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体外肿瘤干细胞富集方式

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摘要 尽管治疗手段越来越多, 癌症仍是全球第二大死亡原因。研究表明, 癌症治疗的困难主要根源于肿瘤的高度异质性及治疗耐药性。研发针对肿瘤干细胞(cancer stem cells, CSCs)的药物可以降低癌症患者死亡率, 因此靶向CSCs成为新型抗癌治疗手段。但目前来看, 在干细胞及细胞间相互作用的研究中, 仍存在干细胞难以捕获、捕获数量不足、捕获后无法按照预期生长趋势生长等问题, 因此用体外实验准确模拟体内实验, 仍是干细胞及肿瘤研究的瓶颈。该文综述了目前干细胞富集的主要方法及最新进展, 以期为未来CSCs的研究提供可行性的参考。

关键词 肿瘤干细胞; 干细胞富集; 肿瘤; 耐药

In Vitro Tumor Stem Cell Enrichment Modality

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Abstract Cancer remains the second leading cause of death in the world, despite the increasing availability of therapies. Studies have shown that the difficulty in cancer treatment is mainly due to the high heterogeneity of tumors and drug resistance. The development of drugs targeting CSCs (cancer stem cells) can reduce the mortality rate of cancer patients, and thus targeting CSCs has become a new type of anticancer therapy. However, in the study of stem cells and cell-cell interactions, stem cells are still difficult to be captured, insufficiently captured, and unable to grow in accordance with the expected growth trend after capture. Therefore, the accurate simulation of *in vitro* experiments into *in vivo* experiments is still a bottleneck in the study of stem cells and tumors. In this paper, the main methods and recent progress of stem cell enrichment are summarized, in order to provide a feasible reference for the future research of CSCs.

Keywords tumor stem cells; stem cell enrichment; tumor; drug-resistant

随着全球癌症发病率的持续上升[1], 癌症给家庭、社会造成了沉重的经济负担。目前, 手术、化疗和放疗等单一疗法是治疗不同类型癌症普遍接受的治疗方式, 以协同或相加的方式针对癌症机制的治疗是目前抗癌治疗的基石, 特别是对晚期和侵袭性癌症[2]。癌症治疗的困难主要根源于肿瘤的高度异质性、肿瘤细胞自给自足的生长信号, 对反生长信号不敏感, 不受控制的增殖和异常蛋白质的快速积累, 抗凋亡, 永生, 持续的血管生成, 组织侵袭和转移等特点[3]。CSCs理论认为, 癌症细胞的层次结构是由CSCs维持的, 它们产生于组织特异性的干细胞或祖细胞, 能够在组织内产生不同类型的功能细

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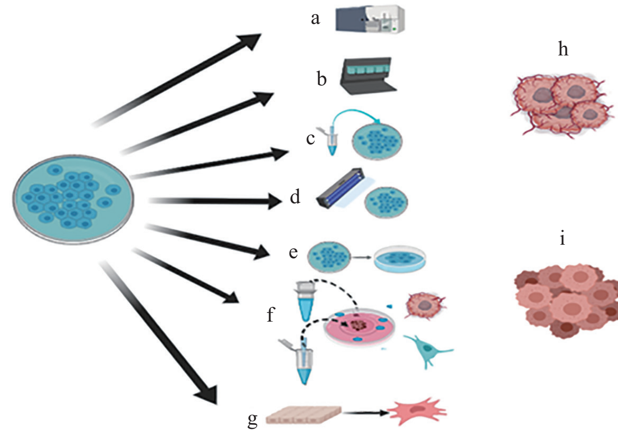

a: 流式分选; b: 磁珠分选; c: 化疗富集; d: 放疗富集; e: 球体形成; f: 3D共培养; g: 上皮–间质转化; h: 肿瘤干细胞; i: 肿瘤细胞。
a: flow cytometry; b: magnetic activated cell sorting; c: chemotherapy enrichment; d: radiotherapy enrichment; e: spherogenesis; f: 3D alginate-based platfoeming; g: epithelial-mesenchymal transition; h: cancer stem cells; i: cancer cells.

图1 干细胞富集方法示意图

Fig.1 Schematic diagram of stem cell enrichment methods

胞, 因此, CSCs为致癌基因如何导致癌细胞的无序行为增加了一个新的维度[4]。研究表明, CSCs是癌症复发的重要因素[5-6], CSCs在放化疗期间保持静止状态, 并可在癌症治疗后重新启动肿瘤生长。耐药干细胞被移植到天然宿主体内后, 仍具有再生和分化的潜力以及耐药性和致瘤性[7]。因此, 无法根除CSCs是解释癌症治疗耐药性、复发和转移的最受支持的理论之一[8], 设计针对CSCs的治疗应该可以降低癌症患者的死亡率, 但目前来看, 在干细胞及细胞间相互作用的研究中, 仍存在干细胞难以捕获、捕获数量不足、捕获后无法按照预期生长趋势生长等问题, 因此用体外实验准确模拟体内实验, 仍是干细胞及肿瘤研究的瓶颈。因此, 寻找合适的方法捕获CSCs, 甚至操控细胞按照组织中的细胞形态变化规律健康生长及构建多功能的干细胞肿瘤模型, 依然是干细胞研究的重中之重。本文旨在通过对目前常用的CSCs富集方式作一综述(图1), 为未来干细胞的研究提供可行的研究策略, 也为CSCs的靶向治疗奠定基础。

1 干细胞富集理论

自干细胞富集理论被提出以来, 干细胞富集方式逐渐被优化。目前, CSCs的富集方法主要包括流式分选及磁珠分选技术、放化疗耐药分选技术、侧群分选技术、肿瘤球培养法、3D细胞共培养技术[9]。其富集机制主要包括: (1) 从肿瘤细胞中泵出药物的ABC转运蛋白的高表达; (2) 增强DNA修复能力; (3) 抗凋亡[7]。近几年来, 随着富集方式的不断多样化、便捷化, 已从多种人类肿瘤如乳腺癌[10]、胰腺癌[11]、前列腺癌[12-14]、头颈癌[13,15]、结肠癌[16]、肝癌[17]和膀胱癌[18-19]等中分离出CSCs。前期研究表明, CSCs分离采用了多种策略: 基于干细胞表面标记、细胞内酶活性、活性氧浓度、线粒体膜电位、启动子驱动的荧光蛋白表达、自体荧光、贴壁或悬浮培养、细胞分裂、侧群细胞鉴定、侵袭性或黏附性、对细胞毒性化合物或缺氧的抵抗力、免疫选择和物理特性[20]。随着人们对富集纯度要求的不断提升, 体内序列稀释肿瘤发生试验(逐渐稀释细胞浓度, 使其全数解离成单细胞悬浮液, 观察每个孔中形成的肿瘤球数量)不断流行, 且该试验被认为是评价CSCs性质的金标准[21]。也有不少学者建立了CSCs模型, 验证了传统富集方式所富集CSCs的干性, 同时也验证了传统富集方式的高效性, 结果揭示了CSCs的高度致瘤性。但应用体内序列稀释肿瘤发生试验处理细胞耗时耗财, 一般不作为CSCs富集的常规方式。因此, 传统富集方式就成为一种不可替代的CSCs富集模式。

2 常用的干细胞富集方式

2.1 基于表面标志物的分选技术

几乎所有实体瘤都具有的特征性表面抗原标志物, 如CD44、CD133、CD27、CD24等, 已成为干细胞研究领域极为活跃的细胞表面标志[15,22], 也被证明是CSCs的特异性标志。据文献报道显示,

肿瘤细胞表面标志与肿瘤的发生、发展、耐药及复发等关系密切[23]。已有研究使用基于抗体的细胞分选技术将细胞分离为CSCs和非CSCs, 以测试这两种不同细胞群对治疗的响应性。目前, 基于CSCs的分选方法主要有流式细胞术(flow cytometry, FCM)和免疫磁珠分离技术(immunomagnetic bead separation techniques, IMB)[24]。FCM是一种具有独特优点的分选纯化技术, 是一项对高速流动的细胞或细胞亚群进行快速定量测定的实验技术, 已被广泛应用于许多领域。该技术方法主要是通过选择适宜的流式细胞仪, 调试参数后, 根据干细胞的形态确定其在流式细胞仪中的大小以及颗粒度等特征, 限定分选条件后, 在FSC/SSC散点图(FSC代表活细胞大小, SSC代表活细胞颗粒度)中设门圈活细胞群, 排除凋亡细胞和碎片, 再按照相应表面标志物质设计分选方案获取目标细胞, 分选结束后记录细胞数量。该方法首先应用于血液学研究, 以收获CD34$^+$细胞总数, 了解收集PBSC(peripheral blood stem cell)过程中血液量对CD34$^+$细胞产量的影响[25]。FCM技术的应用已相对成熟, 在乳腺癌[26]、鼻咽癌[27]、结直肠癌[28-29]、非小细胞肺癌[30-31]、卵巢癌[32-33]等肿瘤中的应用已相当广泛。该分选方式可以通过有序分选使干细胞捕获率提高。但是由于该技术设备价格昂贵, 对无菌条件要求高; 给细胞带来的压力可能影响其生物学行为; 且干细胞是一个稀有群体, 分选过程需要消耗大量细胞, 导致高昂的实验成本[34-35]等因素, 使得应用FCM富集干细胞较为困难。磁活化细胞分选(magnetic activated cell sorting, MACS)或磁激活细胞分选(magnetic activated cell sorting, MACS)技术是一种高效、简便的细胞分离纯化方法。由于该方法涉及免疫磁珠, 也被称为IMS。磁珠上覆盖着一种抗体, 该抗体能与细胞特异表面标志物结合。相较于FAS, MACS的磁珠直径只有50 nm, 因此对细胞的机械压力很小。分选过程是无菌的, 更有利于后续培养[24,36]。磁珠由氧化铁和多糖涂层制成, 对细胞生物学和细胞活力影响也不大。因此, MACS在CSCs富集中的应用也相当广泛。WANG等[37]通过MACS法从人卵巢癌细胞系高效地分离了卵巢癌干细胞, 研究了miRNA在调节CD44$^+$卵巢CSCs的干细胞样特性中的重要作用, 且MACS法在其他肿瘤中也得到了广泛应用。但相对来说, 用该方法处理细胞耗时, 磁珠价格昂贵, 排序单元格有限, 表面标记单一。相关研究表示, 应用MACS技术分离CSCs后通过FCM方法确认所分离的CSCs纯度(细胞表面抗原与抗体一起孵育)是筛选CSCs的可靠方法[38-39]。如前所述, FCM和MACS仍然是临床应用中常用的功能强大的细胞分选方法[40]。

2.2 基于化疗反应性的富集方式

有研究表明肿瘤进展与化疗耐药性相关[41-44]。肿瘤细胞在体外长期暴露于一种化疗药物会被诱导形成多药耐药肿瘤细胞系[45], 耐药的公认机制之一是外排泵转运蛋白的过度表达, 外排转运体可主动将化疗药物泵出肿瘤细胞, 从而抑制细胞内药物积聚, 增加药物外排量, 最终导致多药耐药[46], 也有人提出耐药是通过化疗改变一些关键的凋亡机制(如DNA损伤)和减少细胞内的活性氧(reactive oxygen species, ROS)来逃避化疗应激[47-48]。化疗的主要目的是通过药物介导DNA损伤, 然而CSCs对DNA损伤药物(如顺铂)并不敏感, 反而增强了DNA损伤反应[18]。目前, 有两种理论认为耐药是在细胞水平上产生的化疗耐药表型。一种理论强调了长期接触特定抗癌药物后产生的耐药性。另一种理论认为肿瘤块内存在一小群具有耐药表型的CSCs[49]。在治疗阶段, 肿瘤细胞内的特定反馈通路已被证明可以促进干细胞富集, 化疗诱导更多分化细胞死亡导致伤口愈合反应, 并导致治疗期间CSCs的激活和增殖[50]。这是产生耐药的重要扳机点。研究表明, CSCs具有衰老特性, 这是一种应激反应性细胞周期阻滞, 衰老相关的干性在摆脱细胞周期阻滞后发挥其有害的、高度积极的生长潜力, 这对化疗耐药性和复发至关重要[51]。已发现激活Wnt/β-连环蛋白[52]、Notch[53]、PI3K/Akt/mTOR[54]等信号通路是诱导干细胞富集的关键步骤。因此, 也有不少学者提出抑制相关信号通路可减少干细胞富集达到抗肿瘤的治疗目的[55-57], 但这缺乏可靠的临床试验。目前, 临床上常用的化疗药物有紫杉醇、多西紫杉醇、顺铂、5-氟尿嘧啶、多柔比星、阿霉素和甲氨蝶呤[58-62]等, 这些药物也被用于体外化疗耐药性检测。EI-ASHMAWY等[63]研究发现重复低剂量化疗(所谓节律化疗)可诱导强烈的抗肿瘤作用, 其优点是无毒。因此, 利用体外化疗选择性地富集CSCs可能为选择靶向CSCs的药物提供一种方法。但不可忽略的是, 该方法短期富集效果差[46], 富集纯

度较高的CSCs需花费的时间长，且要结合使用干细胞表面标记物分析富集干细胞的纯度。

2.3 基于放疗反应性的富集方式

放射治疗已广泛应用于癌症治疗多年，它是利用高能量射线照射癌组织，破坏癌细胞染色体，从而达到控制恶性肿瘤生长的一种治疗方法。放疗使处于生长期的敏感细胞被杀死而产生对放射线抵抗的“静止期”细胞，以此增加癌细胞亚群的抗药性，导致癌细胞的生物行为恶化。这是放疗后CSCs富集的结果。相关研究表明，辐照导致了细胞形态变化，促进了上皮–间质转化(epithelial mesenchymal transformation, EMT)表型和干细胞标志物的增加[64]，并诱发了DNA损伤，导致了DNA修复的激活，进而诱导了干细胞表型的获得[65]。JABBARI等[66]将CSCs辐射治疗产生的抵抗力归因于缺氧诱导的细胞对活性氧的防御增强而导致DNA修复水平的升高，也有学者深入研究了辐射抗性的分子基础、CSCs抵抗治疗的机制(DNA修复蛋白的过度表达)及降低CSCs对DNA损伤反应的敏感性[67-68]。据报道利用多次分割剂量射线照射可建立干细胞富集模型[69]，对富集细胞进行干细胞标记物及蛋白表达检测，并通过体内成瘤试验验证其致瘤能力，结果显示了放疗富集干细胞的可行性，该方法类似于上述体内序列稀释肿瘤发生试验。但是辐射会损害干细胞造成干细胞的替代不足及干细胞的增生能力受损，伴随的细胞毒性作用无法修复[70]。因此，该富集方法仍存在一定的局限性，是否可应用于实验研究仍存在很大的争议，本研究组认为在有条件进行上述几种富集方式的情况下，基于放疗反应性的富集方式不作为首选。

2.4 基于肿瘤球形成的富集方式

肿瘤球形成实验最常用于研究CSCs的干性、自我更新和克隆形成性[8]，该实验通过无血清悬浮培养从大块肿瘤中富集最完整的CSCs群体，因此也叫无血清悬浮培养，被认为是从整个肿瘤中富集CSCs亚群的最佳方法[71]。该方法将肿瘤细胞培养于补充有10 ng/mL表皮生长因子(epidermal growth factor, EGF)、10 ng/mL碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)、10 ng/mL Noggin蛋白和1 000 U/mL白血病抑制因子(leukemia inhibitory factor, LIF)的DMEM培养基中，肿瘤细胞于14天后形成集落，使CSCs得到富集和扩增，并保持了其干细胞特性[53]。因此不少学者选择球体形成法来分离CSCs进行耐药机制的研究。ZHAO等[72]研究发现肿瘤球培养物用某些肿瘤生物标记物丰富了CSCs的不同亚群，而单个细胞表面标记物的选择只丰富了一个CSCs亚群，且通过无血清培养基悬浮培养形成的球形细胞可以在形态和功能上分化为内皮细胞，揭示了传统抗血管生成治疗产生耐药性的新机制。由此可见，肿瘤球富集方法丰富了来自大量肿瘤的最完整的CSCs群体。也有人通过肿瘤球富集法从原发性喉癌(primary laryngeal carcinoma, PLC)中分离了CSCs，并通过免疫荧光、流式细胞术及qRT-PCR技术检测了干细胞标记物CD133、CD44、OCT4、SOX2和NANOG4的表达情况[73]。悬浮球培养富集的球形$CD90^+$HepG2干细胞，也被用于研究抗肿瘤免疫效能[74]，对铂耐受的细胞可形成更多的球体，表达CSCs相关标记物[75-76]。后来，GORIČAN等[77]开发了一种新的CSCs富集球体模型(cancer stem cell enrichment sphere model, SCESM)，该模型比传统的自由悬浮球体培养富集球形干细胞花费时间更短，并且具有比常用的MCTS模型更强的CSCs富集能力，SCESM适用于抗CSCs化合物的高温超导分析，要求使用的细胞要适合于CSCs富集，并具有表达干细胞标记物的高基础水平，且已知可以形成致密的球体。将球体使用干细胞培养基进行培养，可以显著提高SCESM球体数量。然而，目前尚不清楚这些球形富集的CSCs在多大程度上代表原位肿瘤中的原始CSCs。要想验证体外富集效能，还需要检测一些CSCs标记物的表达量或进行体内致瘤试验[78-79]，这将使得基于肿瘤球形成的富集方式更具说服力。

2.5 基于肿瘤细胞3D培养的富集方式

研究表明，肿瘤基质细胞与肿瘤细胞关系密切，涉及肿瘤的进展、侵袭、转移和血管生成。QIAO等[6]建立了一种有效的CSCs和基质细胞共培养模型，该模型专注于CSCs和基质细胞之间旁分泌信号的相互作用。该作用导致CSCs生态位的形成，并在癌症放化疗期间促进其存活。上述研究主要基于CSCs和基质细胞共存原理，通过3D藻酸盐体系从体外细胞系中富集CSCs，靶向基质细胞以根除CSCs。该体系由两部分组成：上层是通过钙离子交联形成的海藻酸盐–透明质酸水凝胶微胶囊，下层是通过电荷交换形成的海藻酸盐–壳聚糖水凝胶。简单来说，研究者从生物公司得到氧化的海藻酸盐，并将

其溶解在蒸馏水中, 通过海藻酸盐与钙离子的直接交联制备用于细胞培养的海藻酸盐基水凝胶微胶囊, 采用细胞生长因子EGF和bFGF固定氧化海藻酸盐和低分子量透明质酸后使其与钙离子交联以期制备CSCs培养物。2周后, 使用柠檬酸钠释放CSCs, 并通过胰蛋白酶将CSCs消化成单细胞。将海藻酸盐与壳聚糖直接混合后形成的海藻酸盐–壳聚糖水凝胶, 构成了基质细胞良好的生长环境。由此形成了CSCs富集体系的两部分, 一部分适应CSCs生长, 一部分适应基质细胞生长, 通过此方法不仅分离了CSCs, 也为基质细胞如何影响CSCs提供了可行性研究。也有学者利用橡胶微模具有效地富集了一定量的CSCs, 将癌细胞制成了3D癌细胞球体, 该球体的CSCs生物标记物(CD44、CD44v6、CD133)表达比例明显高于流式细胞术分析[80]。3D球体培养已被用于刺激和维持癌细胞中CSCs亚群的自我更新能力[81], 并介导CD133及CSCs中与自我更新和增殖相关的转录因子的表达[82], LUO等[83]研究发现与从传统肿瘤球培养中获得的CSCs相比, 那些在3D支架上生长的CSCs表现出更强的CSCs特性, 这表明基于生物材料的3D培养平台能更好地维持恶性肿瘤的生长状态。因此, 3D细胞培养能在细胞培养过程中为细胞提供一个更加接近体内生存条件的微环境[84]。基于生物材料的3D培养体系进行的干细胞富集可能为临床提供更有效的预测数据, 也有助于未来的CSCs靶向治疗研究和抗CSCs药物筛选应用。尽管近年来CSCs 3D培养模型的研究取得了进展, 但仍存在局限性, 如缺乏在CSCs研究中被广泛接受和批准的干细胞标记物。类器官培养是一种干细胞研究模型, 主要通过将CSCs嵌入基质胶或细胞外基质, 也可从个体标本中生成CSCs, 以开发个性化的治疗方案, 该研究模型目前应用也相对较为成熟[85-88]。但是构建类器官存在技术困难, 并且通常需要临床标本, 因此并非在每个研究CSCs的实验室中都可以进行类器官研究。因此, 在未来的研究中, 亟需努力去探索上述技术在CSCs研究中的不足, 为肿瘤研究提供更新的技术支持[89]。

2.6 基于Hoechst染色侧群分选的富集方式

这是一种相对传统的CSCs富集方法, 这种方法主要根据细胞排出33342染料的能力对细胞进行分析和分类[90]。由于早期研究对实体肿瘤表面抗原标志物的研究较少, 因此, 侧群分选技术(side population, SP)成为分选CSCs的主要方法。自YU等[91]发现一小部分细胞显示出了明显的Hoechst低染的特征以来, SP的方式逐渐被很多学者应用。国内外研究表明SP细胞与干细胞具有相似的生物学特性[92-93], 因此, SP逐渐成为鉴定CSCs的一种可行性方法。SP的分析方法不依赖于细胞表面标记的表达, 而是基于ABCG2转运蛋白高度表达于干细胞的特性[24]。已有研究发现Hoechst低染的细胞在具有CSCs特性(如自我更新和高致瘤性)的细胞中富集[94-95]。但是随着富集方式的多样化和对干细胞纯度要求的不断提升, SP细胞分离技术逐渐淡出人们的视野, 且SP细胞分离技术存在一定的缺陷, 难以全面反映CSCs的生物学特性[6-7], Hoechst还可能影响细胞的存活, 因此, 近些年来SP细胞分离技术已被许多学者淘汰。

2.7 基于EMT诱导的富集方式

EMT作为转分化程序和肿瘤进展的关键过程, 与CSCs和具有干细胞样特性的细胞的扩增增加呈正相关[96]。研究发现, 肿瘤细胞通过EMT过程获得侵袭性和转移性[97], 并对传统疗法产生耐药性, 且经历过EMT的细胞具有干细胞的特征[98-99]。目前, 乳腺癌[100]、头颈癌[101]、肝癌[102]等肿瘤中的研究均表明具有EMT样特征的细胞同时也可表现出干细胞特征。BASU等[103]从定义上、表型上及功能上详述了CSCs和EMT样细胞之间的联系, 并提出相关证据证明了EMT可降低抗肿瘤治疗疗效、参与肿瘤复发、维持肿瘤休眠状态及介导肿瘤的转移扩散。根据这些发现, 基于ECM天然成分或其类似物的生物材料通常被用于构建CSCs富集平台[36]。E-钙黏蛋白(E-cadherin)是一种膜蛋白, 在肿瘤侵袭、转移和预后中发挥作用, E-cadherin的下调是EMT的一个标志, 利用携带E-cadherin shRNA的慢病毒载体感染肿瘤细胞, 使肿瘤细胞中的E-cadherin表达下调, 诱导形成肿瘤EMT模型, 由此构建了CSCs样细胞富集模型[20]。后来, FRANCESCANGELI等[104]在CRC患者中, 发现了包含一群具有干细胞特征的静止/慢循环细胞(quite/slow circulating cells, QCs/SCCs), 其特征是具有预期的间充质样化疗耐药表型, 该细胞群的发现验证了EMT诱导干细胞的理论。TANG等[105]系统总结了EMT在肿瘤发生研究及CSCs富集、增殖、迁移、侵袭和耐药性方面的作用, 为肿瘤生态位的研究奠定了基础。尽管如此, 通过该方法富集的CSCs不利于长期培养, 它们很容易分化, 因此未

来还需要深入研究以确定该方法的实用性。

3 总结与展望

综上所述, 将CSCs与肿瘤进展联系起来的大多数证据来自肿瘤细胞系和/或动物模型的研究, 提取CSCs并建立预期肿瘤干细胞模型对于干细胞生物标志物及肿瘤耐药及转移的研究至关重要。随着CSCs相关研究的不断深入, 多种新的CSCs分离方法逐渐涌现。由于不同CSCs亚型、代谢以及器官特异性定植和转移之间的差异, 使CSCs富集存在难度, 但是临床上对于CSCs的鉴定尚缺乏统一的标准, 导致CSCs纯度较低。任意一种富集方式都不可避免地会对细胞本身及其生长方式产生影响。因此需要研究者根据具体的实验条件、细胞来源、研究需要等精确地评估每种方法的优缺点, 以选择最有效的方法。体外CSCs富集方法的不断改进有助于更好地模拟体内肿瘤微环境, 并大大提高干细胞靶向治疗效果以及对肿瘤发展和异质性的理解, 也有助于开发和确定CSCs治疗的新药。相信未来针对于CSCs富集的研究将不断创新化、高效化和实用化。

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