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HADHA通过调节PI3K/AKT信号通路抑制人绒毛膜滋养细胞HTR-8/SVneo迁移与侵袭*

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【摘要】 **目的** 探讨羟酰基辅酶A脱氢酶α亚基（hydroxyacyl-CoA dehydrogenase alpha subunit, HADHA）对人绒毛膜滋养细胞HTR-8/SVneo迁移和侵袭能力的影响及其潜在作用机制。**方法** 通过组织免疫荧光染色检测HADHA在6～8周正常早孕绒毛及复发性自然流产绒毛样本中表达水平的差异；慢病毒系统构建*HADHA*过表达及敲低稳转HTR-8/SVneo细胞系，并采用qRT-PCR、Western blot、Transwell、细胞划痕实验评价HADHA对HTR-8/SVneo细胞迁移侵袭能力和相关基因表达的影响；转录组测序及生物信息学分析筛选HADHA可能调控的靶基因及信号通路；加入蛋白激酶B（protein kinase B, AKT）抑制剂明确HADHA调节HTR-8/SVneo细胞迁移侵袭的具体分子机制。**结果** HADHA在复发性自然流产样本的绒毛外滋养层（extravillous trophoblast, EVT）中较正常对照组中高表达。过表达*HADHA*的HTR-8/SVneo细胞中迁移侵袭相关基因*HLA-G*、*MMP*2、*MMP*9、*NCAD*的表达水平降低（$P<0.01$，$P<0.05$），且迁移和侵袭能力减弱（$P<0.05$）；敲低*HADHA*后，基因*HLA-G*、*MMP*2、*MMP*9、*NCAD*的表达水平增高（$P<0.01$，$P<0.05$），且迁移和侵袭能力增强（$P<0.05$）。此外，过表达*HADHA*后p-PI3K、p-AKT水平降低（$P<0.05$），PI3K/AKT信号通路被抑制；敲低*HADHA*后PI3K/AKT信号通路被激活。在*HADHA*敲低稳转细胞系中加入AKT抑制剂MK-2206后，细胞迁移侵袭能力较对照敲低组减弱（$P<0.01$，$P<0.05$）。**结论** HADHA通过抑制PI3K/AKT信号通路抑制HTR-8/SVneo细胞的迁移与侵袭。

【关键词】 HADHA HTR-8/SVneo细胞 迁移 侵袭 PI3K/AKT

HADHA Inhibits the Migration and Invasion of HTR-8/SVneo Cells by Regulating PI3K/AKT Signaling Pathway

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【**Abstract**】 **Objective** To explore the effects of hydroxyacyl-CoA dehydrogenase alpha subunit (HADHA) on the migration and invasion of HTR-8/SVneo cells, a human trophoblast cell line, and its potential mechanism of action. **Methods** Immunofluorescence staining was done to evaluate the expression levels of HADHA in samples of normal villi and recurrent spontaneous abortion (RSA) villi at 6-8 weeks. Lentiviral infection system was used to construct stable HTR-8/SVneo cell lines with *HADHA* overexpression and knockdown. Western blot, qRT-PCR, Wound-healing assay, and Transwell assay were used to determine the effect of HADHA on the migration and invasion of HTR-8/SVneo cells and the expression of relevant genes. Transcriptome sequencing and bioinformatics analysis were done to screen for the potential target genes and signaling pathways regulated by HADHA. The specific molecular mechanism of how HADHA regulates the migration and invasion of HTR-8/SVneo cells was examined by adding the inhibitor of protein kinase B (PKB/AKT). **Results** HADHA was highly expressed in extravillous trophoblasts (EVT) of RSA villus samples as compared with samples from the normal control group. In HTR-8/SVneo cells overexpressing *HADHA*, the expression levels of migration and invasion-related genes, including *HLA-G*, *MMP*2, *MMP*9, and *NCAD*, were decreased ($P<0.01$, $P<0.05$), and the migration and invasion abilities of HTR-8/SVneo cells were weakened ($P<0.05$). *HADHA* knockdown increased the expression levels of *HLA-G*, *MMP*2, *MMP*9, and *NCAD* ($P<0.01$, $P<0.05$), and promoted the migration and invasion of HTR-8/SVneo cells ($P<0.05$). In addition, *HADHA* overexpression decreased the phosphorylation levels of PI3K and AKT ($P<0.05$) and inhibited the PI3K/AKT signaling pathway. *HADHA* knockdown activated the PI3K/AKT signaling pathway. When MK-2206, an AKT inhibitor, was added to stable HTR-8/SVneo cell lines with *HADHA* knockdown, the migration and invasion of the cells were significantly reduced. **Conclusion** HADHA inhibits the migration and invasion of HTR-8/SVneo cells by inhibiting the PI3K/AKT signaling pathway.

【**Key words**】 HADHA HTR-8/SVneo cell Migration Invision PI3K/AKT

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复发性自然流产(recurrent spontaneous abortion, RSA)是人类早期妊娠最常见的并发症,其发生率约为1%~5%[1]。RSA的发生与自身免疫性疾病、子宫解剖缺陷以及子宫内膜功能障碍等因素密切相关[2],但RSA中涉及的细胞和分子生物学机制尚未被完全阐明。

绒毛外滋养层(extravillous trophoblast, EVT)侵入母体蜕膜以及母体子宫螺旋动脉是妊娠过程中的关键事件,其成功发生对于胚胎植入、母胎间物质交换以及妊娠维持等过程至关重要[3]。在胎盘发育的早期阶段,EVT细胞主要由锚定于绒毛尖端的细胞滋养层(cytotrophoblast, CTB)细胞分化而来,具有高度迁移性和侵袭性。研究表明,滋养细胞迁移和侵袭不足会导致胎盘功能障碍,诱发RSA[4]。

羟酰基辅酶A脱氢酶α亚基(hydroxyacyl-CoA dehydrogenase alpha subunit, HADHA)是线粒体三功能蛋白(mitochondrial trifunctional protein, MTP)的α亚基,其酶活性缺乏或基因突变会导致婴儿猝死综合征、心肌病、低血糖等疾病的发生[5-6]。研究发现,HADHA可通过介导脂质重编程来调节肝癌细胞的侵袭,也可通过影响细胞自噬、增殖及凋亡等途径参与癌症的发病机制[7-9],但HADHA在滋养细胞迁移和侵袭中的作用尚不清楚。因此,我们探索了HADHA对人绒毛膜滋养细胞HTR-8/SVneo迁移侵袭能力的影响及其潜在调控机制,旨在为RSA等不良妊娠结局的防治提供线索。

1 材料与方法

1.1 材料

1.1.1 临床样本 本研究所用所有早孕绒毛样本均来源于重庆医科大学附属第一医院。2021年9–11月共收取6~8周正常妊娠行人工流产术的绒毛样本6例,复发性流产绒毛样本6例。研究对象纳入标准:①年龄为25~35岁;②既往无不良生活习惯;③无长期药物暴露史;④无不良妊娠史;排除标准:①不符合纳入标准者;②本身患有多囊卵巢综合征、子宫宫颈机能不全、糖尿病及抗磷脂抗体综合征等疾病的参与者。本研究已取得重庆医科大学伦理委员会批准(批准号20200728),且样本收集已征得患者知情同意。

1.1.2 细胞系 HTR-8/SVneo细胞为加拿大皇后大学Charles H.Graham教授所赠,人胚胎肾细胞HEK293T细胞为重庆医科大学张军老师课题组所赠。

1.1.3 主要试剂与仪器 主要试剂:胎牛血清购自中国上海LONSERA公司;PEI MAX转染试剂购自美国Polysciences公司;兔抗人HADHA抗体购自英国Abcam公司;小鼠抗人β-actin抗体购自北京博奥森生物技术有限公司;兔抗人基质金属蛋白酶(matrix metalloproteinase, MMP)2抗体、兔抗人MMP9抗体、青霉素-链霉素溶液、0.25%胰酶消化液、RIPA裂解液、蛋白酶体抑制剂PMSF、抗荧光淬灭剂、DAPI均购自中国碧云天生物技术有限公司;小鼠抗人人类白细胞抗原-G(human leukocyte antigen-G, HLA-G)抗体、兔抗人N-钙黏蛋白(N-cadherin, NCAD)抗体均购于美国Proteintech公司;兔抗人PI3K、兔抗人p-PI3K、兔抗人蛋白丝氨酸/苏氨酸激酶(AKT)、兔抗人p-AKT抗体均购自美国CST公司;ECL化学发光显色液购自苏州新赛美生物科技有限公司;Triton-100X购自美国Sigma-Aldrich公司;所有引物均由深圳华大基因股份有限公司合成;辣根过氧化物酶二抗、山羊抗兔FITC或山羊抗小鼠TRITC二抗均购自美国Themo Fisher公司;基质胶和Transwell小室均购自美国Corning公司;Trizol和PrimeScript™ RT Master Mix均购自日本Takara公司;2×SYBR Green qPCR Master Mix购自上海陶素生化科技有限公司;AKT抑制剂MK-2206购自美国MedChemExpress公司。

主要仪器:PCR仪、实时荧光定量PCR仪、化学发光显色仪均购自美国Bio-Rad公司;激光共聚焦扫描显微镜购自日本Nikon公司;光学显微镜购自日本Olympus公司;二氧化碳恒温细胞培养箱购自美国Thermo Fisher公司。

1.2 方法

1.2.1 细胞培养 人绒毛膜滋养细胞HTR-8/SVneo培养条件为:RPMI 1640培养基(含体积分数10%胎牛血清和体积分数1%青霉素-链霉素溶液)。HEK293T细胞培养条件为:DMEM高糖培养基(含体积分数10%胎牛血清和体积分数1%青霉素-链霉素溶液)。所有细胞均置于37 ℃、体积分数20%O_2、体积分数5%CO_2的细胞培养箱中进行培养。

1.2.2 重组质粒构建 ①引物设计:*HADHA*过表达引物采用Primer 5.0软件设计,并分别在上游和下游引物5′端加上*Eco*RⅠ和*Bam*HⅠ酶切位点和保护碱基;*HADHA*敲低引物序列从Sigma网站获得。引物序列信息见表1;②目的质粒、载体双酶切:过表达载体pCDH-CD513B-1-PHB和敲低载体pSIH1-H1-puro分别用限制性内切酶*Bam*HⅠ和*Eco*RⅠ进行双酶切,酶切体系各20 μL;③重组反应:将线性化载体DNA与纯化的酶切产物进行重组反应连接,体系为10 μL。其中,目的片段4 μL,酶切载体1 μL,Solution Ⅰ 5 μL;④重组产物转化:将连接产物于感受态细胞中进行转化;⑤将所得菌液均匀涂布在带有相应抗性的LB平板上,于37 ℃孵箱培养16~18 h。

表 1　引物序列

Table 1　Sequence of primers

Gene	Forward primers (5′→3′)	Reverse primers (5′→3′)
HADHA-OE	GATTCTAGAGCTAGCGAATTCATGGTGGCCTGCCGG	GATCCTTCGCGGCCGCGGATCCTCACTGGTAGAACTTCTTGTTAGGG
HADHA-shRNA	GATCCGTGAAGTGTGACTTCGAATTACTCGAGTAATTCGAAGTCACACTTCACTTTTTG	AATTCAAAAAGTGAAGTGTGACTTCGAATTATCTGACAGGAAGTAATTCGAAGTCACACTTCAC

1.2.3　质粒提取　质粒提取步骤参照康为世纪质粒提取试剂盒说明书进行, 具体方法如下: 菌液室温9 000 r/min离心10 min, 弃上清; 加入500 μL Buffer P1重悬菌液后, 加入500 μL Buffer P2裂解菌体; 加入500 μL Buffer E3终止裂解后, 室温13 300 r/min离心5 min; 取上清于Endo-Remover FM过滤柱中, 室温13 300 r/min离心1 min; 加入450 μL异丙醇后, 取上清于Spin Columns DL吸附柱中, 13 300 r/min离心2 min; 750 μL Buffer PW洗膜后, 对膜加入Endo-Free Buffer EB溶解DNA, 并测量DNA浓度。

1.2.4　慢病毒包装系统构建稳转细胞系　①慢病毒包装: 将HEK293T细胞接种于60皿中, 待细胞密度为70%～90%时进行目的质粒、慢病毒包装质粒(psPAX2, pVSVG)共转染, 转染8 h后更换为新鲜的受体细胞培养基, 继续培养36 h后收集病毒液; ②慢病毒感染: 将HTR-8/SVneo细胞接种于60皿中, 待细胞密度为80%左右时, 加入病毒液及助转染试剂polybrene(终质量浓度为8 μg/mL), 12 h后更换为正常培养基; ③药物筛选: 高质量浓度(6～8 μg/mL)嘌呤霉素筛选12 h后, 于培养基中加入1 μg/mL嘌呤霉素长期维持, 获得*HADHA*过表达(*HADHA*-OE)和*HADHA*敲低(*HADHA*-shRNA)稳转细胞系。分组情况: *HADHA*过表达组(*HADHA*-OE)及其相应对照组(CON-OE), *HADHA*敲低组(*HADHA*-shRNA)及其相应对照组(CON-shRNA)。

1.2.5　Western blot　细胞用PBS清洗后, 加入适量RIPA裂解液, 于冰上裂解10 min, 按Buffer∶裂解液=1∶4的比例加入适量5×Protein Loading Buffer, 100 ℃金属浴, 10 min使蛋白变性后, 将其保存于−80 ℃。将提取的蛋白样品通过SDS-PAGE凝胶电泳进行分离, 并通过100 V恒压电转至PVDF膜上; 用5%脱脂牛奶37 ℃封闭1 h, 将膜与一抗ACTB (1∶1 000)、HADHA (1∶1 000)、HLA-G (1∶1000)、MMP2 (1∶500)、MMP9 (1∶500)、NCAD (1∶1 000)、AKT (1∶1 000)、p-AKT (1∶1 000)、PI3K (1∶1 000)、p-PI3K (1∶1 000) 于4 ℃孵育过夜。第二天, 清洗PVDF膜3次后, 将其与HRP偶联的羊抗兔IgG或羊抗鼠IgG于37 ℃孵育1 h, 清洗PVDF膜, 并通过ECL化学发光显色液显影。以ACTB作为内参蛋白, 并通过Quantity One软件进行蛋白灰度值分析。

1.2.6　实时荧光定量PCR（qRT-PCR）　Trizol法提取细胞RNA后, 采用PrimeScript™ RT Master Mix将其逆转为cDNA。cDNA通过SYBR Green Supermix进行基因表达水平的检测, 并用$2^{-\Delta\Delta Ct}$法计算基因相对表达水平。*GAPDH*为内参基因。所有引物均采用Primer 5.0软件设计, 引物序列信息见表2。

1.2.7　组织免疫荧光　将绒毛样本进行石蜡包埋、5 μm连续切片及37 ℃过夜烤片。将切片进行二甲苯脱蜡, 梯度酒精水化后, 用EDTA抗原修复液进行抗原热修复。将自然冷却至室温的切片用0.3% TritonX-100通透15 min, 5%BSA于37 ℃水浴封闭1 h后与一抗工作液4 ℃孵育过夜。第二天, 切片室温复温30 min后进行PBS洗涤, 再与荧光二抗37 ℃孵育1 h。PBS洗涤切片, 细胞核用DAPI染色15 min后, 进行指甲油封片。

1.2.8　细胞划痕实验　将细胞铺于6孔板中, 用含10%FBS的完全培养基培养至细胞密度为95%以上后进行细胞划痕。细胞用无菌PBS清洗后, 更换为无血清细胞培养基继续培养。分别在划痕0 h、12 h、24 h、48 h、72 h后通过倒置显微镜进行拍照, 并通过ImageJ软件计算划痕面积。

1.2.9　Transwell实验　基质胶提前于冰上解冻, 并按基质胶∶培养基=1∶9的比例稀释基质胶。将基质胶(60 μL/室)均匀铺于小室中, 置于37 ℃培养箱静置1 h。将培养好的细胞进行胰酶消化, 并用无血清培养基进行重悬, 经细胞计数板计数后, 于每个小室上室中均匀接种5.5×10^4个细胞, 24孔板下室中加入500 μL含10%FBS的完全培养基, 37 ℃、5%CO_2培养箱继续培养24 h。PBS清洗小室3次, 冰甲醇冰上固定10 min; PBS清洗3次, 并用湿棉棒轻轻擦拭小室内侧, 外侧用苏木素染色25 min; PBS清洗3次后, 显微镜下观察并计数。

1.2.10　转录组测序及分析　细胞用Trizol裂解、液氮速冻后储存于−80 ℃。转录组测序交由北京诺禾致源生物科技技术公司完成。为确保分析质量, 原始数据的完整性通过MD5校验, 并通过Illumina平台过滤以获得高质量片段(clean reads)。采用倍比法(FC)和配对样本*t*检验获取差异表达基因(DEGs), 筛选差异基因需同时满足下列条件: ①*P*-value≤0.05; ②|$\log_2$FC|<1。使用Sangerbox在线分析工具(http://sangerbox.com)绘制火山图、基因本体

表2 定量引物序列

Table 2 The sequence of primers for for qRT-PCR

Gene	Forward primers (5′→3′)	Reverse primers (5′→3′)
HADHA	CTGCCCAAAATGGTGGGTGT	GGAGGTTTTAGTCCTGGTCCC
ASCL2	CTCGACCTATGAGCCTCAG	AATCTCCAAGTGTTGGTGC
MMP2	ATACCATCGAGACCATGCG	ATACCATCGAGACCATGCG
MMP9	TGCAACGTGAACATCTTCG	GAATCGCCAGTACTTCCCA
HLA-G	TGAGATGGAAGCAGTCTTCC	TGAGATGGAAGCAGTCTTCC
NCAD	CCTGCTTATCCTTGTGCTGA	CCTGGTCTTCTTCTCCTCCA
TNC	CATCGGAAAGATTTCTATAGACACC	AACTGAAGTGGCTTCAGCA
RELN	AAAGGAGTCTTACTGCGCT	TTTGTTTGCGAGTGAGGAC
FGF1	TATACGGCTCACAGACACC	TCTCTGCATGCTTCTTGGA
CCND1	CATTGAACACTTCCTCTCCA	AACTTCACATCTGTGGCAC
MMP1	ACAGTAAGCTAACCTTTGATGC	TTGTGCGCATGTAGAATCTG
LAMA2	AGTTCTGACACATGGTCCT	TTTCTTGAAAGCCCGAACTG
MEP1B	AAACTTTGATGTAGATGGCGG	TCAAGTCTGATGTCACCCTC
IGFBP5	GAGCAAGTCAAGATCGAGAG	CTTCTTCACTGCTTCAGCC
STK31	TTCAGTTTTGGGGAATCTTGACC	ACCAGACACTTTTCAACGCTG
GAPDH	AGATCATCAGCAATGCCTCCT	TGGTCATGAGTCCTTCCACG

HADHA: Hydroxyacyl-CoA dehydrogenase alpha subunit; *ASCL2*: Achaete-scute family BHLH transcription factor 2; *MMP*: Matrix metalloproteinase; *HLA-G*: Human leukocyte antigen-G; *NCAD*: N-cadherin; *TNC*: Tenascin C; *RELN*: Reelin; *FGF1*: Fibroblast growth factor 1; *CCND1*: Cyclin D1; *LAMA2*: Laminin subunit alpha 2; *MEP1B*: Meprinasubunit beta; *IGFBP5*: Insulin like growth factor binding protein 5; *STK31*: Serine/threonine kinase 31; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

(GO)富集分析图和京都基因与基因组百科全书(KEGG)通路图。

1.2.11 统计学方法 每组试验至少重复3次,且所有数据均以$\bar{x}\pm s$表示。两组之间的比较采用非配对t检验,多组间的比较采用单因素方差分析。$P<0.05$为差异有统计学意义。采用Graphpad Prism 5.01软件完成。

2 结果

2.1 HADHA在正常早孕绒毛中的表达模式

组织免疫荧光分析HADHA在6~8周正常早孕及复发性自然流产绒毛中的表达模式发现,HADHA在正常早孕绒毛样本的EVT细胞中低表达,而在复发性自然流产绒毛样本EVT细胞中相对高表达(图1)。

2.2 过表达*HADHA*抑制HTR-8/SVeno细胞的迁移和侵袭

慢病毒系统构建*HADHA*过表达HTR-8/SVeno细胞系(*HADHA*-OE)的Western blot结果显示,细胞上皮型标志因子NCAD,侵袭相关因子MMP2、MMP9以及EVT标志物HLA-G在*HADHA*-OE组的表达较CON-OE组显著下调(图2A)。qRT-PCR与Western blot所得结果一致($P<0.05$)(图2B)。细胞划痕结果显示,*HADHA*-OE组细胞迁移能力较CON-OE组降低($P<0.01$)(图2C、图2D)。Transwell结果显示,*HADHA*-OE组细胞侵袭能力较CON-OE组降低($P<0.05$)(图2E、图2F)。以上结果表明,*HADHA*基因过表达会抑制HTR-8/SVeno细胞迁移侵袭。

2.3 敲低*HADHA*促进HTR-8/SVeno细胞的迁移和侵袭

Western blot和qRT-PCR结果显示,HADHA的表达水平在*HADHA*-shRNA组中较CON-shRNA组降低了89.1%,提示*HADHA*敲低稳转细胞系(*HADHA*-shRNA)构建成功($P<0.001$)。此外,NCAD、MMP2、MMP9以及HLA-G的蛋白及mRNA表达水平在*HADHA*-shRNA组均较CON-shRNA组升高($P<0.01$, $P<0.05$)(图3A、图3B)。细胞划痕愈合实验结果显示,*HADHA*-shRNA组细胞的迁移率为CON-shRNA组的2.05倍($P<0.01$)(图3C、图3D)。*HADHA*-shRNA组细胞是侵袭数目为CON-shRNA组的1.92倍($P<0.05$)(图3E、图3F)。以上结果表

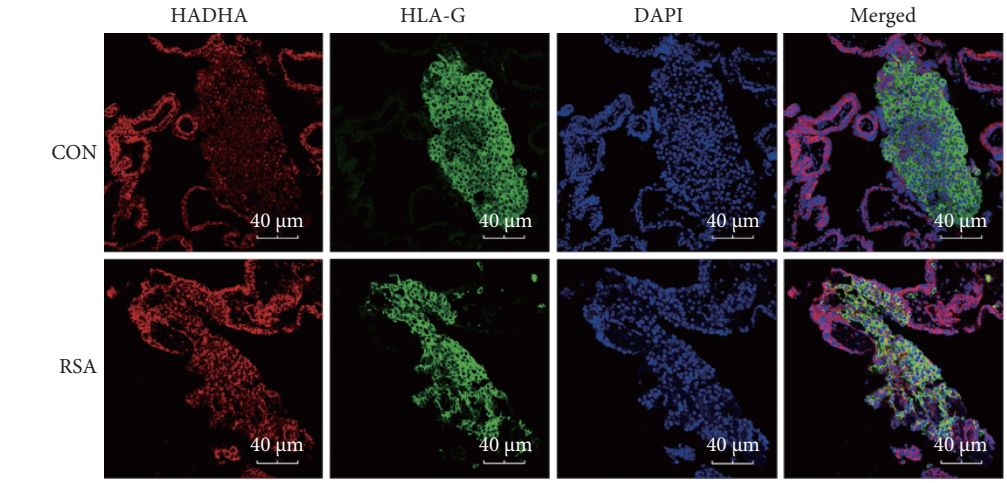


图 1 HADHA在正常及复发性流产绒毛中的表达模式

Fig 1 The expression pattern of HADHA in normal and RSA villi

Immunofluorescence staining of human normal (CON) and RSA villus tissue. HADHA (red) is the gene of interest, HLA-G (green) is an EVT marker, and DAPI (blue) indicates the nucleus. Scale bar=40 μm. HADHA and HLA-G denote the same as those in table 2.

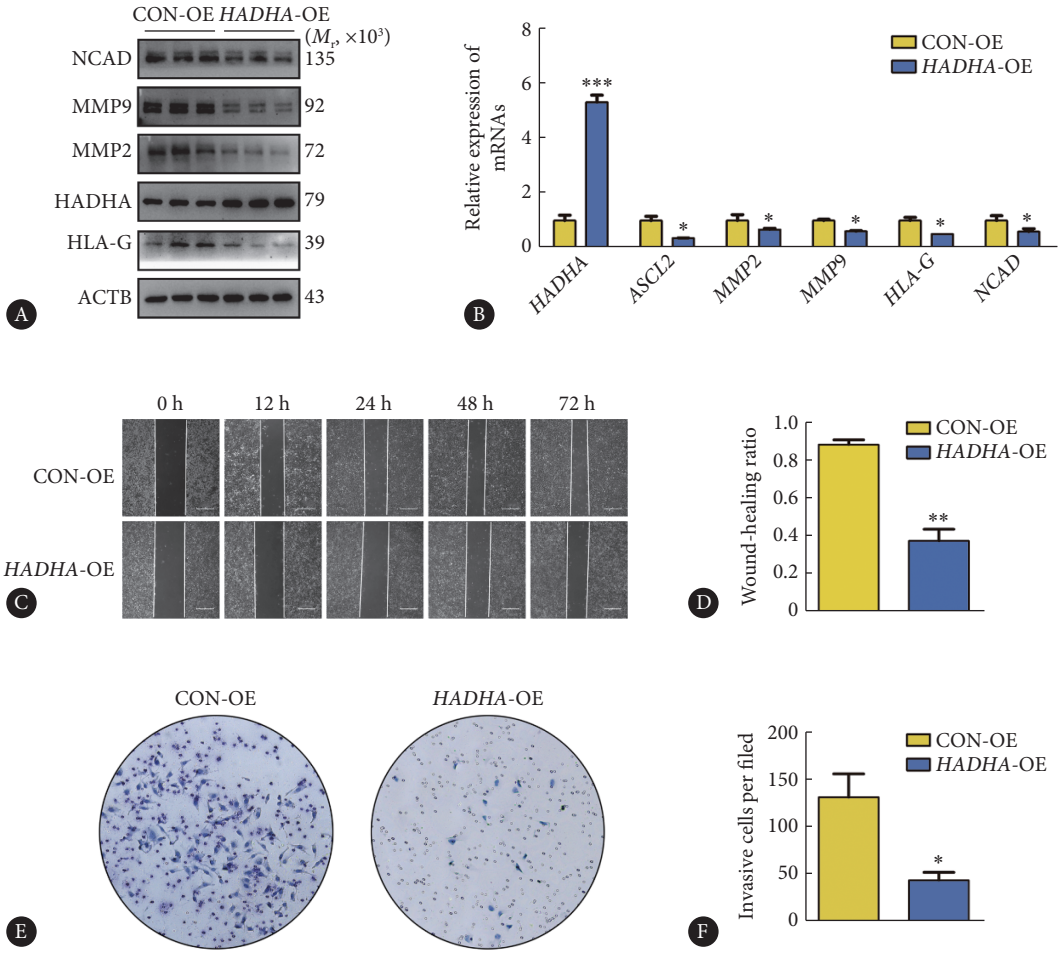


图 2 过表达*HADHA*抑制HTR-8/SVeno细胞的迁移和侵袭

Fig 2 *HADHA* overexpression inhibits the migration and invasion of HTR-8/SVeno cells

A: Western blot (ACTB was the internal reference protein); B: qRT-PCR (*GAPDH* was the internal reference gene, n=3); C: Cell wound-healing assay (Scale bars=400 μm); D: Statistical analysis for scratch area (n=3); E: Transwell (Scale bars=50 μm); F: Statistical analysis for the number of invasive cells (n=3). * P<0.05, ** P<0.01, *** P<0.001, vs. CON-OE. ACTB: β-actin; HADHA, HLA-G, MMP2, MMP9, ASCL2, and NCAD denote the same as those in table 2.

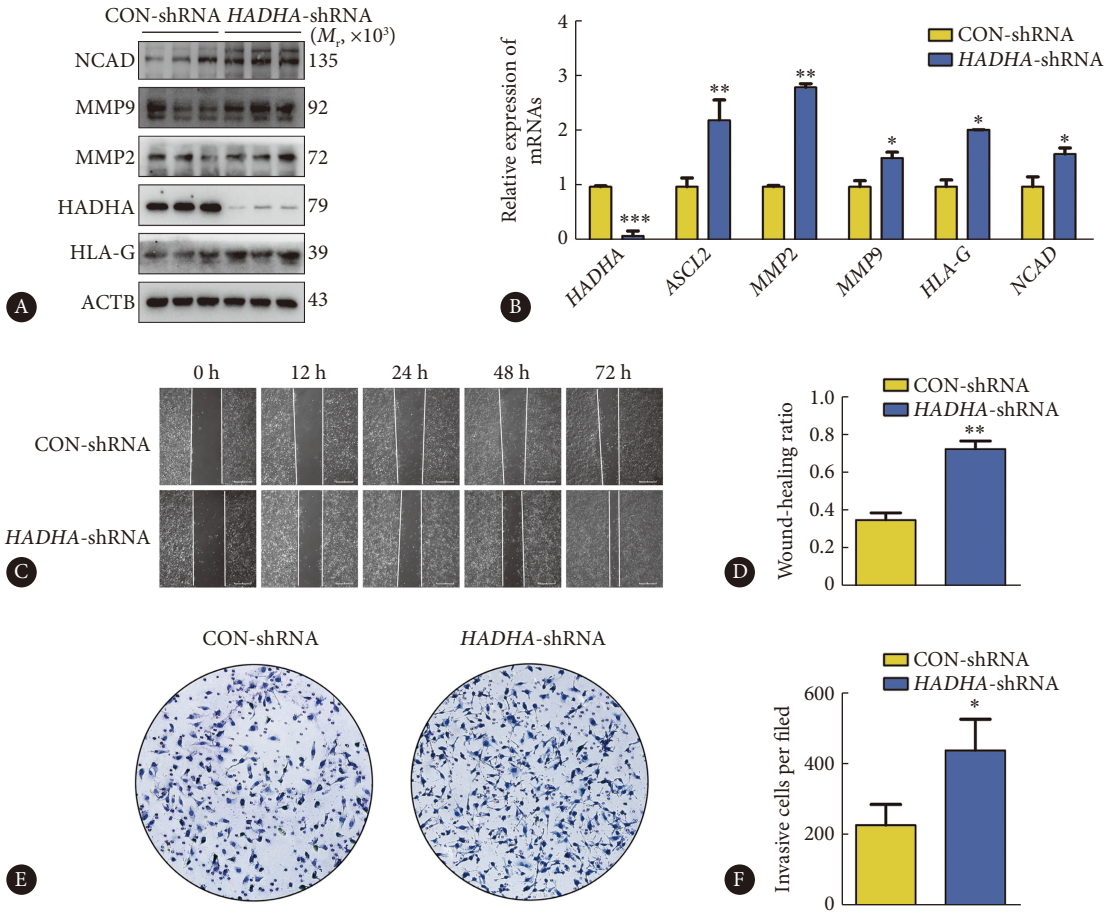


图 3 敲低*HADHA*促进HTR-8/SVeno细胞的迁移和侵袭

Fig 3 *HADHA* knockdown promotes the migration and invasion of HTR-8/SVeno cells

A-F denote the same as those in Fig 2; ACTB, HADHA, HLA-G, MMP2, MMP9, ASCL2, NCAD denote the same as those in table 2. * P<0.05, ** P<0.01, *** P<0.001, vs. CON-shRNA.

明, *HADHA*敲低会促进HTR-8/SVeno细胞迁移侵袭。

2.4 HADHA下游靶基因及信号通路的生物信息学筛选

为了进一步明确HADHA可能调控的下游靶基因及相关信号通路, 我们将*HADHA*敲低及其对照HTR-8/SVeno细胞进行了转录组测序及生物信息学分析。转录组测序共筛选到了269个差异表达基因, 其中133个基因上调表达, 136个基因下调表达(图4A)。对差异表达基因进行GO聚类分析发现, 这些基因主要富集于AKT活性调节及信号转导、二十烷酸转运等生物学过程; 主要富集于含胶原的细胞外基质、中间纤维等细胞组分; 主要富集于信号受体激活剂活性、受体-配体活性、细胞骨架结构组成等分子功能(图4B)。KEGG信号通路主要富集于白介素17(interleukin-17, IL-17)、PI3K/AKT、丝裂原活化蛋白激酶(mitogen-activated protein kinase, MAPK)等信号通路(图4C)。

2.5 qRT-PCR验证HADHA潜在调控的下游靶基因

对转录组测序筛选到的迁移侵袭相关基因进行进一步验证的qRT-PCR结果显示, *HADHA*-shRNA组迁移侵袭相关基因*RELN*、*FGF*1、*CCND*1、*MMP*1、*LAMA*2、*MEP*1*B*、*IGFBP*5、*STK*31的mRNA水平较CON-shRNA组升高, 与转录组测序结果一致(P<0.01, P<0.05)(图5A ~ 5I)。与之对应, *HADHA*-OE组中*RELN*、*MMP*1、*CCND*1等基因的mRNA水平较CON-OE组降低(P<0.01, P<0.05)(图6A ~ 6I)。以上结果表明, HADHA可能通过影响*RELN*、*CCND*1等基因的表达抑制HTR-8/SVneo (Scale bars=400 μm)细胞迁移侵袭。

2.6 HADHA负调控PI3K/AKT信号通路

KEGG通路富集分析结果发现, *RELN*、*CCND*1等基因主要富集于PI3K/AKT信号通路(图4C)。因此, 我们在HADHA敲低细胞系中明确了HADHA对PI3K/AKT信号通路的调控作用。Western blot结果显示, *HADHA*-shRNA组p-PI3K、p-AKT表达水平较CON-shRNA组升高, PI3K/AKT信号通路被激活(P<0.05)(图7A ~ 7C); 与之对应, *HADHA*过表达PI3K/AKT信号通路被抑制(P<

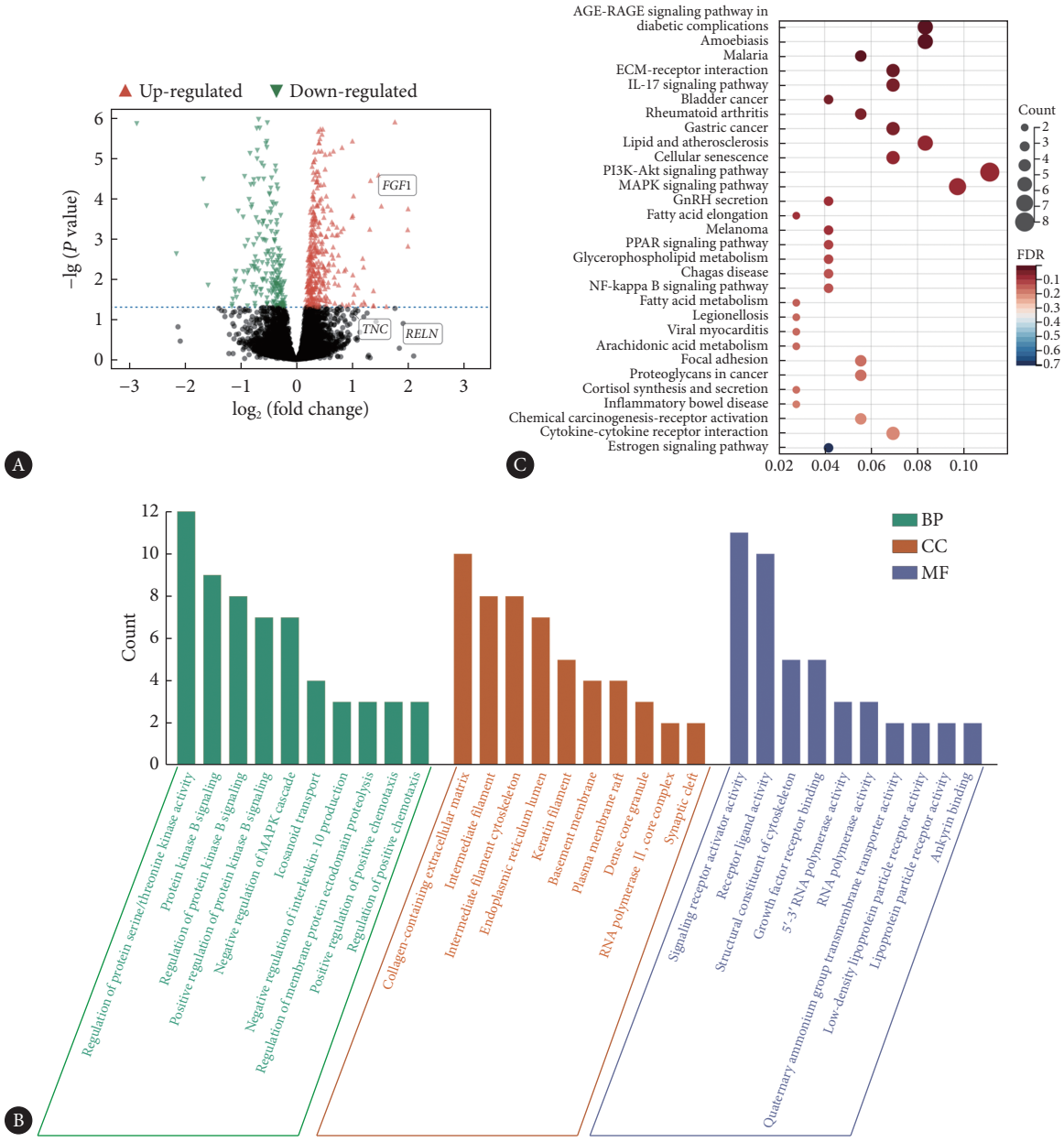


图 4 HADHA下游靶基因及信号通路的生物信息学筛选

Fig 4 Bioinformatics screening of downstream target genes and signaling pathways of HADHA

A: Volcano plot of differentially expressed genes in control and *HADHA* knockdown HTR-8/SVneo cells, Green indicates down-regulated differentially expressed genes, red indicates up-regulated differentially expressed genes, and black indicates genes that were not expressed differentially in the two groups (*FGF*1: Fibroblast growth factor 1; *TNC*: Tenascin C; *RELN*: Reelin); B: Histogram of GO enrichment analysis of differentially expressed genes (BP: Biological process; CC: Cellular component; MF: Molecular function); C: KEGG pathway enrichment analysis of differentially expressed genes (FDR: False discovery rate).

0.05)(图7D ~ 7F)。

2.7　HADHA通过PI3K/AKT信号通路调控HTR-8/SVneo细胞迁移侵袭

进一步在*HADHA*敲低稳转HTR-8/SVneo细胞系中探究了AKT通路抑制剂MK-2 206对细胞迁移侵袭能力的影响。Western blot结果显示, p-AKT以及迁移侵袭相关因子HLA-G、MMP2、NCAD的蛋白表达水平在*HADHA*-shRNA组较CON-shRNA组升高, 而这种效应在MK-2 206处理后降低, 提示抑制PI3K/AKT信号通路会抑制HTR-8/SVneo细胞迁移和侵袭(P<0.01, P<0.05)(图8A、8B)。qRT-PCR与Western blot结果一致(P<0.01, P<0.05)(图8C)。细胞划痕愈合实验结果显示, H*ADHA*-shRNA组中细胞迁移能力较CON-shRNA组强, 在经MK-2206处理后, 迁移能力则减弱(P<0.05)(图8D)。Transwell结果显示, *HADHA*-shRNA组细胞侵袭能力较CON-shRNA组增强, 而在经MK-2206处理后, 侵袭能力减弱(P<0.01, P<0.05)

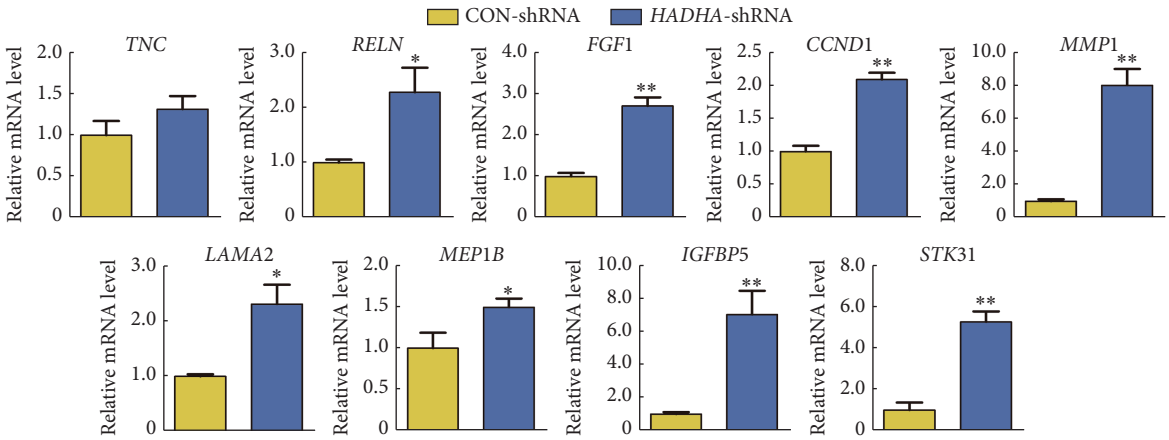


图 5 qRT-PCR验证HADHA潜在调控靶基因（*n*=3）

Fig 5 Validation of potential regulatory target genes of HADHA detected by qRT-PCR (*n*=3)

* *P*<0.05, ** *P*<0.01, vs. CON-shRNA. *TNC*, *RELN*, *FGF*1, *CCND*1, *MMP*1, *LAMA*2, *MEP*1*B*, *IGFBP*5, and *STK*31 denote the same as those in table 2.

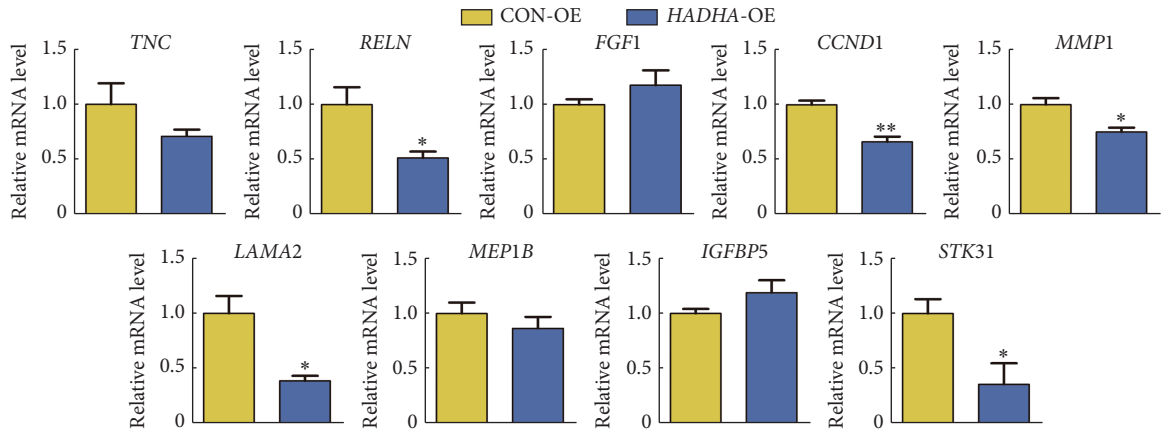


图 6 qRT-PCR验证HADHA潜在调控靶基因（*n*=3）

Fig 6 Validation of potential regulatory target genes of HADHA detected by qRT-PCR (*n*=3)

* *P*<0.05, ** *P*<0.01, vs. CON-OE. *TNC*, *RELN*, *FGF*1, *CCND*1, *MMP*1, *LAMA*2, *MEP*1*B*, *IGFBP*5, and *STK*31 denote the same as those in table 2.

(图8E)。综上, HADHA可能通过负调控PI3K/AKT信号通路抑制HTR-8/SVneo细胞的迁移与侵袭。

3 讨论

RSA的发生与滋养细胞的迁移侵袭能力受损密切相关[10], 而滋养细胞迁移侵袭又会受到多种因素的调控。研究表明, miR-27a-3p通过负调控泛素特异性蛋白酶25(ubiquitin-specific proteases-25, USP25)抑制滋养细胞迁移侵袭进而参与RSA的发病机制[11]。癌蛋白Stathmin-1表达受损可能导致滋养细胞侵袭异常并引起RSA的发生[4]。M1型巨噬细胞中分离出的细胞外囊泡能够通过靶向肿瘤坏死因子受体相关因子6(tumor necrosis factor receptor-associated factor 6, TRAF6)抑制滋养细胞迁移侵袭, 进而引起RSA的发生[12]。

在本研究中, 我们发现*HADHA*过表达会抑制滋养细胞迁移与侵袭。LIU等[8]研究发现HADHA过表达显着抑制细胞生长, 诱导细胞凋亡, 并减少细胞质脂滴(lipid droplets, LD)的形成; 此外, 它还抑制了异种移植小鼠的肿瘤生长并减少了LD的形成。这些数据表明, HADHA过表达可能通过某种途径抑制细胞的侵袭。

PI3K/AKT是一条经典的信号通路, 在调节细胞迁移侵袭、自噬、凋亡等过程中发挥了重要作用[13-15]。已有文献表明, 受体酪氨酸激酶样孤儿受体1(receptor tyrosine kinase-like orphan receptor1, ROR1)等基因可通过激活PI3K/AKT信号通路促进人滋养细胞迁移侵袭[16-18]。在本研究中, 我们发现, *HADHA*敲低会促进HTR-8/SVneo细胞迁移侵袭, 且PI3K/AKT信号通路被激活, 证实HADHA可能是PI3K/AKT信号通路的负调控因子。

此外, 转录组数据分析结果还显示, HADHA可能参与调控MAPK、核因子κB(nuclear factor kappa-B, NF-κB)以

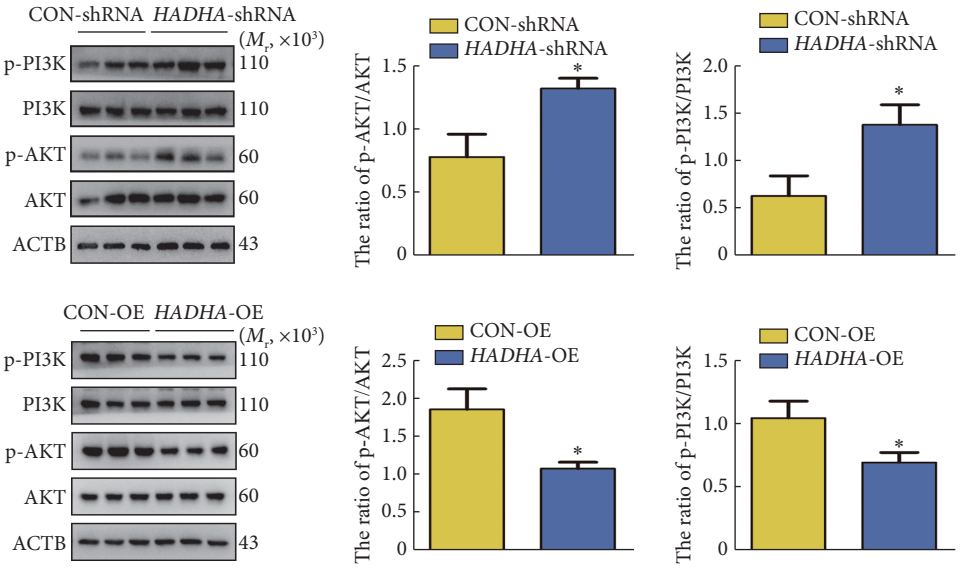
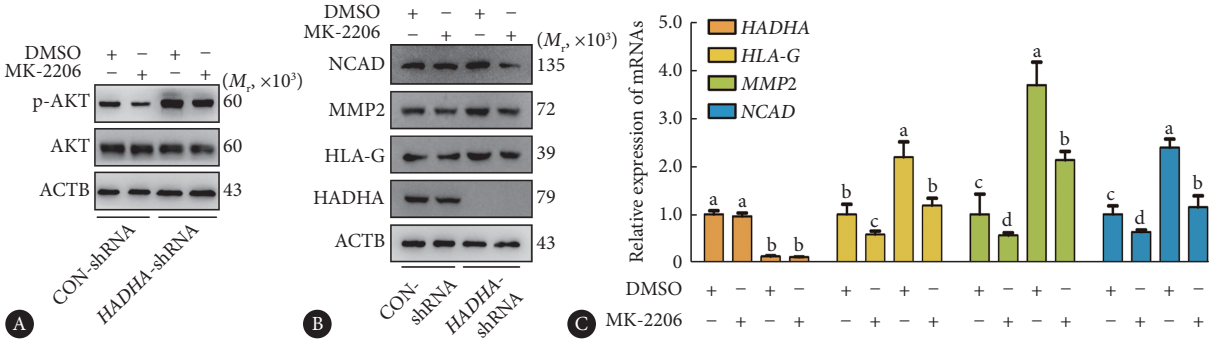


图 7 HADHA负调控PI3K/AKT信号通路（ *n*=3 ）

Fig 7 HADHA negatively regulates the PI3K/AKT signal pathway (*n*=3)

* *P*<0.05, vs. CON-shRNA. ACTB: β-actin; p-PI3K: Phosphorylation-phosphatidylinositol-3-kinase; p-AKT: Phosphorylation-protein kinase B.



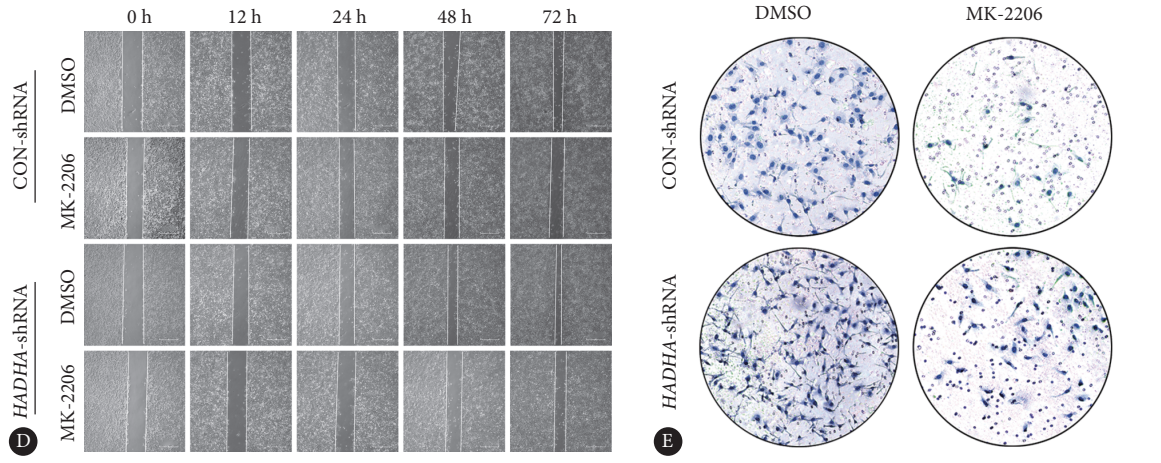


图 8 HADHA通过PI3K/AKT信号通路调控HTR-8/SVneo细胞迁移侵袭

Fig 8 HADHA regulates the migration and invasion of HTR-8/SVneo cells through PI3K/AKT signaling pathway

A, B: Western blot was used to detect the expression of AKT, p-AKT, HLA-G, NCAD, and MMP2 in both control and HADHA knockdown cells; C: qRT-PCR to meausre the mRNA level of *HADHA*, *HLA-G*, *MMP2*, and *NCAD* (*P*<0.05, between a,b,c,d groups [*n*=3]); D: Wound-healing assay to measure the migration of control and *HADHA* knockdown cells (Scale bars=400 μm); E: Transwell for measuring the invasion of control and *HADHA* knockdown cells. ACTB: β-actin; p-AKT: Phosphorylation-protein kinase B; HLA-G: Human leukocyte antigen-G; MMP2: Matrix metalloproteinase-2; MMP9: Matrix metalloproteinase-9; NCAD: N-cadherin; DMSO: Dimethylsulfoxide.

及过氧化物酶体增殖物激活受体(peroxisome proliferators-activated receptors, PPARs)等信号通路。研究表明,MAPK、NF-κB信号通路的激活均可促进滋养层细胞的迁移和侵袭[19-20]。值得注意的是,MAPK、NF-κB等信号通路的激活也被报道能够参与胎盘滋养细胞融合过程[21-22]。因此,HADHA是否能够通过影响迁移侵袭以外的其他途径参与RSA的发生值得深入探究。

综上所述,本研究发现HADHA在HTR-8/SVneo细胞中的低表达能够通过激活PI3K/AKT信号通路促进细胞迁移和侵袭。这一现象提示HADHA可能作为RSA等妊娠不良结局的诊断或治疗的靶标。

* * *

利益冲突 所有作者均声明不存在利益冲突

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