



脆性X智力障碍蛋白靶向DDX5调控Wnt/ β -catenin信号通路促进乳腺癌细胞上皮-间质转化发生*

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【摘要】 目的 探讨脆性X智力障碍蛋白(fragile X mental retardation protein, FMRP)促进乳腺癌细胞迁移、上皮-间质转化(epithelial mesenchymal transition, EMT)发生及其可能的作用机制。方法 采用RT-PCR、Western blot方法,分析FMRP在正常乳腺上皮细胞(MCF-10A)和4种乳腺癌细胞系(MCF-7、BT474、MDA-MB-231、HCC1937)中mRNA和蛋白表达,免疫组化染色检测FMRP在乳腺癌组织中的表达;GEO数据库分析FMRP基因在乳腺癌中的表达及与临床预后的关系;采用慢病毒感染和siRNA干扰技术分别构建FMRP过表达及干扰载体,转染人乳腺癌细胞系MCF-7,设置对照组(Control)、干扰空载体组(NC)、敲低载体组(siFMRP)以及过表达空载体组(Lv-NC)、过表达载体组(Lv-FMRP);划痕实验和Transwell实验检测细胞迁移和侵袭能力,Western blot检测各组细胞中EMT标志物[上皮标志物E-cadherin,间质标志物N-cadherin、vimentin、ZEB1(zinc finger E-box binding homeobox 1)、Slug(snail family zinc finger 2)]表达;免疫共沉淀联合质谱分析(IP-MS)验证FMRP与DEAD box RNA解旋酶-5(DEAD-box helicase5, DDX5)蛋白的互作关系,利用蛋白合成抑制剂放线菌酮(cycloheximide, CHX)、蛋白酶体抑制剂MG132检测FMRP对DDX5蛋白表达的调控作用;同时转染siDDX5载体,观察DDX5是否可以逆转FMRP过表达对细胞迁移、EMT的影响;采用免疫荧光染色检测 β -catenin的定位及表达,Western blot检测Wnt/ β -catenin信号通路核心标志物蛋白表达。结果 FMRP在乳腺癌组织及细胞中呈高表达($P<0.05$),FMRP高表达组总生存和无进展生存低于FMRP低表达组($P<0.05$);敲低FMRP后MCF-7细胞迁移能力减弱,过表达FMRP促进细胞迁移($P<0.05$);敲低FMRP后E-cadherin表达升高,N-cadherin、vimentin、ZEB1、Slug表达降低,抑制EMT发生,而过表达FMRP则促进EMT进程($P<0.05$);FMRP与DDX5蛋白互作,且通过阻断泛素-蛋白酶体途径促进DDX5蛋白稳定性;敲低DDX5逆转FMRP过表达对细胞迁移及EMT的促进作用($P<0.05$),且有效抑制 β -catenin核转位,降低 β -catenin核分布;进一步发现DDX5下调后p- β -catenin、GSK3 β 、Axin2蛋白表达升高,C-myc蛋白表达降低($P<0.05$),而联合FMRP过表达逆转上述蛋白的表达($P<0.05$)。结论 FMRP靶向DDX5通过激活Wnt/ β -catenin信号通路促进乳腺癌细胞迁移和EMT进程。

【关键词】 脆性X智力障碍蛋白 DEAD box RNA解旋酶-5 上皮-间质转化 Wnt/ β -catenin信号通路 乳腺癌

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【Abstract】 **Objective** To investigate the role of fragile X mental retardation protein (FMRP) in promoting cell migration and epithelial-mesenchymal transition (EMT) in breast cancer (BC) and the potential mechanisms involved. **Methods** The mRNA and protein expressions of FMRP in MCF-10A, a normal human breast epithelial cell line, and four breast cancer cell lines, including MCF-7, BT474, MDA-MB-231, and HCC1937, were analyzed by RT-PCR and Western blot. The expression of FMRP in BC tissues was measured by immunohistochemistry (IHC). FMRP expression in BC and its relationship with clinical prognosis were analyzed using GEO database. Lentiviral infection and siRNA interference were used to construct FMRP overexpression and interference vectors, respectively, and the human breast

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cancer cell line MCF-7 was subsequently transfected. A Control group, an interference empty vector group (the NC group), a knockdown vector group (the siFMRP group), an overexpression empty vector group (the Lv-NC group), and an overexpression vector group (the Lv-FMRP group) were set up. The migration and invasion abilities of the cells were assessed by scratch assay and Transwell assay. The expression of EMT markers, including E-cadherin, an epithelial marker, N-cadherin, an mesenchymal markers, vimentin, zinc finger E-box binding homeobox 1 (ZEB1), and snail family zinc finger 2 (Slug), in the cells of each group was determined by Western blot. The interaction between FMRP and DEAD-box RNA helicase-5 (DDX5) protein was analyzed by immunocoprecipitation combined with mass spectrometry (IP-MS). The regulatory effect of FMRP on DDX5 protein expression was assessed using the protein synthesis inhibitor cycloheximide (CHX) and proteasome inhibitor MG132. In addition, transfection with siDDX5 vector was conducted to observe whether DDX5 could reverse the effects of FMRP overexpression on cell migration and EMT. The localization and expression of β -catenin were determined by immunofluorescence staining, and the expression of core markers of Wnt/ β -catenin signaling pathway was examined by Western blot. **Results** FMRP was highly expressed in BC tissues and cells ($P<0.05$), and overall survival (OS) and recurrence-free survival (RFS) of the FMRP high expression group were significantly lower than those of the FMRP low expression group ($P<0.05$). The migration ability of MCF-7 cells was weakened after FMRP knockdown, while overexpression of FMRP promoted cell migration ($P<0.05$). After FMRP knockdown, the expression of E-cadherin was increased, while the expression levels of N-cadherin, vimentin, ZEB1, and Slug were decreased, which inhibited the occurrence of EMT. In contrast, the overexpression of FMRP promoted the EMT process ($P<0.05$). FMRP interacted with DDX5 protein and promoted DDX5 protein stability by blocking the ubiquitin-proteasome pathway. DDX5 knockdown reversed the effect of FMRP overexpression to promote cell migration and EMT ($P<0.05$), effectively inhibited β -catenin nuclear translocation, and decreased β -catenin nuclear distribution. Furthermore, it was found that the expression of p- β -catenin, GSK3 β and Axin2 protein was increased and the expression of C-myc protein was decreased after DDX5 downregulation ($P<0.05$). On the other hand, the expression of these proteins was reversed by combined FMRP overexpression ($P<0.05$). **Conclusion** FMRP targets DDX5 and promotes BC cell migration and EMT via the activation of the Wnt/ β -catenin signaling pathway.

【Key words】 FMRP DDX5 EMT Wnt/ β -catenin signal pathway Breast cancer

乳腺癌(breast cancer, BC)是全球女性首发的恶性肿瘤,截至2021年BC发病率占新增癌症病例数的12%,且发病率及死亡率逐年上升,严重危害女性健康^[1-2]。现阶段,手术切除、放化疗、内分泌治疗、靶向治疗等方法已被应用临床,但疗效和预后欠佳^[3]。因此,深入探索BC发生演变的分子机制以及识别早期诊断的治疗靶点,对于BC的临床治疗具有重要意义。

肿瘤转移是肿瘤患者治疗失败和死亡的主要原因^[4-5]。上皮-间质转化(epithelial mesenchymal transition, EMT)是上皮细胞失去细胞间黏附连接获得间质特征并具有主动侵袭转移能力的过程^[6-7]。研究表明,EMT是恶性肿瘤侵袭和转移的起始步骤,与肿瘤的发生发展密切相关^[8-9]。脆性X智力障碍蛋白(fragile X mental retardation protein, FMRP)是一种高度保守的RNA结合蛋白(RNA-binding protein, RBP),在脆性X综合征(fragile X syndrome, FXS)中缺失或功能失调,对大脑功能调节至关重要^[10-11]。近年来,FMRP在恶性肿瘤进展中的作用越来越受到研究者的重视。研究表明,FMRP在黑色素瘤、肝癌及结直肠癌中发挥癌基因作用,有效促进肿瘤细胞的侵袭转移^[12-14]。本课题组之前的研究发现,FMRP通过激活RAS/MAPK信号通路抑制结直肠肿瘤细胞铁死亡^[15],但FMRP在BC中的

调控作用及其潜在的分子机制尚不清楚。DEAD box RNA解旋酶-5(DEAD-box helicase 5, DDX5)蛋白是RNA解旋酶家族成员,文献报道DDX5激活 β -catenin并促进其核转位,诱导EMT发生^[16]。故笔者猜测FMRP是否通过结合DDX5参与调控乳腺癌EMT进程。本研究拟基于肿瘤组织样本及公共数据库分析FMRP在BC中的表达及对患者远期预后的影响,进一步探究FMRP结合DDX5对BC细胞迁移和EMT的影响及可能的作用机制,旨在为BC的治疗和预后提供潜在靶点。

1 材料与方法

1.1 材料与试剂

收集宁夏医科大学总医院肿瘤外科乳腺癌组织及癌旁正常组织样本,所有样本经宁夏医科大学总医院伦理委员会批准(批准号KYLL-2023-0150),所有参与研究的受试者均签署了书面知情同意书。

正常乳腺上皮细胞(MCF-10A)和4种乳腺癌细胞系(MCF-7、BT474、MDA-MB-231、HCC1937)均购自中科院上海细胞库;胎牛血清购自GIBCO公司;DMEM培养基、RPMI1640培养基、MCF-10A专用培养基、青链霉素混合液购自Biological Industries公司;环己酰亚胺(CHX)

及MG132购自MCE公司; siRNA及引物由上海生工合成, LipofectamineRNA iMAX助转染试剂、Trizol试剂购自赛默飞公司; 反转录试剂盒及qRT-PCR试剂盒购自TaKaRa公司, 全蛋白质、核蛋白及胞浆蛋白定量试剂盒购自南京凯基公司。

1.2 实验设计

首先分析了FMRP在正常乳腺上皮细胞(MCF-10A)和4种乳腺癌细胞系(MCF-7、BT474、MDA-MB-231、HCC1937)中mRNA和蛋白表达; 将收集的乳腺癌患者肿瘤组织和癌旁组织进行免疫组化染色, 观察FMRP蛋白的表达; 通过查询GEO数据库(<https://www.ncbi.nlm.nih.gov/geo/>)并下载GSE10780数据集和GSE3494数据集, 分析FMRP基因在BC中的表达及与临床预后的关系。根据RT-PCR及Western blot中显示的FMRP表达情况选择MCF-7细胞, 采用小干扰转染技术建立3个FMRP敲低细胞株(siFMRP-1、siFMRP-2和siFMRP-3), 并设置未经任何处理的MCF-7细胞为Control组及转染干扰空载体的MCF-7细胞为空载体组(NC), 根据RT-PCR及Western blot中显示的敲低结果, 本研究选择siFMRP-2和siFMRP-3进行后续实验。划痕实验和Transwell迁移实验检测Control组、NC组、siFMRP-2组、siFMRP-3组细胞迁移能力, Western blot检测这4组细胞中FMRP、EMT通路、Wnt/ β -catenin信号通路蛋白表达。

进一步构建FMRP过表达的MCF-7细胞模型(Lv-FMRP组), 并以过表达空载体组(Lv-NC组)为参照, 同样方法检测2组细胞迁移能力及FMRP、EMT通路蛋白表达。

通过免疫共沉淀联合质谱分析(IP-MS)发现FMRP与DDX5蛋白存在互作, 分别用蛋白合成抑制剂CHX、蛋白酶体抑制剂MG132处理MCF-7细胞, Western blot检测FMRP对DDX5蛋白表达的调控作用。

此外, 构建DDX5敲低细胞株(siDDX5-1、siDDX5-2), 并设置Control组(未经任何处理的MCF-7)及NC组(转染干扰空载体的MCF-7细胞), 采用免疫荧光染色检测4组细胞 β -catenin的定位及表达, 蛋白核质分离实验检测细胞胞质和胞核中 β -catenin蛋白水平, Western blot检测细

胞中DDX5及Wnt/ β -catenin信号通路蛋白表达。

因为siDDX5-1、siDDX5-2敲低载体验证效率接近, 后续功能回复实验本研究随机选择了siDDX5-1进行验证。本研究设置Control组(未经任何处理的MCF-7)、Lv-FMRP组、DDX5敲低组(siDDX5)及FMRP过表达联合DDX5敲低组(Lv-FMRP+siDDX5), 划痕实验和Transwell迁移实验检测4组细胞迁移能力, Western blot检测4组细胞中EMT及Wnt/ β -catenin信号通路蛋白表达。

具体检测方法见1.3。

1.3 实验方法

1.3.1 细胞培养及转染

MCF-10A细胞利用MCF-10A专用培养基, MCF-7、MDA-MB-231、HCC1937细胞利用DMEM高糖培养基, BT474细胞利用RPMI1640培养基, 各细胞系均含10%胎牛血清(Gibco, USA)及1%双抗, 于37℃、体积分数5%CO₂培养箱中培养。根据FMRP的基因和蛋白表达情况选择MCF-7细胞, 利用iMAX助转染试剂转染小干扰FMRP载体及小干扰DDX5载体(siFMRP、siDDX5)和干扰空载体, 转染过程严格按照试剂说明书操作, siRNA序列见表1。按照MOI值为20转染FMRP过表达慢病毒载体(Lv-FMRP)和阴性对照慢病毒(Lv-NC), 转染12 h后更换为DMEM完全培养基。待细胞密度为80%时进行常规传代, 并使用2 μ g/mL嘌呤霉素进行筛选, 以获得稳定转染细胞株。

1.3.2 RT-PCR验证FMRP和DDX5 mRNA表达

收集各组细胞, 采用Trizol试剂提取总RNA, 按照TaKaRa反转录试剂盒合成cDNA, 使用TaKaRa荧光定量PCR试剂盒进行mRNA定量检测, 利用LightCyder480仪器进行分析; FMRP上游引物为: 5'-GGTCAAGGAATGGGTCGAGG-3', 下游引物为: 5'-AGTTCGTCTCTGTGGTCAGAT-3'; DDX5上游引物为: 5'-GAGGTTCAAGTCGTTCCAGG-3', 下游引物为: 5'-CCCTTTTGCCCGCAGAGTAT-3'; GAPDH上游引物为: 5'-TGTTGCCATCAATGACCCTT-3', 下游引物为: 5'-CTCCACGACGTACTCAGCG-3'。以GADPH作为内参, 采用2^{- $\Delta\Delta$ C_t}法计算目的条带FMRP或DDX5 mRNA的表达水平。

表 1 siRNA序列
Table 1 The sequences of siRNA

Gene	Sense (5'-3')	Anti-sense (5'-3')
siFMRP-1	GAGGAUGAUAAAGGGUGAGUUTT	AACUCACCCUUUAUCAUCCUCTT
siFMRP-2	CGAGAUUUAUGAACAGUUUATT	UAAACUGUUAUGAAAUCUCGTT
siFMRP-3	GCGUUUGGAGAGAUUACAAAUTT	AUUUGUAAUCUCUCCAAACGCTT
siDDX5-1	CCUGGAAGACUGAUUGACUUUTT	AAAGUCAAUAGUCUCCAGGTT
siDDX5-2	UCCACAUCAAUCAAGCCAUTT	AUGGCYGAUGAUUGAUGUGGATT

1.3.3 Western blot检测

收集各组细胞,选择全蛋白提取试剂盒提取总蛋白,BCA定量检测法测定蛋白浓度,经电泳、转膜后,5%脱脂奶粉封闭1 h,按实验设计,分别加入FMRP(1:1000)、DDX5(1:1000)、N-cadherin(1:1000)、E-cadherin(1:1000)、 β -catenin(1:1000)、p- β -catenin(1:500)、Slug(snail family zinc finger 2)(1:500)、波形蛋白(vimentin)(1:500)、ZEB1(zinc finger E-box binding homeobox 1)(1:1000)、GSK3 β (glycogen synthase kinase 3 beta)(1:1000)、APC(activated protein C)(1:1000)、C-myc(v-myc avian myelocytomatosis viral oncogene homolog)(1:1000)及GAPDH(1:2000)一抗,4℃孵育过夜。TBST漂洗结束后,用对应的二抗孵育目的条带。最后加入曝光液覆盖条带,ECL印迹分析系统进行曝光显影。以GAPDH为对照,Image J软件分析各组条带灰度值。以目的蛋白灰度值与GAPDH灰度值的比值作为目的蛋白的相对表达量。

1.3.4 免疫组织化学染色

将收集的乳腺癌患者肿瘤组织和癌旁组织进行石蜡包埋并切成5 μ m厚的切片。将组织切片脱蜡、抗原修复、封闭、4℃孵育FMRP(1:100)一抗,再加入二抗孵育。然后用DAB对载玻片进行免疫染色,并用苏木精进行复染,常规脱水、干燥、封片。采用Image软件计算相对积分光密度值。

1.3.5 生信分析

GEO数据库采用乳腺癌芯片阵列GSE10780分析FMRP基因在乳腺癌组织及癌旁组织中的表达水平,经过表达分析后样本中共包含42个肿瘤样本,143个正常样本,组间使用Wilcoxon Test计算显著性,比较FMRP基因的表达情况。生存分析由Kaplan-Meier Plotter数据库完成,以FMRP基因表达量中位数为标准,将乳腺癌队列GSE3494划分为FMRP高表达组和FMRP低表达组,并进行生存分析。总生存(OS)分析共纳入236例患者,无进展生存(RFS)分析共纳入247例患者。

1.3.6 划痕实验检测细胞迁移能力

取对数生长期细胞,消化后将各组细胞接种于6孔板,待细胞完全贴壁后,用移液器枪头划伤贴壁细胞,PBS清洗漂浮细胞,利用倒置显微镜分别在0、48 h时间段拍照,记录各时间段细胞划痕面积,并计算划痕宽度进行统计分析。划痕宽度为48 h细胞间距离均值与0 h细胞间距离均值的比值。

1.3.7 Transwell迁移实验

先将Transwell小室(Millipore, USA)放入24孔板中,

取对数生长期细胞进行消化,使用无血清培养基调整细胞密度至 1×10^4 /孔,将100 μ L细胞悬液接种于Transwell小室上室,下室加入600 μ L含10%胎牛血清的培养基作为诱导剂,37℃、体积分数5%CO₂条件下培养48 h。取出小室,固定细胞,结晶紫染色,PBS冲洗,晾干。显微镜下观察视野内小室迁移的细胞并随机选取5个视野拍照计数。

1.3.8 CO-IP验证FMRP与DDX5蛋白结合

为了探究FMRP调控BC细胞转移的分子机制,利用IP-MS技术筛选FMRP可能互作的结合蛋白。向细胞沉淀中加入IP细胞裂解液(含蛋白酶抑制剂和磷酸酶抑制剂),收集细胞提取蛋白上清。按照抗体说明书加入FMRP(1:30)一抗过夜,加入Protein A/G琼脂糖珠,4℃摇动2 h,收集沉淀。加入2 $\times$ SDS蛋白上样缓冲液将琼脂糖珠-抗原抗体复合物悬起,充分混匀。煮沸8 min,离心去琼脂糖珠,将上清电泳,上清也可以暂存在-40℃之后电泳。通过SDS-PAGE分离免疫共沉淀样品,从凝胶上切下条带,用胰蛋白酶消化,进行质谱分析。

1.3.9 蛋白酶体途径降解实验

取生长状态良好的Lv-NC组或Lv-FMRP组细胞,加入CHX(50 μ g/mL)处理细胞0、2、4、6 h后,Western blot检测DDX5的蛋白表达水平。用MG132(10 μ mol/L)分别处理敲减/未敲减FMRP表达的MCF-7细胞,24 h后Western blot检测DDX5的蛋白表达水平。

1.3.10 胞质胞核分离实验

取培养细胞 5×10^6 mL⁻¹,用预冷的PBS洗涤2遍,加入预冷的Buffer A与Buffer B混匀,放置冰上30 min,3000 r/min离心10 min。收集上清至新的离心管中,即为胞浆蛋白。在离心沉淀物(细胞核)中加入Buffer C,放置冰上30 min,每间隔10 min涡旋剧烈振荡15 s,14000 r/min离心30 min,上清即得胞核蛋白,上述提取的胞浆蛋白和胞核蛋白进行BCA法蛋白定量检测,分装并保存于-80℃,用于后续Western blot分析。

1.3.11 免疫荧光染色检测 β -catenin亚细胞定位

为了阐明DDX5在Wnt/ β -catenin通路中的作用,利用免疫荧光染色检测 β -catenin的亚细胞定位。将各组细胞以 1×10^3 /孔接种于放入细胞爬片的12孔板中,多聚甲醛固定,Triton-X100破膜,BSA室温封闭,按照抗体说明书加入稀释抗体 β -catenin(1:200),4℃过夜,最后加入FITC标记山羊抗兔IgG(1:500)和DAPI染色,荧光显微镜下观察并采集图像。

1.4 统计学方法

采用Graphpad 9和SPSS 22.0进行数据处理和统计学分析,计量资料以 $\bar{x} \pm s$ 表示。两组间比较采用t检验,多

组均数比较采用单因素方差分析(One-Way ANOVA), 组间两两比较使用LSD-*t*法, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 FMRP在BC中的表达增高且与患者不良预后相关

RT-PCR和Western blot结果显示: BC细胞系中FMRP表达均高于MCF-10A, 其中MCF-7、BT474与MCF-10A的差异有统计学意义(图1A、1B, $P < 0.05$ 或 $P < 0.01$)。

免疫组织化学结果显示, 乳腺癌患者病理组织切片中, FMRP主要定位于细胞质, 且癌组织中FMRP表达高于癌旁组织(图1C)。GSE10780数据集结果显示, FMRP在癌组织中的表达高于癌旁组织(图1D, $P = 0.024$), GSE3494数据集结果显示, FMRP高表达组患者的预后生存较低表达组更差(图1E, $P = 0.044$, $P = 0.031$)。

2.2 敲低FMRP抑制BC细胞迁移能力和EMT发生

结果如图2A、2B所示, 与Control组MCF-7细胞相比,

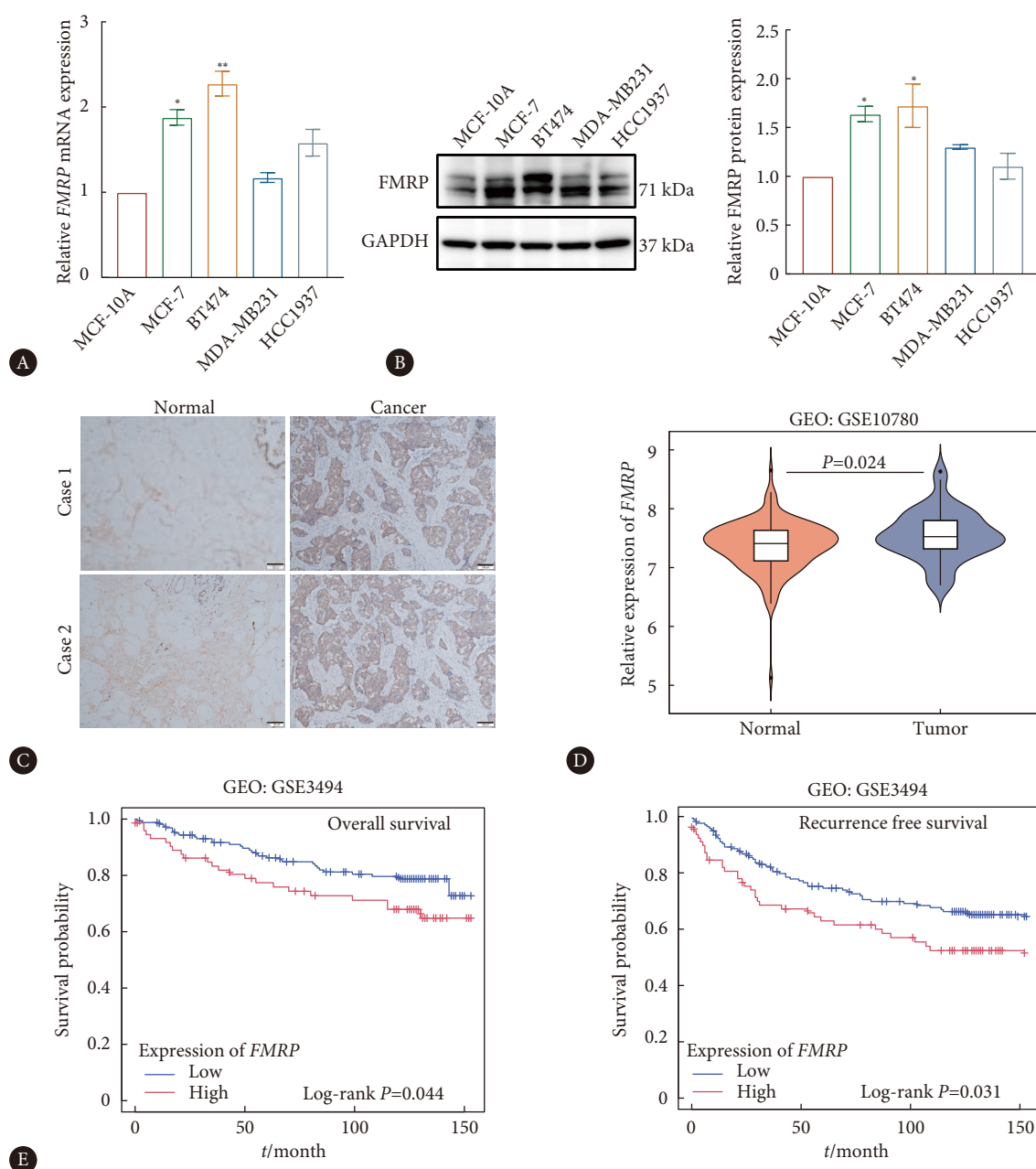
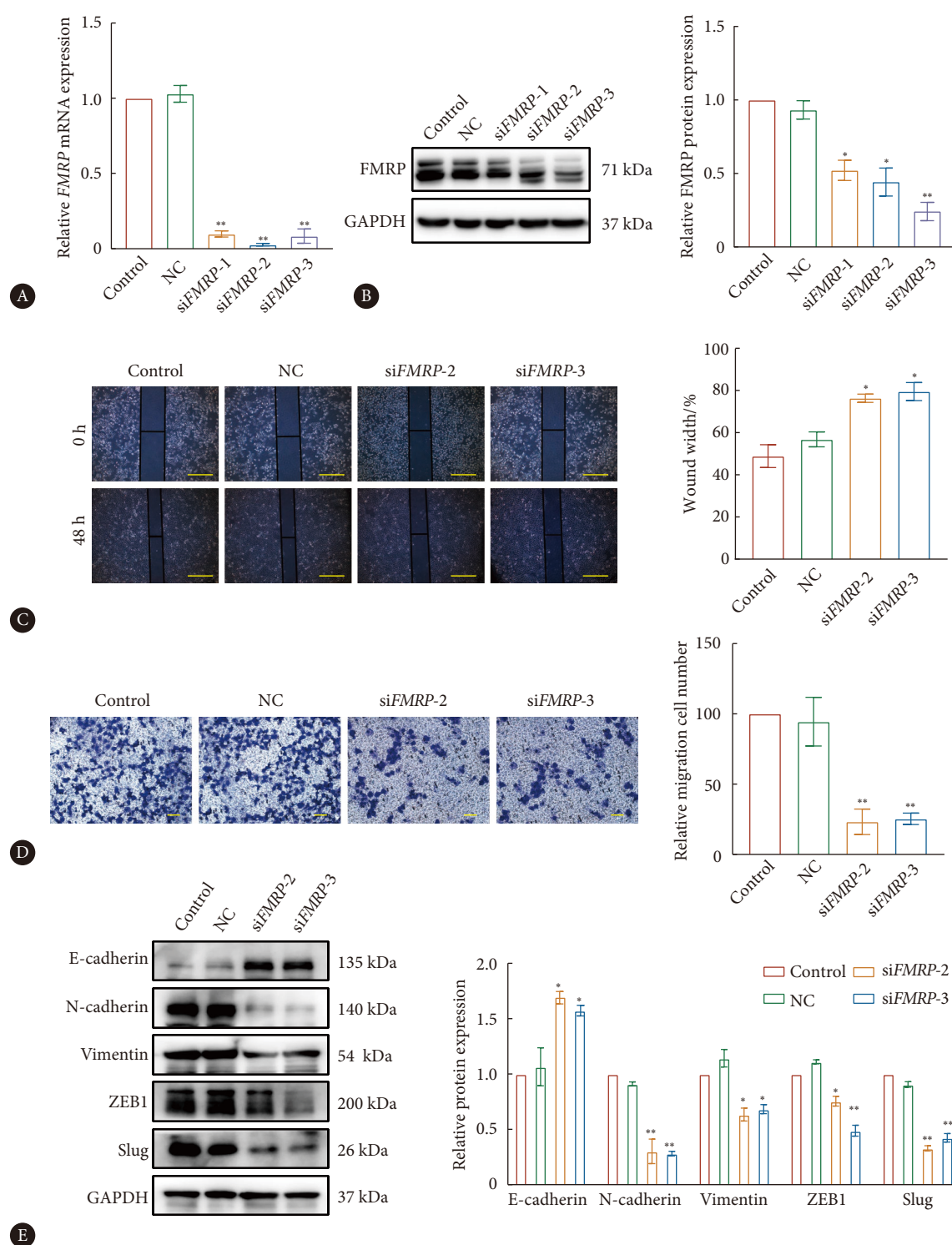


图 1 FMRP在BC中的表达及与预后的关系

Fig 1 The expression of FMRP in BC and its relationship with the prognosis

A-B, Analysis of the expression levels of FMRP in different BC cell lines by RT-PCR and Western blot ($n = 3$); C, IHC analysis of the expression of FMRP in BC tissues (scale bar = 200 μm); D, the expression of FMRP in BC tissues and normal tissue was exhibited for GSE10780 ($n = 185$); E, Kaplan-Meier Plotter analysis for the overall survival ($n = 236$) and recurrence-free survival ($n = 247$) in BC patients with different levels of FMRP expression from GSE3494. * $P < 0.05$, ** $P < 0.01$, vs. MCF-10A group.

图2 敲低*FMRP*对细胞迁移及EMT的影响Fig 2 The effect of *FMRP* knockdown on cell migration and EMT

A-B, The levels of *FMRP* were analyzed by RT-PCR and Western blot after *FMRP* knockdown; C, cell migration was determined by scratch assay (scale bar=200 μ m); D, cell invasion was determined by Transwell assay (scale bar=50 μ m); E, the protein levels of E-cadherin, N-cadherin, vimentin, ZEB1 (zinc finger E-box binding homeobox 1), and Slug (snail family zinc finger 2) were analyzed by Western blot after *FMRP* knockdown. * $P<0.05$, ** $P<0.01$, vs. control group. $n=3$.

3个*FMRP*敲低组*FMRP*表达均降低($P<0.05$)。划痕实验和Transwell实验结果显示:与Control组相比,*FMRP*敲低组MCF-7细胞迁移能力均下降(图2C、2D, $P<0.05$);进一

步探究*FMRP*对EMT的影响, Western blot结果显示,敲低*FMRP*促进上皮标志物E-cadherin表达,抑制间质标志物N-cadherin、vimentin、ZEB1、Slug表达(图2E, $P<0.05$)。

以上结果表明敲低*FMRP*显著抑制BC细胞迁移能力和EMT发生。

2.3 *FMRP*过表达促进BC细胞迁移及EMT进程

相比于Lv-NC组, Lv-*FMRP*组*FMRP*的mRNA和蛋白表达升高(图3A、3B, $P<0.01$), 表明模型构建成功。划痕实验和Transwell实验结果显示, 过表达*FMRP*促进MCF-7细胞迁移能力(图3C、3D, $P<0.05$); Western blot结果显示, 与Lv-NC组相比, 过表达*FMRP*抑制E-cadherin表达, 促进N-cadherin、vimentin、ZEB1和Slug蛋白表达(图3E, $P<0.05$)。

2.4 *FMRP*与DDX5蛋白互作并促进其稳定性

质谱筛选结合蛋白KEGG通路富集分析显示与Wnt

信号通路、细胞黏附、紧密连接、细胞周期及凋亡密切相关(图4A); 查阅文献并结合质谱结果, 初步筛选DDX5作为候选蛋白用于后续研究。CO-IP实验结果证实*FMRP*与DDX5蛋白结合, 与质谱结果一致(图4B)。RT-PCR和Western blot分析*FMRP*对DDX5表达的调控作用, 结果发现: *FMRP*下调不影响DDX5 mRNA水平, 但有效降低DDX5蛋白表达(图4C、4D)。DDX5表达水平随着CHX作用时间延长而减少, 但过表达*FMRP*抵消CHX对DDX5蛋白的降解作用(图4E, $P<0.05$), 说明*FMRP*增加DDX5半衰期促进其稳定性。敲低*FMRP*可下调DDX5蛋白表达, 联合MG132作用可以挽救*FMRP*敲低对DDX5的抑制作用(图4F, $P<0.05$)。上述结果表明, *FMRP*通过阻

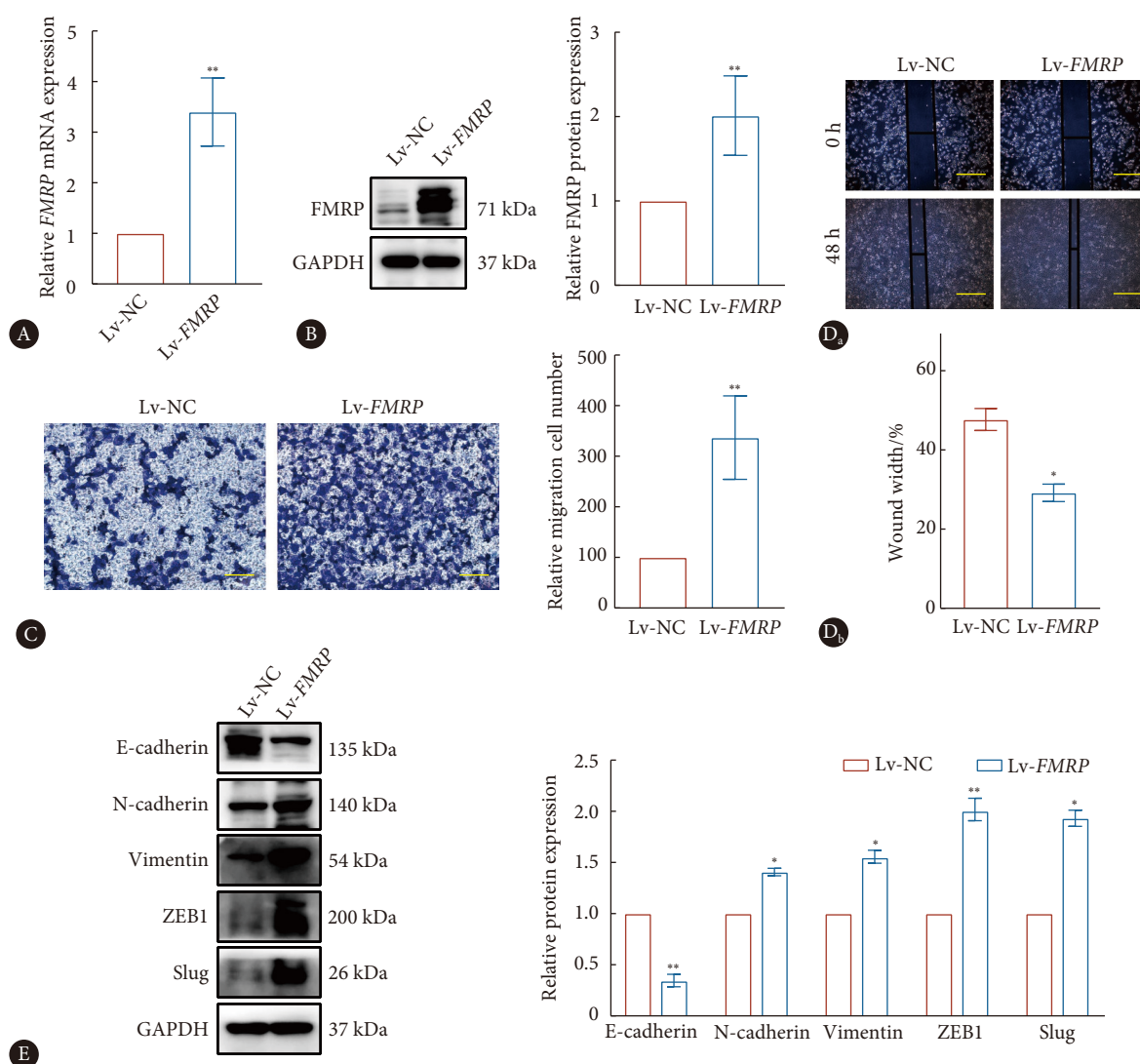


图3 过表达*FMRP*对细胞迁移及EMT的影响

Fig 3 The effect of *FMRP* overexpression on cell migration and EMT

A-B, The expression levels of *FMRP* were analyzed by RT-PCR and Western blot after the overexpression of *FMRP*; C, cell invasion was determined by Transwell assay (scale bar=50 μ m); D, cell migration was determined by scratch assay (a, scale bar=200 μ m) and wound width was caculated (b); E, the protein levels of E-cadherin, N-cadherin, vimentin, ZEB1, and Slug were analyzed by Western blot. * $P<0.05$, ** $P<0.01$, vs. Lv-NC group. $n=3$.

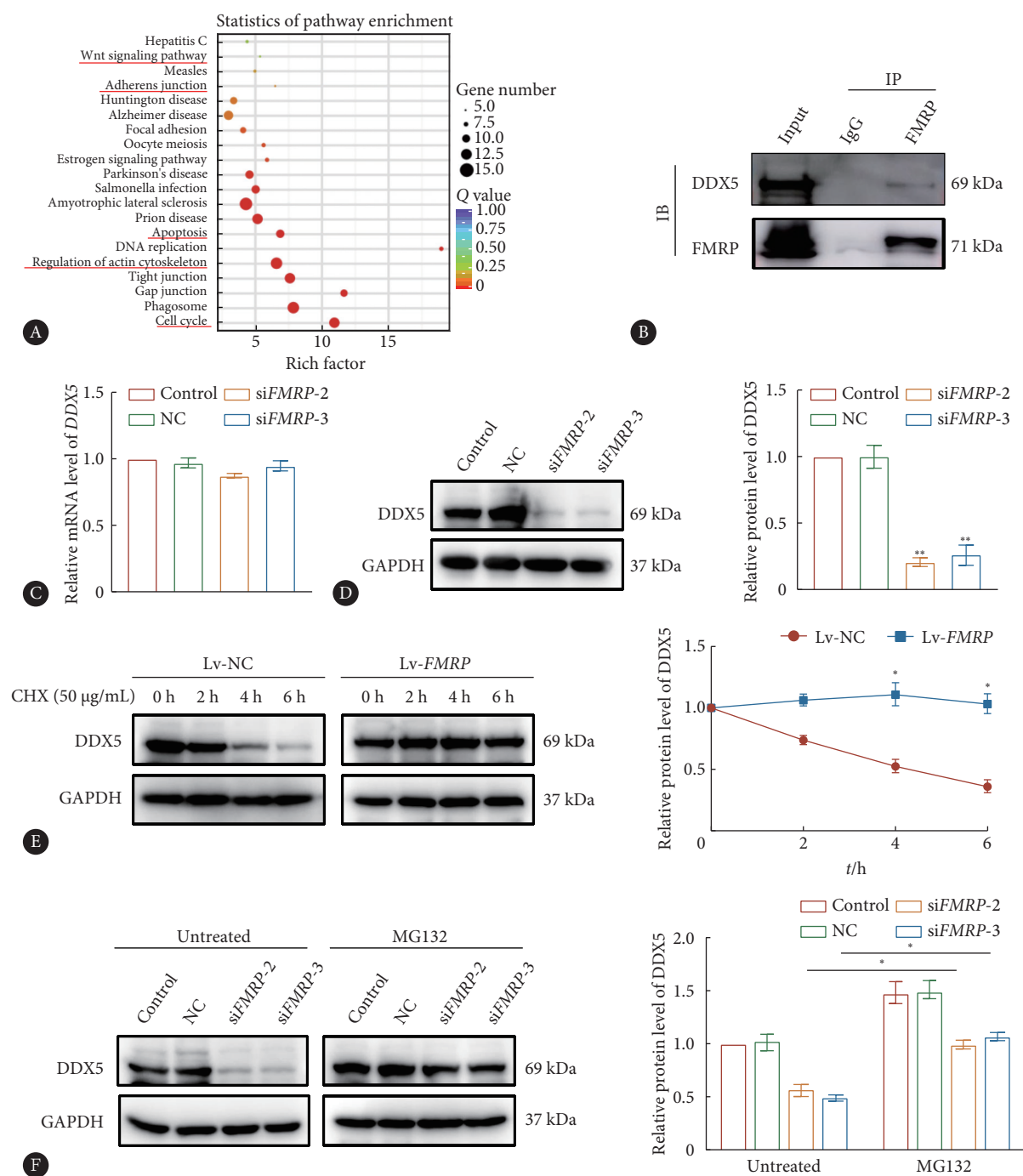


图4 FMRP与DDX5互作并促进其稳定性

Fig 4 FMRP interacts with the DDX5 protein and enhances its stability

A, KEGG enrichment analysis of signaling pathways involving FMRP interacting proteins; B, Western blot confirms the interaction between FMRP and DDX5; C-D, RT-PCR and Western blot analysis of the expression levels of DDX5 after the knockdown of *FMRP*; E, the expression levels of DDX5 were analyzed by Western blot after *FMRP* overexpression combined with CHX (50 μ g/mL); F, the expression levels of DDX5 were analyzed by Western blot after *FMRP* knockdown combined with MG132 (10 μ mol/L). * $P < 0.05$, ** $P < 0.01$, vs. control group or Lv-NC group. $n = 3$.

断泛素-蛋白酶体降解途径提高DDX5蛋白的稳定性。

2.5 DDX5敲低逆转FMRP过表达对BC细胞迁移及EMT的促进作用

RT-PCR和Western blot结果显示: 与Control组相比, DDX5敲低组 (siDDX5) 其mRNA和蛋白水平均降低

(图5A、5B, $P < 0.05$); 划痕实验及Transwell实验结果表明: 与Control组相比, siDDX5抑制MCF-7细胞迁移及侵袭能力, 但联合Lv-FMRP可以逆转敲低DDX5对细胞恶性表型的抑制作用(图5C、5D, $P < 0.05$)。进一步检测EMT标志物表达, 结果表明: 与Control组相比, 敲低DDX5促进E-

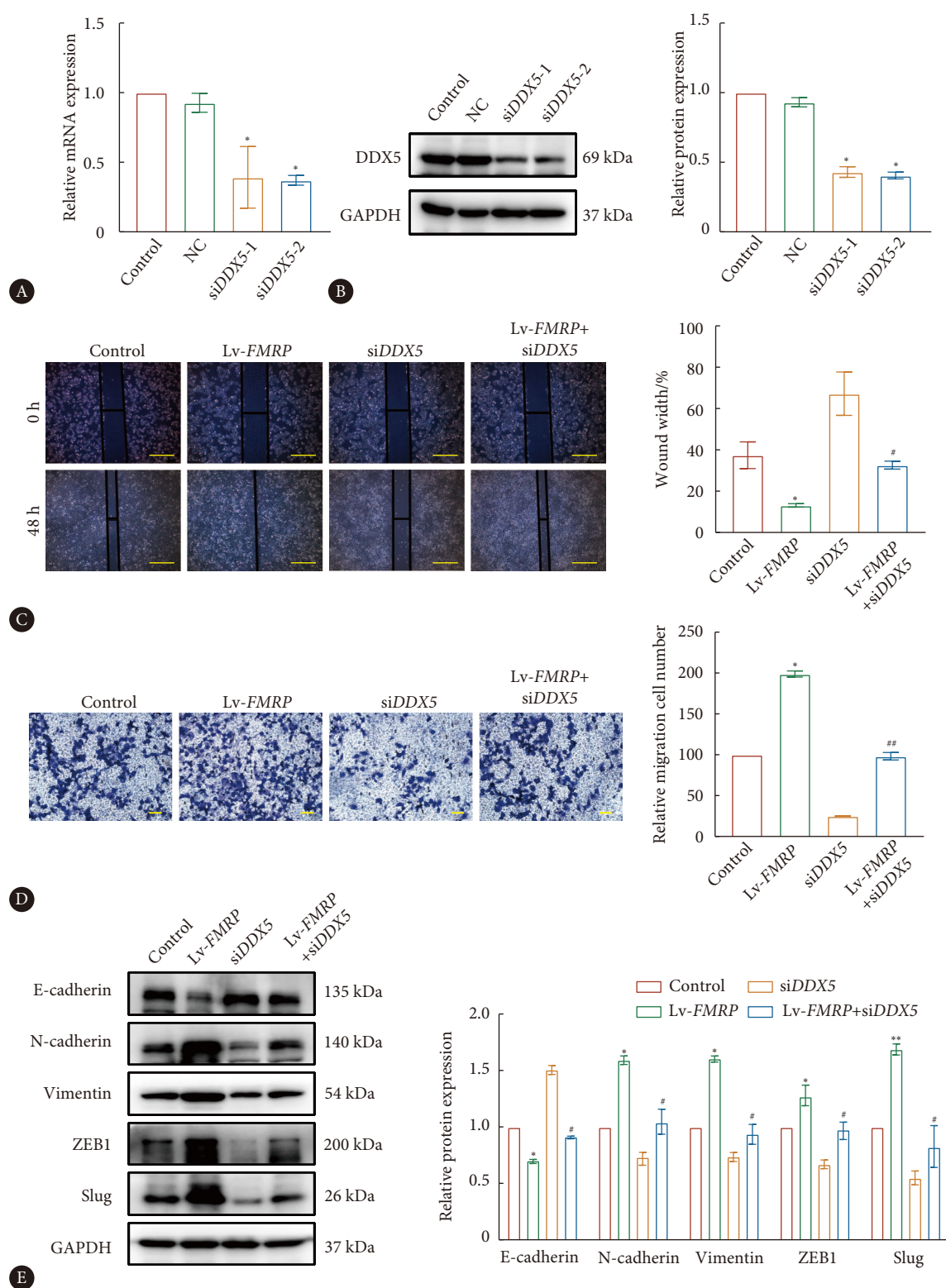


图 5 FMRP靶向DDX5对细胞迁移及EMT的影响

Fig 5 The effects of DDX5 targeting by FMRP on cell migration and EMT

A-B, The expression levels of DDX5 was analyzed by RT-PCR and Western blot after the knockdown of DDX5; C, cell migration was determined by scratch assay (scale bar=200 μ m); D, cell invasion was determined by Transwell assay (scale bar=50 μ m); E, the protein levels of E-cadherin, N-cadherin, vimentin, ZEB1, and Slug were analyzed by Western blot after FMRP overexpression and DDX5 knockdown. * $P<0.05$, ** $P<0.01$, vs. control group; # $P<0.05$, ## $P<0.01$, vs. siDDX5 group. $n=3$.

cadherin表达,抑制N-cadherin、vimentin、ZEB1和Slug蛋白表达,而联合Lv-FMRP可以逆转上述蛋白的相对表达

(图5E, $P<0.05$)。以上结果表明FMRP靶向DDX5促进BC细胞迁移、侵袭及EMT发生。

2.6 FMRP靶向DDX5激活Wnt/ β -catenin信号通路

结果显示: 对照组中 β -catenin胞浆胞核均有表达, 而siDDX5组中 β -catenin胞核表达明显减少(图6A)。同时蛋白核质分离实验结果发现, 敲低DDX5导致胞质和胞核中 β -catenin蛋白水平均降低(图6B), 提示DDX5下调显著抑制 β -catenin转入细胞核。进一步利用Western blot检测

Wnt/ β -catenin通路核心及下游蛋白表达, 结果显示: 与Control组相比, siDDX5组p- β -catenin、APC、GSK3 β 蛋白表达升高, C-myc蛋白表达降低(图6C, $P<0.05$), 但联合Lv-FMRP组有效逆转敲低DDX5对Wnt/ β -catenin信号通路的抑制作用(图6D, $P<0.05$)。上述结果表明FMRP通过靶向DDX5促进Wnt/ β -catenin信号通路激活。

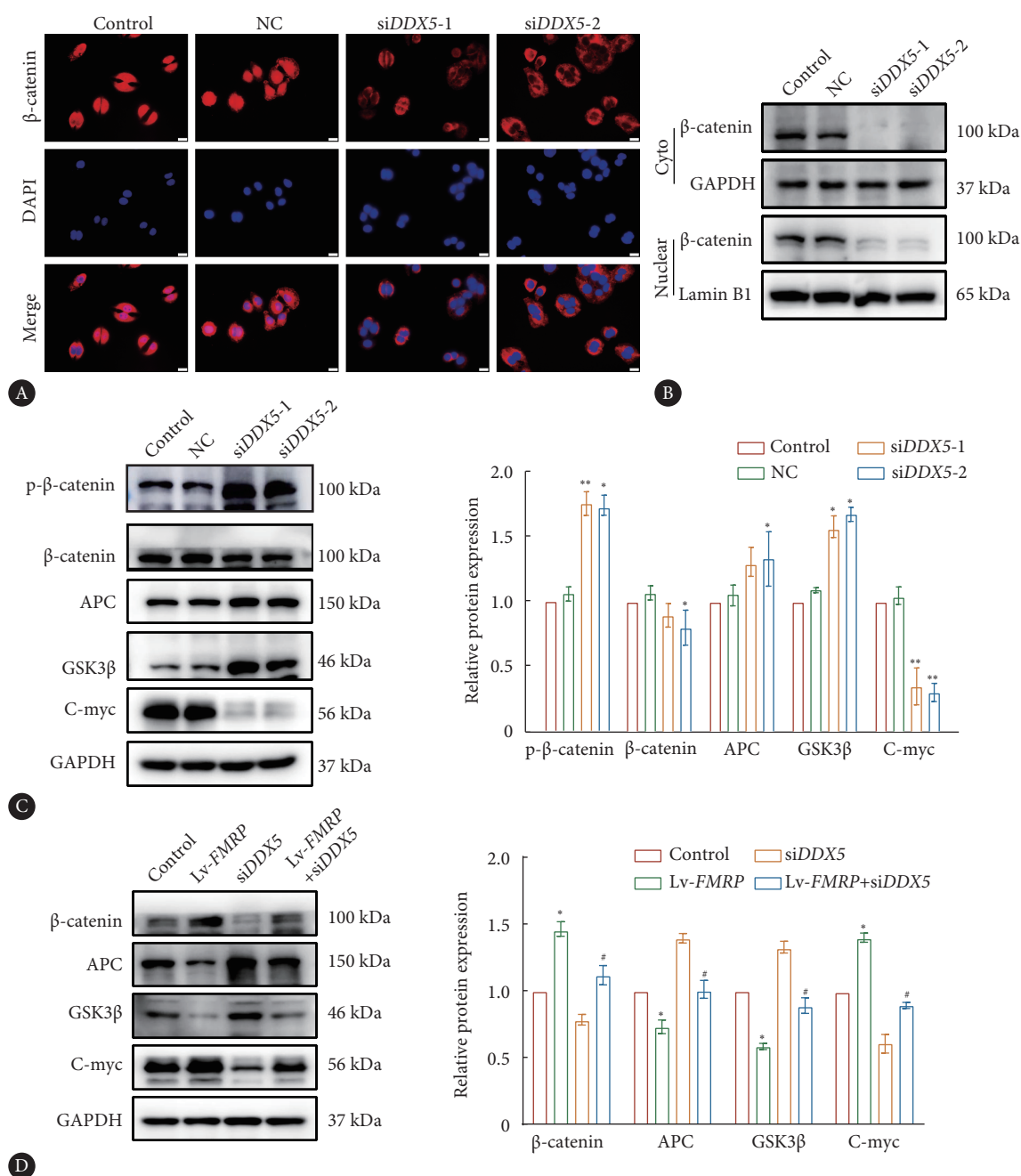


图6 FMRP靶向DDX5激活Wnt/ β -catenin 信号通路

Fig 6 FMRP targets DDX5 to activate the Wnt/ β -catenin signaling pathway

GSK3 β : glycogen synthase kinase 3 beta; APC: activated protein C; C-myc: v-myc avian myelocytomatosis viral oncogene homolog. A, Immunofluorescence analysis of the localization of β -catenin after DDX5 knockdown (scale bar=20 μ m); B, Western blot analysis of the cytoplasmic and nuclear expression of β -catenin; C, Western blot analysis of the protein expression levels of Wnt/ β -catenin signaling pathway after DDX5 knockdown; D, Western blot analysis of the protein expression levels of Wnt/ β -catenin signaling pathway after FMRP overexpression and DDX5 knockdown. * $P<0.05$, ** $P<0.01$, vs. control group. # $P<0.05$, vs. siDDX5 group. $n=3$.

3 讨论

FMRP是一种在脑组织中高度表达且普遍存在的蛋白质,其缺失是导致FXS发生的关键调节因子^[17]。近年来,FMRP在肿瘤生物学领域引起了研究者的广泛关注,发现FMRP异常表达与恶性肿瘤增殖、转移及预后密切相关^[12-14]。本研究通过分析公共数据库结合临床样本,发现FMRP在BC中高表达,且与患者不良预后呈负相关。通过体外实验发现过表达FMRP促进BC细胞迁移侵袭及EMT表型,而干扰FMRP则呈现相反趋势,这与LUCA等^[18]研究基本一致,提示FMRP可能作为癌基因在BC转移进展中发挥重要作用。肿瘤转移是大多数癌症患者死亡的主要原因,原位肿瘤细胞通过EMT过程增加自身侵袭性,侵袭至周围组织导致转移发生。然而,FMRP促进BC细胞转移的具体作用机制尚不清楚,需要进一步探索。

研究发现,FMRP可与多种mRNA或蛋白互作结合,参与神经调控、突触活动、细胞应激等生物过程^[19-20]。本研究通过IP-MS结合CO-IP分析筛选了FMRP互作蛋白DDX5,后续验证发现FMRP不影响DDX5 mRNA表达,但与DDX5蛋白表达呈正相关。鉴于FMRP下调降低DDX5蛋白表达,本研究结果发现干预FMRP通过泛素蛋白酶体降解途径促进DDX5蛋白降解。进一步通过回复实验证明,DDX5下调可逆转FMRP过表达对BC细胞迁移和EMT的促进作用,表明FMRP可能通过靶向DDX5表达实现促进BC恶性转移的调控作用。

DDX5是RNA解旋酶家族的主要成员,参与mRNA翻译及降解过程,在细胞分裂和增殖过程中发挥重要作用^[21-22]。研究发现,DDX5在恶性肿瘤中高表达且参与调控肿瘤细胞增殖、侵袭转移、EMT等恶性过程^[23-24]。为了深入探究FMRP靶向DDX5调控BC进展的作用机制,本研究通过免疫荧光染色结合核质分离实验,发现DDX5下调促使 β -catenin核定位表达减弱,这之前文献报道DDX5激活 β -catenin促进其核转位基本一致^[16]。Wnt/ β -catenin信号通路异常激活与肿瘤细胞EMT、转移、干性维持等多个生物学过程密切相关^[25-27],既往文献报道DDX5通过促进胞质APC/Axin/GSK-3 β 复合物解离保护 β -catenin降解^[16]。因此,本研究进一步分析了FMRP是否与DDX5结合影响Wnt/ β -catenin信号通路表达,结果发现敲低DDX5导致Wnt通路被抑制,联合FMRP过表达可以逆转DDX5对Wnt/ β -catenin信号通路的抑制作用,提示FMRP促进BC细胞转移和EMT发生可能是通过激活Wnt/ β -catenin信号通路实现的。

综上所述,本研究明确了FMRP过表达促进BC细胞

转移和EMT的生物学功能,其机制可能与靶向DDX5激活Wnt/ β -catenin信号通路相关。本研究为BC进展的分子机制提供新思路,表明FMRP可能成为BC治疗与评估预后的潜在靶点。

* * *

作者贡献声明 曹佳负责论文构思、正式分析和初稿写作,王晶负责调查研究,石斌负责提供资源和监督指导,马小兰负责研究方法、软件和可视化,吴伟超负责调查研究和研究项目管理,王南负责数据审编、经费获取和审读与编辑写作。所有作者已经同意将文章提交给本刊,且对将要发表的版本进行最终定稿,并同意对工作的所有方面负责。

Author Contribution CAO Jia is responsible for conceptualization, formal analysis, and writing--original draft. WANG Jing is responsible for investigation. SHI Bin is responsible for resources and supervision. MA Xiaolan is responsible for methodology, software, and visualization. WU Weichao is responsible for investigation and project administration. WANG Nan is responsible for data curation, funding acquisition, and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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Declaration of Conflicting Interests All authors declare no competing interests.

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