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MiRNA–218 对 PAG1 的调控及其对卵巢癌干细胞的影响

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[摘　要] **目的：**研究miR-218在卵巢癌干细胞(ovarian cancer stem cells，OCSCs)中的表达，并探讨其对PAG1的调控作用和对OCSCs的影响及作用机制。**方法：**收集卵巢癌患者新鲜肿瘤组织，分离培养原代卵巢癌细胞，用流式细胞仪筛选出CD133$^+$表型的OCSCs。采用Western印迹法检测干性特异相关基因Nanog和Sox2蛋白的表达。qRT-PCR检测miR-218在OCSCs中的表达。miR-218 mimic转染OCSCs，分为miR-218 mimic组和miR-218 NC组，MTS检测各组OCSCs增殖；软琼脂克隆实验检测各组OCSCs肿瘤球形成能力的影响；miR-218过表达慢病毒质粒及对照质粒vector感染OCSCs，经1.0 μg/mL嘌呤霉素筛选稳定过表达miR-218或vector的OCSCs细胞，注射到BALB/c裸鼠中，观察miR-218对OCSCs体内成瘤能力的影响。Targetscan 7.2预测软件及双荧光素酶报道基因试验，分析miR-218对PAG1基因的靶向作用，qRT-PCR检测miR-218 mimic组和miR-218 NC组OCSCs中PAG1 mRNA的表达。Western印迹法检测miR-218 mimic组和miR-218 NC组OCSCs中pSrc和pAKT蛋白的表达。**结果：**Nanog和Sox2蛋白在CD133$^+$表型OCSCs中的表达显著高于CD133$^-$表型OCSCs中的表达；miR-218在CD133$^+$表型OCSCs中的表达显著低于CD133$^-$表型OCSCs中的表达(P<0.05)。miR-218 mimic组OCSCs增殖减慢，OCSCs肿瘤球形成能力下降。$OCSCs_{miR\text{-}218}$裸鼠成瘤体积显著低于$OCSC_{svector}$组(P<0.05)。TargetScan7.2软件预测和双荧光素酶报道基因试验证实PAG1为miR-218的靶基因，miR-218 mimic组PAG1 mRNA的表达减少。Western印迹法检测miR-218 mimic组OCSCs中pSrc和pAKT蛋白的表达显著减少。**结论：**MiR-218在OCSCs中低表达，可能通过靶向下调PAG1，抑制pSrc和pAKT蛋白的表达影响OCSCs的干性。

[关键词] 卵巢癌；miRNA-218；PAG1；干性

Regulation of miRNA-218 on PAG1 and its effect on ovarian cancer stem cells

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Abstract **Objective:** To investigate the expression of miR-218 in ovarian cancer stem cells (OCSCs), and to explore its regulation on PAG1 and its effect on OCSCs. **Methods:** Fresh tumor tissues of ovarian cancer patients were collected, and primary ovarian cancer cells were isolated and cultured. The OCSCs of CD133$^+$ phenotype were

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selected by flow cytometry. Western blot was used to detect the expression of stem specific genes Nanog and Sox2. qRT-PCR was used to detect the expression of miR-218 in OCSCs. miR-218 mimic was transfected into OCSCs and divided into miR-218 mimic group and miR-218 NC group. MTS was used to detect the proliferation of OCSCs in each group. Soft agar clone assay was used to detect the effect of tumor formation ability of each group; OCSCs were infected with viral plasmid and control plasmid vector, and OCSCs cells stably overexpressing miR-218 or vector were screened by 1.0 μg/mL puromycin and injected into BALB/c nude mice to observe the tumorigenic ability of miR-218 in OCSCs. Targetscan 7.2 predictive software and dual luciferase reporter gene assay were used to analyze the targeting effect of miR-218 on PAG1 gene. qRT-PCR was used to detect the expression of PAG1 mRNA in miR-218 mimic group and miR-218 NC group. Western blot was used to detect the expression of pSrc and pAKT proteins in miR-218 mimic group and miR-218 NC group. **Results:** The expression of Nanog and Sox2 protein in $CD133^+$ phenotype OCSCs was significantly higher than that in $CD133^-$ phenotype OCSCs; the expression of miR-218 mRNA in $CD133^+$ phenotype OCSCs was significantly lower than that in $CD133^-$ phenotype OCSCs ($P<0.05$); the proliferation of OCSCs in the miR-218 mimic group was slowed down, and the tumor formation ability of OCSCs was decreased. The tumor formation volume of $OCSCs_{miR\text{-}218}$ nude mice was significantly lower than that of $OCSCs_{vector}$ group ($P<0.05$). TargetScan 7.2 software prediction and dual luciferase reporter gene assay confirmed that PAG1 is a target gene of miR-218, and the expression of PAG1 mRNA is decreased in miR-218 mimic group. The expression of pSrc and pAKT protein in OCSCs of miR-218 mimic group was significantly reduced by Western blot. **Conclusion:** MiR-218 is down-regulated in OCSCs, which may affect the dryness of OCSCs by down-regulating PAG1 and inhibiting the expression of pSrc and pAKT proteins.

Keywords ovarian cancer; miR-218; PAG1; stem

卵巢癌是一种恶性程度较高的肿瘤，为女性生殖系统中第三大常见肿瘤，预后较差，据文献[1]报道卵巢癌病死率居女性恶性肿瘤之首。有研究[2]指出虽然近年来医疗诊断及治疗水平不断提升，但是卵巢癌的发病率仍居高不下。肿瘤干细胞是肿瘤发生的源头，促进肿瘤的发生发展[3]。越来越多的研究[4-5]已经证实卵巢癌中同样存在肿瘤干细胞，卵巢癌干细胞(ovarian cancer stem cells，OCSCs)不仅是卵巢癌发生的起始细胞，同时与卵巢癌的复发、转移及耐药均密切相关，是导致患者不良预后的关键因素。研究[6-7]表明微小RNA(miRNA)在肿瘤细胞各种生命活动中发挥至关作用，而在卵巢癌中发现miRNA可以调控OCSCs干性特征，促进肿瘤细胞的恶性进展。研究[8-9]报道miRNA-218在卵巢癌组织和细胞系中的表达降低，并抑制卵巢癌细胞的增殖和侵袭及促进卵巢癌细胞的凋亡。而miR-218调控OCSCs的干性及作用机制未见有报道，因此本文以分离培养的OCSCs为研究对象，研究miR-218调控的下游靶基因及对OCSCs干性的影响，阐明miR-218调控OCSCs促进肿瘤发生的潜在机制，为发掘卵巢癌新的治疗靶点，改善卵巢癌患者的预后提供新的实验依据。

1 材料与方法

1.1 主要试剂

IV型胶原酶购自美国Sigma公司；碱性成纤维细胞生长因子和表皮生长因子均购自美国Gibco公司；TRIzol试剂、反转录试剂盒及qRT-PCR试剂盒等均购自日本TaKaRa公司；MTS增殖检测试剂盒购自南京凯基生物技术有限公司；miR-218 mimic、突变型和野生型PAG1的3'-非翻译区(3'-UTR)荧光素酶报告载体(PAG1-wt和PAG1-mut)及miR-218过表达质粒均购自上海吉凯基因公司；RIPA高效裂解液及PVDF膜均购自北京索莱宝科技有限公司；蛋白浓度检测BCA试剂盒购自美国Thermo公司；4周龄雌性BALB/c裸鼠购自北京维通利华实验动物技术有限公司；兔抗人Sox2抗体(11064-1-AP)、兔抗人pSrc抗体(27130-1-AP)购自美国Proteintech公司；兔抗人Nanog抗体(ab21624)、鼠抗人pAKT抗体(ab81283)、鼠抗人GAPDH抗体(ab9484)购自美国Abcam公司。

1.2 临床样本

取经2位病理科医师诊断为卵巢癌的新鲜组织

标本1例，该患者为十堰市太和医院首次确诊，手术前未经放化疗等任何形式的治疗，未合并影响生命健康的其他重大疾病。手术切除后的肿瘤组织在无菌条件下立即放入组织保存液中，进行下一步的分离培养或冻于−80 ℃冰箱中备用。根据赫尔辛基宣言和十堰市太和医院伦理指南进行手术标本的处理，并签署患者知情同意书。

1.3 肿瘤细胞分离和 OCSCs 的培养

将新鲜肿瘤组织于无菌环境中用眼科剪去除正常组织和脂肪后，用含有抗真菌药物和抗生素的缓冲液冲洗干净。用眼科剪将组织剪成米粒大小的组织，放入含有1 mg/mL IV型胶原酶的无血清DMEM-F12培养基中进行消化，37 ℃摇床上摇1 h。用含10%胎牛血清的DMEM-F12完全培养基终止消化后，用无菌微米孔径尼龙细胞过滤器过滤组织碎块，2 000 r/min离心5 min。完全培养基洗IV型胶原酶1次后，将细胞重悬在含有表皮生长因子和碱性成纤维细胞生长因子的无血清培养基中培养。约2周时细胞球开始形成，用流式细胞仪分选出$CD133^+$的OCSCs，在血清培养基中悬浮扩大培养。

1.4 qRT-PCR

将待测细胞PBS洗涤两次后，PBS完全吸干后加入1 mL的TRIzol裂解液，冰上裂解10 min，细胞吹打充分裂解后转移至EP管中，加入200 μL氯仿，震荡混合数次静置5 min，4 ℃，以12 000 r/min离心30 min，吸取上层水相层溶液至新的EP管中，并与等体积的异丙醇混匀，以12 000 r/min，4 ℃离心15 min，用75%的乙醇洗涤沉淀两次，晾干后加入适量的DEPC水溶解RNA。将总RNA反转录为cDNA。以反转的cDNA作为模板按照说明书进行qRT-PCR。分析各样品的循环阈值(threshold cvcle，Ct)，采用$2^{-\Delta\Delta Ct}$法分析各组实验数据。

1.5 细胞转染及裸鼠移植瘤模型的构建

按说明书将脂质体2000与miR-218 mimics、对照各组转染试剂转染至OCSCs中，分为miR-218 mimics组和NC组，放至培养箱中培养。miR-218过表达慢病毒质粒及其对照质粒vector，均以复感染指数MOI(病毒/细胞数量)30感染OCSCs，96 h后更换含1.0 μg/mL嘌呤霉素的新鲜培养基培养，并隔天换液，筛选至所有细胞均可观察到荧光。将构建的成功的稳转细胞株$OCSCs_{miR\text{-}218}$和$OCSCs_{vector}$分别注射到裸鼠右侧腋下，注射量为每只6×10^6个/100 μL，每组6只裸鼠。游标卡尺每3 d测量1次肿瘤的体积，4周后处死裸鼠，该实验的所有操作均符合动物伦理学。

1.6 MTS 实验

取转染48 h的各组转染细胞，以每孔1.5×10^3个细胞、150 μL培养基接种于96孔培养板，置培养箱中培养，每组设置6个复孔，在铺板后第0，24，48和72 h时，每孔加入30 μL MTS试剂，37 ℃细胞培养箱中孵育2 h。用酶标仪测取490 nm处的OD值，以时间h为横坐标，OD值为纵坐标，绘制细胞存活曲线。重复3次实验。

1.7 软琼脂克隆形成实验检测克隆形成能力

1 mL 20%无血清培养基与1 mL 1.2%无菌的软琼脂1:1充分混匀后，均匀铺至6 cm培养皿中，冷却至固态作为底层胶。同时0.5 mL 20%无血清培养基与0.5 mL 0.6%高压灭菌的软琼脂1:1充分混匀后作为上层胶，37 ℃预热后与1×10^5个/100 μL各组细胞混匀后，铺至固态的底层胶上，放至培养箱中培养，每3 d往皿中加入1 mL培养基，每天在显微镜下观察并拍照。

1.8 荧光素酶报告基因实验

实验分4组：PAG1-wt与miR-218 mimics共转染组(PAG1-wt+miR-218)；PAG1-wt与NC共转染组(PAG1-wt+NC)；PAG1-mut与miR-218 mimics共转染组(PAG1-mut+miR-218)；PAG1-mut与NC共转染组(PAG1-mut+NC)，上述4组分别共转染到OCSCs中，收集转染48 h的细胞，按照双荧光素酶报道基因检测试剂盒的说明书检测荧光素酶活性。

1.9 Western 印迹法检测蛋白表达

各组细胞PBS洗3次，加入100 μL含蛋白酶抑制剂的RIPA冰上裂解10 min，刮刀刮至EP管中，超声裂解30 min后4 ℃，12 000 r/min离心30 min。将上清移至新的EP管中，检测蛋白浓度，加入Loading buffer煮沸变性后，样品进行SDS-PAGE电泳，蛋白湿转至PVDF膜上，8%牛奶室温封闭1h，TBST洗2次，加入稀释好的一抗室温孵育2 h或4 ℃过夜，TBST洗3次后，二抗室温孵育2 h，ELC化学发光法曝光条带。

1.10 统计学处理

采用SPSS 19.0软件进行统计分析，数据以均数±标准差($\bar{x}\pm s$)表示，采用配对t检验比较两组间

差异。P<0.05为差异有统计学意义。

2 结果

2.1 OCSCs 的分选

将新鲜卵巢癌组织原代分离培养的肿瘤球利用流式细胞仪进行分选，结果筛选出$CD133^+$的细胞约占全部细胞的1.18%。并将$CD133^+$的细胞扩大培养后提取蛋白，Western印迹结果表明：肿瘤干细胞特异性相关基因Nanog，Sox2蛋白在$CD133^+$细胞中的表达显著高于$CD133^-$细胞(图1)，同时qRT-PCR结果显示miR-218在$CD133^+$细胞中的表达为0.34±0.08，显著低于在$CD133^-$细胞中的表达(1.00±0.10，P<0.05)。

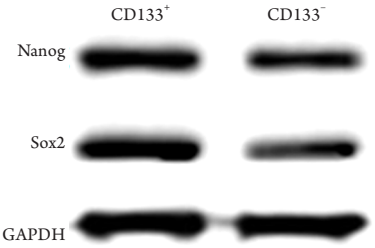

图1 Western印迹法检测肿瘤干细胞相关基因Nanog，Sox2蛋白在$CD133^+$细胞中的表达显著高于$CD133^-$细胞

Figure 1 Western-blot detection of tumor stem cell related-genes Nanog, Sox2 protein expression in $CD133^+$ cells was significantly higher than that in $CD133^-$ cells

2.2 MTS 检测 miR-218 对 OCSCs 增殖的影响

为研究miR-218的表达对OCSCs增殖能力的影响，分别转染miR-218 mimic和NC 48 h后，细胞计数铺板，采用MTS法测定miR-218 mimic和NC组OCSCs的增殖能力，结果显示：与NC组比较，miR-218 mimic组细胞增殖能力明显减慢(P<0.05，图2)。

2.3 软琼脂克隆实验检测 miR-218 对 OCSCs 克隆形成能力的影响

为研究miR-218的表达对OCSCs克隆形成能力的影响，分别转染miR-218 mimic和NC 48h后，细胞计数铺板，采用软琼脂克隆实验测定miR-218 mimic和NC组OCSCs的克隆形成能力，结果显示：miR-218 mimic和NC组肿瘤克隆球形成的数目分别为(5.89±1.27)个、(12.34±2.15)个。MiR-218 mimic肿瘤克隆球形成能力明显低于NC组(P<0.05，图3)。

2.4 MiR-218 对 OCSCs 体内成瘤能力的影响

$OCSCs_{miR\text{-}218}$组裸鼠体积显著低于$OCSCs_{vector}$组(图4A，B)，差异具有统计学意义(P<0.05)。

2.5 MiR-218 对 PAG1 的调控

TargetScan7.2软件在线预测显示PAG1启动子区有miR-218的结合位点，提示PAG1是miR-218直接调控的靶基因(图5A)。进一步双荧光素酶报道基因试验显示：与PAG1-wt+NC共转染组(1.00±0.05)比较，PAG1-wt+miR-218共转染组的荧光素酶活性显著降低(0.38±0.07，t=12.89，P<0.001)；而PAG1-mut+NC(1.00±0.10)和PAG1-mut+miR-218(0.87±0.06)组之间的荧光素酶活性差异无统计学意义(t=1.85，P>0.05；图5B)。qRT-PCR结果显示：与NC转染组比较，miR-218 mimic组OCSCs中PAG1 mRNA表达水平显著下降(P<0.05，图5C)。

2.6 Western 印迹法检测 pSrc 和 pAKT 蛋白表达

为研究miR-218的表达对OCSCs中pSrc和pAKT蛋白表达的影响，分别转染miR-218 mimic和NC 48 h后收集细胞提取蛋白，Western印迹法结果显示与NC组比较，miR-218 mimic组细胞中pSrc和pAKT蛋白表达水平均明显降低(图6)。

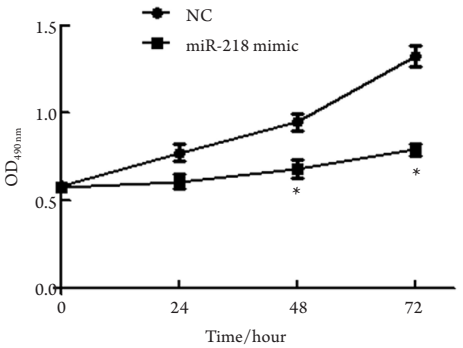

图2 MTS检测miR-218的表达对OCSCs增殖能力的影响，miR-218显著抑制OCSCs的增殖能力

Figure 2 MTS detects the effect of miR-218 expression on the proliferative capacity of OCSCs, and miR-218 significantly inhibits the proliferation of OCSCs

*P<0.05 vs NC组。

*P<0.05 vs NC.

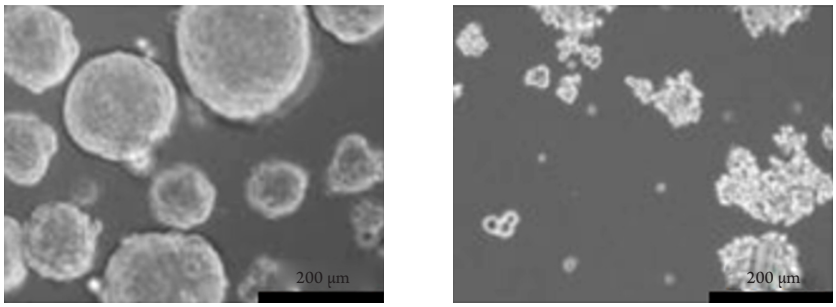

图3 软琼脂克隆形成实验检测miR-218的表达对OCSCs克隆形成能力的影响，miR-218显著抑制OCSCs的克隆球形成能力

Figure 3 Soft agar colony formation assay detects the effect of miR-218 expression on the clone formation ability of OCSCs, miR-218 significantly inhibited the clone formation ability of OCSCs

图4 MiR-218的表达对OCSCs体内成瘤能力的影响

Figure 4 Effect of miR-218 expression on tumorigenic ability of OCSCs in vivo

(A)裸鼠解剖瘤体大小；(B)游标卡尺测量裸鼠成瘤体积，OCSCs$_{miR-218}$细胞株的裸鼠成瘤体积显著低于OCSCs$_{vector}$。*P<0.05 vs vector。

(A) Anatomical tumor size of nude mice; (B) Vernier calipers measured the tumor formation volume of nude mice, and the tumor formation volume of nude mice of OCSCs$_{miR-218}$ cell line was significantly lower than that of OCSCs$_{vector}$. *P<0.05 vs vector.

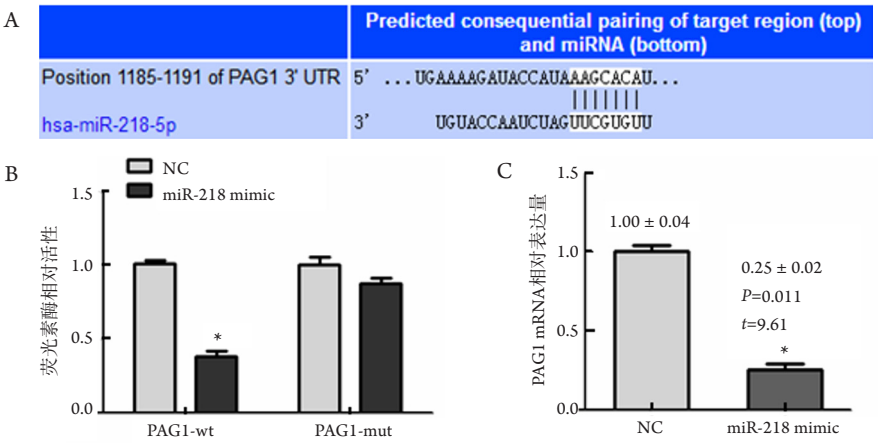

图5 MiR-218对PAG1的调控

Figure 5 Regulation of PAG1 by miR-218

(A)TargetScan7.2软件预测PAG1启动子区有miR-218的结合位点；(B)PAG1-wt+miR-218共转染组的荧光素酶活性显著降低，PAG1-mut+miR-218共转染组的荧光素酶活性无显著差异；(C)miR-218 mimic转染组的PAG1 mRNA表达水平显著降低。与NC组比较，*P<0.05。

(A) TargetScan7.2 software predicted a binding site for miR-218 in the PAG1 promoter region; (B) Luciferase activity was significantly reduced in the PAG1-wt+miR-218 co-transfection group, there was no significant decrease in luciferase activity in PAG1-mut+miR-218 co-transfection group; (C) Expression level of PAG1 mRNA was significantly decreased in miR-218 mimic transfection group. Compared with the NC group, *P<0.05.

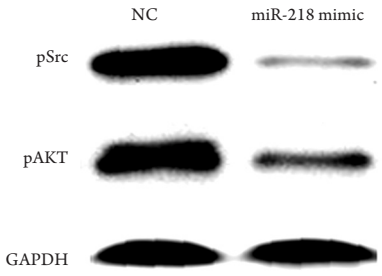

图6 MiR-218表达显著抑制OCSCs中pSrc和pAKT蛋白的表达
Figure 6 MiR-218 significantly inhibited the expression of pSrc and pAKT in OCSCs

3 讨论

据文献[10]报道：卵巢癌作为女性常见恶性肿瘤之一，因其具有早期症状不明显、较早出现转移、耐药复发等特性，使卵巢癌治疗效果欠佳，患者病死率较高。而研究[5]认为肿瘤干细胞与肿瘤的发生及导致肿瘤患者预后不良的复发转移的机制相关，因此研究肿瘤干细胞的调控机制有望成为改善卵巢癌患者预后的关键。miRNA为一类不具备编码蛋白质功能的RNA，但可以充当癌基因和抑癌基因的角色，调控肿瘤的发生发展。miRNA可以调控肿瘤干细胞。Tung等[11]研究数据表明：miR-34c-5p下调AREG的表达阻断AREG-EGFR-ERK信号途径，抑制OCSCs干性和耐药性。Chen等[12]报道：STAT3-miR-92a-DKK1信号途径在卵巢肿瘤中促进肿瘤干细胞样细胞的产生，阻断其信号转导具有治疗卵巢癌的潜力。另外还有研究[13-14]认为miR-218属于SLIT基因家族，在肺癌、胃癌等肿瘤中表达下调，被报道具有抑癌基因的作用。Li等[15]指出miRNA-218在卵巢癌组织和细胞系中均为低表达，可通过靶向RUNX2抑制卵巢癌细胞的增殖和侵袭，以及通过抑制Wnt/β-catenin信号通路促进卵巢癌细胞的凋亡。Guan等[16]发现miR-218在前列腺癌中可以抑制前列腺癌干细胞特性。Wu等[17]研究发现人脑胶质瘤中miR-218-5p抑制胶质瘤干细胞$A2B5^+CD133^-$亚群的干细胞特性和侵袭能力，而miR-218是否调控OCSCs未见有报道。

有研究[18-19]指出：肿瘤干细胞是存在于肿瘤细胞中类似干细胞具有较强的自我分化和更新能力的一类细胞群，已经在多种肿瘤组织中分离出了肿瘤干细胞，其中在卵巢癌中$CD133^+$的亚群肿瘤细胞已经被鉴定为OCSCs。本研究从卵巢癌新鲜肿瘤组织中分离培养出占总细胞1.18%的$CD133^+$细胞，符合肿瘤干细胞为肿瘤细胞较小群体的特性，并采用Western印迹法验证肿瘤干细胞特异性基因Nanog和Sox2的蛋白在$CD133^+$细胞中的表达显著高于$CD133^-$，表明筛选出的$CD133^+$细胞为OCSCs，将其扩大培养作为后续研究的工具细胞。有研究[8]认为miR-218在卵巢癌组织和细胞中表达均低，而在OCSCs中的表达未知。我们采用qRT-PCR检测发现miR-218同样低表达于OCSCs中，进一步采用MTS、软琼脂克隆形成实验分别检测转染miR-218 mimic对OCSCs增殖和克隆形成能力的影响，结果表明miR-218显著抑制OCSCs的增殖和克隆形成能力，裸鼠移植瘤实验显示稳定过表达miR-218的OCSCs成瘤体积较小，即体内成瘤能力降低，体内和体外实验均表明miR-218具有抑制OCSCs干性的生物学功能。但其作用机制仍需进一步探讨。

据文献[20]报道：miRNA通常在基因转录后水平调控蛋白的表达，即抑制RNA的翻译或增加miRNA的不稳定性负调控其靶基因，但每个miRNA均可以与数百个靶基因mRNA的3'非翻译区结合，包括保守的和非保守的靶基因，促使miRNA在各种生命活动中发挥重要作用。因此miR-218可能同样通过负调控其靶基因抑制OCSCs的干性。TargetScan7.2在线软件预测富含鞘糖脂微结构域1相关磷酸化蛋白(phosphoprotein associated with glycosphingolipid-enriched microdomains 1，PAG1)基因3'非翻译区有miR-218的结合位点，同时双荧光素酶报道基因试验证实PAG1为miR-218直接调控的靶基因，miR-218 mimic转染的OCSCs中PAG1 mRNA的表达下降，miR-218负调控其表达。研究[21]报道PAG1表达下降可促进放射性抗拒喉癌细胞的凋亡，使细胞周期阻滞在G_2/M期，逆转喉癌细胞对放疗的抗性。而放疗抗性与肿瘤干细胞是密切相关的，同时PAG1又称Csk(C末端Src激酶)结合蛋白，是一种普遍存在于细胞膜上的脂筏相关支架蛋白。有研究[22]指出PAG1作为重要的Src家族激酶(Src family tyrosine kinase，SFK)调节剂，在肿瘤中发挥的作用取决于其表达水平。另有研究[23]发现在乳腺癌中PAG1高表达激活Src促进肿瘤细胞的增殖、侵袭和转移，并通过Src和AKT信号通路可以促使脂肪来源的间充质干细胞相关乳腺癌细胞获得化学药物耐药性。同时Tomar等[24]研究表明Csk在卵巢癌中处于低甲基化和高表达状态，与患者无进展存活和总体存活显著相关。因此采用Western印迹法检测miR-218 mimic转染的OCSCs中pSrc和pAKT蛋白的表达，发现过表达miR-218

抑制pSrc和pAKT蛋白的表达。表明miR-218通过负靶向调控PAG1基因阻断Src和AKT信号通路抑制OCSCs的干性。

综上，miR-218在OCSCs中的表达下调，过表达miR-218显著抑制OCSCs增殖、软琼脂克隆形成及体内成瘤能力。该过程可能是miR-218通过负靶向调控PAG1，抑制Src和AKT信号通路实现对OCSCs干性的抑制作用。本研究揭示了miR-218调控OCSCs的干性功能及其作用机制，为治疗卵巢癌和改善患者的预后提供了新的研究思路。

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