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肿瘤微环境影响间充质干细胞增殖及其分子机理

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摘要 间充质干细胞(mesenchymal stem cells, MSCs)是一类具有靶向迁移特性的潜在抗瘤细胞。肿瘤微环境中的生长因子和趋化因子可能对MSCs的生物学行为产生显著影响, 但MSCs在肿瘤微环境中的生物学行为变化规律及其机理还并不清楚。该文探究了大鼠肝癌细胞条件培养基(conditioned medium from hepatocellular carcinoma cells, HCC-CM)对大鼠间充质干细胞(rat mesenchymal stem cells, rMSCs)增殖的影响及其机理。研究发现, HCC-CM能显著促进rMSCs的增殖和基质衍生因子-1(stromal derived factor-1, SDF-1)的表达。Western blot检测发现, HCC-CM在15 min至2 h均显著上调rMSCs ERK1/2磷酸化, PD98059可完全消除HCC-CM诱导的ERK1/2磷酸化并同时阻断HCC-CM诱导的rMSCs增殖。趋化因子C-X-C受体4(Chemokine C-X-C motif 4, CXCR4)抑制剂AMD3100部分抑制ERK1/2磷酸化, 同时也部分抑制了HCC-CM促rMSCs增殖。结果揭示了HCC-CM对rMSCs增殖的影响以及ERK1/2信号分子和SDF-1/CXCR4在HCC-CM促rMSCs增殖过程中的作用, 为全面了解MSCs在重塑的肿瘤微环境中生物学行为的变化规律奠定了基础。

关键词 肝癌细胞; 条件培养基; 间充质干细胞; 增殖; 分子机理

Effects of Tumor Microenvironment on Proliferation of Mesenchymal Stem Cells and Its Molecular Mechanism

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Abstract Mesenchymal stem cells (MSCs) have been demonstrated to be a promising targeted antitumor agent. The various tumor-cell-secreted cytokines and chemokines may have a significant impact on MSCs. However, the changes of MSCs cellular characteristics as well as involved molecular mechanism under tumor microenvironment are not fully understood. In this study, we investigated the conditioned medium from hepatocellular carcinoma cells (HCC-CM) on proliferation of rat mesenchymal stem cells (rMSCs) and the possible signal molecules in this procedure. We found that HCC-CM significantly promoted the proliferation and the expression of stromal cell-derived factor-1 (SDF-1) in rMSCs. Western blot showed that ERK1/2 was strongly activated following the stimuli of HCC-CM from 15 min to 2 h. PD98059 significantly inhibited HCC-CM-induced rMSCs ERK1/2 phosphorylation and proliferation. Moreover, AMD3100, the inhibitor of CXCR4, partially disrupted phosphorylation of ERK1/2 as well as HCC-CM-induced proliferation. These results demonstrated that ERK1/2 molecule and SDF-1/

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CXCR4 play a significant role in the HCC-CM-induced rMSCs proliferation. This study also provides an insight into the molecular mechanism of MSCs proliferation in response to conditioned medium of tumor cells and lays a foundation for fully understanding the cellular characteristics of MSCs in the remolded tumor microenvironment.

Key words hepatoma cells; conditioned medium; mesenchymal stem cells (MSCs); proliferation; molecular mechanism

肿瘤微环境的构成除了恶性的上皮细胞外, 还包括周围其他类型的非癌症细胞、细胞外基质以及可溶的信号分子等[1]。间充质干细胞(mesenchymal stem cells, MSCs)是一类可受到肿瘤细胞分泌的细胞因子或趋化因子招募定向迁移到肿瘤细胞的多能干细胞[2-4]。MSCs与肿瘤细胞的相互作用会重塑肿瘤微环境[5], 进而导致肿瘤细胞和MSCs显著的生物学行为变化。重塑的肿瘤微环境既可通过MSCs的免疫抑制功能帮助肿瘤细胞逃离免疫监督, 促进肿瘤细胞恶性增殖[6]; 又可通过阻断Wnt信号通路, 抑制肿瘤细胞的增殖[7-8]; 抑瘤或致瘤主要取决于肿瘤细胞的类型和分化水平等。但重塑的肿瘤微环境对MSCs生物学特性的影响并不清楚, 其机理也鲜有研究。

细胞信号转导是涉及多种信号分子的网络系统, 它能将化学信号或物理信号转换为生物信号, 进而促发一系列细胞水平的响应[9]。丝裂原活化蛋白激酶(mitogen-activated protein kinases, MAPKs)信号分子具有接受从细胞膜上受体传递下来的信息并进一步向核内传递的能力, 控制基因转录和蛋白表达[10]。胞外信号调节激酶1/2(extracellular-signal-regulated kinases 1/2, ERK1/2)是MAPKs家族的主要成员, ERK1和ERK2均包含苏氨酸–谷氨酸–酪氨酸(Thr-Glu-Tyr)二级结构域, 该结构极易受到多种生长因子的影响[11], 其活化环的激活能通过Ras-Raf-1-MEK1/2传递信息最终激活ERK1/2信号[10]。ERK1/2信号的激活与MSCs的增殖密切相关, Liang等[12]报道低剂量电离辐射激活rMSCs ERK1/2信号促进其增殖。除了物理刺激, 化学刺激也能转变为细胞信号通过ERK1/2分子影响rMSCs增殖, 如成纤维细胞生长因子2(fibroblast growth factor 2, FGF-2)、FGF-4和血小板衍生因子(platelet derived growth factor, PDGF)等[13-14]。以上研究说明, 多种因素均可能刺激rMSCs的增殖, 且该过程与ERK1/2信号分子密切相关。

SDF-1是一种可与CXCR4协同作用并向下游传递信号的基质细胞分泌蛋白, 在细胞增殖、凋亡、迁移甚至分化等方面具有重要的作用[15-18]。尽管SDF-1/CXCR4扮演着信号通路入口的角色, 但对其研究还并不深入。ERK1/2是能接受SDF-1/CXCR4信号的主要信号分子, Gao等[19]报道了外源加入的SDF-1通过ERK1/2信号通路促进MSCs迁移, 但SDF-1/CXCR4与ERK1/2信号分子在增殖方面是否存在关联, 这类研究还非常有限。本文通过大鼠肝癌细胞条件培养基(HCC-CM)培养rMSCs, 建立肝癌细胞与MSCs共存微环境模型, 进而研究HCC-CM对rMSCs增殖的影响以及ERK1/2信号分子和SDF-1/CXCR4在此过程中的作用。

1 材料与方法

1.1 材料

1.1.1 *动物与细胞* SD(Sprague Dawley)雄性大鼠购于重庆第三军医大学实验动物中心, 2月龄, 150~200 g。大鼠肝癌细胞CBRH-7919购于中国科学院上海生命科学研究所细胞资源中心, 大鼠肝细胞BRL-3a由首都医科大学北京佑安医院郑素军博士提供。

1.1.2 *主要试剂* DMEM-LG低糖培养基、EDTA和胰蛋白酶(Hyclone公司); Percoll淋巴细胞分离液(GE Healthcare Europe); 类标准胎牛血清(兰州民海生物工程有限公司); MEK/ERK1/2抑制剂PD98059(碧云天生物技术研究所); MTT(Sigma公司); EdU细胞增殖检测试剂盒(广州锐博生物科技有限公司); RNA提取试剂盒(北京百泰克生物技术有限公司); 引物(Invitrogen公司); 逆转录试剂盒、PCR相关试剂和SDS-PAGE凝胶配置试剂盒(TaKaRa公司); CXCR4抑制剂AMD3100、兔抗大鼠CXCR4抗体(Santa Cruz公司); 兔抗大鼠p-ERK1/2、兔抗大鼠t-ERK1/2抗体(Cell Signaling Technology公司)。

1.2 方法

1.2.1 *细胞分离与培养* 将2月龄SD大鼠脱颈处死,

无菌条件下剪开大鼠股骨和胫骨, 用无血清DMEM-LG培养基吹出骨髓, 加入预先装有1.073 g/mL淋巴细胞分离液的离心管中。通过密度梯度离心法分离大鼠骨髓间充质干细胞(rat mesenchymal stem cells, rMSCs), 将分离得到的rMSCs置于含有10%胎牛血清以及100 U/mL青霉素和100 μg/mL链霉素的完全培养基中培养, P2-P4代rMSCs用于实验研究。大鼠肝癌细胞CBRH-7919和大鼠肝细胞BRL-3a培养条件同rMSCs。

1.2.2 条件培养基的制备 将常规培养的CBRH-7919用PBS清洗3次, 之后加入无血清培养基孵育24 h, 收集肝癌细胞条件培养基(conditioned medium from hepatocellular carcinoma cells, HCC-CM), 1 500 r/min离心5 min、过滤、分装、–20 °C保存备用。同样的方法制备大鼠正常肝细胞BRL-3a条件培养基(conditioned medium from rat normal hepatocyte cells, NCM)。

1.2.3 抑制ERK1/2激活以及SDF-1/CXCR4信号轴 将MEK/ERK1/2抑制剂PD98059和CXCR4小分子抑制剂AMD3100溶解于DMSO中, –20 °C保存。抑制实验中, PD98059和AMD3100的工作浓度分别为50 μmol/L和10 μg/mL, 用抑制剂预处理1 h后, 在抑制剂存在下评价HCC-CM对rMSCs ERK1/2磷酸化以及增殖的影响。

1.2.4 MTT检测 将rMSCs接种于6孔板中, 24 h后饥饿处理24 h, 之后分别加入1 000 μL无血清培养基、HCC-CM、NCM, 继续培养24 h后加入100 μL MTT(5 mg/mL)孵育5 h, 弃废液后每孔加入1 000 μL DMSO溶解甲瓒, 吸取150 μL至96孔板, 用酶联免疫检测仪于490 nm波长检测吸光度值。

1.2.5 EdU检测 将rMSCs接种于24孔板中, 24 h后饥饿处理24 h。配置适量30 μmol/L EdU培养基, 无血清培养组作为对照组, 10%血清完全培养基组作为阳性对照组。细胞在各组EdU培养基中孵育24 h后通过4%多聚甲醛固定细胞。Apollo®和Hoechst33342分别对新生细胞核以及全部细胞核进行染色, 荧光显微镜下观察并照相记录, 利用ImageJ2对结果进行分析处理。

1.2.6 RT-PCR 使用RNA提取试剂盒提取细胞总RNA, 根据逆转录试剂盒说明书执行逆转录。在RT-PCR反应中, *SDF-1*、*CXCR4*和*β-actin*的引物序列见表1。PCR扩增条件为: 97 °C预变性3 min; 97 °C变性15 s, 55.5 °C退火30 s, 72 °C延伸15 s, *SDF-1*、*CXCR4*和*β-actin*的循环次数分别为29、33和21; 72 °C延伸7 min。PCR产物通过Gold View核酸染料染色的1.5%琼脂糖凝胶电泳分离。以*β-actin*为内参评价*SDF-1*和*CXCR4*基因水平表达的变化。

1.2.7 Western blot 加入含有蛋白酶抑制剂以及磷酸酶抑制剂混合物I和II的细胞裂解液提取蛋白, 通过10% SDS-聚丙烯酰胺凝胶电泳(PAGE)分离蛋白质, 电泳后电转至PVDF膜。5%脱脂牛奶室温孵育1 h后分别与CXCR4、磷酸化ERK1/2(p-ERK1/2)和总ERK1/2(t-ERK1/2)抗体4 °C孵育过夜, 与辣根过氧化物酶标记的二抗(山羊抗兔)室温孵育1 h。ECL发光液显色后通过Bio-Rad显影仪采集分析图像。以β-actin为内参评价CXCR4蛋白水平的表达变化, 以t-ERK1/2为内参评价p-ERK1/2的表达变化。

1.2.8 统计分析 数据通过Excel分析, 以平均值±标准差($\bar{x}\pm s$)表示。rMSCs无血清处理组增殖率、mRNA表达率和蛋白表达率均定义为100%, 两组间比较采用*t*检验分析, $P<0.05$表示具有显著性差异, $P<0.01$表示具有极显著性差异。

2 结果

2.1 大鼠肝癌细胞条件培养基(HCC-CM)促进rMSCs增殖

表1 RT-PCR引物

Table 1 Primers used for RT-PCR

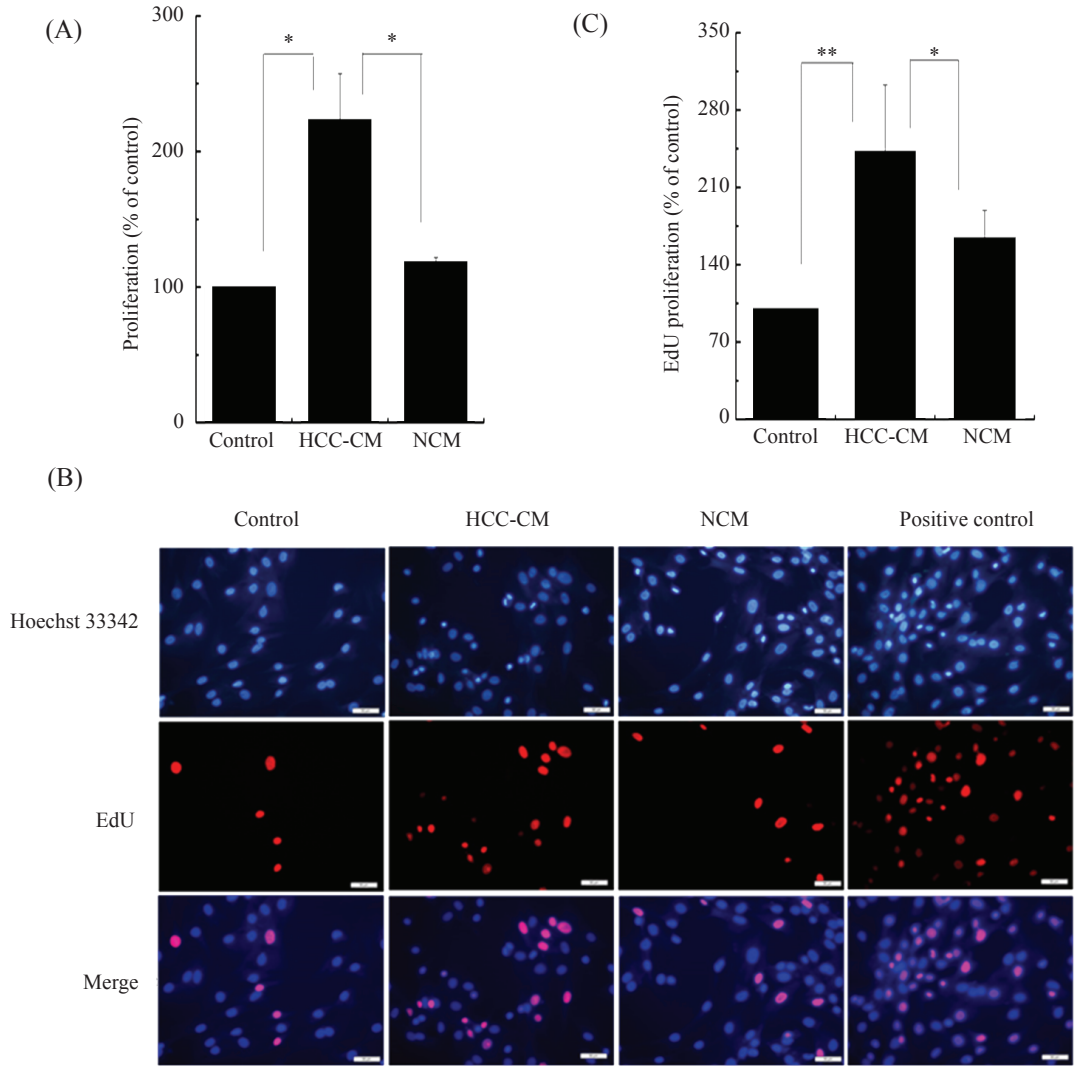
基因 Gene	上游引物(5′→3′) Upstream primer(5′→3′)	下游引物(5′→3′) Downstream primer(5′→3′)	产物长度(bp) Length(bp)
SDF-1	TTT GAG AGC CAT GTC GCC A	TGT CTG TTG TTG CTT TTC AGC C	104
CXCR4	GGG TTG GTA ATC CTG GTC	ATG ATG TGC TGG AAC TGG	446
β-actin	CTG CCG CAT CCT CTT CCT C	CTC CTG CTT GCT GAT CCA CAT	398

通过MTT法检测发现, HCC-CM刺激rMSCs 24 h后, HCC-CM处理组相比对照组增殖率显著上调(P<0.05)(图1A)。EdU检测发现, HCC-CM处理rMSCs 24 h后, 新生rMSCs占全部rMSCs的比例显著高于对照组(P<0.01)(图1B和图1C)。本实验同时发现, NCM对于rMSCs增殖也存在作用, 但不及HCC-CM促rMSCs增殖效果显著。

2.2 HCC-CM激活ERK1/2信号促进rMSCs增殖

为了确定HCC-CM是否能通过激活ERK1/2信号诱导rMSCs增殖, Western blot检测了HCC-CM刺激rMSCs 15, 30, 60, 90, 120 min的磷酸化ERK1/2(p-ERK1/2)表达。结果显示, 在各检测时间点p-ERK1/2表达量相比对照组均显著增加(图2A和图2B)。

研究发现, MEK/ERK1/2抑制剂PD98059处理HCC-CM刺激的rMSCs后, ERK1/2磷酸化被完全抑制(图2A和图2B)。该结果提示HCC-CM激活rMSCs的ERK1/2信号通路。同时, PD98059有效抑制了HCC-CM诱导的rMSCs增殖(图2C和图2D)。因此, HCC-CM促进rMSCs增殖是通过ERK1/2信



A: HCC-CM促rMSCs增殖, *P<0.05, n=3。B: 新生细胞的细胞核整合入EdU, Apollo®染色激发红光, Hoechst33342复染细胞核激发蓝光(标尺=50 μm); C: EdU评价HCC-CM促rMSCs增殖, *P<0.05, **P<0.01, n=3。

A: induced proliferation of rMSCs upon exposure to HCC-CM was detected, *P<0.05, n=3; B: EdU was confined to nuclei and labeled with Apollo® Dye, dividing cells had incorporated EdU (red) and counterstained with Hoechst33342 (blue) (scale bar=50 μm); C: induced proliferation in rMSCs was detected using EdU proliferation assay, *P<0.05, **P<0.01, n=3.

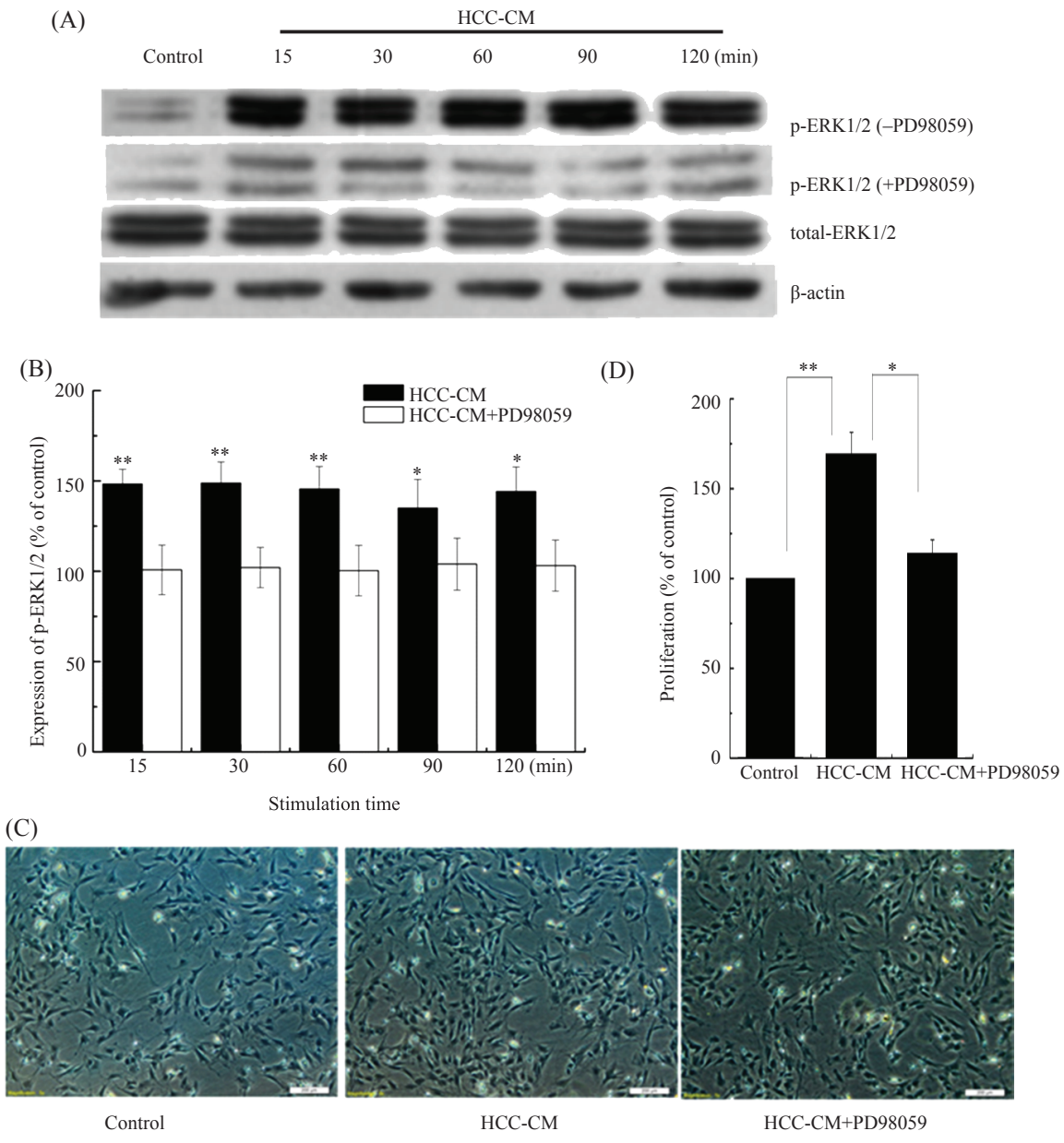
图1 HCC-CM促rMSCs增殖

Fig.1 HCC-CM induced rMSCs proliferation

号分子实现的。

2.3 HCC-CM刺激rMSCs SDF-1的表达

通过RT-PCR检测发现, *SDF-1*基因在大鼠肝癌细胞CBRH-7919以及大鼠正常肝细胞BRL-3a中未见表达(图3A)。但在HCC-CM刺激下, rMSCs中*SDF-1* mRNA表达相比对照组显著上调(图3B和图



A: PD98059完全抑制HCC-CM诱导的rMSCs ERK1/2磷酸化; B: PD98059抑制HCC-CM诱导rMSCs ERK1/2磷酸化的统计学分析。黑色栏代表HCC-CM刺激rMSCs后, ERK1/2磷酸化相比无血清对照组显著上调, **P*<0.05, ***P*<0.01, *n*=3。白色栏代表HCC-CM加入PD98059后, ERK1/2磷酸化与对照组(无血清加入PD98059)相比无显著性差异, *n*=3; C: PD98059显著抑制HCC-CM诱导的rMSCs增殖(标尺=200 μm); D: PD98059抑制HCC-CM诱导rMSCs增殖的统计学分析, **P*<0.05, ***P*<0.01, *n*=3。

A: PD98059 completely inhibited HCC-CM-induced phosphorylation of ERK1/2 (p-ERK1/2) in rMSCs; B: the densitometric analysis of HCC-CM-induced p-ERK1/2 with or without PD98059. Blank bar chart represented the percentual increase of p-ERK1/2 expression in HCC-CM-incubated group over untreated control, **P*<0.05, ***P*<0.01, *n*=3. While white bar chart represented the increase of p-ERK1/2 expression in HCC-CM-incubated group with PD98059 over untreated control, *n*=3; C: PD98059 significantly inhibited HCC-CM-induced rMSCs proliferation (bar=200 μm); D: the densitometric analysis of HCC-CM-induced rMSCs proliferation with or without PD98059, **P*<0.05, ***P*<0.01, *n*=3.

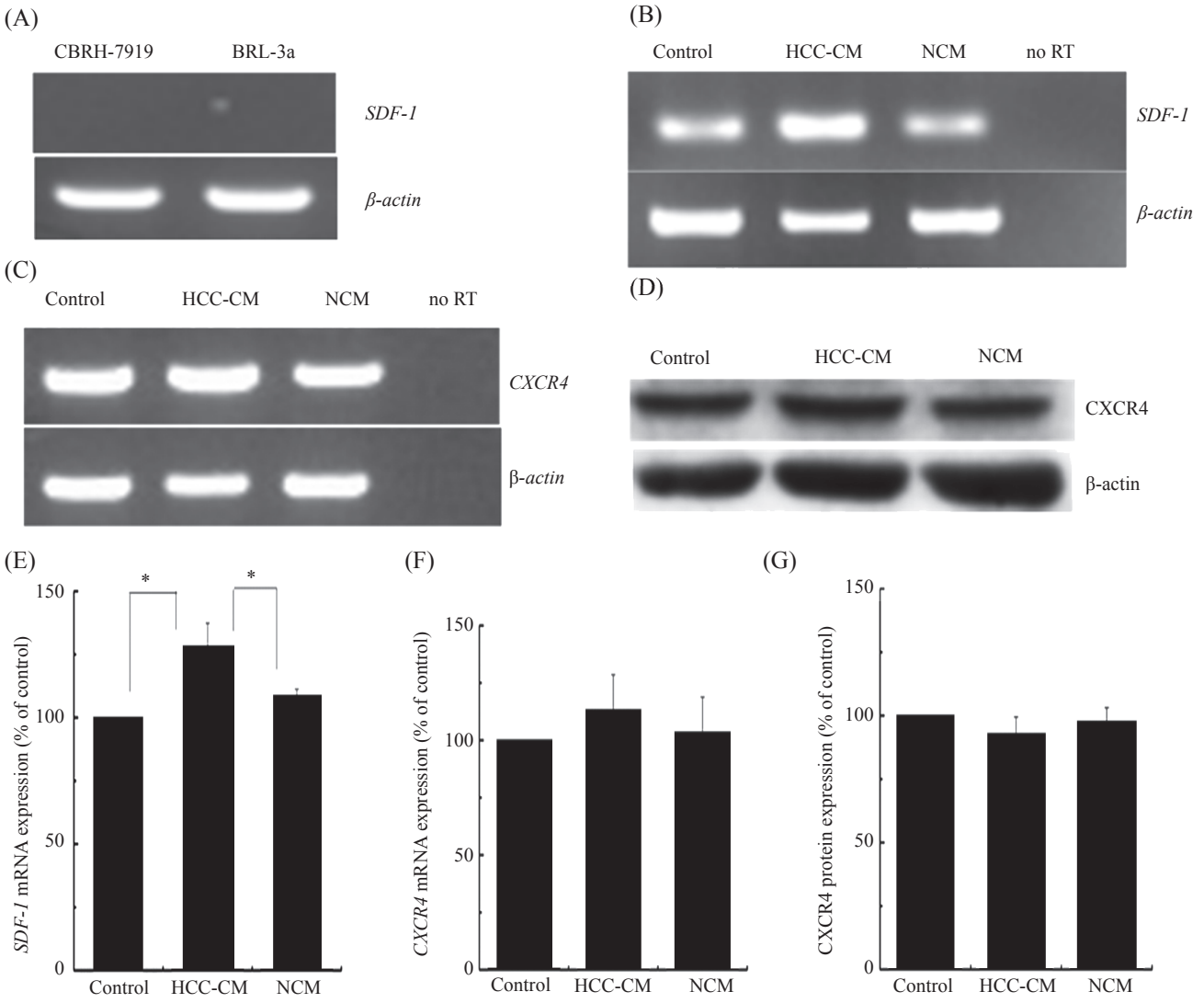
图2 HCC-CM通过ERK1/2信号分子促进rMSCs增殖

Fig.2 HCC-CM promoted rMSCs proliferation via ERK1/2 molecule

3E)。SDF-1主要通过其受体CXCR4发挥作用, 因此我们进一步关注了HCC-CM是否影响rMSCs膜上受体CXCR4的表达。RT-PCR(图3C和图3F)和Western blot(图3D和图3G)检测结果显示, HCC-CM不影响rMSCs CXCR4的表达。

2.4 AMD3100部分抑制HCC-CM诱导的rMSCs ERK1/2磷酸化和增殖

研究发现, CXCR4抑制剂AMD3100部分抑制HCC-CM诱导的rMSCs ERK1/2磷酸化(图4A和图4B), 说明ERK1/2信号可能是由包括CXCR4在内的多种受体或信号分子介导的下游信号通路之一。同时, AMD3100部分消除HCC-CM诱导的rMSCs增殖(图4C和图4D)。以上结果提示, SDF-1/CXCR4部分调控HCC-CM促rMSCs增殖。

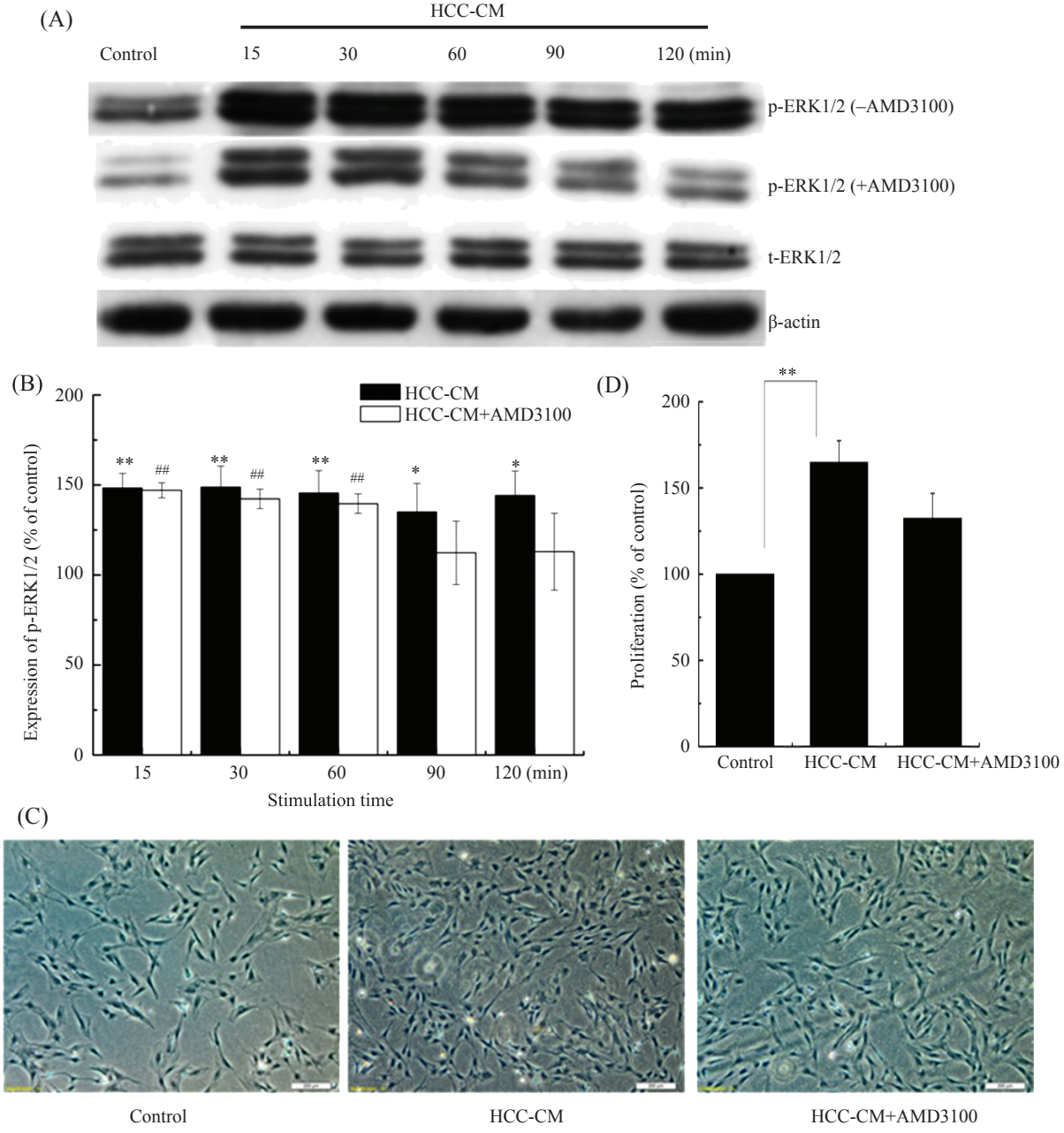


A: CBRH-7919和BRL-3a细胞不表达*SDF-1*基因; B: HCC-CM上调rMSCs *SDF-1*表达, *n*=3; C: HCC-CM不影响rMSCs *CXCR4*基因水平表达, *n*=3; D: HCC-CM不影响rMSCs CXCR4蛋白水平表达, *n*=3; E: HCC-CM上调rMSCs *SDF-1* mRNA水平表达的统计学分析, **P*<0.05, *n*=3; F: HCC-CM不影响rMSCs *CXCR4* mRNA水平表达的统计学分析, *n*=3; G: HCC-CM不影响rMSCs CXCR4蛋白水平表达的统计学分析, *n*=3。

A: no mRNA expression of *SDF-1* was detected in CBRH-7919 and BRL-3a; B: HCC-CM induced the expression of *SDF-1* in rMSCs, *n*=3; C: the expression of *CXCR4* was not altered upon the presence of HCC-CM in rMSCs, *n*=3; D: the expression of CXCR4 was not altered upon the presence of HCC-CM in rMSCs, *n*=3; E: the densitometric analysis of HCC-CM-induced *SDF-1* mRNA upregulation, **P*<0.05, *n*=3; F: the densitometric analysis of HCC-CM-induced *CXCR4* mRNA expression, *n*=3; G: the densitometric analysis of HCC-CM-induced CXCR4 protein expression, *n*=3.

图3 SDF-1/CXCR4的表达情况

Fig.3 The expression of SDF-1/CXCR4



A: AMD3100部分抑制HCC-CM诱导的rMSCs ERK1/2磷酸化; B: AMD3100部分抑制HCC-CM诱导rMSCs ERK1/2磷酸化的统计学分析。黑色栏代表HCC-CM刺激rMSCs后, ERK1/2磷酸化相比无血清对照组显著上调, **P*<0.05, ***P*<0.01, *n*=3。白色栏代表加入AMD3100后, ERK1/2磷酸化相比对照组(无血清加入AMD3100)仍存在显著性差异, $^{\#\#}P$<0.01, *n*=3; C: AMD3100部分抑制HCC-CM诱导的rMSCs增殖(标尺=200 μm); D: AMD3100部分抑制HCC-CM诱导rMSCs增殖的统计学分析, ***P*<0.01, *n*=4 。

A: AMD3100 partially inhibited HCC-CM-induced p-ERK1/2 in rMSCs; B: the densitometric analysis of HCC-CM-induced p-ERK1/2 with or without AMD3100. Blank bar chart represented the percentage of increase of p-ERK1/2 expression in HCC-CM-incubated group over untreated control, **P*<0.05, ***P*<0.01, *n*=3. While white bar chart represented the increase of p-ERK1/2 expression in HCC-CM-incubated group with AMD3100 over untreated control, $^{\#\#}P$<0.01, *n*=3; C: AMD3100 partially inhibited HCC-CM-induced rMSCs proliferation (bar=200 μm); D: the densitometric analysis of HCC-CM-induced rMSCs proliferation with or without AMD3100, ***P*<0.01, *n*=4.

图4 SDF-1/CXCR4部分调控HCC-CM促rMSCs ERK1/2磷酸化以及增殖

Fig.4 SDF-1/CXCR4 partially regulated HCC-CM-induced rMSCs p-ERK1/2 and proliferation

3 讨论

肿瘤微环境能调控肿瘤细胞和MSCs的增殖、分化等多种生物学行为[5], 但对其相互作用规律及其机理, 研究者还缺乏系统的认识。本实验发

现, HCC-CM显著促进rMSCs的增殖, 同时NCM对rMSCs增殖也具有作用。BRL-3a分泌增殖刺激活性因子(multiplication stimulating activity, MSA)至其条件培养基中[20], 这可能是NCM促rMSCs增殖的原因之一。

胞外刺激影响基因转录和蛋白表达是通过信号分子相互作用完成的[10]。MAPKs信号分子能通过诱导基因表达控制细胞主要的生物学行为[21]。C-Jun氨基末端激酶(c-Jun N-terminal kinase, JNK)信号分子、p38信号分子和ERK1/2信号分子共同组成MAPKs信号家族。JNK信号分子在炎症、分化、凋亡等方面扮演着重要角色[22-23], 也能控制肌动蛋白骨架以及细胞铺展[24-25]。P38信号分子在炎症、凋亡和分化等方面的作用已为人们广泛所知[26]。作为MAPKs中研究最为广泛的ERK1/2信号分子, 既在MSCs迁移和增殖方面具有重要作用[12,27-28], 又能调节MSCs自我更新、分化等行为[29-30]。在本实验中, 虽然PD98059显著抑制HCC-CM促rMSCs增殖, 但HCC-CM促rMSCs增殖效果并未完全降至对照组水平。该结果提示, 其他信号分子可能参与了以ERK1/2为信号主体的促rMSCs增殖过程。

CXCR4是细胞膜上能与SDF-1结合并传递信号的主要受体, ERK1/2是与增殖密切相关的信号分子, 因此探究SDF-1/CXCR4与ERK1/2分子在增殖方面的协同作用极具价值。一直以来, 人们认为CXCR4是SDF-1的唯一受体[31], 但近年来新受体CXCR7逐渐进入人们的视野。Hartmann等[32]证实多种类型细胞的ERK1/2磷酸化是通过CXCR7信号受体完成的, 但也有报道指出CXCR7只有在与其他受体形成二聚体时才能发挥作用[33]。本研究中, 在HCC-CM共存微环境下, rMSCs *SDF-1* mRNA表达相比对照组显著上调, 但抑制SDF-1/CXCR4不能完全阻断HCC-CM促rMSCs增殖。因此, 我们认为HCC-CM刺激rMSCs自分泌SDF-1, 进而部分通过SDF-1/CXCR4介导的信号促rMSCs增殖。

AMD3100不仅能抑制SDF-1/CXCR4信号[34], 也有文献报道AMD3100可能是CXCR7的变构激活剂[35]。Sierro等研究发现, 在SDF-1刺激下, 转染CXCR7的HEK293细胞组ERK1/2磷酸化水平比只表达CXCR4的HEK293细胞组更加显著[36]。本实验中, AMD3100作用于rMSCs后, 部分抑制其下游与增殖密切相关的ERK1/2磷酸化, 同时也部分影响HCC-CM促rMSCs增殖。SDF-1促rMSCs增殖是否也通过CXCR7传递, AMD3100对SDF-1/CXCR7的精确影响机制还需要深入研究。

综上, 本实验研究结果显示, HCC-CM对rMSCs增殖有较强的促进作用, ERK1/2和SDF-1/CXCR4是参与该过程的关键信号分子, SDF-1/CXCR7对rMSCs增殖的影响及其机理还需进一步研究。

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