

# Embryonic and Cancer Stem Cells - two views of the same landscape

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## 1. Introduction

According to the Cancer Stem Cell (CSC) theory, there is a small subset of neoplastic cells in the tumors, which retain unlimited proliferative capacity. These cells give also rise to a differentiated cancer progeny that does not have spreading potential but makes up the bulk of the tumor. Thus, CSCs would be the ultimate cause of tumor growth, maintenance and recurrence (Figure 1).

CSCs are experimentally defined by the ability to recapitulate the heterogeneity of the original tumor when transplanted into immunocompatible or nude mice. In 1867 Julius Cohnheim proposed that tumors are derived from embryonal cells that rest in the adult tissues. Later on, in the middle of the following century, Furth and Kanh (1937) and Pierce and Dixon (1959) proved the stem cell properties of a subset of cells in leukemia and testicular germ cell tumors (TGCTs) and Till and McCulloch (1961) transplanted colony forming units (CFU) from the bone marrow into lethally irradiated mice. Additionally, the group of Barry Pierce showed the *in vitro* modulation of cancer cell differentiation (Pierce & Verney, 1961) and the *in vivo* cloning of single embryonal carcinoma (EC) cells, proving their pluripotency (Kleinsmith & Pierce, 1964). The differentiation of cloned leukemic cells (Pluznik & Sachs, 1965) and the reprogramming of embryonal carcinoma (EC) cells when injected into early embryos (Brinster, 1974) were also outstanding results. All these discoveries paved the way for the isolation of embryonic stem (ES) cells (Evans and Kaufman, 1981; Martin, 1981) and one of the above groups also studied the differentiation of CSCs from distinct tumors, like squamous cell carcinoma, chondrosarcoma and adenocarcinoma (Pierce & Wallace, 1971; Pierce, 1974; Pierce et al., 1977) seeding the concept of cancer differentiation therapy (Pierce & Speers, 1988). A new progress in this matter was done with the isolation of CSCs in human acute myeloid leukemia (Lapidot et al., 1994) and, particularly, with the discovery of new markers for progenitor cells in several solid tumors, such as breast (Al-Hajj et al., 2003), brain (Singh et al., 2003) and colon (O'Brien et al., 2007) cancer.

During the evolution of CSC research, there have been several technical improvements that have undoubtedly contributed to the success in the engraftment of the tumor cells in the host mice and the subsequent tumor formation. Firstly, it has been proved that the immune system of the host notably affects the survival of the transplanted cells, and thus, the use of

mutant mice with less effective immune systems increases the calculated CSC number within the studied tumor. Similarly, CSCs have been shown to be more prone to successfully form tumors when transplanted accompanied by either carcinoma-associated fibroblasts or *Matrigel*® (Hwang et al., 2008, Quintana et al., 2008).

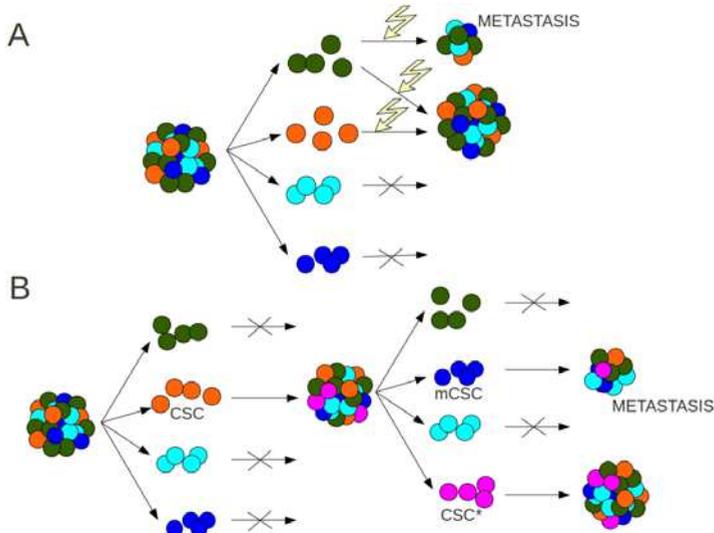


Fig. 1. Hierarchical and stochastic models of CSC in solid tumor growth. (A) According to the stochastic model of cancer progression, every cell present in the neoplasia is able to generate all the cell lineages of the tumor. Nonetheless, the malignant potential of the cells may depend on external factors. (B) The CSC hypothesis states that only a small population of cells, is responsible for tumor growth, due to their self-renewal capacity and unlimited proliferative potential. As tumor progresses, distinct CSCs (CSC\*) may originate due to additional mutations or epigenetic modifications. Some of these new CSCs may undergo the epithelial-mesenchymal transition, retaining stem cell characteristics, giving rise to migrating CSCs (mCSCs), the ultimate cause of metastasis

It is important to consider that the CSC model has not yet been proven true for all the existing tumor types. Thus, it is also thinkable that some cancers follow the random or stochastic model that states that every cell within the tumor may have the ability to seed tumors under the required conditions. The importance of getting to a better distinction between tumors that follows one model or the other lies on the increase of the effectiveness of cancer treatments, which will be condemned to failure if right cells are not targeted.

## 2. Cancer stem cell origin

Most cancers are believed to arise from a single cell that undergoes malignant transformation triggered by genetic mutations or epigenetic modifications, followed by clonal selection of cells that gain the ability to adapt to the microenvironment. Although CSCs have been described and even isolated in several tumor types, the identity of the pre-cancerous cell that first acquires the tumorigenic potential is still a matter of debate. On one

hand, tissue-specific stem cells residing in normal tissues or organs, may acquire malignant characteristics and turn into CSCs. This hypothesis is supported by the fact that tumorigenic modifications require years to accumulate. Given that stem cells present the longest life span in the organism, they are the ideal targets of the neoplastic process. Furthermore, adult stem cells share many functional characteristics with CSCs, e.g. self-renewal and the potential to differentiate into several cell types.

It is likewise possible that the first mutagenic "hit" affects somatic cells responsible for the support of the specialized stem cell niche. This specific microenvironment is necessary for the maintenance of the stem cell identity. Hence, alterations in one or more of the supporting cells may cause that non-tumorigenic stem cells receive aberrant signals which trigger their transformation into malignant cells.

Nevertheless, adult stem cells may not be the source of every type of cancer. CSCs could arise from normal somatic cells, that as a consequence of genetic mutations are re-programmed into "defective" stem cells. This is likely the case for T-lymphoid leukemia that develops from T-cells in which the T-Cell Receptor (TCR) gene rearrangement has already occurred. This genomic modification happens during T-Cell maturation in the thymus and is unique and irreversible. Interestingly, T-Cell leukemias present clonal TCR rearrangements and thus, it seems clear that in this case tumors arise from somatic cells that undergo de-differentiation and recover stem cell properties (Schmidt & Przybylski, 2001).

Experiments that comprise the analysis of cell lines cultured in 3D systems, have shown the ability of certain cells to adopt a stem cell-like phenotype under particular culture conditions. Liu and coworkers reported that embryonic fibroblasts from retinoblastoma (Rb) knockout mice, are able to form spherical structures that express specific stem cell markers when cultured in suspension, and some cells in the spheres adopted CSCs characteristics (Liu et al., 2009). This process has also been reported in the 293T cell line, derived from human embryonic kidney (Debeb et al., 2010). Furthermore, Meyer and colleagues have suggested the possibility of reprogramming differentiated cancer cells into CSCs (Meyer et al., 2009). They have reported that non-invasive CD44+/CD24- breast cancer cells are able to give rise to malignant CD44+/CD24- progeny *in vivo* and *in vitro*.

In 2006, Takahashi and Yamanaka reported that the overexpression of a cocktail of 4 transcription factors (Oct4, Sox2, c-Myc and Klf4) in adult fibroblasts turned them into pluripotent stem cells (Takahashi & Yamanaka, 2006). Recent studies have demonstrated that these so-called induced pluripotent stem (iPS) cells and ES cells exhibit similar gene expression signatures and potentiality. Because they are genetically modified cells, the idea of using these first generation iPS cells in therapeutic treatments has been seriously questioned. Furthermore, two of the four genes used in the cocktail have been shown to behave as oncogenes (Geoghegan & Byrnes, 2008). In order to solve these issues, new methods for iPS generation have been established, which instead of transfecting cells, are based on the use of recombinant proteins (Zhou et al., 2009).

Taken together, these experiments reveal the capacity of somatic cells to acquire pluripotency. Thus, it could also be possible that alterations in the expression of one or more of those genes triggers the transformation of a given normal somatic cell into a malignant cell capable of forming a tumor. Indeed, the overexpression of Oct4 can lead to epithelial dysplasias by blocking the differentiation of progenitor cells (Hochedlinger et al., 2005). Moreover, several tumor types, for example bladder carcinoma, lung adenocarcinoma, ovarian carcinoma and testis tumors present abnormally higher levels of Oct4 when compared to their normal counterpart tissues. Similarly, Klf4, Sox2 and c-myc appear

upregulated, either alone or together, in a variety of hematological malignancies and solid tumors, including brain, breast, bladder, lung, pancreas, colon and kidney cancer (Schoenhals et al., 2009).

Interestingly, non-CSCs present in the tumor may as well gain stem cell properties due to the acquisition of further genetic and epigenetic modifications and in response to changes in the tumor microenvironment. Therefore, the cell population responsible for tumor growth at a given tumor stage, may not be the same during tumor evolution or metastasis (Roesch et al., 2010). In this regard, the previously mentioned CSC model proposes that CSCs are the only cells within the tumor that can acquire the ability to spread and grow in distant sites.

### 3. Cancer types in which CSCs have been identified

Although the existence of CSCs has been reported in several tumor types, so far it has not been described any marker that exclusively labels CSCs. Normally, these cells are isolated using antibodies specific for normal stem cells of the same tissue from which the tumor is originated. Flow-cytometry-based cell-sorting, enables the isolation of a side population which is transplanted into host mice to test their tumorigenic potential and conclude whether indeed, it is enriched in CSCs. CD24, CD44, CD133, epithelial specific antigen (ESA) and ATP-binding cassette B5 (ABCB5) are some of the cell surface markers that have been proved to be differentially expressed by those CSC enriched populations (Table 1).

Tumor type	Surface markers used to purify the CSCs	References
Accute myeloid leukemia	CD34+/CD38-	Lapidot et al., 1994
Breast cancer	CD44+/CD24-/CD24low	Al-Hajj et al., 2003
Brain cancer	CD133+	Singh et al., 2003
Head and neck squamous cell carcinoma	CD44+	Prince et al., 2007
Colon cancer	CD133+	O'Brien et al., 2007
Pancreatic adenocarcinoma	CD44+/CD117+	Li et al. 2007
Melanoma	ABCB5+	Schatton et al., 2008
Ovarian cancer	CD44+/CD24+/ESA+	Zhang et al., 2008
Prostate cancer	CD133+/ $\alpha$ 2 $\beta$ 1integrin/ CD44+	Maitland & Collins, 2008

Table 1. Distinct surface markers have been extensively used to isolate CSC-enriched subpopulations from different cancer types

Leukemia and TGCTs were the first tumor types in which CSCs were experimentally described (Furth & Kanh, 1937; Pierce & Dixon, 1959). Furth and Kahn proved that a single cell from a murine cancer cell line was able to transmit leukemia. Regarding TGCTs, Kleinsmith and Pierce (1964) dissociated teratocarcinoma-derived embryoid bodies and transplanted single cells into host mice. Around 2.5% of the grafted cells had the ability to form new tumors, albeit their differentiation potential was highly variable. Later on, Stevens

(1968) managed to generate testicular teratocarcinomas by grafting 3 and 6-day embryos into testicles of adult mice and to serially transplant the formed tumors. It was not until 1994 when surface markers were used to isolate an enriched fraction of tumor-initiating cells. In that year, Lapidot and coworkers (1994) separated a subpopulation of CD34+/CD38- cells from human acute myeloid leukaemia (AML) patients and verified their CSC properties after transplanting them into severe combined immune-deficient (SCID) mice observing that they were able to recapitulate the disease.

Regarding solid tumors, it is likely that additional technical issues, such as the difficulty in obtaining homogeneous single cell suspensions for their further separation, delayed the achievement of a well-characterized CSCs population from a human tumor. The first solid malignancy from which CSCs were isolated was breast cancer. Al-Hajj and colleagues described a CD44+/CD24-/low cell population that was significantly enriched in tumor-initiating cells (Al-Hajj et al., 2003). Shortly after these findings, CSCs of brain tumors were also isolated using CD133 as a marker and characterized by the expression of other markers for non-pathological neural stem cells (Singh et al., 2003). Interestingly, different pathologic subtypes of brain tumors, like medulloblastomas, astrocytomas and gangliogliomas, share common CSCs. However, the self-renewal capacity of CSCs varies depending on the tumor subtype and its aggressiveness.

Prince and colleagues (2007) developed an immunodeficient mouse model to test the tumorigenic potential of different populations of cancer cells derived from primary human head and neck squamous cell carcinoma (HNSCC). They reported that a population of CD44+ cancer cells was capable of giving rise to new tumors that reproduced the original tumor heterogeneity and could be serially passaged. Moreover, these CSCs had a primitive cellular morphology and did express the basal cell markers Cytokeratin 5 and 14. On the contrary, CD44- cells did not show tumorigenic potential and were similar to differentiated squamous epithelium as assessed by the expression of differentiation markers such as Involucrin.

Later on, it was established that in colon cancer, CSCs are characterized by the expression of the CD133 marker (O'Brien et al., 2007). In humans, colon cancer-initiating cells represent only around 0.06% of all cells within the tumor, but 0.4% of them are CD133+. Thus, it is still necessary to find additional cell surface markers in combination with CD133 in order to further purify the CSC fraction from this type of cancer.

In 2007, Li and colleagues analyzed the tumor initiating ability of different subpopulations from primary human pancreatic adenocarcinoma. They reported that those pancreatic cancer cells with a CD44+/CD24+/ESA+ phenotype, that represent around 0.2 to 0.8% of pancreatic cancer cells, had a 100-fold increased tumorigenic potential compared to non-tumorigenic cancer cells. Furthermore, the genetic analysis of pancreatic CSCs revealed an increased expression of the signaling molecule Sonic Hedgehog (Li et al., 2007).

Schatton and colleagues (2008) identified an ACBB5+ subpopulation of melanoma cells that represents 0.0001% of the total tumor cells and shows high capacity to re-establish the malignancy after xenotransplantation into mice. In addition, they proved that the specific targeting of these CSCs using monoclonal antibodies against ABCB5, resulted in inhibition of tumor growth. However, Quintana and collaborators (2008) have recently shown that the amount of CSCs in human melanoma may change dramatically depending on the conditions of the xenotransplantation assay. This finding raises the question whether tumors in which CSCs are rarely detected, may in fact have a higher number of these cells. For example, tumor microenvironment as well as site of inoculation may have an influence on the tumorigenic potential of presumptive CSCs. The frequency of CSCs in human

melanoma reported by this group is indeed much higher than reported for any other cancer type that follows the CSC model. Although efforts were made to characterize those melanoma-seeding cells, they could not find phenotypic differences between tumorigenic and non-tumorigenic cell populations.

Zhang and colleagues (2008) have also identified the surface phenotype of CSCs from ovarian adenocarcinoma. These cells are characterized by a higher expression of CD44 and CD117 (c-kit), Bmi-1, stem cell factor, Notch-1, Nanog, nestin, ABCG2 and Oct4 when compared to non-malignant ovarian tumor cells (Zhang et al., 2008). Finally, Maitland and Collins (2008) have reported that tumor-initiating cells are enriched in a CD133+/ $\alpha$ 2 $\beta$ 1integrin+/CD44+ subpopulation from human prostate cancer. Moreover, prostate CSCs have a unique genetic fingerprint that makes them useful to predict the tumor staging and clinical outcome. Remarkably, these putative CSCs do not express androgen receptor, which makes them refractory to the widely used androgen-based therapies (Maitland & Collins, 2008).

#### 4. CSC and ES cell similarities

Three different types of non-malignant stem cells have so far been described *in vivo*: ES cells, chord blood/placental stem cells and adult stem cells. Among them, ES cells are the only ones that show pluripotency, being capable of giving rise to cell derivatives of the three germ layers: ecto-, endo- and mesoderm. ES cells are obtained from the inner cell mass (ICM) of the blastocyst and can be cultured *in vitro* using specific conditions to prevent their differentiation. In turn, adult and chord blood/placental stem cells are multipotent and can differentiate into a limited number of cell lineages (Rogers & Casper, 2003).

The ability of cancer cells to grow indefinitely led to the belief that CSCs were similar to adult stem cells. However, detailed gene expression analysis of both cell types reveals that actually, CSCs share more characteristics with ES cells. In fact, long before the CSC hypothesis was enunciated, it was observed that tumor and embryo development share multiple common features. For instance, many tumors have been histologically classified due to their differentiation state, being this characteristic also relevant to prognosis. This is the case of TGCTs that are characterized by the presence of embryonic and extra-embryonic tissues, together with embryonal carcinoma (EC) cells, a population of pluripotent stem cells. EC cells, the CSCs of TGCTs, are considered the pathological counterpart of ES cells, due to their ability to lose their malignant phenotype and participate in normal embryo development when transplanted into blastocysts. In the resulting chimeric mice EC cells appear in tissues derived from the three germ layers, including germ cells (Brinster, 1974; Mintz & Illmensee, 1975). Based on these similarities, EC-derived post-meiotic neurons have been implanted in damaged regions of the brain in a clinical trial (Hara et al., 2008). Nonetheless, due to the karyotypic instability of EC cells, these regeneration therapies have some safety concerns.

##### 4.1 Gene signature in CSC and ES cells

A key goal in cancer research is to identify the molecular mechanisms by which CSCs arise and acquire their stem-like characteristics. Nowadays, extensive databases, mainly obtained by microarray analysis, have been generated, offering the possibility of comparing the expression profile of a huge number of both tumor and normal cells or tissues. Based on the fact that ES and cancer cells share properties such as self-renewal and differentiation

capacity, there has been a recent increased interest in finding out whether CSCs and ES cells present a similar gene signature. As previously mentioned, Oct4, Sox2, c-Myc and Klf4 comprise the gene cocktail that induces pluripotency in somatic cells by a process known as "somatic cell reprogramming" (Takahashi & Yamanaka, 2006). Thus, it is believable that alterations in the expression of one or more of these ES-defining genes may trigger the transformation of a normal somatic cell into a malignant CSC.

Oct4 is the commonly used synonym for POU5F1 (POU class 5 homeobox 1). This transcription factor is active from the fertilized oocyte throughout the whole preimplantation period of embryo development. Oct4-deficient mouse embryos fail to form the ICM, lose pluripotency and differentiate into trophoblast. Therefore, the level of Oct4 expression in mice is crucial for regulating pluripotency and early cell differentiation since one of its main functions is to maintain the undifferentiated state of the embryo (Zaehres et al., 2005). Moreover, an Oct4 overexpression leads to epithelial dysplasias by blocking the differentiation of progenitor cells (Hochedlinger et al., 2005). It is likewise known that several tumor types, as for example bladder carcinoma, lung adenocarcinoma and testis tumors among others, present increased levels of Oct4 expression compared to their normal counterpart tissues (Schoenhals et al., 2009). It has been recently reported the existence of a subpopulation of cells from ovarian cancer that express Lin 28 and Oct4 genes, both highly expressed in human ES cells. In fact, the up-regulation of these genes in tumor samples is correlated with advanced tumor grade (Peng et al. 2010). Interestingly, the CSC population isolated from ovarian cancer has an up-regulated expression of Oct4 (Zhang et al., 2008). The expression of these two factors seems to be essential for cell growth, since their inhibition using iRNA results in a significant reduction in cell growth and survival (Peng et al., 2010).

Sox2, also known as Sry (sex determining region Y), is a key transcription activator during early embryonic development and its activity is also important in adult stem cells, since it has been reported to maintain the proliferative potential of neural stem cells (Episkopou, 2005). Oct4 forms an heterodimer with Sox2 that drives the expression of several pluripotent-specific genes, including Nanog, FGF-4, UTF1, Fbx15 and Lefty1, together with Oct4 and Sox2 themselves. The expression of at least three of these genes (Nanog, Sox2 and Oct4) is essential to maintain the pluripotent ES cell phenotype. With the possible exception of Lefty1, the expression level of each of the genes regulated by the Oct4/Sox2 complex is substantially reduced upon differentiation of both ES and EC cells, due to the down-regulation of Sox2 and Oct4 (Boer et al., 2007). It has also been reported that the CSCs of multiple myeloma express high levels of Sox2, together with Oct4 and c-Myc, being the latter a well-known proto-oncogene. c-Myc was first described in Burkitt's lymphoma patients, and its function has been proved to be crucial for early embryo development and adult stem cell maintenance. For example, antisense DNA inhibition of c-Myc expression in preimplantation mammalian embryos results in developmental arrest at the eight-cell morula stage (Paria et al., 1992). Over-expression of c-Myc has been described in several hematological malignancies, such as leukemia, lymphoma, smoldering myeloma and multiple myeloma. Wang and colleagues (2008) studied the importance of this transcription factor in CSCs using glioma CD133+ cells as a model. Inhibition of c-myc using lentivirally transduced short hairpin RNA (shRNA) resulted in cell cycle arrest in the G(0)/G(1) phase, reduced proliferation and increased apoptosis. Furthermore, glioma CSCs with decreased c-Myc expression levels failed to form neurospheres *in vitro* or tumors when xenotransplanted into brains of immunocompromised mice. Overall, c-Myc seems to play an essential role in the regulation of the stem cell characteristics of CSCs. Finally, the transcription

factor Klf-4 (Krüppel-like factor 4), necessary for somatic cell reprogramming, is also required for ES cell self-renewal and maintenance of pluripotency (Zhang et al., 2010). Klf4 appears up-regulated in most hematological malignancies, such as acute lymphoblastic leukemia, hairy cell leukemia and multiple myeloma, being yolk sac tumors, a germ cell tumor subtype, one of the few solid tumors that present elevated levels (Schoenhals et al., 2009).

A better understanding of the acquisition and regulation of the self-renewal and proliferation potential by CSCs will improve the design and generation of anti-neoplastic drugs and accelerate the discovery of novel molecular targets for clinical application. This knowledge may as well help to use the existing treatments in a more CSC-specific and effective manner.

#### 4.2 Signaling pathways shared between CSCs and ES cells

Besides the gene signature that defines the ES cells, there are some signals that can extrinsically regulate several stem cell properties such as self-renewal. Many studies show that embryonic development and tumorigenesis share common regulatory mechanisms. Thus, it would not be surprising to find out that the cellular process, which leads to the generation of both, ES and CSCs, is regulated by the same factors. Hereby, we list the most important pathways that modulate ES function, mentioning their relationship with CSCs.

The Notch signaling pathway is highly conserved in mammals, playing an important role in embryonic development and adult tissue repair. It is known that Notch signaling is down-regulated during the cellular differentiation, being a promoter of stem cell survival, proliferation and undifferentiation of some adult stem cells, like for example hematopoietic and brain stem cells. It has been reported that the inhibition of this signaling pathway in some cancer types, such as medulloblastoma, reduces their proliferation *in vitro* and their capacity of forming tumors when transplanted into mice (Fan et al., 2006). These authors showed the blockage of Notch signaling by gamma-secretase inhibitors (GSI) reduced neurosphere growth and clonogenicity *in vitro*, whereas the activation of this pathway increased tumor growth. Furthermore, the expression of glioblastoma CSC markers such as CD133, Nestin, Bmi1, and Olig2 was reduced when Notch signaling was blocked. Xenograft transplantation experiments revealed that GSI-pretreated cells did not form tumors and even the implantation of drug-impregnated polymer beads in the tumor beds also effectively reduced tumor growth and significantly prolonged survival (Fan et al., 2010). Thus, it is likely that Notch pathway inhibition depletes CSCs through reducing proliferation and increasing apoptosis associated with decreased AKT and STAT3 phosphorylation.

Regarding the Wnt pathway, it is known to play an essential role in cell proliferation and stem cell maintenance. Sato and coworkers (2004) demonstrated that the overactivation of the Wnt/b-catenin pathway was responsible of the maintenance of pluripotency in ES and adult stem cells, as assessed by the expression of markers such as Oct4, Nanog and Rex1. Mutations within this signaling pathway occur frequently in human cancers. In fact, experiments in mice have shown that the overactivation of the Wnt/b-catenin pathway promotes oncogenic transformation of different cell types. For instance, mice that overexpress Wnt1 under de MMTV (mammary tumor virus) promoter develop salivary and mammary gland tumors and the accumulation of b-catenin due to mutations in APC gene may be linked to the appearance of colorectal tumors in humans (Markowitz et al., 2009).

Fibroblast growth factors (FGFs) are intercellular signaling molecules that, among other functions, participate in embryonic development, affecting several cell functions such as proliferation, differentiation, survival, adhesion and migration (Szebenyi & Fallon, 1999).

The FGF signaling pathway regulates the specification events that occur in the early embryo, when the cells composing the ICM of the blastocyst give rise to the three germ layers. Since ES cells are derived from the ICM it is believable that these cells conserve the same pathways. One of the most interesting members of the FGF pathway is FGF-2, also known as basic-FGF or bFGF. It has been reported that elevated expression levels of FGF-2 in the microenvironment of metastatic prostate tumors induce chemotherapy-resistance in the malignant cells. It has been speculated that the dysregulation of this factor in tumor cells is acquired as an adaptation to gain self-renewal and unlimited proliferative ability, two of the main features that define ES cells (Villegas et al., 2010).

During normal embryo development, the Hedgehog (Hh) signaling pathway is related to the epithelial-mesenchymal transition (EMT) by means of the up-regulation of the E-cadherin repressor SNAI1. EMT is related to tumor progression in correlation with the loss of the epithelial characteristics and the acquisition of metastatic phenotype (Hay, 1995). According to the CSC theory, metastatic cells retain stem cell properties, thus cells that undergo EMT have to conserve stem cell properties. To describe these cells, Brabletz and colleagues (2005) proposed the term migrating CSCs (mCSCs).

A better knowledge of the pathways that regulate ES cell functions is crucial to understand how CSCs self-renewal, survival, proliferation and metastasis are regulated. This will help to target specifically those stem cells that may be the source of recurrent tumors and are able to escape from the majority of the currently used cancer therapies (Takebe & Ivy, 2010).

## 5. Testis germ cell tumors as a CSC model

Early pathologists noted that certain germinal tumors contained a mixed population of adult tissues, but looked like a “caricature” of them (Aréchaga, 1993). Therefore, these malignancies were named teratocarcinomas, being “terato” a prefix meaning “monster”. Testis germinal cell tumors (TGCTs) form a heterogeneous group of neoplasias that are usually classified according to their histological aspect. Teratocarcinomas are characterized by the presence of embryonic and extra-embryonic tissues together with a population of pluripotent stem cells, named EC (Kleinsmith & Pierce, 1964). EC cells represent the most aggressive cell population in germ line tumors and are as well responsible for the transplantability of the tumors between immunocompatible animals. Interestingly these cells are frequently referred as the “malignant counterpart” of ES cells, due to the similar features that both cell types share (Aréchaga, 1993, Andrews et al., 2005). For example, as previously noted, when injected into a blastocyst, EC cells lose their malignancy and participate in the normal development of the embryo, just like ES cells (Brinster, 1974). Furthermore, it has been described that when cultured, ES cells acquire karyotypic changes that resemble those of EC cells, being their general *in vitro* behavior also similar.

TGCTs derive from a precursor lesion called carcinoma *in situ* (CIS) of the testis, which is found forming a single row at the basement membrane of seminiferous tubules. It was described for the first time when “atypical spermatogonia” were found in testis samples of patients that later developed TGCTs (Skakkebaek, 1972). It is known that CIS cells are already present in a latent state at the moment of birth, but it is not until puberty, when triggered by hormonal changes, the cells start to proliferate and to invade the testis stroma (Díez-Torre et al., 2004).

Two possible origins for the CIS of the testis have been proposed. Taking in consideration that germ cell tumors appear mostly in the gonads and that the transplantation of mouse embryo genital ridges into testis originates tumors (Stevens, 1967), it seems that CIS cells

result from germ cells that are unable to differentiate correctly. However, ES cell origin is supported by the fact that these tumors can be also generated by the transplantation of early embryos (Stevens, 1968) and that the gene expression pattern and the differentiation potentiality of the tumor cells is similar to that of the ES cells. Furthermore, the existence of, in terms of histology, identical tumors that appear in extra-gonadal locations, suggests its non-germinal origin. However, it is possible that these rare neoplasias develop from primordial germ cells (PGCs) that are retained during their migration towards the gonads. In fact, extra-gonadal germ cell tumors mostly arise in the body mid-line, which is the path that PGCs follow in their migration.

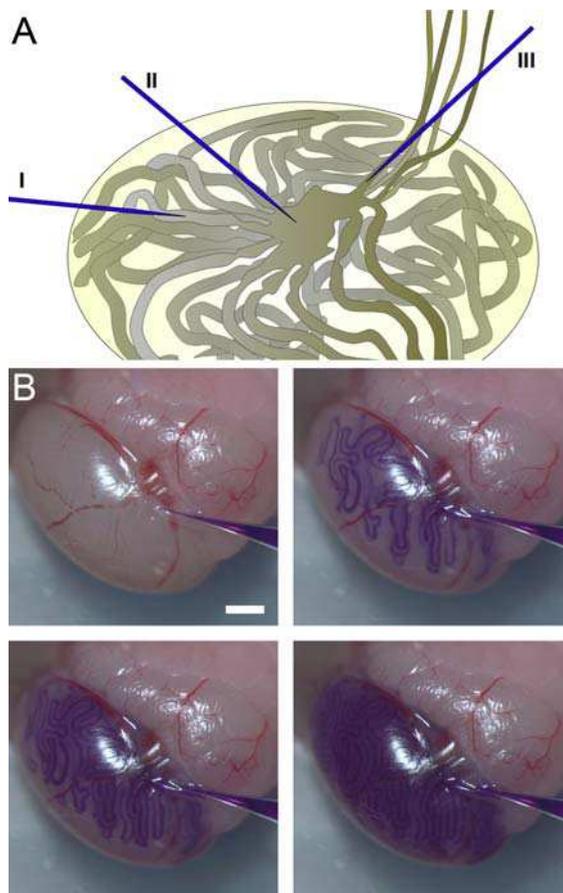


Fig. 2. Technique of cell transplantation into the seminiferous tubules. (A) Scheme showing the three different approaches for cell transplantation into the seminiferous tubules. The sharpened micropipette can be inserted directly in the seminiferous tubules (I), through the efferent ducts (II) or into the *rete testis*. (B) Time lapse photographic series of the microinjection of a blue-colored cell suspension. The microinjection pipette is inserted through the efferent ducts in this case. The process finishes when almost all tubules are filled. Scale bar represents 1mm

Since non-human mammals rarely develop TGCTs until now no suitable animal models are available to study this malignancy. Leroy Stevens (1973) made one of the first attempts to establish an animal model of TGCTs describing an inbred subline of mice termed 129-terSv which shows a high incidence of those tumors. However, most of the spontaneous tumors were teratomas, the differentiated and benign variant of teratocarcinoma, and thus not adequate for the study of human TGCTs, which generally are malignant (Stevens, 1973). In the middle 90s of the last century Brinster and Zimmerman (1994) developed the technique of cell transplantation into the seminiferous tubules of azoospermic animals. Their initial approach consisted of microinjecting a spermatogonia-enriched cell suspension into single seminiferous tubules. Later on, two more effective approaches were described, namely the microinjection into the *rete testis* and through the efferent ducts (Figure 2 A and B; Ogawa et al., 1997). Using this technique, it was possible to determine that germinal cells transplanted from one animal into another are capable of nesting in the seminiferous epithelium and differentiate into fertilization-competent spermatozoa. At the same time, Brinster and Avarbock (1994) tested the ability of the seminiferous compartment of reprogramming ES cells and driving their differentiation towards the germ cell lineage. However, ES transplantation resulted in tumors. Later on, a TGCT model using this technique was developed. It consists of the transplantation of EC cell lines into the seminiferous tubules of germ-depleted mice (Li et al., 2008a). The experiments involved the transplantation of two human lines derived from TGCTs, the JKT-1 seminoma cell line and the 833K EC cell line. Transplantation of both resulted in tumors that expressed TGCT markers.

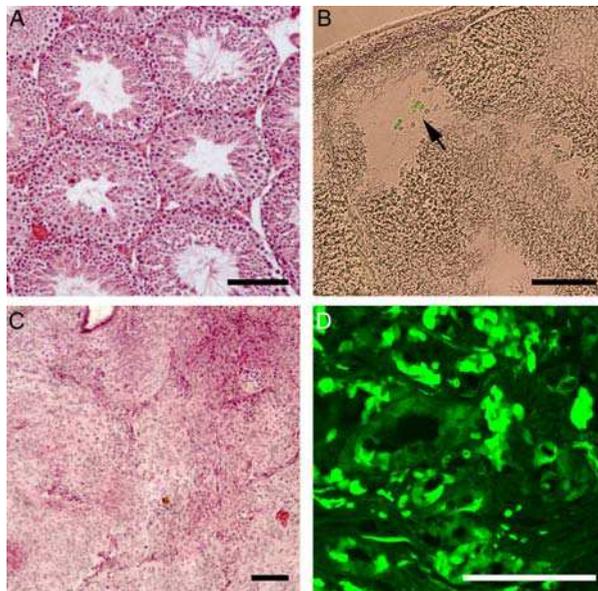


Fig. 3. Histology of an experimental testis teratocarcinoma. (A) Normal testicular tissue is composed of dense packed seminiferous tubules. (B) Cryostat section of a mouse testis few minutes after transplantation of autofluorescent ES cells (arrow) into the seminiferous tubules. (C) 5 weeks after transplantation teratocarcinoma tumors are formed in the transplanted testis, (D) Most of the neoplastic tissue fluorescent. Scale bars represent 50 $\mu$ m

Besides the similarities between EC and ES cells (Andrews et al., 2005), several studies have also shown that ES cells share many phenotypic and genetic similarities with the CIS of the testis (Almstrup et al., 2006; Rajpert-De Meyts, 2006; Looijenga, 2009). For example, it has been estimated that nearly 50% of the genes up-regulated in human ES cells are also expressed in CIS cells. Among these genes pluripotentiality-related and undifferentiation-related genes can be found. These observations led us to establish a TGCT model based in the transplantation of ES cells into the seminiferous tubules, with the belief that this model mimics more accurately than others the early stages of TGCT development (Silván et al., 2009a; Silván et al., 2010a). To follow the fate of the transplanted cells, we generated a stable GFP-transfected ES cell line, named AB1<sup>GFP</sup>. Short after the transplantation ES cells can be seen in the lumen of the seminiferous tubules (Figure 3 A and B). Around 24 to 36 hours later, ES cells are found integrated in the seminiferous epithelium, close to the basal membrane. Interestingly, this localization is similar to that of the spontaneous CIS of the testis. Five weeks after the ES cell transplantation, mature teratocarcinoma are completely formed (Figure 3 C). Most of the structures of the formed tumors were found to be derived from the transplanted cells, as can be determined by their fluorescence (Figure 3 D).

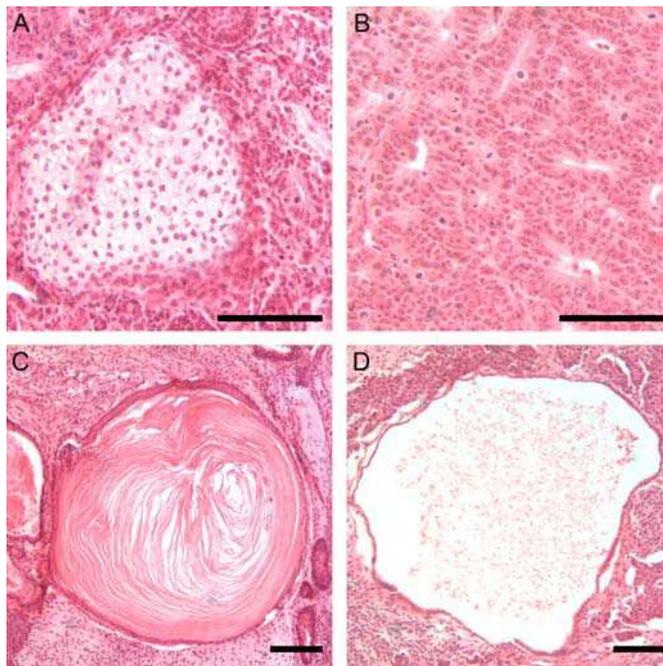


Fig. 4. Experimental teratocarcinomas showed different histological patterns of differentiation, such as cartilage (A), neural (B), epidermoid (C) and adenomatous areas (D). Scale bars represent 50µm

This transplantation procedure mimics better than others the early stages of TGCT development. At the histological level, experimental teratocarcinomas highly resembled the spontaneous TGCTs, showing structures derived from the three germ layers (Figure 4 A-D). In addition, regions with undifferentiated appearance (Figure 3C), which would be similar

to the EC component of spontaneous non-seminoma tumors, could as well be found (Silván et al., 2010a).

Although TGCT treatment has a high success rate (Gerl et al., 1996; Bosl, 1999), early diagnosis and identification of the causes of the high incidence among young men are still unknown. The experimental model that we have developed allows the study of the pre-invasive state of testicular teratocarcinomas and is potentially useful for the screening of novel therapeutic drugs, such as inhibitors of angiogenesis. Furthermore, prior to the transplantation procedure, donor cells can be modified, up- or down-regulating one or more genes, thereby providing a functional assay to evaluate the effect of these genes in EC transformation. Hence, we believe this model can help in the study of the cellular and molecular mechanisms involved in CSC establishment.

## 6. CSCs, ES cells and hypoxia

It is well known that adult tissues contain stem cell populations for cell renewal and an increasing number of evidences support that these cells are localized in specific *niches* characterized by low oxygen levels (Mazumdar et al., 2009). Since stem cells are the only cell lineage that is not replaced during the whole animal's life span, it is likely that the hypoxic microenvironment, where these cells are found, protects them from potential damages caused by oxygen (Diabira & Morandi, 2008). *In vitro* studies that tested the effect of oxygen showed a direct relationship between oxygen levels and the regulation of stem cell proliferation, differentiation and survival. More precisely, hypoxia has been reported to increase the proliferation of neural crest stem cells (Morrison et al., 2000), stimulate the survival of chondrocytes (Schipani et al., 2001) and disrupt adipocyte differentiation (Yun et al., 2002). Applying observations to the cancerous tissue, it is likely that CSCs are as well located in low oxygenated regions. In fact, due to their rapid growth and defective vascularization, solid tumors frequently present regions with reduced oxygen supply and necrosis. Consequently, tumor hypoxia has been correlated with bad prognosis factors, such as malignancy stage and resistance to radio- and chemotherapy (Jubb et al., 2010; Bertout et al., 2008).

Cellular response to hypoxia leads to several changes in gene expression triggered by a group of transcription factors known as Hypoxia Inducible Factors (HIFs). These factors, which belong to the basic helix-loop-helix (bHLH) and PAS (PER-ARNT-SIM) families, are composed by two subunits: one of them is variable (HIF-1 $\alpha$ , -2 $\alpha$  or -3 $\alpha$ ) and oxygen sensitive, but rapidly stabilized in response to hypoxic conditions. The other subunit, HIF-1 $\beta$ , also known as ARNT (aryl hydrocarbon receptor nuclear translocator), is constant and constitutively expressed (Wang & Semenza, 1995). When the cell is exposed to low oxygenation conditions, degradation of the alpha subunit is inhibited, binds the  $\beta$  subunit and the resulting complex translocates into the nucleus. There, it activates specific genes through the recognition of promoter regions known as hypoxia response elements (HREs). The up-regulation of these hypoxia-related genes mediates a number of changes at both cellular and systemic level.

Oxygen availability regulates several physiological processes, such as cell proliferation, differentiation and migration, being low oxygen levels, one of the main causes of cellular stress. The relationship between hypoxia and tumor growth was first reported as the radioprotective effect of anoxia in normal tissues was demonstrated using whole-body anoxia in newborn rodents (reviewed by Gray et al., 1953). Later on, the study of ex-utero

mouse embryo growth demonstrated that hypoxia is needed for a proper embryonic development (Morris & New, 1979). In fact, during early development ES-precursor cells are exposed to a hypoxic microenvironment due to the absence of vasculature in early embryos (Mitchell & Yochim, 1968). Because of this common hypoxic environment, HIF factors play a critical role in normal development and tumor growth, invasion and metastasis (Harris, 2002).

The study of HIF knockout mice has demonstrated the key role of these transcription factors during the embryonic and fetal development. HIF-1 $\alpha$  and HIF-1 $\beta$  null mice embryos die as the result of defects in vascular development (Ryan et al., 1998; Maltepe et al., 1997). The effects of HIF-2 $\alpha$  disruption range from embryonic lethality due to aberrant vasculature (Peng et al., 2000) to postnatal lethality because of multiorgan failure and altered mitochondrial metabolism (Scortegagna et al., 2003). The relationship of hypoxia with solid tumor progression has been repeatedly reported in clinical studies that prove that those patients with hypoxic tumors have a significantly poorer clinical outcome (Vaupel, 2008; Liu et al., 2010; Jubb et al., 2010). The causes of this worst prognosis include an increased ability for tumor invasion and metastasis and a higher resistance to radio- and chemotherapy (Brizel et al., 1999; DeClerck & Elble, 2010) as the result of a hypoxia-dependent expression of drug-resistance genes (Wartenberg et al., 2003), the selection of apoptosis-resistant clones (Graeber et al., 1996) and the disruption of DNA repairing mechanisms (Chan et al., 2009).

The link between low oxygen levels and tumor progression, together with the identification of CSCs and the known role of hypoxia as a key component of the stem cell microenvironment has led to the hypothesis that hypoxia maintains the undifferentiated state of CSCs and thus contributes to cancer growth, invasion and metastasis. Stem cell niches constitute the adequate environment for the maintenance of the undifferentiated state and for regulating stem cell differentiation into specific cell lineages (Moore & Lemischka, 2006). It has been recently speculated that hypoxia may regulate stem cell localization and maintenance inducing the expression of paracrine factors in a HIF-dependent manner. That is the case of endothelial progenitor cells. Hematopoietic stromal cells expressing the SDF-1 chemokine attract the stem cells expressing CXCR4 at low oxygenation conditions (Ceredini et al., 2004). This example illustrates the importance of stroma cells in the regulation of the stem cell microenvironment. The differentiation status of the stroma cells is crucial for the maintenance of stem cells. In bone marrow, for instance, when osteoblasts are removed during early differentiation, there is a critical reduction in bone marrow-derived hematopoietic stem cells (Visnjic et al., 2004). Nevertheless, this effect on hematopoiesis is significantly reduced when osteoblasts are removed at later stages of differentiation (Corral et al., 1998). Further evidences, such as the expression of hematopoietic stem cell maintaining factor angiopoietin-1 by undifferentiated CD146<sup>+</sup> osteoprogenitor cells but not in differentiated cells, point to immature stroma cells as key supporters of the stem cell niche (Sacchetti et al., 2007). The crosstalk between tumor cells and the surrounding stroma cells has a crucial effect on tumor growth, invasion and metastasis (Diez-Torre et al., 2010). The importance of this interaction suggests that stroma cells may provide the adequate microenvironment to maintain CSCs. In fact, hypoxia may regulate CSC maintenance and differentiation through three different mechanisms and can act directly on CSCs inhibiting them to differentiate. Low oxygen levels can also induce or maintain an immature state in tumor stroma cells and, finally, it can up-regulate the expression of paracrine factors that mediate the interaction between tumor and stroma cells in a way that stimulates CSC homing, proliferation and invasion.

Karnoub and colleagues (2007) reported that mouse stroma cells surrounding human breast cancer xenografts contain mesenchymal stem cells able to form fibroblastoid colonies *in vitro* whereas this ability is absent in those cells obtained from adjacent normal stroma. The same study demonstrated that the presence of bone marrow-derived mesenchymal stem cells is sufficient to induce the acquisition of a highly aggressive phenotype by poorly metastatic human breast carcinoma cells when injected in host mice. Thus, immature stroma cells may be recruited by the tumor microenvironment, being these undifferentiated cells involved in the acquisition of an aggressive phenotype by breast carcinoma cells.

Surprisingly, it has been reported that brain CSCs are preferentially located in the proximity of tumor associated endothelial cells. Although this result may partially contradict the hypoxic niche-theory, *in vitro* co-culture experiments have shown that endothelial cells secrete paracrine factors that promote CSC growth and stemness. Thus, the role of endothelial cells within the tumor stem cell niche could be independent of their vascular function (Calabrese et al., 2007). This idea is in concordance with the well known abnormality of tumor-associated blood vessels that lead to the formation of hypoxic or anoxic regions into the solid tumors and the presence of endothelial precursors without vascular functionality near the tumor cells (Silván et al., 2010b). Further research on the interactions between CSCs and endothelial cells in tumor hypoxic regions could provide valuable information for a better understanding of the tumor stem cell niche.

The role of hypoxia in the induction and maintenance of CSC undifferentiation has been recently studied in two different tumor types, neuroblastoma and breast carcinoma (Axelson et al., 2005). In both tumors, a correlation between poorly differentiated regions and tumor aggressiveness has been observed. In the case of neuroblastoma, the analysis of gene expression profiles of cultured tumor cells and neuroblastoma xenografts has demonstrated that hypoxia down-regulates the expression of mature neuron marker genes whereas induces the expression of c-Kit and Notch, genes associated with a neural crest-like phenotype (Jögi et al., 2002). Similar effects have been reported in ductal breast carcinoma (Helczynska et al., 2003) and cultures of EC cell lines (Silván et al., 2009b). However, the role of hypoxia in tumor progression is not limited to the maintenance of the undifferentiated state of CSCs, it has also been shown that low oxygen availability increases tumor cell proliferation and apoptosis resistance in lung adenocarcinoma (Chen et al., 2007a), melanoma (Bedogni et al., 2008) and thymus lymphomas (Bertout et al., 2009) through the alteration of Notch1 signaling. The relationship between hypoxia and tumor progression is also mediated through the overlapping of HIFs and some oncogene signaling pathways, such as cMyc and p53 (Mazumdar et al., 2009).

All these observations point out that oxygenation level plays a critical role in the stem cell microenvironment, and that, together with other niche components such as stromal cell contacts, extracellular matrix proteins, growth factors and temperature, is directly involved in CSC behavior, and thus, cancer progression and metastasis.

## 7. Importance of CSCs for cancer therapy

Many of the currently used cancer therapies are directed against rapidly dividing cells, which represent most of the tumor cell population. However, in a significant number of cases these therapies fail to eliminate the stem cell fraction of the tumor, what leads to tumor relapse and the selection of a more aggressive and therapy-resistant cancer cells and thus implies a worst clinical outcome. The development of therapies that specifically target CSCs

seems to be needed in order to achieve the complete tumor remission and prevent metastasis. The identification of the different cancer cell populations through DNA and tissue microarray analyses, the study of *in vitro* obtained cancer spheroids or the selection of more aggressive and therapy-resistant tumor cell lines by successive xenograft transplantation, are some of the strategies that are being followed with the aim of identifying those agents that selectively eliminate CSCs (Ischenko et al., 2008). It has to be considered that CSCs share many features with normal adult stem cells, and therefore, it has to be checked that the new therapies are effective against CSCs without being harmful for healthy adult progenitor cells. The results obtained by recent research works support the feasibility of this objective.

As we have previously mentioned, recent evidence demonstrates that the stem cell fraction of several tumor types show a higher resistance to radio and chemotherapy (DeClerck & Elble, 2010; Elliot et al., 2010). Different mechanisms have been suggested to explain the acquisition of this resistance. One of these explanations is related to the slow proliferation rate shown by stem cells, which stay in the G<sub>0</sub> phase of the cell cycle during long periods of time. This feature protects them from drugs that target actively dividing cells, the so-called cell-cycle specific agents. Moreover, CSCs show an increased expression of adenosine triphosphate-binding cassette proteins, which are known to outflow chemotherapeutic drugs. In fact, one of these proteins, the breast cancer-resistance protein (ABCG2), has been used to identify and isolate the side population of breast and other cancer types by flow cytometry due to its ability to extract Hoechst dye from the cell (Kim et al., 2002).

Additional mechanisms used by CSCs to escape chemotherapy are the up-regulation of drug metabolizing enzymes, for example the ALDH1 enzyme that metabolizes cyclophosphamide (Smalley et al., 2005), and the over-expression of survival promoting factors, such as the apoptosis inhibitors Survivin and Bcl-2 family proteins (Litingtung et al., 1999). Obviously, some of these stem cell markers have a prognostic significance. The expression of ALDH1 in breast carcinoma, for example, has been associated with poor clinical outcome and an increased risk of recurrence (Ginestier et al., 2007)

The results of several studies on the resistance of CSCs to radio- and chemotherapy suggest that the limited success of the current therapies in some tumor types could be related to their inability to target the CSC population. Indeed, it has been recently reported that chemotherapy leads to an increase of the breast CSC population, characterized by a CD44+/CD24- phenotype (Yu et al., 2007; Li et al., 2008b). Interestingly, in one of these neoadjuvant therapeutic trials, it was shown that targeting Her-2 with *Lapatinib*® produces a reduction of the CSC population and that this effect leads to a significantly increased pathologic complete response rate (Li et al., 2008). Since Her-2 is a known stem cell regulator, this observation constitutes important evidence in favor of the CSC hypothesis and suggests that the effectiveness of Her-2 inhibitors, like *Trastuzumab*® and *Lapatinib*®, could be connected with its ability to target the CSC fraction.

The encouraging results obtained with Her-2 inhibitors point to other proteins and signaling pathways that regulate self-renewal of stem cells, such as Wnt, Notch, Bmp4 and Hh, as potential targets for future therapeutic compounds. In this regard, targeting of Hh signaling by *Cyclopamine*®, a steroid-like compound, has been shown to inhibit the growth of ovarian carcinoma (Chen et al., 2007b) and the EMT in pancreatic cancer cell lines, leading to a significant reduction of *in vitro* invasiveness and metastatic potential *in vivo* (Feldmann et al., 2007). Interestingly, a combined treatment with *Cyclopamine*® and EGFR inhibitor not only resulted in a reduction of tumor cell invasion *in vitro* but it also increased the rate of

apoptotic death in prostate carcinoma cells (Mimeault et al., 2006) and several human pancreatic cancer cell lines (Hu et al., 2007). Moreover, it has been reported that *Cyclopamine*® increases the cytotoxic effect of both radio- and chemotherapy in different pancreatic cancer cell lines (Shafae et al., 2006). The inhibition of Hh signaling may have interesting applications in the treatment of glioma. This idea is supported by the fact that the treatment with this drug targets CSCs *in vitro* and leads to a reduction of glioma tumor formation *in vivo* (Clement et al., 2007). Another interesting effect caused by the blocking of the Hh pathway has been observed in multiple myeloma. In this poor prognosis tumor, the inhibition of Hh significantly reduced the myeloma stem cell clonal expansion and, additionally, stimulated the complete differentiation of these stem cells without affecting the growth of other plasma cells (Peacock et al., 2007).

Some promising results related to blocking of the Notch signaling pathway have been already obtained. The treatment of medulloblastoma cells with GSI-18, a inhibitor of Notch signaling, produces a remarkable reduction of the CD133+ stem cell population and the complete eradication of the side population detected by flow cytometry in medulloblastoma cell mass (Fan et al., 2006). Interestingly enough, the suppression of the stem cell fraction correlates with the loss of tumorigenic potential of the cell mass when transplanted into nude mice. Given that Notch signaling has been reported to participate in pancreatic differentiation, the targeting of this pathway might also be effective in the treatment of pancreatic cancer (Murtaugh et al., 2003).

There are several studies that emphasize the importance of Bmp4 has a potential target for anti-CSC therapies, this possibility is linked to the role of Bmp4 in the regulation of adult stem cell expansion (Hua et al., 2006). It has been reported, for example, that Bmp4 induces a decrease in CD133+ glioblastoma CSC population *in vitro* and *in vivo* (Piccirillo et al., 2006).

PTEN is another factor known to participate in the maintenance of stem cell populations and thus a potential target for cancer therapies. It is a tumor suppressor gene whose expression is usually down-regulated in many tumor types (Di Cristofano & Pandolfi, 2000). PTEN activity is mediated through the inhibition of PI3K/AKT signaling pathways, which include the downstream target mTOR, and in this way modulates several processes related to cell proliferation, growth and survival (Seeliger et al., 2007). A study performed with adult hematopoietic cells demonstrated that the conditional deletion of PTEN in these cells induces an expansion of leukemic cancer cells whereas significantly reduces the proportion of normal hematopoietic stem cells. Additionally, the treatment with *Rapamycin*®, an mTOR inhibitor, in order to overtake the PTEN deletion, led to the recovery of normal stem cell population and a reduction in CSCs (Yilmaz et al., 2006).

A different strategy to target CSCs based on the cytotoxic effect of IFN- $\alpha$  has been recently reported (Moserle et al., 2008). More precisely, it has been demonstrated that IFN- $\alpha$  presents a remarkable antiproliferative and pro-apoptotic activity on ovarian cancer cells containing a high proportion of side population cells. Similarly, *in vivo* gene therapy with human IFN- $\alpha$  led to a significant regression of large side population containing tumors, whereas no evident effects were observed in those tumors with a poor side population fraction. This result may have an important therapeutic significance since the IFN- $\alpha$  activity seems specifically target tumors that usually present a higher resistance to conventional treatments and, thus, with high number of CSCs.

It has also been reported that the resistance to radiation of glioblastoma CSCs correlates with an over-expression of DNA damage response genes (Bao et al., 2006). In fact, treatment

of this malignancy with radiotherapy leads to an increment of CD133+ glioblastoma cells. A similar observation has been reported for colorectal cancer, in which the CSC population is enriched after chemotherapy. Moreover, the CSCs that remain are responsible for tumor relapse and the acquisition of a more aggressive phenotype (Dylla et al., 2008). Colorectal CSC resistance to chemotherapy agents like 5-fluorouracil and *Oxaliplatin*® seems to be mediated by CSC-derived IL-4, that acts as an autocrine apoptosis inhibitor (Todaro et al., 2007). Indeed, the pre-treatment of the colorectal tumor with IL-4 blocking antibodies results in a significant increase in the treatments effectiveness.

Finally, it is important to note that those CSCs present in the tumor at one particular time point, may change during the progression of the malignancy, resulting in a "moving target" (Clarke et al., 2006; Roesch et al., 2010). Thus, the adequate therapy of a neoplasia may depend of a balanced situation of CSCs rate of proliferation, stroma reaction and degree of differentiation of the tumor parenchyma. In conclusion, the development of novel therapies that target specifically the CSC population, may be effective for the treatment of those cancer types that can be interpreted according to the CSC theory.

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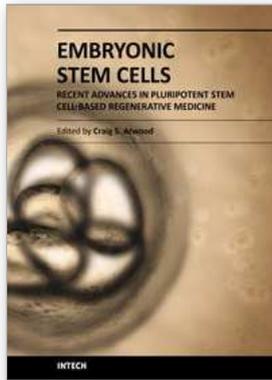
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