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The cancer stem cell phenotype as a determinant factor of the heterotypic nature of breast tumors

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ABSTRACT

Gathering evidence supports the existence of a population of cells with stem-like characteristics, named cancer stem cells (CSC), which is involved not only in tumor recurrence but also in tumorigenicity, metastization and drug resistance. Several markers have been used to identify putative CSC sub-populations in different cancers. Notwithstanding, it has been acknowledged that breast CSC may originate from non-stem cancer cells (non-SCC), interconverting through an epithelial-to-mesenchymal transition-mediated process, and presenting several deregulated canonical and developmental signaling pathways. These support the heterogeneity that, directly or indirectly, influences fundamental biological features supporting breast tumor development. Accordingly, CSC have increasingly become highly relevant cellular targets.

Abbreviations: ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; ESC, embryonic stem cell; JAK, Janus kinase; mTOR, mammalian target of rapamycin; NCL, nucleolin; non-SCC, non-stem cancer cell; PI3k, phosphatidylinositol-4,5-bisphosphate 3-kinase; STAT, signal transducers and activator of transcription; TIC, tumor initiating cells; WNT, wingless-related integration site.

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In this review, we will address the stemness concept in cancer, setting the perspective on CSC and their origin, by exploring their relation and regulation within the tumor microenvironment, in the context of emerging therapeutic targets. Within this framework, we will discuss nucleolin, a protein that has been associated with angiogenesis and, more recently, with the stemness phenotype, becoming a common denominator between CSC and non-SCC for multicellular targeting.

1. Tumor microenvironment as a key player in cancer development

Cancer remains a stressful condition in the western world, having surpassed heart diseases in 1999 as the pathology with the highest mortality (Jemal et al., 2006). Globally, lung cancer stands as the leading cause of death amongst patients with tumors in the respiratory system, whereas colon cancer stands out in digestive diseases. If one accounts for gender, a substantially different reality emerges, revealing breast cancer as the leading cause of death, accounting for 23% of all cancer cases among women (Jemal et al., 2011). Epidemiologic data from the United States of America suggest that every 1 in 8 women will develop breast cancer over their life time (DeSantis et al., 2014; Siegel et al., 2013). Among those, some will derive from familial inheritance. In this respect, *BRCA1* and *BRCA2* mutations confer high risk of breast cancer development, accounting for 40% of the familial cases (Shuen and Foulkes, 2011). Over the years, such realities have unleashed a tremendous effort from the scientific community in order to address this enormous health problem. Such efforts have been unraveling novel insights about tumor biology, adding novel cellular and molecular players present in the tumor microenvironment that play a significant role in cancer development.

In fact, a multitude of different cell types, including cancer cells, fibroblasts, endothelial cells or cells from the immune system are identified within the tumor microenvironment. They carry dissimilar roles, thus contributing to the heterotypic nature of tumors (Hanahan and Weinberg, 2011). This layout suggests an intrinsic interaction between those cells, which ultimately provides a fostering ground for the acquisition of several features that support tumor development. Indeed, such features were rationally summarized by Douglas Hanahan and Robert Weinberg in a seminal manuscript from 2000 (Hanahan and Weinberg, 2000), which originally included six important hallmarks of the disease and that were further upgraded in 2011 (Hanahan and Weinberg, 2011): (I) sustained proliferative signaling, (II) evading growth suppressors, (III) enabling replicative immortality, (IV) resisting cell death, (V) angiogenesis induction, (VI) activation of invasion and metastization, (VII) immune system evasion, (VIII) deregulation of cellular energetics, and also enabling characteristics as (IX) genome instability and mutation and (X) tumor-promoting inflammation. These features account for the major known deregulated pathways in cancer/tumor cells, which control metabolism, including nucleic acid turnover, cellular proliferation and fate, and cell signaling (Hanahan and Weinberg, 2011).

2. Cancer stem cells, tumor initiation and progression

Along with those instrumental processes for drug resistance and tumor survival, the role of a rather illusive cell type, resembling cells from embryonic development has been unveiled. The acknowledgement of common signaling pathways between stem cells and subpopulations of tumor cells set developmental biology and cancer closer than one would think, and gave rise to the cancer stem cell concept (Reya et al., 2001).

2.1. Lessons from stem cells: cellular reprogramming

Pluripotent stem cells have the potential to differentiate into any type of cells of the three germ layers (endoderm, mesoderm and ectoderm) (De Miguel et al., 2010), besides displaying self-renewal capability (De Miguel et al., 2010; Evans and Kaufman, 1981; Thomson et al., 1998). In an attempt to identify pluripotent cells, such as embryonic stem cells (ESC), several markers have been established, including upregulated levels of *NANOG*, *OCT4* (a.k.a. *POU5F1*), *TDGF* and *GDF3*, which are strongly regulated developmental genes (Adewumi et al., 2007; De Miguel et al., 2010). NANOG and OCT4, as well as SOX2, are regulatory transcription factors essential for self-renewal and pluripotency maintenance of stem cells (Pan and Thomson, 2007; Stadtfeld and Hochedlinger, 2010). They control several downstream gene targets, including *STAT3*, essential for self-renewal (Boyer et al., 2005; Niwa et al., 1998; Stuart et al., 2014). Tight levels of OCT4, control the transition between pluripotency and differentiation (Radzisheuskaya et al., 2013). Takahashi et al. demonstrated that it is possible to reprogram somatic cells such as adult fibroblasts, first from mouse, and later from human, into a state of pluripotency. Upon promoting the expression of four key transcription factors – OCT4, SOX2, c-MYC and KLF4 (OSKM) –, induced pluripotent stem cells (iPS) were generated (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). These cells closely resembled ESC, showing similar expression patterns of stem cell markers, like *NANOG* or *GDF3*, and demonstrating oriented differentiation capacity (Takahashi et al., 2007).

Overall, this suggested that cells with self-renewal potential can be generated from terminally differentiated somatic cells, thus reverting hierarchical developmental organization. This guided reintroduction of stemness in somatic cells somewhat represents a gain of function, a feature often occurring during cancer development.

2.2. The stemness concept in cancer

Tumors are biological entities that can be interpreted as an aberrant dysfunctional organ initiated by a tumorigenic cancer cell with the capacity to proliferate indefinitely by acquired mutations (Reya et al., 2001; Visvader, 2011). Viewed as an organ, tumors present functional heterogeneity in the microenvironment demonstrated by the existence of different populations of cells, including cancer cells with diverse phenotype. In order to accommodate that functional heterogeneity, a hierarchical organization model of tumor development, known as the cancer stem cell model, was proposed. This model postulates the existence of a sub-population of stem-like cells (the *cancer stem cells* – CSC) in the tumor microenvironment that is responsible for sustained tumor growth (Kreso and Dick, 2014; Reya et al., 2001; Visvader, 2011; Visvader and Lindeman, 2008, 2012). CSC have been defined operationally by their capacity to generate new tumors in immunocompromised mice, upon isolation from an established tumor (Scheel and Weinberg, 2012). However, the observation that not all cells of a putative CSC population are able to seed tumors, led to the introduction of the concept of Tumor Initiating Cells (TIC). Their abundance (established by *in vivo* limiting dilution experiments)

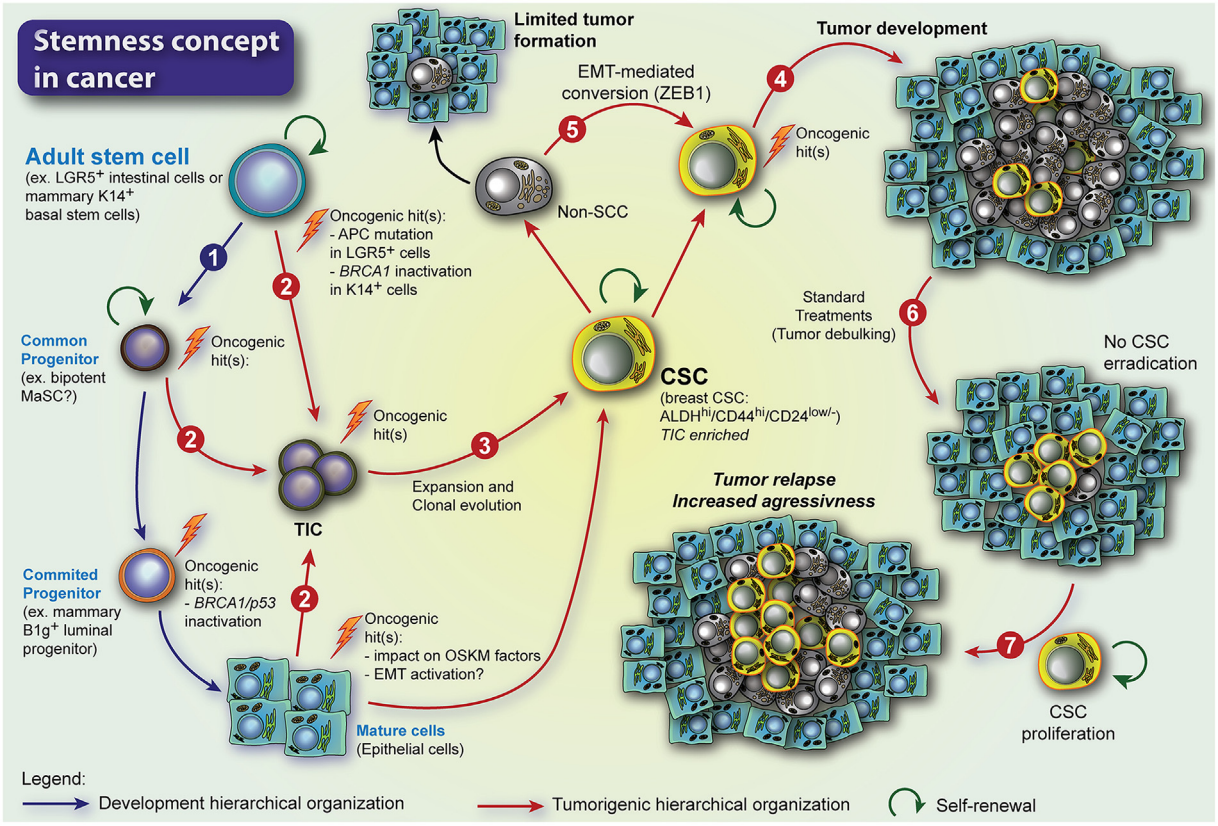

Fig. 1. From stem cells to tumor initiating cells and cancer stem cells. In normal developmental cellular hierarchy, **stem cells** originate **progenitor** cells, which generate progressively more committed progeny, culminating in the establishment of **mature cells** composing the different tissues **(1)**. In tumor development, either one of those cells (stem, progenitor or mature cells) may suffer different oncogenic hits **(2)**, eventually turning them into **Tumor Initiating Cells (TICs)**. As these cells expand **(3)**, subsequent oncogenic hits (occurring epigenetically, for example) lead to the generation of **Cancer Stem Cells (CSCs)**. CSCs are therefore the drivers sustaining tumorigenesis **(4)**, a role that cannot be undertaken by any other tumor cell (like, non-Stem Cancer Cells, **non-SCC)** unless they convert into a CSC phenotype **(5)**. Either way, standard treatments are many times only capable to debulk tumor mass, but cannot eliminate the drug resistant CSCs **(6)**. Residual CSCs are in turn capable of repopulating the tumor area, often leading to tumor resistance and increased disease aggressiveness **(7)**. *Abbreviations: B1g, beta 1 integrin; EMT, epithelial-to-mesenchymal transition; K14, keratin 14; OSKM, OCT4/SOX2/KLF4/MYC.*

reflects the tumor-forming ability of a given putative CSC population (Fig. 1) (Scheel and Weinberg, 2012).

Conceptually and in the absence of pre-established disease, TICs might either be a normal adult stem cell, subsequent to several abnormal transformations, a partially differentiated cell, like a common progenitor, or a differentiated cell that has gone through a series of oncogenic hits, thus acquiring a stem-like character (Hanahan and Weinberg, 2011; Reya et al., 2001; Visvader and Lindeman, 2012). As these cells expand, acquired mutations during neoplastic progression may result in the development of CSC, which are responsible for sustained tumor growth and maintenance, as well as in an enrichment of cells capable of tumor initiation (Fig. 1) (Visvader, 2011). Despite abnormal, CSC share features of normal stem cells such as self-renewal and differentiation capacity (Reya et al., 2001; Vallier et al., 2009; Visvader and Lindeman, 2012).

2.2.1. *Bridges between stem cells and cancer stem cells*

The cancer stem cell existence was firstly reported by Bonnet et al. (Bonnet and Dick, 1997) in acute myeloid leukemia by implicating aberrant hematopoietic cells expressing the $CD34^+/CD38^-$ phenotype (a.k.a. SCID leukemia initiating cells – SL-IC) in disease development, which were also able to differentiate into leukemic blasts. Later on, cancer stem cells were also implicated in solid tumor development by functional comparison of breast cancer cells expressing different levels of CD44 (receptor for hyaluronic acid and monitoring extracellular changes) and CD24 (cell–cell and matrix interaction) (Al-Hajj et al., 2003). Additionally, aldehyde dehydrogenase (ALDH) levels and/or activity (also present in normal adult breast stem cells), was correlated with poor clinical outcome, metastasis, tumor relapse and drug resistance (Croker and Allan, 2011; Ginestier et al., 2007; Liu and Wicha, 2010; Marcato et al., 2011). Those three markers (CD44, CD24, and ALDH) remain consensual for the identification of putative breast CSC (Badve and Nakshatri, 2012). However, such consensus is cautiously maintained as they may, in fact, identify breast CSC with different degrees of differentiation according to tumor histological origin (Ricardo et al., 2011).

Further on, putative CSC have been unveiled in different tumor types using a series of different surface markers (Stuelten et al., 2010; Visvader and Lindeman, 2012). Nevertheless, the evolving landscape is highly complex, since tumors of different histological origins may present different densities of putative CSC (understood as tumorigenic). These cells may further evolve under genetic and epigenetic control, presenting different degrees of hierarchical organization (Kreso and Dick, 2014). Studies with patient-derived breast cancer xenografts revealed the existence of clonal selection, varying from extreme selective engraftment of minor genetic clones to moderate, polyclonal engraftment (Eirew et al., 2015). This poses an enormous challenge for the identification of markers, which is often supported by functional characterization using *in vivo* tumorigenic assays or *in vitro* sphere suspension cultures (Kreso and Dick, 2014).

Another relevant work supporting the CSC concept, with direct stem cell involvement in cancer initiation and development, was established in a mouse model of intestinal cancer and involved a specific *APC* (adenomatous polyposis coli) gene mutation in LGR5$^+$ crypt intestinal stem cells (Barker et al., 2009; Schepers et al., 2012; Schuijers and Clevers, 2012; Snippert et al., 2010). Long-lived crypt LGR5-overexpressing intestinal stem cells are responsible for crypt homeostasis (Schuijers and Clevers, 2012). As such, these LGR5$^+$ stem cells divide symmetrically, later acquiring either stem or a transient-amplifying phenotype in a neutral stochastic competition (Snippert et al., 2010). Barker and colleagues demonstrated that specific mutation of *APC* gene in LGR5$^+$ stem cell resulted in the formation of intestinal microadenomas, with sustained hierarchical organization in early lesions (Barker et al., 2009). Furthermore, using *lineage tracing* experiments, it was identified a LGR5$^+$ subpopulation of adenoma cells (precursor of intestinal cancer) that generates additional LGR5$^+$ cells, thus fueling adenoma growth (Schepers et al., 2012). These results enabled one to clearly perceive stem cell or stem cell-like phenotype involvement in cancer initiation and progression.

The LGR5 marker has also been suggested for breast cancer patient stratification, as its expression was associated with poor overall patient survival (Chen et al., 2013; Yang et al., 2015). Recent evidence suggests the existence of bipotent mammary stem cells (MaSC) in postnatal gland. They are capable of generating both myoepithelial (basal) and luminal cell lineages, thus contributing to physiological ductal tree homeostasis (Rios et al., 2014). In fact, targeted deletion of *BRCA1* gene in basal and luminal cell lineages enabled the development of adenomyoepitheliomas (rare in humans) and basal-like tumors, respectively (Fig. 1) (Molyneux et al., 2010). However, manipulation of *BRCA1* gene, which mutations rarely occur in sporadic basal-like tumors, does not rule out that basal breast cancers may arise from other cell type than luminal lineage cells. Thus, the precise cell origin remains to be identified (Visvader, 2011). Notwithstanding, the existence of bipotent MaSC in adult gland may support breast cancer development from lineage precursors, owing to their deregulation either by spontaneous/intrinsic or extrinsically acquired mutations (Fig. 1) (Rios et al., 2014).

Regardless their origin, it is clear that putative CSC, namely those from breast cancer, have a significant role in reshaping tumor microenvironment, a feature supported by aberrant pathway activation, like WNT signaling, or activated epithelial-to-mesenchymal transition, a cornerstone in cancer metastasis (Scheel and Weinberg, 2012; Takebe et al., 2011).

2.2.2. Acquisition of cancer stem cell traits

Breast CSC, among other less studied cell subpopulations, are considered instrumental for disease development and progression, as well as evasion to therapy and subsequent relapse. Their origin is yet unknown. Nevertheless, recent data suggested that breast stem-like cancer cells were generated by defined reprograming factors (OSKM cocktail) from non-tumorigenic human mammary epithelial cells (Nishi et al., 2014). Indeed, SOX2 seems to be highly expressed in early stage breast tumors, controlling xenograft tumor initiation (Leis et al., 2012). This is in accordance with the generation of SOX2-overexpressing cancer stem-like cells from luminal breast cancer cells, upon nuclear reprogramming induced by OSKM factors (Corominas-Faja et al., 2013). Interestingly, notwithstanding SOX2 overexpression, levels of OCT4 and NANOG were low, a condition also observed in early stage breast tumors (Corominas-Faja et al., 2013; Leis et al., 2012).

Nevertheless, in a different sample cohort of human breast tumors, NANOG overexpression in 9% of the cases (TMN I/II stage) has been associated with poor prognosis, correlating with highly proliferative early stage tumors (Nagata et al., 2014). Additionally, it has been reported that radiation, concomitantly with steroid hormones (which increase proliferation rate of progenitor cells), led to an increase in ALDH$^+$ cell population (putative breast CSC) (Vares et al., 2013).

It thus likely that breast cancer cells with stem-like traits could be generated through acquired mutations, leading to uncontrolled reactivation of pluripotency-associated programs in adult somatic cells (or even MaSC). This process could be, in part, initiated/modulated by environmental stimuli such as xenobiotics or radiation. Notwithstanding, cells of origin may rely on pluripotency reactivation at different extents (for example, pluripotency markers NANOG and OCT4 present a limited expression in early stage tumors), which may account for different phenotypic signatures among breast tumors.

2.2.3. Prognostic value of putative breast cancer stem cell markers

Indeed, the expression of current CSC markers, such as ALDH, CD44 or CD24, differs among breast cancer molecular subtypes. ALDH has a scattered distribution in each subtype, and basal-like tumors enclose higher percentage of CD44$^+$/CD24$^{-/low}$ cells than the luminal type (Ricardo et al., 2011). Nevertheless, those markers convey an important prognostic value.

ALDH1 overexpression has been associated with poor clinical outcome in breast cancer patients (Ginestier et al., 2007). This could be related with the selection of cells with increased metastatic potential enabled by ALDH1 activity, in accordance with the metastatic predictive value of ALDH1A3 (Croker et al., 2009; Marcato et al., 2011). Additionally, CD44$^+$/CD24$^{-/low}$ phenotypic cells are predominant in triple-negative invasive breast carcinomas, an aggressive molecular subtype associated with poor clinical outcome (Idowu et al., 2012). Nevertheless, this depiction does not always hold true as there are indications that CD44$^-$/CD24$^+$ phenotype relates with poor prognosis in early invasive breast carcinoma (Ahmed et al., 2012). Thus the relevance of the referred markers of breast CSC may vary according to the histologic type and/or tumor stage, as well as to the degree of differentiation of tumor cells, which highlights the heterogeneity of the tumor microenvironment. In fact, the diverse cellular components of the tumor microenvironment unlock many regulatory restraints of CSC, providing the soil for them to proliferate and evolve (Korkaya et al., 2011).

2.3. Cancer stem cells and microenvironment regulation

Cells from the tumor microenvironment, such as fibroblasts, endothelial cells or even mesenchymal stem cells have been suggested to regulate breast CSC, upon secreting different signaling molecules associated with survival, proliferation or differentiation (Liu et al., 2011). One such example is the chemokine receptor CXCR1, necessary for breast CSC self-renewal and survival (Ginestier et al., 2010). In addition, endothelial cell signaling has also been suggested to regulate CSC self-renewal in breast cancer (Korkaya et al., 2011). Although relevant in physiological conditions in diverse cellular functions, these signals may translate into activation of fundamental signaling pathways, often deregulated in CSC, like NOTCH, Hedgehog and WNT/β-Catenin (Bolos et al., 2013; Cai et al., 2013; Karamboulas and Ailles, 2013; Lamb et al., 2013; Nagamatsu et al., 2014; Takebe et al., 2011) or the canonical JAK/STAT, or PI3k/AKT pathways (Ithimakin et al., 2013; Korkaya et al., 2009; Lin et al., 2013; McCubrey et al., 2006), supporting their survival and self-renewal.

2.3.1. WNT and β-catenin: proliferation of CSC

In WNT/β-Catenin signaling, the transcription activator β-Catenin is maintained in low levels by continuous degradation. Upon WNT binding to Frizzled receptors, β-Catenin is released from the multiprotein destruction complex (including APC and GSK-3β)

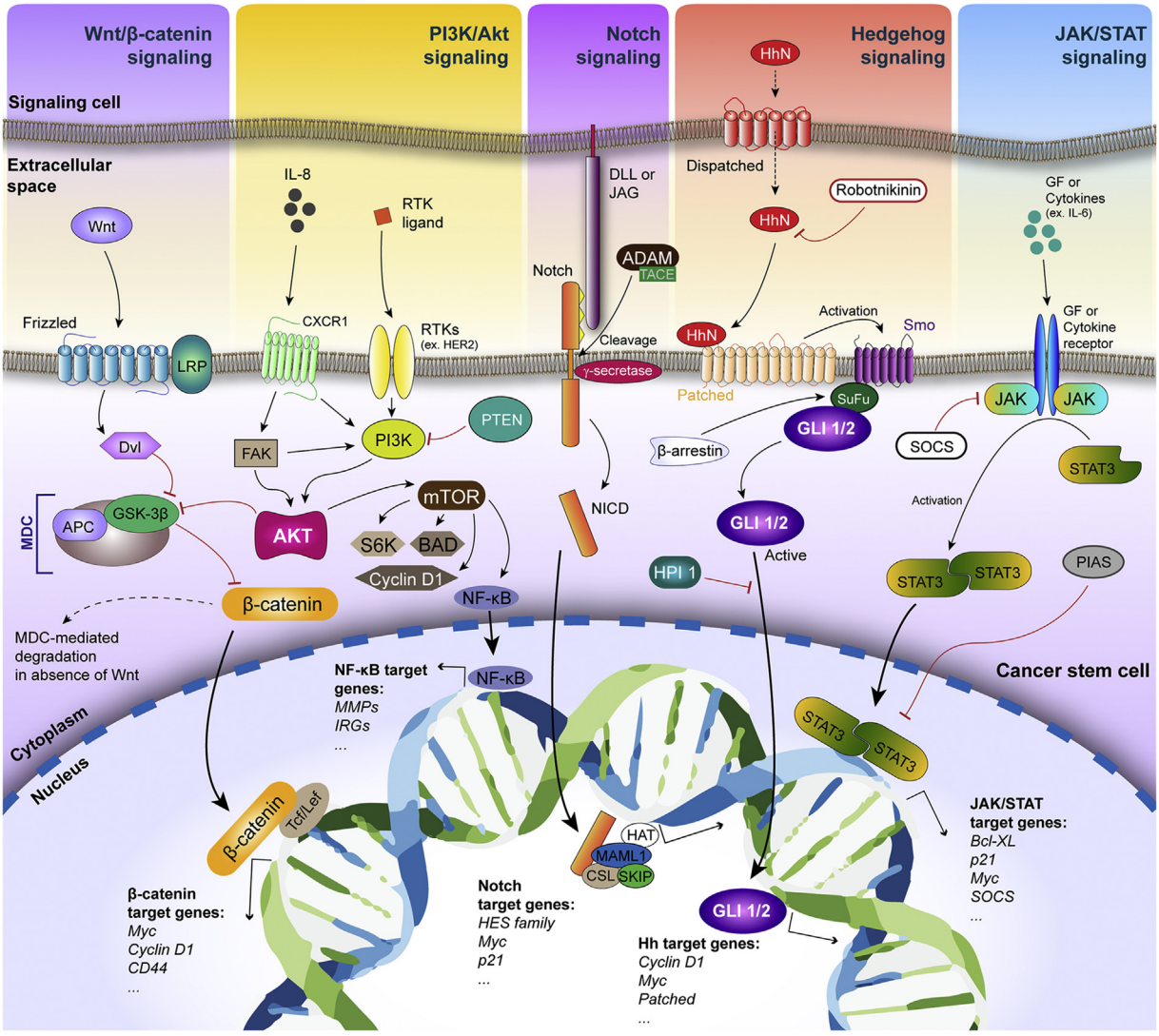

Fig. 2. Fundamental pathways deregulated in cancer stem cells. Developmental signal transduction pathways, including NOTCH, WNT and Hedgehog are highly associated with CSC phenotype. Additional signal transduction is mediated by receptor tyrosine kinases (RTK), like HER2, and PI3k/AKT pathway or JAK/STAT pathway. Regulation of CSC through interleukins adds an additional layer to the regulatory network. Abbreviations: *ADAM, A disintegrin and metalloproteinase; CSL, CBF1/Su(H)/Lag-1; DLL, Delta-like ligand; Dvl, Disheveled; FAK, focal adhesion kinase; GF, growth factor; HAT, histone acetyltransferase; HhN, Hedgehog ligands; HPI 1, hedgehog protein inhibitor 1; IRGs, inflammation-related genes; JAG, jagged ligands; LRP, low-density lipoprotein receptor-related protein; MAML1, Mastermind-like 1; MDC, multiprotein destruction complex; MMPs, metalloproteinases; NICD, NOTCH intracellular domain; PIAS, protein inhibitors of activated STATs; RTK, receptor tyrosine kinase; SKIP, ski-interacting protein; Smo, smoothened; SOCS, suppressor of cytokine signaling; SuFu, suppressor of fused, TACE, TNF-α converting enzyme; Tcf/Lef, T-cell factor/lymphoid enhancing factor.*

and translocated from the cytoplasm to the nucleus, thus activating several proliferation-related target genes (Fig. 2) (Takebe et al., 2011). During development it controls cell fate whereas in adults supports tissue self-renewal (Takebe et al., 2011). Indeed, WNT deregulated activity in LGR5^{+} crypt stem cells, upon *APC* mutation, a negative WNT signaling regulator, leads to intestinal cancer (Barker et al., 2009; Schuijers and Clevers, 2012). Whereas *APC* mutations are clearly associated with colorectal cancer, aberrant WNT signaling activation at the cell surface may be responsible for WNT-associated breast tumorigenesis, as only a few breast tumors present that mutation (King et al., 2012). In fact, WNT/β-Catenin has been shown to downregulate let-7 miRNAs family leading to Lin28-mediated expansion of breast cancer stem cells (Cai et al., 2013). Additionally, WNT over-activation was observed in breast cancer stem cells when compared to normal stem cell populations, and, upon signaling inhibition, led to a reduction in ER-dependent mammosphere formation (Lamb et al., 2013).

2.3.2. NOTCH and Hedgehog: cell-to-cell signaling and CSC polarity

Notch signaling regulates cell-to-cell communication during embryogenesis as well as proliferation. Signal transduction is mediated by transmembrane NOTCH receptors family (NOTCH1-4), upon binding of Delta-like or Jagged ligands expressed at the surface of an adjacent cell (Fig. 2). Upon receptor-ligand binding, γ-secretase cleaves and releases the Notch intracellular domain, which further translocates into the nucleus and activates the transcription of target genes, like oncogenic *MYC* and *P21* (Fig. 2) (Takebe et al., 2011).

In breast cancer, NOTCH1 overexpression has been associated with tumor growth, increased cell migration and invasion of luminal-type breast cancer cells (Bolos et al., 2013). A similar effect was described for NOTCH4 on triple-negative breast cancer cells (Nagamatsu et al., 2014). Importantly, NOTCH4 has been involved in breast CSC regulation, presenting a higher expression in CSC than NOTCH1 (Harrison et al., 2010). The activity of the latter seems to

impact the phenotype of different tumor cells, as NOTCH1 silencing induced growth arrest and apoptosis in both breast CSC and non-SCC (Suman et al., 2013). Overall, the involvement in cell signaling of different receptors of NOTCH family is evident, a complexity that, according to above, might relate with the histological origin of the tumors.

Hedgehog pathway is another developmental pathway that has been linked to cancer biology (Amakye et al., 2013; Takebe et al., 2011). Controlling tissue polarity and stem cell maintenance, Hedgehog cascade is initiated by binding of HhN ligands to Patched, leading to activation of SMO (smoothened) and release of *GLI1/2*, which is then translocated to the nucleus thus activating *CYCLIN D1*, *MYC*, among others (Fig. 2) (Amakye et al., 2013). Loss of *PTCH1* (Patched encoding gene) and amplification of *GLI1* have been described in breast cancer (Karamboulas and Ailles, 2013). In fact, overexpression of *GLI1* led to breast tumor formation in a mouse model, which associated with expansion of a population of progenitor cells (Fiaschi et al., 2009), thus relating Hedgehog regulation and stem-like cancer cells.

2.3.3. PI3k/AKT and JAK/STAT: proliferation and metabolism of CSC

Despite the clear involvement of the described developmental pathways in CSC phenotypic control, canonical signaling pathways, such as PI3k/AKT (Martelli et al., 2010), also regulate CSC behavior. Indeed, an overlap between PI3k/AKT and WNT/β-Catenin signaling regulating normal and malignant progenitor cell populations was demonstrated (Korkaya et al., 2009).

Upon activation of PI3k through growth factor binding to the corresponding receptor tyrosine kinase, including the human epidermal growth factor receptor, this pathway relays signals, mediated by AKT/mTOR, to a wide range of downstream targets, including S6K, BAD, CYCLIN D1, NF-κB or GSK-3β (Baselga, 2011). These are responsible for the control of protein synthesis, cell survival, cell cycle/proliferation and metabolism (Baselga, 2011; Chalhoub and Baker, 2009; Sulzmaier et al., 2014). In addition, loss of PTEN has been implicated in the sustained activation of PI3k/AKT pathway, an effect due to the negative regulatory nature of this phosphatase, responsible for the conversion of phosphatidylinositol triphosphate to phosphatidylinositol biphosphate (Baselga, 2011; Chalhoub and Baker, 2009). Despite this intricate relation, PI3k/AKT pathway mutations and PTEN loss may have distinct roles in the pathogenesis as their distribution varies according to different breast cancer subtypes (Stemke-Hale et al., 2008).

Recently, the p85α regulatory subunit of PI3k was described as a positive regulator of the expression of epidermal growth factor receptor (EGFR) and cell malignant transformation. p85α is essential for C-JUN activation and further upregulation of nucleolin transcription, which leads to an increased *EGFR* mRNA stability (mRNA degradation is inhibited) and EGFR protein expression, resulting in cell malignant transformation (Xie et al., 2016).

Not less important is the canonical JAK/STAT (Janus Kinase/Signal Transducers and Activators of Transcription) pathway. The elements within this signaling cascade present several isoforms that, upon alternative activation, may elicit different effects related to cell cycle or apoptosis (Rawlings et al., 2004). Nevertheless, activation of JAK, leads to direct activation of STATs (Fig. 2), which, in turn, directly regulate transcription of different genes, setting forth a direct mechanism of external signal translation into a transcriptional response (Rawlings et al., 2004). This cascade is under control of different mechanisms, including a negative-feedback loop promoted by SOCS, which is expressed upon STAT activation (Rawlings et al., 2004; Timofeeva and Tarasova, 2012). Major target genes include *BCL-XL*, *P21* or *C-MYC*, involved in apoptosis or cell cycle control (Rawlings et al., 2004).

Altogether, an overlap between different features associated both with embryonic development and cancer is becoming evident. This sets forth a hierarchical organization of tumor cells where an intricate interaction between embryonic and classical signaling networks modulates CSC behavior and properties, and reshape tumor landscape.

2.4. Epithelial-to-mesenchymal transition and breast CSC

Epithelial-to-mesenchymal transition (EMT) may represent the "gain-of-function" process, enabling stem-like features in cancer cells (or pre-malignant cells). EMT (a concept that originally emerged in embryology as a mean of tissue remodeling) is the program by which epithelial cells acquire the ability to invade adjacent and/or distant tissues, enabling their dissemination and further metastization (Hanahan and Weinberg, 2011; Scheel and Weinberg, 2012). It is typically associated with the loss of epithelial adhesion molecules, among which E-cadherin stands out, under the control of transcription repressors SNAIL, SLUG, ZEB1 and TWIST (Hanahan and Weinberg, 2011; Scheel and Weinberg, 2012). Indeed, it was recently shown that ZEB1 promoter enabled a swift shift from a non-SCC to a CSC state, in response to environmental stimulus like TGF-β (Chaffer et al., 2013). This challenges the unidirectional hierarchical CSC concept, demonstrating that non-SCC and CSC can readily interconvert upon environmental stimuli, a process governed, in part, by EMT (Marjanovic et al., 2013). In addition, the conversion seems to be common to both normal epithelial cells as well as cancer cells (Chaffer et al., 2011). Thus, this suggests that cancer cells may rely on physiological programs to acquire highly aggressive phenotypic traits, which include motility and invasiveness, central to metastasis (Scheel and Weinberg, 2012).

The demonstration of protein kinase A-mediated mesenchymal-to-epithelial transition activation supports the modulation of the epigenetic reprogramming and cell differentiation into an epithelial state, leading to a loss of their tumor-initiating ability (Pattabiraman et al., 2016). These results suggested that enforcing an epithelial state and preventing EMT may overcome the metastatic and drug resistance phenotype associated with CSC (Pattabiraman et al., 2016). Nevertheless, the CSC phenotype may be modulated by hallmarks of cancer like angiogenesis (Conley et al., 2012).

2.5. Stemness and angiogenesis

Angiogenesis, understood as the formation of new vascular vessels from pre-existing ones, represents an essential mechanism for homeostasis maintenance, contributing to phenomena like wound healing (Tonnesen et al., 2000). However, cancer subverts the intrinsic beneficial nature of those processes, in an attempt to cope with nutrient scarcity as solid tumors grow (Ferrara and Kerbel, 2005; Hanahan and Weinberg, 2011; Welti et al., 2013).

Several cellular and molecular signaling components are involved in the tumor angiogenic process. Vascular endothelial growth factor (VEGF), a potent pro-angiogenic factor, is central for vessel development, with cancer cells and mesenchymal progenitor cells functioning as main secretion drivers (Carmeliet and Jain, 2011; Melero-Martin and Dudley, 2011; Welti et al., 2013). In part, and concomitantly with pro-angiogenic Ang2 and metalloproteinase activity, VEGF signaling enables the selection, formation and migration of the tip cell from endothelial cells, the leading driver of vessel sprouting (Carmeliet and Jain, 2011; Welti et al., 2013). In response to hypoxia, HIF-1α and HIF-2α activation lead to the expression of pro-angiogenic VEGF in an attempt to increase oxygen supplies (Carmeliet and Jain, 2011; Welti et al., 2013). In this respect, CSC have been shown to locate at the vicinity of blood vessels in human samples of head and neck squamous cell carcinoma

(Krishnamurthy et al., 2010). Additionally, in malignant gliomas, tumors that are highly dependent on angiogenesis, the putative CSCs population demonstrated the ability to produce proangiogenic factors, such as VEGF, to stimulate angiogenesis (Bao et al., 2006) Furthermore, the development of tumor hypoxia has shown to increase breast CSC proliferation, an effect mediated by HIF-1α (Conley et al., 2012).

It is thus apparent the existence of a CSC-angiogenesis loop in which defective angiogenesis favors the balance towards CSC, which subsequently increases pro-angiogenic factor secretion, like VEGF, to stimulate angiogenesis (Carmeliet and Jain, 2011), potentially limiting the effect of anti-angiogenic therapies (Conley et al., 2012).

3. Emerging molecular and cellular targets: nucleolin-based multiple targeting

Previous sections evidenced cancer as an intricate cascade of aberrantly activated physiological processes. Even though this provides several levels for therapeutic intervention, it also represents an enormous challenge, since most processes are gateways to circumvent treatment and often lead to drug resistance (Singh and Settleman, 2010). Thus, it is apparent that only a concomitant and specific targeting of paramount molecular and cellular entities, as the tumor vasculature and the cancer stem cell niche, may enable groundbreaking therapeutic improvements by hampering the foundations for the progression and evolution of the tumor microenvironment.

3.1. Current strategies targeting angiogenesis and cancer stem cells

Angiogenesis and its cellular and molecular components have been, in some cases, successfully targeted with anti-angiogenic therapies, based on the rationale that cutting off tumor nourishment impairs tumor growth (Ferrara and Kerbel, 2005; Welti et al., 2013). This is the case of bevacizumab. However, anti-angiogenic therapy is highly affected by poor efficiency and development of resistance, in part aided, by the increase in the number of resistant tumor cells, as CSC (Carmeliet and Jain, 2011; Conley et al., 2012).

As emerging therapeutic targets, CSC represent a paramount target owing to their association with drug resistance, disease recurrence and metastasis. Therefore, multiple approaches to eradicate CSC are under development, including the design of inhibitors of embryonic signaling pathways, such as WNT, NOTCH and Hedgedhog, which control stemness features like CSC self-renewal and expansion (Takebe et al., 2015; Vazquez-Martin et al., 2011). For example, through Notch silencing, breast cancer stem cell expansion was arrested (Suman et al., 2013). Targeting a downstream effector of NOTCH, γ-secretase, using GSIXII inhibitor, in combination with the Bcl-2-homology 3 domain (BH3) mimetic inhibitor, ABT-737, enhanced breast cancer cell death in *ex vivo* human breast cancer samples, as compared to GSIXII alone (Seveno et al., 2012).

Other drugs are under phase I and/or phase II clinical development, often combined with standard chemotherapy, including VS-6063 (targeting focal adhesion kinase), tarextumab (targeting NOTCH receptors) and BBI608 (targeting STAT3 and β-catenin) for mesothelioma, pancreatic cancer and gastric cancers, respectively (Kaiser, 2015). The PI3k and mTORC1/2 dual inhibitor VS-5584 preferentially targets cancer stem cells (Kolev et al., 2015) and was under phase I clinical development against advanced non-hematologic malignancies and lymphoma (terminated).

Nevertheless, the strategy of targeting only one of those cellular elements, including CSC, may be undermined by their plasticity and adaptability (Badve and Nakshatri, 2012). To circumvent this issue, drug combinations targeting multiple cellular components of the tumor microenvironment may represent a disruptive strategy.

3.2. Nucleolin-based multiple cellular targeting

Alongside the aforementioned markers, nucleolin (NCL) has been associated with tumor proliferation. Nucleolin is a nucleolar protein involved in chromatin structure as well as transcription, ribosome assembly and nucleous-cytoplasm transport, playing a central role in cell cycle, influencing microtubule nucleation, and nucleolus structure (Fig. 3) (Gaume et al., 2015; Ginisty et al., 1999; Srivastava and Pollard, 1999; Ugrinova et al., 2007).

In an oncological context, NCL has also been described as highly overexpressed on the surface of cancer and endothelial cells of tumor angiogenic vessels (Christian et al., 2003; Ginisty et al., 1999; Hovanessian et al., 2010). In this context, NCL has been shown to mediate the antiangiogenic and anti-lymphangiogenic properties of endostatin (Shi et al., 2007; Zhuo et al., 2010). Furthermore, NCL has been shown to synergize with EGFR and mutant Ras to promote tumor growth and malignant transformation, upon enhancing the translation of Sp1 transcription factor and MMP9 (Farin et al., 2011; Fogal et al., 2009; Hsu et al., 2015; Hung et al., 2014). At a different level, extranuclear NCL has been suggested as a poor prognostic factor in lung and esophageal cancer (Qi et al., 2015; Xu et al., 2016).

At the cell membrane, NCL modulates influx of calcium and internalization of different ligands as part of the nucleus-cytoplasm-membrane shuttling (Fig. 3) (Berger et al., 2015; Borer et al., 1989; Losfeld et al., 2009).

Different strategies targeting surface NCL, currently under preclinical development, have been devised including the anti-NCL pseudopeptides as N6L or HB-19 (Birmpas et al., 2012; Krust et al., 2011) or even a recombinant immunoagent (Palmieri et al., 2015). Additionally, taking advantage of NCL overexpression in different cell subpopulations within solid tumors, multi targeting strategies have also been developed. Liposomes containing doxorubicin and functionalized with the nucleolin-binding F3 peptide significantly increased cytotoxicity against cancer and endothelial cells from tumor blood vessels relative to the non-targeted sample. In addition, it enabled a marked reduction of the viable rim area of a breast tumor model relative to the non-targeted controls or free drug (Moura et al., 2012). A similar strategy explored NCL for the delivery of anti-*PLK1* siRNA to both cell types (Gomes-da-Silva et al., 2013a; Gomes-da-Silva et al., 2013b; Gomes et al., 2013).

In a solid tumor, the overexpression of nucleolin is not limited to cancer and endothelial cells, which is somehow in relation with the additional roles assigned to the protein within embryonic stem cell biology. Indeed, NCL interaction with OCT4 was documented during cell cycle progression upon phosphorylation in ESC (Johansson et al., 2010). Of utmost importance, NCL expression was described to be essential for maintenance of embryonic stem cell homeostasis and self-renewal, through direct repression of *p53* mRNA translation (Fig. 3) (Chen et al., 2012; Cinghu et al., 2014; Takagi et al., 2005; Yang et al., 2011). As such, it is evident that NCL is supporting cell functions both at developmental stage and in cancer. A correlation between mRNA levels of NCL and pluripotency markers NANOG and OCT4 in breast CSCs and mouse ESC, has been demonstrated (Fonseca et al., 2015). Additionally, NCL surface expression enabled the isolation of highly tumorigenic triple-negative breast cancer cells, similarly to $ALDH^{hi}/CD44^{hi}$ population, which are, currently, consensual markers of breast CSC (Fonseca et al., 2015). This has revealed NCL as a common denominator between CSCs and non-SCC, thus representing an opportunity for targeting purposes. In fact, the intracellular delivery of a synergistic drug combination of doxorubicin and C6-ceramide (a pro-apoptotic sphingolipid described to inhibit the PI3k/AKT signaling cascade) mediated by

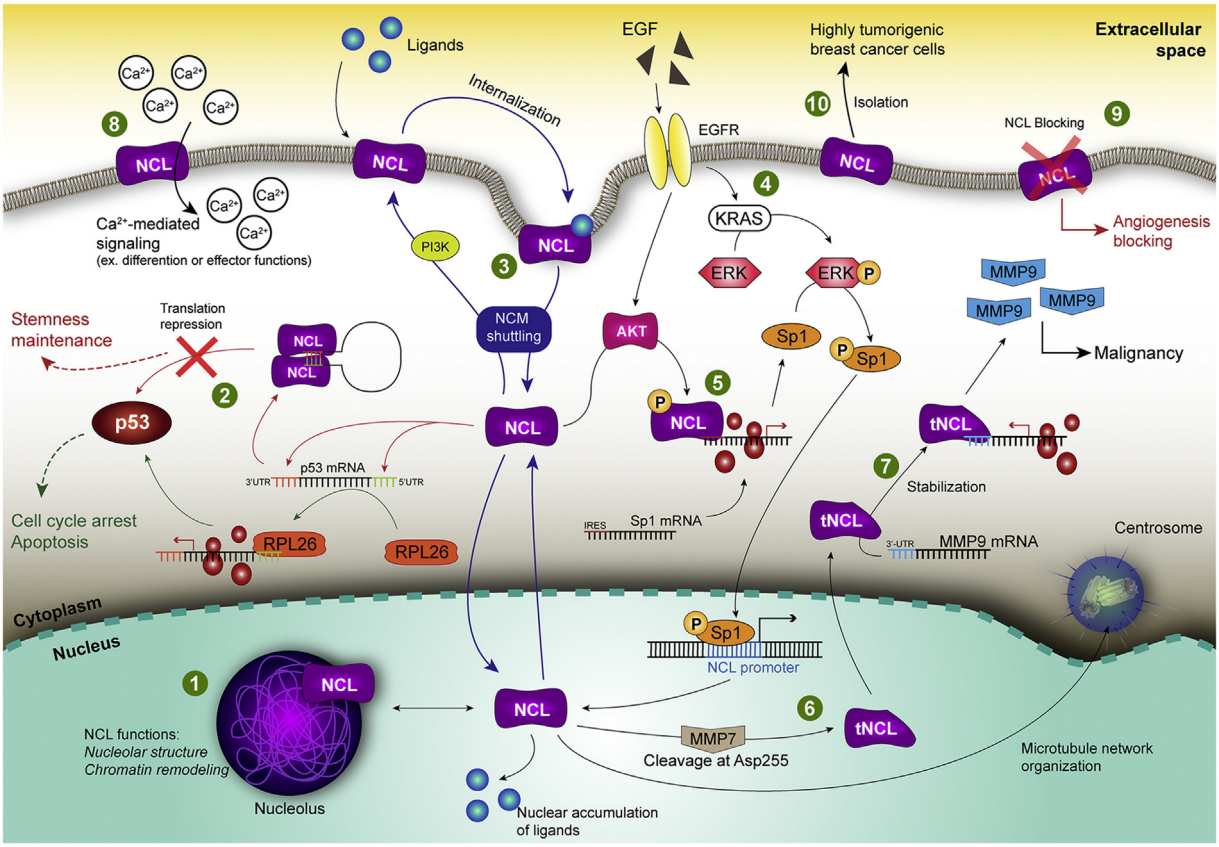

Fig. 3. Diagram representing the different roles of nucleolin. Within the cell, nucleolin exerts physiological functions as chromatin remodeling **(1)**, as well as stemness maintenance of embryonic stem cells through blocking of ribosomal protein L26-mediated p53 expression **(2)**. In cancer cells, nucleolin shuttling, between the nucleus and the cell membrane, mediates the internalization and nuclear localization of different ligands **(3)**. Additionally, nucleolin overexpression is dependent, in some cancer cell types, on the activation of the epidermal growth factor receptor and Sp1 transcription factor **(4)**. Nucleolin stabilizes the mRNA and modulates the translation of the latter **(5)**, closing a positive feedback loop. Meanwhile, nucleolin may be cleaved by MMP7 **(6)** to enhance the production of MMP9, leading to malignant transformation **(7)**. Furthermore, surface nucleolin mediates the internalization of calcium **(8)** as well as the anti-angiogenic activity of different ligands as endostatin **(9)**. Surface nucleolin also enables the selection of highly tumorigenic cells that generate tumors at the same extent as putative breast CSC **(10)**. Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; *NCL, nucleolin; NCM, nucleus-cytoplasm-membrane shuttling; RPL26, ribosomal protein L26; tNCL, truncated NCL.*

nucleolin, enabled 100% of cell death (Fonseca et al., 2014; Fonseca et al., 2015).

4. Conclusion

Cancer treatment represents a massive challenge owing to intricate relationships established in the tumor microenvironment, where cells, signaling molecules and signaling cascades cooperate in an unstable genetic playground, leading to constant variability and therapeutic evasion.

Despite the acknowledgment of the existence of a subpopulation of cells with stem-like characteristics, involved in drug resistance, metastases and tumor recurrence, their clear identification in many cancers remains elusive. Different markers have enabled the identification of breast cancer cells with aberrant activation of canonical and developmental pathways associated with stemness, and further suggested the involvement of adult stem cells in carcinoma development. In fact, it has been proposed that the risk of developing cancer in different tissues is proportional to the number of normal stem cell divisions in adult tissues (Tomasetti and Vogelstein, 2015). Accordingly, there is likely a component of cancer risk that is owed to randomness ("bad luck") in the acquisition on mutations in noncancerous stem cells, besides environmental or inherited factors (Tomasetti and Vogelstein, 2015).

Overall, the information herein discussed clearly supports the cancer stem cell model, which anticipates hierarchical organization but also constant clonal evolution of tumor cells. This imposes a daunting challenge in the identification of tumor initiating and maintaining cells, as well as in the therapeutic strategies to adopt. Accordingly, the exploitation of markers that are common to different tumor cell types, as nucleolin in breast cancer, may render an important contribution for the development of novel therapeutic tools to address important unmet medical needs.

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