胰腺癌干细胞中受OCT4调控的表面标志研究

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摘要 *OCT4*和*Nanog*被公认是人ESC的自我更新调控基因, 其中*OCT4*能够转录调控多种表面蛋白的表达, 如SEMA6A。该文将人胰腺癌细胞株Panc-1、Bxpc-3、Aspc-1和Cfpac-1培养在无血清条件下, 采用EGF、IGF-1和FGF-10诱导球体形成。用免疫荧光法分别检测这4种人胰腺癌细胞株及其球体细胞以及15例胰腺癌组织标本和13例正常胰腺组织标本中*OCT4*和*Nanog*的表达, 结果显示, 4种人胰腺癌细胞株在无血清-DF12培养基中5~10 d即可形成悬浮生长的球体。*OCT4*和*Nanog*在4种细胞株均有表达, 且球体细胞中表达明显高于亲代细胞。在胰腺癌组织中仅有少量表达自我更新基因, 在正常胰腺组织中微量表达。此外, 还检测到Panc-1球体细胞表面高表达SEMA6A。由此可见, 自我更新基因*OCT4*和*Nanog*在胰腺癌细胞中的表达和CSC有关, 其表面蛋白SEMA6A作为胰腺癌干细胞表面标志物值得进一步研究。

关键词 胰腺癌; 肿瘤干细胞; 自我更新; 球体; 分子标志物

癌干细胞(cancer stem cells, CSC)被认为是恶性肿瘤的起始(initiating)细胞, 能通过自我更新(self-renewal)获得无限增殖能力, 并可分化形成肿瘤内各种癌细胞类型[1]。鉴于此, CSC作为肿瘤诊治的重要靶标已日益引起业界的高度关注, 成为当前肿瘤研究的前沿领域。胰腺癌恶性程度高, 预后极差, 且发病率近年来有明显上升趋势。因而对其细胞起源及癌变机制的研究一直是胰腺肿瘤治疗的主攻方向之一。胰腺癌干细胞的研究目前尚处于起始阶段, 其细胞属性(cell identity)尚未阐明, 如Hermann等[2]和Li等[3]两个研究组分别以$CD133^+$和$CD44^+CD24^+ESA^+$为特异性标志物对胰腺癌CSC进行了分离和鉴定, 但得到的结果尚缺乏验证。自我更新是所有干细胞(包括CSC)的共性特征, 因此胰腺癌干细胞的鉴定必须基于此特征。近年来关于胚胎干细胞(embryonic stem cells, ESC)自我更新和全能(pluripotent)分化机理的研究已取得显著进展。资料显示, ESC自我更新主要在基因转录水平受到精细调控, 如*OCT4*和*Nanog*被公认是人ESC的核心调控基因[4]。因而, 以此类基因作为鉴定CSC的依据, 并找到其调控的相应表面蛋白作为特异性标志物, 是值得探索的研究新方向。

1 材料与方法

1.1 肿瘤细胞及组织标本

人胰腺癌细胞株Panc-1、Bxpc-3、Aspc-1、Cfpac-1购自中国科学院上海生命科学研究院细胞库。肿瘤细胞在10%胎牛血清-DMEM培养基(均为Hyclone公司产品)中传代培养, 取活跃增殖期细胞进行实验。15例胰腺癌组织标本及13例正常胰腺组织标本均取自2007年1月—2009年12月华山医院普外科施行的胰腺癌根治手术, 其中男性10例, 女性5例, 年龄在48~72岁, 平均为60.1岁。病理报告显示肿瘤细胞低分化7例、中分化7例、高分化1例。临床分期(TNM) Ⅰ期1例、Ⅱ期8例、Ⅲ期6例。

1.2 球体培养及分离

用无血清-DF12培养基(DMEM/F12培养基, Hyclone公司)将细胞调整至2×10^4/ml, 取5 ml细胞培养在超低吸附培养皿(Corning公司)中, 分别加入20 ng/ml重组人胰岛素样生长因子-1(insulin-like growth factor-1, IGF-1, Serotec公司产品), 20 ng/ml重

收稿日期: 2011-01-23 接受日期: 2011-03-01

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组人表皮生长因子(epidermal growth factor, EGF, Serotec公司产品), 20 ng/ml重组人纤维母细胞生长因子-10(fibroblast growth factor-10, FGF-10, PeproTech公司)。间隔2天添加上述细胞因子一次, 直至球体形成。收集细胞, 800 r/min离心2 min后取球体细胞沉淀。

1.3 细胞的免疫荧光检测

将洁净灭菌盖玻片每片滴加0.1 mg/ml多聚赖氨酸(poly-D-Lyshe, PDL, Sigma公司)250 μl, 涂匀干燥。待测细胞调整至1×10^6/ml, 将200 μl细胞滴在PDL盖玻片上培养过夜。细胞经4%多聚甲醛固定, 0.25% Triton X-100通透后染色, 第一抗体分别为Santa Cruz公司OCT4特异性鼠多克隆IgG(sc-5279)和Nanog特异性羊多克隆IgG(sc-30331), 稀释度均为1:50。在湿盒内4 ℃温育过夜后经PBS(pH7.4)淋洗去除非结合的抗体, 然后用FITC标记的驴抗鼠多克隆IgG、Rodamine标记的驴抗羊多克隆IgG(1:100稀释, Santa Cruz公司)室温染色2 h, PBS淋洗并经Hoechst33342染核后封片, 在Olympus IX51型荧光显微镜下观察并摄片。SEMA6A免疫荧光检测第一抗体为Santa Cruz公司SEMA6A特异性鼠多克隆IgG(sc-74274)(1:50稀释), 第二抗体为Rodamine标记的驴抗鼠多克隆IgG(1:100稀释), 方法同前。

1.4 组织切片的免疫荧光检测

将病理科的石蜡组织标本经切片、烘片、二甲苯脱蜡、酒精脱二甲苯、柠檬酸盐修复后染色。检测*OCT4*和*Nanog*的表达, 免疫荧光染色方法同上。

2 结果

2.1 胰腺癌细胞球体的形成

将人胰腺癌Panc-1、Bxpc-3、Aspc-1和Cfpac-1细胞培养在无血清-DF12培养基中, 经EGF、IGF-1和FGF-10刺激5~10 d即可形成悬浮生长的球体(图1)。胰腺癌球体细胞经吹打分散后连续传代培养3个月, 仍能形成球体(资料未列)。

2.2 胰腺癌细胞株亲代和球体细胞内自我更新基因*OCT4*、*Nanog*的表达

将4种人胰腺癌Panc-1、Bxpc-3、Aspc-1、Cfpac-1细胞和经无血清培养法得到的相应球体细胞分别收集, 进行特异性免疫荧光检测。可见自我更新基因*OCT4*和*Nanog*在4种细胞株中均有少量表达, 球体细胞中阳性率呈现明显增高(图2), 提示经无血清培养使具有自我更新功能的胰腺癌细胞得以富集。

2.3 胰腺癌组织及正常胰腺组织中*OCT4*和*Nanog*的表达

为了评估上述干细胞标志表达的临床相关性, 我们检测了13例正常胰腺组织标本及15例胰腺癌组织标本。结果显示, 在正常胰腺组织中少量细胞表达*OCT4*和*Nanog*, 胰腺癌组织中阳性细胞增多(图3)。

2.4 球体细胞内OCT4及表面蛋白SEMA6A表达分析

为了寻找胰腺癌球体细胞中受*OCT4*等干细胞基因调控的表面标志, 我们选择SEMA6A进行探讨。该膜蛋白在人ESC(hESCs)中受*OCT4*调控。图4结果显示, Panc-1球体细胞中95%以上表达OCT4蛋白, 此类细胞同时检测到SEMA6A表达, 提示在胰腺癌CSC样细胞中*SEMA6A*可能也是*OCT4*调控的靶基因。

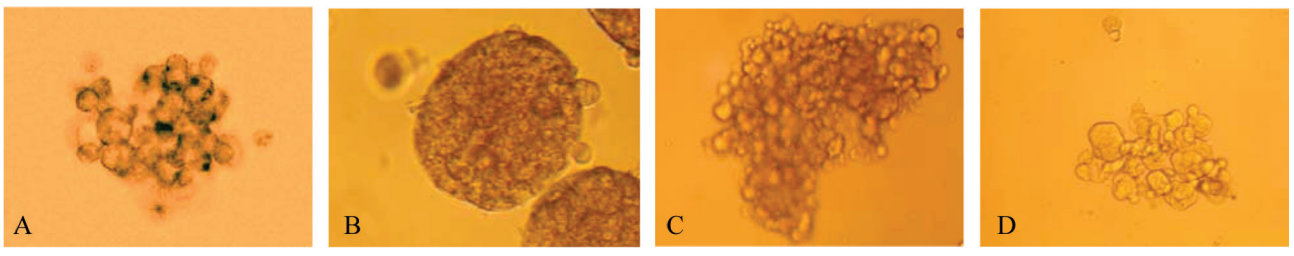

图1 胰腺癌细胞株球体形成(×200)

A: Panc-1球体细胞; B: Bxpc-3球体细胞; C: Aspc-1球体细胞; D: Cfpac-1球体细胞。

Fig.1 Tumorspheres generated from pancreatic cancer cell lines (×200)

A: Panc-1 sphere; B: Bxpc-3 sphere; C: Aspc-1 sphere; D: Cfpac-1 sphere.

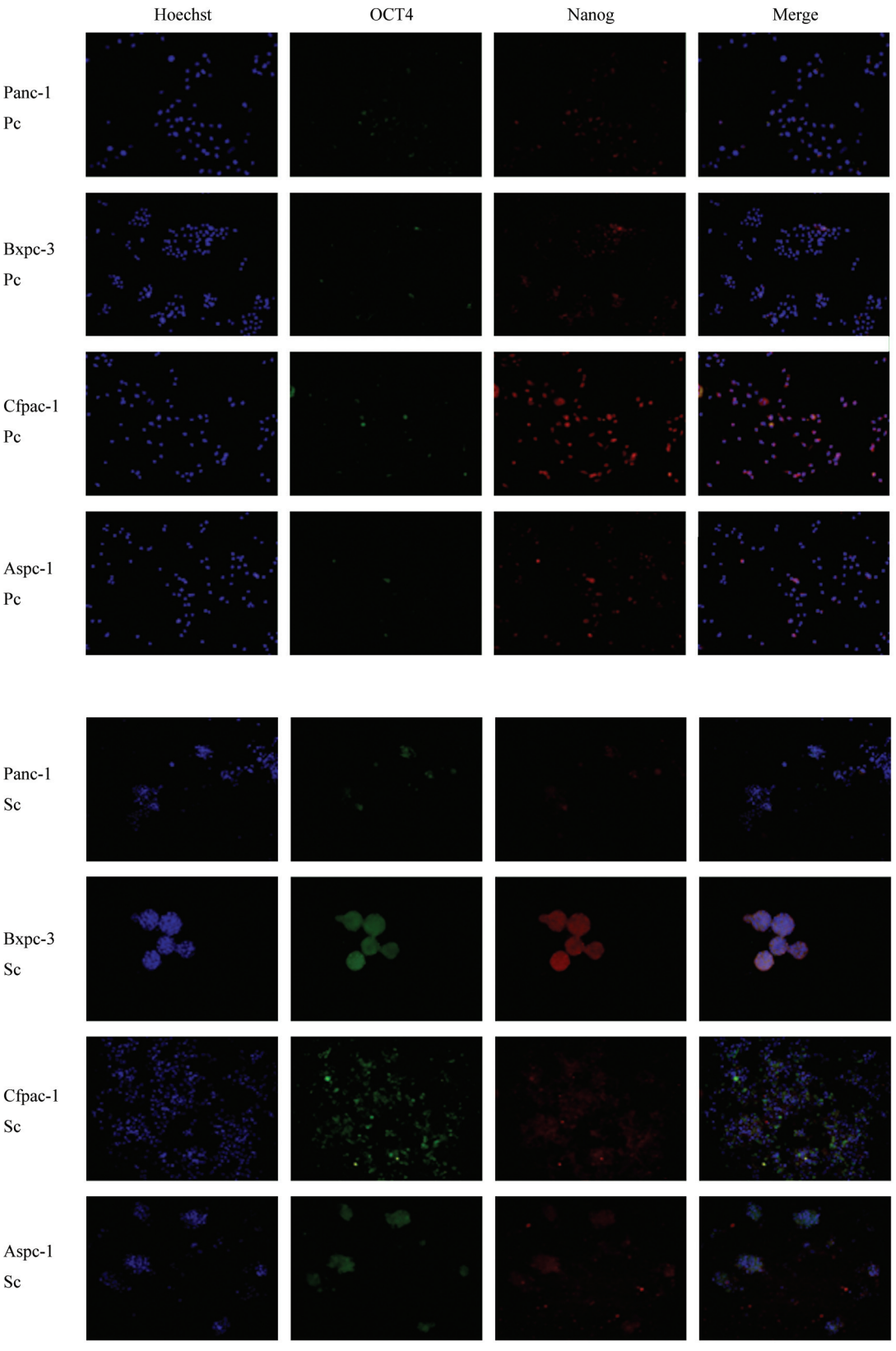

图2　免疫荧光检测胰腺癌细胞中自我更新基因的表达

用免疫荧光法分别检测胰腺癌亲代和球体细胞中*OCT4*(绿色荧光)和*Nanog*(红色荧光)的表达, 用Hoechst33342染核。Pc: 亲代细胞; Sc: 球体细胞。

Fig.2　The immunofluorescent staining for the expression of self-renewal genes in pancreatic cancer cells

The human pancreatic cancer cell lines and their sphere-forming cells were stained with DNA dye (Hoechst33342) or specific antibodies for the expression of *OCT4* (green) and *Nanog* (red). Pc: parental cells; Sc: sphere cells.

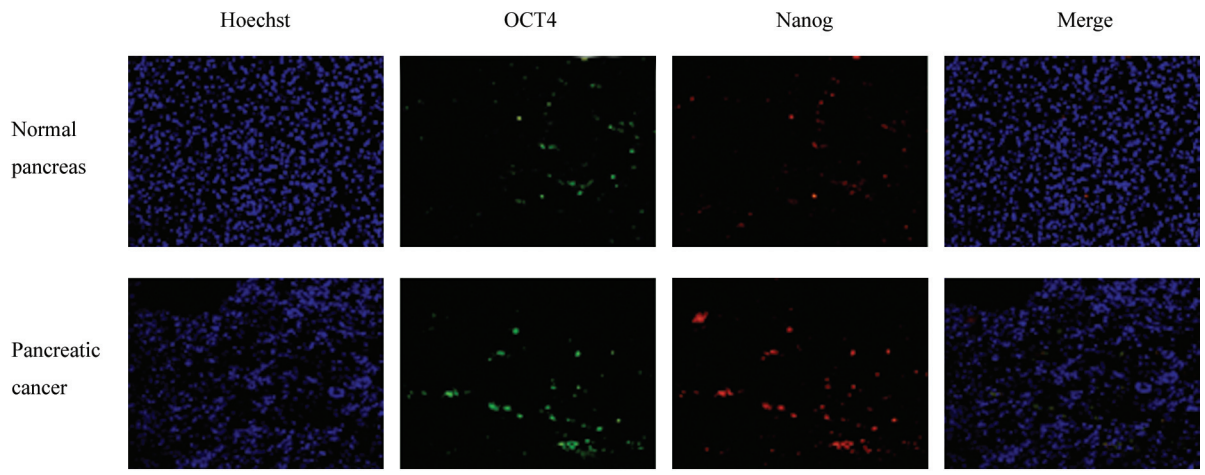

图3　免疫荧光检测胰腺组织中自我更新基因的表达

用免疫荧光法分别检测13例正常胰腺组织和15例胰腺癌组织中*OCT4*(绿色荧光)和*Nanog*(红色荧光)的表达, 用Hoechst33342染核。

Fig.3　The immunofluorescent staining for the expression of self-renewal genes in pancreatic tissues

13 cases of normal pancreatic tissues and 15 cases of pancreatic cancer tissues were stained with DNA dye (Hoechst33342) or specific antibodies for the expression of *OCT4* (green) and *Nanog* (red).

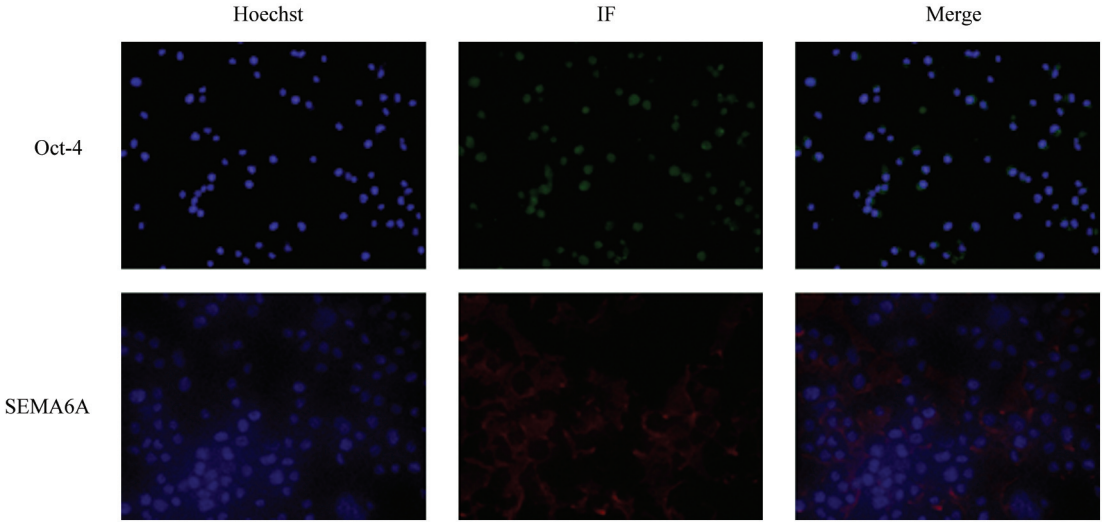

图4　免疫荧光检测胰腺癌Panc-1球体细胞中*Oct4*和*SEMA6A*的表达

用免疫荧光法检测胰腺癌Panc-1球体细胞中*OCT4*(绿色荧光)和*SEMA6A*(红色荧光)的表达, 用Hoechst33342染核。

Fig.4　The immunofluorescent staining for the expression of *OCT4* and *SEMA6A* in Panc-1 spheres

The Panc-1 spheres were stained with DNA dye (Hoechst33342) or specific antibodies (IF) for the expression of *OCT4* (green) and *SEMA6A* (red).

3　讨论

癌干细胞是指恶性肿瘤中能够自我更新及多向分化(multilineage differentiation)成肿瘤内各类癌细胞的原始未分化癌细胞[5]。这类细胞可以在无血清培养条件下经表皮生长因子(epidermal growth factor, EGF)等多种生长因子诱导形成悬浮生长的细胞团块, 称为球体(spheres)[6]。Glinsky等[7]指出, CSC具有失巢凋亡(anoikis)抗性, 因而在无血清培养条件下可以悬浮生长, 如脑瘤、乳腺癌、结肠癌、前列腺癌、肺癌和黑色素瘤的CSC均具有球体形成能力[6,8]。国内研究者[9]采用无血清培养使胰腺癌Panc-1细胞株增殖形成球体, 并通过克隆形成实验和动物移植实验证实后者具有高致瘤性。美国研究者[2]也证明具有高致瘤性的$CD44^+CD24^+ESA^+$胰腺癌细胞能够形成球体, 而非致瘤性的$CD44^-CD24^-ESA^-$细胞无法形成球体。

OCT4属于POU(Pit-Oct-Unc)转录因子家族(又称POU5f1), 其特征是特异性结合靶基因启动子中的核苷酸八聚体(Octamer)序列(ATGCAAAT), 从而启动基因表达。在ESC中, *OCT4*与*Nanog*和*Sox2*构成的核心调节环(core regulatory network)通过16种主调节(master regulators)基因, 控制2 260种mRNA的转录, 后者构成了ESC特异性功能基因组(functional genomics)[10]。实验证明, 选择性敲除(knockdown)上述核心调节环的任何一个基因均导致ESC失去自我更新能力而分化[11,12]。在CSC研究中, Chen等[13]发现特异性干扰*OCT4*基因表达可使原来具有肺癌起始能力的LC-CD133$^+$细胞丧失肿瘤侵袭、球体形成和化疗耐药的能力, 认为*OCT4*在肺癌CSC维持自我更新功能中有关键作用。Ben-Porath等[14]发现分化差、侵袭力强的肿瘤比高分化肿瘤更高表达ESC相关基因如*OCT4*、*Nanog*、*Sox2*和*c-Myc*, 在临床分期高、雌激素受体(ER)阴性、预后差的乳腺癌病人中常有过度表达。我们也观察到胰腺癌球体细胞除表达OCT4外, 还同时表达Nanog, 而在胰腺癌组织中二者仅有少量表达, 在正常胰腺组织中微量表达。提示CSC与ESC在自我更新的调控机制上可能遵循共同的自然法则, 其自我更新调控基因*OCT4*和*Nanog*可以作为胰腺癌干细胞鉴定和进一步纯化的标志。

但OCT4和Nanog均为核蛋白, 不适宜用于活细胞分选, 因而有必要筛查受上述基因调控的细胞表面标志物。Assou等[15]通过hESCs基因表达谱的比较分析, 发现此类细胞特异性表达SEMA6A。SEMA6A属于进化保守的Semaphorin家族。该家族由20余种成员组成, 分为分泌型和膜结合型2个亚族, SEMA6A属于后者。此类膜蛋白的作用机制与Notch类似, 即SEMA6A通过与相邻细胞表面的特异性受体plexin结合, 引起受体介导的细胞内信号转导, 如活化PI3K-Akt酪氨酸激酶级联反应。在胚胎发育过程中, SEMA6A表达局限于原始未分化细胞, 如ESC等。Katoh等[16]证明*SEMA6A*基因的启动子区域存在OCT4和Nanog的特异性结合位点, 因而其表达受ESC转录因子调控。胰腺癌球体细胞同时表达ESC转录因子*OCT4*和*Nanog*及其转录靶基因*SEMA6A*, 提示此类癌细胞具有原始未分化细胞特性。此类细胞有可能属于迄今未报道的胰腺癌CSC。因此, 以上述标志为基础分析胰腺癌CSC的细胞属性, 值得进一步探讨。

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A Preliminary Study on the Surface Marker of Human Pancreatic Cancer Stem Cell Regulated by OCT4

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Abstract *OCT4* and *Nanog* are two core transcriptional factors to regulate the self-renewal in human embryonic stem cells (hECSs). The expression of SEMA6A membrane protein in hESCs is regulated by OCT4. In this paper, we induced the sphere formation in Panc-1, Bxpc-3, Aspc-1 and Cfpac-1 pancreatic cancer cell lines by culturing the cells in the serum-free conditions supplemented with EGF, IGF-1 and FGF-10. Their expression of self-renewing genes, *OCT4* and *Nanog,* were measured by immunofluorscent staining. The same assay was also done in these cell lines including 15 cases of pancreatic cancer tissues and 13 cases of normal pancreas. The float-growing spheres were developed after 5 to 10 days culture in all the cell lines tested. The expression of *OCT4* and *Nanog* in the sphere-forming cells was much higher than their relevant counterparts in cell lines. These stemness markers were also found in the pancreatic cancer tissues and at much lower level in normal pancreas. Furthermore, the stem-like spheres in Panc-1 were observed to express SEMA6A, a surface marker known to be the OCT4 downstream target. In conclusion, the expression of self-renewing genes, *OCT4* and *Nanog*, in pancreatic cancer cells implies their relevance to the cancer stem cells. The SEMA6A protein regulated by OCT4 may represent an invaluable surface marker for studying the putative pancreatic cancer stem cells.

Key words pancreatic cancer; cancer stem cells; self-renewal; sphere; molecule marker

Received: January 23, 2011 Accepted: March 1, 2011

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