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细胞基质Ⅰ型胶原通过调控β-catenin促进头颈鳞癌细胞转移和增殖*

左建宏[1,2] 李新辉[1] 李茂玉[1] 万恂恂[1] 曾谷清[1] 贺秋艳[1] 李建璜[1] 肖志强[1]**
([1]中南大学湘雅医院卫生部肿瘤蛋白质组学重点实验室，长沙 410008；[2]南华大学公共卫生学院，衡阳 421001)

摘要 为探讨细胞基质Ⅰ型胶原 (Col-Ⅰ)对头颈鳞癌细胞转移和增殖能力的影响，以头颈鳞癌细胞株为对象，运用 transwell 小室检查细胞的体外迁移能力，用倒置显微镜成像系统检测细胞的运动速度和扩散能力，Western blotting 或 / 和免疫细胞荧光染色检测细胞表面黏附分子 E-cadherin 的表达和 β-catenin 的细胞定位，采用 MTT 以及 PCNA 的表达水平评价细胞增殖. 结果显示，Col-Ⅰ能够促进头颈鳞癌细胞迁移、提高细胞运动速度、加快细胞扩散，其可能机制是通过上调 β- 连环蛋白磷酸化，从而下调黏附蛋白 E-cadherin 的表达. Col-Ⅰ还能促进头颈鳞癌细胞的增殖，其可能机制是增加 β-catenin 的核移位，提高细胞周期蛋白 D1 (cyclin D1)表达. 研究结果表明，Col-Ⅰ通过上调 β-catenin 磷酸化水平，以及促进 β-catenin 核移位，从而增强头颈鳞癌细胞的转移和增殖能力.

关键词 Ⅰ型胶原，肿瘤微环境，头颈部鳞癌细胞，转移，增殖
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头颈部鳞癌(head and neck squamous cell carcinoma，HNSCC)是一种严重威胁人类生命的恶性上皮肿瘤，已经成为全世界第六大常见的肿瘤[1-2]. 全球 HNSCC 年新发病例大约 65 万人，且每年至少有 35 万人死于 HNSCC[3]. 据报道，美国仅 2007 年就有 45 660 例诊断为 HNSCC，死于 HNSCC 的人数高达 11 210 人[4]. 尽管早期 HNSCC 经手术或放疗后均能获得良好的效果，但遗憾的是，60%的 HNSCC 病例就诊时已属晚期(Ⅲ、Ⅳ期)，5 年生存率仍很低[5]. 主要原因是 HNSCC 常发生局部或远端淋巴结转移，而且常常是临床难以发现 HNSCC 的微转移[6]，这也是导致疗效和预后差、易于复发和死亡率高的重要原因[7-8]. 因此，研究 HNSCC 转移机制对于提高 HNSCC 疗效具有重要意义.

肿瘤微环境(tumor microenvironment)是肿瘤在其发生发展过程中所处的内环境，它在肿瘤的侵袭、转移、黏附、增殖、血管新生以及肿瘤耐药等方面具有十分重要的作用[9-12]. 由于肿瘤组织低氧或缺氧[13]、pH 值下调、感染等原因导致炎症细胞聚集以及炎症因子和蛋白质水解酶的产生，最终形成了肿瘤组织特定的微环境[10,14-15]. 肿瘤微环境是一个十分复杂的系统，主要由肿瘤细胞本身、免疫细胞、成纤维细胞、炎症细胞、组织液及细胞外基质等共同组成[16-17]. 肿瘤微环境的研究是当今肿瘤研究的热点，但在国内外对其研究还处于初级阶段.

细胞基质Ⅰ型胶原(collagen typeⅠ，Col-Ⅰ)是肿瘤微环境中的重要组成成分，由肿瘤细胞与周围的间质细胞相互作用并诱导其表达，其受体主要是 β1 -integrin. Col-Ⅰ具有促进细胞生长、分化、黏附、迁移等多种胞转移和增殖能力，研究其分子机制，为 HNSCC 转移的防治提供科学依据.

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** 通讯联系人.
Tel: 0731-84327239, E-mail: zqxiao2001@yahoo.com.cn
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1　材料和方法

1.1　材料

鼠抗人 E-cad 抗体(ZYMED 公司产品)；鼠抗人 β-catenin 抗体、鼠抗人 PCNA 抗体(BD 公司产品)；鼠抗人 Cyclin D1 抗体(Santa Cruz biotechnology 公司产品)；鼠抗人 β1-integrin 抗体(Abcam 公司产品)；兔抗人 p-β-catenin 抗体(Santa Cruz biotechnology 公司产品)；HRP 标记的山羊抗小鼠 IgG 抗体以及 FITC 标记的山羊抗小鼠 IgG 荧光抗体(Santa cruz biotechnology 公司产品)；Collagen type Ⅰ和 Fibronectin (Sigma 公司产品)．中南大学卫生部肿瘤蛋白质组学重点实验室用细胞株 HSC-3、SCC10A、HA376 和 SCC74A 用含 10%小牛血清的 DMEM 培养基于 37℃、5% CO_2 培养箱中培养．

1.2　方法

1.2.1　细胞迁移试验．在 transwell (8 μm pore size; Corning Costar Corp 公司产品) 膜的底部用 Collagen type Ⅰ(Col-Ⅰ，1 mg/L)，Fibronectin (Fn，10 mg/L) 预包被，然后在 transwell 的上室加入 2×10^5 个细胞，下室加入不含血清的 DMEM 培养液，细胞在 37℃、5% CO_2，孵育 3 或 6 h，用 4%多聚甲醛固定，结晶紫染色 5 h，上室未转移的细胞用棉签抹去．用 20×物镜的显微镜随机计数 10 个视野，取其均值．

1.2.2　细胞运动试验．将细胞置于用 Col-Ⅰ(1 mg/L) 或 fibronectin (Fn，10 mg/L) 包被的 6 孔细胞培养皿中，然后用 Zeiss Axiovert 倒置显微成像系统每隔 20 min 拍照 1 次，持续 12 h.

1.2.3　细胞扩散试验．用 Trypsin-EDTA 消化收集细胞，4℃、1 000 r/min 离心 5 min，弃去上清，加入含有 0.5%小牛血清 DMEM 培养液，用细胞筛网收集单个细胞，并将细胞稀释成浓度为 2×10^4 个 /ml，再将细胞加入预先用 polyHEMA 处理的细胞培养皿中，在 5% CO_2、37℃条件下培养 12 h．离心收集细胞多聚体，置于用 Col-Ⅰ(1 mg/L)预处理的 6 孔细胞培养板，然后用 Zeiss Axiovert 倒置显微成像系统每隔 1 h 拍照 1 次，总计 15 h.

1.2.4　Western blotting．收集细胞，用 PBS 洗涤 3 次, 加入裂解液[50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl，1% Triton X-100，0.5%脱氧胆酸钠，0.1% SDS，10 mmol/L $MgCl_2$，和蛋白酶抑制剂混合物 (Roche Molecular Biochemicals)]裂解细胞，离心收集上清．BCA (Pierce 公司产品) 法测蛋白质的浓度，SDS-PAGE，转膜，依次孵育 一抗和二抗，ECL (Amersham Biosciences) 检测蛋白质信号．

1.2.5　免疫细胞荧光染色．取对数生长细胞，用 0.25%胰蛋白酶消化，制成单细胞悬液．将细胞接种到预先放置 Col-Ⅰ或 Fn 包被的 6 mm × 22 mm 盖玻片的培养皿中，在 5% CO_2、37℃条件下培养 24 h，取出盖玻片，PBS(0.01 mol/L, pH 7.4)洗 2 次，4% PFA 固定 10 min．用 PBS 振荡洗 5 min，滴加适当稀释的特异性抗体，置于湿盒内，37℃保温 30～60 min 或置冰箱过夜，PBS 振荡洗 3 次，每次 5 min．滴加适当稀释的 FITC 标记的二抗，置于湿盒内，37℃保温 45 min．PBS 振荡洗 2 次，每次 5 min，然后用蒸馏水振荡洗 1 次，再用 DAPI 复染，荧光显微镜下观察．

1.2.6　流式细胞术检测细胞 β1-Integrin 受体的表达．在每个流式检测管中加入 50 μl 1∶1 000 稀释的 β1-Integrin 抗体，在空白管中加入 50 μl PBS，再在各管中分别加入 50 μl 细胞悬液(约 10^6 个细胞)，并轻轻混匀．冰浴孵育 1 h．孵育完成后，每流式检测管中加 2 ml PBS、4℃下 1 000 r/min 离心 5 min，弃去上清，重复洗涤 3 次．每管加入 50～100 μl 稀释好的 FITC 标记二抗后，于冰浴或在 4℃冰箱中避光孵育 45 min，洗涤细胞 3 次(方法同上)，并上机检测．

1.2.7　MTT 检测细胞增殖．以每孔 500 个细胞接种于用 Col-Ⅰ(1 mg/L) 或 fibronectin (Fn，10 mg/L) 包被的 96 孔培养板中，每孔体积 200 μl，每组 5 孔，同时设空白对照(仅加培养液)，置 CO_2 培养箱中孵育，每隔 48 h 检测一次各组，每孔加入噻唑蓝(MTT)溶液(5 g/L) 20 μl，37℃继续孵育 4 h，终止培养，小心吸弃孔内培养上清液，加入 150 μl DMSO，振荡 10 min，使结晶物充分溶解，以空白对照孔调零，ELx800 酶标仪上 562 nm 测定各孔吸光度值(*A* 值)，以相对应 *A* 比值表示细胞增殖能力大小．

2　结　　果

2.1　Col-Ⅰ促进头颈鳞癌细胞的迁移和运动

在 Col-Ⅰ基质上，无论在 3 h 还是在 6 h 时间点，HNSCC 细胞 HSC-3 穿越 transwell 底部膜进入下室的细胞数明显高于在 Fn 细胞基质上 (图 1a，

$P < 0.001$)，表明 Col-Ⅰ促进细胞迁移．同时，细胞在 Col-Ⅰ细胞基质上运动速度也明显快于在 Fn 基质上的速度(图 1b，$P < 0.05$)．细胞的运动轨迹也反映了细胞在 Col-Ⅰ基质上运动速度明显增加(图 1b).

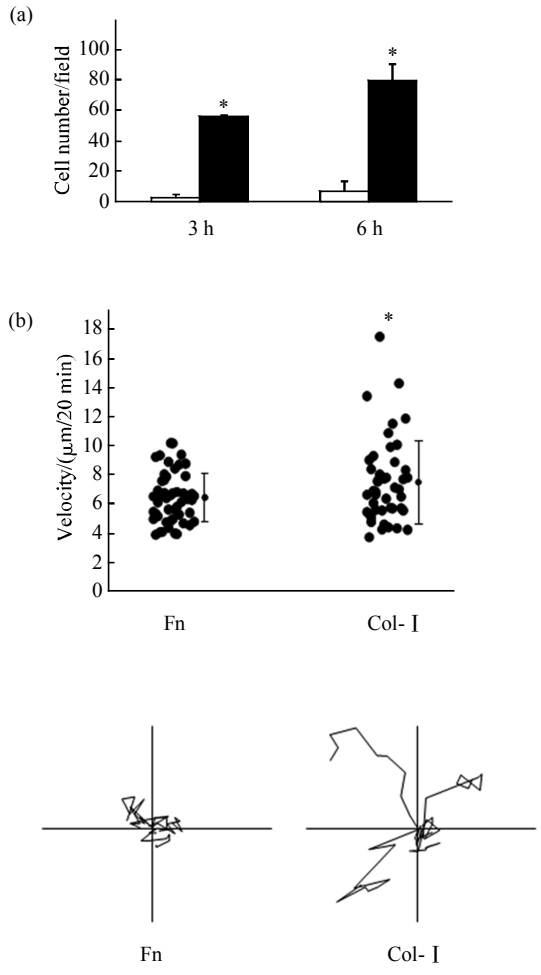

Fig. 1　Collagen type Ⅰ (Col-Ⅰ) promotes cell migration

(a) HSC-3 cells were subjected to 3 h and 6 h migration assays using transwells coated with fibronectin (Fn) or Col-Ⅰ substrates. The number of cells that migrated through the transwell membranes was estimated by counting at least 10 random microscopic fields. Columns, mean; bars, SD (*$P < 0.001$). □: Fn; ■: Col-Ⅰ. (b) Cell velocity on Fn or Col-Ⅰ-coated substrates was measured by time-lapse video microscopy (Top; *$P < 0.05$). Representative cell tracks are shown (bottom).

2.2　Col-Ⅰ促进头颈鳞癌细胞的扩散

为了进一步证实 Col-Ⅰ基质对细胞黏附和转移的影响，我们进行了多细胞聚集体(multicellular aggregates，MCA)扩散实验(cell scattering assay)．结果显示：当 HNSCC 细胞 HSC-3 的 MCA 置于 Col-Ⅰ预处理的 6 孔培养板 30 min时，它能黏附于细胞培养板，在 6 h 时间点，MCA 形成单层细胞，并逐步扩散，至 15 h 时间点时，除在已形成单层细胞的基础上进一步分散外，还能看到扩散到远处的单个细胞，而在 Fn 细胞基质上，细胞未见明显变化(图 2a).

为了进一步量化上述结果，我们应用了 NIH 图像分析软件测量 MCA 分散形成的面积，计算并绘成图(图 2b)．结果更清晰地显示：HSC-3 的 MCA 随着时间的推移，在 Col-Ⅰ预处理的 6 孔培养板上，细胞扩散形成的面积从第 6 小时开始明显比在 Fn 基质上大，并具有统计学意义(*$P < 0.05$，**$P < 0.001$)．上述结果表明：Col-Ⅰ能加快 HSC-3 细胞的扩散.

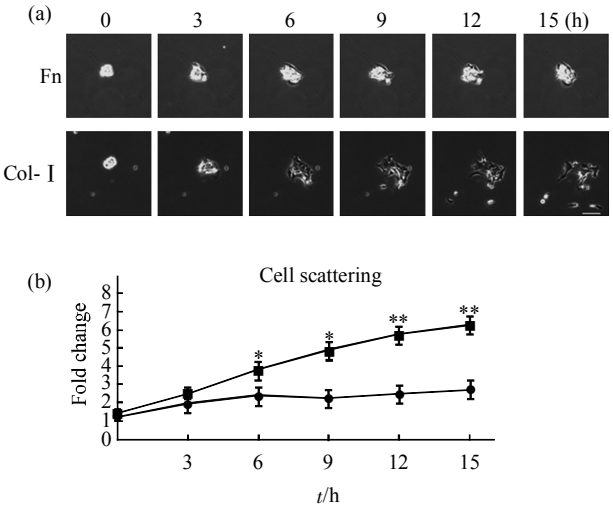

Fig. 2　Collagen type Ⅰ increases cell scattering

(a) Cells as indicated were detached and subjected to forced cell aggregation for 12 h. Cell aggregates were then collected and seeded onto Col-Ⅰ-coated (1 mg/L) or Fn (10 mg/L) culture plates and monitored by time-lapse video microscopy. HSC-3 cells on Col-Ⅰ substrates show an immediate out-migration from aggregates whereas the cells on Fn substrates were severely delayed. Bar, 100 μm. (b) Dispersion of cell aggregates shown in (a) were then assessed for scattering as described in **Materials and methods**. The data were expressed as the relative scatter index. Points, mean; bars, SE (*$P < 0.05$; **$P < 0.001$). ●—●: Fn; ■—■: Col-Ⅰ.

2.3　Col-Ⅰ通过下调 E-cadherin 促进头颈鳞癌细胞迁移

为了探讨 Col-Ⅰ促进细胞迁移和运动的可能机制，采用免疫细胞荧光染色和 Western blotting 检测 Col-Ⅰ对 HNSCC 细胞株 HSC-3 和 SCC10A 细胞 E-cadherin 表达的影响．结果显示，与在 Fn 基质上培养细胞比较，在 Col-Ⅰ基质上培养细胞的 E-cadherin 表达明显下调(图 3a, 图 3b).

为了进一步验证 Col-Ⅰ是通过下调 E-cadherin 促进细胞迁移能力，本研究选用了不表达 E-cadherin 的 HNSCC 细胞株 HA376 和 SCC74A (图 3c)进行细胞迁移实验．结果表明：与 Fn 相比较，Col-Ⅰ虽然轻微地提高了细胞的迁移能力，但并无显著意义(图 3d).

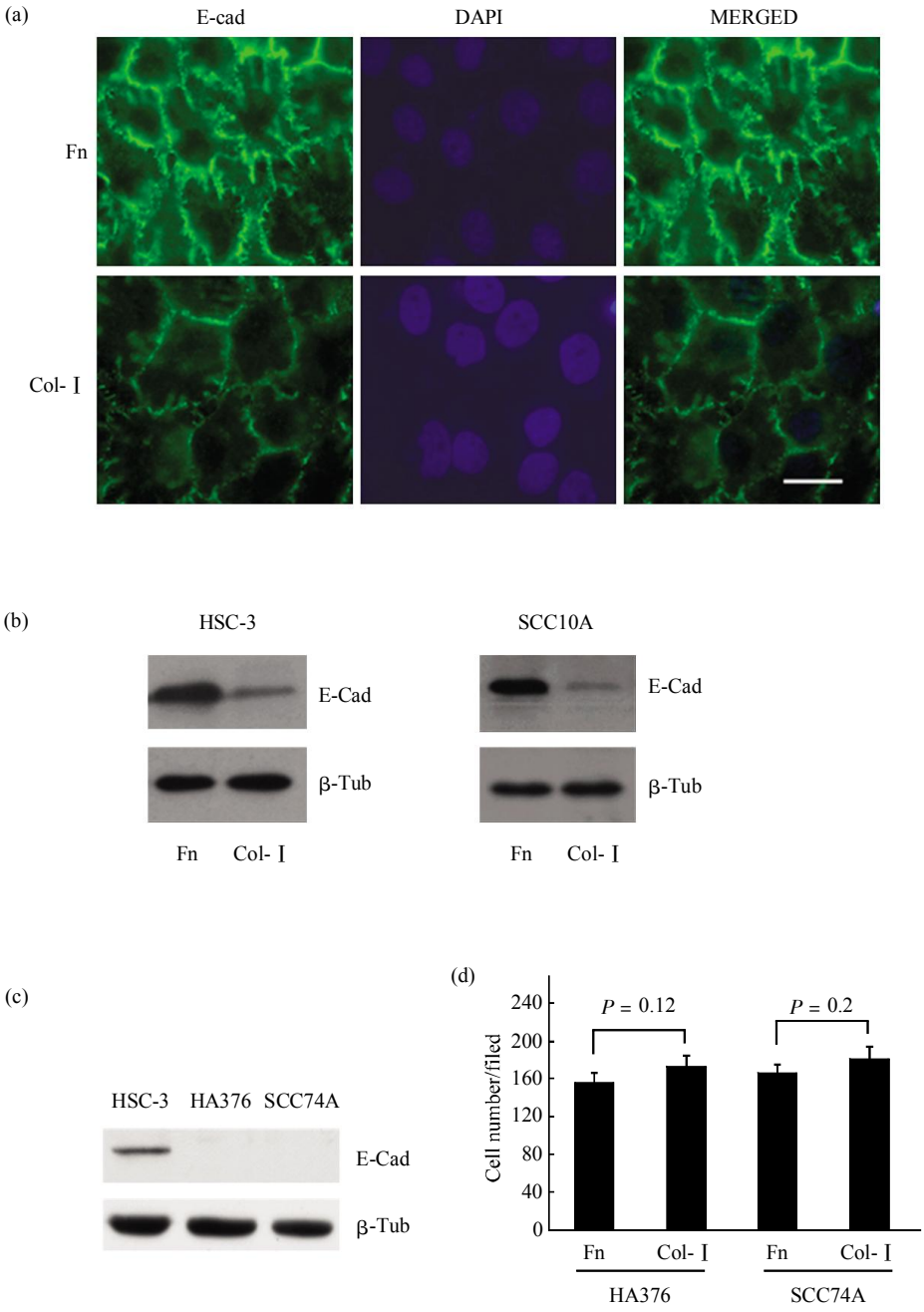

Fig. 3　Collagen type Ⅰ downregulates expression of E-cadherin

(a) E-cadherin expression of HSC-3 was detected by immunostaining for the cells on Fn and Col-Ⅰ substrates. (b) E-cadherin expression was analyzed by immunoblotting for the cells of HSC-3 and SCC10A on Fn and Col-Ⅰ substrates. β-Tubulin (Tub) was used as a loading control. (c) E-cadherin expression was detected by immunoblotting for the cells of HA376 and SCC74A. β-Tubulin (Tub) was used as a loading control. (d) HA376 and SCC74A cells were subjected to migration assays using transwells coated with Fn or Col-Ⅰ substrates.

2.4　Col-Ⅰ促进头颈鳞癌细胞 β-catenin 酪氨酸磷酸化

为了探讨 Col-Ⅰ下调头颈鳞癌细胞 E-cadherin 的机制，采用 Western blotting 检测在 Col-Ⅰ和 Fn 细胞基质上培养的 HSC-3 细胞 β-catenin 酪氨酸磷酸化水平．结果显示：与 Fn 比较，在 Col-Ⅰ基质

上培养细胞的 β-catenin 酪氨酸磷酸化水平明显升高(图 4a).

为了进一步证实 Col-Ⅰ促进 β-catenin 磷酸化，应用抗 β1-Integrin(Col-Ⅰ的受体)抗体封闭 β1-Integrin 受体，阻断由 Col-Ⅰ和 β1-Integrin 结合所介导的下游信号通路[18, 27, 32-33]，再采用 Western blotting 检测 HSC-3 细胞的 β-catenin 酪氨酸磷酸化水平．结果显示：β1-Integrin 在 HSC-3 细胞表面大量表达(图 4b)，与非特异性的鼠 IgG 抗体比较，抗 β1-Integrin 抗体封闭 β1-Integrin 受体后，β-catenin 酪氨酸磷酸化明显下调(图 4c).

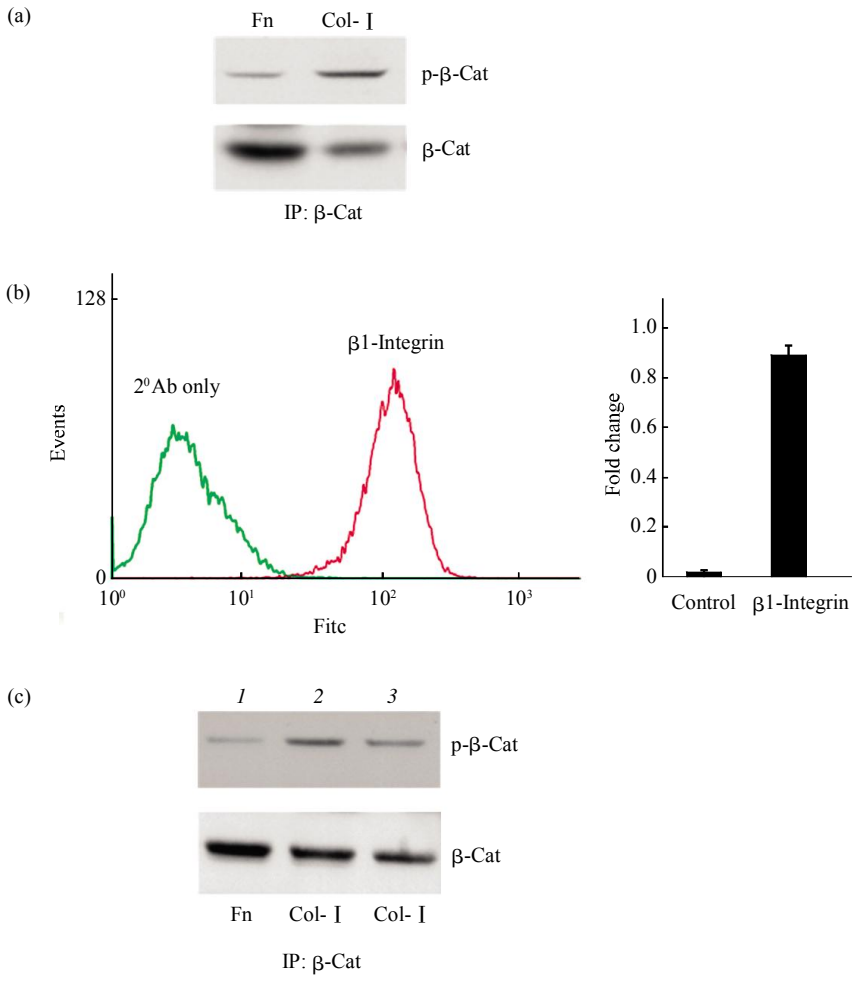

Fig. 4　Collagen type Ⅰ activates β-catenin by β1-integrin

(a) p-β-catenin and β-catenin were analyzed by immunoblotting for the cells on Fn and Col-Ⅰ substrates. (b) Flow cytometry analysis of integrin surface expression on HSC-3 cells. Right, fold change in mean fluorescence intensity. (c) The influence of β1-Integrin on activating phosphorylation was investigated by blocking of β1-integrin using a neutralizing antibody. *1*: HSC-3; *2*: ms IgG Ab; *3*: Anti-β1-Integrin Ab.

2.5　Col-Ⅰ促进头颈鳞癌细胞的增殖

MTT 实验结果显示：HSC-3 细胞在 Col-Ⅰ基质上的增殖速度明显高于在 Fn 基质上的增殖速度，在第 3 天和第 5 天，与对照组比较具有显著意义($P < 0.05$，图 5a)．另外，免疫细胞荧光染色结果显示：在 Col-Ⅰ基质上，PCNA 阳性细胞数目明显多于在 Fn 基质上的数目(图 5b)．结果提示：Col-Ⅰ具有促进头颈鳞癌细胞增殖的作用.

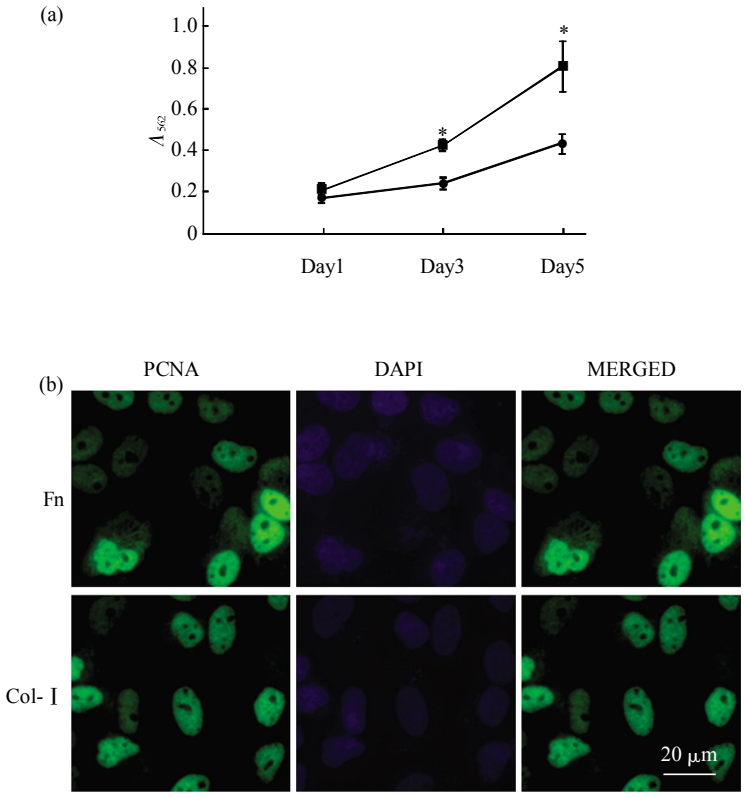

Fig. 5　Collagen type Ⅰ increases the cell proliferation

(a) The analysis of cell proliferation was by MTT. Bars represent absorption of the cells. Error bars, SE. (*P < 0.001). ●—●: Fn; ■—■: Col- Ⅰ. (b) HSC-3 cells grown on the substrates were costained with PCNA antibody and DAPI (bar, 20 μm).

2.6　Col- Ⅰ促进头颈鳞癌细胞 β-catenin 的核移位并上调 Cyclin D1 的表达

为了探讨细胞在 Col- Ⅰ基质上增殖加快的可能机制，采用 Western blotting 检测在 Col- Ⅰ基质上培养的 HSC-3 细胞 β-catenin 的细胞分布．结果显示：β-catenin 在细胞核内的表达明显增加(图 6a)，

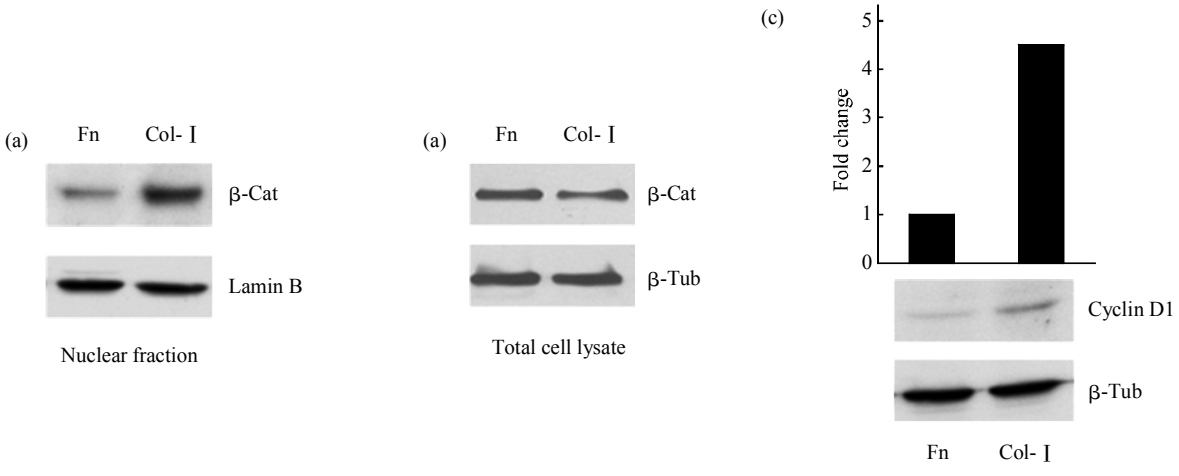

Fig. 6　Collagen type Ⅰ increases the expression of cyclin D1 and nuclear localization of β-catenin

(a) β-catenin from nuclear extracts of HSC-3 cells on different substrates were analyzed by Western blotting. The lamin B was used as a loading control. (b) A general change β-catenin in total protein lysate was analyzed by Immunoblots. (c) Cyclin D1 expression was analyzed by immunoblotting for the cells on Fn and Col-I substrates. β-Tubulin (Tub) was used as a loading control.

而 β-catenin 在细胞内的表达总量未见明显增加(图 6b). 由于 β-catenin 核内分布增加是促进细胞增殖的一个重要机制[34-35]，因此研究结果提示，Col-Ⅰ促进头颈鳞癌细胞增殖可能与 Col-Ⅰ促进 β-catenin 核移位有关.

β-catenin 核移位可诱导 β-catenin-Lef/TCF- 依赖的基因表达增加，Cyclin D1 是 Lef/Tcf 的一个重要的靶基因，同时也是一种重要的细胞周期蛋白[34-35]. 因此，采用 Western blotting 检测在 Col-Ⅰ基质上培养的 HSC-3 细胞 Cyclin D1 的表达. 结果显示：在 Col-Ⅰ基质上 Cyclin D1 蛋白的表达明显上调，其表达量是在 Fn 基质上的 4 倍(图 6c). 结果提示，Col-Ⅰ促进头颈鳞癌增殖可能与其促进 β-catenin 核移位、上调 Cyclin D1 蛋白的表达有关.

3 讨 论

转移是头颈部鳞癌(HNSCC)一个重要的生物学特征，也是 HNSCC 患者死亡的主要原因之一[3-4]. 转移是一个涉及多基因、多阶段、多步骤的复杂过程. 近年的许多证据表明，肿瘤转移与肿瘤的微环境紧密相关[15, 36-37]. Col-Ⅰ是肿瘤微环境中的一种重要组分[38-39]. 因此，研究 Col-Ⅰ在 HNSCC 转移的作用，具有十分重要的理论和实际意义.

本研究显示：Col-Ⅰ能增强 HNSCC 细胞的迁移、运动和扩散以及增殖，因此具有促进 HNSCC 细胞转移的潜能. 由于恶性肿瘤细胞之间黏附降低是癌细胞侵袭转移行为的重要分子基础，而钙黏蛋白 (cadherin) 是介导同型细胞间黏附的一种重要黏附蛋白，具有维持正常上皮完整性和极性的功能[40-41]，当其表达降低或黏附功能受损时，肿瘤细胞易于从原发灶脱离，发生侵袭转移[42-43]. 我们的结果显示，Col-Ⅰ能降低 HNSCC 细胞 E-cadherin 的表达，这可能是 Col-Ⅰ促进 HNSCC 细胞转移的机制之一. 另外，本研究还显示，Col-Ⅰ能上调 β-catenin 的磷酸化水平，由于 β-catenin 磷酸化能使 E-cadherin 的表达降低[30-31]，因此，我们推测 Col-Ⅰ通过上调 HNSCC 细胞 β-catenin 的磷酸化水平而导致 E-cadherin 表达下调.

关于 Col-Ⅰ促进 HNSCC 细胞增殖的机制，本研究显示，Col-Ⅰ能促进 β-catenin 核移位. 由于 β-catenin 核移位能激活 β-catenin-Lef /Tcf 转录因子，而 Cyclin D1 是 β-catenin-Lef /Tcf 转录因子的靶基因[34-35]. 因此，我们检测 Col-Ⅰ能否上调 HNSCC 细胞 Cyclin D1 的表达. 结果显示：Col-Ⅰ在增加 β-catenin 在核内表达的同时，也诱导 Cyclin D1的表达. 结果提示，β-catenin 核移位，激活 β-catenin-Lef/Tcf 转录因子导致靶基因 Cyclin D1 的表达可能是 Col-Ⅰ促进 HNSCC 增殖的机制之一.

本研究结果发现：Col-Ⅰ作为肿瘤微环境中的一种重要的细胞基质，具有降低 HNSCC 细胞黏附，促进细胞转移和增殖的功能，其可能机制涉及上调 β-catenin 的磷酸化水平以及促进 β-catenin 核移位. 研究结果为揭示 HNSCC 转移的分子机制，研究肿瘤微环境与 HNSCC 转移的关系奠定了理论和实验基础.

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Collagen Type Ⅰ Promotes Head and Neck Squamous Carcinoma Cell Metastasis and Proliferation *Via* β-Catnenin*

ZUO Jian-Hong[1,2)], LI Xin-Hui[1)], LI Mao-Yu[1)], WAN Xun-Xun[1)], ZENG Gu-Qing[1)], HE Qiu-Yan[1)], LI Jian-Huang[1)], XIAO Zhi-Qiang[1)]

([1)] *Key Laboratory of Cancer Proteomics of Ministry of Health of China, Xiangya Hospital, Central South University, Changsha* 410008, China; [2)] *School of Public Health, University of South China, Hengyang* 421001, China)

Abstract To detect whether Col-Ⅰ has some contribution to migration and proliferation of head and neck squamous carcinoma cell (HNSCC) in tumor microenvironment, transwells were applied to measure cell migration, cell velocity and cell scattering were analyzed by Zeiss Axiovert inverted microscope. The expression of E-cadherin as one of cell surface adhesion molecules and cell location of β-catenin were detected by Western blotting and/or immunofluorescence, then proliferation was analyzed by MTT and the PCNA expression. Results showed that Col-Ⅰ promoted cell metastasis, cell velocity and cell scattering, which E-cadherin was repressed possibly by the increased phosphorylation of β-catenin. The nuclear translocation of β-catenin led to an increasing expression of cyclin D1 and proliferation. Together, a conclusion is made that Col-Ⅰ promotes HNSCC metastasis *via* upregulation of phosphorylation of β-catenin and proliferation by the nuclear translocation of β-catenin.

Key words collagen type Ⅰ, tumor microenvironment, head and neck squamous carcinoma cell, metastasis, proliferation

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**Corresponding author.

Tel: 86-731-4327239, E-mail: zqxiao2001@yahoo.com.cn

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