在体外模拟的骨微环境中下调BMP9对人乳腺癌SK-BR-3细胞增殖、迁移的影响及其可能机制

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摘要 探讨在体外模拟的乳腺癌骨转移微环境中, 采用RNAi技术下调骨形态发生蛋白9(bone morphogenetic protein 9, *BMP9*)基因对人乳腺癌SK-BR-3细胞增殖、迁移的影响及可能机制。用Transwell小室将空白组SK-BR-3细胞、感染腺病毒AdRFP的对照组SK-BR-3/RFP细胞、感染腺病毒AdsiBMP9的实验组SK-BR-3/siBMP9细胞分别与人骨髓基质HS-5细胞间接共培养后, MTT增殖实验、划痕愈合实验、Transwell迁移实验分别检测下调BMP9对SK-BR-3细胞增殖、迁移的影响; RT-PCR和Western blot法检测相关因子的变化。结果显示, 在共培养体系中, SK-BR-3/siBMP9组的BMP9显著低于对照组(P<0.05); 下调BMP9可有效促进SK-BR-3/siBMP9细胞增殖(P<0.05), SK-BR-3/siBMP9细胞的划痕愈合率、穿膜细胞数也明显升高(P<0.05); SK-BR-3/siBMP9组血管内皮细胞生长因子(VEGF)、结缔组织生长因子(CTGF) mRNA和蛋白表达均显著上调(P<0.05), 且Western blot结果显示p-AKT显著上调(P<0.05)。综上, 在骨转移微环境中, 干扰BMP9表达可促进人乳腺癌SK-BR-3细胞增殖及迁移, 其作用机制可能与上调VEGF、CTGF表达有关, 且这个过程可能涉及PI3K/AKT通路的激活。

关键词 骨形态发生蛋白9; 乳腺肿瘤; 骨微环境; 共培养

Effects of Down-regulating BMP9 on Proliferation and Migration of Human Breast Cancer SK-BR-3 Cells and Its Possible Mechanism in Simulated Bone Microenvironment *in vitro*

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Abstract To investigate the effects of siRNA-mediated down-regulation of human bone morphogenetic protein 9 (BMP9) on proliferation and migration of human breast cancer SK-BR-3 cells and its possible mechanism in simulated bone microenvironment *in vitro*, SK-BR-3 cells as blank group, SK-BR-3/RFP cells infected with adenovirus RFP as control group, and SK-BR-3/siBMP9 cells infected with adenovirus siBMP9 as experimental group were indirectly co-cultured with human bone marrow stromal cells HS-5 with Transwell chamber. Effects of down-regulating BMP9 on SK-BR-3 cell proliferation and migration were investigated by MTT, wound-healing test and

收稿日期: 2013-06-19 接受日期: 2013-09-05

国家自然科学基金(批准号: 81172017)和国家重点基础研究发展计划(973计划)(批准号: 2011CB707906)资助的课题

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Received: June 19, 2013 Accepted: September 5, 2013

This work was supported by the National Natural Science Foundation of China (Grant No.81172017) and the 973 Program of the Ministry of Science and Technology of China (Grant No.2011CB707906)

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网络出版时间: 2013-10-22 09:30 URL: http://www.cnki.net/kcms/detail/31.2035.Q.20131022.0930.002.html

transwell migration test; The expression of related factors were screened by RT-PCR and Western blot. The result showed that in the co-culture system, the expression of BMP9 was decreased in SK-BR-3/siBMP9 group (P<0.05); Down-regulating BMP9 could promote the proliferation of SK-BR-3/siBMP9 cells (P<0.05), and the wound-healing rate and the number of migratory cells were remarkably increased (P<0.05); Compared with the control group, the expressions of VEGF and CTGF were significantly up-regulated at mRNA and protein levels in SK-BR-3/siBMP9 group (P<0.05), and Western blot showed p-Akt was higher than the control group (P<0.05). All together, down-regulating BMP9 can promote the proliferation and migration of breast cancer cells SK-BR-3 in microenvironment of bone metastasis, which may be related to up-regulating the expressions of VEGF and CTGF, and PI3K/Akt pathway may be involved in this process.

Key words bone morphogenetic protein 9; breast tumor; bone microenvironment; co-culture

乳腺癌是女性最常见的恶性肿瘤之一, 极易发生溶骨性转移, 从而引发一系列病理性骨折, 其预后差, 致死率高。早在1889年, Paget等[1]就提出了著名的“土壤与种子”学说, 阐明了肿瘤细胞与肿瘤微环境的相互作用、相互促进的关系, 乳腺癌溶骨性转移就与骨微环境关系密切。骨微环境包括骨细胞、基质细胞、免疫细胞等, 它们与肿瘤细胞相互作用, 使转移的肿瘤细胞发生基因和表型改变, 并为其在骨组织的生长提供了适宜的土壤[2], 乳腺癌细胞和骨微环境相互作用, 从而导致溶骨性损伤。在这些相互作用中, 溶骨性损伤与乳腺癌细胞的恶性程度及间充质干细胞的成骨分化密切相关, 乳腺癌细胞能抑制间充质干细胞成骨分化, 间充质干细胞也能促进乳腺癌细胞的侵袭、转移[3]。

骨形态发生蛋白(bone morphogenetic proteins, BMPs)是一组可诱导骨和软骨形成的分泌性蛋白, 除BMP1外, 其余均属于转化生长因子-β(transforming growth factor-β, TGF-β)超家族成员。近年来发现BMPs也参与肿瘤生长、分化、转移等多种生物学行为[4]。目前, BMP9被认为是BMPs家族中成骨作用最强的成员, 发现较晚, 研究较少。有文献报导BMP9可以促进卵巢癌细胞增殖[5], 抑制前列腺癌细胞的生长、侵袭及迁移[6], 抑制骨肉瘤细胞增殖及迁移[7], 但BMP9对乳腺癌的生物学作用研究较少。本实验前期研究发现, 外源性BMP9能抑制乳腺癌MDA-MB-231细胞的生长、迁移、侵袭[8]及溶骨损伤[9], 并能抑制乳腺癌细胞与骨髓基质细胞的相互作用[10]。为了进一步确认BMP9对乳腺癌的作用, 本研究拟选用内源性BMP9高表达的人乳腺癌SK-BR-3细胞为研究对象, 利用基因干扰技术, 制备干扰BMP9的重组腺病毒并感染SK-BR-3细胞沉默其BMP9的表达, 并与人骨髓基质细胞HS-5构建体外共培养模型, 旨在探讨BMP9在乳腺癌骨转移微环境中的生物学作用, 为深入了解乳腺癌骨转移的分子生物学机制及开发新的治疗靶点奠定基础。

1 材料与方法

1.1 材料

1.1.1 *细胞与腺病毒* 人骨髓基质细胞HS-5购自美国模式培养物集存库(ATCC); 人乳腺癌细胞系SK-BR-3、腺病毒AdRFP由美国芝加哥大学分子肿瘤实验室何通川教授惠赠, 腺病毒AdsiBMP9由重庆医科大学检验医学院本实验室构建[11]。

1.1.2 *主要试剂* 高糖型DMEM培养液购自Hyclone公司; 胎牛血清购自Gibco公司; Transwell小室购自Millipore公司; MTT试剂购自Solarbio公司; 反转录试剂盒购自TaKaRa公司; 引物由宝生物工程(大连)有限公司合成; Western blot及蛋白质提取相关试剂购自上海碧云天公司; VEGF抗体、β-actin抗体购自Santa Cruz公司; CTGF抗体购自Abcam公司; AKT抗体和p-AKT抗体购自Cell Signaling公司。

1.2 方法

1.2.1 *细胞培养* SK-BR-3细胞、HS-5细胞分别用含10% Hyclone及10% Gibco胎牛血清的高糖DMEM培养基培养, 内含100 U/mL青霉素和100 μg/mL链霉素。当细胞融合率达70%~80%时, 用0.25%胰酶消化, 传代, 置于37 °C、5% CO_2的细胞培养箱中培养。

1.2.2 *腺病毒感染及共培养体系建立* 将SK-BR-3细胞按1.2×10^5/孔接种于六孔板, 8 h后细胞贴壁, 加入滴度均为1×10^{10} IU/mL的AdsiBMP9或AdRFP重

组腺病毒0.3 μL/孔直接感染SK-BR-3细胞, 同时将HS-5细胞按1.0×10^5/室接种于Transwell小室。8~12 h待HS-5细胞贴壁后, 将SK-BR-3细胞和HS-5细胞均换新鲜无血清培养基, 将Transwell小室悬挂于六孔板上, 共培养开始。将共培养体系置于培养箱中, 腺病毒感染36 h后在荧光显微镜下观察病毒感染率, 共培养3天后提取下室SK-BR-3细胞的总RNA和总蛋白, 期间不换液, 小室膜孔径为0.4 μm, 上下室能相互通透。实验组: SK-BR-3/siBMP9+HS-5, 对照组: SK-BR-3/RFP+HS-5, 空白组: SK-BR-3+HS-5。

1.2.3 MTT法检测细胞增殖能力 设AdsiBMP9实验组、AdRFP阴性对照组、空白对照组三组, 每组设6个平行孔。共培养1~4 d后分别检测MTT, 将共培养体系取出, 避光加入MTT(5 mg/mL) 500 μL/孔, 继续孵育4 h, 终止培养; 吸弃孔内培养, 每孔加入3.75 mL DMSO, 震荡混匀10 min至结晶物充分溶解; 吸取200 μL/孔溶解物移至96孔板, 在酶联免疫检测仪上波长492 nm处检测各孔的光密度(*D*)值。

1.2.4 划痕实验检测细胞横向迁移能力 待共培养体系中SK-BR-3细胞汇合达80%时加入腺病毒, 同时在小室中接种HS-5。8~12 h后吸弃六孔板中培养基, 用自制Marker笔划痕, PBS洗2次, 加入新鲜无血清培养基, 于倒置显微镜下摄片, 沿划痕边缘等间距取三处测量划痕宽度, 取均值。HS-5换新鲜无血清培养基, 并将小室悬挂于六孔板上，共培养开始。分别在12 h、24 h取出六孔板摄片, 在相同观察点测量划痕宽度。划痕愈合率(%)=(0 h划痕宽度–24 h划痕宽度)/0 h划痕宽度×100%。

1.2.5 Transwell实验细胞纵向迁移能力 将共培养体系中各组SK-BR-3细胞用胰酶消化, 无血清培养基重悬。将含有3×10^{4}细胞的200 μL细胞悬液加入上室, 下室中加入700 μL含20%胎牛血清的培养基, 每组设置3个复孔, 于37 ℃、5% CO_2条件下培养24 h。取出Transwell小室, 湿棉签拭去小室膜上未穿膜的细胞, 自然风干至保持膜表面稍湿润即可, 用结晶紫染液染色20 min。低倍镜随机选取5个视野计数穿膜细胞数目, 取平均值。

1.2.6 反转录聚合酶链反应(RT-PCR)检测相关基因变化 Trizol法提取共培养体系中SK-BR-3细胞总RNA, 取2 μg总RNA反转录合成cDNA, 以1 μL cDNA为模板进行PCR反应; PCR条件: 预变性94 ℃, 5 min; 变性94 ℃, 30 s, 退火52~58 ℃, 30 s, 延伸72 ℃, 30 s, 29~34个循环; 最后延伸72 ℃, 10 min (引物序列见表1)。

1.2.7 Western blot检测相关蛋白变化 提取共培养体系中SK-BR-3细胞总蛋白, 检测蛋白浓度, 加热变性后于–20 ℃保存。取50 μg蛋白上样进行SDS-PAGE凝胶电泳, 将分离后的蛋白用湿转法转膜至PVDF膜上, 5%牛血清白蛋白37 ℃封闭1.5 h, 依次结合相应一抗(1:1 000) 4 ℃孵育过夜, TBST洗膜10 min, 重复3次后加入辣根过氧化物酶标记的相应二抗(1:5 000) 37 ℃孵育1 h, TBST洗膜10 min, 重复3次后加入化学发光显色液成像, 检测相应蛋白条带。

1.2.8 数据统计 所有实验独立重复3次, RT-PCR及Western blot结果均用Quantity One进行灰度分析, 用目的基因吸光度值/内参吸光度值的均数±标准差表示, 统计软件为SPSS 17.0, 采用student′s *t*检验及方差分析进行统计学分析, $P<0.05$表示差异具有统计学意义。

表1 RT-PCR引物序列及产物长度

Table 1 Sequence of primers and product length

基因 Gene	引物序列 Sequence of primers	产物长度 Product length
GAPDH	Forward: 5′- CAG CGA CAC CCA CTC CTC-3′	120 bp
	Reverse: 5′- TGA GGT CCA CCA CCC TGT-3′	
BMP9	Forward: 5′- CTG CCC TTC TTT GTT GTC TT-3′	322 bp
	Reverse: 5′- CCT TAC ACT CGT AGG CTT CAT A-3′	
VEGF	Forward: 5′- GAT GTC CAC CAG GGT CTC-3′	150 bp
	Reverse: 5′- CTT GCC TTG CTG CTC TAC-3′	
CTGF	Forward: 5′- GCG GCT TAC CGA CTG GA-3′	170 bp
	Reverse: 5′- AGG CGG CTC TGC TTC TC-3′	

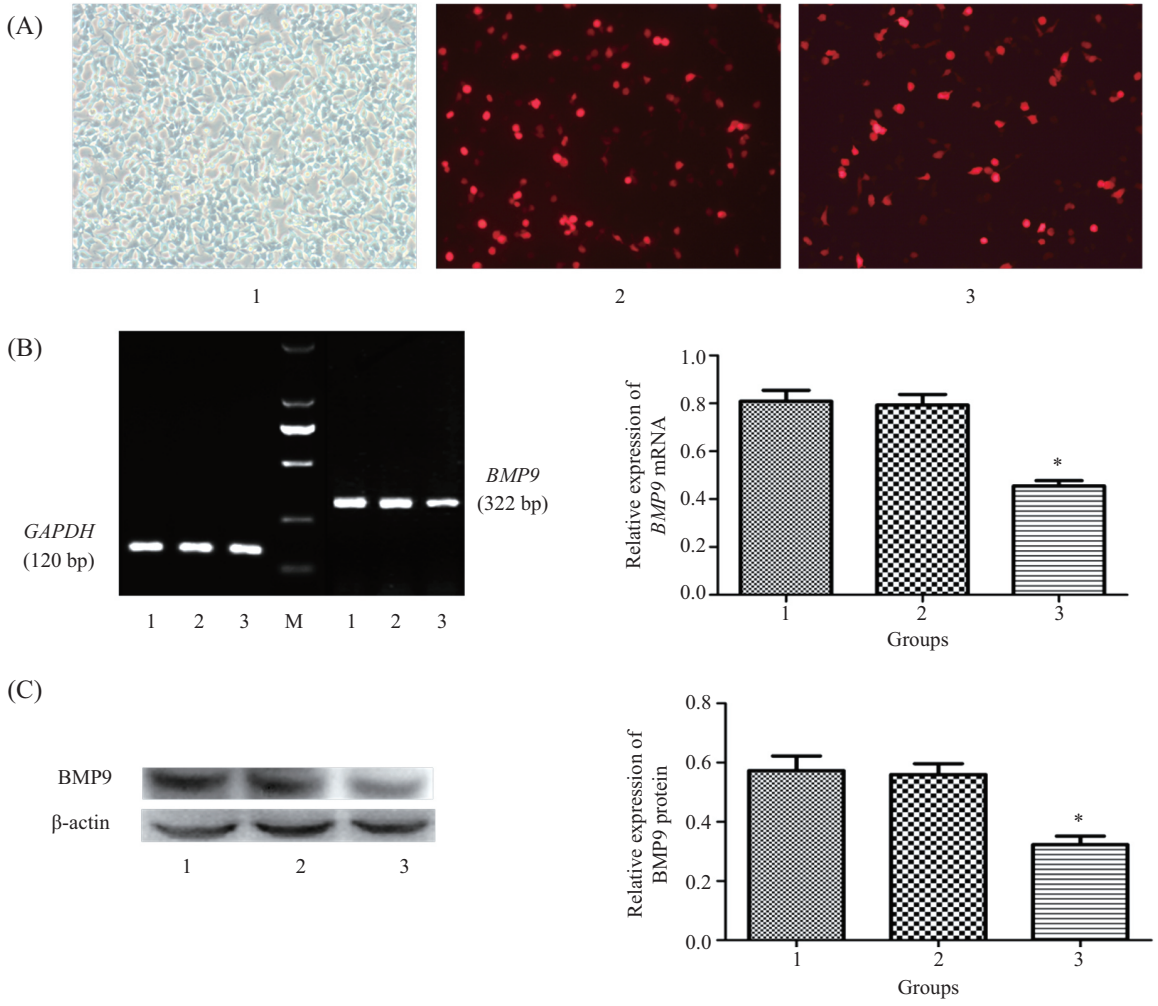
2 结果

2.1 共培养体系中AdsiBMP9腺病毒有效下调SK-BR-3内源性BMP9的表达

荧光显微镜下检测感染腺病毒36 h后, 共培养体系中空载病毒AdRFP及实验组病毒AdsiBMP9在SK-BR-3中感染效率一致, 均约为40%(图1A)。共培养3天后提取共培养体系中SK-BR-3细胞的总RNA和总蛋白, RT-PCR在mRNA水平上检测共培养体系中各组SK-BR-3的*GAPDH*表达基本一致, SK-BR-3/siBMP9+HS-5组的*BMP9*表达显著低于对照组(P<0.05)(图1B)。Western blot在蛋白水平上进一步证实腺病毒AdsiBMP9能有效下调SK-BR-3内源性BMP9的表达(P<0.05)(图1C)。

2.2 共培养体系中干扰BMP9表达促进SK-BR-3细胞增殖

每天同一时间点采用MTT法检测光密度(D)值, 共培养体系建立的第3天测得SK-BR-3/siBMP9+HS-5组的D值为(0.937±0.031), 明显高于SK-BR-3/RFP+HS-5组(0.752±0.026)(P<0.05), 而SK-BR-3/RFP+HS-5组与SK-BR-3+HS-5组(0.786±0.028)无显著性差异, 说明SK-BR-3/siBMP9+HS-5组的细胞增殖率在第3天即明显高于对照组(P<0.05)。第4天, SK-BR-3/siBMP9+HS-5组的细胞增殖率也显著高于对照组(P<0.05)(图2)。

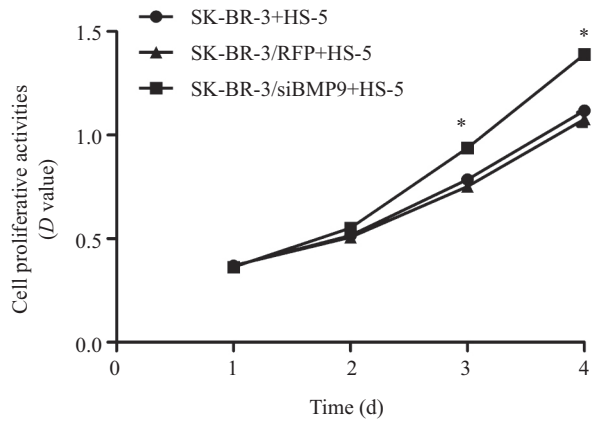


A: AdRFP及AdsiBMP9在共培养体系中SK-BR-3细胞中的表达(100×); B: RT-PCR检测各组细胞中*BMP9* mRNA的表达; C: Western blot检测各组细胞中BMP9蛋白的表达; *P<0.05与SK-BR-3/RFP+HS-5比较。1: SK-BR-3+HS-5; 2: SK-BR-3/ RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5; M: DL2000 marker.

A: the expression of AdRFP and AdsiBMP9 in SK-BR-3 cells of co-culture (100×); B: the expression of *BMP9* mRNA in different groups was detected by RT-PCR; C: the expression of BMP9 protein in different groups was detected by Western blot; *P<0.05 compared with SK-BR-3/RFP+HS-5. 1: SK-BR-3+HS-5; 2: SK-BR-3/ RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5; M: DL2000 marker.

图1 共培养体系中各组SK-BR-3细胞BMP9的表达

Fig.1 The expression of BMP9 in different groups of SK-BR-3 cells in co-culture



*P<0.05, 与SK-BR-3/RFP +HS-5比较。

*P<0.05 compared with SK-BR-3/RFP+HS-5.

图2 共培养体系中下调BMP9对SK-BR-3细胞增殖能力的影响

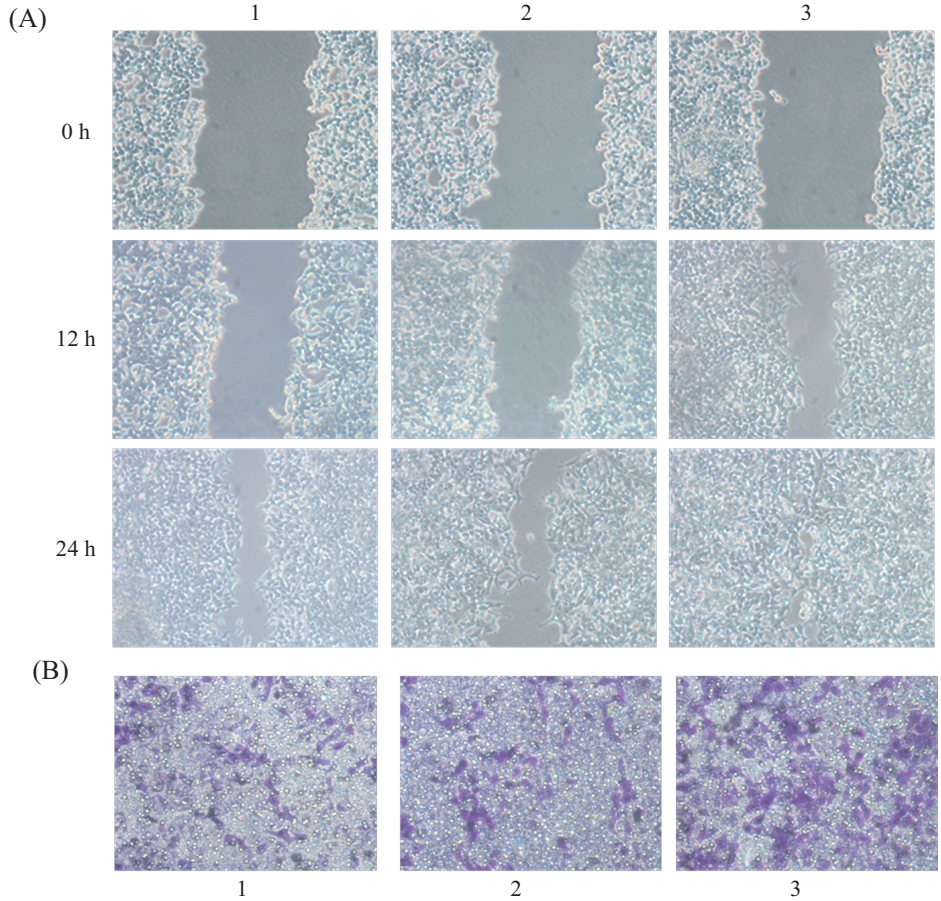
Fig.2 Effect of down-regulating BMP9 on the proliferation capability of SK-BR-3 cells in co-culture

2.3 共培养体系中干扰BMP9表达促进SK-BR-3细胞迁移

划痕实验检测siBMP9对SK-BR-3细胞横向迁移能力的影响。24 h后SK-BR-3/siBMP9+HS-5组的划痕愈合率(93.3%±2.8%)显著高于SK-BR-3/RFP+HS-5组(67.8%±3.3%)(P<0.05), 而SK-BR-3/RFP+HS-5组与SK-BR+HS-5组(74.3%±2.3%)无显著性差异(图3A)。无基质胶的Transwell实验检测干扰BMP9后对SK-BR-3细胞纵向迁移能力的影响。在低倍镜下观察, SK-BR-3/siBMP9+HS-5组的穿膜细胞数(121.0±4.3)显著高于SK-BR-3/RFP+HS-5组(69.0±3.1)(P<0.05), 而SK-BR-3/RFP+HS-5组与SK-BR+HS-5组(73.0±4.7)无显著性差异(图3B)。

2.4 共培养体系中干扰BMP9表达上调VEGF、CTGF及p-AKT

共培养3 d后提取共培养体系中SK-BR-3细胞

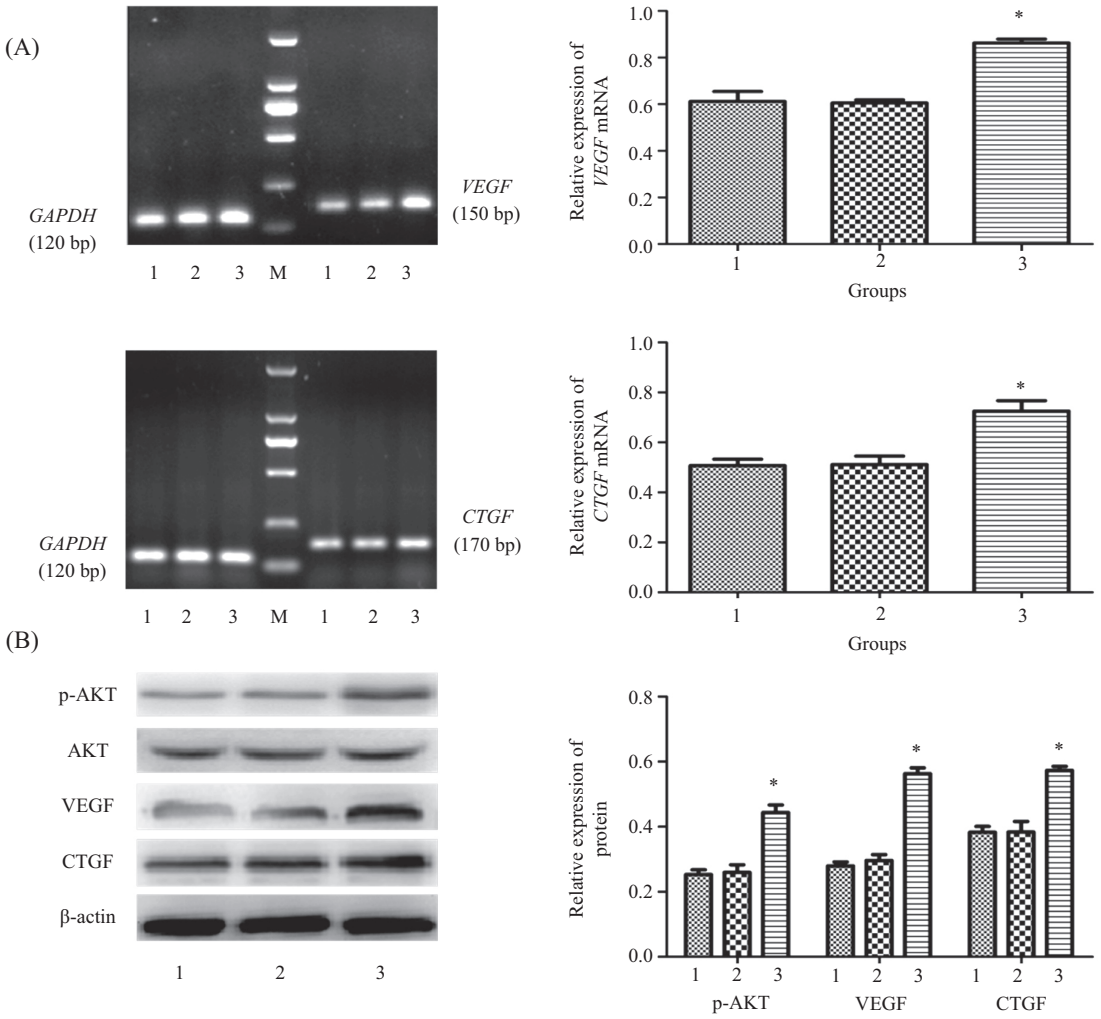


A: 划痕实验检测横向迁移能力(100×); B: Transwell实验检测纵向迁移能力(100×)。1: SK-BR-3+HS-5; 2: SK-BR-3/RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5。*P<0.05, 与SK-BR-3/RFP+HS-5比较。

A: the lateral migration capability was detected by wound assay (100×); B: the vertical migration capability was detected by Transwell assay (100×). 1: SK-BR-3+HS-5; 2: SK-BR-3/RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5. *P<0.05 compared with SK-BR-3/RFP+HS-5.

图3 共培养体系中下调BMP9对SK-BR-3细胞迁移能力的影响

Fig.3 Effect of down-regulating BMP9 on the migration capability of SK-BR-3 cells in co-culture



A: RT-PCR检测共培养体系中各组SK-BR-3细胞*VEGF*、*CTGF* mRNA的表达水平; B: Western blot检测共培养体系中各组SK-BR-3细胞VEGF、CTGF及p-AKT蛋白表达水平; **P*<0.05, 与SK-BR-3/RFP+HS-5组比较。1: SK-BR-3+HS-5; 2: SK-BR-3/RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5; M: DL2000 marker.

A: expression of *VEGF* and *CTGF* mRNA level in different groups of SK-BR-3 cells in co-culture by RT-PCR; B: expression of VEGF, CTGF and p-AKT protein level in different groups of SK-BR-3 cells in co-culture by Western blot; **P*<0.05 compared with SK-BR-3/RFP+HS-5 group. 1: SK-BR-3+HS-5; 2: SK-BR-3/RFP+HS-5; 3: SK-BR-3/siBMP9+HS-5; M: DL2000 marker.

图4 RT-PCR和Western blot检测共培养体系中各组SK-BR-3细胞相关因子表达

Fig.4 Expression of the related factors expression level in different groups of SK-BR-3 cells in co-culture by RT-PCR and Western blot

的总RNA和总蛋白。 RT-PCR在mRNA水平上检测增殖、迁移相关基因的变化, 结果显示, SK-BR-3/siBMP9+HS-5组的*VEGF*及*CTGF*显著高于SK-BR-3/RFP+HS-5组(*P*<0.05), 而SK-BR-3/RFP+HS-5组与SK-BR-3+HS-5组无显著性差异(图4A)。Western blot在蛋白水平上检测SK-BR-3/siBMP9+HS-5组的VEGF及CTGF也显著高于对照组(*P*<0.05)(图4B)。同时Western blot结果显示各组细胞AKT表达一致, 而SK-BR-3/siBMP9+HS-5组的p-AKT显著高于对照组(*P*<0.05)(图4B)。

3 讨论

BMPs是一组可诱导成骨分化的细胞因子, 以自分泌或旁分泌的形式作用于自身及周围细胞[12]。近年来有研究发现BMPs在很多肿瘤中异常表达, 乳腺癌是女性恶性肿瘤之一, 发病率逐年升高, 因此探讨其与BMPs的关系成了当前的研究热点。BMP2抑制乳腺癌细胞的凋亡及促进迁移和侵袭[13]; BMP4也可通过上调MMP1和CXCR4的表达促进乳腺癌细胞的侵袭[14]; BMP7与乳腺癌上皮细胞向间质细胞的转变有关, 过表达BMP7可抑制乳腺癌的侵袭能力及骨

转移能力[15-16]。而BMP9是成骨作用最强的BMPs成员, 发现较晚, 故BMP9对乳腺癌的作用报道较少。

乳腺癌极易转移到骨、肺、肝等组织, 以骨组织最常见, 致死率高。转移到骨中的乳腺癌细胞分泌的甲状旁腺激素相关蛋白(PTHrP)可以招募并激活骨中的破骨细胞, 并促进成骨细胞分泌破骨细胞分化因子(RANKL)活化破骨细胞造成骨吸收[17], 而骨吸收释放大量的TGF-β等又能直接刺激乳腺癌细胞产生骨转移相关因子, 进一步加重骨损伤。过度的骨破坏为骨转移的乳腺癌细胞增殖及扩张提供了良好的微环境, 形成恶性循环[18], 乳腺癌骨转移正是通过打破骨微环境的动态平衡而造成溶骨性损伤。前期研究发现, 在乳腺癌骨转移微环境中外源表达的BMP9能调节乳腺癌细胞与骨髓基质细胞的相互作用, 并促进间充质干细胞成骨分化, 故本实验拟构建体外共培养模型模拟骨转移微环境, 从基因沉默的角度进一步探究下调内源性BMP9表达是否能作用于乳腺癌的增殖及迁移从而影响溶骨性损伤, 并初步探究其可能机制。

肿瘤发生侵袭转移是一个多基因调控的多步骤过程, 与肿瘤细胞增殖、黏附、迁移等多个环节密切相关。本研究通过MTT实验、划痕实验、Transwell迁移实验发现在共培养体系中, 干扰内源性BMP9可以有效促进乳腺癌SK-BR-3细胞的增殖及迁移。BMP9主要通过经典的BMPs/Smad信号通路发挥生物学功能[19], 还可通过非经典的PI3K/AKT通路发挥作用, 已有文献报道BMP9通过下调PI3K/AKT信号通路抑制骨肉瘤细胞的增殖和侵袭能力[20]。而本研究Western blot结果显示, 在共培养体系中, 干扰乳腺癌SK-BR-3细胞内源性BMP9也能激活PI3K/AKT通路, 使p-AKT显著上调。VEGF是血小板衍生生长因子PDGF家族成员之一, 与肿瘤新生血管生成密切相关, 能特异性促进细胞的增殖及迁移过程。有研究证实, 趋化因子基质细胞衍生因子-1(CXCL12)及受体CXCR4介导的乳腺癌转移依赖其自身分泌的VEGF[21], PTEN通过PI3K/AKT/VEGF/eNOS途径影响肿瘤血管生成[22]。CTGF是即刻早期基因CCN家族成员之一, 也是TGF-β的下游靶介质, 具有趋化细胞、诱导黏附、调节细胞增殖、迁移等功能。研究证实, CTGF在乳腺癌中高表达, 并能影响乳腺癌细胞增殖和迁移能力[23], 同时有报道在肝癌细胞中抑制PI3K/AKT通路可下调TGF-β介导的CTGF的表达[24]。而本研究用RT-PCR及Western blot在mRNA及蛋白水平上检测到在共培养体系中, 干扰乳腺癌SK-BR-3细胞内源性BMP9能显著上调VEGF及CTGF的表达。因此, 在骨转移微环境中干扰内源性BMP9能促进乳腺癌细胞的增殖和迁移, 其作用机制可能与干扰BMP9上调VEGF及CTGF的表达有关, 而这个过程可能涉及PI3K/AKT通路的激活, 其涉及到的具体信号通路机制仍有待进一步证实。

总之, 本研究从基因沉默的角度探讨了在模拟骨转移微环境的共培养体系中BMP9对乳腺癌SK-BR-3细胞增殖及迁移的影响及其可能机制。结合前期成果, BMP9同时有成骨和抑癌作用, 有望成为抑制乳腺癌溶骨性骨转移的一个潜在治疗靶点。

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