Chapter 2
Cancer Stem Cells—Biopathology with Reference to Head and Neck Cancers

Miguel Ángel González Moles

Abstract Researchers have proposed that tumor development is based exclusively on the activity of cancer stem cells (CSCs), the so-called CSC hypothesis. This new model of carcinogenesis may offer insights into the high mortality of oral cancer, the poor response to treatment, and the elevated risk of multiple tumors in patients with oral squamous cell carcinoma (OSCC). Greater knowledge is needed of the molecular pathways that participate in maintaining the stem cell (SC) state in order to understand the mechanisms involved in tumor and metastasis development. This endeavor requires the development of specific markers to identify SCs and CSCs in tissues and to determine topographic relationships with their lineage. This chapter provides an update on the literature related to stem cells and oral cancer, centering on the CSC hypothesis in this context and on progress in the identification of oral stem cells.

2.1 Introduction

In the conventional clonal evolution model of carcinogenesis, an accumulation of genetic and epigenetic changes can have an impact on any oral epithelial cell, producing a clone with proliferative advantages that can develop invasiveness. According to the recently proposed cancer stem cell (CSC) hypothesis, only long-surviving cells, such as stem cells (SCs), can accumulate sufficient oncogenic alterations for carcinogenesis to be triggered. Hence, CSCs (transformed SCs) would be involved in the onset, progression, and spread of the tumor. If this is the case, the poor outcomes of oral cancer therapies may be attributable to their targeting of the whole tumor mass rather than specific cells responsible for its development and growth. On the other hand, CSCs have been attributed with molecular defense mechanisms and other features that may protect them against

M.Á. González Moles (✉)
Department of Oral Medicine, School of Dentistry,
University of Granada, Granada, Spain
e-mail: magonzal@ugr.es

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conventional radio-chemotherapeutic treatment (Maitland and Collins 2005; Wicha et al. 2006). Targeted therapies that eliminate the CSC population offer the potential for cure. Given this promise, it is not surprising that the CSC hypothesis has attracted so much attention in recent years (Thomas et al. 2006).

There is therefore a need to develop novel therapeutic strategies for oral cancer that target CSCs. This task requires a greater understanding of the molecular pathways that maintain stemness. This chapter reviews current knowledge on SCs and CSCs in oral cancer, analyzing the protective anti-cancer mechanisms of normal stem cells and exploring techniques to distinguish normal from malignant SCs.

2.2 Stem Cells

SCs are characterized by their self-renewal capacity and their ability to differentiate into any type of mature cell (Reya et al. 2001; Shakib et al. 2011). Embryonic SCs can differentiate into cells of the three germinal lines to generate all types of tissue (Martin 1981; Evans and Kaufman 1981), while adult SCs are undifferentiated cells that only differentiate into the cell types present in the host tissue, which they can then regenerate (Zhang et al. 2012). The CSC is a recently proposed type of SC that differentiates in an aberrant manner and can produce tumor cell populations that are phenotypically different. According to the CSC hypothesis, CSCs drive tumorigenesis and tumor growth. However, knowledge of the proportions of CSCs and normal SCs in healthy or tumor tissue is hampered by the lack of specific markers of SCs and CSCs, especially in oral and head and neck squamous cell carcinomas (HNSCCs) (Boman and Wicha 2008). It was recently reported that an HNSCC contains less than one CSC for every 2500 cells (Ishizawa et al. 2010).

Their capacity for self-renewal allows SCs to persist throughout the life of the individual. They must be able to renew and maintain a balance between self-renewal and differentiation, preserving tissue homeostasis (Reya et al. 2001). If cancer is indeed a regulatory disorder of SC self-renewal, it is important to determine the molecular mechanisms that regulate normal SC self-renewal in order to understand the processes underlying tumor cell proliferation.

2.3 Spatial Ordering of Stem Cells and Progeny in Oral Epithelium in Relation to Antitumor Mechanisms

Normal SCs represent a small proportion of the cells in the oral epithelium, and their proliferation rate is low (Boman and Wicha 2008). In order to maintain the epithelial structure, there is consequently a need for a proliferative hierarchy with a certain spatial ordering. According to the hierarchical model of oral epithelium proliferation, in which the SCs divide asymmetrically, (Fig. 2.1), a normal SC

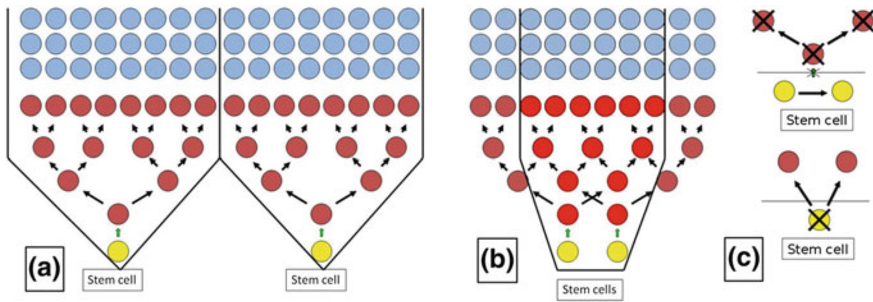

Fig. 2.1 **a** At each division, the normal SC (*yellow circles*) generates an identical SC and an ATC. The ATC (*brown circles*) divides 3–5 times until the terminal differentiation of all daughter cells. This asymmetric hierarchical proliferation predicts a theoretical spatial ordering of the progeny as inverted pyramidal structures (proliferative units—within *black lines*). **b** Areas of intersection (*red circles*) with neighboring proliferative units, in which the epithelial renewal would depend on the coordinated and alternating activity of different SCs. **c** above: symmetrical division of an SC giving rise to two SCs; below: Symmetrical division of an SC giving rise to two ATCs with loss of the SC.)

persists at each division and generates an amplifying transitory cell (ATC). The ATC can divide three to five times until the terminal differentiation of all daughter cells (Potten 1981; Ghazizadeh and Taichman 2001; Janes et al. 2002). The self-renewal capacity of ATCs is lower than that of SCs, and their principal role is to increase the number of differentiated cells resulting from the single division of a SC. Thus, if an ATC can divide three times before terminal differentiation, one SC would only have to divide once to generate one SC and eight ATCs for terminal differentiation. Accordingly, the oral epithelium contains three compartments: basal SC compartment; parabasal ATC compartment; and a more superficial epithelial compartment in which cells undergo terminal differentiation. This asymmetric division pattern may be an important antitumor protection mechanism, given that the ATC population is responsible for the greatest proliferation, carries the highest mutation risk, and finally develops terminal differentiation and desquamation (Janes et al. 2002) (see below).

Oral epithelial SCs can also divide symmetrically, generating two ATCs (losing the existing SC) or two cells with SC properties. This symmetrical division pattern may have evolved for the recovery of basal SC populations lost through traumatic or other lesions of the epithelium (Mackenzie 2006). The asymmetric hierarchical proliferation may predict a theoretical spatial ordering of the progeny of an oral SC as inverted pyramidal structures or “proliferative units” (Fig. 2.1). The vertex of the unit is one SC in the bottom stratum; the next stratum contains one ATC, followed by two, four, and eight ATCs in progressively higher strata, followed by a stratum of non-replicative cells undergoing differentiation. The areas between proliferative units would be pyramidal; in the usual ki-67-assessed epithelial proliferation pattern, however, parabasal proliferative areas are linear rather than pyramidal (Fig. 2.2). This may be attributable to the ability of new ATCs to expand laterally,

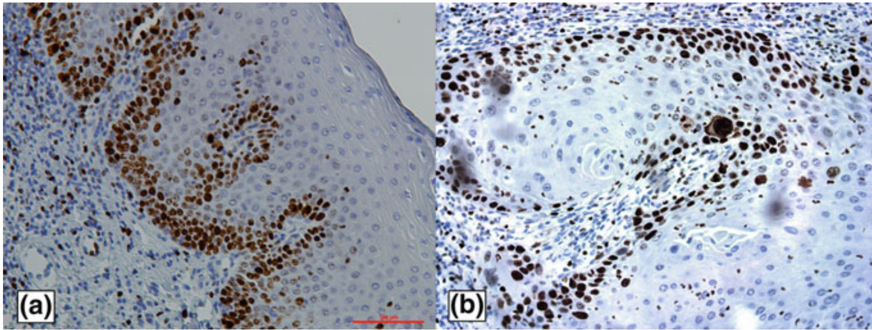

Fig. 2.2 **a** Although areas between proliferative units are predicted to be pyradimidal, the usual ki-67-assessed pattern of epithelial proliferative cells reveals linear rather than pyramidal parabasal proliferative areas (immunohistochemical technique, original magnification 20×). **b** Proliferative cells occupy basal and parabasal layers in premalignant and malignant epithelia (immunohistochemical technique, original magnification 20×)

occupying parabasal layers, meaning that the progeny of ATC division does not always pass to more superficial layers. This pattern would be more similar to the columnar organization observed on the dorsal skin of mice (Janes et al. 2002). In a human skin model developed by Janes et al. (2002), ATCs form a network around clusters of SCs containing around 40 cells. In common with our model, the assumption was made that each proliferative unit is maintained by more than one SC and that ATCs migrate laterally in parabasal layers. The linear rather than pyramidal proliferative pattern may also indicate areas of intersection between proliferative units and neighboring units, where epithelial renewal would be governed by the coordinated and alternating activity of different SCs, with ATCs proliferating in the parabasal layer responsible for epithelium renewal. In fact, epithelial renewal areas influenced by more than one SC may represent a further antitumor mechanism, allowing a reduction in the proliferation of each SC while maintaining epithelial homeostasis. It is not easy to determine the number of SCs, but all basal cells are very unlikely to be SCs. The nature of non-SC basal cells and their function in maintaining epithelial homeostasis has not yet been elucidated; however, they may be intermediate cells between SCs and ATCs with a low proliferation rate, possibly playing a role in the structural maintenance of SCs in the basal epithelial layer, as proposed in the human epidermis (Jensen et al. 1999).

2.4 Cancer Stem Cells

It is currently thought that a small group of tumor cells, known as CSCs, have the ability to self-renew and generate a phenocopy of the original tumor (Clarke et al. 2006; Sampieri and Fodde 2012), being progenitors of tumor bulk cells and driving tumor growth.

The idea that CSCs are responsible for tumorigenesis derives from the heterogenous composition of tumors, including OSCCs. Histologic studies reveal distinct areas of tumors with different degrees of differentiation; while genetic studies show a similar heterogeneity, with areas that differ in gene expression and therefore in protein expression; the cells in a tumor differ in proliferation rate and capacity to generate new tumors. As already noted, it is proposed that a small cell population of CSCs with a distinctive phenotype is responsible for the growth of a new tumor (Reya et al. 2001; Bánkfalvi et al. 2002; Tremmel et al. 2003; Pardal et al. 2003; Costea et al. 2006; Zhou and Jiang 2008; Visvader and Lindeman 2008; Mărgăritescu et al. 2011). Their existence was also brought to mind by the similarity in histologic profile and proliferation pattern between well-differentiated tumors and their epithelium of origin. The former are commonly organized in three compartments, as in normal epithelium (basal CSC, ATC, and innermost differentiated cell compartment). This similarity in proliferative hierarchy with non-tumor oral epithelia suggests that tumor growth is maintained by a single type of tumor cell, the CSC.

Bonnet and Dick (1997) found that a small group of tumor cells with CD34+/CD38 phenotype in human acute myeloid leukemia (AML) was able to generate AML when transplanted to NOD/SCID mice, but the other tumor cells were not. They proposed that the human AML is organized as a hierarchy of cells derived from primitive hematopoietic cells. Likewise, breast cancer tumors were generated in NOD/SCID mice by a small group of CD44+/CD24− cells but not by the 100-fold more numerous CD44− cells (Al-Hajj et al. 2003). CSCs have subsequently been evidenced in other solid tumors (Singh et al. 2003; Fang et al. 2005; O'Brien et al. 2006; Ho et al. 2007; Hermann et al. 2007; Yang et al. 2008) including HNSCC and OSCC, in which only a small subset of CD44+ cells was able to generate a tumor (Zhang et al. 2012).

2.5 Stochastic Model of Tumor Origin and the CSC Hypothesis

According to the stochastic model, malignant transformation is due to the random mutation of any cell, generating a tumor through the clonal progression of mutant cell progeny that possess a proliferative advantage and produce a genomic instability that leads to the accumulation of epigenetic/genetic events and the selection of progressively more aggressive subclones (Nowell 1976; Wicha et al. 2006; Campbell and Polyak 2007). Development by these subclones of different phenotypes and proliferative capacities would be responsible for tumor heterogeneity. In this model, cancer is considered a proliferative disease (Shakib et al. 2011).

The CSC hypothesis, supported by the aforementioned experimental evidence (Nowell 1976; Lapidot et al. 1994; Bonnet and Dick 1997; Al-Hajj et al. 2003; Singh

et al. 2003; Singh et al. 2004; Kim et al. 2005; Fang et al. 2005; Collins et al. 2005; O'Brien et al. 2006; Campbell and Polyak 2007; Prince et al. 2007; Dalerba et al. 2007; Hermann et al. 2007; Yang et al. 2008) has two components. The first component is related to the cell origin of tumors and the increased risk of accumulating multiple mutations during the long life of SCs. The malignant transformation of a normal cell is estimated to require 3–6 oncogenic events (Kinzler and Vogelstein 1996; Hahn and Weinberg 2002). A genetic model of progression was proposed in OSCC, correlating summative genetic alterations with phenotypic progression to malignancy (Califano et al. 1996; Califano et al. 2000; Gollin 2001) and indicating that the accumulation of sufficient mutations to develop OSCC would only be possible in cells with a long life. Furthermore, SCs and CSCs both possess self-renewal capacity, and self-renewal dysregulation is an early and key event in carcinogenesis. The self-renewal machinery is activated in normal SCs, and maintenance of this activation would appear more likely in comparison to a de novo activation in a more differentiated cell (Sawyers et al. 1991; Sell and Pierce 1994; Reya et al. 2001). The suggestion is that CSCs appropriate the self-renewal machinery in normal SCs. Animal studies have implicated dysregulation of the molecular pathways involved in self-renewal (Wnt, Notch and Hedgegog) in tumorigenesis (Wicha et al. 2006). Notch and Hedgehog activation enhances self-renewal in hemopoietic stem cells (HSCs) (Varnum-Finney et al. 2000; Karanu et al. 2000; Bhardwaj et al. 2001) and is observed in leukemias and other cancers (Unden et al. 1996; Diévart et al. 1999; Nam et al. 2002; Nickoloff et al. 2003; Benson et al. 2004; Olsen et al. 2004; Karhadkar et al. 2004). The pool of HSCs and progenitor hematopoietic cells is augmented by Wnt, with its downstream activator ß-catenin, increasing the cell proliferation rate in human bone marrow (Austin et al. 1997; Van Den Berg et al. 1998), while keratinocyte proliferation was stimulated by increased ß-catenin and Wnt protein levels in culture (Reya et al. 2001). Activation of the Wnt pathway has been observed in colon, skin, and oral cancer (Gat et al. 1998; Liu et al. 2010; Iwai et al. 2010; Fujii et al. 2011; Ravindran and Devaraj 2012).

The second component of the CSC hypothesis is that CSCs are responsible for maintaining tumor growth, supported by findings that SC transplantation into NOD/SCID mice not only enhanced cell self-renewal but also promoted tumor growth through their aberrant differentiation capacity, reproducing the heterogeneity of the original tumor (Wang and Dick 2005).

If the CSC hypothesis is correct, normal SCs must possess powerful anticancer mechanisms to reduce the risk of malignant transformation. One mechanism may be their asymmetric division pattern, giving rise to ATCs for proliferation, differentiation, and desquamation and preventing the accumulation of mutations in ATCs that suffer initial oncogenic events. The rate of SC division is also low in asymmetric division, reducing the risk of mutation at each mitosis, and can be even lower if renewal areas are influenced by more than one SC. A further anti-cancer mechanism may involve the selective segregation of DNA in the asymmetric division of the SC, as observed in bowel and breast SCs, with secretion of the new synthesized DNA strand from the SC compartment into ATCs (Potten et al. 2002; Dontu and Wicha 2005; Mackenzie 2006). In fact, the genome of adult SCs must be

highly stable, because they must be maintained and preserved by DNA repair mechanisms over an individual's lifetime. Thus, a high incidence of cancer is observed in syndromes with DNA repair defects (e.g., ataxia telangiectasia and xeroderma pigmentosum) (Mackenzie 2006). Some results suggest that CSCs may undergo altered behavior, including therapeutic resistance as a result of chromosomal instability due to chromosome segregation defects (Kaseb et al. 2016).

It is also possible, although less likely, that CSC may originate from the fusion of an HSC with a differentiated epithelial cell, acquiring the self-renewal capacity of the HSC (Bjerkvig et al. 2005), producing genomic instability, and promoting the accumulation of oncogenic events. A further possibility is the fusion of an HSC with a mutated epithelial somatic cell, giving rise to a mutant cell with SC features that can accumulate further oncogenic events. The fusion of HSCs with epithelial cells has been demonstrated *in vitro* (Wagers and Weissman 2004) and in animal models of stomach cancer (Houghton et al. 2004) but not in OSCC. The dedifferentiation of a mature cell might also result in a CSC, with oncogenic events leading mature epithelial cells to retrieve their self-renewal capacity and lose their terminal differentiation capacity (Gat et al. 1998; Zhu and Watt 1999). These cells may acquire additional mutations that produce their transformation (Perez-Losada and Balmain 2003). However, the acquisition of SCs requires the reprogramming of differentiated cells, for which identity maintenance is essential; therefore, this reprogramming would require the involvement of powerful regulators of the transcriptional and/or epigenetic machinery (Abollo-Jiménez et al. 2010). Four transcription factors participate in reprogramming in oncogenesis, and the early inhibition of somatic genes involved in differentiation by c-Myc appears to be crucial. Histone deacetylase inhibitors can replace c-Myc in reprogramming (Huangfu et al. 2008; Iglesias-Linares et al. 2010), participating in suppression of the differentiated cell's gene expression program. The transcription factors OCT-4, Sox-2, Klf-4, and 4YTF become involved in reprogramming at a later stage (Abollo-Jiménez et al. 2010).

It was recently reported that differentiated cancer cells can become CSC-like via epithelial mesenchymal transition (EMT) , in which epithelial cells acquire mesenchymal characteristics under the influence of specific environmental stimuli and can invade surrounding tissues and spread to distant organs (see Chap. 1). EMT is promoted by the activity of transcription factors such as Snail, Twist 1, and ZEB 1 (Batlle et al. 2000; Yang et al. 2004; Mani et al. 2008; Sánchez-Tilló et al. 2010), which change epithelial cell polarity and repress E-cadherin expression, among other effects (Cano et al. 2000). EMT was found to be involved in the acquisition by differentiated cells of SC properties in breast (Morel et al. 2008) and nasopharyngeal (Xia et al. 2010) cancer and HNSCC, in which Bmi-1, another transcription factor involved in SC self-renewal, was induced by Twist 1 and E-cadherin expression was repressed (Park et al. 2004; Valk-Lingbeek et al. 2004; Widschwendter et al. 2007; Spivakov and Fisher 2007; Zhang et al. 2012). E-cadherin downregulation in suprabasal oral premalignant epithelia has been implicated in multiple tumor development, which may indicate a reprogramming process related to molecular mechanisms involved in self-renewal and the acquisition of CSC-like characteristics

by partially-differentiated oral epithelial cells (ATCs) (González-Moles et al. 2012a, b).

Finally, CSCs can be produced by neosis, a new type of cell division seen in cancers, in which DNA damage can generate senescent multinucleated cells that evade apoptosis, divide, and give rise to SC-like cells (Sundaram et al. 2004); their division produces mononuclear "Raju" cells with extended mitotic life and the capacity to generate transformed cell lines (Sundaram et al. 2004; Rajaraman et al. 2005), similar to observations in senescent oral keratinocytes in culture (Kang et al. 2000). Nevertheless, although multinucleate cells are common in OSCCs and premalignant epithelia, it is not known whether neosis can give rise to CSCs in OSCC (Costea et al. 2006).

Proliferative patterns observed by our group (González-Moles et al. 2010) in OSCC and non-tumor epithelia adjacent to invasive carcinomas (González-Moles et al. 2000; González-Moles et al. 2010) suggested different origins for CSCs and precancerous SCs. Basal proliferation can sometimes be observed in premalignant epithelia and tumor nests neighboring non-neoplastic epithelia. Given the basal localization of SCs, this may indicate that the usual asymmetrical division of normal SCs is replaced with a symmetric division, in which a premalignant SC or CSC generates two premalignant SCs or two CSCs that stay in the basal layer of premalignant epithelia or at the periphery of well-differentiated tumor nests, respectively. The over-accumulation of premalignant SCs would exceed the capacity of the basal layer, gradually leading to their occupation of parabasal layers. Likewise, the basal and suprabasal proliferation of CSCs can also produce an expansive growth in well-differentiated OSCC nests (Fig. 2.2). On the other hand, elevated basal and suprabasal proliferation rates in some epithelia might also be attributed to premalignant or malignant SCs that still proliferate asymmetrically but have suffered mutational events that enhance their proliferation rate. Asymmetric division of these CSCs would lead to a malignant transitory amplifying compartment that is able to differentiate, accounting for the maintenance of tumor nests with a similar appearance to that of normal or premalignant epithelia. Another frequent observation in premalignant epithelia is a scantly proliferative basal layer with highly proliferative parabasal cell layers (2–3 layers) (Fig. 2.2), which was associated by our group with multiple tumor development (González-Moles et al. 2010). This observation may indicate that the tumor does not only originate in normal basal SCs but can sometimes originate in ATCs, whose high proliferation rate may increase the mutation risk, altering the reprogramming process; this would allow ATCs to gain greater self-renewal capacity and maintain an elevated proliferation rate without completely losing their differentiation capacity, i.e., CSC-like characteristics. In the presence of genomic instability, this would increase the risk of new oncogenic events.

Proliferative cells are very occasionally observed in epithelium above the transient amplifying compartment, which may possibly correspond to the reprogramming of differentiated cells. The virtual absence of proliferative cells in superficial layers of normal epithelium corroborates the difficulty of reprogramming differentiated cells, which requires major molecular changes.

2.6 Identification of Stem Cells and CSCs

There is a lack of SC markers, limiting investigation of the participation of SCs in carcinogenesis. They can be only identified by analyzing their replicative behavior *in vitro* and detecting long-lived cells in tissues (Janes et al. 2002).

In *in vitro* clonal trials, keratinocytes in low-density cultures produce different types of colonies depending on their precursors. Large, compact, actively growing colonies are sometimes observed, known as holoclones (Barrandon and Green 1987), comprising small cells from individual founding cells that can be repeatedly passaged. Other keratinocytes divide a few times, lose their proliferation capacity, and develop terminal differentiation, producing abortive colonies (paraclones) of large flattened cells. Finally, keratinocytes can generate colonies (meroclones) of cells in a state of transition between holoclones and paraclones. Cells forming holoclones tend to be classified as SCs, while cells forming paraclones are considered ATCs. As in the normal epithelium, malignant keratinocytes can give rise to malignant holoclones that differ from malignant paraclones, comprising smaller, more adhesive, and more rapidly clonogenic cells. The behavior and expression patterns of markers of cells that make up malignant holoclones are similar to those of normal epithelial SCs and possess the essential characteristics of a CSC (Locke et al. 2005). The *in vitro* clonal assay is a reproducible method for identifying and isolating SCs and CSCs and evaluating their response to therapeutic agents (Janes et al. 2002).

Long-surviving cells in the oral mucosa can be identified by labeling and flow cytometry using β-1 integrin, α-6 integrin, CD71, E-cadherin, β-catenin, epithelial-specific antibody, and CD44 as surface makers, most of which show elevated expression in holoclones from OSCC cell lines. However, there are few reliable markers of oral SCs (Tudor et al. 2004). Many are also expressed by non-SCs, e.g., β-1 integrin (Jones et al. 1995) and CD44 (González-Moles et al. 2004), while some are intracellular and undetectable by flow cytometry (Kaur et al. 2004).

The most frequently applied method for identifying CSCs in culture is to use flow cytometry to detect cells able to excrete the vital DNA dye Hoechts 33342 (Goodell et al. 1996; Wang et al. 2007; Loebinger et al. 2008; Zhou et al. 2008; Sung et al. 2008; Zhang et al. 2009; Chen et al. 2009; Yajima et al. 2009; Song et al. 2010; Yanamoto et al. 2011), recording dye-retaining cells and a distinctive small non-dyed population of cells (side population [SP]) expressing SC markers. The capacity of SPs to excrete Hoechts 33342 dye depends on the cell membranous activity of the superfamily of ABC transporter pumps. The human ABCG2 member is considered a CSC marker (Seigel et al. 2005; Ho et al. 2007; Shi et al. 2008; Wang et al. 2009). SP cells have been identified in various tumors (Kondo et al. 2004; Hirschmann-Jax et al. 2004; Haraguchi et al. 2006; Chiba et al. 2006; Szotek et al. 2006) and in normal tissue (Shimano et al. 2003; Kim and Morshead 2003; Majka et al. 2005; Larderet et al. 2006). In HNSCC, SP cells are highly tumorigenic (Wang et al. 2007; Zhang et al. 2009; Song et al. 2010; Wan et al. 2010; Tabor et al. 2011), expressing SC markers (e.g., ABCG2, (Mack and Gires 2008; Song et al. 2010; Tabor et al. 2011), Bmi-1(Wan et al. 2010; Tabor et al. 2011), CD44, and

Oct4 (Zhang et al. 2009), and showing an abnormal Wnt signaling pathway(Song et al. 2010; Tabor et al. 2011). The SP population is highly variable in OSCC cell lines, ranging from 0.2 to 10% of the cancer cell population (Zhang et al. 2009).

However, these procedures are not suitable for routine use and do not enable the topographic localization of SCs in healthy or tumor tissue for evaluating their proliferative activity or spatial relationships with their progeny. Some promise has been shown by the following markers:

2.6.1 β-1 Integrin

β-1 Integrin is a potential oral SC marker, is expressed in basal keratinocytes and downregulated when cells leave the basal layer (Cotsarelis et al. 1999; Janes et al. 2002). Human keratinocytes can be classified by the rate of their binding with type IV collagen, the natural ligand of β-1 integrin, into two types. Fast-adhering keratinocytes resemble SCs (Jones et al. 1993; Jones 1996), whereas slow-adhering keratinocytes, with low levels of integrin β-1, behave as late ATCs (Jones et al. 1993; Jones 1996), suggesting that integrin β-1 is required to maintain the keratinocytes in an undifferentiated state (Adams and Watt 1989; Levy et al. 2000; Hombach-Klonisch et al. 2008). The main shortcoming of integrin β-1 as a SC marker is its lack of specificity, given that around 20–45% of basal keratinocytes show high integrin β-1 expression.

2.6.2 Transcription Factors Oct3/4, Sox and Nanog

Oct3/4 (Nichols et al. 1998; Niwa et al. 2000), Sox (Avilion et al. 2003) and Nanog (Chambers et al. 2003; Mitsui et al. 2003), play a fundamental role in maintaining the pluripotency and self-renewal of embryonic and adult SCs (Boyer et al. 2005; Loh et al. 2006; Campbell et al. 2007), promoting self-renewal through interaction with Stat-3, Hesx-1, and Zic-3, and signaling molecules TCF-3, FGF-2, and LEFTV2 (Boyer et al. 2005; Loh et al. 2006). Oct3/4 is regarded as one of the best indicators of stemness (de Jong and Looijenga 2006; Marynka-Kalmani et al. 2010). HNSCC cells forming holoclones express high levels of these factors (Lim et al. 2011), suggesting that cancerous cells expressing these factors have SC-like behavior (Zhang et al. 2012).

2.6.3 CD 133

The protein CD 133 (Miraglia et al. 1997; Yin et al. 1997) was used as a marker of HSCs (Yin et al. 1997; Chiou et al. 2008) and subsequently as a marker of epithelial

(Weigmann et al. 1997; Corbeil et al. 2000), neural (Uchida et al. 2000; Lee et al. 2005), prostate (Shepherd et al. 2008), and kidney (Bussolati et al. 2005) SCs. Studies in OSCC (Zhang et al. 2010) have demonstrated that a small proportion of tumor cells (1–3%) are CD133+ and are highly clonogenic and tumorigenic, showing increased resistance to chemotherapy (Zhang et al. 2010). CD133+ cells from OSCC form holoclones and possess self-renewal capacity (Chiou et al. 2008), while CD133 is expressed by CSCs in the Hep-2 laryngeal cancer line (Zhou et al. 2007; Wei et al. 2009). Therefore, CD133 may be a useful CSC marker in HNSCC and OSCC (Zhang et al. 2012), although some authors did not detect CD133+ cells in oral cancer (Märgäritescu et al. 2011).

2.6.4 CD44

CD44 (Aruffo et al. 1990; Screaton et al. 1992) provides anchorage for MMP-9, which is essential for metalloproteinase activity and may favor tumor invasiveness. CD44 was the first CSC marker to be used in breast cancer, and Prince et al. (2007) reported a group of CSC-enriched CD44+ cells in HNSCC that could be serially passaged in vivo, reproducing the original tumor. These CD44+ cells expressed high Bmi-1 levels and displayed self-renewal and differentiation capacities. However, questions have been raised about the value of CD44 as a CSC marker in OSCC because of its expression by a much larger number of tumor and normal oral epithelial cells (González-Moles et al. 2004; Mack and Gires 2008; Clay et al. 2010; Oliveira et al. 2011) than the small number of CSCs considered to be present in oral tissues. There is also debate about the usefulness of CD44 as a marker of OSCC progression and prognosis. Thus, some researchers associated higher CD44 expression with greater tumor aggressiveness (Okamoto et al. 2002; Chen et al. 2010; de Jong et al. 2010; Joshua et al. 2012), while others associated reduced or loss of CD44 expression with a negative prognosis (Sato et al. 2000; Carinci et al. 2002; González-Moles et al. 2002, 2004; Kosunen et al. 2007; Clay et al. 2010). The loss of CD44 expression was found by our group to be an early event in oral carcinogenesis, favoring the acquisition of invasive capacity by premalignant epithelial clones and associated with a greater thickness of tumor invasion, behaving as an independent predictor of poor survival. Association of the loss of a stem cell marker with a worse prognosis appears inconsistent with the notion that tumor growth depends on CSCs, although this may be explained by an ability of some CSC properties (e.g., expression of some adhesion molecules) to be lost and re-expressed, favoring invasion and the reestablishment of stable, cohesive metastatic colonies. This has been reported for the expression of E-cadherin adhesion molecule in EMT and in the mesenchymal-to-epithelial transitional stage (MET) (Kudo et al. 2004; Zhang et al. 2012). It may be possible to improve the value of CD44 as a SC marker by combining it with DHAL activity markers. DHAL, a family of intracellular enzymes, participates in cell differentiation, detoxification, and drug resistance through the oxidation of intracellular aldehydes

(Moreb et al. 1996; Magni et al. 1996; Sophos and Vasiliou 2003; Chute et al. 2006). DHAL1, the prototype of the family, is expressed in HSCs and hematopoietic progenitor cells (Magni et al. 1996; Chute et al. 2006), and in normal and malignant breast and lung SCs (Ginestier et al. 2007; Sullivan et al. 2010). Some studies in HNSCC have described ALDH-positive cells with CSC-like behavior and increased tumorigenic capacity (Chen et al. 2009; Clay et al. 2010; Krishnamurthy et al. 2010; Chen et al. 2011). It was reported that the specificity of CD44 as a CSC marker in HNSCC is increased by combining it with ALDH (Chen et al. 2009; Clay et al. 2010), and the production of a tumor in NOD/SCID mice was found to require the transplantation of tenfold fewer ALDH+/CD44+ cells in comparison to cells positive for CD44 alone (Prince et al. 2007). According to these findings, ALDH expression may isolate a subset of CSC-enriched CD44+ cells, although both markers are not always expressed in CSCs, and there is a small proportion of CSCs that are positive for ALDH and negative for CD44 (Clay et al. 2010).

2.6.5 E-Cadherin

E-cadherin binds to actin in the cytoskeleton through interaction with catenins (Hajra and Fearon 2002). The downregulation of E-cadherin, an invasion suppressor molecule (Vleminckx et al. 1991), predicts a worse prognosis (Hoteiya et al. 1999). E-cadherin underexpression was observed by our group in precancerous fields associated with multiple tumor development (González-Moles et al. 2012a, b). Loss of E-cadherin expression allows the release of ß-catenin, which acts as a transcription factor, activating the Wnt pathway (Takes et al. 2001). E-cadherin can be downregulated by hypermethylation of CDH1 promoter, enhancing the metastatic potential of hypermethylated clones. E-cadherin-methylated tumor cells may be focally dissociated at invasion fronts, spread to lymph nodes and then become demethylated, reacquiring E-cadherin expression (Kudo et al. 2004). Hence, methylation is reversible in OSCC progression, an important issue because E-cadherin may favor the growth of metastatic tumor cells nests by compacting cellular aggregates and activating apoptosis evasion pathways (Kantak and Kramer 1998). As noted above, E-cadherin downregulation is essential in EMT, promoting invasiveness and conferring tumor cells with SC properties (Yu et al. 2012), while E-cadherin recovery is important for MET. Hence, the state of a cell and its SC characteristics can be modified to gain an invasive or metastatic advantage. However, despite its role in the physiology and biopathology of SCs, doubts remain about the value of E-cadherin as a CSC marker in OSCC (Zhou and Jiang 2008), mainly because of its low specificity (González-Moles et al. 2012a, b).

A range of other molecules have been tested for CSC markers. These include CD147, CD97, CD117, Ep CAM, CK19 and ESA but have shown poor specificity for the identification of oral SCs (Hombach-Klonisch et al. 2008; Zhou and Jiang 2008; Richard and Pillai 2010).

2.7 Conclusions

The CSC hypothesis opens up a wide field for future research, and there is an urgent need to identify specific markers for the detection of cancer stem cells in routine laboratory tests. The availability of reliable markers would improve knowledge of the cell types that generate a tumor, their distribution in tissues, and their relationships with their progeny, allowing exploration of the prognostic relevance of their proliferative activity and invasive capacity. This type of research has potentially important therapeutic implications, given the possibility of designing effective therapies that specifically target CSCs.

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