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*Nanog*多潜能转录因子在肺腺癌干细胞中的转录功能分析

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摘要 综合shRNA介导的基因沉默和全基因组表达芯片杂交技术, 筛查了肺腺癌肿瘤干细胞(cancer stem cells, CSC)中靶向灭活*Nanog*多潜能基因后的差异表达基因。发现*Nanog*沉默引起了1 605个基因差异表达, 其中95个基因属于受*Nanog*转录调控的靶基因。对差异表达基因进行KEGG分析后发现, *Nanog*的一个重要功能是转录抑制蛋白酶体编码基因表达。此外, 研究还发现在此类细胞的特征性基因表达谱中, 25个基因的表达受*Nanog*调控, 包括15个基因在*Nanog*沉默后表达上调和10个基因表达下调。上述研究结果揭示, *Nanog*多潜能转录因子在肺腺癌的CSC中具有转录调控功能。

关键词 肺腺癌; 肿瘤干细胞; 多潜能基因; Nanog; 表达沉默

An Analysis on the Transcriptional Activity of *Nanog* Pluripotent Transcription Factor in Lung Adenocarcinoma Stem Cells

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Abstract An integrated analysis of shRNA-mediated gene knockdown and cDNA-based microarray was performed to screen out the genes expressed differentially after inactivation of *Nanog* pluripotent gene in lung adenocarcinoma stem cells (CSC). The results showed that *Nanog* inactivation elicited 1 605 genes that were expressed differentially, among which 95 genes belong to the known *Nanog* downstream target genes. With KEGG analysis, a critical function of *Nanog* was found to repress the expression of genes encoding for the components of proteasome. Moreover, in the unique gene signature of these CSCs, 25 genes were shown to be regulated by *Nanog*. Its inactivation elicited the up-regulation of 15 genes and the down-regulation of 10 genes. These data indicated that *Nanog* pluripotent factor is functional in the gene transcription in lung adenocarcinoma stem cells.

Key words lung adenocarcinoma; cancer stem cell; pluripotent gene; Nanog; knockdown

近年来研究揭示, 恶性肿瘤中存在干细胞的层级(hierarchy)结构, 其中的恶性干细胞称为肿瘤干细胞(cancer stem cells, CSC), 具有显著的致瘤、转移和耐药特性, 是影响疾病转归和患者生存的一类主要细胞[1-3]。肺腺癌是肺癌最常见的组织类型之一, 此类肿瘤的CSC虽然分属多个表型不同的细胞亚群,

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但共性特征是高表达干细胞多潜能(pluripotent)基因尤其是*OCT4*和*Nanog*[4]。台湾研究者Chious等[5]曾报道, 异位(ectopic)表达*OCT4*和*Nanog*可以促使肺腺癌细胞逆向分化成为高致瘤性的CSC, 回顾性临床研究则证明, 在原发肿瘤中存在*OCT4*和*Nanog*阳性细胞的患者预后差。

前文作者[6]报道, 在肺腺癌CSC中*OCT4*具有转录功能, 靶向灭活*OCT4*后可以引起2 138个基因差异表达。但目前还不清楚*Nanog*在此类细胞中的转录功能。基于此, 本文采用shRNA介导的*Nanog*基因沉默技术, 筛查了靶向灭活该基因后差异表达的基因群。

1 材料与方法

1.1 材料

胎牛血清、DMEM及DMEM/F12培养基购自HyClone公司。免疫荧光检测抗体: 兔抗人OCT4多克隆抗体(sc-9081)、兔抗人HIF1α(sc-10790)、羊抗人Nanog多克隆抗体(sc-30331)、羊抗人Slug多克隆抗体(sc-10437)、荧光素Rhodamine标记的驴抗兔及抗羊IgG抗体均购自Santa Cruz公司。生长因子: 胰岛素样生长因子-1(insulin-like growth factor-1, IGF-1)和表皮生长因子(epidermal growth factor, EGF)购自Serotec公司。纤维母细胞生长因子-10(fibroblast growth factor-10, FGF-10)购自PeproTech公司。*Nanog*特异性shRNA慢病毒(sc-43958-v)购自Santa Cruz公司。Trizol试剂及Platinum™ Taq酶试剂购自Invitrogen公司。RvertAid™首链cDNA合成试剂购自Fermantas公司。

1.2 方法

1.2.1 细胞培养 肺腺癌SPC-A1细胞购自中国科学院上海生命科学研究院生化细胞所细胞库。细胞培养条件为10%胎牛血清、100 U/mL青霉素和100 μg/mL链霉素的DMEM培养基、37 °C、5% CO_2培养箱中培养。实验选择对数增殖期细胞, 经0.25%胰酶消化后收集。

1.2.2 无血清球体悬浮培养 实验方法见前文[7-8]。简述之, 将1×10^{5}细胞(5 mL)置于超低吸附培养皿(Corning公司)中, 分别加入IGF-1、EGF和FGF-10(各20 ng/mL), 间隔2 d添加生长因子一次直至球体形成(培养5~7 d)。收集悬浮生长细胞后100 ×*g*离心2 min取沉淀中的球体(Spheres)。

1.2.3 基因表达沉默 *Nanog*表达沉默按生产商提供的配套试剂及实验指南操作。简述之: 6孔板(Costa公司)内每孔种植3.0×10^5 *Nanog*$^+$细胞(1 mL培养基), 培养24 h后每孔加入2 mL聚凝胺(Polybrene, 终浓度为10 μg/mL), 作用30 min后加入30 μL慢病毒。感染后次日更换培养基, 第2日开始用10 μg/mL嘌呤霉素(Puromycin)筛选, 2周后获得抗性细胞克隆。

1.2.4 免疫荧光检测 将细胞(1×10^{5}细胞, 500 μL培养基)接种于置有无菌盖玻片的24孔板(Costa公司)中, 培养过夜后经4%多聚甲醛固定30 min, PBS洗3次(每次洗3 min, 下同), 0.25% Triton X-100通透15 min, 1% BSA封闭非特异性结合3 h, PBS洗3次后加入一抗(1:100稀释), 设阴性对照(用PBS代替一抗), 4 °C过夜。PBS洗3次去除非结合抗体后加二抗(1:100稀释), 室温避光1 h, PBS洗3次, 滴加Hoechest 33342(1:50稀释)染核5 min, PBS洗2次, 加封片剂于Olympus IX51型荧光显微镜下观察。

1.2.5 RNA提取及RT-PCR检测 按生产商操作指南, 采用Trizol试剂提取细胞内总RNA后, 取10 μg RNA反转录成cDNA。PCR反应液包括5 μL 10×PCR buffer, 1.5 μL 25 mmol/L $MgCl_2$, 4 μL 2.5 mmol/L dNTP, 1.25 U Taq酶, 1 μL 20 μmol/L上游及下游引物和5 μL cDNA, 用DEPC-H_2O补充至50 μL体积。PCR反应条件为95 °C预变性, 5 min; 94 °C变性45 s, 57 °C退火45 s, 72 °C延伸45 s, 共40个循环; 72 °C延伸7 min。扩增产物在2%琼脂糖凝胶中电泳20 min。引物序列采用Primer express 2.0软件设计, *Nanog*基因的上游和下游引物序列分别为5′-ggc cga aga ata gca atg gt-3′及5′-aat ttg gct gga act gca tg-3′, 扩增产物为300 bp。*OCT4*基因的上游和下游引物分别为5′-aag aga aag cga acc agt atc g-3′及5′-agt gaa gtg agg gct ccc a-3′, 扩增产物为300 bp。*OCT4*剪辑变异体*OCT4B*的上游和下游引物序列分别为5′-gta ggt tct tga atc ccg aat g-3′及5′-tgc ttt gca ata ctc ctg aag at-3′, 扩增产物为350 bp。*OCT4A*的上游和下游引物序列分别为5′-acc tgg cta agc ttc caa gg-3′及5′-cat cgg cct gtg tat atc cc-3′, 扩增产物为400 bp。内参基因*18s rRNA*的上游和下游引物序列分别为5′-gct ctt agc tga gtg tcc cg-3′及5′-cct ccg act ttc gtt ctt gat-3′, 扩增产物为280 bp。

1.2.6 基因表达芯片检测及生物信息学分析 以*Nanog*$^+$球体细胞为对照组, *Nanog*-KD细胞为实验组, 提取总RNA后根据生产商提供的配套试剂及实验指

南, 采用human-12T Illumina Beadchip进行全基因组表达筛查。差异表达基因按照差异分值(DiffScore)遴选, 差异分值体现一个基因在两个样本中的表达差异程度, 该数值≥13时显示二个样本间探针检测信号的差异具有统计学显著意义(P<0.05)。然后, 依据GenBank注译基因从差异表达基因中获得功能已知基因群, 并将结果上传至KEGG(Kyoto Encyclopedia of Genes and Genomes)数据库进行生物信息学分析。

2 结果

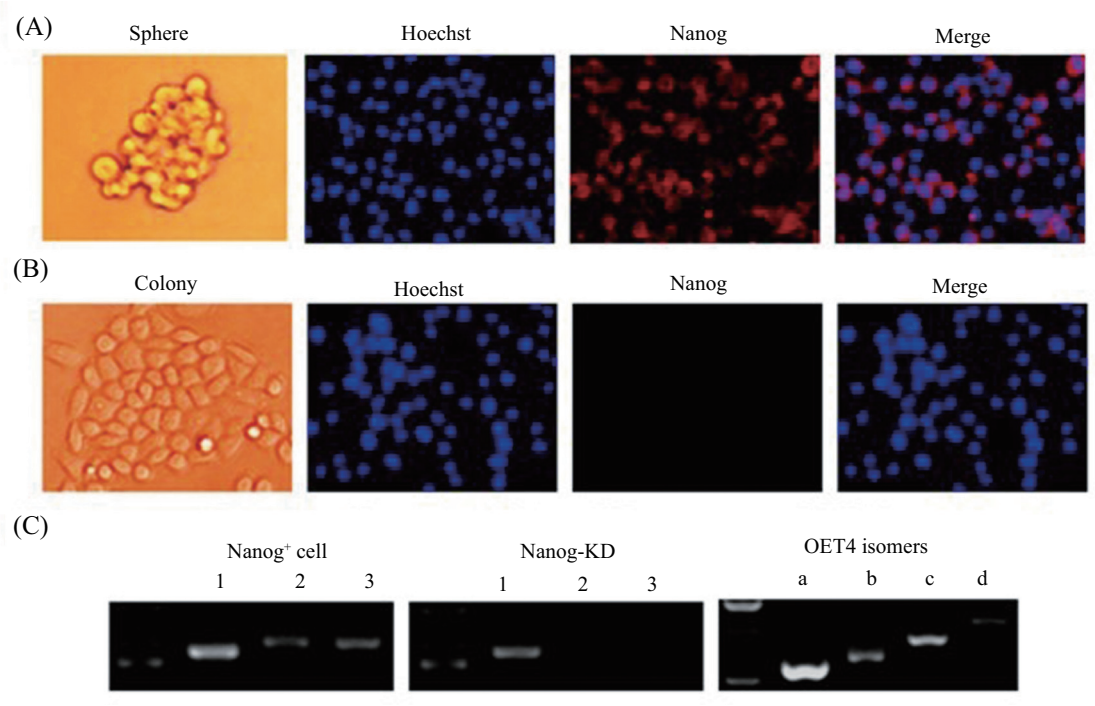
2.1 *Nanog*$^+$细胞的富集与基因表达沉默

作者曾报道[7-8], SPC-A1细胞在无血清培养条件下能够形成悬浮生长的肺球体(pulmosphere), 此类成球(sphere-forming)细胞表达*OCT4*和*Nanog*等干细胞特征性基因并具有明显的致瘤及转移能力。本文采用免疫荧光检测技术, 证实肺球体细胞中Nanog蛋白呈阳性表达(图1A)。

以上述*Nanog*$^+$细胞为实验对象, 通过转染*Nanog*特异性shRNA慢病毒并用嘌呤霉素筛选获得了抗性细胞克隆, 免疫荧光检测显示此类抗性细胞(称为Nanog-KD)中Nanog蛋白表达转阴(图1B)。RT-PCR检测证实, *Nanog*$^+$细胞阳性表达*Nanog*和*OCT4*特异性mRNA, 而上述转录本在Nanog-KD细胞中的表达显著下调(图1C)。此外, *OCT4*基因能够转录形成*OCT4A*和*OCT4B*剪辑变异体, 其中仅*OCT4A*具有多潜能转录因子功能[9], 为了明确*Nanog*$^+$细胞中的*OCT4*变异体, 我们应用OCT4编码基因*POU5f1*的外显子特异性引物进行了PCR扩增, 结果显示此类细胞表达*OCT4A*, 但*OCT4B*也能检测到(图1C)。

2.2 *Nanog*基因沉默细胞的全基因组表达筛查结果

分别提取Nanog$^+$细胞和Nanog-KD细胞的总RNA后进行基因芯片表达筛查, 结果显示与Nanog$^+$细胞比较, Nanog-KD细胞中存在1 605个差异表达基因, 其中1 284个基因表达上调, 321个基因表达下调。表1列举了差异表达最显著的前20个基因(top 20)。



A: 分离后的肺球体(sphere)细胞显示Nanog蛋白阳性表达; B: 肺球体细胞转染特异性shRNA慢病毒后, 筛选获得的抗性细胞集落(colony)不表达Nanog; C: RT-PCR检测*18s rRNA*(1)、*OCT4*(2)和*Nanog*(3) mRNA的表达。对*18s rRNA*(a)以及*OCT4*变异体*OCT4*(b)、*OCT4B*(c)和*OCT4A*(d)也进行了分析。

A: the isolated pulmosphere showed positive expression of Nanog protein; B: after transfection with specific ShRNA lentivirus into pulmospheres, the resistant colony was negative for Nanog; C: the expression of *18s rRNA* (1), *OCT4* (2) and *Nanog* (3) mRNA were determined by RT-PCR assay. The *18s rRNA* (a), and *OCT4* isomers *OCT4* (b), *OCT4B* (c), *OCT4A* (d) were also analyzed.

图1 肺腺癌干细胞及其基因沉默细胞中*Nanog*表达分析

Fig.1 *Nanog* expression in lung adenocarcinoma stem cells and their gene-knockdown cells

人胚胎干细胞中存在1 687个受Nanog转录调控的基因[10], 此类基因在实体肿瘤CSC中的表达状况目前还不了解。据此我们筛查了肺腺癌CSC中的*Nanog*靶基因。结果发现, 有95个此类靶基因在*Nanog*沉默后差异表达, 其中73个基因表达上调, 22个基因下调。表2列举了59个差异表达最显著的基因, 这些基因的差异分值均≥20(即检测信号值在二个样本间的差异具有统计学极显著意义, $P<0.01$)。

此外, 前文我们通过*OCT4*表达沉默技术筛查获得了一组OCT4$^+$细胞的特征性基因[6], 本文结果发现, 该组基因中有25个基因在*Nanog*沉默后的表达也发生显著改变, 其中包括*MUC*1和*CA9*(表3)。我们已经证明, 在肺腺癌细胞群中MUC1$^+$亚群不表达OCT4, 而CA9$^+$亚群属于OCT4$^+$细胞[6,11]。因此, 至少从表型分析的角度, Nanog在抑制肺腺癌CSC分化中起重要作用, 其表达沉默将使其失去自我更新能力(如下调OCT4表达, 图1和图2)继而促使细胞分化。

2.3 靶向灭活*Nanog*上调蛋白酶体基因表达

除了潜在的分化调控作用外, Nanog的另一项重要功能是转录抑制26S蛋白酶体(proteasome)编码基因PSMB2和PSMB5表达(表2)。对差异表达基因进行KEGG分析后发现, *Nanog*$^+$细胞内不仅*PSMB2*和*PSMB5*基因表达降低, 而且其他19个蛋白酶体(proteasome)编码基因也呈明显低表达(表4)。

在有氧培养条件下, 细胞内HIF1蛋白的半衰期仅为5~15 min, 通常检测阴性, 通过蛋白酶体降解是HIF1快速代谢的一个主要途径。前文我们报道, 肺腺癌细胞中OCT4表达受HIF1调控, 靶向灭活*HIF1α*基因引起OCT4表达转阴。由此推测, 此类细胞内HIF1α蛋白检测阳性与Nanog抑制蛋白酶体基因表

表1 *Nanog*沉默后差异表达最显著的前20个(top 20)基因

Table 1 The top 20 differentially expressed genes after *Nanog* knockdown

基因 Genes	基因全称 Gene name	Nanog$^+$ 细胞* Nanog$^+$ cell*	Nanog-KD* Nanog-KD*	差异分值 Diffscore
ANXA8L2	Annexin A8-like 2	90.810	1 309.193	335.375
CITED4	Cbp/p300-interacting transactivator 4	118.780	2 082.158	335.375
TNNC1	Troponin C type 1 (slow)	89.563	2 495.420	335.375
IGFBP1	Insulin-like growth factor binding protein 1	33.333	409.187	178.869
MUC1	Mucin 1, cell surface associated, transcript variant 5	113.302	1 324.072	166.161
SCARA3	Scavenger receptor class A, member 3, transcript variant 2	22.298	262.889	127.151
EDN1	Endothelin 1	6.517	663.251	122.795
JSRP1	Junctional sarcoplasmic reticulum protein 1	46.190	846.567	122.795
NNMT	Nicotinamide N-methyltransferase	26.174	556.791	122.795
PMS2L4	Postmeiotic segregation increased 2-like 4 pseudogene	403.694	2 159.106	122.795
SERPINE2	Serpin peptidase inhibitor, clade E (nexin), member 2	1 497.676	72.547	–208.394
QPRT	Quinolinate phosphoribosyltransferase	1 221.995	–2.831	–140.890
RAC2	Ras-related C3 botulinum toxin substrate 2	461.219	1.976	–133.809
PEPD	Peptidase D	6 229.578	1 138.234	–129.903
TUBB2B	Tubulin, beta 2B	748.702	150.004	–126.629
NDUFA4L2	NADH dehydrogenase 1 alpha subcomplex, 4-like 2	1 374.642	166.017	–124.603
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	1 277.152	186.957	–124.603
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	672.577	2.892	–122.928
SPOCK1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan	398.264	35.866	–122.027
CA9	Carbonic anhydrase IX	3 867.584	522.182	–117.225

*芯片检测信号值。

*Signal value detected by microarray.

表2 *Nanog*沉默后差异表达最显著的*Nanog*靶基因

Table 2 The top differentially expressed *Nanog* target genes after *Nanog* knockdown

基因 Genes	基因全称 Gene name	$Nanog^+$ 细胞* $Nanog^+$ cell*	Nanog-KD* Nanog-KD*	差异分值 Diffscore
CYR61	Cysteine-rich, angiogenic inducer, 61	200.889	1 456.931	107.940
CABLES1	Cdk5 and Abl enzyme substrate 1, transcript variant 1	26.528	447.512	102.954
RAB17	RAB17, member RAS oncogene family	30.671	387.252	90.677
CITED2	Cbp/p300-interacting transactivator	85.817	434.677	87.768
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	117.266	519.602	80.234
SGK	Serum/glucocorticoid regulated kinase	172.734	830.182	65.326
FZD2	Frizzled homolog 2 (*Drosophila*)	262.060	902.760	65.026
CDK6	Cyclin-dependent kinase 6	517.984	1 822.018	60.810
CTGF	Connective tissue growth factor	75.233	614.668	59.813
CLDN1	Claudin 1	120.992	429.858	52.003
ID2	Inhibitor of DNA binding 2, helix-loop-helix protein	12.558	211.684	51.058
IER5L	Immediate early response 5-like	48.785	234.810	47.320
EXOSC5	Exosome component 5	134.357	512.578	46.887
LHX4	LIM homeobox 4	–7.594	116.845	39.948
PSMB2	Proteasome subunit, beta type, 2	2 002.895	3 920.010	38.496
XK	X-linked Kx blood group (McLeod syndrome)	27.757	168.797	37.546
HSPC111	Hypothetical protein HSPC111	107.136	297.744	36.582
PSEN2	Presenilin 2 (Alzheimer disease 4), transcript variant 2	70.063	251.863	35.048
PDHB	Pyruvate dehydrogenase (lipoamide) beta	1 453.483	3 157.462	34.763
SAT2	Spermidine/spermine N1-acetyltransferase family member 2	68.038	228.822	34.460
WDR6	WD repeat domain 6	630.067	1 420.588	32.062
TTF2	Transcription termination factor, RNA polymerase II	281.476	593.497	31.189
NUDT5	Nudix (nucleoside diphosphate linked moiety X)-type motif 5	385.156	821.628	30.108
SP2	Sp2 transcription factor	65.662	206.119	28.831
CCL2	Chemokine (C-C motif) ligand 2	–17.614	78.344	27.572
PSMB5	Proteasome (prosome, macropain) subunit, beta type, 5	1 369.711	3 054.064	27.254
S100A11	S100 calcium binding protein A11	1 121.765	1 984.193	27.182
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer	20.743	143.853	26.827
JUP	Junction plakoglobin, transcript variant 2	582.952	1 347.664	26.641
C1QTNF6	C1q and tumor necrosis factor related protein 6	14.599	111.746	26.316
BLCAP	Bladder cancer associated protein	295.131 6	655.441	26.141
MRPL51	Mitochondrial ribosomal protein L51	1 383.077	2 787.112	26.047
C14orf112	Chromosome 14 open reading frame 112	531.5285	1 230.973	25.930
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	68.748	255.973	24.917
SUPT6H	Suppressor of Ty 6 homolog (*S.cerevisiae*)	49.736	181.339	24.656
PHF5A	PHD finger protein 5A	577.517	1 093.236	24.095
FBXO31	F-box protein 31	128.244	277.636	22.120
CLN3	Ceroid-lipofuscinosis, neuronal 3, transcript variant 1	96.190	250.019	21.755
CSTF3	Cleavage stimulation factor, 3′ pre-RNA, subunit 3, 77 kDa	193.151	472.384	21.732
RPS26	Ribosomal protein S26	1 951.687	2 282.887	21.611
BMP2	Bone morphogenetic protein 2	270.453	527.448	21.100
WARS	Tryptophanyl-tRNA synthetase, transcript variant 2	154.408	357.144	20.571
KLHL5	Kelch-like 5 (*Drosophila*), transcript variant 3	208.587	471.376	20.379
CHCHD6	Coiled-coil-helix-coiled-coil-helix domain containing 6	83.875	208.046	20.359
DHRS3	Dehydrogenase/reductase (SDR family) member 3	489.419	79.930	–96.680
SPRED2	Sprouty-related, EVH1 domain containing 2	227.047	15.680	–69.513

(续表2)

基因 Genes	基因全称 Gene name	Nanog$^+$ 细胞* Nanog$^+$ cell*	Nanog-KD* Nanog-KD*	差异分值 Diffscore
PTGS2	Prostaglandin-endoperoxide synthase 2	409.485	65.725	–65.649
STRA6	Stimulated by retinoic acid gene 6 homolog(mouse)	288.512	40.748	–61.828
STC1	Stanniocalcin 1	211.926	17.284	–60.782
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	592.575	154.990	–44.237
ETV5	Ets variant gene 5(ets-related molecule)	289.658	59.649	–36.864
SEMA3A	Semaphoring 3A	139.130	13.319	–36.317
RNF24	Ring finger protein 24	352.961	138.922	–32.090
RAB38	RAB38, member RAS oncogene family	227.029	60.171	–31.100
DHCR7	7-dehydrocholesterol reductase	868.080	409.099	–28.971
COL7A1	Collagen, type VII, alpha 1	3 854.172	2 095.417	–24.158
FKBP14	FK506 binding protein 14, 22 kDa	462.096	226.273	–22.712
PRNP	Prion protein, transcript variant 3	629.122	284.946	–22.467
COL4A6	Collagen, type IV, alpha 6, transcript variant A	91.045	14.376	–21.893

*芯片检测信号值。

*Signal value detected by microarray.

表3 *Nanog*沉默后OCT4$^+$细胞特征性基因的差异表达

Table 3 The OCT4$^+$ cell unique genes differentially expressed after *Nanog* knockdown

基因 Genes	基因全称 Gene name	OCT4$^+$ 细胞* OCT4$^+$ cell*	Nanog-KD* Nanog-KD*	差异分值 DiffScore
OLR1	Oxidized low density lipoprotein(lectin-like) receptor 1	3 359.097 0	4 689.731 0	16.128 1
NNMT	Nicotinamide N-methyltransferase	26.174 5	556.791 2	122.795 3
C8orf4	Chromosome 8 open reading frame 4	26.095 4	127.441 1	19.671 1
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	210.162 9	2 108.313 0	65.693 4
LEPREL1	Leprecan-like 1	128.362 4	360.442 0	40.052 8
IER5L	Immediate early response 5-like	48.785 2	234.810 2	47.320 4
ABCA13	ATP-binding cassette, sub-family A (ABC1), member 13	–30.533 4	85.275 8	39.401 1
MUC1	Mucin 1, cell surface associated, transcript variant 1	113.301 8	1 324.072 0	166.160 5
SUPT6H	Suppressor of Ty 6 homolog (*S.cerevisiae*)	49.736 4	181.339 1	24.655 7
SLC3A2	Solute carrier family 3, member 2, transcript variant 6	415.656 2	759.074 2	20.258 1
XK	X-linked Kx blood group(McLeod syndrome)	27.757 1	168.796 6	37.545 9
DOLK	Dolichol kinase	131.290 6	408.087 1	29.237 3
FAM100A	Family with sequence similarity 100, member A	4.136 8	154.200 3	54.317 4
SFTA1P	Surfactant associated 1 (pseudogene), non-coding RNA	9.380 1	281.862 5	73.246 7
HYI	Hydroxypyruvate isomerase homolog *(E.coli*)	61.862 2	258.551 7	52.909 7
GBP2	Guanylate binding protein 2, interferon-inducible	353.151 3	18.072 7	–80.932 3
CA9	Carbonic anhydrase IX	3 867.584 0	522.181 5	–117.225 0
ALOX5	PREDICTED: arachidonate 5-lipoxygenase	170.998 7	24.284 4	–24.442 3
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	672.577 0	2.892 5	–122.928 0
SLC29A4	Solute carrier family 29 (nucleoside transporters), member 4	270.055 1	73.685 2	–26.463 3
MALL	Mal, T-cell differentiation protein-like	266.751 4	–7.174 5	–96.716 1
WFDC3	WAP four-disulfide core domain 3, transcript variant 2	239.222 0	76.429 6	–29.183 6
LOC100132761	PREDICTED: hypothetical protein LOC100132761	141.025 7	41.808 7	–23.341 7
ETV5	Ets variant gene 5 (ets-related molecule)	289.658 4	59.648 8	–36.864 4
NDUFA4L2	NADH dehydrogenase 1 alpha subcomplex, 4-like 2	–36.864 4	166.017 0	–124.603 0

*芯片检测信号值。

*Signal value detected by microarray.

表4 *Nanog*沉默后的蛋白酶体编码基因表达

Table 4 The expression of proteasome-encoding genes after *Nanog* knockdown

基因 Genes	基因全称 Gene name	Nanog$^+$ 细胞* Nanog$^+$ cell*	Nanog-KD* Nanog-KD*	差异分值 DiffScore
PSMB10	Proteasome subunit, beta type, 10	367.490	1 248.450	63.745
PSMB3	Proteasome subunit, beta type, 3	1 628.930	5 280.882	55.621
PSMA2	Proteasome subunit, alpha type, 2	43.7320	337.840	54.195
PSME3	Proteasome activator subunit 3(PA28 gamma)	149.099	559.727	51.441
PSMB2	Proteasome subunit, beta type, 2	2 002.895	3 920.010	38.496
PSMB6	Proteasome subunit, beta type, 6	2 558.932	5 906.578	34.288
PSMB7	Proteasome subunit, beta type, 7	2 955.526	6 283.536	28.673
PSMD8	Proteasome 26S subunit, non-ATPase, 8	298.303	709.836	27.826
PSMB5	proteasome subunit, beta type, 5	1 369.711	3 054.064	27.254
PSMC4	Proteasome 26S subunit, ATPase, 4	416.674	909.644	24.664
PSMD11	Proteasome 26S subunit, non-ATPase, 11	46.229	180.964	21.648
PSMA3	Proteasome subunit, alpha type, 3	293.954	625.863	21.457
PSME2	Proteasome activator subunit 2(PA28 beta)	851.892	2 162.467	21.020
PSMC1	Proteasome 26S subunit, ATPase, 1	1 697.621	3 021.525	20.193
PSMG1	Proteasome assembly chaperone 1	272.133	558.801	19.491
PSMA5	Proteasome subunit, alpha type, 5	2 214.329	4 736.350	18.039
PSMB8	Proteasome subunit, beta type, 8	234.218	548.984	17.106
PSMD5	proteasome 26S subunit, non-ATPase, 5	109.848	241.193	15.999
PSMC2	Proteasome 26S subunit, ATPase, 2	963.885	1 764.094	13.742
PSMA6	Proteasome subunit, alpha type, 6	1 268.852	2 065.238	13.275
PSMD7	Proteasome 26S subunit, non-ATPase, 7	1 218.455	1 938.582	13.109

*芯片检测信号值。

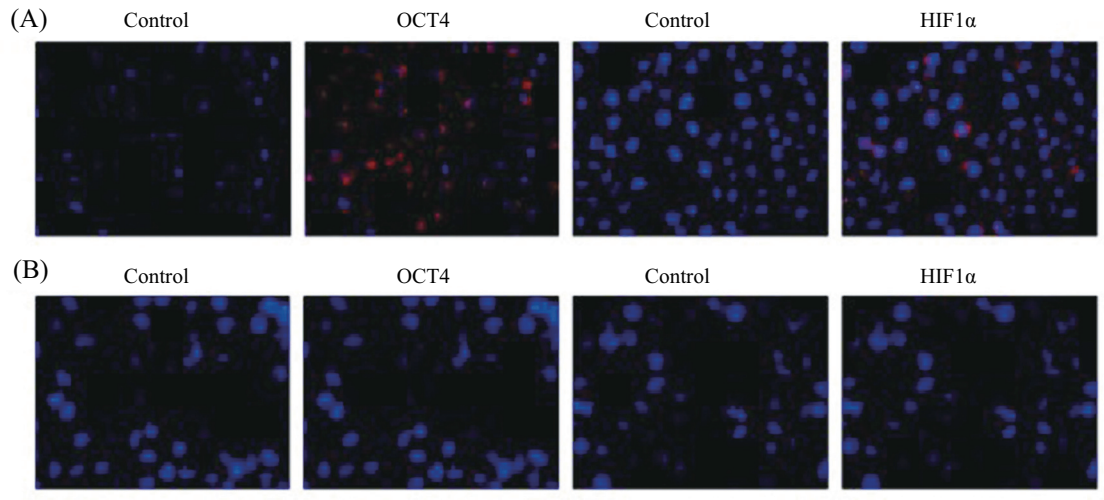
*Signal value detected by microarray.

达有关, 而*Nanog*基因沉默将增强蛋白酶体活性从而促使HIF1降解。为了验证该假设, 我们采用免疫荧光技术检测了Nanog-KD细胞中HIF1和OCT4蛋白, 结果与我们的预期吻合(图2)。

3 讨论

*Nanog*是胚胎干细胞不可缺少的一种多潜能基因。借助染色质免疫沉淀–基因芯片(CHIP-on-Chip)检测技术, Boyer等[10]报道在人胚胎干细胞中有1 687个基因的启动子(promoter)存在Nanog特异性结合位点, Nanog与之相互作用可以启动或抑制基因表达。肺腺癌CSC也表达*Nanog*基因[4], 但目前不清楚在此类细胞中该转录因子调控的靶基因。本研究提供的数据显示, Nanog在肺腺癌中控制1 605个基因表达, 其中95个基因属于已知的*Nanog*靶基因。实验结果揭示, 在肺腺癌CSC中Nanog具有转录功能, 但其调控的靶基因群与胚胎干细胞存在较大差异。

目前认为, Nanog在控制干细胞自我更新和多潜能分化中起着关键作用。实验证明, 在胚胎干细胞中*Nanog*基因过表达可以维持未分化的增殖状态而无需饲养细胞的辅助, 其表达沉默则诱导胚外内胚层分化[13-14]。这种调控机制与Nanog转录诱导OCT4表达有关[15]。CSC是恶性肿瘤内具有自我更新并分化形成各类癌细胞潜能的一类未分化细胞[2], 但其自我更新及分化的调控机制目前仍不完全清楚。本文结果显示, *Nanog*基因沉默不仅伴随着OCT4蛋白表达显著下调, 而且也引起Nanog靶基因的差异表达。值得注意的是, 该组靶基因中有多个基因已知参与调控远端气道(细支气管和肺泡)上皮分化, 如*ID2*、*FZD2*和*ETV5*等。实验研究揭示, ID2是胚胎期肺脏



A: *Nanog*$^+$肺球体细胞表达OCT4和HIF1α; B: Nanog-KD细胞不表达上述蛋白。

A: the *Nanog*$^+$ pulmospheres positively expressed OCT4 and HIF1α; B: the Nanog-KD cells were negative for both proteins.

图2 *Nanog*$^+$及其基因沉默细胞中OCT4和HIF1α表达

Fig.2 The expression of OCT4 and HIF1α in *Nanog*$^+$ cells and their gene-knockdown counterparts

远端呼吸上皮的谱系(Lineage)特异性标志, 其中肺泡上皮的发育受Wnt信号调控, 后者需要特异性FZD受体参与[16-18]。而ETV5(又称ERM)的主要功能之一是与甲状腺转录因子1(thyroid transcription factor 1, TTF-1)协同调控AT2细胞(II型肺泡细胞, Alveolar type 2 cells)特异性标志C型表面蛋白(surfactant protein C, SP-C)表达[19], *ETV5*下调将促使肺泡上皮分化。因此, *Nanog*基因沉默使得OCT4明显下调以及多种气道上皮分化标志差异表达, 提示此类细胞已失去了自我更新能力并发生分化。但这些基因的差异表达是否引起细胞形态的相应变化, 仍需要深入细致的分析, 这方面的研究尚在进行之中。

此外, 本文通过生物信息学分析, 发现Nanog调控OCT4表达的一个新颖作用机制, 即Nanog可能通过调控蛋白酶体基因表达影响HIF1α-OCT4轴。OCT4蛋白由位于6号染色体6p21.33区的*POU5f1*基因编码, 该基因的启动子在进化上(从啮齿类如小鼠到人类)高度保守, 通常将其分为4个同源保守区(homology conserved regions), 即CR1-CR4, 其中CR3和CR4在*OCT4*表达中起主要调控作用[20-21]。最近研究揭示, 在CR3和CR4转录调控区内含有多个低氧反应元件(hypoxia responsive elements, HRE, 碱基序列: G/ACGTG), 该基序可被HIF特异性识别并结合。Covello等[22]率先报道, HIF2α能够与HRE结合并诱导OCT4表达。但随后的研究揭示, HIF1α和HIF2α通过识别相同的HRE基序均能够调控OCT4表达[23-24]。通过基因沉默技术我们也证明在肺腺癌CSC中存在类似的调控机制[6]。这些结果说明癌细胞尤其是CSC中存在HIF1α-OCT4调控轴, 但在含氧状态下, 细胞内HIF1蛋白通过蛋白酶体降解途径快速代谢[25], 因而可以预期CSC维持HIF1α-OCT4轴功能需要对蛋白酶体基因表达进行精细调控。蛋白酶体是胞浆内的一种多蛋白细胞器, 由20S核心颗粒(core particle, CP)和19S调控颗粒(regulatory particle, RP)组成, 后者又分为Lid和Base二个亚颗粒。蛋白酶体的3种蛋白水解酶活性(caspase-like、trypsin-like和chymotrypsin-like)分别由CP中PSMB1、PSMB2和PSMB5等蛋白介导[26-27]。Pan等[28]最近报道, 当肺腺癌细胞形成悬浮生长的球体时, OCT4和Nanog高表达而上述蛋白水解酶活性明显低下, 此结果提示蛋白酶体活性与HIF1α-OCT4轴之间存在内在联系, 但其机制尚不清楚。本文结果显示, 肺腺癌细胞中HIF1α蛋白持续阳性表达与Nanog抑制多种蛋白酶体基因表达有关, 因而*Nanog*基因沉默可能通过增强蛋白酶体活性, 从而促使HIF1α降解导致OCT4转阴。*Nanog*沉默细胞内HIFα和OCT4蛋白水平下调现象支持该假设, 但证明上述Nanog-HIF1α-OCT4调控机制还需要深入系统的分析。

解析Nanog与OCT4之间的关系将为探索肺腺癌CSC的特异性靶向治疗提供新的研究思路, 即HIF可能

是肺腺癌CSC靶向治疗的重要治疗靶标, 抑制其表达和/或促使其降解将能够使得*OCT4*和*Nanog*多潜能基因表达转阴, 从而促使CSC分化。鉴于HIF抑制剂中已有多个药物获得美国食药监管局(FDA)批准进入临床试验[28], 进一步评估此类药物的体内抗癌效应及毒副作用, 将为靶向抑制肺腺癌CSC提供新颖治疗手段。

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