表达，此时细胞生长密度约75%；低表达实验组表达量约为76%，对照组约70%；过表达实验组荧光数量为73%，对照组约68%。见图2。



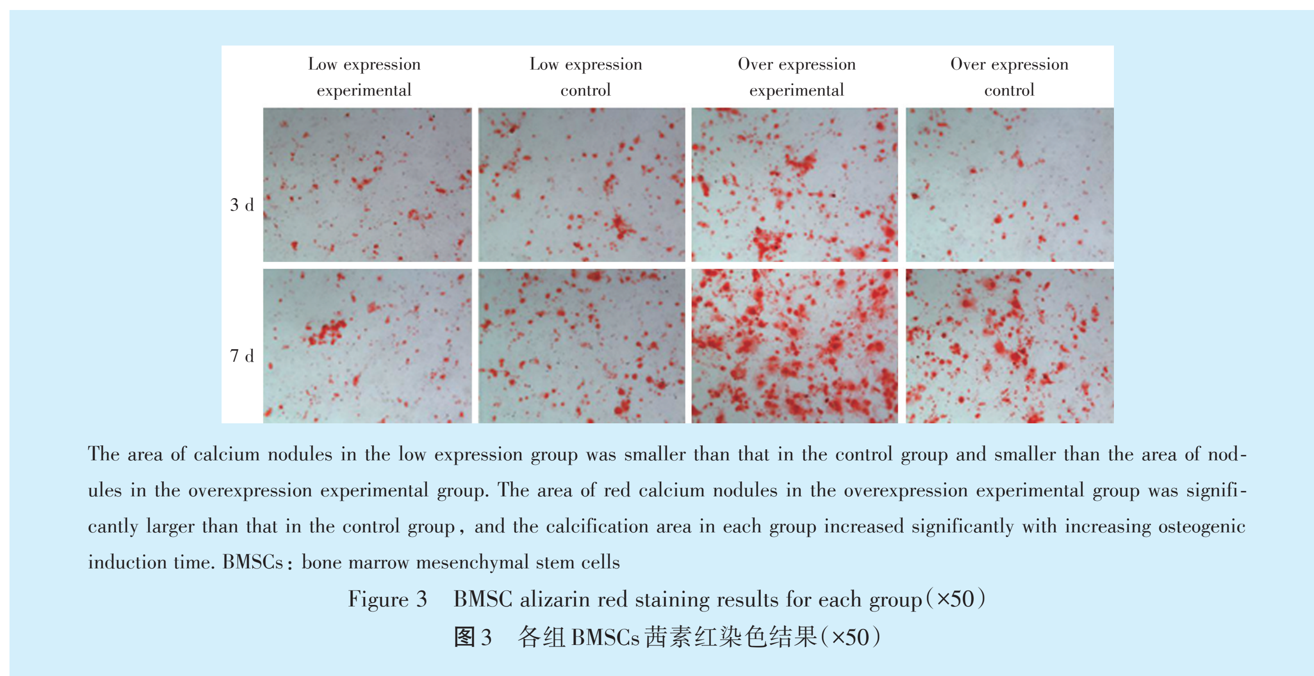
BMSCs that were successfully transfected with DNA plasmid showed green fluorescence expression, and the cell growth density was approximately 75%. The expression level of the low expression experimetal group was approximately 76%, and that of the control group was approximately 70%. The fluorescence level of the overexpression experimetal group was 73%, and that of the control group was approximately 68%. BMSCs: bone marrow mesenchymal stem cells

Figure 2 BMSC transfected with plasmid after 48 h (× 100)

图2 质粒转染BMSCs 48 h后(× 100)

2.4 茜素红染色结果

各组成骨诱导3 d和7 d后茜素红染色显示：低表达实验组钙结节面积小于其对照组，也少于同一时间段过表达组的结节数量；过表达实验组红色钙结节面积大于其对照组，随着成骨诱导时间的增加，各组钙化面积也增大（图3）。



The area of calcium nodules in the low expression group was smaller than that in the control group and smaller than the area of nodules in the overexpression experimental group. The area of red calcium nodules in the overexpression experimental group was significantly larger than that in the control group, and the calcification area in each group increased significantly with increasing osteogenic induction time. BMSCs: bone marrow mesenchymal stem cells

Figure 3 BMSC alizarin red staining results for each group (×50)

图3 各组BMSCs茜素红染色结果（×50）

2.5 骨分化与血管生成相关因子mRNA结果

过表达实验组中HIF-1α的mRNA表达量高于其对照组（$P < 0.001$），低表达实验组中HIF-1α的表达减低（$P < 0.001$）；结果显示：低表达组中Runx2、PDGF-BB和TGF-β的mRNA表达显著降低（$P < 0.001$）；过表达组中Runx2、PDGF-BB和TGF-β的mRNA表达水平显著高于其对照组（$P < 0.001$），差异均具有统计学意义（表2）。

表2 各组成骨分化与血管生成相关因子mRNA表达

Table 2 mRNA expression of osteogenic differentiation and angiogenesis related factors $\bar{x} \pm s$

Gene names	Low expression experimental group	Low expression control group	t	P	Over expression experimental group	Over expression control group	t	P
HIF-1α	0.373 ± 0.020	1.023 ± 0.231	22.522	< 0.001	1.563 ± 0.128	1.013 ± 0.171	0.896	< 0.001
Runx2	0.618 ± 0.012	1.025 ± 0.229	32.776	< 0.001	25.462 ± 0.406	1.023 ± 0.204	6.278	< 0.001
PDGF-BB	0.097 ± 0.011	1.009 ± 0.155	16.531	< 0.001	3.322 ± 0.111	1.009 ± 0.147	1.705	< 0.001
TGF-β	0.447 ± 0.013	1.023 ± 0.222	9.203	< 0.001	1.734 ± 0.016	1.006 ± 0.112	17.389	< 0.001

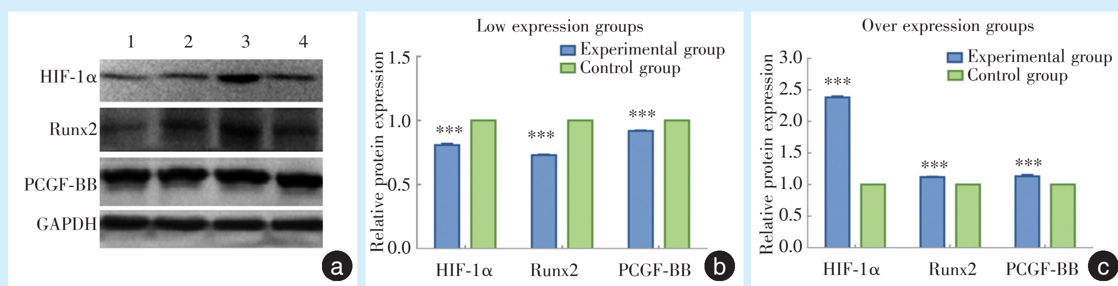
HIF-1α: hypoxia inducible factor-1α; Runx2: runt-related transcription factor 2; PDGF-BB: platelet derived growth factor-BB; TGF-β: transforming growth factor-β

2.6 骨分化与血管生成相关因子Western blot结果

过表达实验组中HIF-1α的蛋白表达高于其对照组（$P < 0.001$），低表达实验组中HIF-1α的表达低于其对照组（$P < 0.001$）；结果显示：低表达实验组中Runx2，PDGF-BB的表达显著降低（$P < 0.001$），过表达实验组中Runx2，PDGF-BB的表达显著增高（$P < 0.001$），差异具有统计学意义。（图4）

3 讨 论

新骨再生和血管生成是骨组织工程中修复骨缺损的两个基本环节，研究表明，HIF-1α途径的激活可以作为血管生成的关键介质，而血管生成正是骨骼再生所必需的[6]。本研究选用HIF-1α作为细胞因子探讨其在组织工程骨中的应用。本实验茜素红染色结果显示，HIF-1α的表达水平与BMSCs钙结节形成面积成正比，表明了HIF-1α水平对



1: low expression experimental group; 2: low expression control group; 3: over expression experimental group; 4: over expression control group; HIF-1α: hypoxia inducible factor-1α; Runx2: runt-related transcription factor 2; PDGF-BB: platelet derived growth factor-BB; TGF-β: transforming growth factor-β; $^{***}P < 0.001$

Figure 4 Protein expression of osteogenic differentiation and angiogenesis related factors

图4 各组成骨分化与血管生成相关因子表达

BMSCs的成骨能力有重要的影响。

磷脂酰肌醇3-激酶(phosphatidylinositol 3-kinase,PI3K)/蛋白激酶B(protein kinase B,AKT)作为与HIF-1α密切相关的信号通路,其传导途径是一种原型存活途径,其中Akt是下游靶标,可在细胞生长或存活中起重要作用。激活后Akt可能通过抗凋亡和促凋亡底物的磷酸化产生抗凋亡作用[7]。其在血管生成中起重要作用,并对正常细胞的增殖、黏附和迁移也起关键作用,能够直接或间接的参与血管生成的全过程。Xie等[8]研究发现通过受体酪氨酸激酶(receptor tyrosine kinase,RTK)激活PI3K/Akt途径后,可使HIF-1α的表达增强。

转化生长因子-β(transforming growth factor-β,TGF-β)可诱导血管内皮细胞和血管平滑肌的迁移和分化,既有血管抑制作用也有促血管生成活性,在血管生成中发挥双重作用[9-10]。TGF-β可同时调控成骨细胞与破骨细胞间的作用,促进细胞外基质及Ⅰ型胶原的合成,从而参与骨重建。本实验结果发现,BMSCs高表达HIF-1α后,TGF-β的mRNA与蛋白表达水平也显著增高,而HIF-1α低表达后TGF-β的mRNA与蛋白表达水平显著降低。Lv等[11]将HIF-1α干扰RNA质粒转染至小鼠NIH-3T3细胞以沉默内源性HIF-1α,结果显示沉默HIF-1α后细胞中TGF-β的表达没有增加。

PDGF-BB为一种贮藏在血小板内的碱性蛋白质,是间充质细胞迁移和增殖的关键调节因子[12],通过在血管周围细胞发挥作用从而促进血管的成熟。本实验结果显示,BMSCs过表达HIF-1α后显示PDGF-BB的mRNA和蛋白表达均增加。Pang等[13]在研究中发现PI3K/Akt信号通路参与了PDGF-BB诱导的气道平滑肌细胞增殖与迁移。Mermis等[14]将HIF-1α特异性siRNA敲低,结果显示HIF-1α转染细胞PDGF-BB在艾滋病病毒GP-120蛋白的存在下的表达显著降低,与本实验结果一致。

Runx2作为HIF-1α直接靶点,能够将间充质干细胞、成骨细胞和前成骨细胞中的相关成骨基因进行定向转录和翻译,可用于检测成骨分化的能力[15]。有报道指出HIF-1α上调可以增强Runx2的表达,Runx2参与体内成骨过程[16];Qu等[17]发现沉默HIF-1α的表达后可显着抑制Runx2的表达。本实验结果显示分别上调和下调BMSCs中的HIF-1α后,Runx2的mRNA和蛋白表达水平分别呈上升和下降趋势,这与Xu[16]的研究结果一致。

综上所述,HIF-1α作为促进成骨分化及血管生成能力的重要因子,通过调控Runx2、PDGF-BB、TGF-β的水平影响BMSCs的成骨分化及血管生成能力。

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