研究论文

BMP2诱导MEFs成骨分化中Notch信号的作用及机制研究

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摘要 该文研究了Notch信号在骨形态发生蛋白2(bone morphogenetic protein 2, BMP2)诱导小鼠胚胎成纤维细胞(mouse embryonic fibroblasts, MEFs)成骨分化中的作用及机制。利用过表达Notch配体之一DLL1的腺病毒(adenovirus-delta-like 1, Ad-DLL1)、显性负性突变型Notch1受体的腺病毒(adenovirus-dominant-negative mutant of Notch1, Ad-dnNotch1)或γ-分泌酶抑制剂{N-[N-(3,5-difluorophena-cetyl-L-alanyl)]-S-phenylglycine t-butyl ester, DAPT}处理MEFs, 细胞化学染色和/或活性测定检测碱性磷酸酶(alkaline phosphatase, ALP)表达、钙盐沉积; qRT-PCR、Western blot、荧光素酶分别检测BMP2信号I、II型受体和成骨基因表达、Smad1/5/8蛋白磷酸化水平及Smad结合元件(Smad-binding element, SBE)转录活性。结果显示, DLL1促进BMP2介导MEFs早晚期成骨分化, 并上调ALK2等受体的mRNA水平、Smad1/5/8的磷酸化水平及SBE转录活性; 与之相对应, dnNotch1和DAPT抑制上述指标。Notch经典靶基因发状分裂相关增强子1(hairy/enhancer-of-split related with YRPW motif 1, *Hey1*)可促进BMP2诱导成骨分化, 并逆转DAPT对BMP2诱导成骨分化的抑制作用。该研究结果提示, Notch信号促进BMP2诱导MEFs成骨分化, 可能是通过激活BMP2/Smads通路实现的, 这一过程中*Hey1*发挥了重要作用。

关键词 Notch信号通路; 骨形态发生蛋白2; 骨髓间充质干细胞; 成骨分化

Role of Notch Signaling in BMP2-Induced Osteogenic Differentiation of MEFs and Its Mechanism

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Abstract This study was aimed to investigate the role of Notch in bone morphogenetic protein 2 (BMP2)-induced osteogenic differentiation in mouse embryonic fibroblasts (MEFs). The over-express DLL1 (one of the Notch ligands) adenoviruses (adenovirus-delta-like, Ad-DLL1), dominant-negative mutant of Notch1 (one of the Notch receptors) adenoviruses (adenovirus-dominant-negative mutant of Notch1, Ad-dnNotch1) and specific γ-secretase inhibitor {N-[N-(3,5-difluorophena-cetyl-L-alanyl)]-S-phenylglycine t-butyl ester, DAPT} were used to infect or treat MEFs, respectively. The early osteogenic index alkaline phosphatase (ALP) and late osteogenic

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index calcium deposits were detected by cytochemical staining and/or activity determination. The expression levels of BMP2 receptors and osteogenesis-related genes, the phosphorylation level of Smad1/5/8, and the transcriptional activity level of Smad-binding element (SBE) were determined by qRT-PCR, Western blot and luciferase reporter assay, respectively. The results showed that, compared with control group, DLL1 could obviously promote BMP2-mediated osteogenic differentiation (P<0.05). DLL1 could also increase the mRNA levels of *ALK2* and other BMP2 signaling receptors, the phosphorylation level of Smad1/5/8 and the transcriptional activity level of SBE in MEFs (P<0.05). Correspondingly, dnNotch1 and DAPT exert negative effects on the indexs mentioned above (P<0.05). On the other hand, *Hey1*, the classical target gene of Notch signal, could promote BMP2-induced osteogenic differentiation. We furthermore found that *Hey1* could reverse the inhibitory effect of DAPT on BMP2-induced osteogenic differentiation (P<0.01). These results indicated that Notch signaling could remarkably enhances BMP2-induced osteogenesis in MEFs and might exerts its effect though affecting the activation of BMP2/Smads signaling, *Hey1* plays an important role in this process.

Keywords Notch signaling pathway; bone morphogenetic protein 2; mesenchymal stem cells; osteogenic differentiation

骨形态发生蛋白2(bone morphogenetic protein 2, BMP2)是众多BMPs中的一员, 具有很强的诱导间充质干细胞(mesenchymal stem cells, MSCs)成骨分化的能力, 是骨组织工程中最常用的生长因子, 也是少数已应用于临床的BMPs之一。近几年的研究发现, BMP2在临床中成骨效应远不如预期, 提高BMP2浓度来增强成骨作用, 易导致溶骨性改变、异位成骨、局部水肿等不良反应[1-3]。到目前为止, 尚没有摸索出安全有效的方法来解决这一困境[4-7]。解决这一临床应用问题, 尚需要进一步探讨阐明BMP2诱导MSCs成骨分化的机制, 以期发现调控其成骨作用的关键步骤、细胞因子或信号通路。本课题组前期研究发现, Notch信号通路在BMP9和BMP4诱导的MSCs成骨分化过程中发挥着必不可少的作用[8-12], 但其在BMP2介导的成骨分化中的作用和机制并不明确。小鼠胚胎成纤维细胞(mouse embryonic fibroblasts, MEFs)在体内外具备MSCs的特征, 被认为是研究MSCs相关实验的重要细胞[13-15]。本研究分别用γ-分泌酶抑制剂{N-[N-(3,5-difluorophena-cetyl-L-alanyl)]-S-phenylglycine t-butyl ester, DAPT}(Notch抑制剂)、显性负性突变型Notch1腺病毒(adenovirus-dominant-negative mutant of Notch1, Ad-dnNotch1)(可竞争抑制Notch1受体活性)及过表达DLL1腺病毒(adenovirus-delta-like 1, Ad-DLL1)(上调Notch配体DLL1)处理MEFs, 从BMP2受体、胞质Smads蛋白质水平以及成骨相关基因表达等多方面探讨Notch信号在BMP2诱导MEFs成骨分化中的作用及可能分子机制, 为BMP2乃至其他BMPs的临床应用策略提供理论依据。

1 材料与方法

1.1 实验材料

1.1.1 细胞株及腺病毒 MEFs、腺病毒Ad-RFP、Ad-GFP、Ad-BMP2、Ad-DLL1、Ad-dnNotch1及Ad-Hey1由本实验室保存。人结肠癌细胞(human colorectal cancer cell)株HCT116由重庆医科大学分子实验室保存。

1.1.2 主要试剂和仪器 DMEM高糖培养基购自Hyclone公司。胎牛血清FBS购自Gibco公司。磷酸盐粉、兔抗鼠总Smad1/5/8抗体购自北京中杉金桥生物技术有限公司。胰蛋白酶购自Invitroge公司。DAPT_565784购自Calbiochem公司。碱性磷酸酶(alkaline phosphatase, ALP)底物购自BD公司。ALP染色试剂盒、维生素C、β-磷酸甘油、茜素红S、牛血清白蛋白(bovine serum albumin, BSA)购自Sigma公司。引物由Invitrogen公司合成。TRIzol购自Invitrogen公司。RT-PCR试剂盒、Real-time PCR试剂盒购自TaKaRa公司。Western blot及IP细胞裂解液、兔抗鼠p-Smad1/5/8抗体购自Santa Cruz公司。鼠抗鼠β-actin单抗隆抗体购自上海碧云天生物技术有限公司。羊抗鼠、羊抗兔二抗购自Cell Signaling Technology公司。Smad结合元件(Smad-binding element, SBE)荧光素酶质粒由重庆医科大学分子实验室保存。荧光素酶检测试剂购自Promega公司。

倒置荧光显微镜购自日本Nikon公司。凝胶成像系统、荧光定量PCR仪购自美国Bio-Rad公司。

1.2 实验方法

1.2.1 细胞培养与处理 用DMEM培养基(含10%胎牛血清、1%青霉素/链霉素)培养MEFs和HCT116细胞, 置CO_2培养箱(37 ℃、5% CO_2)中培养。用0.25%胰蛋白酶消化进行传代培养。

1.2.2 条件培养基的制备 HCT116细胞汇合至50%左右时, 加入Ad-BMP2或Ad-GFP, 4 h后用DMEM培养基(无血清无抗生素)换液, 于1天和2天后收集培养基各1次, 1 000 r/min低温离心5 min后放置于4 ℃备用。

1.2.3 qRT-PCR 细胞处理3天后, TRIzol提取总RNA, 逆转录为cDNA, 以*GAPDH*作为内参, 按照Real-time PCR试剂盒操作说明检测相关基因的表达。引物信息见表1。反应体系为10 μL: 上游引物(0.40 μL)、下游引物(0.40 μL)、SYBR Green I(5.00 μL)、cDNA(1.00 μL)、ddH_2O(3.20 μL)混匀; 反应程序: 94 ℃预变性3 min; 92 ℃ 20 s; 68 ℃ 30 s; 72 ℃ 20 s, 延伸末尾采集荧光, 40个循环。融解曲线采集(65~99 ℃), 数据采用$2^{-\Delta\Delta Ct}$分析。

1.2.4 碱性磷酸酶活性测定与染色 将MEFs接种(按30%的密度)于24孔板中, 6 h后加入Ad-GFP、Ad-RFP、Ad-dnNotch1或Ad-DLL1, 6 h后换液。待荧光出现后加入Ad-BMP2, 在继续培养细胞的第7天进行ALP染色及活性测定。(1)ALP染色方法: 负压吸引器弃去培养板内旧培养基, 用4%甲醛固定细胞, 每孔加入250 μL配制好的ALP染液, 避光(20 min), 观察染色结果。(2)ALP活性定量分析: 负压吸引器弃去培养板内旧培养基, 每孔加入配置好的1×细胞裂解液100 μL, 裂解10 min。吸取细胞裂解物, 收集到1.5 mL EP管中, 13 000 r/min离心5 min。另一EP管中加入20 μL配置好的ALP底物, 吸取5 μL细胞裂解物上清至此管中, 震荡, 静置(60 min), 上机检测, 记录结果。

1.2.5 钙盐沉积染色实验 将MEFs接种(按30%的密度)于24孔板中约6 h后, 加入Ad-GFP、Ad-RFP、Ad-dnNotch1或Ad-DLL1, 6 h后换液。待荧光出现后加入Ad-BMP2, 并于6 h换液的同时加入相应工作浓度的维生素C及β-磷酸甘油。细胞培养的第14天进行茜素红S染色。茜素红S染色方法: 负压吸引器弃去培养板内旧培养基, 0.05%戊二醛固定细胞, 10 min后, 去离子水洗涤3次, 加0.04%茜素红S进行染色, 10 min后会有红色堆积物形成, 用去离子水终止反应, 洗涤1次, 观察并保存结果。

1.2.6 蛋白质提取及Western blot检测 将MEFs接种(30%的密度)于细胞培养板中培养6 h后, 加入Ad-RFP、Ad-dnNotch1或Ad-DLL1, 6 h后换液。待荧光出现后加入BMP2条件培养基, 诱导培养1 h, 进行蛋白质提取。蛋白质提取方法: 用冰磷酸盐缓冲液(phosphate buffered saline, PBS)洗涤细胞3次, 加入含蛋白酶抑制剂和磷酸酶抑制剂的细胞裂解液(200 μL)

表1 引物序列(小鼠)

Table 1 The sequence of primers for PCR (mouse)

基因 Gene	正向引物(5′→3′) Forward primer (5′→3′)	反向引物(5′→3′) Reverse primer (5′→3′)
DLL1	CCG GCT GAA GCT ACA GAA AC	AGC CCC AAT GAT GCT AAC AG
NECD	GCA GAA CAA CAA GGA GGA GAC T	GAG GTC CTT AGC TTC CTT GCT AC
OSX	GGG AGC AGA GTG CCA AGA	TAC TCC TGG CGC ATA GGG
Runx2	GGT GAA ACT CTT GCC TCG TC	AGT CCC AAC TTC CTG TGC T
Colla 1	CGG CTC CTG CTC CTC TTA	TTC ATT GCA TTG CAC GTC AT
OCN	TCT GAC AAA GCC TTC ATG TCC	AAA TAG TGA TAC CGT AGA TGC
ALK1	ACC TGG GAC TGG CTG TGA	GCA GTC TGT GCG GAT GTG
ALK2	GTG GCT CCG GTC TTC CTT	AGC GAC ATT TTC GCC TTG
ALK3	TGG ACA TTG CTT TGC CAT C	CGT AGC TGG GCT TTT GGA
ALK6	CGT GAC ACT CCC ATT CCT C	TGG GCC CAT CAA CAA AAT
ActRII	GGC TCC AGA GGT GTT GGA	CCA TCT GCA GCA GTG CAA
ActRIIβ	GGG ACC ATG ATG CAG AGG	GGT GAC GGA GGT CAC CAG
BMPRII	AGC GTC ACA AGC CTG TCC	TTT GTG GCG TGC AAA TGT
GAPDH	GGC TGC CCA GAA CAT CAT	ATG ATG TTC TGG GCA GCC

裂解5 min(于冰上), 将细胞全部移入1.5 mL EP管中, 低温离心机5 000 r/min离心20 min, 吸上清并加入5×蛋白质上样缓冲液(50 μL), 沸水浴(10 min), 分装后–20 ℃保存。Western blot方法: 按说明书中的比例配制好浓缩胶和分离胶, 恒压(100 V)下进行凝胶电泳, 恒流(210 mA)下转膜, BSA封闭后按操作说明加入一抗和二抗, 孵育, 洗膜(3次), 最后化学法发光显影。

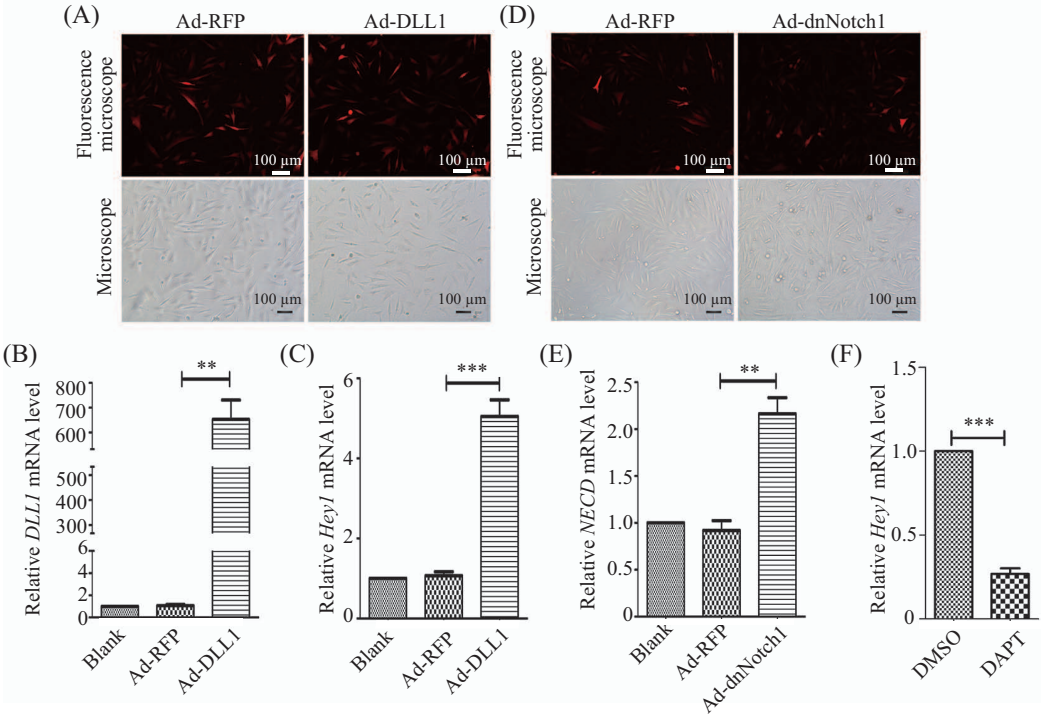
1.2.7 SBE荧光素酶检测 将MEFs接种(30%的密度)于细胞培养板中, 继续培养约6 h后用脂质体2000将SBE质粒转染入细胞内。转染过程: 将3 μg的质粒与15 μL的脂质体混匀后, 加入到250 μL的培养基(无血清无抗生素)中, 15 min后加入到2.5 mL培养基(无血清无抗生素)中, 继续培养6 h后换液。过夜, 将转染后的细胞接种(30%的密度)于细胞培养板中, 加入Ad-DLL1或Ad-BMP2后换液, 裂解细胞(36 h后), 1 000 r/min离心15 min, 轻轻吸取上清50 μL与底物(20 μL)反应, 检测SBE的转录活性。

1.2.8 统计学分析 数据采用SPSS 17.0进行统计分析, 实验独立重复3次, 数据以平均值和标准差($\bar{x}\pm s$)表示, 多组间比较采用单因素方差分析, 组间两两比较采用t检验, $P<0.05$为差异具有显著性。

2 结果

2.1 Ad-DLL1、Ad-dnNotch1及DAPT对Notch信号通路的影响

分别用Ad-DLL1或Ad-dnNotch1或10 μmol/L DAPT等处理MEFs, 细胞培养至第3天时采用qRT-PCR检测Notch靶基因*Hey1*或Notch1胞外段(Notch1 extracellular domain, NECD)的表达, 以了解Ad-DLL1、Ad-dnNotch1及DAPT对Notch信号通路的影响。结果显示, 在MEFs中, Ad-DLL1可显著上调*Hey1*表达, 提示Notch信号通路的激活。Ad-dnNotch1明显增加了*NECD*的表达, 提示Ad-dnNotch1可竞争性抑制Notch1受体的表达, 进而抑制Notch信号通路。DAPT下调Notch靶基因*Hey1*的mRNA水平, 提示DAPT能抑制Notch信号通路的活性(图1)。

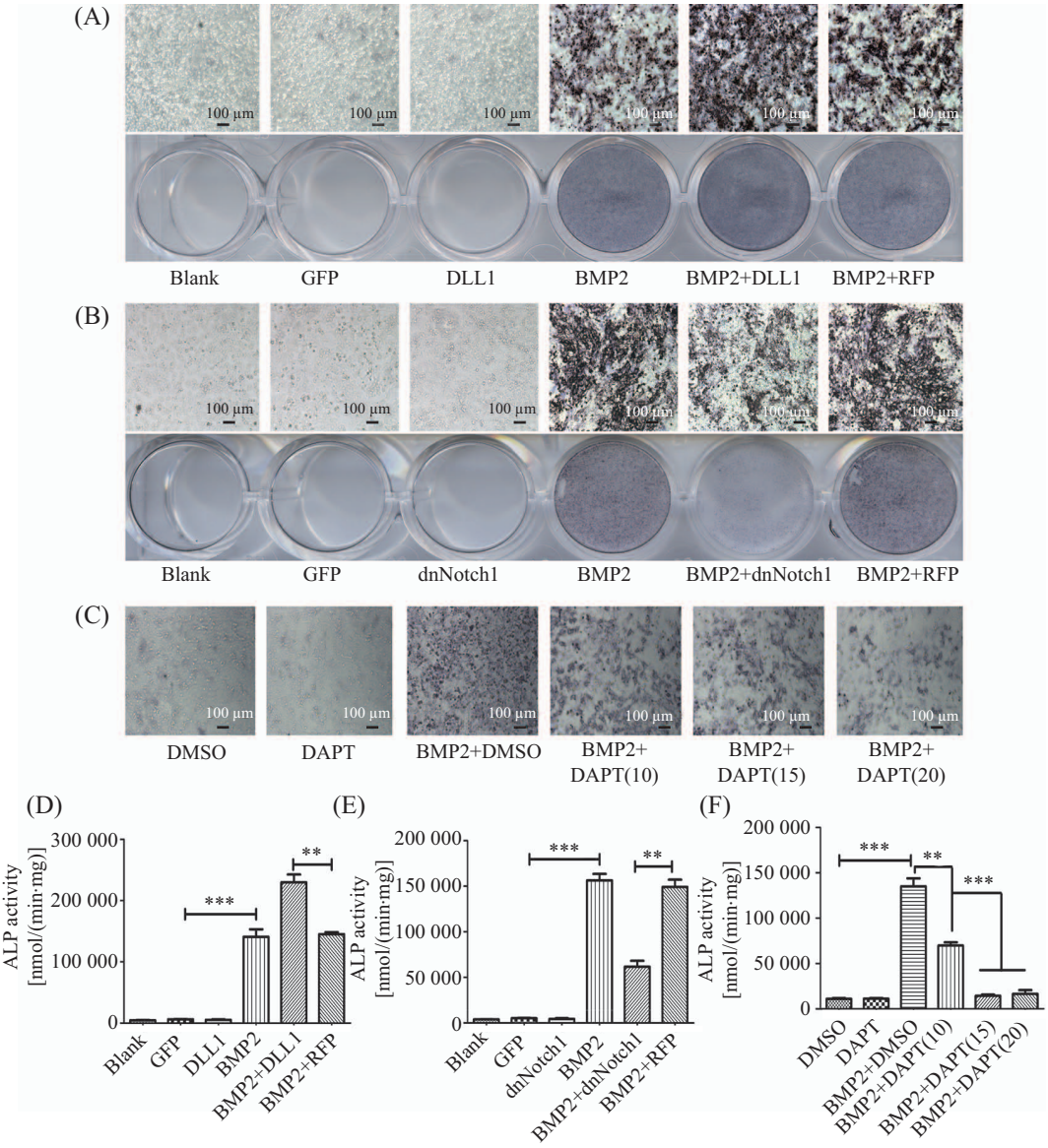


A: 腺病毒Ad-RFP和Ad-DLL1感染36 h荧光图; B: qRT-PCR检测Ad-DLL1对*DLL1* mRNA水平的影响; C: qRT-PCR检测Ad-DLL1对*Hey1* mRNA水平的影响; D: 腺病毒Ad-RFP和Ad-dnNotch1感染36 h荧光图; E: qRT-PCR检测Ad-dnNotch1对*NECD* mRNA水平的影响; F: qRT-PCR检测DAPT对*Hey1* mRNA水平的影响。**$P<0.01$, ***$P<0.001$。

A: fluorescent photos after Ad-RFP and Ad-DLL1 infected for 36 h; B: qRT-PCR was used to detect the effect of Ad-DLL1 on the mRNA level of *DLL1*; C: qRT-PCR was used to detect the effect of Ad-DLL1 on the mRNA level of *Hey1*; D: fluorescent photos after Ad-RFP and Ad-dnNotch1 infected for 36 h; E: qRT-PCR was used to detect the effect of Ad-dnNotch1 on the mRNA level of *NECD*; F: qRT-PCR was used to detect the effect of DAPT on the mRNA level of *Hey1*. **$P<0.01$, ***$P<0.001$.

图1 相关腺病毒/试剂对Notch信号通路的影响

Fig.1 The effects of related adenoviruses/reagents on Notch signaling pathway



A: ALP染色检测DLL1对BMP2诱导的早期成骨分化的影响; B: ALP染色检测dnNotch1对BMP2诱导的早期成骨分化的影响; C: ALP染色检测DAPT对BMP2诱导的早期成骨分化的影响; D: ALP活性分析检测DLL1对BMP2诱导的早期成骨分化的影响; E: ALP活性分析检测dnNotch1对BMP2诱导的早期成骨分化的影响; F: ALP活性分析检测DAPT对BMP2诱导的早期成骨分化的影响。*P<0.05, **P<0.01, ***P<0.001。

A: ALP cytochemical staining was used to detect the effect of DLL1 on BMP2-induced early osteogenic differentiation; B: ALP cytochemical staining was used to detect the effect of dnNotch1 on BMP2-induced early osteogenic differentiation; C: ALP cytochemical staining was used to detect the effect of DAPT on BMP2-induced early osteogenic differentiation; D: ALP activity determination was used to detect the effect of DLL1 on BMP2-induced early osteogenic differentiation; E: ALP activity determination was used to detect the effect of dnNotch1 on BMP2-induced early osteogenic differentiation; F: ALP activity determination was used to detect the effect of DAPT on BMP2-induced early osteogenic differentiation. *P<0.05, **P<0.01, ***P<0.001.

图2 ALP活性及染色检测Notch信号对BMP2诱导的早期成骨分化的影响

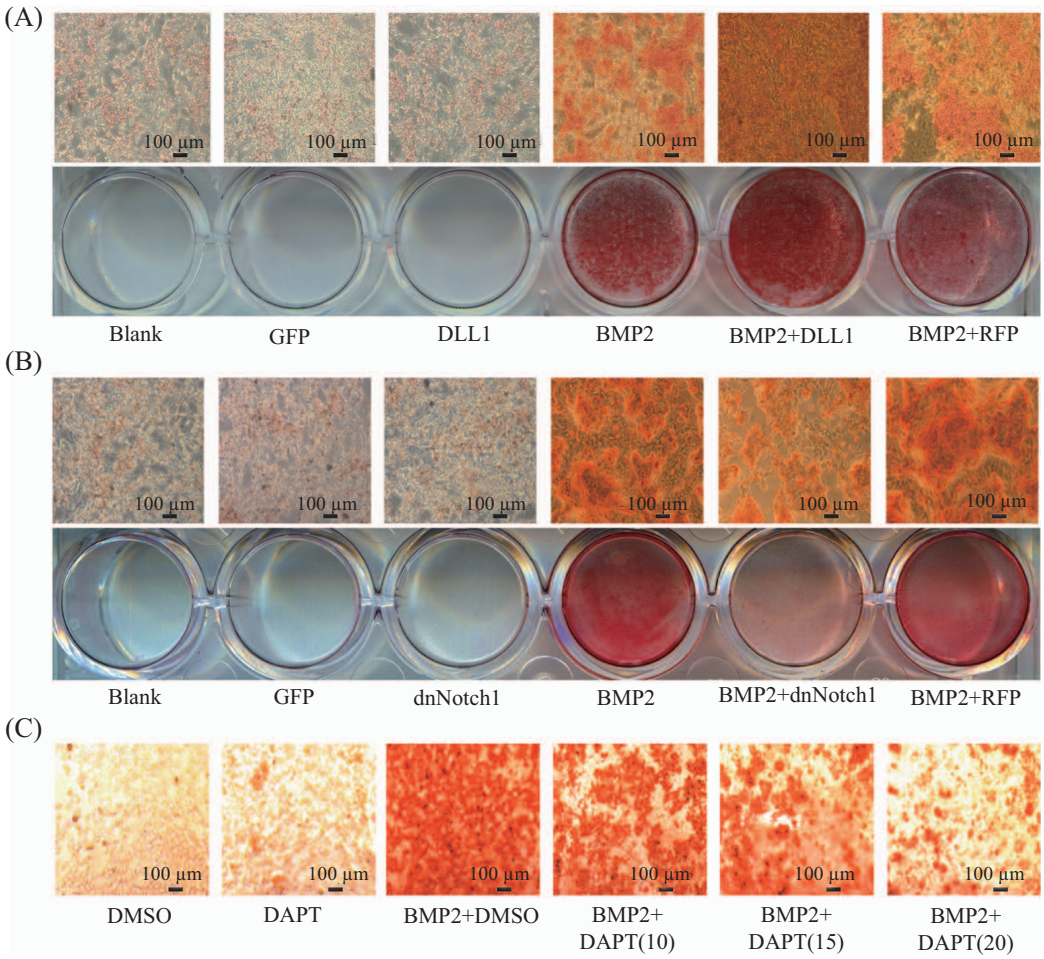
Fig.2 The effects of Notch signaling on BMP2-induced early osteogenic differentiation detected by ALP activity and cytochemical staining

2.2 Notch信号对BMP2诱导MEFs早期成骨的影响

BMP2诱导MEFs成骨分化时, 分别用Ad-DLL1或Ad-dnNotch1或10 μmol/L、15 μmol/L、20 μmol/L的DAPT等处理MEFs。细胞培养7天后, 检测早期成骨指标ALP的表达。结果表明, DLL1促进BMP2诱导的MEFs的ALP表达; 相对应地, dnNotch1和DAPT抑制BMP2诱导的MEFs的ALP表达(图2)。

2.3 Notch信号对BMP2诱导MEFs晚期成骨的影响

BMP2诱导MEFs成骨分化时, 分别用Ad-DLL1或Ad-dnNotch1或10 μmol/L、15 μmol/L、20 μmol/L



A: 茜素红S染色检测DLL1对BMP2诱导的晚期成骨指标钙盐沉积的影响; B: 茜素红S染色检测dnNotch1对BMP2诱导的晚期成骨指标钙盐沉积的影响; C: 茜素红S染色检测DAPT对BMP2诱导的晚期成骨指标钙盐沉积的影响。

A: alizarin red S staining was used to detect the effect of DLL1 on BMP2-induced late osteogenic index calcium deposits; B: alizarin red S staining was used to detect the effect of dnNotch1 on BMP2-induced late osteogenic index calcium deposits; C: alizarin red S staining was used to detect the effect of DAPT on BMP2-induced late osteogenic index calcium deposits.

图3 茜素红S染色检测Notch信号对BMP2诱导的晚期成骨分化的影响

Fig.3 The effects of Notch signaling on BMP2-induced late osteogenic differentiation detected by alizarin red S staining

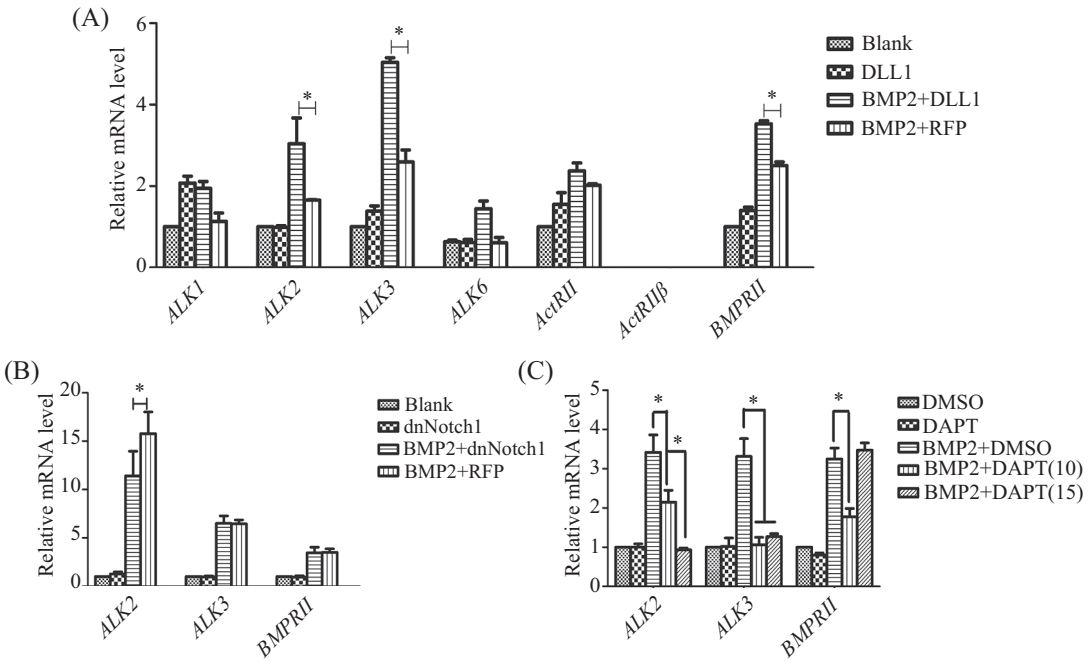
的DAPT等处理MEFs, 细胞培养14天后检测晚期成骨指标钙盐的沉积。茜素红S染色结果表明, DLL1促进BMP2诱导的钙盐沉积; 与之相对应, dnNotch1和DAPT抑制BMP2诱导的钙盐沉积(图3)。

2.4 Notch信号对BMP2受体表达的影响

BMP2诱导MEFs成骨分化时, 用Ad-DLL1等腺病毒处理细胞, 培养3天后, 采用qRT-PCR检测DLL1对BMP2信号通路I型受体(*ALK1*、*ALK2*、*ALK3*、*ALK6*)和II型受体(*ActRII*、*ActRIIβ*、*BMPRII*) mRNA水平的影响。结果显示, 与对照组相比, DLL1能明显上调BMP2介导的*ALK2*、*ALK3*、*BMPRII* mRNA水平(图4A)。我们进一步检测了dnNotch1及DAPT对BMP2介导的*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响。BMP2诱导MEFs成骨分化时, 用Ad-dnNotch1或10 μmol/L、15 μmol/L DAPT等处理MEFs 3天, 采用qRT-PCR检测*ALK2*、*ALK3*、*BMPRII* mRNA水平。结果显示, dnNotch1抑制BMP2介导的*ALK2*的表达; DAPT抑制BMP2介导的*ALK2*、*ALK3*、*BMPRII*的表达。以上结果表明, Notch信号影响BMP2相应受体的表达(图4B和图4C)。

2.5 Notch信号对BMP2/Smads通路的影响

BMP2诱导MEFs成骨分化时, 分别用Ad-DLL1或Ad-dnNotch1等腺病毒处理MEFs, 细胞培养3天后, 通过Western blot和荧光素酶检测BMP2信号通路中Smad1/5/8磷酸化水平和SBE的转录活性。结果显示, DLL1可明显上调BMP2诱导的p-Smad1/5/8水平; 与之相对应, dnNotch1下调BMP2诱导的p-Smad1/5/8水平; 但两者对Smad1/5/8总蛋白质水



A: qRT-PCR检测DLL1对BMP2信号通路I、II型受体mRNA水平的影响; B: qRT-PCR检测dnNotch1对*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响; C: qRT-PCR检测DAPT对*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响。*P<0.05。

A: qRT-PCR was used to detect the effect of DLL1 on the mRNA levels of type I and type II receptors of BMP2 signaling pathway; B: qRT-PCR was used to detect the effect of dnNotch1 on the mRNA levels of *ALK2*, *ALK3* and *BMPRII*; C: qRT-PCR was used to detect the effect of DAPT on the mRNA levels of *ALK2*, *ALK3* and *BMPRII*. *P<0.05.

图4 qRT-PCR检测Notch信号对BMP2信号通路I、II型受体mRNA表达水平的影响

Fig.4 The effects of Notch signaling on the mRNA levels of type I and type II receptors of BMP2 signaling pathway detected by qRT-PCR

平均没有明显的影响。同时, SBE荧光素酶实验结果显示, DLL1显著促进BMP2介导的SBE转录活性; dnNotch1则表现为抑制。以上结果说明, 在BMP2诱导成骨分化的过程中, Notch具有促进Smad1/5/8蛋白质磷酸化、增强核内SBE转录活性的作用(图5)。

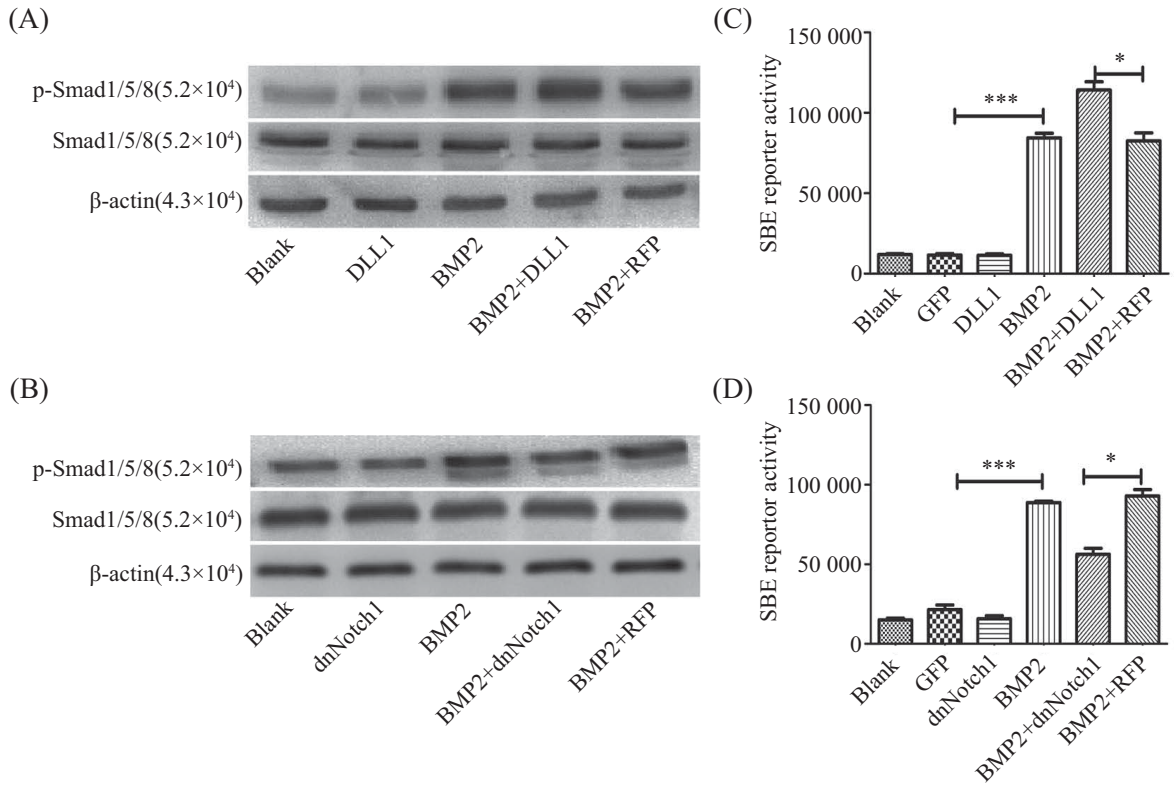
2.6 Notch信号对BMP2诱导的MEFs成骨相关基因表达的影响

BMP2诱导MEFs成骨分化时, 分别用Ad-DLL1或Ad-dnNotch1等腺病毒处理MEFs, 细胞培养3天后, 采用qRT-PCR检测成骨相关基因的表达水平。结果显示, DLL1促进BMP2诱导的成骨关键基因*Runx2*、成骨早期标记基因*Colla 1*及成骨晚期标记基因*OCN*的表达, 但*OSX*无显著变化。dnNotch1抑制BMP2诱导的成骨早期标记基因*Colla 1*及成骨晚期标记基因*OCN*的表达, 成骨关键基因*Runx2*下调, 但无统计学意义, *OSX*无明显变化。以上结果说明, Notch信号通路可调控BMP2介导的成骨相关靶基因的表达, 但不同的配体和受体的作用有一定的区别(图6)。

2.7 *Hey1*在BMP2诱导MEFs成骨过程中的作用

在BMP2诱导MEFs成骨分化时, 分别用10 μmol/L DAPT和/或Ad-Hey1等腺病毒处理MEFs, 细胞培养7天后, 采用ALP活性定量及ALP染色的方法检测早期成骨指标ALP。结果显示, *Hey1*单独没有诱导成骨分化的作用, 但可促进BMP2介导的成骨分化, 且能部分逆转DAPT对BMP2介导的成骨分化的抑制作用(图7)。结果提示, *Hey1*在Notch信号促进BMP2介导的成骨分化中起到重要的作用。

在BMP2诱导MEFs成骨分化时, 用10 μmol/L DAPT和/或Ad-Hey1等腺病毒处理MEFs, 细胞培养3后, 采用qRT-PCR检测*Hey1*对BMP2信号通路受体*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响, 通过Western blot检测BMP2信号通路中Smad1/5/8磷酸化水平。结果显示, *Hey1*单独对*ALK3*和*BMPRII* mRNA水平及Smad1/5/8磷酸化水平无明显影响, 却可抑制*ALK2*表达; 另一方面, *Hey1*上调BMP2介导的*ALK2*、*ALK3*和*BMPRII* mRNA水平及Smad1/5/8磷酸化水平, 且能逆转DAPT对BMP2介导的上述指标的抑制作用

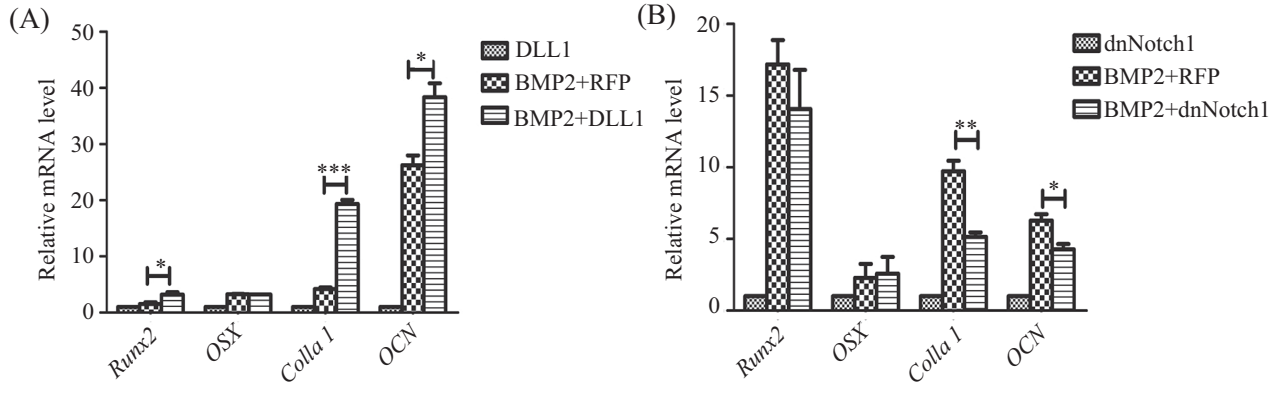


A: Western blot检测DLL1对BMP2通路Smad1/5/8蛋白质磷酸化水平的影响; B: Western blot检测dnNotch1对BMP2通路Smad1/5/8蛋白质磷酸化水平的影响; C: 荧光素酶实验检测DLL1对SBE转录活性的影响; D: 荧光素酶实验检测dnNotch1对SBE转录活性的影响。*P<0.05, ***P<0.001。

A: Western blot was used to detect the effect of DLL1 on the phosphorylation level of Smad1/5/8 in classical BMP2 signaling pathway; B: Western blot was used to detect the effect of dnNotch1 on the phosphorylation level of Smad1/5/8 in classical BMP2 signaling pathway; C: luciferase reporter assay was used to detect the effect of DLL1 on the SBE transcriptional activity; D: luciferase reporter assay was used to detect the effect of dnNotch1 on SBE transcriptional activity. *P<0.05, ***P<0.001.

图5 Western blot和荧光素酶实验检测Notch信号对BMP2经典信号通路中Smad1/5/8磷酸化及SBE转录活性的影响

Fig.5 The effects of Notch signaling on the phosphorylation level of Smad1/5/8 and SBE activity in classical BMP2 signaling pathway detected by Western blot and luciferase reporter assay

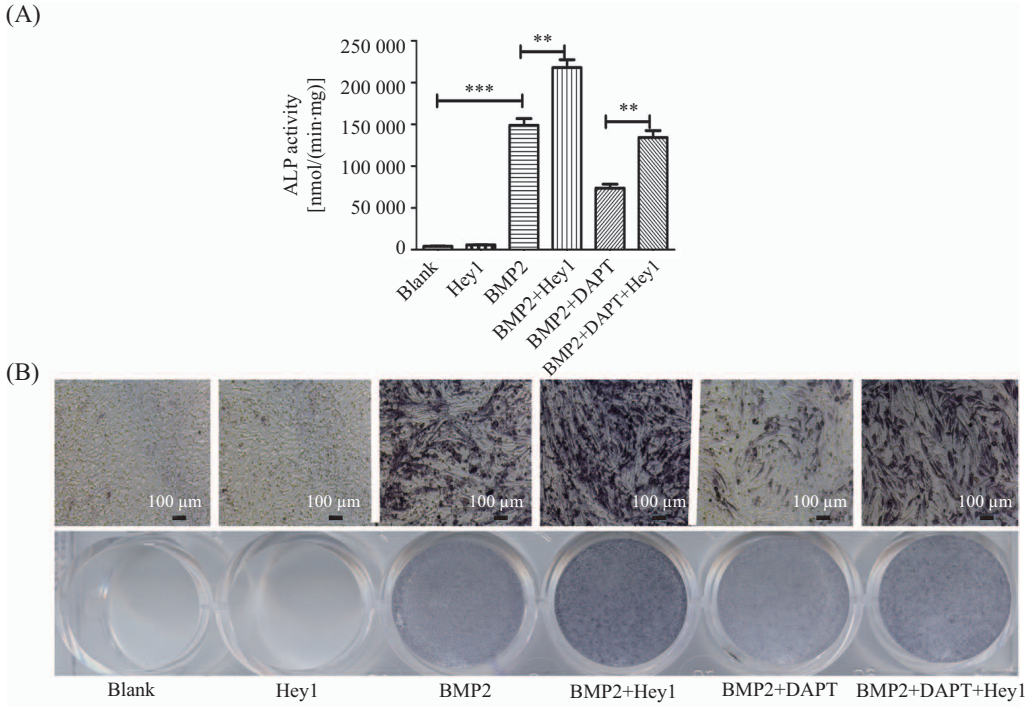


A: qRT-PCR检测DLL1对BMP2诱导的成骨相关基因mRNA水平的影响; B: qRT-PCR检测dnNotch1对BMP2诱导的成骨相关基因mRNA水平的影响。*P<0.05, **P<0.01, ***P<0.001。

A: qRT-PCR was used to detect the effect of DLL1 on the mRNA levels of BMP2-induced osteogenesis-related genes; B: qRT-PCR was used to detect the effect of dnNotch1 on the mRNA levels of BMP2-induced osteogenesis-related genes. *P<0.05, **P<0.01, ***P<0.001.

图6 qRT-PCR检测Notch信号对BMP2诱导的成骨相关基因mRNA水平的影响

Fig.6 The effects of Notch signaling on the mRNA levels of BMP2-induced osteogenesis-related genes detected by qRT-PCR

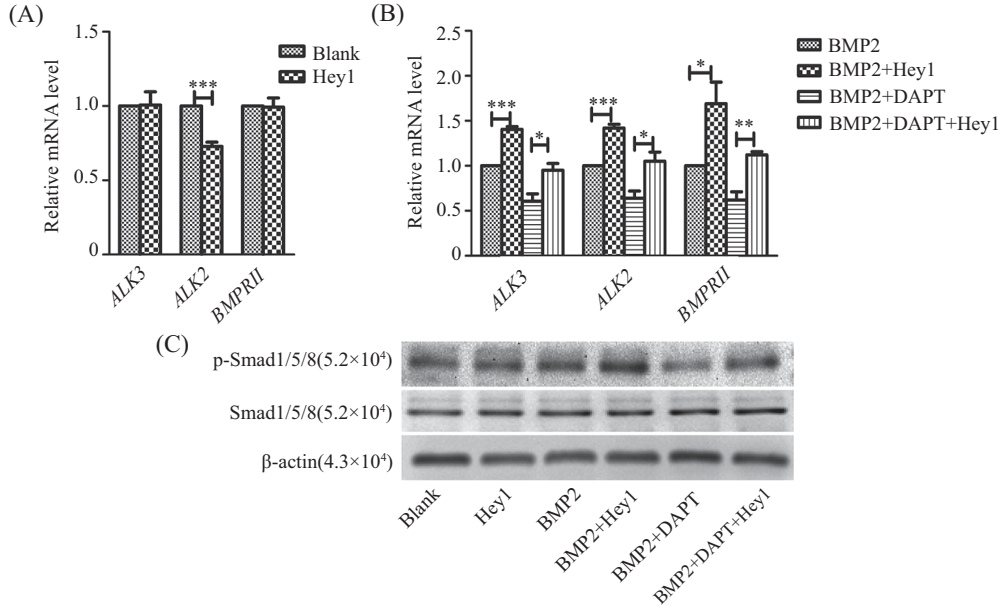


A: ALP活性分析检测Hey1对BMP2早期成骨分化的影响, ***P*<0.01, ****P*<0.001; B: ALP染色检测Hey1对BMP2早期成骨分化的影响。

A: ALP activity determination was used to detect the effect of *Hey1* on BMP2-induced early osteogenic differentiation, ***P*<0.01, ****P*<0.001; B: ALP cytochemical staining was used to detect the effect of *Hey1* on BMP2-induced early osteogenic differentiation.

图7 ALP活性及染色检测*Hey1*对BMP2诱导的早期成骨分化的影响

Fig.7 The effects of *Hey1* on BMP2-induced early osteogenic differentiation detected by ALP activity and cytochemical staining



A: qRT-PCR检测Hey1对BMP2信号通路受体*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响, ****P*<0.001; B: BMP2诱导成骨条件下, qRT-PCR检测Hey1对DAPT抑制的*ALK2*、*ALK3*、*BMPRII* mRNA水平的影响, **P*<0.05, ***P*<0.01, ****P*<0.001; C: Western blot检测Hey1对BMP2通路Smad1/5/8蛋白质磷酸化水平的影响。

A: qRT-PCR was used to detect the effect of Hey1 on the mRNA levels of *ALK2*, *ALK3* and *BMPRII*, the BMP2 signaling pathway receptors, ****P*<0.001; B: qRT-PCR was used to detect the effect of Hey1 on the mRNA levels of DAPT-inhibited *ALK2*, *ALK3* and *BMPRII* during BMP2 induced osteogenic differentiation, **P*<0.05, ***P*<0.01, ****P*<0.001. C: Western blot was used to detect the effect of Hey1 on the phosphorylation level of Smad1/5/8 of BMP2 signaling pathway.

图8 qRT-PCR及Western blot检测*Hey1*对BMP2经典信号通路的影响

Fig.8 The effects of *Hey1* on classical BMP2 signaling pathway detected by qRT-PCR and Western blot

(图8)。结果说明, *Hey1*是Notch信号影响BMP2信号的关键中介因子。

3 讨论

BMP2是成骨BMPs(还包括BMP4、6、7和9)中的一员[16-18]。BMP2最初发现时就与骨和软骨形成有关, 主要由骨祖细胞、成骨细胞、软骨细胞、血小板和血管内皮细胞合成[19], 具有很强的诱导MSCs成骨分化的能力, 是目前少数已应用于临床的BMPs之一。临床应用发现, BMP2促成骨效应不如预期, 且易导致溶骨性改变、异位成骨、局部水肿等不良反应[1-3]。改善这一临床应用情况, 需要进一步明确BMP2诱导MSCs成骨分化机制。国内外研究发现, BMP2主要通过经典的Smads通路调控下游靶基因, 诱导成骨分化, 且已经明确Wnt[20]、MAPK[21]、Notch[22-23]等信号可调控这一过程, 但具体机制不明确。

Notch信号是在生物进化上相对保守的信号转导系统, 决定着细胞的命运、调控着各种不同组织中的细胞增殖、分化和凋亡[22,24]。经典的Notch信号通路包括Notch受体(Notch1-4)、配体(Jagged1、Jagged2、DLL1、DLL3、DLL4)及CSL-DNA结合蛋白三部分。Notch配体与受体结合, Notch受体在γ内分泌酶的作用下释放NICD, 转入细胞核内, 结合转录因子CSL, 调控下游靶基因*Hey1*等表达[25]。关于Notch信号在BMP2成骨分化中作用的研究, 国内外早有报道, 但研究结果相互矛盾: 有研究显示Notch信号促进BMP2成骨[22,26], 但也有研究提出Notch信号在BMP2成骨中发挥抑制作用[27-28], 甚至有研究认为, BMP2诱导MSCs成骨分化与Notch无关[29-30]。本课题组前期研究发现, Notch能促进BMP9和BMP4诱导MSCs成骨分化[8-12]。因此, 在BMP2诱导成骨分化中Notch信号的作用值得深入研究。

我们采用具有MSCs特征的MEFs, 利用多种手段分别下调和上调Notch信号, 检测早期成骨指标ALP表达和晚期成骨指标钙盐沉积。结果显示, 下调Notch(DAPT和dnNotch1)明显抑制BMP2介导的早晚期成骨分化, 上调Notch(DLL1)则促进BMP2介导的早晚期成骨分化, 提示Notch有促进BMP2诱导MEFs成骨分化的作用, 这一结论与Tezuka等[22]学者关于BMP2的研究结果相吻合。目前关于Notch信号促进BMP2诱导MSCs成骨分化的机制研究不多且不够系统[22,26,31-32]。鉴于此, 我们对Notch信号在BMP2受体、Smads蛋白、成骨相关基因等各方面的作用进行了较为系统的研究。结果显示, DLL1可上调BMP2受体*ALK2*、*ALK3*、*BMPRII*的mRNA水平及Smad1/5/8的磷酸化水平, 并可增强SBE的转录活性, 促进BMP2诱导的成骨相关基因*Runx2*、*Colla 1*、*OCN*的表达, 而dnNotch1对上述指标均发挥负向调控作用。以上研究结果提示, Notch信号可能通过上调BMP2的受体表达, 促进Smad1/5/8磷酸化, 增强SBE转录活性, 促进成骨相关基因*Runx2*等的表达来促进BMP2介导的成骨分化。

*Hey1*是Notch的经典靶基因。Sharff等[33]学者的研究发现, *Hey1*在BMP9诱导的MSCs成骨分化中发挥着重要作用, 那么在Notch促进BMP2成骨分化过程中, *Hey1*起到怎样的作用呢? 我们采用Ad-Hey1和/或DAPT处理BMP2诱导的MEFs, 检测ALP表达, 结果显示, *Hey1*单独没有诱导成骨分化的作用, 却可明显促进BMP2介导的成骨分化, 这与Sharff等[33]、王淼等[34]在BMP9的研究中的结果相吻合。我们的研究还发现, *Hey1*可逆转DAPT(下调Notch)对BMP2成骨分化的抑制作用, 提示*Hey1*在Notch促进BMP2诱导MEFs成骨过程中起到重要作用。那么, *Hey1*是在哪一个环节影响BMP2成骨的呢? 我们采用Ad-Hey1和/或DAPT处理BMP2诱导的MEFs, 检测BMP2受体表达及Smad1/5/8的磷酸化水平, 结果显示, *Hey1*单独对*ALK3*和*BMPRII* mRNA水平及Smad1/5/8磷酸化水平无明显影响, 却可抑制*ALK2*表达; 进一步生物信息学分析(结果未展示)显示, *ALK2*启动子上有*Hey1*的结合位点, 这与Giacopelli等[35]研究一致。同时, 我们也发现, 在BMP2诱导成骨时, *Hey1*能上调*ALK2*、*ALK3*和*BMPRII* mRNA水平及Smad1/5/8磷酸化水平, 且能逆转DAPT对BMP2介导的上述指标的抑制作用。这些现象都提示, *Hey1*在BMP2促进成骨过程中有着重要的作用, 但机制复杂, 需要进一步阐明。

综上所述, 本研究明确了BMP2诱导MEFs成骨分化中, Notch信号的促进作用, 并进一步揭示这一作用可能是通过激活BMP2/Smads通路来完成。在这一过程中*Hey1*发挥着重要的作用, 但其具体机制还需进一步研究。值得注意的是, 本研究与我们在其他成骨BMPs(包括BMP9和BMP4)中的研究

结果非常相似, 即Notch信号促进BMP2/4/9诱导的MSCs早晚期成骨分化。在这一过程中, Notch信号可影响BMPs受体表达(尤其是ALK2的表达), 上调Smad1/5/8磷酸化和促进成骨基因表达。系列研究提示, Notch信号通路在成骨BMPs中发挥着重要的作用, 其机制的阐明将为成骨BMPs和MSCs在临床中的应用提供重要的理论依据。

参考文献 (References)

1 Tian H, Zhao J, Brochmann EJ, Wang JC, Murray SS. Bone morphogenetic protein-2 and tumor growth: Diverse effects and possibilities for therapy. Cytokine Growth Factor Rev 2017; 34: 73-91.

2 Scarfi S. Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair. World J Stem Cells 2016; 8(1): 1-12.

3 Poon B, Kha T, Tran S, Dass CR. Bone morphogenetic protein-2 and bone therapy: successes and pitfalls. J Pharm Pharmacol 2016; 68(2): 139-47.

4 Aro HT, Govender S, Patel AD, Hernigou P, Perera de Gregorio A, Popescu GI, *et al*. Recombinant human bone morphogenetic protein-2: a randomized trial in open tibial fractures treated with reamed nail fixation. J Bone Joint Surgery Am 2011; 93(9): 801-8.

5 Lyon T, Scheele W, Bhandari M, Koval KJ, Sanchez EG, Christensen J, *et al*. Efficacy and safety of recombinant human bone morphogenetic protein-2/calcium phosphate matrix for closed tibial diaphyseal fracture: a double-blind, randomized, controlled phase-II/III trial. J Bone Joint Surg Am 2013; 95(23): 2088-96.

6 Fu R, Selph S, McDonagh M, Peterson K, Tiwari A, Chou R, *et al*. Effectiveness and harms of recombinant human bone morphogenetic protein-2 in spine fusion: a systematic review and meta-analysis. Ann Intern Med 2013; 158(12): 890-902.

7 Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, Yoon ST, *et al*. Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. J Bone Joint Surg Am 2013; 95(17): 1537-45.

8 曹俊杰, 李爱芳, 卫亚琳, 廉 静, 唐 敏. Notch信号参与BMP4诱导的间充质干细胞成骨分化及其机制的初步探讨. 中国生物工程杂志(Cao Junjie, Li Aifang, Wei Yalin, Lian Jing, Tang Min. Role of Notch signaling in BMP4-induced osteogenic differentiation of mesenchymal stem cells and its mechanisms. China Biotechnology) 2017; 37(04): 48-55.

9 廉 静, 杨伦韵, 曹俊杰, 罗进勇, 何百成, 唐 敏. DLL1在骨形态形成蛋白9诱导骨髓间充质干细胞成骨分化中的作用. 第三军医大学学报 (Lian Jing, Yang Lunyun, Cao Junjie, Luo Jingyong, He Baicheng, Tang Min. Delta-like1 promotes osteogenic differentiation of mesenchymal stem cells induced by BMP9. Journal of Third Military Medical University) 2016; 38(1): 38-43.

10 杨伦韵, 张晓艳, 廉 静, 曹俊杰, 罗进勇, 唐 敏. Notch1在BMP9诱导iMEF细胞成骨分化中的作用. 第三军医大学学报(Yang Lunyun, Zhang Xiaoyan, Lian Jing, Cao Junjie, Luo Jingyong, Tang Min. Role of Notch1 signaling in the process of BMP9-induced osteogenetic differentiation of immortalized mouse embryonic fibroblasts. Journal of Third Military Medical University) 2015; 37(1): 39-45.

11 谢佳瑛, 胥文春, 徐道晶, 张晓艳, 唐 敏. Notch信号参与BMP9诱导的间充质干细胞成骨分化及其机制的初步探讨. 中国生物工程杂志(Xie Jiaying, Xu Wenchun, Xu Daojing, Zhang Xiaoyan, Tang Min. Role of Notch signaling in BMP9-induced osteogenesis differentiation of mesenchymal stem cells and its mechanisms. China Biotechnology) 2012; 32(11): 14-22.

12 刘 强, 翁亚光, 徐道晶, 罗 敏, 唐 敏. Notch信号通路在骨形态发生蛋白9诱导的多潜能干细胞成骨分化中的作用. 第三军医大学学报(Liu Qiang, Weng Yaguang, Xu Daojing, Luo Min, Tang Min. Role of Notch signaling pathway in bone morphogenetic protein9-induced osteogenic differentiation of multipotent stem cells. Journal of Third Military Medical University) 2010; 32(23): 2492-6.

13 Garreta E, Genove E, Borros S, Semino CE. Osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts in a three-dimensional self-assembling peptide scaffold. Tissue Eng 2006; 12(8): 2215-27.

14 Lengner CJ, Lepper C, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Primary mouse embryonic fibroblasts: a model of mesenchymal cartilage formation. J Cell Physiol 2004; 200(3): 327-33.

15 Saeed H, Taipaleenmaki H, Aldahmash AM, Abdallah BM, Kassem M. Mouse embryonic fibroblasts (MEF) exhibit a similar but not identical phenotype to bone marrow stromal stem cells (BMSC). Stem cell Rev 2012; 8(2): 318-28.

16 Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, *et al*. TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. J Biol Chem 2010; 285(38): 29588-98.

17 Wu N, Zhao Y, Yin Y, Zhang Y, Luo J. Identification and analysis of type II TGF-beta receptors in BMP-9-induced osteogenic differentiation of C3H10T1/2 mesenchymal stem cells. Acta Biochim Biophys Sin (Shanghai) 2010; 42(10): 699-708.

18 Zhao YF, Xu J, Wang WJ, Wang J, He JW, Li L, *et al*. Activation of JNKs is essential for BMP9-induced osteogenic differentiation of mesenchymal stem cells. BMB Rep 2013; 46(8): 422-7.

19 Carreira AC, Alves GG, Zambuzzi WF, Sogayar MC, Granjeiro JM. Bone morphogenetic proteins: structure, biological function and therapeutic applications. Arch Biochem Biophys 2014; 561: 64-73.

20 刘光源, 田发明, 张 柳, 刘家寅. BMP2信号通路与经典Wnt信号通路及相互交联对成骨分化的调控. 中国骨质疏松杂志(Liu Guangyuang, Tian Faming, Zhang Liu, Liu JiaYin. The regulation of osteogenic differentiation by BMP2 and the canonical Wnt signaling pathway and the crosstalk between them. Chinese Journal of Osteoporosis) 2014; 20(5): 551-6.

21 Pan H, Li X, Wang J, Zhang K, Yang H, Li Z, *et al*. LIM mineralization protein-1 enhances bone morphogenetic protein-2-mediated osteogenesis through activation of ERK1/2 MAPK pathway and upregulation of Runx2 transactivity. J Bone Miner Res 2015; 30(8): 1523-35.

22 Tezuka K, Yasuda M, Watanabe N, Morimura N, Kuroda K, Miyatani S, *et al*. Stimulation of osteoblastic cell differentiation

by Notch. J Bone Miner Res 2002; 17(2): 231-9.
23 Wang C, Wang J, Li J, Hu G, Shan S, Li Q, *et al*. KDM5A controls bone morphogenic protein 2-induced osteogenic differentiation of bone mesenchymal stem cells during osteoporosis. Cell Death Dis 2016; 7(8): e2335.
24 de la Pompa JL. Notch signaling in cardiac development and disease. Pediatr Cardiol 2009; 30(5): 643-50.
25 Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006; 7(9): 678-89.
26 Nobta M, Tsukazaki T, Shibata Y, Xin C, Moriishi T, Sakano S, *et al*. Critical regulation of bone morphogenetic protein-induced osteoblastic differentiation by Delta1/Jagged1-activated Notch1 signaling. J Biol Chem 2005; 280(16): 15842-8.
27 Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, *et al*. Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat Med 2008; 14(3): 306-14.
28 Deregowski V, Gazzerro E, Priest L, Rydziel S, Canalis E. Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenetic protein signaling. J Biol Chem 2006; 281(10): 6203-10.
29 Zhu F, Sweetwyne MT, Hankenson KD. PKCdelta is required for Jagged-1 induction of human mesenchymal stem cell osteogenic differentiation. Stem Cells 2013; 31(6): 1181-92.
30 Dishowitz MI, Zhu F, Sundararaghavan HG, Ifkovits JL, Burdick JA, Hankenson KD. Jagged1 immobilization to an osteoconductive polymer activates the Notch signaling pathway and induces osteogenesis. J Biomed Mater Res A 2014; 102(5): 1558-67.
31 Wang S, Kawashima N, Sakamoto K, Katsube K, Umezawa A, Suda H. Osteogenic differentiation of mouse mesenchymal progenitor cell, Kusa-A1 is promoted by mammalian transcriptional repressor Rbpj. Biochem Biophys Res Commun 2010; 400(1): 39-45.
32 de Jong DS, Steegenga WT, Hendriks JM, van Zoelen EJ, Olijve W, Dechering KJ. Regulation of Notch signaling genes during BMP2-induced differentiation of osteoblast precursor cells. Biochem Biophys Res Commun 2004; 320(1): 100-7.
33 Sharff KA, Song WX, Luo X, Tang N, Luo J, Chen J, *et al*. *Hey1* basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of mesenchymal progenitor cells. J Biol Chem 2009; 284(1): 649-59.
34 王 淼, 王 南, 权正学, 罗小辑. *Hey1*表达对BMP-9诱导下C3H10T1/2细胞的成骨分化及增殖影响. 中国修复重建外科杂志(Wang Miao, Wang Nan, Quang Zhengxue, Luo Xiaoji. Effect of *Hey1* expression on osteogenic differentiation and proliferation of C3H10T1/2 cells induced by bone morehogenetic protien 9. Chinese Journal of Reparative and Reconstructive Surgery) 2016; 30(03): 279-85.
35 Giacopelli F, Cappato S, Tonachini L, Mura M, Di Lascio S, Fornasari D, *et al*. Identification and characterization of regulatory elements in the promoter of ACVR1, the gene mutated in Fibrodysplasia Ossificans Progressiva. Orphanet J Rare Dis 2013; 8(1): 145-61.
36 刘大鹏, 艾合麦提, 古 丽, 穆合甫力. 体外培养骨髓基质细胞增殖和分化的相互关系. 中国临床康复(Liu Dapeng, Ai Hemaiti, Gu Li, Mu Hefuli.Correlation between proliferation and differentiation of bone marrow stroma cells cultured *in vitro*. Chinese Journal of Clinical Rehabilitation) 2005; 9(38): 10-3.
37 Agathocleous M, Harris WA. Metabolism in physiological cell proliferation and differentiation. Trends Cell Biol 2013; 23(10): 484-92.
38 Cao J, Wei Y, Lian J, Yang L, Zhang X, Xie J, *et al*. Notch signaling pathway promotes osteogenic differentiation of mesenchymal stem cells by enhancing BMP9/Smad signaling. Int J Mol Med 2017; 40: 378-88.