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·论　著·

肿瘤相关肝星状细胞促进肝癌细胞上皮间质转化和迁移

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[摘要]　**目的**　建立人肝癌组织中肿瘤相关肝星状细胞(tHSCs)分离、培养方法,探讨tHSCs对肝癌细胞生物学行为的影响。**方法**　采用密度梯度离心法从新鲜切除的人肝癌组织样本中分离tHSCs,锥虫蓝染色检测细胞活力,细胞免疫荧光鉴定活化tHSCs表达α-平滑肌肌动蛋白的特征,光镜观察培养的tHSCs形态学变化;tHSCs无血清培养后,收集条件培养液,分别与肝癌细胞系QGY-7701、BEL-7402、SMMC-7721共培养,并以肝星状细胞系LX-2做对照,采用MTT法、细胞划痕实验、蛋白质印迹等方法检测肝癌细胞增殖、迁移和上皮间质转化(EMT)相关指标的表达。**结果**　从肝癌组织中分离的tHSCs活率在95%以上,纯度接近100%,在体外可进行传代培养与冻存。tHSCs能诱导3个受试肝癌细胞系产生典型的EMT样形态学变化及其分子标记物表达改变,表现为上皮标记物E-钙黏蛋白表达减少($P<0.05$),间质标记物波形蛋白表达增加($P<0.05$)。与此同时,tHSCs能促进培养的3个肝癌细胞系增殖和迁移。**结论**　从人肝癌组织中分离的原代tHSCs活力和纯度高,可用于tHSCs的生物学功能实验研究。tHSCs在诱导肝癌细胞产生EMT样改变的同时,体外促进肝癌细胞的增殖和迁移。

[关键词]　肝肿瘤;肝星状细胞;上皮间质转化;细胞增殖;细胞运动
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Tumor-associated hepatic stellate cells significantly promote epithelial-mesenchymal transition and migration of hepatocellular carcinoma cells

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[**Abstract**]　**Objective**　To establish a method for isolating and purifying tumor-associated hepatic stellate cells (tHSCs) from human hepatocellular carcinoma (HCC) tissues and to study their impact on the biological behaviors of HCC cells *in vitro*. **Methods**　The tHSCs were isolated from the fresh human HCC tissue by collagenase/pronase digestion, followed by density gradient centrifugation using Nycodenze. The viability of the isolated cells was determined by trypan blue staining assay. The tHSCs were identified by immunocytochemical staining of α-SMA, a specific marker for activated hepatic stellate cells. The morphologic changes of the tHSCs were observed under microscope. The conditioned medium (CM) of tHSCs cultured in serum-free DMEM was collected, and then used for co-culture with HCC cell lines QGY-7701, BEL-7402, and SMMC-7721. CM of human hepatic stellate cell line LX-2 was used as control. Cell viability, proliferation, migration potential and EMT-related protein expression changes were assessed by using the MTT assay, wound migration assay and Western blotting analysis, respectively. **Results**　The viability of isolated tHSCs was over 95%, with a purity about 100%, and the isolated tHSCs could be passaged and cryopreserved. tHSCs significantly induced typical EMT-like morphological changes and altered expression of molecular markers in the three HCC cell lines, with the epithelial marker E-cadherin significantly decreased ($P<0.05$) and the stromal marker Vimentin significantly increased ($P<0.05$). Meanwhile, tHSCs efficiently enhanced the proliferation and migration of HCC cells *in vitro*. **Conclusion**　We have established a method for isolating and purifying tHSCs from human HCC tissues, which can help future study on tHSCs. tHSCs can enhance the proliferation and migration of HCC

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cells through inducing EMT-like phenotype.

[**Key words**] liver neoplasms; hepatic stellate cells; epithelial-mesenchymal transition; cell proliferation; cell movement
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肿瘤微环境是一个复杂的综合系统，细胞外基质(ECM)和多种间质细胞是其主要组成成分[1]。肝脏特有的肝星状细胞(hepatic stellate cells，HSCs)是一种具有多向潜能的间质细胞，占肝脏非实质细胞约 13%[2]。肝脏损伤时，静息型 HSCs 被活化，转变成高增殖性肌成纤维细胞样细胞，表现为富含维生素 A 的脂滴减少和 α-平滑肌肌动蛋白(α-SMA)阳性。由于能够产生大量的 ECM，活化 HSCs 被认为是肝纤维化等慢性肝病发生发展的中心环节[2]。研究显示，活化 HSCs 与肝癌发生发展有关[3-4]。但有关研究均采用 HSCs 细胞系或来自于正常肝组织的 HSCs，而这些 HSCs 的表型与肝癌组织中的 HSCs 并不完全相同[5]。本研究从新鲜人肝癌组织中分离 HSCs(肿瘤相关 HSCs，tHSCs)，经鉴定均为活化 HSCs，并且能够诱导肝癌细胞产生上皮间质转化(epithelial-mesenchymal transition，EMT)样改变，体外促进肝癌细胞增殖和迁移。

1　材料和方法

1.1　*材料*　(1)新鲜人肝癌组织取自东方肝胆外科医院肝外四科肝癌切除样本，立即放入预冷的 DMEM 培养液中。(2)肝癌细胞株 QGY-7701、BEL-7402、SMMC-7721 和 HSCs 系 LX-2 购自中国科学院典型培养物保藏委员会细胞库/中国科学院上海生命科学研究院细胞资源中心。(3)主要试剂：DMEM 培养基干粉和胎牛血清(美国 Gibco 公司)；链酶蛋白酶、DNA 酶和Ⅳ型胶原酶(德国 Roche 公司)；碘海醇(Nycodenz，挪威 Axis-Shield 公司)；α-SMA、E-钙黏蛋白(E-cadherin)和波形蛋白(Vimentin)单克隆抗体(英国 Abcam 公司)。

1.2　*tHSCs 分离和培养*　新鲜人肝癌组织采用不含 Ca^{2+} 的 D-Hanks、胶原酶和链酶蛋白酶依次灌洗，然后置于含有胶原酶、链酶蛋白酶和脱氧核糖核酸酶的消化液中剪碎，37℃震荡 30 min。经筛网过滤制成细胞悬液，450×g 离心 5 min，弃上清。用 DMEM 培养液重悬细胞沉淀，并通过 50×g 速度离心 3 min 分离肝细胞。收集上清，以 400×g 速度离心 5 min×2 次，富集非实质细胞。细胞沉淀用 6 mL 含有 28.7% Nycodenz 的 DMEM 培养液重悬，并在 4℃以 1 400×g 速度离心 15 min。在 Nycodenz 层和 DMEM 层之间吸取细胞，DMEM 洗涤 2 次，在含 20%胎牛血清的 DMEM 培养基常规培养。

1.3　*tHSCs 条件培养液(tHSC-CM)和 LX-2 条件培养液(LX2-CM)收集*　在 6 孔培养板中分别接种 tHSCs或 LX-2，10^5 个细胞/孔，24 h 后用无血清 DMEM 清洗 2 次，然后在 2 mL 无血清 DMEM 中培养 24 h，收集培养液上清。

1.4　*条件培养液培养肝癌细胞*　肝癌细胞株 QGY-7701、BEL-7402 和 SMMC-7721 在常规培养液中接种培养 24 h 后改用 tHSC-CM 或 LX2-CM 进行培养。

1.5　*主要检测方法*　细胞增殖实验采用四甲基偶氮唑蓝法(MTT 法)[6]。细胞划痕实验参照文献[7]。蛋白表达分别采用细胞免疫荧光染色和蛋白质印迹法。后者结果经 ECL 显色后在 X 线片上曝光。运用 Quantity One 软件进行灰度扫描分析[7]。

1.6　*统计学处理*　采用 SPSS 12.0 软件进行统计分析。计量资料以 $\bar{x} \pm s$ 表示，组间均数比较采用 Student's t 检验。检验水平(α)为 0.05。

2　结　果

2.1　*分离的 tHSCs 活率、活化、纯度和形态学鉴定*　锥虫蓝染色检测分离的细胞活率在 95%以上。在激发波长 328 nm 的荧光显微镜下，富含维生素 A 的脂滴能自发产生蓝绿色荧光。图 1A 和 1B 显示，富含维生素 A 脂滴的 tHSCs 少见。细胞(爬片)免疫荧光染色结果显示，几乎所有的 tHSCs 均表达 α-SMA(图 1C～1E)。相差显微镜下可观察到新鲜分离的 tHSCs 呈圆球形，少量细胞胞质内含有折光性颗粒(图 1F)。2～3 d 后，细胞开始伸展并伸出伪足，呈现肌成纤维细胞样(图 1G)。5～7 d 后，细胞继续伸展部分细胞并相互融合呈片状(图 1H)，14 d 后，细胞铺满瓶底(图 1I)。以上结果表明，分离的 tHSCs 纯度接近 100%，并且几乎都处于活化状态。

2.2　*tHSCs 诱导肝癌细胞产生 EMT 样形态学变化*　在 tHSC-CM 中培养 48 h 和 72 h，肝癌细胞系 QGY-7701、SMMC-7721 和 BEL-7402 都发生明显的 EMT 样改变，表现为细胞之间松散，且呈现棒状、变长的成纤维细胞样形态。而 LX2-CM 的作用则不明显(图 2)。

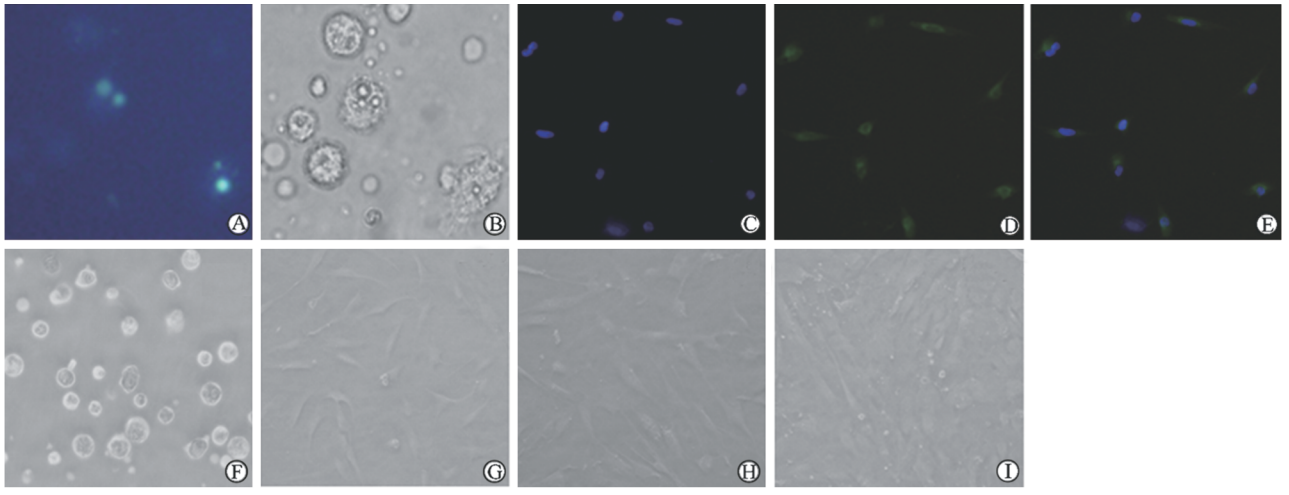

图 1　原代 tHSCs 分离培养和鉴定

Fig 1　Isolation and identification of primary tumor-associated hepatic stellate cells (tHSCs)

A,B: Vitamin A autofluoresence of primary tHSCs (A: 328 nm, B: White light); C-E: Primary tHSCs shows expression of α-smooth muscle actin (α-SMA). Cells were costained with 4,6-diamino-2-phenyl indole (DAPI) to identify nuclei (C: DAPI, D: α-SMA-fluorescein isothiocyanate, E: Merge); F-I: Primary tHSCs were isolated from primary hepatocellular carcinoma tissue. Images were taken after 0 d (F), 3 d (G), 7 d (H) and 14 d (I). Original magnification: ×200

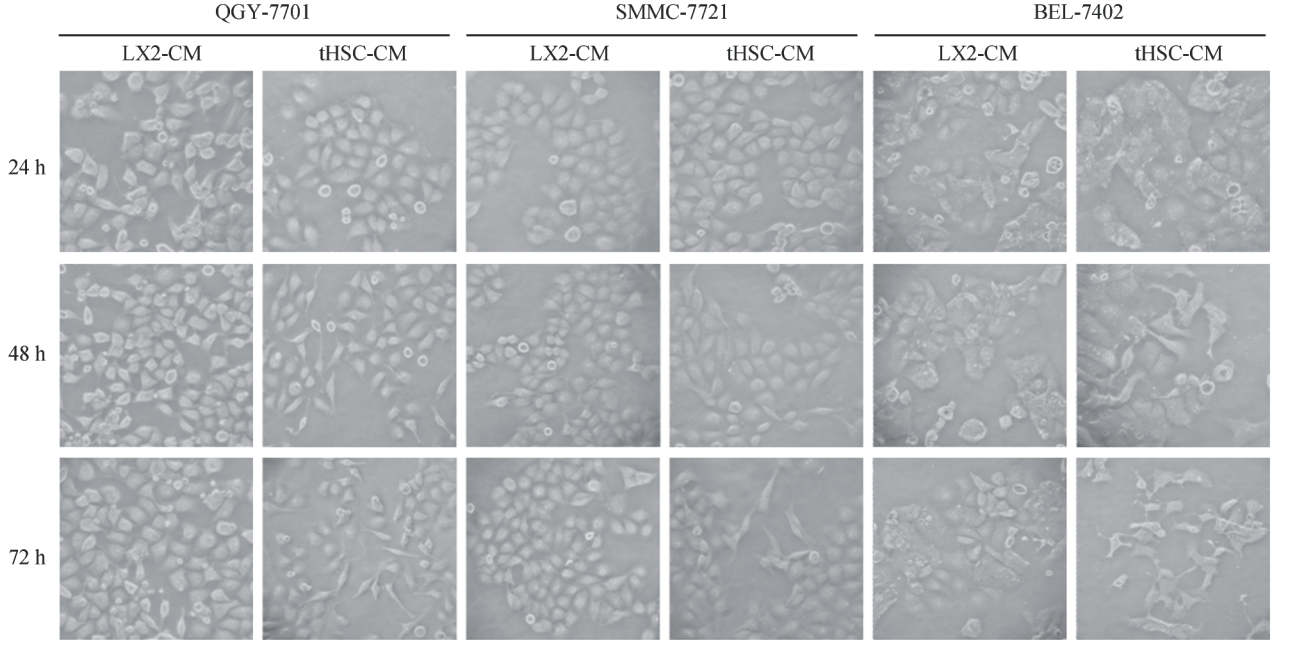

图 2　tHSC-CM 诱导肝癌细胞发生 EMT 样改变

Fig 2　Epithelial-mesenchymal transition (EMT) change of hepatocellular carcinoma (HCC) cell lines induced by conditioned media of tumor-associated hepatic stellate cells (tHSC-CM)

Phase contrast images of HCC cells undergoing EMT induced by primary tHSC-CM. Stimulated cells displayed an altered mesenchymal morphology. Original magnification: ×200

2.3　tHSCs 导致肝癌细胞 EMT 分子标记物表达变化　在 tHSCs 诱导肝癌细胞产生 EMT 样形态学变化的同时,蛋白质印迹检测结果显示,肝癌细胞表达上皮标记物 E-钙黏蛋白减少,而间质标记物波形蛋白表达则增加(图 3)。

2.4　tHSCs 促进肝癌细胞增殖和迁移　MTT 法检测结果显示,tHSC-CM 能提高肝癌细胞体外增殖能力,并在 72 h 内具有时间依赖性(图 4)。细胞划痕实验结果显示,tHSC-CM 可促进肝癌细胞迁移(图 5)。

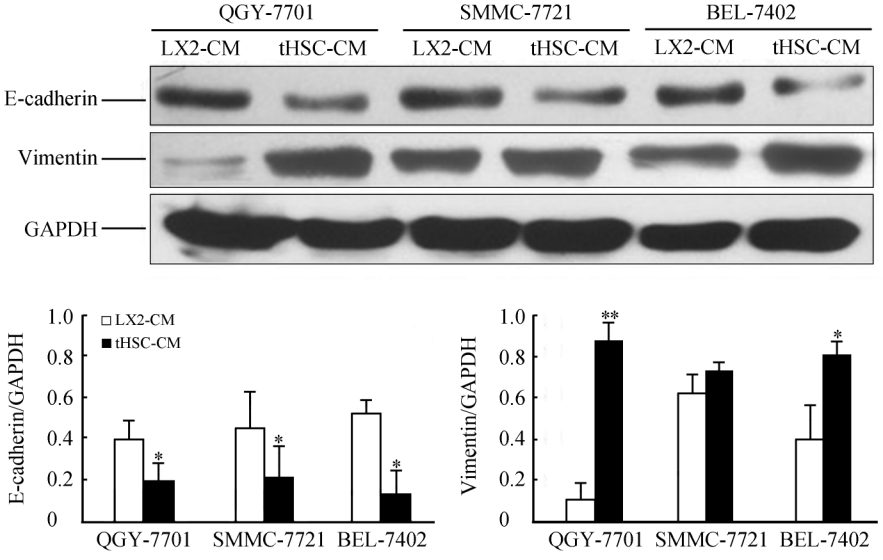

图 3　不同肝癌细胞系中 EMT 标记物表达变化

Fig 3　Expression of epithelial-mesenchymal transition (EMT) marker in hepatocellular carcinoma (HCC) cell lines

tHSC-CM: Conditioned media of tumor-associated hepatic stellate cell. $^{*}P<0.05$, $^{**}P<0.01$ vs LX2-CM group; $n=3$, $\bar{x}\pm s$

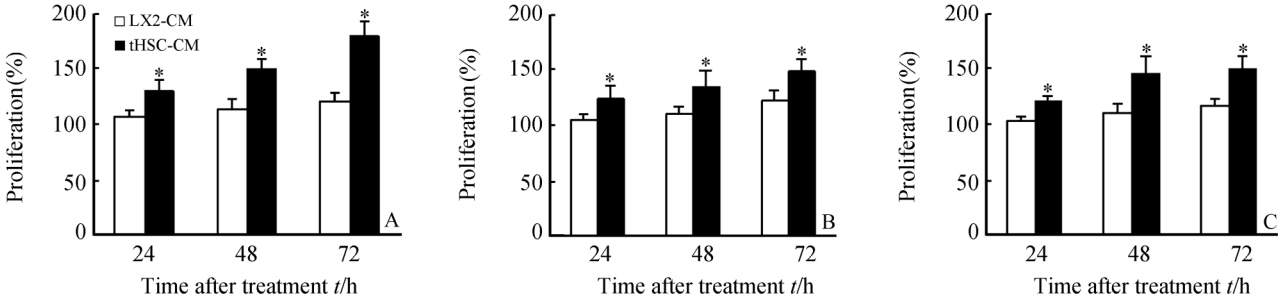

图 4　tHSC-CM 促进不同肝癌细胞增殖

Fig 4　Conditioned media of tumor-associated hepatic stellate cell (tHSC-CM) promoted proliferation of hepatocellular carcinoma (HCC) cell lines

A: QGY-7701; B: SMMC-7721; C: BEL-7402. MTT assays showed that primary tHSC-CM significantly promoted growth of HCC cell lines *in vitro*. $^{*}P<0.05$ vs LX2-CM group; $n=3$, $\bar{x}\pm s$

3　讨　论

HSCs 是存在于肝脏中的一种非实质细胞，主要位于肝脏的 Disse 间隙内，约占肝脏所有细胞的 5%～8%，占肝脏所有非实质细胞的 13%[2]。活体原位灌注消化是分离大鼠 HSCs 的一个常用方法，但由于肝脏质地不同等因素不适用于人肝癌组织原代 HSCs 的分离。我们借鉴国内外 HSCs 分离、培养的经验[8]，摸索出从肝癌组织中成功分离并培养原代 tHSCs 的实验方法，初步认为分离肝癌组织原代 HSCs 需要特别注意以下两点。首先，选取肝癌组织以质地柔软的白色鱼肉状组织为宜，尽量避免具有过多出血点和质地较硬的肝癌组织，否则灌注不充分，影响消化结果。其次，尽量减少消化和离心时间，最大程度地降低对细胞的损伤，以消化时间控制在 20～30 min 内为宜。细胞形态学和分子生物学鉴定结果表明，我们从肝癌组织中分离的原代 HSCs 活力和纯度高，并且几乎所有 HSCs 均处于活化状态，从而为进一步开展 tHSCs 的生物学功能研究奠定了基础。

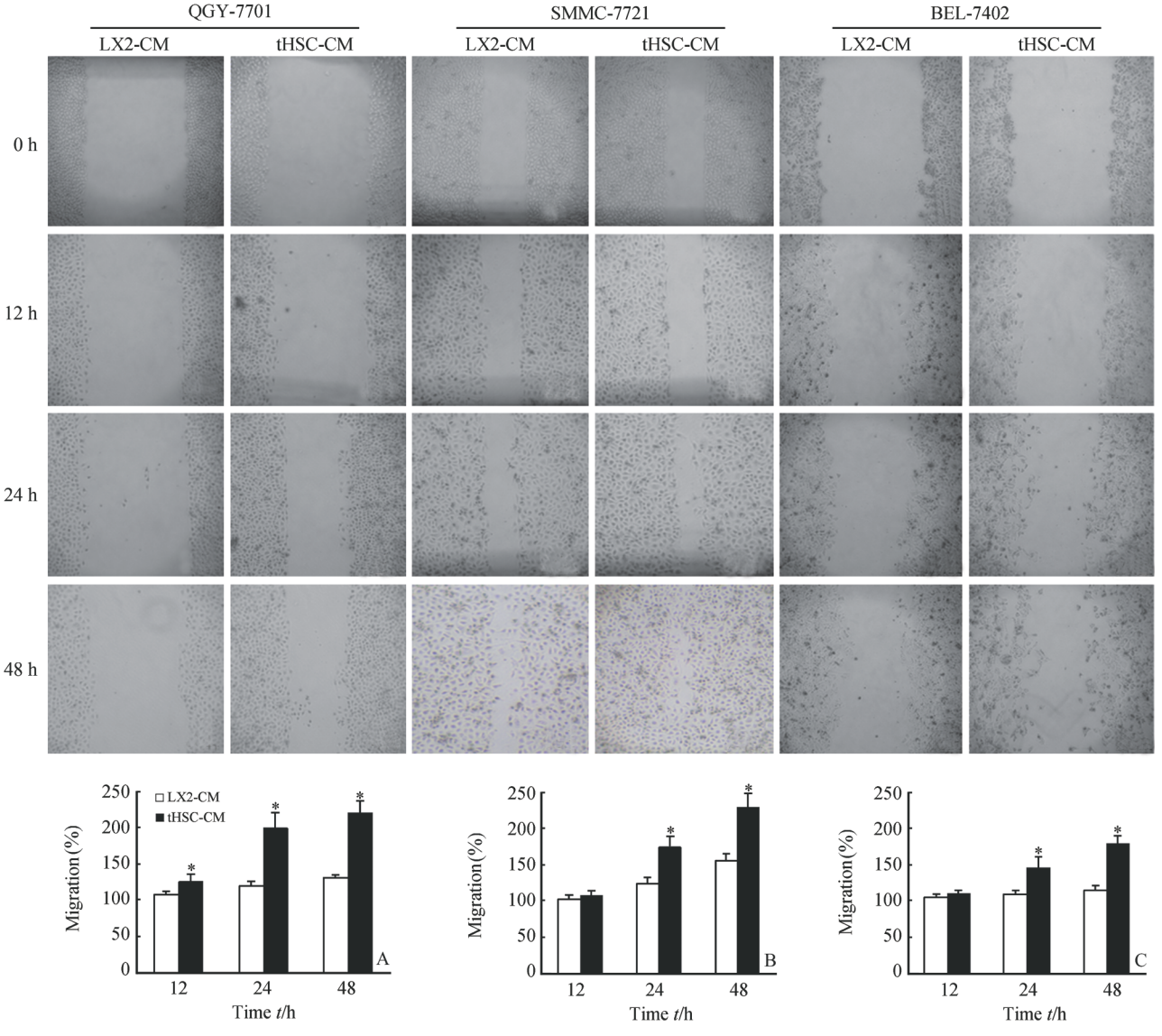

图 5　tHSC-CM 促进肝癌细胞迁移能力

Fig 5　Conditioned media of tumor-associated hepatic stellate cell (tHSC-CM) promoted migration of hepatocellular carcinoma (HCC) cell lines

A: QGY-7701; B: SMMC-7721; C: BEL-7402. Representative images of monolayer scratch assays indicated that primary tHSC-CM increased the migratory potential of different HCC cell lines. Images were taken after 0, 12, 24 and 48 h of culture. * $P<0.05$ vs LX2-CM group; $n=3$, $\bar{x}\pm s$

由于能够产生大量的 ECM,HSCs 增殖活化已被广泛认为是肝纤维化等慢性肝病发生发展的中心环节[2,9-13]。近年来研究显示,活化的 HSCs 和肝癌发生发展有着密切的关系[4,14-16]。推测肝癌细胞产生的一些细胞因子能够促进 HSCs 活化,而活化的 HSCs 不仅能够产生各种细胞因子,促进肝癌细胞增殖与分化[16-19],而且能够分泌可溶性蛋白引起间质改变,为肿瘤侵袭提供微环境的支持[20]。然而,现有的 HSCs 与肝癌关系的研究都仅限于 HSCs 系或者从正常肝组织中分离培养的原代 HSCs。而这些 HSCs 的表型并不完全相同于 tHSCs[5]。本研究首次应用 tHSCs 研究其对肝癌细胞生物学行为的影响,发现与 LX-2 相比,tHSCs 不仅能诱导肝癌细胞产生 EMT 样改变,而且还能促进肝癌细胞的增殖和迁移。这进一步说明 tHSCs 可能受到肝癌细胞的作用而被活化,使其表型明显不同于普通 HSCs。之所以选择 24 h 培养基,是因为 ELISA 检测结果显示 24 h 培养基中的多种细胞因子水平(生长因子-α 和-β、肝细胞生长因子、血管内皮生长因子、血小板衍生生长因子等)含量高于 48 h 和 72 h。

已知 HSCs 可分泌上述细胞因子，而这些细胞因子已被证实具有促进肿瘤细胞 EMT、增殖和侵袭的作用[3-4,21]。因此，推测在肝癌微环境中，tHSCs 通过分泌大量细胞因子参与肝癌细胞 EMT，从而增强肝癌细胞增殖和侵袭能力。

4　利益冲突

所有作者声明本文不涉及任何利益冲突。

[参 考 文 献]

[1] Mbeunkui F, Johann D J Jr. Cancer and the tumor microenvironment: a review of an essential relationship [J]. Cancer Chemother Pharmacol, 2009, 63: 571-582.

[2] Geerts A. On the origin of stellate cells: mesodermal, endodermal or neuro-ectodermal? [J]. J Hepatol, 2004, 40: 331-334.

[3] Friedman S L. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver[J]. Physiol Rev, 2008, 88: 125-172.

[4] Leonardi G C, Candido S, Cervello M, Nicolosi D, Raiti F, Trabali S, et al. The tumor microenvironment in hepatocellular carcinoma[J]. Int J Oncol, 2012, 40: 1733-1747.

[5] Xia Y, Chen R, Song Z, Ye S, Sun R, Xue Q, et al. Gene expression profiles during activation of cultured rat hepatic stellate cells by tumoral hepatocytes and fetal bovine serum [J]. J Cancer Res Clin Oncol, 2010, 136: 309-321.

[6] Li J, Liu P, Zhang R, Cao L, Qian H, Liao J, et al. Icaritin induces cell death in activated hepatic stellate cells through mitochondrial activated apoptosis and ameliorates the development of liver fibrosis in rats[J]. J Ethnopharmacol, 2011, 137: 714-723.

[7] Zhou Y M, Cao L, Li B, Zhang R X, Sui C J, Yin Z F, et al. Clinicopathological significance of ZEB1 protein in patients with hepatocellular carcinoma[J]. Ann Surg Oncol, 2012, 19: 1700-1706.

[8] Xu L, Hui A Y, Albanis E, Arthur M J, OByrne S M, Blaner W S, et al. Human hepatic stellate cell lines LX-1 and LX-2: new tools for analysis of hepatic fibrosis [J]. Gut, 2005, 54: 142-151.

[9] Gong W, Pecci A, Roth S, Lahme B, Beato M, Gressner A M, et al. Transformation-dependent susceptibility of rat hepatic stellate cells to apoptosis induced by soluble Fas ligand[J]. Hepatology, 1998, 28: 492-502.

[10] Gressner A M. Transdifferentiation of hepatic stellate cells (Ito cells) to myofibroblasts: a key event in hepatic fibrogenesis[J]. Kidney Int Suppl, 1996, 54: S39-S45.

[11] Purps O, Lahme B, Gressner A M, Meindl-Beinker N M, Dooley S. Loss of TGF-beta dependent growth control during HSC transdifferentiation[J]. Biochem Biophys Res Commun, 2007, 353: 841-847.

[12] Schnabl B, Choi Y H, Olsen J C, Hagedorn C H, Brenner D A. Immortal activated human hepatic stellate cells generated by ectopic telomerase expression[J]. Lab Invest, 2002, 82: 323-333.

[13] Schnabl B, Purbeck C A, Choi Y H, Haqedorn C H, Brenner D. Replicative senescence of activated human hepatic stellate cells is accompanied by a pronouced inflammatory but less fibrogenic phenotype[J]. Hepatology, 2003, 37: 653-664.

[14] 王　伟，官　阳，杨木兰，张春明. 活化肝星状细胞与肝细胞肝癌发生及转移的相关性[J]. 临床与实验病理学杂志，2007，23：492-494.

[15] Amann T, Bataille E, Spruss T, Muhlbauer M, Gabele E, Scholmerich J, et al. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma[J]. Cancer Sci, 2009, 100: 646-653.

[16] Coulouarn C, Corlu A, Glaise D, Gu non I, Thorqeirsson S S, Cl ment B. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepacellular carcinoma[J]. Cancer Res, 2012, 72: 2533-2542.

[17] Faouzi S, Lepreux S, Bedin C, Dubuisson L, Balabaud C, Bioulac-Saqe P, et al. Activation of cultured rat hepatic stellate cells by tumoral hepatocyte[J]. Lab Invest, 1999, 79: 485-493.

[18] King K Y, Goodell M A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response[J]. Nat Rev Immunol, 2011, 11: 685-692.

[19] Pinzani M, Gentilini A, Caligiuri A, De Franco R, Pelleqrini G, Milani S, et al. Transforming growth factor-beta 1 regulates platelet-derived growth factor receptor beta subunit in human liver fat-storing cells[J]. Hepatology, 1995, 21: 232-239.

[20] Jung J O, Gwak G Y, Lim Y S, Kim C Y, Lee H S. [Role of hepatic stellate cells in the angiogenesis of hepatoma][J]. Korean J Gastroenterol, 2003, 42: 142-148.

[21] Pinzani M, Marra F. Cytokine receptors and sigaling in hepatic stellate cells[J]. Semin Liver Dis, 2001, 21: 397-416.

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