Biomarker discovery for renal cancer stem cells

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Abstract

Characterised by high intra- and inter-tumor heterogeneity, metastatic renal cell carcinoma (RCC) is resistant to chemo- and radiotherapy. Therefore, the development of new prognostic and diagnostic markers for RCC patients is needed. Cancer stem cells (CSCs) are a small population of neoplastic cells within a tumor which present characteristics reminiscent of normal stem cells. CSCs are characterised by unlimited cell division, maintenance of the stem cell pool (self-renewal), and capability to give rise to all cell types within a tumor; and contribute to metastasis in vivo (tumourigenicity), treatment resistance and recurrence. So far, many studies have tried to establish unique biomarkers to identify CSC populations in RCC. At the same time, different approaches have been developed with the aim to isolate CSCs. Consequently, several markers were found to be specifically expressed in CSCs and cancer stem-like cells derived from RCC such as CD105, ALDH1, OCT4, CD133, and CXCR4. However, the contribution of genetic and epigenetic mechanisms, and tumor microenvironment, to cellular plasticity have made the discovery of unique biomarkers a very difficult task. In fact, contrasting results regarding the applicability of such markers to the isolation of renal CSCs have been reported in the literature. Therefore, a better understanding of the mechanism underlying CSC may help dissecting tumor heterogeneity and drug treatment efficiency.

Keywords: cancer stem cells; tumor-initiating cells; renal cell carcinoma; biomarkers

Received 4 September 2017; Revised 27 November 2017; Accepted 13 December 2017

No conflicts of interest were declared.

Introduction

Renal cell carcinoma (RCC), a malignant tumor affecting the adult kidney, accounts for 2% of all cancers. It affects 64,000 people every year, with a mortality of 20%, and is among the 10 most common cancers worldwide [1]. Arising from renal tubular epithelial cells, RCC is the most frequent malignancy affecting the adult kidney (87%) [2].

RCC is a very heterogeneous class of tumors [3]. According to the classification proposed by the World Health Organization in 2016, which combines histological and genetic characteristics and clinical implications, RCC can be subdivided into three different entities [3,4]. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC and represents up to 80% of all RCCs [5,6]. Papillary renal cell carcinoma (pRCC) accounts for 10–15% of all RCCs [7–9], whereas chromophobe RCC makes up only the remaining 5% [10].

RCC is characterised by being asymptomatic at early stage and showing a poor response to radiotherapy and chemotherapy once metastatic, making this tumor type very difficult to diagnose and treat [11]. Due to the much higher prevalence of ccRCCs, very few clinical trials have been carried out considering other histological RCC subtypes. Therefore, most of the drugs have been developed based on ccRCC, but they are currently applied to all RCC patients. Treatment of advanced or metastatic RCC patients is achieved primarily by targeted therapies [tyrosine kinase inhibitors (TKIs)] and 5-year survival for these patients is 12% [12]. Despite all the progress made in the development of novel anti-cancer compounds, the management and treatment of RCC patients still remains a crucial aspect in the clinic.

In particular, intra-tumor and inter-tumor heterogeneity is one of the major limitations in the treatment of epithelial tumors [13]. Two different tumor models were proposed to play a role in tumor development, progression, and tumor heterogeneity. The clonal evolution model or stochastic model implies the presence of a tumor cell population carrying different mutations which have accumulated over time and then selected...
under different selective pressures [14]. Every cell within a tumor has potentially the same likelihood to facilitate tumor formation and progression. Selection, clonal expansion and genetic instability are the key elements driving the stochastic approach [15]. The cancer stem cell (CSC) model, or hierarchical model, instead, proposes that tumor growth and propagation is driven by a small phenotypically distinct subset of cells with pluriproliferative features within the total cancer cell population [16,17]. According to this model, the tumor bulk is established by a pool of CSCs that have both stem cell potential and the ability to give rise to progeny with self-limited proliferative capacity [18]. As a result of this model, elimination of the entire CSC population will result in tumor eradication, whereas leaving even only one cell behind will lead to tumor recurrence [15,19].

Nevertheless, it is becoming increasingly clear that genetic and epigenetic factors are not the only two factors contributing to tumor heterogeneity. The tumor microenvironment (TME), stromal cells, soluble molecules, and extracellular vesicles (i.e. exosomes) play an important role in modulating metastatic properties and sensitivity of tumor cells to therapy [20,21]. Therapy itself may act as selection mechanism that shapes tumor evolution. More recently, a unifying model of clonal evolution applied to CSCs was proposed by Kreso et al., whereby CSCs can acquire mutations and generate new stem cell branches and, at the same time, tumor cells in the non-CSC subpopulation can undergo epithelial-mesenchymal transition (EMT) and acquire CSC-like features contributing to tumor heterogeneity [17] (Figure 1). Processes such as inflammation, hypoxia, angiogenesis, and EMT occurring in the TME contribute to the maintenance of CSC fate. Due to cellular plasticity, it is important to note that the cell of origin – the normal cell that acquires the first genetic hit(s) that culminate in the initiation of cancer – does not necessarily refer to the CSC population as the hierarchical model would suggest. CSCs are the cellular subset within the tumor that uniquely sustains malignant growth. Cells-of-origin and CSCs refer to tumor-initiating cells (TICs) and cancer-propagating cells, respectively [22].

CSCs are a small population of neoplastic cells within a tumor presenting characteristics reminiscent of normal stem cells (NSCs). In particular, they are capable of giving rise to all the cell types present in the tumor tissue which they derive from (differentiation). They are characterised by unlimited cell
division and maintenance of the stem cell pool (self-renewal). They can give rise to tumor and contribute to metastasis formation in vivo (tumourigenicity). Moreover, CSCs are recognised to be the major cause of tumor recurrence and resistance to therapy.

Dick and co-authors performed the first experimental study on CSCs in 1994. They isolated CD34+/CD38- cells from acute myeloid leukemia (AML) patients and showed that they could initiate AML in vivo upon transplantation into NOD/SCID mice [23,24]. Subsequently, several others have showed the presence of CSCs in colorectal cancer, breast cancer, glioblastoma, melanoma, lung cancer, liver, and prostate cancer [25–33]. Growing evidence suggests that renal cancer, as many other solid tumors, possesses a rare population of cells capable of self-renewal that contribute to metastasis and resistance to therapy [34]. Therefore, the identification of a specific subpopulation of cells within a tumor that either initiate or maintain tumourigenesis is of utmost importance for understanding tumor biology and in the development of novel therapies. In this review, we outline potential CSC markers in RCC as well as advantages and pitfalls in the identification of these tumor-propagating cells.

Cancer stem cell biomarkers

To date, several markers have been found to be specifically expressed in CSCs and cancer stem-like cells derived from RCC. A summary of these putative CSC markers is given in Table 1.

CD105

CD105 (endoglin) is a transmembrane glycoprotein encoded by the endoglin gene located on chromosome 9q34. This protein is composed of two constitutively phosphorylated subunits of 95 kDa each, forming a 180 kDa homodimeric mature protein [54]. CD105 is an accessory protein of the TGFβ complex. Upon activation of the TGFβ complex, the binding of endoglin results in the activation of Smad proteins leading to the regulation of various cellular processes such as cell proliferation, migration, differentiation, and angiogenesis [55]. Endoglin is predominantly expressed in endothelial cells where it is activated by hypoxia and TGFβ stimulation, whereas it is decreased by tumor necrosis factor α (TNFα) [56].

Interestingly, in breast, prostate, and gastric cancer, CD105 was found in endothelial cells forming immature tumor vasculature. In ccRCC, a subpopulation of cells representing <10% of the tumor mass showed CD105 upregulation. CD105+ cells isolated by magnetic sorting displayed potent capability to grow as spheres and initiate tumors and metastases recapitulating the clear cell histological pattern in mice [48,57]. These cells also expressed mesenchymal markers CD44, CD90, CD29, CD73, and Vimentin; embryonic stem cell markers Oct3/4, Nanog and Nestin, and the embryonic renal marker Pax2 [48]. However, they did not express CD133, also known as human tubular progenitor cell marker [58]. CD105+ CSCs were able to differentiate into epithelial and endothelial cells and generate CD105- cells. Additionally, immunohistochemical analysis of tumoural CD105 was found to correlate positively with nuclear grade and tumor stage, whereas endothelial expression correlated negatively with clinicopathological features [59]. Thus, CD105 has been proposed as the main marker for CSC identification in RCC.

CSCs have been found to secrete higher amount of exosomes and CSC-derived exosomes have been found involved in promoting angiogenesis in xenograft mice with renal cancer [57], metastatic niche formation in lung carcinoma [60] as well as invasion, migration and tumor growth in many other tumor types [61–65]. Interestingly, CD105+ CSCs can release microvesicles and exosomes containing pro-angiogenic mRNAs (VEGF, FGF, MMP2, and 9) that trigger angiogenesis and promote the formation of a premetastatic niche in vivo [57]. Extracellular vesicles (EVs) derived from renal CSCs impaired T cell activation and dendritic cell differentiation by HLA-G promoting escape from the immune system [66].

Nevertheless, the use of CD105 as a renal CSCs marker has been questioned in many studies, where CD105+ cells also showed CSC-like features [36].

CD133

Prominin-1 (CD133) is a transmembrane glycoprotein of 865 amino acids (120 kDa) encoded by the gene PROM1 on chromosome 4p15 [67]. This protein exists in different isoforms and its regulation is complex [68,69]. Expressed by almost all cell types, CD133 localises in the plasma membrane suggesting its involvement in membrane remodeling and signal transduction [68]. Phosphorylation of CD133 results in the activation of PI3K/AKT signalling pathway [70,71]. Hypoxia, mTOR inhibition and TGFβ1 increased CD133 expression in lung cancer, pancreatic cancer, and hepatocellular carcinoma (HCC). Oct4 and Sox2 have been found to bind to the promotor region of CD133 inducing its activation in lung cancer cell lines. Along with its expression in...
| Sample     | Assay                        | Putative marker of the study | Positive markers          | Negative markers                  | CSC features                                           | Reference |
|------------|------------------------------|------------------------------|---------------------------|-----------------------------------|--------------------------------------------------------|-----------|
| 769P       | Side population              | ABCB1                        | ABCC1, ABCG2              | Clonogenic, tumourigenicity, resistance to chemo and radiotherapy | Huang et al [35]                                     |           |
| 786O       | Sphere formation assay       | CD73                         |                           |                                   | Song et al [36]                                       |           |
| 786O       | Flow cytometry               | Rh123                        |                           |                                   | Lu et al [37]                                         |           |
| 786O, 769P, A704, Caki1, Caki2 | Flow cytometry              | USP21                        | ALDH                       |                                   | Peng et al [38]                                       |           |
| ACHN       | Side population              | ALDH1                        | CD105, CD133              | Sphere formation, clonogenic, proliferation, invasion | Ueda et al [39]                                       |           |
| ACHN, Caki1 | Sphere formation assay       | Oct4, Nanog, Pax2            | CD24, CD34, CD11, CD19, CD45 |                                   | Lichner et al [40]                                   |           |
| ACHN, Caki1 | Flow cytometry               | CD105                        | CD24, CD34, CD11, CD19, CD45 |                                   | Khan et al [41]                                       |           |
| ACHN, Caki1 | MACS                        | CD133 “/CD24”, Oct4, Notch1, Notch2, Jagged1, Jagged2, DLL1, DLL 4 |                   |                                   | Xiao et al [42]                                       |           |
| ACHN, Caki1, SMKTR2, SMKTR3, RenCo | Side population      | DNAJB8                        |                           |                                   | Nishizawa et al [43]                                 |           |
| ACHN, Caki1, Caki2, 786O, 769P | Flow cytometry           | ALDH1                        | CD24                      |                                   | Micucci et al [44]                                   |           |
| HEK293T    | Sphere formation assay       | Oct4, Nanog, Pax2            | CD24                      |                                   | Debeb et al [45]                                      |           |
| RCC xenograft | Sphere formation assay     | CXCR4                        |                           |                                   |                                                       |           |
| RCC26, RCC53 | Flow cytometry            | CXCR4, CD24, CD29, CD44, CD73, Nanog, Oct4, Sox2 | CD90, CD105, CD133, CXCR1, Vimentin, β-catenin | | Varna et al [46]                                      |           |
| RCCs       | Flow cytometry               | CD105                        |                           |                                   | Gassenmeier et al [47]                               |           |
| RCCs       | Flow cytometry               | CD105, CD44, CD73, CD29, Nanog, Oct4, Vimentin, Nestin | CD133                     |                                   | Bussolati et al [48]                                 |           |
| RCCs       | Flow cytometry               | CD133 “/CD24”, CD73, CD44, CD29, Vimentin |                           |                                   | Bruno et al [49]                                      |           |
| RCCs       | Flow cytometry               | CD133 “/CD24”                |                           |                                   | Galleggiante et al [50]                               |           |
| RCCs       | Side population              | CD133                        |                           |                                   | Addla et al [51]                                      |           |
| RenCo      | Sphere formation assay       | DNAJB8                        |                           |                                   | Yamashita et al [52]                                  |           |
| SK-RC-42   | Sphere formation assay       | Oct4, Nanog, BMI, β-catenin  | MHC-II, CD80              |                                   | Zhong et al [53]                                      |           |

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stem and progenitor cells within normal tissues, CD133 has been proposed as a putative CSC marker across different tumor types [68].

CD133+ cancer cells were able to form spheres, gave rise to tumors in vivo and exhibited chemoresistance properties in colorectal carcinoma (CRC), HCC, lung cancer, glioblastoma, pancreatic cancer, and ovarian cancer. On the contrary, sorted CD133+ cells from RCC patients did not show tumorigenic capability in vivo although they expressed stem cell markers such as CD44, CD29, Vimentin, and Pax2 [58]. When co-transplanted with renal carcinoma cells, CD133+ progenitors significantly enhanced tumor development and growth. The same result was obtained using CD133+ cells derived from normal kidney tissue [72]. Of note, CD105+ cells did not express CD133, suggesting that CD133+ cells may represent renal resident adult progenitor cells rather than CSCs.

Interestingly, CD133+/CD24+ cells derived from RCC cell lines ACHN and Caki1 displayed sphere formation capability, enhanced invasion and migration properties, high colony formation efficiency in soft agar, and resistance to sorafenib and cisplatin [42].

Another interesting publication identified CD133 and CXCR4 co-expressing CSCs in spheres derived from RCC xenografts and tumor tissues. Increased expression of these markers was found in RCC patients after sunitinib treatment [46]. Nevertheless, whether the CD133 and CXCR4 positive or negative cells had detectable levels of CD105 was not assessed. Additionally, the gene expression profile as well as the tumorigenic potential of the spheres was not deciphered. Moreover, CD133+ cells were also able to give rise to tumors in immunodeficient mice in glioblastoma and CRC [73,74].

Last, CD133 expression was found to correlate strongly with nuclear HIF1α in RCC patients [75,76]. CD133 mRNA levels in blood can be useful for identifying metastasis, predicting recurrence, and stratifying the patients into different risk groups for possible adjuvant treatment [77]. However, CD133 expression analysed by IHC in RCC patients was inconsistent and varied among different studies [55,78]. Because of the complex epigenetic and microenvironmental modulation together with higher protein processing and post-translation modifications, the applicability of CD133 as a CSC marker is limited [68].

CD44

CD44 is a transmembrane glycoprotein of 85 kDa (742 aa) encoded by the CD44 gene located on chromosome 11. CD44 exists in more than 20 isoforms due to RNA alternative splicing, giving rise to different proteins in different cancer tissue subtypes. Due to the wide variety of isoforms, CD44 is involved in diverse biological processes such as cell–cell interaction, cell adhesion, migration, proliferation, differentiation, and angiogenesis [79].

Although other extracellular matrix (ECM) components such as collagen, growth factors, and metalloproteinases can interact with CD44, the extracellular glycosaminoglycan hyaluronan (HA) represents its primary ligand [80]. Binding of CD44 to HA promotes multiple signalling pathways including activation of receptor tyrosine kinases (RTK), TGFβ, MAPK, PI3K/AKT supporting cell proliferation, survival, invasion, and ultimately homing of CSCs in many tumor types [79,81]. In addition, CD44 has been found to be involved in the regulation of stem cell features via the Wnt/β-catenin signalling pathway and protein kinase C (PKC) [82]. Because of its tight interaction with the ECM, CD44 plays an essential role in modulation of the CSC niche. CSCs can synthesise HA to attract tumor-associated macrophages (TAMs) in the CSC niche. On the other hand, stromal cells will produce growth factors that regulate stem cell activity [79]. Enhanced CD44 expression was observed in RCC cell lines after co-culture with macrophages. This effect was the result of activation of the NFκB pathway by the TNFα derived from TAMs [83]. TNFα enhanced migration and invasion of ccRCC cells together with down-regulation of E-cadherin expression and up-regulation of matrix metalloproteinase 9 (MMP9) and CD44 expression [84]. Interestingly, spheres derived from HEK293T, ACHN, Caki-1, and 786O renal cancer cell lines as well as CD105+ cells isolated from RCC specimens showed the presence of a CD44+ population with self-renewal properties, sphere formation capability and resistance to therapy [37,40,45]. Moreover, CD44 expression was found to be associated with Fuhrman grade, primary tumor stage, histological subtype, and poor prognosis in RCC patients [55,84,85]. Therefore, CD44 expression may serve as a prognostic and predictive as well as potential CSC marker for RCC [78].

In view of the involvement of CD44 in enhancing stem cell features in cancer cells and mediating crosstalk with the TME, CD44-based therapeutic strategies have been developed [82]. Monoclonal antibodies against CD44 are now in clinical trial for patients affected by AML, whereas knockdown of CD44 has been shown to increase sensitivity to chemotherapy in cell cultures derived from HCC, lung, breast and pancreatic cancers [80,86].
CD24

CD24 is a small cell surface protein molecule composed of only 27 amino acids, resulting in a molecular weight ranging between 20 and 70 kDa depending on the glycosylation status. It is encoded by the CD24 gene located in the chromosome 6q21. CD24 is expressed in a wide variety of cell types, including hematopoietic cells [87]. Nevertheless, it is preferentially expressed in progenitor and stem cells. CD24 was shown to be an important maker for cancer diagnosis and prognosis in breast, non-small cell lung, colon, ovarian, and prostate cancer [87–89]. CD24 upregulation has been also found associated with CSCs and CSC features in many solid tumors. On the contrary, breast CSCs showed low CD24 levels, suggesting that the role of CD24 in stem cells may be tissue dependent [90–92]. Interestingly, high CD24 expression was observed in CSCs derived from the RCC cell line Caki2 [93], although contrasting results were reported when analysing the expression of CD24 together with the CSC marker CD44. Nevertheless, CD24 expression was found to correlate with tumor grade, overall survival, and disease-free survival in RCCs suggesting its prognostic significance [88]. Lazzeri et al identified a subpopulation of cells exhibiting self-renewal properties, expression of stem cell transcription factors, and the ability to regenerate kidney tissue upon injury. These cells derived from the human embryonic kidney expressed both CD24 and CD133 indicating they may represent putative normal kidney stem cells [94].

Because of the very limited research studies conducted on CD24 in RCC, we can conclude that, to date, no clear observation that CD24 can be used as a CSC marker in RCC has been made.

CXCR4

The CXC-chemokine receptor 4 (CXCR4 or CD184) is a seven transmembrane G protein-coupled receptor (GPCR) on the cell membrane. It is encoded by the CXCR4 gene located on chromosome 2q24. CXCR4 selectively binds to the CXC chemokine stromal cell-derived factor 1 (SDF1 or CXCL12) leading to the activation of a variety of biological processes such as proliferation, survival, migration, stemness, and angiogenesis [95]. A number of signalling pathways are involved in the signal transduction. For instance, PLC/MAPK, PI3K/AKT, JAK/STAT, and the Ras/Raf pathway.

CXCR4 was found expressed in many different tumor tissues. It has been shown in breast, small cell lung cancer, neuroblastoma, and renal cancer that CXCR4+ cells migrate towards tissues expressing high levels of SDF1 to metastasise [42,96,97]. Therefore, CXCR4/SDF1 is involved in cell-stroma interactions creating a permissive niche for metastasis [55]. Further, SDF1 stimulates adhesion of bone marrow progenitor/stem cells through CD44, demonstrating again a link between CD44 and CXCR4 signalling and TME [82].

Recent studies showed that CXCR4+ cells derived from several RCC cell lines (RCC26 and RCC53; Caki1, Caki2, 786O, and 769P) express high levels of stem cell-associated genes and exhibit resistance to therapy (TKIs) and enhanced capability to form spheres in vitro and tumors in vivo compared to CXCR4+ cells [44,47]. Conversely, inhibition of CXCR4 by ADM3100 or small interfering RNA (siRNA) impaired tumor formation [44,47]. Interestingly, loss of pVHL in ccRCCs as well as hypoxia led to increased CXCR4 and MMPs expression indicating HIF1α may be responsible for expansion of the CXCR4 population [98]. Supporting evidence showed that CD133+/CXCR4+ cells co-expressed HIF1α and were located in perinecrotic areas in RCCs [46]. Moreover, hypoxia promoted CD133+/CXCR4+ cells tumorigenicity whereas HIF2α was shown to be involved in the expansion of CXCR4+ CSCs in four RCC cell lines [44]. The translational relevance of CXCR4 expression in the clinic was investigated in 2673 RCC patients by meta-analysis revealing a negative correlation between CXCR4 expression and overall survival (OS), cancer free survival, and disease free survival [99]. Taken together, these results indicate that CXCR4 may be explored as a potential CSC marker in RCC, perhaps in combination with a second marker. Nevertheless, care should be taken when choosing the appropriate marker for CSC isolation since too restrictive a selection may lead to failure in targeting all the stem-like cells present in the tumor population.

ALDH1

Aldehyde dehydrogenase 1 (ALDH1) is a cytosolic enzyme involved in the dehydrogenation of aldehydes to their corresponding carboxylic acids [100]. It is encoded by the ALDH1 gene located on chromosome 9q21. ALDH1 plays an important role in cellular differentiation, proliferation, mobility, mobility, embryonic development, and organ homeostasis [39].

ALDH1 has been initially proposed and used as a marker to isolate stem cells from normal tissues such as brain and bone marrow with potential applications in the area of regenerative medicine [101,102]. More recently, the activity of cytosolic ALDH1 has also been shown to be a reliable marker of CSCs in several types of solid tumor, including breast, colon,
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pancreas, lung, liver, prostate, and bladder [103,104]. Nevertheless, its prognostic significance in RCC is still unclear [105], although ALDH1 was found to correlate with tumor grade in RCC by Ozbek and co-authors [106].

High expression of ALDH1 was found in the side population (SP) derived from the RCC cell line ACHN compared to the non-SP. Analysis of the ALDH1 \(^+\) cells revealed enhanced sphere formation capability, self-renewal properties, tumourigenicity and high expression of stemness genes in the ALDH1 \(^+\) cells compared to ALDH1 \(^-\) cells. Moreover, drug treatment and hypoxic conditions were shown to increase the ALDH1 \(^+\) cell population in vitro [39].

Interestingly, a recent study investigated ALDH1 expression patterns in 24 types of normal human tissue as well as in primary epithelial tumor specimens and epithelial cancer cell lines, showing that ALDH1 may not be a suitable CSC marker for all tumor types especially in tissues where ALDH1 is constitutively highly expressed such as liver and pancreas [107]. Therefore, growing evidence suggests that ALDH1 is not only a putative stem cell marker, but may actually play multiple functional roles in regulating stem cell function [100].

**ABCB5**

The drug efflux transporter ABCB5 (ATP-binding cassette, sub-family B, member number 5), is an integral membrane glycoprotein encoded by the **ABCB5** gene located on chromosome 7p21. It is composed of 812 amino acids and has an overall molecular weight of 90 kDa. This protein is involved in the transport of small ions, sugar, peptides, and organic molecules across the plasma membrane against a concentration gradient by hydrolysis of ATP [108]. Because of its function, ABCB5 has been considered responsible for mediating therapeutic resistance [109].

ABCB5 has been found to be overexpressed in CSCs derived from melanoma, liver, and colorectal cancers. Moreover, it was found to be associated with tumor progression, chemoresistance, and recurrence in many other tumor types [110]. For instance in renal cell cancer, **ABCB1** was found expressed in all cells and these tumors rarely respond to primary chemotherapy treatment [111]. Therefore, ABCB5 is exploited for distinguishing between stem cells (side population) and non-stem cells using flow cytometry.

**Others**

DNAJB8 is a member of the heat shock family of proteins (HSP40) that regulate chaperone activity. It is encoded by the **DNAJB8** gene located on chromosome 3q21. DNAJB8 is commonly expressed in the testis. Recently, Nishizawa et al., showed that DNAJB8 is expressed in different cancer cells including RCCs. In particular, the expression of DNAJB8 correlated with the SP compartment, and overexpression of the protein increased SP cells. Interestingly, DNAJB8 immunisation completely abolished tumor formation in mice, indicating that DNAJB8 can be a target for immunotherapy [43].

MicroRNAs (miRNAs) are non-coding small RNA molecules (22 nucleotides) involved in regulation of gene expression by translational repression, mRNA cleavage, and deadenylation. The role of miRNAs in CSCs has been described for different tumor types [112]. Six miRNAs involved in TGF\(\beta\) and Wnt signalling pathways showed the most significant variations in expression by RT-PCR between spheres and parental cells derived from two metastatic RCC cell lines, ACHN and Caki1. Among those, miR17 was significantly downregulated in Caki1 and ACHN spheres. Inhibition of miR17 resulted in enhanced sphere formation indicating that TGF\(\beta\) signalling plays an important role in renal CSCs and that miR17 impairs the signalling cascade by targeting the TGF\(\beta\) signalling pathway [40].

Galleggiante et al isolated a subpopulation of cancer cells expressing CD133 and CD24 from 40 RCC samples. This population showed stem cell properties such as self-renewal, differentiation, tumourigenicity and expression of stemness-related transcription factors. CD133\(^+\)/CD24\(^+\) cells appeared to be more undifferentiated compared to the corresponding tubular adult renal progenitor cells. Interestingly, these cells also expressed on the cell membrane the amino acid transporter CTR2 which was found to be involved in resistance to cisplatin [50].

Rhodamine 123 (Rh123) is a fluorescent dye that permeates the cell membrane and accumulates in the mitochondria proportionally to the mitochondrial membrane potential [113]. 786O cells were stained with Rh123 and sorted by flow cytometry into two population: Rh123\(^{high}\) and Rh123\(^{low}\). Rh123\(^{high}\) exhibited high proliferative activity, differentiation, resistance to radiation, tumourigenic potential, and spheroid formation in soft agar, indicating Rh123 as an alternative method to isolate CSCs [37].

Finally, high CD73 expression was observed in spheres derived from the 786O RCC cell line. Moreover, CD73\(^+\) cells displayed high levels of stemness-related transcription factors, resistance to radiotherapy, and tumourigenicity in vivo [36].
Isolation techniques

Different approaches for CSC isolation have been developed in recent years (Figure 2).

Antigen-based methods require labelling of the cells based on the expression of specific markers. These include magnetic bead-conjugated antibodies (MACS) [114,115], fluorescence-activated cell sorting (FACS) [116,117], and SP analysis [118,119]. Antigen selection often relies on markers which have been found relevant in developmental biology, embryonic, and hematopoietic stem cell studies. Nevertheless, dissociation of the tumor tissue into a single cell suspension may damage surface antigens limiting the efficiency of isolating CSCs using cellular marker-based methods [118]. Further, cells can lose viability upon enzyme treatment and after sorting procedures [120]. Cell sorting itself has proven to be imprecise with 1–3% of tumourigenic cells contaminating the non-tumourigenic population [19]. In addition, no generally applicable markers are known to date, and excessively permissive or restrictive labelling may have implications when developing therapeutic strategies targeting CSCs based on marker expression [19]. The identification and characterisation of putative CSC markers may also be achieved using functional assays [58]. In order to recreate the in vivo CSC niche using in vitro culture conditions, three-dimensional cell culture models were developed. Two different methods can be adopted for culturing CSCs in 3D: anchorage-independent and anchorage-dependent.

While the anchorage-independent system takes advantage of the ability of CSCs to grow in suspension, the anchorage-dependent system uses scaffolds in order to enable cells to mimic their interaction with the ECM microenvironment and promote features of stemness. These methods are represented by spheroid [121,122] and organoid cultures [123–125], and the sphere formation assay [53,117,122] and hanging drops [126,127], respectively.

The ECM plays crucial roles in establishing the CSC niche and in mediating tumor drug resistance. It is composed of collagens, laminins, fibronectin, proteoglycans, and all the non-cellular components present in the tissues [128]. Different scaffolds can be used in these 3D CSC culture assays in order to mimic the ECM. Natural scaffolds include collagen, gelatin, elastin, fibrinogen, agarose, and alginate. Combinations of materials are also possible. Synthetic scaffolds can overcome the risk of contamination, degradation and batch-to-batch variations compared to natural scaffolds. These are mainly polymeric microparticles (e.g. hydrogels, PLGA, and PLC) [129].

Antigen-based methods

Magnetic bead-conjugated antibodies

MACS allows the isolation and enrichment of stem cells without further staining. Cells are labelled using antibodies conjugated to magnetic nanoparticles.

Figure 2. Identification and isolation of CSCs. Several potential CSC markers are shown. CD105, TBR, CD133, CD44, CD24, CXCR4, and ABCB5 are some of the most studied membrane CSC markers; whereas miRNAs, DNAJB8, ALDH1 stand out among the intracellular CSC markers. Based on these markers, FACS and MACS have been adopted as isolation methods for the separation of CSCs from other tumor cells. More recently, other techniques exploiting CSC properties have been developed with the aim of discovering potentially new biomarkers; these include the sphere assay, spheroid and organoid formation and hanging drops.
Labelled cells are then transferred into a column placed in a strong magnetic field. During this step, cells expressing the antigen will bind to the magnetic beads and stay in the column, whereas all the other cells that are negative for the antigen will flow through [114,115]. The population of interest can be subsequently eluted from the column.

**Fluorescence-activated cell sorting**

FACS is an alternative isolation method capable of sorting cells using fluorescently labelled antibodies targeting selected surface proteins or intracellular markers via direct or indirect immune fluorescence staining. Flow cytometry allows a sample of cells or particles in suspension to be separated through a narrow liquid stream. As the sample passes through a laser it allows for detection of size, granularity, and fluorescent properties of individual cells/particles [130]. Generally, FACS separation uses fluorochromes directly conjugated with either primary or secondary antibodies with different emission wavelengths. Although MACS is simpler and requires less complicated equipment than FACS, it is monoparametric and cannot isolate cells via multiple markers simultaneously [116,117].

**Side population analysis**

Hoechst SP analysis is one of several strategies used in stem cell studies [131]. SP is defined as a small fraction of cancer cells within a tumor exhibiting stem-like properties. The ability to discriminate the SP is based on the differential efflux of Hoechst 33342 by the multi-drug resistance ABC transporters [132]. CSCs possess higher activity and/or higher amounts of the ABC pumps, which are also responsible for the efflux of chemotherapeutic agents resulting in chemoresistance of CSCs [109]. Therefore, SP stands out as the portion of cells able to extrude the dye against a concentration gradient when compared to cells not having stem cell features [119].

Identification of CSCs is achieved by specifically inhibiting ABC pumps using verapamil (100 μM) or reserpine (5 μM). Hoechst is excited at 405 nm and the blue signal is collected with a 450/40 nm band-pass filter, whereas the red fluorescence is collected with a 610/20 nm filter. Due to the high capability to extrude Hoechst dye, the side population can be defined as the population negative for Hoechst blue and Hoechst red [118]. Nevertheless, analysis of the SP has raised many concerns due to the dynamic nature of the dye efflux property as well as toxicity of the Hoechst dye making this technique highly variable [120,131].

SP isolation can also be achieved by using rhodamine 123 (Rh123) [37,133,134]. Rh123 is a mitochondrial dye that stains mitochondria with increasing intensity as cells become activated [37]. Rh123 fluorescence intensity is an index of mitochondrial mass, number and activation state, and multi-drug efflux pump activity [135]. Decreased intra-cellular accumulations of Rh123 results from the efflux of the dye.

Because non-stem cells may also express some of the ABC transporters, the isolation of CSCs through SP analysis is imperfect. While the SP may contain some non-stem cells, conversely some stem cells may not be located in the SP fraction [109].

**Functional assays**

**Sphere formation assay**

The sphere formation assay exploits the capability of CSCs to grow in vitro as spheroids in an anchorage-independent manner due to their mesenchymal phenotype. Cells are plated onto ultra-low attachment plates under serum-free medium conditions. Recent studies have demonstrated that CSC expansion requires medium lacking serum, which is believed to stimulate cellular differentiation [53,117,122]. The medium composition may vary but it is generally composed of DMEM/F12 medium supplemented with stem cell growth factors and/or hormones (i.e. bFGF, EGF, HGF, insulin, androgen, and progesterone). Therefore, cancer cells with stem cell-like features are able to proliferate and form spherical structures, whereas all the others will die. As a result of asymmetrical cell division, each CSC is capable of forming a sphere composed of both cells that have stem cell features as well as more differentiated cells. By passaging the spheres, CSCs can be enriched [53,117,122]. This results in an increased sphere number from one passage to the other. Compared to MACS and FACS, the sphere formation assay may retain clonal variations within the CSC pool by avoiding marker selection. Nevertheless, several critical parameters can impair CSC isolation and investigation using the sphere formation assay. These are: inappropriate seeding cell densities which can impact sphere formation and sphere clonality; the presence of quiescent CSCs which cannot be expanded using this method; and finally, possible overestimation of the stem cell frequency [136].

**Hanging drops**

In the hanging drop system, droplets of cell suspension are deposited onto a dish or into special plates (e.g. the GravityPLUS™ Hanging Drop System,
InSphero AG, Switzerland and Perfecta3D® hanging drop plates, Sigma-Aldrich). Upon inversion of the tray, cells accumulate and aggregate by gravity on the surface of the liquid drop. This method is mainly used in the study of embryonic stem cells [126,127]. The Hanging Drop system allows efficient formation of uniform-size spheroids in a relative short time, making it a very useful tool for high-throughput screening studies.

Tumor spheroids

Incorporation of ECM proteins into serum-free medium may induce CSC-ECM interactions, normoxic/hypoxic conditions, metabolic gradients, and cooperation with stromal cell components through co-culture [129]. In the tumor spheroid model, a defined number of tumor cells are encapsulated into macro-beads derived from natural or synthetic scaffolds, and allowed to float into the medium until spheroids are formed. Another interesting version is based on plating tumor cells into soft agar [121,122]. An initial layer of agar composed of 0.6% agarose is deposited on the bottom of the plate. Once it has settled, a second layer of 0.3% agarose containing a tumor single cell suspension is placed onto the 0.6% agarose. An additional feeder layer of 0.3% agarose is then added. Agarose concentration may be adapted depending on the cancer type.

Tumor organoids

Organoids are formed by distributing dissociated tumor cells into Matrigel drops. Matrigel is a gel-based natural compound that consists of laminin, collagen IV, and enactin. Matrigel drops containing tumor cells are dispersed into normal tissue culture plates, and the cell-matrix mixture is incubated at 37°C before adding the medium. Different ratios of cell suspension/Matrigel can be used depending on tumor type. Organoid medium is composed of aD-MEM/F12 supplemented with stem cell growth factors promoting Wnt signalling pathway (B27 supplement, N-acetyl-L-cysteine, EGF, A-83, Noggin, and R-spondin 1) [123–125].

Transplantation assay

To finally evaluate the tumorigenic potential of a tumor cell population expressing CSC features, cancer cells are serially transplanted into immunocompromised mice (serial tumor transplantsations) at low cell density (limiting dilution assay). Cancer cells capable of developing tumors repeatedly, with recapitulation of the histological features and heterogeneity of the parental tumor, are defined as TICs [13]. The terms TIC and CSC are often used interchangeably, although TIC more specifically refers to the cell-of-origin. Nevertheless, the capability of a cancer cell to form tumors in vivo and to recapitulate the tumor heterogeneity of the corresponding parental tumor is one of the most known features of a CSC. Cancer cells capable of growing as 3D cultures but that do not have tumourigenic potential cannot be considered CSCs. Importantly, immune-deficient mice are not completely devoid of an immune system, and reactions involving host cytokines and immune cells may still take place when CSCs are transplanted into immunocompromised mice [19,137,138]. Additionally, cells need to adapt to the mouse milieu shaping CSC survival and properties. Therefore, optimisation of the transplantation assay as well as critical interpretation of the results should be adopted when studying CSC properties in vivo.

Lineage tracing

To determine the cell(s) of origin of cancer, normal cells are labelled under cell-specific promoters followed by induction of genetic modifications. In this way, a single cell or a population of cells is marked and their signature is transmitted to all the progeny [139]. Investigation of the cellular source of a tumor can be achieved by identifying and tracing over time these transformed cells responsible for forming the tumor. In parallel, this technique can be adopted to resolve the cell fate of tumor subpopulations in established tumors or to determine how cells behave in the context of the intact tissue or organism [13]. More importantly, genetic lineage tracing allows in vivo visualisation of stem cells [129].

Normal kidney stem cells

At the top of the hierarchy of cellular organisation, normal adult stem cells maintain tissue homeostasis and facilitate regeneration [140]. The kidneys carry out many different functions in the human body, including secreting hormones, absorbing minerals, filtering blood, and producing urine. Hence, impaired kidney function can ultimately lead to life threatening complications [141]. Tissue homeostasis in the kidney is limited and further diminished by age or disease [142]. This results in 20 million people worldwide suffering for chronic kidney disease (CKD) [143]. Therefore, identifying stem cell populations in the fetal and adult kidney is important for developing effective therapeutic applications and understanding stem cell biology within kidney tissue.
Cancer stem cells in renal cell cancer

Here, we briefly describe the application of embryonic and adult renal stem cell markers with the aim to translate this knowledge to CSC biomarker discovery.

Wilms’ tumor (WT) has proved to be the best model system for studying embryonic renal stem cells. These tumors result from differentiation arrest of embryonic progenitor cells committed to the nephric lineage [144]. Comparative gene expression profiling of WT and fetal human kidneys showed high concordance in the expression of the following markers: Pax2, Six1/2, NCAM, Fzd7, and Fzd2 [145,146]. Nevertheless, embryonic renal stem cells are entirely exhausted during nephrogenesis and the expression of these genes is rapidly lost during differentiation [147], limiting their utility to uncover purely embryonic-specific renal stem cell markers.

Adult renal stem cells have been investigated using BrdU staining [148]. BrdU is incorporated into dividing cells during the pulse phase, but further cell divisions will quickly dilute the stain leaving only cells that divide infrequently such as stem cells labelled with BrdU [149]. Nevertheless, some limitations must be considered such as adult stem cells dividing infrequently and not being labelled and, on the other hand, kidney has normally a limited mitotic index resulting in impaired signal dilution.

Following BrdU staining, a subset of cells expressing CD133 and CD24 were isolated from the urinary pole of Bowman’s capsule and from the proximal tubules, in particular in the S3 segment. These cells also expressed stemness markers (i.e. Sox2, CD44, Oct4, and Vimentin), and could be discriminated by differential CD106 expression [150,151]. Stem cells derived from the Bowman’s capsule were shown to move and differentiate from the urinary pole to the vascular pole acquiring podocyte traits (PDX marker) and losing stem cell markers (CD133 and CD24), whereas stem cells from the distal end of the proximal tubules were able to migrate within this segment [150–152]. The renal papilla offers a perfect niche for stem cells due to its hypoxic and hyperosmotic microenvironment [153]. Interestingly, Nestin and CD133 have been found to be expressed in stem cells derived from papillae [154].

Several studies have demonstrated that resident adult kidney stem cells are not the major player involved in tissue repair in the proximal tubule [155]. While differentiated cells undergo EMT and proliferate to repopulate the damaged area, some investigators have proposed that other non-resident stem cells such as bone marrow-derived stem cells or MSCs may be also involved in the process [147]. Nevertheless, these cells are defined as renal progenitor cells rather than renal stem cells due to their limited differentiation capability and lack of self-renewal properties.

Conclusions

Tumor relapse and metastasis are the primary causes of poor survival in ccRCC patients. CSCs are thought to be responsible for tumor propagation, metastasis formation, and treatment failure in many solid tumors, including renal cancer [25–34]. According to the CSC hypothesis, conventional therapies (i.e. radiation and chemotherapy) usually eliminate the majority of cells present in the tumor bulk while sparing the CSC pool [18]. This results in tumor recurrence. Therefore, understanding the mechanisms underlying metastasis and drug-resistance associated with CSCs may help to identify new therapeutic options.

Various approaches have been developed with the aim to successfully isolate and characterise CSCs, leading to the identification of a variety of CSC markers [156]. However, contrasting results have been reported in the literature on the use of these biomarkers [36,73,74,107]. Several studies have shown that CSC markers are not unique across tumor types; therefore, knowledge on relevant markers for NSCs or CSCs from other tumor types may not be useful in renal cancer. Growing evidence suggests that distinct CSC subpopulations may coexist within a heterogeneous tumor and new CSC (sub-)clones can be generated, selected and compete with each other similarly to the stochastic model during tumor progression and treatment, resulting in greater intra- and inter-CSC clone variability [140]. Therefore, some biomarkers can be relevant and applicable in certain phases during tumor development and progression, whereas they become obsolete in others.

Many scientists have raised concerns about the stem cell hypothesis. In particular, the fact that CSCs are considered as a rare slow-cycling subpopulation of cells questioned the possibility of their involvement in treatment failure, in support of mechanisms of acquired or intrinsic resistance [19]. Many studies demonstrate a higher CSC content than would be expected under the hypothesis of CSCs being a small subset, which may be explained by inefficient isolation methods affecting functional assays as well as xenograft rates [120]. Finally, if CSCs are slowly proliferating one could argue that CSCs are lost during in vitro manipulation, whereas these cells remain a constant fraction of the total population [109].
these concerns finds their explanation in the plastic nature of CSCs as well as technical issues.

CSC traits are sustained by interaction with the TME (niche) [21]. The CSC niche is an anatomically distinct TME present within a tumor that supports and sustains CSC properties [157,158]. It is composed of ECM, cancer-associated fibroblasts, mesenchymal stem cells, endothelial, and immune cells [137]. Stem cell niches are often localised in hypoxic regions where low O2 levels induce slow cycle proliferation and minimise DNA damage due to ROS [44,46]. Processes such as inflammation, hypoxia, angiogenesis and EMT taking place within the TME contribute to the maintenance of CSC fate by acting on the most known pathways regulating CSCs: Wnt, SHH, Notch, TGFβ, and growth factor-receptor tyrosine kinase (RTK) [118,137,158,159]. Interestingly, tumor cells in the non-CSC compartment can spontaneously undergo EMT and acquire a CSC-like phenotype and surface marker expression [160]. At the same time, CSCs display different stemness features depending on the microenvironment, and these features may be transient [138,157]. The entire process has to be considered reversible, plastic, and dynamic.

Therefore, understanding the mechanisms underlying the properties of CSCs, and the integration of genomic and functional assays exploiting such features, may advance CSC studies as well as promote the identification of new biomarkers for renal CSCs. CSC assays should take into consideration the contribution of the niche. Moreover, optimisation of the transplantation assay using highly immune-deficient mice humanised with human TME and growth factors together with complementary lineage tracing analysis is of utmost importance for advancing CSC studies [137]. Last, combination of therapies specifically targeting CSCs by not only acting on CSC surface markers but also inhibiting CSC-related signalling pathways, delivering CSC-specific therapeutics as well as targeting the CSC niche together with conventional chemotherapy and radiotherapy, may ultimately lead to improved RCC patient survival [55,137].

**Author contributions statement**

C.C. conceived and designed the manuscript. C.C. and H.M. acquired and interpreted literature data. C.C. wrote the manuscript, whereas H.M. assisted in the manuscript drafting and revision.

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