

· 医学与免疫生物技术 ·

FOXG1 在结直肠癌侵袭及转移中的作用及机制

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摘要: 构建叉头框 G1 (Forkhead box G1, FOXG1) 的慢病毒干扰 (shRNA) 质粒及表达质粒, 通过敲低和过表达 FOXG1 探讨其对结直肠癌细胞上皮-间质转化 EMT 的作用及其机制。应用 Western blotting 检测 FOXG1 在 RKO、SW480、SW620、LOVO、DLD-1 五种结直肠癌细胞中蛋白的表达水平, 设计并合成 FOXG1 的 shRNA 片段 (shFOXG1), 运用 DNA 重组技术获得重组质粒, 经双酶切技术及测序方法鉴定后进行慢病毒的包装、纯化及稳定转染, 经筛选后获得稳定的结直肠癌细胞株, 通过 Western blotting 和 qRT-PCR 技术检测 FOXG1 敲低和过表达效率及 EMT 关键因子 E-cadherin、Vimentin、Fibronectin、Snail、Twist mRNA 和蛋白的变化, 光学显微镜观察敲低后细胞形态学变化, 通过划痕实验检测迁移能力变化, Transwell 检测侵袭迁移能力的变化。5 种结直肠癌细胞中, FOXG1 在 RKO 细胞中蛋白表达量最高, 而在 DLD-1 细胞中表达量最低, 与对照组相比较, 在 RKO 细胞中敲低 FOXG1, 细胞形态由长梭型变成了类圆形或者多边形, 细胞极性和紧密连接增加, 细胞迁移距离明显降低, 侵袭转移穿过小室的细胞数也明显减少, EMT 关键因子 E-cadherin 表达增高, Vimentin、Fibronectin、Snail、Twist 表达降低, 过表达 FOXG1 组则相反。FOXG1 在结直肠癌中高表达, 这种基因的高表达能够促进结直肠癌细胞的侵袭和转移, 对结直肠癌细胞的 EMT 起着重要的调控作用。

关键词: FOXG1, 结直肠癌, EMT, 转移

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Role and mechanism of FOXG1 in invasion and metastasis of colorectal cancer

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Abstract: This study was aimed to investigate the effect of Forkhead Box G1 (FOXG1) on the epithelial-mesenchymal transition (EMT) of colorectal cancer (CRC) cells and the underlying mechanism. For this purpose, FOXG1 lentiviral interference (shRNA) plasmid and expression plasmid were constructed. Western blotting was used to analyze the expression of FOXG1 protein in five CRC cells, namely RKO, SW480, SW620, LoVo and DLD-1. The shRNA fragment of FOXG1 (shFOXG1) was designed and synthesized. Recombinant plasmids were obtained with the aid of DNA recombination technique. Double digestion and sequencing were used to identify the recombinant plasmids, and then lentivirus packaging, purification and stable transfection were carried out. Additionally, stable CRC cell lines were screened out. The changes of FOXG1 knockdown and overexpression efficiency, E-cadherin, Vimentin, Fibronectin, Snail, Twist mRNA and protein were investigated respectively by Western blotting and qRT-PCR analysis. Furthermore, the changes of cell morphology after knockdown and cell migration ability were evaluated respectively with optical microscopy, scratch test and Transwell assay. FOXG1 had the highest protein expression in RKO and the lowest in DLD-1 among the five CRC cells. Compared with those of the control group, the cell morphology in FOXG1 knockdown RKO group was changed from spindle into round or polygonal shape, cell polarization was enhanced and tight junction assembly was accelerated while cell migration distance was noticeably decreased. Moreover, the number of cells invaded and migrated through chambers was significantly reduced. Among these key factors of EMT, the expression of E-cadherin was increased while the expressions of Vimentin, Fibronectin, Snail and Twist were decreased. The opposite was the case in the overexpressed FOXG1 group. The overexpression of FOXG1 in CRC promoted the invasion and metastasis of CRC cells and played a crucial role in regulating the EMT. Thus, FOXG1 might be a novel therapeutic target in CRC treatment.

Keywords: FOXG1, colorectal cancer, EMT, metastasis

结直肠癌是全球范围内常见的恶性消化道肿瘤之一，其发病率和死亡率还在逐年上升^[1]。目前结直肠癌的治疗措施主要为手术切除，但由于结直肠癌细胞具有较高的侵袭和转移的生物学特性，故影响结直肠癌患者治疗效果的主要因素为远处转移，以肝转移最为常见^[2-3]。因此找寻新的肿瘤标志物及治疗靶标对早期诊断和及时制止其侵袭转移，对患者的预后具有至关重要的意义。

上皮-间质转化 (Epithelial-mesenchymal transition EMT) 是一种常见的病理生理学现象，是指上皮样细胞获得间质细胞特性的过程，发生 EMT 时，细胞结构改变、极性消失、细胞间连接减少而运动能力则增强，表现出类似间质细胞的表型和迁

移能力，如上皮样标志物 E-cadherin 表达降低，而间质样标志如 Vimentin、Fibronectin 则增高^[4-8]，近年来 EMT 被认为是肿瘤转移的起始环节，成为肿瘤转移研究的热点^[9-11]，因此对 EMT 的研究有助于增进对肠癌转移的认识。

叉头框 G1 (Forkhead box G1, FOXG1) 基因，又名脑因子 1 基因，是叉头框 (Forkhead box, FOX) 基因家族里的重要一员，位于 14q12，其编码的转录因子常特异表达于人类脑组织，常与神经系统的发育性病变有关^[12-17]，有文献报道 FOXG1 在卵巢癌、肝癌中高表达，并可作为不良预后的分子指标^[18-19]，即 FOXG1 通过抑制 p21^{WAF1/CIP1} 转录，进而增强 TGF- β 介导的促肿瘤

增殖能力抗增殖反应,但 FOXG1 在结直肠癌中尚无报道,且 FOXG1 是否能促进肿瘤的侵袭转移也未见报道。本研究首次分析了 FOXG1 在结直肠癌侵袭转移中的作用及机制。

1 材料与方法

1.1 材料

各类肠癌细胞由本实验室保存,DMEM 培养基、胎牛血清、胰酶、Lipofectamine 2000 转染试剂等均购自 Gibco 公司;兔抗人 FOXG1 抗体购自 Abcam 公司;兔抗人 E-cadherin、Vimentin、鼠抗人 Fibronectin 抗体购自 Cell Signaling 公司;分子克隆和慢病毒包装等试剂由本实验室保存;Trizol 试剂购自 Invitrogen 公司;逆转录聚合酶链式反应试剂盒购自 TaKaRa 公司;所有引物均由金斯瑞公司负责合成。

1.2 方法

1.2.1 细胞培养

人结肠癌细胞系 RKO、SW480、SW620、LOVO、DLD-1 培养于含 10%胎牛血清 1640 培养基中(含青链霉素)在 37 °C、5% CO₂ 的培养箱中孵育,当细胞密度达 80%左右进行传代,本实验采用对数生长期中的细胞进行实验。

1.2.2 Western blotting 检测

提取总蛋白,制 10%胶进行 SDS-PAGE,转膜 2 h,5%脱脂牛奶封闭 1 h,一抗孵育 4 °C 过夜,一抗比例:GAPDH (1:1 000)、FOXG1 (1:700)、E-cadherin (1:1 000)、Vimentin (1:1 000) Fibronectin (1:1 000),PBST 洗 1 h 每 10 min 换一次液,二抗室温孵育 1.5 h,PBST 再洗 1 h,拍照存图。

1.2.3 质粒的构建及慢病毒转染 RKO 细胞

设计并合成 3 对 FOXG1 的 shRNA 片段(shFOXG1),运用 DNA 重组技术获得重组质粒,经双酶切技术及测序方法鉴定正确后进行慢病毒

的包装,包装后存放于-80 °C,细胞分为加入病毒的 PLKO.1-shFOXG1 实验组及未经处理的阴性对照组。转染前将细胞接种于 6 孔板中,当细胞密度达 50%左右进行细胞稳定转染,转染前 1 h 常规换液,经嘌呤霉素筛选约 1 个月后可获得稳定的细胞株。

1.2.4 RT-PCR 检测

以 Trizol 法常规提取细胞中的总 RNA,按试剂盒进行反转录反应合成 cDNA,再以 cDNA 为模板进行实时荧光定量 PCR 反应,反应条件为 95 °C 60 s;95 °C 15 s,60 °C 60 s 共 40 个循环,以 GAPDH 作为内参,Bio-Rad 凝胶成像仪采集图片,以 $Folds=2^{-\Delta\Delta C_t}$ 来表示实验组和对照组中目的基因的表达倍数比^[20],各引物序列见表 1。

1.2.5 划痕实验

将细胞接种于 6 孔板中,待细胞融合率达到 70%~80%时,使用枪头比着直尺用力划线,用 PBS 轻轻冲洗划下的细胞,加无血清培养基继续培养,分别在 0 h 和 48 h 拍照存图。

表 1 目的基因的引物序列

Table 1 The primers of related genes

Gene name	Primer (5'-3')
GAPDH	F: CTTTGGTATCGTGGAAGGACTC R: GTAGAGGCAGGGATGATGTTCT
FOXG1	F: GAGCGACGACGTGTTTCATC R: GCCGTTGTAACCTCAAAGTGCTG
E-cadherin	F: TGCCCAGAAAATGAAAAAGG R: GTGTATGTGGCAATGCGTTC
Vimentin	F: GAGAAGCTTTGCCGTTGAAGC R: GCTTCCTGTAGGTGGCAATC
Fibronectin	F: CAGTGGGAGACCTCGAGAAG R: TCCCTCGGAACATCAGAAAC
TWIST	F: GCCAGGTACATCGACTTCCTCT R: TCCATCCTCCAGACCGAGAAGG
SNAIL	F: CACTATGCCGCGCTCTTTC R: GGTCGTAGGGCTGCTGGAA

F: forward; R: reverse.

1.2.6 Transwell 检测

无血清培养基与 Matrigel 胶按 1:1 配成基质胶,按 10 μL /室加入包被 Transwell 小室底部膜的上室面,室温风干,上室面加入含 10 000 个细胞的细胞悬液,下室面加 900 μL 的含 20% 的 FBS 的培养基,置于 37 $^{\circ}\text{C}$ 、5% CO_2 的培养箱中孵育 48 h 后用 4% 多聚甲醛进行固定 30 min, PBS 洗涤 2 次,用结晶紫燃料染色 30 min,轻擦上室面细胞,显微镜下计数穿过上室面得细胞数,随机取 5 个视野拍照取均值。细胞迁移试验:用未被包被的 Transwell 小室,转移时间为 24 h,所铺细胞量为含 5 000 个细胞的细胞悬液。其余步骤同侵袭实验。

1.2.7 统计学方法

计量资料用 $\bar{x} \pm s$ 表示,采用 SPSS 13.0 进行两独立样本的 t 检验, $P < 0.05$ 有统计学意义。

2 结果与分析

2.1 FOXG1 在 5 种肠癌细胞系中的表达及与侵袭能力的相关性分析

在 RKO、SW480、SW620、LOVO、DLD-1 五种肠癌细胞系中,通过 Western blotting 检测发现,FOXG1 在 RKO 细胞中蛋白表达量最高,而在 DLD-1 细胞中表达最低(图 1A),由文献得知这 5 种结直肠癌细胞的侵袭力如下:LOVO>SW620>RKO>SW480>DLD-1^[21],通过对 FOXG1 蛋白相对表达量与相应细胞侵袭能力作相关性分析发现,在肠癌细胞中 FOXG1 的表达量与侵袭能力呈正相关(图 1B)。

2.2 敲低 FOXG1 可抑制 RKO 细胞侵袭和迁移能力

重组质粒构建成功,经双酶切技术及测序方法鉴定为正确后进行慢病毒的包装、纯化及稳定转染,经筛选后获得了稳定的结直肠癌细胞株,再通过 3 种含 shFOXG1 沉默序列的慢病毒分别感染 RKO 细胞后,Western blotting 检测发现,与对

照组相比,shFOXG1-3 干扰片段对 FOXG1 干扰沉默效果最明显(图 2A)。划痕实验结果表明在 RKO 细胞中干扰 FOXG1 后,其迁移能力显著变弱(图 2B)。Transwell 结果表明干扰 FOXG1 后,其侵袭和迁移能力也显著下降(图 2C),由于 shFOXG1-3 干扰片段对 FOXG1 干扰沉默效果最明显,故后续实验均以 shFOXG1-3 慢病毒转染细胞,并命名为干扰组(Interfered group),单独使用时简称为 shFOXG1。

2.3 过表达 FOXG1 促进 DLD-1 细胞侵袭和迁移能力

在 DLD-1 细胞过表达 FOXG1 后,与对照组相比较,FOXG1 蛋白显著升高(图 3A)。RT-qPCR 进一步验证过表达效率(图 3B)。Transwell 结果表明,过表达 FOXG1 促进了 DLD-1 细胞的侵袭和迁移能力(图 3C)。

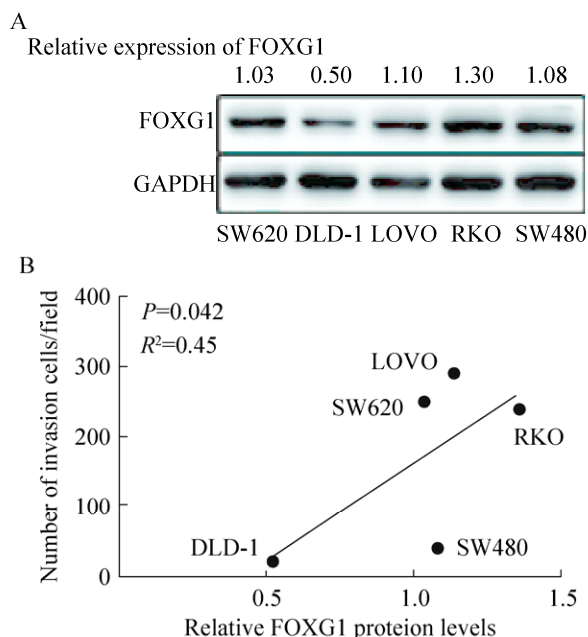


图 1 在结直肠癌细胞中 FOXG1 的表达与侵袭能力呈正相关

Fig. 1 FOXG1 expression of colorectal cancer cells was positively correlated with their invasion ability. (A) FOXG1 expression in colorectal cancer cell lines were analyzed by Western blotting. (B) The correlation between FOXG1 expression and cell invasive abilities was analyzed (Pearson correlation assay).

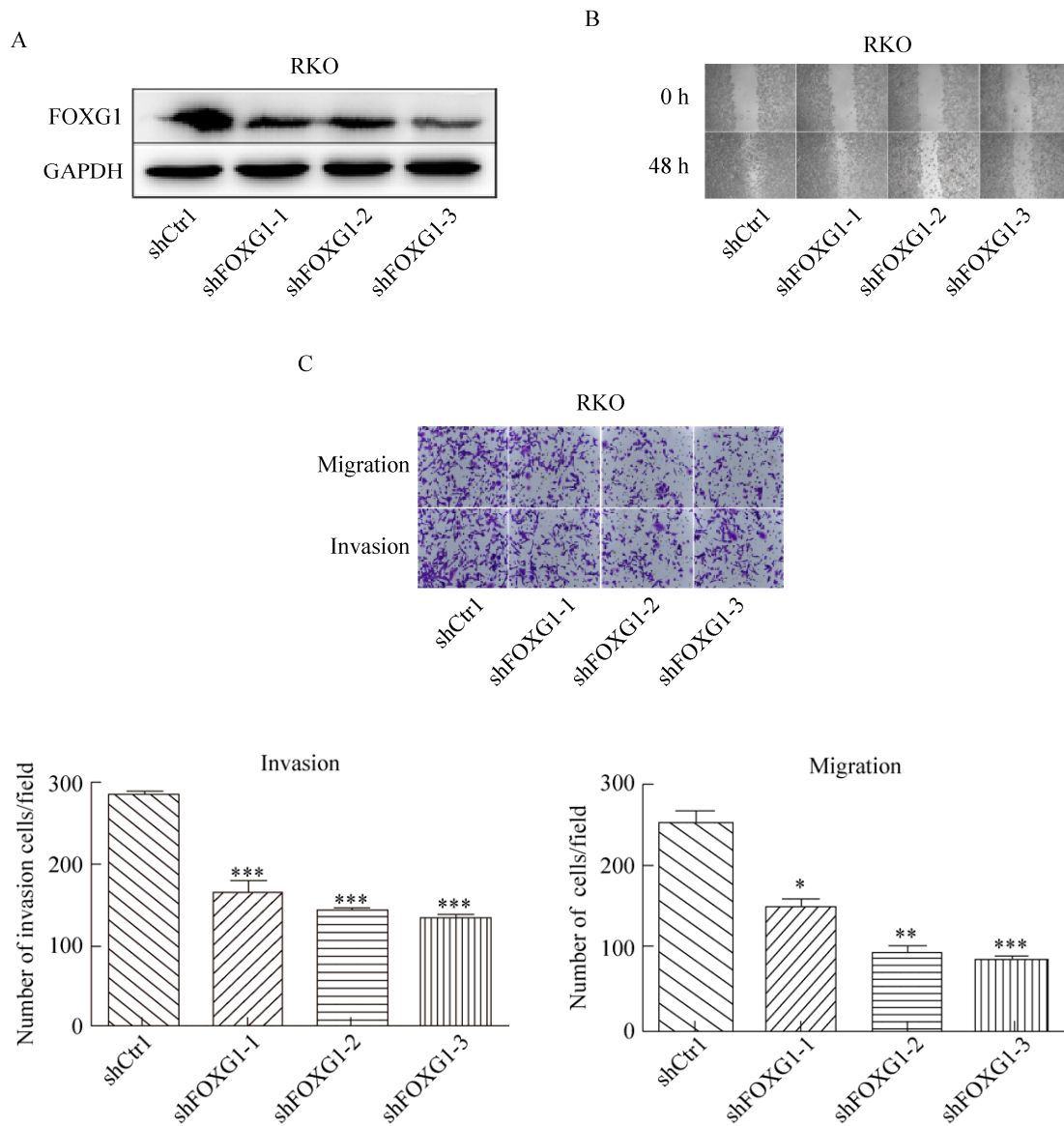


图2 干扰 FOXG1 抑制 RKO 细胞侵袭和迁移能力

Fig. 2 Knockdown of FOXG1 weakened invasion and migration abilities of RKO cells. (A) The interference efficiency of FOXG1 protein was detected by Western blotting after three virus-infected RKO cells containing shFOXG1 silencing sequence ($P < 0.05$). (B) Healing ability was tested by scratch test. (C) Transwell assay show invasion and migration abilities of FOXG1 Knockdown RKO cells compared with corresponding control cells. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

2.4 FOXG1 对维持结肠癌 EMT 具有重要作用

干扰 FOXG1 后细胞形态由典型的长梭型变成类圆形或多边形，生长方式也由较为分散转为较密集的生长 (图 4A)。干扰 FOXG1，Western

blotting 检测分析发现上皮样标志物 E-cadherin 表达在 mRNA 和蛋白水平都增加，而间质类标志物 Vimentin、Fibronectin 表达降低 (图 4B)。而过表达 FOXG1 则相反 (图 4C)。

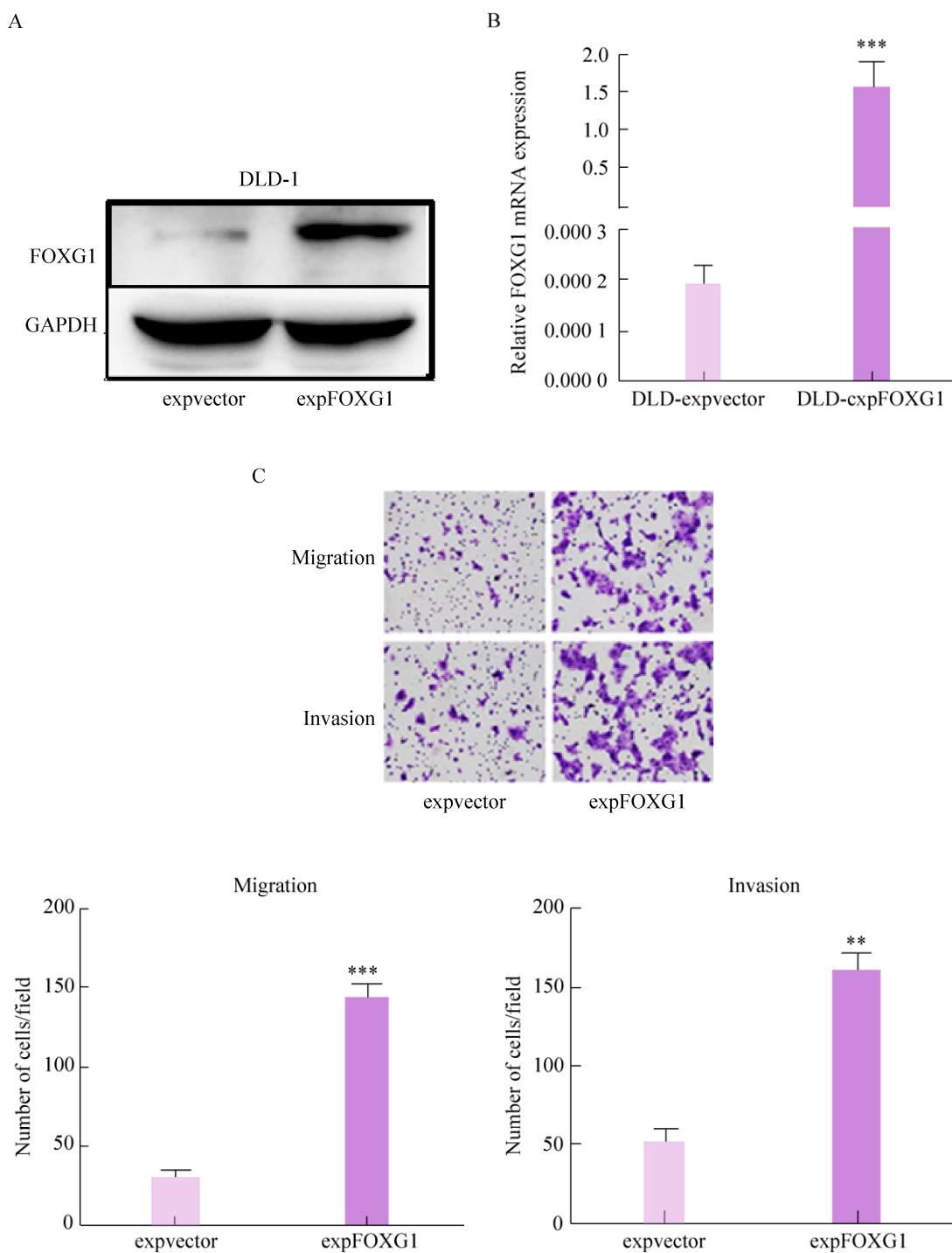


图 3 过表达 FOXG1 促进 DLD-1 细胞侵袭和迁移能力

Fig. 3 Overexpression of FOXG1 promotes invasion and migration abilities of DLD-1 cells. (A) The overexpression efficiency of FOXG1 protein was detected by Western blotting. (B) The overexpression efficiency of FOXG1 mRNA was detected by RT-qPCR. (C) Transwell assay show invasion and migration abilities of FOXG1 overexpression DLD-1 cells compared with corresponding control cells. **: $P < 0.01$; ***: $P < 0.001$.

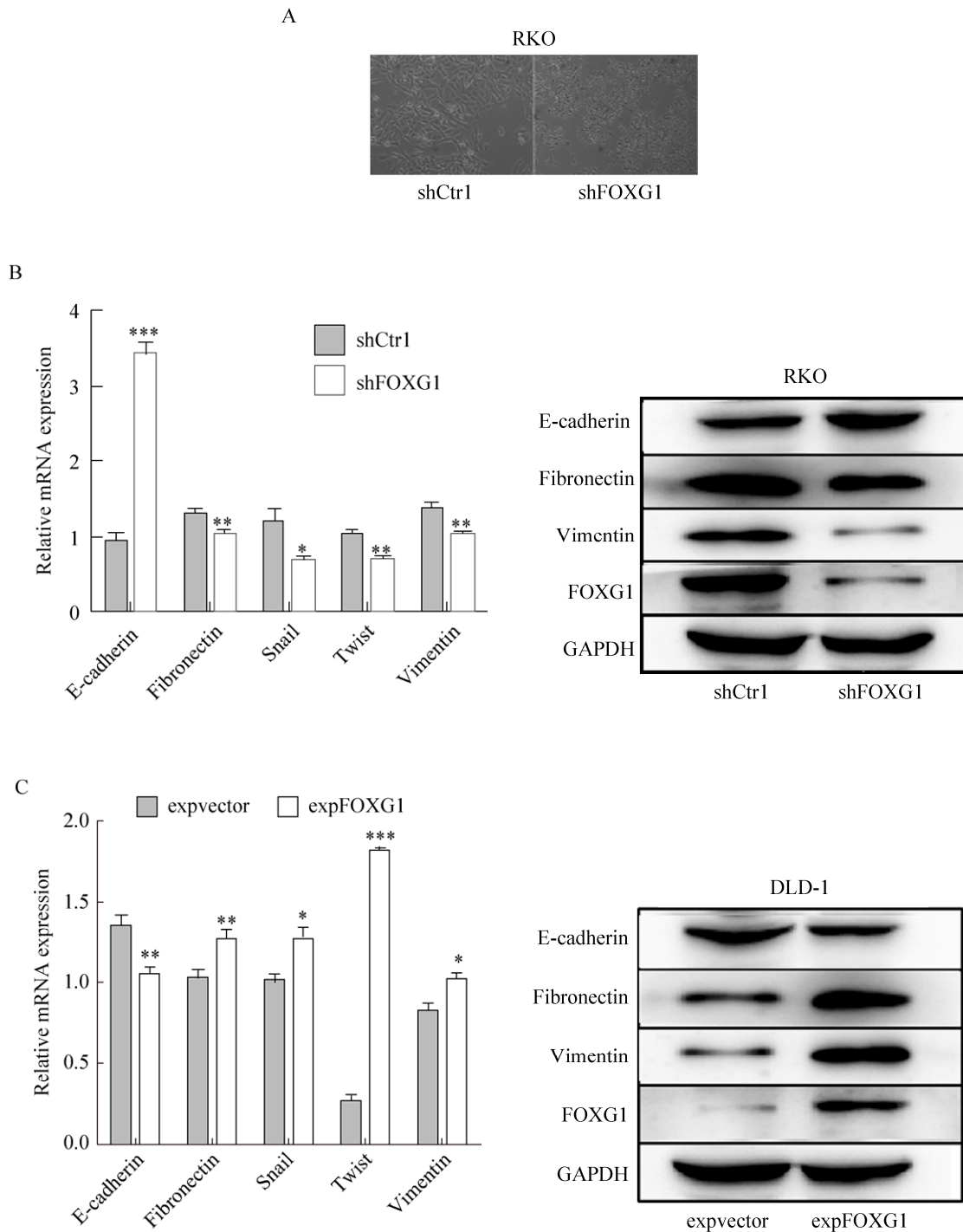


图 4 FOXG1 对维持 EMT 具有重要作用

Fig. 4 FOXG1 plays an important role in maintaining EMT. (A) Morphological changes in RKO cells after stable FOXG1 expression deletion. (B) Expression changes of EMT markers were analyzed by RT-qPCR and Western blotting in RKO cells after FOXG1 knockdown. (C) Expression changes of EMT markers were analyzed by RT-qPCR and Western blotting in DLD-1 cells after overexpression of FOXG1. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

3 讨论

研究表明 FOX 基因家族与肿瘤 EMT 相关,如 FOXQ1、FOXC1、FOXC2、FOXM1 能够降低 E-cadherin 表达,促进肿瘤的转移并发生 EMT^[22-23]。FOXG1 是 FOX 基因家族里的重要一员,位于 14q12,其编码的转录因子常特异表达于人类脑组织,常与神经系统的发育性病变有关^[12],它涉及神经细胞的分化、增殖、转移和凋亡,对端脑的发育有重要作用^[24],其异常表达常致 FOXG1 的相关疾病^[25-26],如发育迟缓、语言障碍、智力低下等。有研究表明,FOXG1 在卵巢癌、肝癌中高表达并作为促癌基因,通过对 P21 的抑制促进癌细胞的增殖^[18-19],这提示我们 FOXG1 可能与肿瘤的发生发展有关。而 FOXG1 在结直肠癌中表达情况尚无报道,且之前对于 FOXG1 在某些实体瘤的研究主要局限于增殖方向,FOXG1 是否能促进肿瘤的其他生物学功能还不明确,且大量研究表明 EMT 与结直肠癌的侵袭和转移有关^[27],故本研究在细胞水平上探索了在直结肠癌中 FOXG1 与 EMT 和转移的关系。基于上述,查阅 TCGA 数据库得知在结直肠癌组织中 FOXG1 的表达高于正常组织,同时本研究检测了 5 种结直肠癌中 FOXG1 的蛋白表达水平,并作了蛋白相对表达量与细胞侵袭能力相关性分析 (Pearson 相关性检验),最终确定干扰和过表达的细胞系即对 RKO 细胞进行干扰,DLG-1 细胞进行过表达,并检测对结直肠癌细胞侵袭转移能力的影响。结果表明慢病毒干扰载体 PLKO.1-shFOXG1 成功沉默了 FOXG1 的表达后,RKO 细胞的侵袭转移能力下降,干扰 FOXG1 后 RKO 细胞形态由典型的长梭型变成类圆形或多边形,细胞形态变得饱满,细胞间的紧密连接增多生长也变得较为密集,且上皮类标志物 E-cadherin 表达增高,而间质类标志物 Vimentin、Fibronectin 表达降低,过表达组则相反,这些结果表明 FOXG1 可能是肿瘤细胞

维持间质样表型所必需的,但具体机制还需进一步研究。

综上所述,本研究证明 FOXG1 能促进结直肠癌细胞侵袭和转移,并促进其发生 EMT,有望成为一个新的治疗靶点,但其具体机制还需进一步探究。

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