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MASH1介导肾上腺髓质嗜铬细胞发生神经元转分化

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[摘 要] **目的：**肾上腺髓质嗜铬细胞(adrenal medulla chromaffin cells，AMCCs)在神经生长因子(nerve growth factor，NGF)诱导下向神经元转分化，引起肾上腺素分泌减少，其可能参与了支气管哮喘的发病。神经分化发育的关键因子哺乳动物无刚毛-鳞甲同源物(mammalian achaete scute-homologous 1，MASH1)在神经元转分化的AMCCs中升高。本研究拟探讨MASH1对AMCCs神经元转分化的影响及机制。**方法：**分离并培养大鼠AMCCs，用siMASH1、MASH1过表达质粒转染，再用NGF和/或地塞米松、MAPK激酶-1抑制剂PD98059刺激48 h。在光镜及电镜下观察AMCCs形态。用免疫荧光法检测酪氨酸羟化酶和肾上腺素合成关键酶苯乙醇胺-N-甲基转移酶(phenylethanolamine-N-methyltransferase，PNMT)水平，蛋白质印迹法检测AMCCs中PNMT、MASH1、外周蛋白、细胞外调节蛋白激酶(extracellular regulated protein kinases，ERK)及磷酸化的细胞外调节蛋白激酶(phosphorylated extracellular regulated protein kinases，pERK)、JMJD3的蛋白质水平，Real-time RT-PCR法检测*MASH1*、*JMJD3*的mRNA水平，ELISA法检测细胞上清液中肾上腺素水平。**结果：**培养的细胞经免疫荧光染色示酪氨酸羟化酶和PNMT均为阳性，鉴定为AMCCs。NGF处理后AMCCs出现细胞突起，pERK、外周蛋白、MASH1蛋白表达量均显著上升(均$P<0.05$)；PNMT蛋白表达和肾上腺素分泌水平下降(均$P<0.01$)。MASH1干扰逆转了NGF的作用，使AMCCs中PNMT蛋白和肾上腺素水平升高，外周蛋白及细胞突起数量减少($P<0.01$)。过表达MASH1可使细胞突起、外周蛋白表达量显著增加(均$P<0.01$)，PNMT蛋白和肾上腺素水平下降(均$P<0.01$)。与NGF组对比，NGF+PD98059组AMCCs中MASH1、JMJD3蛋白及mRNA水平均下降(均$P<0.05$)。经过PD98059和地塞米松处理后，NGF促进AMCCs转分化的作用受到抑制，细胞突起数量及肾上腺素水平均下降(均$P<0.05$)；此外，NGF激活的pERK/MASH1通路活性也受到抑制。**结论：**MASH1是AMCCs发生神经元转分化的关键因子，NGF可能通过pERK/MASH1通路诱导转分化。

[关键词] MASH1；嗜铬细胞；神经元；神经生长因子；地塞米松；转分化

MASH1 induces neuron transdifferentiation of adrenal medulla chromaffin cells

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ABSTRACT **Objective:** Nerve growth factor (NGF) induces neuron transdifferentiation of adrenal medulla chromaffin cells (AMCCs) and consequently downregulates the secretion of epinephrine (EPI), which may be involved in the pathogenesis of bronchial asthma. Mammalian achaete scute-homologous 1 (MASH1), a key regulator of neurogenesis in the nervous system, has been proved to be elevated in AMCCs with neuron transdifferentiation in vivo. This study aims to explore the role of MASH1 in the process of neuron transdifferentiation of AMCCs and the mechanisms.

Methods: Rat AMCCs were isolated and cultured. AMCCs were transfected with siMASH1 or MASH1 overexpression plasmid, then were stimulated with NGF and/or dexamethasone, PD98059 (a MAPK kinase-1 inhibitor) for 48 hours. Morphological changes were observed using light and electron microscope. Phenylethanolamine-N-methyltransferase (PNMT, the key enzyme for epinephrine synthesis) and tyrosine hydroxylase were detected by immunofluorescence. Western blotting was used to test the protein levels of PNMT, MASH1, peripherin (neuronal markers), extracellular regulated protein kinases (ERK), phosphorylated extracellular regulated protein kinases (pERK), and JMJD3. Real-time RT-PCR was applied to analyze the mRNA levels of *MASH1* and *JMJD3*. EPI levels in the cellular supernatant were measured using ELISA.

Results: Cells with both tyrosine hydroxylase and PNMT positive by immunofluorescence were proved to be AMCCs. Exposure to NGF, AMCCs exhibited neurite-like processes concomitant with increases in pERK/ERK, peripherin, and MASH1 levels (all $P<0.05$). Additionally, impairment of endocrine phenotype was proved by a signifcant decrease in the PNMT level and the secretion of EPI from AMCCs (all $P<0.01$). MASH1 interference reversed the effect of NGF, causing increases in the levels of PNMT and EPI, conversely reduced the peripherin level and cell processes (all $P<0.01$). MASH1 overexpression significantly increased the number of cell processes and peripherin level, while decreased the levels of PNMT and EPI (all $P<0.01$). Compared with the NGF group, the levels of MASH1, JMJD3 protein and mRNA in AMCCs in the NGF+PD98059 group were decreased (all $P<0.05$). After treatment with PD98059 and dexamethasone, the effect of NGF on promoting the transdifferentiation of AMCCs was inhibited, and the number of cell processes and EPI levels were decreased (both $P<0.05$). In addition, the activity of the pERK/MASH1 pathway activated by NGF was also inhibited.

Conclusion: MASH1 is the key factor in neuron transdifferentiation of AMCCs. NGF-induced neuron transdifferentiation is probably mediated via pERK/MASH1 signaling.

KEY WORDS MASH1; chromaffin cells; neuron; nerve growth factor; dexamethasone; transdifferentiation

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肾上腺髓质嗜铬细胞(adrenal medulla chromaffin cells，AMCCs)和交感神经元有共同的来源，均起源于交感神经的神经嵴细胞和多能Schwann细胞前体[1-2]。与交感神经元不同，AMCCs具有可塑性[3]。神经生长因子(nerve growth factor，NGF)可以促进未成熟的大鼠AMCCs向神经元转分化，转分化后的AMCCs与交感神经元没有区别[3]。不仅如此，NGF干预还能刺激新生大鼠和成年大鼠的AMCCs出现类似于神经元的细胞突起[1, 4]。由于AMCCs具有神经表型和内分泌表型的双重潜能，其有望成为一种新的治疗神经系统疾病的手段[5]。

支气管哮喘(以下简称“哮喘”)患者的外周血中NGF水平升高，肾上腺素水平降低[6]。本课题组前期实验[7]证实：哮喘患者体内升高的NGF能够诱导AMCCs向神经元转分化，从而引起AMCCs分泌肾上腺素不足。循环中肾上腺素水平下降将引起患者在哮喘急性发作时出现持续的气道平滑肌痉挛。因此，AMCCs发生转分化的过程被认为是哮喘的发病机制之一[7-11]。然而，AMCCs发生神经元转分化的具体机制尚不清楚，研究其机制将有助于探究哮喘的发病过程。在哺乳动物中，哺乳动物无刚毛-鳞甲同源物(mammalian achaete-scute homologue-1，MASH1)是一个含碱性螺旋-环-螺旋结构域的转录因子，它在NGF诱导的发生神经元转分化的AMCCs中表达升高[7]，在神经分化和发育过程中发挥关键作用[12]。其是否参与AMCCs神经元转分化过程目前尚无研究。本研究旨在通过体外试验探究MASH1在AMCCs神经元转分化中的作用及机制，为后续AMCCs在哮喘或者神经系统疾病中的应用提供基础。

1 材料与方法

1.1 材料

NGF及地塞米松(dexamethasone，DEX)购自美国Sigma公司，MAPK激酶-1抑制剂PD98059、细胞外调节蛋白激酶(extracellular regulated protein kinases，ERK)及磷酸化的细胞外调节蛋白激酶(phosphorylated extracellular regulated protein kinases，pERK)抗体购自美国Cell Signaling Technology公司，苯乙醇胺N-甲基转移酶(phenylethanolamine N-methyl transferase，PNMT)抗体购自美国Millipore公司，酪氨酸羟化酶(tyrosine hydroxylase，TH)抗体和JMJD3抗体购自美国Abcam公司，MASH1抗体购自美国BD Biosciences公司，胎牛血清购自美国Gibico公司，DMEM细胞培养基购自美国HyClone公司，转染脂质体Lipofectamin 2000和TRIzol购于美国Invitrogen公司，ELISA试剂盒购自美国IBL-America公司，反转录试剂盒购于美国Fermentas公司，Real-time PCR试剂盒购于日本Takara公司。

1.2 AMCCs的原代培养

本实验经中南大学湘雅医院伦理委员会批准(审批号：201403261)。从6周龄雄性SPF级SD大鼠体内摘除双侧肾上腺，机械分离出肾上腺髓质后再使用胶原酶消化，采用Percoll非连续等密度梯度离心法分离和纯化AMCCs[1, 11]。使用含有10%胎牛血清的DMEM的培养基对AMCCs进行重悬，并以(5~6)×10^6/mL的密度接种在涂有胶原蛋白的聚苯乙烯培养皿中。24 h后更换培养基，此后每48 h更换1次。AMCCs共培养72 h，期间使用MTT法估计细胞的存活率。将细胞分为8组，分别为空白对照组(Control组)、神经生长因子(2.5S，100 ng/mL)组(NGF组)[1,13]、二甲基亚砜(0.05%)对照组(DMSO组)、DEX(10 μmol/L)组(DEX组)[1, 14]、NGF+DEX组、MAPK激酶-1抑制剂PD98059(10 μmol/L)组(PD98059组)[13]、NGF+PD98059组和NGF+DMSO组。光镜下计数细胞突起数量，用MTT法检测细胞相对存活率。各组细胞经刺激48 h后，收集其细胞和上清液进行后续分析。

1.3 光镜下观察AMCCs形态

将AMCCs平铺在6孔板的无菌玻璃盖板上，细胞密度为1×10^5/mL。用4%多聚甲醛固定细胞，PBS清洗，然后进行HE染色。长度超过细胞直径2倍的突起定义为细胞突起[14]。在光镜(×200)下观察AMCCs的形态，并进行细胞突起计数，每张玻片上大约计数200个AMCCs。

1.4 免疫荧光验证AMCCs

原代培养的AMCCs用4%多聚甲醛溶液在培养板中固定20 min，再用PBS清洗，0.1% TritonX-100渗透15 min，加入PNMT(1:100)和TH(1:100)的抗体在4 ℃下孵育过夜。过夜后用PBS清洗，在室温下加入荧光标记二抗避光孵育1 h。阴性对照用含有5%牛血清蛋白的PBS代替一抗。使用荧光显微镜(Leica 2000)观察细胞并摄片。

1.5 电镜下观察AMCCs形态

AMCCs在2%戊二醛中固定3 h后，将标本在1%四氧化锇中再次固定1 h，然后用50%、70%、90%、100%乙醇进行逐级脱水，再以还氧树脂包埋剂812(Epon812)、十二烯基丁二酸酐(DDSA)进行包膜成块。对标本进行超薄(70 nm)切片，再用醋酸双氧铀和柠檬酸铅染色，在H-7500透射电子显微镜

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(Hitachi)下观察细胞并摄片。

1.6 SiMASH1和MASH1质粒的构建和转染

本研究设计了3种用于MASH1沉默的干扰序列，其中具有最佳沉默效率的序列是5'-CUCUAUGGC-GGUUCGCUU-3'(正向)和5'-ACUUAGAUUCAAUG-UUCUCA-3'(反向)。干扰对照序列(si-NC)5'-UUCUC-CGAACGUGUCACGUTT-3'(正向)和5'-ACGUGACA-CGUUCGGAGAATT-3'(反向)由Gene Pharma公司(上海)合成。MASH1序列(基因名称*ASCl1*，序列大小为719 bp)由Genscript公司(中国)采用全基因化学合成方法合成。在*Hin*dIII和*Age*I限制性酶切位点之间插入克隆载体pEGFP-N1构建MASH1质粒(pMASH1)。其中pEGFP-N1被用作表达载体，制备感受态细菌，转化和筛选重组载体质粒，扩增细菌，再使用质粒提取试剂盒提取MASH1质粒。所有质粒都被送到Gene Pharma公司进行基因测序，验证无误后于−80 ℃冰箱冷冻。AMCCs在6孔板中培养12~24 h，当细胞丰度为50%~60%时，更换为无血清、无抗生素的新鲜培养液，通过Lipofectamine 2000试剂用siMASH1、si-NC、pMASH1或pEGFP-N1质粒对AMCCs进行瞬时转染。空白对照细胞只用Lipofectamine 2000进行转染。将AMCCs按2×10^5个/孔，转染质粒4 μg/孔加入培养液中，转染siRNA的终浓度为100 nmol/L，于培养箱内培养12 h后换为含10%胎牛血清的DMEM培养基继续培养，48 h后收集细胞用于后续检测。

1.7 蛋白质印迹法检测AMCCs中靶蛋白表达水平

从各组AMCCs中提取30 μg总蛋白质，在10% SDS-PAGE凝胶上分离，并将蛋白质电转到PVDF膜(Millipore)上。将PVDF膜在4 ℃下与PNMT(1∶1 000)、外周蛋白(1∶1 000)、MASH1(1∶250)、JMJD3(1∶100)、pERK(1∶1 000)和ERK(1∶1 000)抗体孵育过夜，清洗后用辣根过氧化物酶结合的二抗在室温下孵育1 h。通过增强化学发光试剂进行显色曝光，并使用Glyko Bandscan 5.0软件进行量化。目的蛋白质的相对含量以目的蛋白质条带灰度值与内参(β-actin)条带灰度值的比值表示，将该比值除以同一胶片上对照组的灰度值作为该目的蛋白质表达量的半定量结果。实验重复5次。

1.8 Real-time PCR法检测AMCCs中*MASH1*及*JMJD3* mRNA水平

使用TRIzol试剂从AMCCs中提取总RNA。参照反转录试剂盒说明书进行操作。*MASH1*基因用上游引物5'-CAA-CAAGAAGATGAGCAAGGTG-3'和下游引物5'-AAC-CCGCCATAGTTCAAGT-3'进行扩增，*JMJD3*基因用上游引物5'-GGATGACCTCTATGCGT-CCAAT-3'和下游引物5'-CGTTCTTCACCTCGTTCC-ACTC-3'进行扩增。在7900HR Fast Real-time PCR system (Applied Biosystems)中使用SYBR Green PCR试剂盒进行扩增。PCR扩增程序包括94 ℃预热5 min，变性(94 ℃，30 s)、退火(60 ℃，30 s)和延伸(72 ℃，1 min)35个循环，最后72 ℃延伸10 min。PCR产物在2%琼脂糖凝胶上电泳以确认大小。结果以实验组与对照组β-actin的循环数(Ct值)的比值表示。实验重复5次。

1.9 ELISA法检测细胞上清液中肾上腺素水平

按照ELISA试剂盒说明书操作，采用ELISA法检测细胞上清液中肾上腺素水平。将空白对照孔调零，在450 nm波长处依序测量各孔的OD值。实验重复5次。

1.10 统计学处理

采用SPSS 26.0统计学软件分析数据，计量资料以均数±标准差($\bar{x}\pm s$)表示，样本符合正态分布且方差齐性时组间比较采用单因素方差分析(one-way ANOVA)的SNK-q检验，对不同浓度及干预时间点的组间比较采用重复测量方差分析，以α=0.05为检验水准，P<0.05为差异有统计学意义。

2 结　果

2.1 AMCCs的鉴定

免疫荧光染色示细胞质内TH(图1A，绿色)和PNMT(图1B，红色)共定位双染阳性，鉴定为AMCCs。

2.2 确定NGF刺激的浓度和时间

如图2所示，分离的AMCCs在早期呈圆形，在光镜下AMCCs附着在培养皿上后变成多角形。经过NGF刺激，AMCCs出现长的类似神经元样的细胞突起(图2A)。随着NGF刺激时间的延长(100 ng/mL处理24、48、72 h)，细胞突起数量逐渐增加(图2A)。当NGF刺激24 h后，200 ng/mL NGF组AMCCs的细胞突起数量显著多于50 ng/mL及100 ng/mL NGF组(均P<0.05)。当干预48 h后，100 ng/mL与200 ng/mL NGF组AMCCs的细胞突起数量差异无统计学意义(P>0.05)。MMT法测NGF不同干预时间(24、48、72 h)不同干预浓度(50、100、200 ng/mL)AMCCs的

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相对细胞活性(以0 h的相对细胞活性作为参考值)。结果显示：随着NGF刺激时间的延长，100 ng/mL与200 ng/mL NGF组AMCCs的OD值逐渐下降；在刺激48 h后，200 ng/mL组细胞活性显著低于100 ng/mL NGF组(*P*<0.05，图2C)。根据以上结果，NGF刺激时间选择48 h，刺激浓度为100 ng/mL。

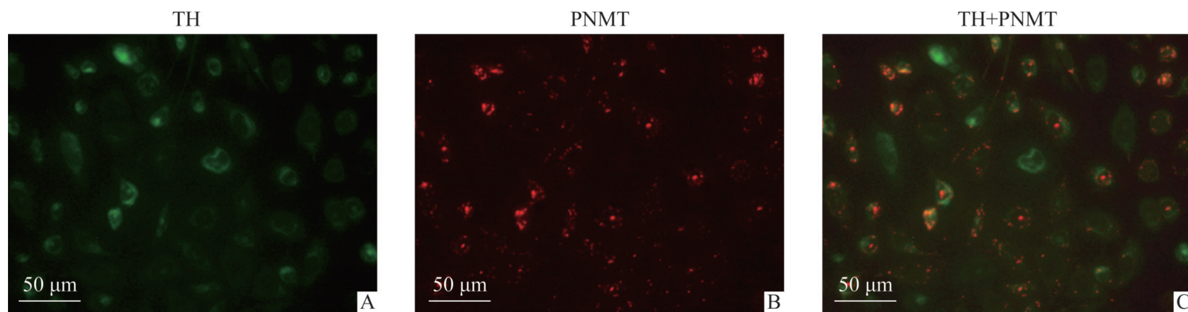

图1 免疫荧光法检测原代大鼠肾上腺髓质嗜铬细胞

Figure 1 Immunofluorescence micrograph of AMCCs of primary culture rat

A and B: Immunoreactivity staining for TH (green, A) and PNMT (red, B). C: Dual staining for TH and PNMT. Cells have TH colocalized with PNMT, being proved to be epinephrine cells. Cells presenting only TH-positive are considered as norepinephrine cells. AMCCs: Adrenal medulla chromaffin cells; TH: Tyrosine hydroxylase; PNMT: Phenylethanolamine N-methyl transferase.

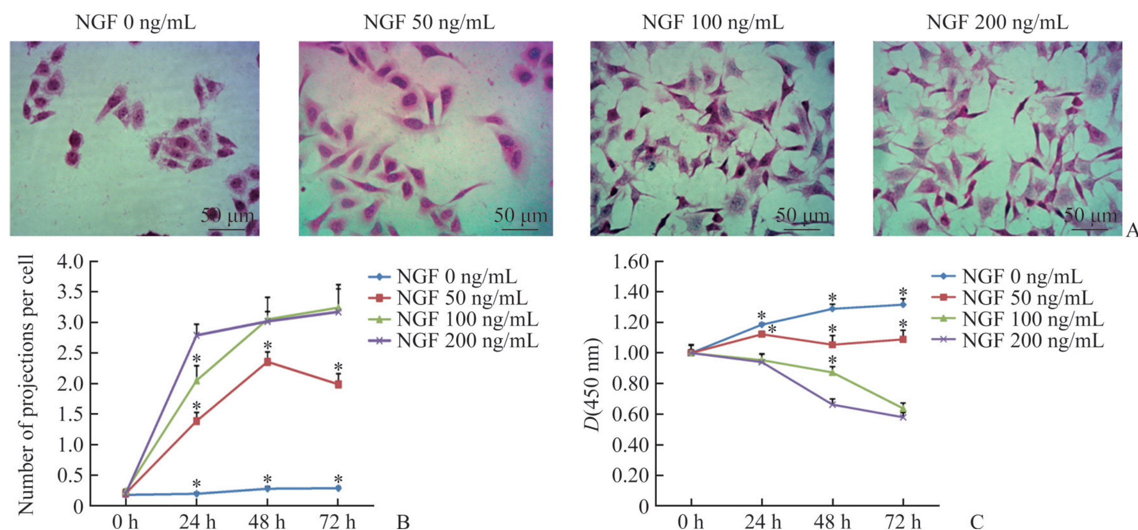

图2 神经生长因子刺激嗜铬细胞出现形态及细胞活性改变(*n*=3)

Figure 2 Nerve growth factor stimulated morphological and cellular activity changes of AMCCs (*n*=3)

A and B: Under the light microscope, AMCCs shows the cell processes in each group after simulation with different concerntrations of NGF for 48 hours. When the intervention time is equal to or more than 48 hours, there is no significant difference in the number of processes between 100 ng/mL and 200 ng/mL NGF groups. C: After 48 hours of NGF stimulation, the relative rate of AMCCs viability in the 200 ng/mL NGF group is significantly lower than that in the 100 ng/mL NGF group. **P*<0.05 vs the 200 ng/mL NGF group. NGF: Nerve growth factor; AMCCs: Adrenal medulla chromaffin cells; *D*: Optical density.

2.3 NGF诱导AMCCs向神经元转分化

经过NGF刺激，AMCCs出现长的细胞突起(图3A)。电子显微镜下显示：对照组的嗜铬颗粒散在分布于AMCCs的细胞质中，在NGF刺激后，AMCCs出现突起的同时嗜铬颗粒也明显减少(图3B)。在NGF组中，细胞突起数量显著增加(*P*<0.01，图3C)。与对照组相比，NGF刺激可使AMCCs中外周蛋白的表达量升高，同时PNMT的表达量下降(*P*<0.01，图3D、3E)。与NGF组相比，NGF+DEX组的AMCCs突起数量显著减少(图3C)，PNMT蛋白表达水平升

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高，同时外周蛋白表达水平下降(P<0.05，图3D、3E)，证实DEX刺激能够部分逆转NGF对AMCCs神经元转分化作用，恢复AMCCs的内分泌表型。

2.4 MASH1高表达是AMCCs神经元转分化的关键

NGF刺激可上调AMCCs中MASH1蛋白的水平(P<0.01，图4)。干扰MASH1后，NGF组的AMCCs中PNMT蛋白的表达水平上升，外周蛋白的表达水平下降(P<0.01，图4A)，AMCCs的细胞突起数量也减少(P<0.01，图4B)。相反，MASH1的过表达与NGF的作用相当。与对照组相比，MASH1过表达组AMCCs的突起数量增加(P<0.01，图4C)，外周蛋白的表达水平升高，PNMT表达水平显著下降(均P<0.01，图4D)。与NGF组、pMASH1组比较，NGF+pMASH1组MASH1表达水平、AMCCs突起数量稍有增加，但差异均无统计学意义(均P>0.05，图4D)。

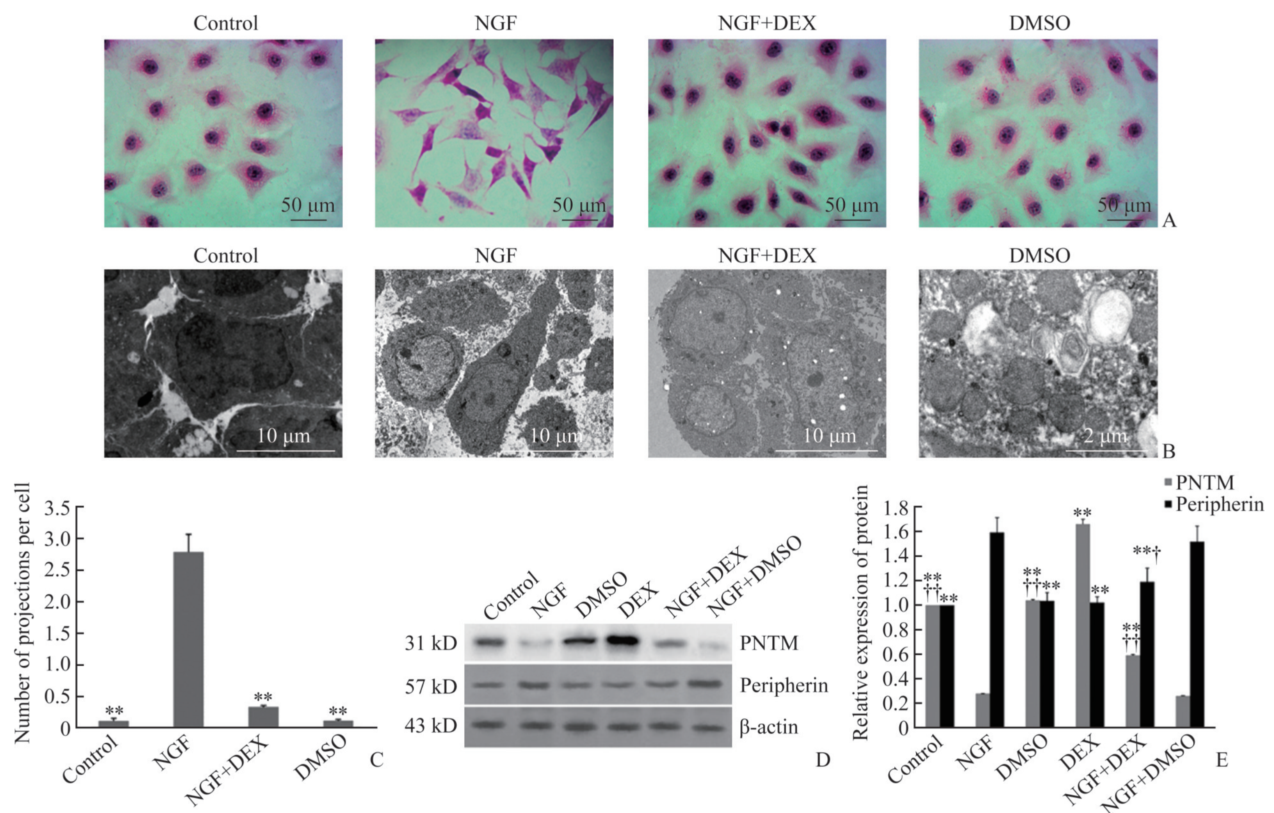

图3　神经生长因子诱导肾上腺髓质嗜铬细胞发生神经元转分化以及地塞米松对其的抑制作用(n=5)

Figure 3　Neuron transdifferentiation of AMCCs induced by NGF and the inhibitory effect of DEX (n=5)

A: After exposure to NGF, AMCCs exhibit long neurite-like processes under the light microscope after hematoxylin and eosin (HE) staining. B: Processes outgrowth and decreased chromaffin granules are shown in the presence of NGF under the electron microscope. C: The number of processes significant increases in the NGF group. A combination of NGF and DEX recoveres the regular shape of AMCCs and suppresses the projections. D and E: AMCCs treated with NGF shows a significant increase in peripherin protein expression and a significant decrease in the PNMT protein level. The protein expression of PNTM is augmented and the level of peripherin is reduced by NGF in combination with DEX. **P<0.01 vs the NGF group, †P<0.05 vs the DEX group, ††P<0.01 vs the DEX group. AMCCs: Adrenal medulla chromaffin cells; NGF: Nerve growth factor; DEX: Dexamethasone; PNMT: Phenylethanolamine N-methyl transferase. DMSO: Dimethylsulfoxide.

2.5 NGF通过激活pERK/MASH1通路诱导AMCCs向神经元转分化

加入PD98059后，NGF+PD98059组的AMCCs突起数量与NGF组相比显著减少(P<0.01，图5A、5B)。NGF刺激使AMCCs中JMJD3和MASH1的蛋白质及mRNA的表达水平升高，pERK的蛋白质表达

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水平也上升(均P<0.05)，上述改变经过PD98059处理后受到抑制(均P<0.05，图5C、5D)。同时，NGF诱导的PNMT表达下调和外周蛋白表达上调作用也被PD98059所逆转(P<0.05，图5D)。这些结果表明pERK/MAPK通路可能参与了NGF诱导的AMCCs中MASH1的激活。此外，DEX干预也抑制了AMCCs中NGF介导的pERK/MAPK信号通路的激活以及JMJD3和MASH1的高表达(均P<0.05，图5E)。

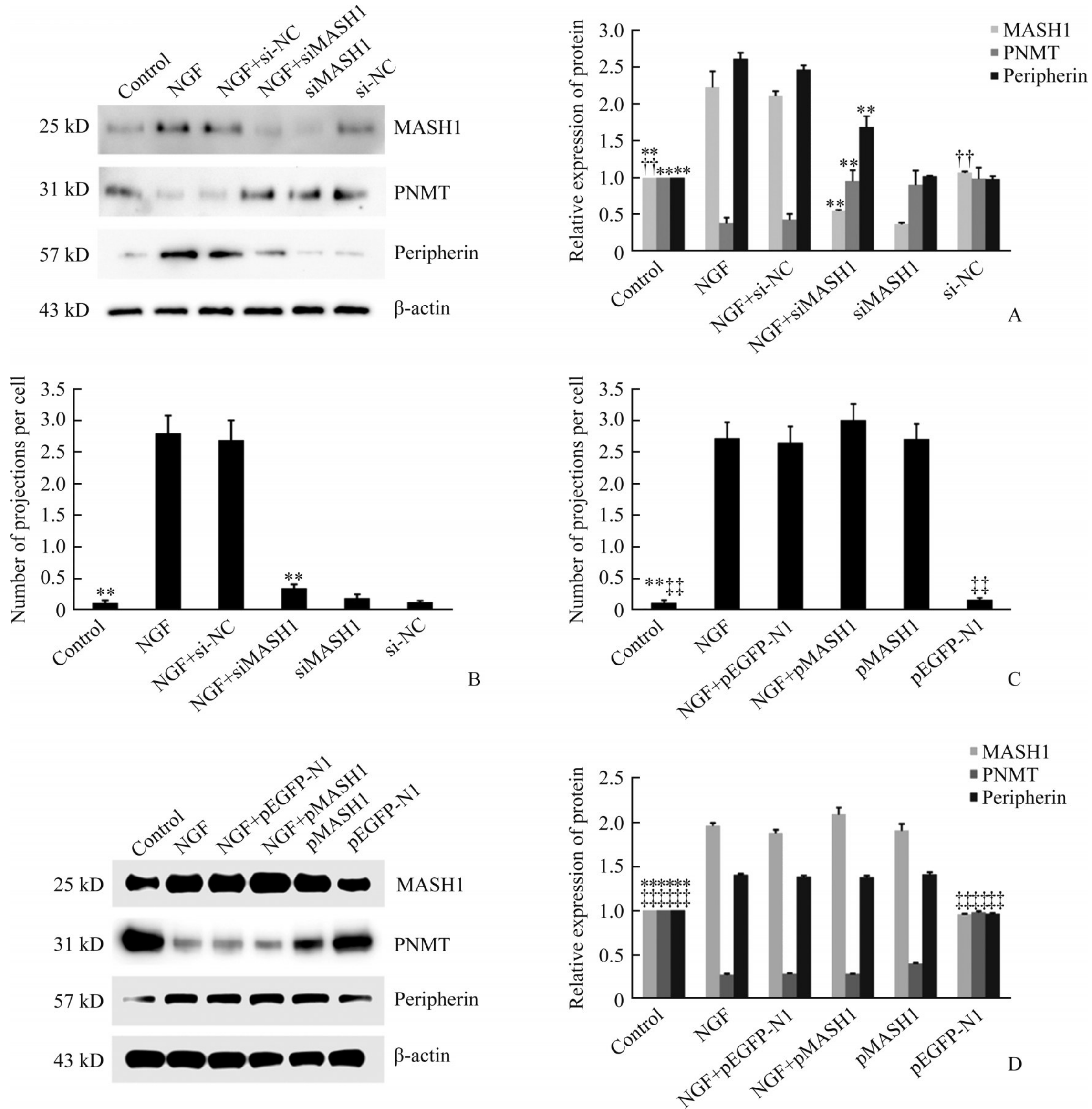

图4 MASH1表达水平的升高可诱导肾上腺髓质嗜铬细胞向神经元转分化(n=5)

Figure 4 Elevated MASH1 mediates neuron transdifferentiation of AMCCs (n=5)

A: NGF upregulates the level of MASH1 in AMCCs. B: MASH1 knockdown in AMCCs increases the protein level of PNMT, conversely reduces the level of peripherin, and significantly decreases the number of projections induced by NGF simulation. C and D: The number of projections increases significantly in the MASH1 overexpression group accompanied by the elevated protein level of PNMT and the decreased protein level of peripherin. **P<0.01 vs the NGF group, ††P<0.01 vs the siMASH1 group, ‡‡P<0.01 vs the pMASH1 group. AMCCs: Adrenal medulla chromaffin cells; NGF: Nerve growth factor; PNMT: Phenylethanolamine N-methyl transferase.

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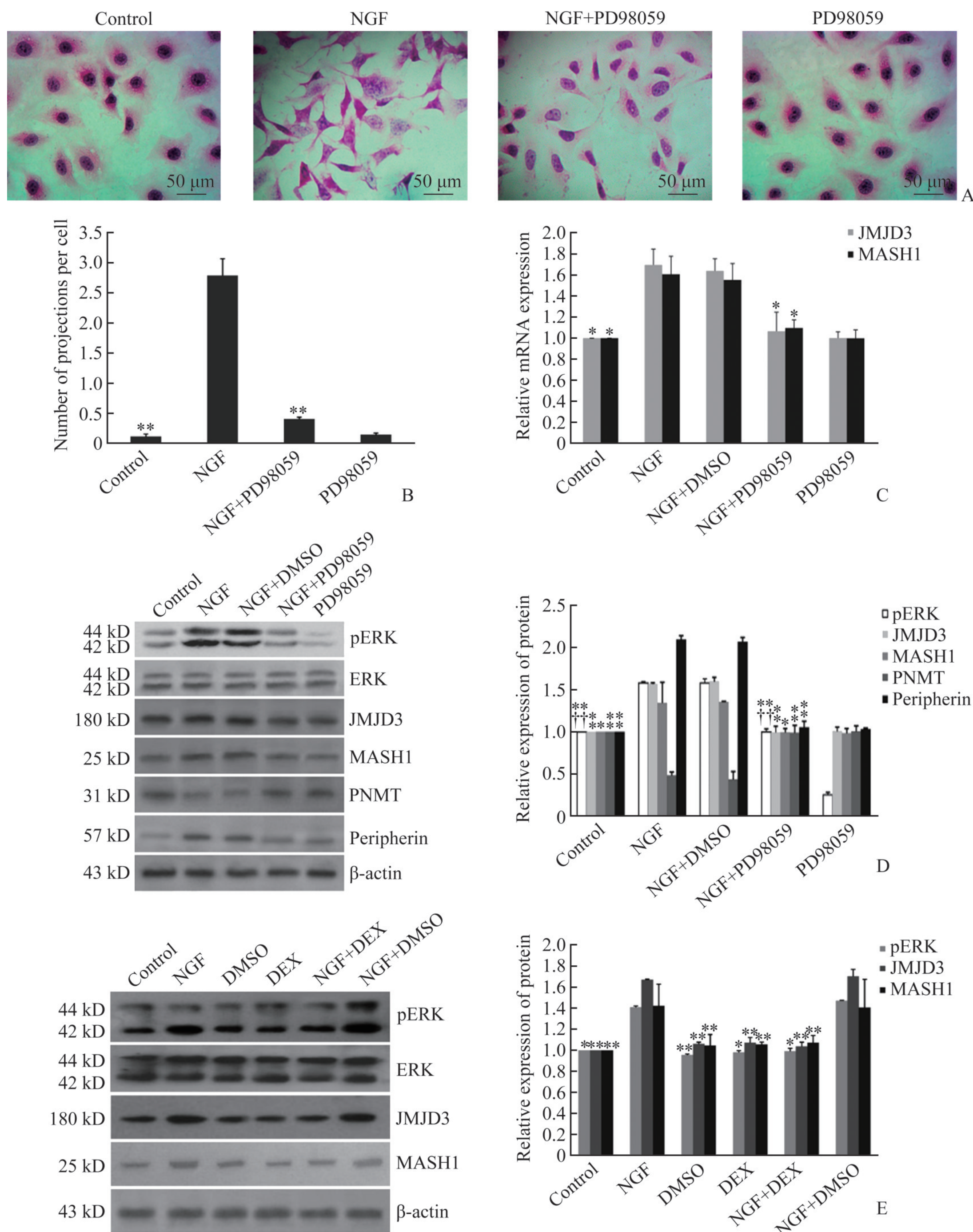

图5　神经生长因子可能通过激活pERK/MASH1信号通路诱导肾上腺髓质嗜铬细胞发生神经元转分化(n=5)

Figure 5　NGF-induced neuron transition of AMCCs by activation of pERK/MASH1 signaling pathway (n=5)

A and B: A rise in neurite elongation by NGF interference is attenuated substantially after exposure to PD98059. C and D: After exposure to NGF, the protein levels of pERK, and protein as well as mRNA levels of JMJD3 and MASH1 in AMCCs are significantly elevated, which is reversed by the treatment of PD98059. The protein expression of PNMT is upregulated and the level of peripherin is downregulated by PD98059 treatment. E: The application of DEX to AMCCs inhibits the activation of pERK/MASH1 signaling by NGF treatment. $^*P<0.05$ vs the NGF group, $^{**}P<0.01$ vs the NGF group, $^{††}P<0.01$ vs the PD98059 group. NGF: Nerve growth factor; AMCCs: Adrenal medulla chromaffin cells; PNMT: Phenylethanolamine N-methyl transferase; DEX: Dexamethasone.

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2.6 NGF通过激活pERK/MASH1信号通路抑制AMCCs分泌肾上腺素

NGF刺激使AMCCs内分泌表型受损，从而减少其肾上腺素的分泌(P<0.01，图6A)。DEX或PD98059处理使AMCCs分泌肾上腺素增多(P<0.01，图6A、6B)。MASH1干扰逆转了NGF对AMCCs中肾上腺素分泌的抑制作用使肾上腺素分泌增加(P<0.01，图6C)。MASH1的过表达与NGF作用类似，肾上腺素的分泌也受到抑制(P<0.01，图6D)。与pMASH1组对比，NGF+pMASH1组肾上腺素分泌显著下降(P<0.01，图6D)。

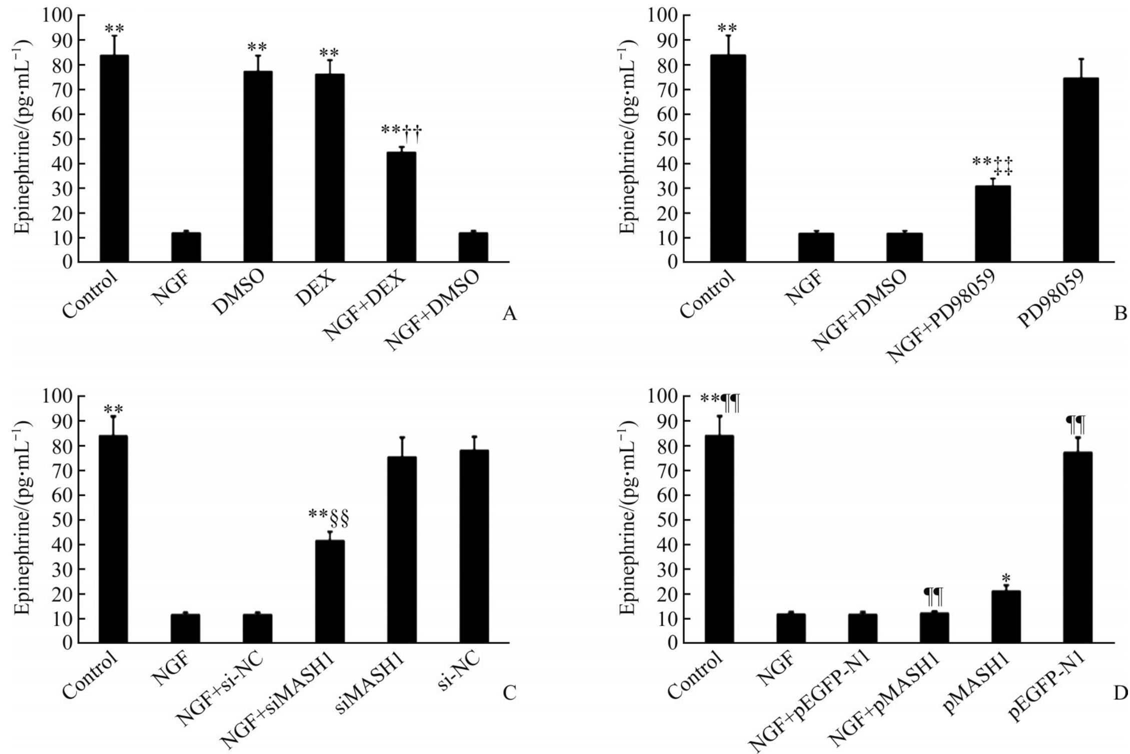

图6 神经生长因子通过激活pERK/MASH1信号通路抑制肾上腺髓质嗜铬细胞分泌肾上腺素(n=5)

Figure 6 NGF inhibits the secreted levels of epinephrine from AMCCs via the activation of pERK/MASH1 signaling (n=5)

A and B: NGF treatment suppresses epinephrine synthesis from AMCCs. DEX and PD98059 inhibit the effect of NGF by elevating the secreted levels of epinephrine. C: MASH1 knockdown upregulates the expression of epinephrine in AMCCs treated with NGF. D: MASH1 overexpression significantly downregulates the level of epinephrine. Compared with the MASHI overexpression, NGF combined with MASH1 overexpression further downregulates the level of epinephrine from AMCCs. *P<0.05 vs the NGF group, **P<0.01 vs the NGF group, ††P<0.01 vs the DEX group, ‡‡P<0.01 vs the PD98059 group, §§P<0.01 vs the siMASH1 group, ¶¶P<0.01 vs the pMASH1 group. NGF: Nerve growth factor; DEX: Dexamethasone; AMCCs: Adrenal medulla chromaffin cells. DMSO: Dimethylsulfoxide.

3 讨 论

既往研究[1, 4, 7, 9-10, 15]均证实AMCCs具有可塑性，其在NGF的诱导下会向神经元转分化。但是，很少有研究涉及AMCCs转分化的机制。有学者[16]提出miR-124a抑制NGF诱导的PC12细胞(大鼠AMCCs瘤克隆的细胞株)中的神经元样突起。本研究表明MASH1可诱导AMCCs发生神经元转分化。

MASH1在哺乳动物的神经分化及发育即从多潜能细胞向神经组细胞分化并产生神经元的过程中发挥重要作用[17-18]。给予NGF可以显著提高MASH1在神经干细胞和Müller细胞中的表达水平[19-20]。本课题组前期在哮喘动物模型中发现MASH1在AMCCs中表达水平显著升高[7]。MASH1是交感神经祖细胞的标志物，其在祖细胞中的表达量较高[21]。存在MASH1缺陷的小鼠交感神经元功能严重受损，而同

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样由交感神经祖细胞发育而来的AMCCs却不受影响[13]，也有研究[22-23]指出MASH1在AMCCs或交感神经元的成熟过程中是必需的。目前大多数研究集中在MASH1对神经元的发育作用，尚无MASH1对成熟AMCCs表型影响的研究。本研究发现MASH1对AMCCs发生神经元表型的转换是必不可少的。在抑制MASH1后，NGF诱导AMCCs分化的作用被抑制；当MASH1过表达时，AMCCs无论从形态上还是功能上均出现了类似NGF刺激的改变。与NGF组相比，过表达MASH1并没有使NGF诱导的神经转分化效果进一步增强，这可能与本研究中使用的NGF浓度较高有关。既往研究[13]表明：小剂量的NGF(10 ng/mL)对AMCCs的主要作用在于促进其增殖，而不是促进其神经突触的生长。因此，本研究根据预实验结果选择较高浓度NGF进行刺激。在预实验中，我们发现当NGF超过100 ng/mL时，即使浓度再增加，AMCCs中细胞突起数量也不会出现显著的增长，说明AMCCs神经转分化的能力是有限的。但是，与pMASH1组对比，NGF+pMASH1组的细胞突起数量稍有增多，同时PNMT表达水平稍降低，NGF+pMASH1组分泌肾上腺素的水平较pMASH1组显著下降，说明MASH1过表达与NGF有一定的协同效应，分泌功能改变较形态上改变更明显。外源性NGF可使MASH1的表达增多，TrkA和PI3K/Akt信号、Notch1/Hes1信号和Wnt/β-catenin信号通路被证实在不同的细胞类型中均参与该过程[19-20, 23-24]。在AMCCs中，MAPK通路的磷酸化水平升高被证明可能是NGF诱导其发生神经元分化的关键通路[7, 25]。本研究结果显示：NGF诱导的MASH1表达与pERK/MAPK通路活化有关；PD98059处理对NGF引起的AMCCs形态学和内分泌功能上的改变均有逆转作用，同时也抑制了JMJD3和MASH1的表达上调。JMJD3是一种特异性组蛋白H3赖氨酸27脱甲基酶，它可以调控一些神经发生的关键因子的表达，包括MASH1[26-27]。JMJD3已被证实通过ERK/MAPK通路调控参与PC12细胞向神经元表型分化的过程[28]。在P19细胞中，JMJD3与MASH1启动子的近端上游区域结合后使MASH1的启动子活性显著增强，从而使MASH1水平升高[27]。在本研究中，各组AMCCs中MASH1的表达水平变化趋势与JMJD3相同。此外，NGF诱导的JMJD3水平的上调明显被PD98059抑制，该发现与在PC12细胞中的结果一致[27-28]。因此，我们推断JMJD3可能是pERK/MAPK信号级联的下游因子、MASH1的上游因子。NGF诱导的AMCCs神经元转分化可能是由pERK/JMJD3/MASH1信号通路所介导的。

肾上腺皮质分泌的糖皮质激素对AMCCs内分泌表型的维持至关重要。既往有研究[14]发现DEX对NGF介导的AMCCs神经元转分化有显著的抑制作用。PNMT是AMCCs内分泌表型的标志物。外周蛋白是神经元特异性中间丝蛋白，是神经元的结构标志物，其在AMCCs中几乎不表达[29]。DEX可诱导AMCCs中PNMT表达增加，抑制外周蛋白的表达[1,14]。本实验也证实了上述研究结果。然而，很少有研究涉及糖皮质激素在AMCCs转分化过程中的作用机制。研究[30]发现用皮质酮(动物体内的糖皮质激素)刺激PC12细胞可显著抑制MAPK经典途径相关基因表达，包括*ERK*。但是，在本实验中pERK/MAPK通路并没有在DEX的干预下出现显著的变化。DEX处理只是抑制了NGF对AMCCs中pERK/MAPK通路的激活作用。在以往研究[30]中，PC12细胞是用皮质酮处理的，对PC12的干预时间从15 min到24 h不等。而本实验是使用DEX这一经典的人工合成的糖皮质激素对AMCCs干预48 h。因此，研究差异可能是干预对象和方法上的不同造成的。此外，DEX对NGF诱导的JMJD3和MASH1表达水平升高也有抑制作用。以上结果提示，DEX可能通过下调pERK/MASH1信号通路活性来发挥对NGF诱导的AMCCs神经元转分化的抑制作用。

本研究有以下局限性：1)只观察到JMJD3和MASH1之间有相同的趋势，并没有证实JMJD3在pERK/MASH1通路中的作用。2)仅在体外试验中证实了MASH1在AMCCs神经元转分化中的作用，虽然本课题组在既往的动物试验中发现哮喘模型大鼠的AMCCs中MASH1表达升高可能参与NGF诱导的AMCCs神经元转分化的过程[7]，但仍缺乏体内试验的结果来证实MASH1的作用及其机制。由于缺乏肾上腺素能神经支配，气道平滑肌的舒缩调节主要是依赖血液循环中的肾上腺素与气道平滑肌上的β_2-肾上腺素能受体结合而实现的。当循环中的肾上腺素水平不足时，在应激情况下可能出现持续的支气管平滑肌痉挛，导致哮喘持续发作。哮喘患者外周循环中NGF水平升高可以通过诱导AMCCs发生神经元转分化而导致AMCCs分泌肾上腺素不足，该过程参与哮喘的发病[7, 9-10, 16]。与NGF类似，MASH1也对AMCCs分泌肾上腺素有抑制作用。敲除*MASH1*基因后，NGF对AMCCs分泌肾上腺素的抑制作用显著减弱。这些结果提示MASH1有可能作为哮喘的潜在治疗目标，后续应继续研究MASH1在哮喘动物模型中的作用。

综上所述，本研究证实pERK/MASH1信号通路参与了NGF诱导的AMCCs向神经元转分化的过程，

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而MASH1是该过程中的一个关键因子。DEX对NGF诱导的pERK/MASH1信号激活有抑制作用，这一发现是对糖皮质激素治疗哮喘的经典抗炎机制的补充。AMCCs具有内分泌及神经双重表型，其可能为神经退行性疾病或神经内分泌疾病的治疗提供细胞来源。

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利益冲突声明：作者声称无任何利益冲突。

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