Review Article

Nestin as a marker of cancer stem cells

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The crucial role of cancer stem cells (CSCs) in the pathology of malignant diseases has been extensively studied during the last decade. Nestin, a class VI intermediate filament protein, was originally detected in neural stem cells during development. Its expression has also been reported in different tissues under various pathological conditions. Specifically, nestin has been shown to be expressed in transformed cells of various human malignancies, and a correlation between its expression and the clinical course of some diseases has been proved. Furthermore, the coexpression of nestin with other stem cell markers was described as a CSC phenotype that was subsequently verified using tumorigenicity assays. The primary aim of this review is to summarize the recent findings regarding nestin expression in CSCs, its possible role in CSC phenotypes, particularly with respect to capacity for self-renewal, and its utility as a putative marker of CSCs.

The role of cancer stem cells (CSCs) in the pathology of malignant diseases has been extensively studied during the last decade. At present, it is widely accepted that CSCs participate in the processes of tumor initiation, progression, metastasis, and relapse.[1,2] The intermediate filament (IF) protein nestin is an extensively studied marker of neural stem cells that is a putative marker of the CSC phenotype, as its expression has been identified in many human malignancies.[3,4] The primary aim of this review is to summarize the recent findings in this interesting field of tumor biology.

Characteristics and Detection of CSCs

Cancer stem cells are defined as a small subpopulation of undifferentiated cells in tumor tissue that are characterized by their capacity for self-renewal and differentiation into various lineages and clones that generate tumor masses. Due to their ability to form a continuously growing tumor, the synonymous terms "tumor-initiating" or "tumorigenic" have been used to describe these cells.[5,6]

Published reports suggest three primary hypotheses regarding the origin of CSCs. The first hypothesis is that CSCs develop from tissue-specific adult stem cells, which show the same capacity for self-renewal and differentiation and survive in the organism for an extended period, rendering these cells as vulnerable to the accumulation of oncogenic mutations. Furthermore, CSCs showed similar primitive phenotypes, such as the expression of specific stem cell markers.[7] The second hypothesis for the origin of CSCs is based on the transformation and dedifferentiation of mature or differentiating cells, which have been reported to reacquire stem cell properties as a consequence of transforming mutations.[8] The third hypothesis is that CSCs and, consequentially, tumorigenesis originate as a result of an "aberrant deposit" of embryonic stem cells in developing tissues during ontogenesis.[1]

Cancer stem cells are typically characterized based on the presence and/or absence of several cellular markers, a combination of which is specific for the CSC phenotype in a respective tumor. These markers include cell surface or membranous proteins (CD15, CD24, CD44, CD133, CXCR4, NCAM, and ABC transporters), cytoplasmic proteins (nestin, Musashi-1, and aldehyde dehydrogenase) or nuclear proteins (Sox-2, Oct3/4, and Nanog) that carry out various structural or metabolic functions in the cell.

The immunodetection of CSC markers on the cell surface has facilitated the identification and isolation of selected CSC subpopulations using appropriate sorting methods (FACS or magnetic-activated cell sorting (MACS)). Other frequently used methods for the isolation of CSCs are based on the functional characteristics of CSCs, for example, the expression of cell adhesion molecules, cytoprotective enzymes, and membrane transporters.[9] These characteristics can be identified using standardized *in vitro* functional assays: detection of the side population, sphere formation assays, and clonogenicity assays, for example. However, *in vivo* tumorigenicity assays using immunodeficient mice represent the gold standard for

the detection of CSCs because this method provides direct evidence of self-renewal and of tumor-forming capacities in an organism. A positive result on this test is considered to confirm the CSC phenotype in the observed cell population.[5]

Characterization of Nestin

Nestin (neuronal stem cell protein) was originally identified using the Rat-401 monoclonal mouse antibody in 1985. This antibody displayed specificity to an antigen that was transiently expressed in specific regions of the developing central nervous system (CNS) and in non-neuronal cells in the peripheral nervous system.[10] Subsequent analysis led to the classification of nestin as a class VI IF protein.[11]

In general, IF represent one of the three main components of cytoskeleton in animal cells. In contrast to microtubules and actin filaments, which consist exclusively of highly conserved globular proteins tubulin and actin, respectively, IF proteins are fibrous and their expression is tissue- or cell-specific. All IF proteins exhibit the same structural organization: a central α-helical rod domain flanked by N- and C-terminal tail domains;[12] therefore, IF are homopolymers or heteropolymers formed of two or more IF proteins. Intermediate filament proteins are classified according their structure and localization as follows: classes I and II encompass acidic and basic cytokeratins; class III embraces vimentin, desmin, glial fibrillary acidic protein, syncoilin, and peripherin; class IV consists of neurofilaments and α-internexin; class V of lamins; and class VI of nestin and synemin.[13] Intermediate filaments are responsible for mechanical integrity of the cell, they serve as an integrating scaffold for other cytoskeletal components and for some organelles. They are also involved in formation of tissue architecture and in the process of tissue regeneration.[14]

The human nestin gene (Fig. 1) is located on the long (q) arm of chromosome 1 at position 23.1. Its promoter resides in a 5′-non-translated region containing two Sp-1-binding sites and lacks a functional TATA box.[15] The nestin gene consists of four exons separated by three introns. Enhancer elements were found in the first and second introns.[16] The enhancer located in the first intron specifically increases nestin expression in myogenic precursors; the mechanism underlying this regulation is likely based on the presence of two E-boxes within the enhancer sequence, to which the transcription factor MyoD cooperatively binds.[17] The second intron contains two neural precursor-specific enhancers, identified as a pan-CNS enhancer and a midbrain-specific enhancer, both of which contain at least two regulatory elements.[18] These two enhancer elements represent binding sites for different types of regulatory molecules, for example, nuclear hormone receptors and transcription factors belonging to the SOX or POU family.[18,19] The expression of the nestin gene is also regulated by epigenetic mechanisms, that is, DNA methylation and histone acetylation. Specifically, histone acetylation appears to be the preferred mechanism of nestin regulation during neural differentiation.[20]

The human nestin protein (Fig. 2) consists of 1621 amino acids and displays a predicted molecular weight of 177.4 kDa. However, nestin is typically detected by Western blotting at a higher apparent molecular weight, ranging from 200 to 240 kDa. This difference can be explained by the presence of multiple phosphorylation sites and glycosylated side chains.[21] The structure of the nestin protein is similar to that of other IF proteins: a conserved 306-amino acid α-helical rod domain, which forms the protein core, 7-amino acid N-terminal and 1308-amino acid C-terminal tail domains. Within the rod domain, helical coils play an important role in dimerization and consecutive filament formation. Previous studies have suggested that nestin is able to form heterodimers only with other IF proteins, most favorably with vimentin.[18,22]

Nestin Expression in Transformed Cells

Nestin expression in the developing and adult human organism under both physiological and pathological conditions has been well described.[4,23–25] Although nestin is primarily regarded as a marker of neural stem/progenitor cells, its expression has also been shown in various embryonic cells and tissues, including skeletal muscle, cardiac muscle, umbilical cord blood cells, Sertoli and interstitial testicular cells, odontoblasts, hair follicle sheath cells, hepatic cells, and renal progenitors.[23] In adults, nestin-positive cells are localized to tissue /organ-specific sites, where they serve as a quiescent resource of cells capable of proliferation, differentiation, and migration after their re-activation.[23] The re-expression or upregulation of nestin was observed in various tissues during reparation processes after several types of injury, including the following: in
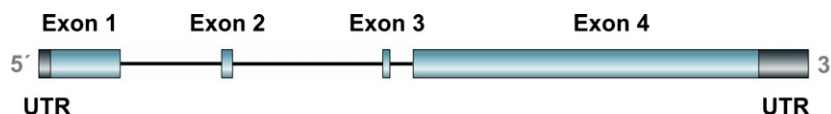
Fig. 1. Exon/intron structure of the human nestin gene. Four exons are depicted in cyan color. The 5′-UTR (black) is located within the first exon; similarly, the 3′-UTR (black) is located within the fourth exon.
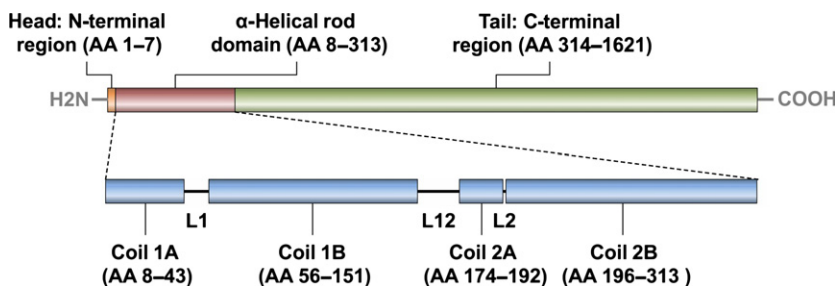
Fig. 2. Domain structure of the human nestin protein. Three domains are depicted: "head" located in the N-terminal region (orange), α-helical rod domain (red), and "tail" in the C-terminal region (green). The rod domain consists of four coils (blue) separated by three linkers (L1, L12, and L2). Numbers of amino acids (AA) in the individual domains and coils are given in brackets.

reactive astrocytes after CNS damage,[26] during the fibrotic response to ischemic heart disease,[27] and in the context of mesangial cell repopulation after induced nephritis.[28] Special attention has been paid to the detailed analyses of nestin expression in many types of human malignancies because nestin may serve as an important diagnostic or prognostic marker.

Increased nestin expression is typical for tumors generated from undifferentiated precursor cells or immature progenitors, which rapidly proliferate during neurogenesis.[29] Although these precursor cells replace nestin with other IF proteins during neuronal differentiation, nestin expression can be transiently re-activated in transformed cells.[25]

In our previous detailed review, we summarized the current understanding of nestin expression in cancer cells and vascular endothelial cells from various types of solid tumors.[24] Unsurprisingly, nestin was detected in neuroectodermal neuroepithelial tumors, including tumors of astrocytic, oligodendroglial, oligoastrocytic, ependymal, embryonic, neuronal, and mixed neuronal-glial origin. Furthermore, nestin expression was found in mesenchymal tumors (e.g. osteosarcoma, rhabdomyosarcoma, gastrointestinal stromal tumor), germ cell tumors (e.g. embryonal carcinoma, germinoma, choriocarcinoma, yolk sac tumor), and epithelial tumors (e.g. pancreatic adenocarcinoma, breast carcinoma, ovarian carcinoma, lung carcinoma).[24,25,30,31]

In some tumor types, increased nestin expression correlates with the tumor grade and indicates an immature and invasive phenotype of transformed cells.[32] Moreover, a correlation between the levels of nestin expression in tumor tissue and the clinical course of the disease was reported for breast carcinoma, ovarian carcinoma, gastrointestinal tumors, germ cell tumors, osteosarcoma, ependymoma, and melanoma.[33–39] Thus, nestin expression is considered to serve as a prognostic marker in these cancer types; however, its prognostic value should be verified in large cohorts of the patients. In contrast, no such relationship was found for pancreatic adenocarcinoma.[40,41] Interestingly, the use of nestin as a predictive marker of a poor response to conventional therapy and to novel therapeutic agents was recently reported for multiple myeloma.[42]

The evidence regarding nestin expression in hematological malignancies is limited to a small number of studies of multiple myeloma. Nestin mRNA was detected in NCAM-positive cells of human myeloma cell lines and in primary myeloma cells.[43] The nestin protein was immunodetected in mature plasma cells from multiple myeloma patients and in myeloma cell lines.[44] Furthermore, an association between increased nestin expression and the pathogenesis of multiple myeloma has been reported.[42]

Nestin as a Putative Marker of CSCs

At present, there is no consensus concerning a "universal" marker for the identification of CSCs in various tumor types.[9] Clearly, a tumor-specific CSC phenotype should be characterized by the co-expression of several markers, both intracellular and surface-associated. As mentioned above, one putative CSC marker is nestin, which is often co-expressed with other stem cell markers (Table 1), such as CD133, Oct3/4 and Sox-2 (Fig. 3).

Identification of nestin in CSCs of neurogenic tumors. The first study of nestin in CSCs revealed its expression in brain tumor stem cells (BTSCs) isolated from different types of human CNS tumors (medulloblastoma, astrocytoma, ependymoma,

and ganglioglioma). All of these CSC populations were formed nestin-positive spheres, which were subsequently grown in vitro. However, nestin was downregulated during the induced differentiation of BTSCs. Moreover, BTSCs isolated from glioblastoma or medulloblastoma initiated tumor growth in the brains of NOD/SCID mice.[29,45] Detailed analyses of cell lines derived from other types of pediatric brain tumors (i.e., ependymoma, medulloblastoma, glioma, and primitive neuroectodermal tumors) revealed that the number of nestin-positive CSCs is increased in spheres exhibiting a CSC phenotype based on functional assays. Furthermore, sphere-derived cells showed a higher capacity for multilineage differentiation and greater resistance to etoposide than monolayer-derived cells.[46] Other evidence of increased nestin expression in spheres was described using glioblastoma and medulloblastoma cell lines[47,48] and cells derived from peripheral nerve sheath tumor.[49] This increase in nestin expression due to sphere formation appears to be a common phenomenon associated with the stemness of cells within the sphere. The studies mentioned above reported the co-expression of nestin with CD133 or both CD133 and Sox-2 as a common CSC phenotype in tumors of neurogenic origin.[29,46–49]

Nestin-positive cells in xenografts from human astrocytoma- and glioblastoma-derived CSCs showed significant co-expression of proliferating cell nuclear antigen (PCNA) indicating proliferation activity of these cells, as well as co-expression of vascular cell adhesion molecule-1, which may affect cell migration and spreading.[50]

In glioma-prone mice with nestin-GFP tagged transgene expressed both in neural stem cells and in relatively quiescent glioma cells, re-initiation of cell division and growth of nestin-positive glioma cells were observed after eradication of proliferating tumor cells by temozolomide.[51] These results are in accordance with cancer stem cell theory concerning relapses after conventional chemotherapy aimed at proliferating tumor cells.

Furthermore, neural stem cells and "more stem-like glioma-initiating cells" showed similar biological features as well as gene expression profiles, particularly enrichment of Ca^{2+} signaling genes. High expression of Ca^{2+} channels (i.e., AMPA-selective glutamate receptor 1) correlated with expression of nestin and brain lipid-binding protein as well as with sensitivity to Ca^{2+} channel blockers in glioma-initiating cells. Nestin, together with brain lipid-binding protein and glutamate receptor 1, are thus considered a novel combination of glioma CSC markers and the enhanced sensitivity to Ca^{2+} could be included in functional tests of glioma CSCs.[52]

Identification of nestin in CSCs from mesenchymal tumors. Surprisingly, the coexpression of nestin with CD133, which was originally described as the CSC phenotype in neurogenic tumors, was also found in tumors of mesenchymal origin. The first evidence of nestin-/CD133-positive cells in rhabdomyosarcoma samples and cell lines (Fig. 3a) was followed by verification of their tumorigenic potential based on functional assays.[53] The same group also described the presence of nestin-/CD133-positive cells in osteosarcoma cell lines based on immunodetection and predicted the coexpression of these markers as a putative CSC phenotype.[54] An additional study of osteosarcoma, chondrosarcoma, and fibrosarcoma cell lines indicated that nestin mRNA is expressed in sphere-forming subpopulations; however, adherent subpopulations of the same cell lines were identified as nestin-negative.[55] Alternatively, one study of established osteosarcoma cell lines reported contrasting results: the same pattern of nestin expression (i.e.,

Table 1. Overview of known cancer stem cell (CSC) phenotypes in various human solid tumors and functional assays used for their identification

Tumor type	Phenotype of CSCs	Functional assays		References
		In vitro	*In vivo*	
Neurogenic tumors				
Medulloblastoma	Nestin, CD133	Neurospheres, differentiation assay	Xenograft formation	(29,45)
	Nestin, Sox2, CD133	Neurospheres, differentiation assay, drug resistance	Xenograft formation	(46)
	Nestin, Sox2, CD133, β-catenin	Medullospheres		(47)
Ependymoma	Nestin, CD133	Neurospheres, differentiation assay		(29)
	Nestin, Sox2, CD133	Neurospheres, differentiation assay, drug resistance	Xenograft formation	(46)
Glioma	Nestin, CD133	Neurospheres, differentiation assay	Xenograft formation	(29,45)
	Nestin, Sox2, CD133	Neurospheres, differentiation assay, drug resistance	Xenograft formation	(46)
Glioblastoma	Nestin, CD133, Musashi-1, Sox2	Neurospheres, differentiation assay, clonogenic assay	Xenograft formation	(48)
CNS primitive neuroectodermal tumor	Nestin, Sox2, CD133	Neurospheres, differentiation assay, drug resistance	Xenograft formation	(46)
Malignant peripheral nerve sheath tumors	CD133, Oct4, nestin, NGFR (CD271)	Spheres, differentiation assay	Xenograft formation	(49)
Mesenchymal tumors				
Rhabdomyosarcoma	Nestin, CD133	Clonogenic assay	Xenograft formation	(53)
Osteosarcoma	Nestin, CD133			(54)
	CD133, Oct3/4, Sox2, Nanog, nestin	Sarcospheres, clonogenic test, *in vitro* tumorigenic assay, side population, differentiation assay		(55)
	ABCA5, CBX3, ABCG2, ALDH	Spheres, clonogenic test, drug resistance	Xenograft formation	(56)
Chondrosarcoma	CD133, Oct3/4, Sox2, Nanog, nestin	Sarcospheres, clonogenic test, *in vitro* tumorigenic assay, side population, differentiation assay	Xenograft formation	(55)
Fibrosarcoma	CD133, Oct3/4, Sox2, Nanog, nestin	Sarcospheres, clonogenic test, *in vitro* tumorigenic assay, side population	Xenograft formation	(55)

Table 1 (Continued)

Tumor type	Phenotype of CSCs	Functional assays		References
		In vitro	*In vivo*	
Epithelial tumors				
Ovarian carcinoma	Nestin, Oct4, Nanog	Spheroids, differentiation assay	Xenograft formation	(57)
	Nestin, Nanog, Oct4, Sox2, ABCG2, CD133, CD117	Spheres, drug resistance	Xenograft formation	(58)
	Nestin, Oct4, Nanog, Sox2, Bmi-1, CD133, CD44, CD24, ALDH1, CD117, ABCG2	Spheroids, drug resistance	Xenograft formation	(59)
Oral squamous cell carcinoma	Nestin, CD133, Oct4, Nanog, ABCG2, CD117	Spheres, soft-agar assay, differentiation assay	Xenograft formation	(60)
Prostate carcinoma	CD49b, CD49f, CD44, deltaNp63, nestin, CD133, Nanog, Oct-4, Bmi-1, Jagged-1, Hes-1, Patched, Smoothened, CD201	Prostaspheres, clonogenic assay		(61)
	CD117, ABCG2, Nanog, Oct4, Sox2, nestin, CD133	Drug resistance	Xenograft formation	(62)
Gallbladder carcinoma	CD133, nestin, Oct4, Nanog	Spheres, drug resistance, differentiation assay	Xenograft formation	(63)
Non-small-cell lung cancer	Nestin, CD133			(64)
Lung cancers	CD44, CD90, Nanog, Oct4	Spheres, irradiation resistance, clonogenic assay	Xenograft formation	(65)
Colon cancer	Nestin, Bmi1	Spheres, soft agar colony formation assay, invasion assay, drug resistance		(68)
Breast cancer	CD44, Oct4, nestin, CD24⁻	Mammospheres		(69)
	CD44, ESA, nestin, CD24⁻	Mammospheres, invasion assay	Xenograft formation	(70)
	Nanog, Oct3/4, nestin, Sox2, CD34			(71)
Gastric adenocarcinoma	Nestin, CD44			(72)
Pancreatic ductal adenocarcinoma	ALDH1A1, ABCG2, nestin	Spheres, invasion assay, side population,		(74)
	CD133, CD44, Oct4, nestin	Drug resistance	Xenograft formation	(76)

CNS, central nervous system.
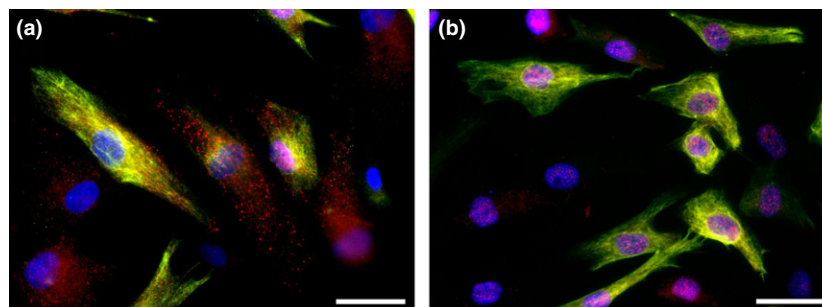
Fig. 3. Co-expression of nestin and other putative cancer stem cells markers in the NSTS-11 rhabdomyosarcoma cell line. Representative double labeling for nestin and CD133 (a) and for nestin and Oct4 (b). CD133 (a) and Oct4 (b) were stained by indirect immunofluorescence using Alexa 568-labeled secondary antibody (red); nestin (a, b) was stained by the same method using Alexa 488-labeled secondary antibody (green); nuclei were counterstained with DAPI (blue). Bar = 25 μm.

downregulation of nestin in adherent cells) was found in spheres and adherent populations of CHA59 cells based on real-time PCR and Western blotting, whereas a reverse pattern (i.e., down-regulation of nestin in sarcospheres) was described in the HuO9 cell line.[56] Interestingly, both spheres and adherent cells were detected as nestin-negative in the Saos-2 cell line,[56] but strong nestin expression was previously identified in the same cell line based on immunofluorescence.[54]

Identification of nestin in CSCs from epithelial tumors. Cells coexpressing CD133 and nestin (a putative CSC phenotype) were also found in several types of epithelial tumors.

Early studies of ovarian adenocarcinoma, including high-grade serous ovarian carcinoma, identified a population of self-renewing CSCs that grew as sphere-forming clusters. Furthermore, these cells were tumorigenic *in vivo*, showing increased chemoresistance and expressed CSC markers, including nestin.[57–59]

Cultivation of oral squamous cell carcinoma cells in defined serum-free medium led to the formation of spheres enriched with cells exhibiting CSC phenotypes, including high expression of nestin and other stem cell markers (i.e., CD133, Oct-4, and Nanog), differentiation capacity, enhanced migratory capacity, and tumorigenicity. However, nestin was also found in sphere-like bodies of basal cells that showed limited proliferation potential and reduced expression of self-renewal genes.[60]

Similarly, stem-like prostate cancer cells expressing typical stem cell markers, such as Nanog, Oct4, Sox-2, CD133, and nestin, were isolated from prostate tumor tissue[61] and from a prostate cancer cell line.[62] In the first study, the obtained "prostaspheres" were maintained *in vitro*.[61] The second study verified the proposed chemoresistance and tumorigenicity of these prostatic CSCs.[62] Additionally, CSCs were obtained from human gallbladder carcinoma through sphere formation, and the expression of nestin, CD133, Oct-4, and Nanog was identified in these cells.[63]

Nestin/CD133-positive cells were also detected in paraffin blocks of 121 non-small-cell lung cancer samples based on immunohistochemistry and immunofluorescence; the co-localization of these two markers was detected in 17% of the samples, although the double-stained cells represented <1% of all positive cells. The authors concluded that CD133/nestin-positivity may serve as a marker of lung CSCs.[64] Other relevant studies did not refer to nestin expression in lung CSCs[65,66] and only mentioned the effect of nestin on cell proliferation.[67]

Treatment of an HCT-117 colon cancer cell line or primary colon cancer cells with interleukin-1β promoted sphere formation, upregulated the typical stemness markers nestin and Bmi1, and increased drug resistance. Moreover, the observed self-renewal status was accompanied by epithelial-mesenchymal transition (EMT), the upregulation of the EMT activator Zeb1, and the downregulation or loss of E-cadherin expression.[68]

Breast CSCs are typically isolated according to their ESA+/CD44+/CD24- phenotype. Interestingly, these cells also express Oct-4 and nestin and display increased tumorigenicity through the formation of mammospheres under *in vitro* conditions.[69] The high expression of nestin is associated with a 100-fold increase in tumorigenicity in comparison with cells showing weak nestin expression and ESA+/CD44+/CD24-phenotype. Conversely, knockdown of nestin in breast CSCs led to cell cycle arrest, apoptosis induction, repression of cell migration, and inhibition of spontaneous EMT. On a molecular level, silencing of nestin disrupted Wnt/β-catenin activation, which is an important signaling pathway in breast CSCs.[70]

Furthermore, Oct-4/nestin-positivity in cancer tissues has been associated with younger age, malignant tumor grades, lymph node metastasis, and shorter survival.[69,70] Co-expression of nestin with other established stem cell markers (e.g., Oct3/4 or Sox-2) was shown in circulating breast cancer cells.[71]

A correlation between the expression of nestin and CD44 was recently found in gastric adenocarcinoma; this study provided the first evidence of the nestin+/CD44+ phenotype in this type of tumor.[72] In pancreatic ductal adenocarcinoma (PDAC), the expression of nestin as a putative marker of CSCs has recently been discussed.[73,74] High levels of nestin together with ALDH1A1 and ABCG2 were detected in metastatic cells from human PDAC injected into NOG mice; moreover, these cells showed typical features of CSCs such as sphere formation and tumorigenicity. Downregulation of nestin by shRNA in these cells resulted in decreased capacity of both sphere formation *in vitro* and metastasizing *in vivo*.[74] Nestin modulates nestin-positive cell invasion, EMT, and metastasis during the progression of PDAC. It was also shown that nestin expression in PDAC cells is regulated by the transforming growth factor-β1/Smad pathway: nestin expression is induced in a Smad4-dependent manner and under hypoxic conditions.[75] Nestin was also detected in spheres together with CD133 and CD44,[73] and the recently isolated Panc-1 stem-like cell line demonstrated coexpression of CD133, CD44, Oct4, and nestin.[76] In contrast, no significant correlation between the CD133 and nestin expression patterns has been detected in PDAC tumor samples,[41] and nestin expression did not correlate with the clinical course of PDAC.[40]

What is the Role of Nestin in CSCs?

In some tumor types, nestin expression correlates with aggressive growth, metastasis, and poor prognosis.[25] Nevertheless, the role of nestin in transformed cells, especially CSCs, aside from its participation in cytoskeleton formation, remains unclear.

One primary hypothesis for the role of nestin in CSCs arose from an *in vivo* experiment: nestin knockout mice showed embryonic lethality and a reduced number of neuronal stem cells. Moreover, the remaining neuronal stem cells exhibited decreased self-renewal capacity and increased cell death, with no apparent defects in proliferation, differentiation, or cytoskeletal integrity.[77] A proposed relationship between nestin expression and self-renewal is supported by the previous finding that the nestin promoter is activated by Notch, which binds to the second intron enhancer element of the nestin gene. The combined activation of Notch and KRAS led to the proliferation of nestin/PCNA-positive cells in lesions along the subventricular zone of mice, suggesting that these lesions may represent premalignant stages of tumorigenesis and that nestin/PCNA-positive cells may exhibit CSC phenotypes.[78]

It has been shown in neuroblastoma cells that E-box sequences in the regulatory second intron of the nestin gene are specific regions to which N-myc transcription factors can bind. Furthermore, an increase in N-myc protein levels subsequently enhances nestin expression and cell proliferation and motility.[79] In addition, N-myc regulates the expression of several genes that encode stem-related factors, including lif, klf2, klf4, and lin28b.[80] Thus, nestin unequivocally belongs to the group of stemness genes regulated by N-myc.

An additional mouse study indicated that nestin-positive CSCs localized to the perivascular niche of medulloblastoma tumor tissue and exhibited radioresistance through the

activation of the AKT/PI3K and p53 signaling pathways. These characteristics are in contrast to those of the rest of the tumor mass, which undergoes apoptosis. In medulloblastoma displaying extensive nodularity, the loss of the *PTEN* gene in combination with Sonic hedgehog overexpression induced the formation of tumors from nestin-positive progenitor cells. Furthermore, the activation of the AKT signaling pathway using ionizing irradiation led to the proliferation of nestin-positive CSCs, whereas the majority of cells in the tumor mass were differentiated.[81]

A cytoprotective function of nestin was shown in neuronal stem cells, as overexpression of nestin inhibited oxidant-induced apoptosis, including the activity of cyclin-dependent kinase 5, which serves as an important regulator of neuronal development and function.[82] However, this mechanism of nestin activity has not been demonstrated in CSCs.

The functions of many proteins are regulated by their cellular localization. Nestin is predominantly localized to the cytoplasm, where it participates in the formation of IFs as integral components of the cytoskeleton in animal cells. However, based on confocal microscopy and immunodetection using ultrathin sections by transmission electron microscopy, strong nestin signals were detected in the nuclei of the GM7 glioblastoma cell line;[83] this cell line was later characterized as positive for both nestin and CD133.[84] Subsequently, the nuclear localization of nestin was found in two neuroblastoma cell lines and in one medulloblastoma cell line out of a total of 11 neurogenic patient-derived cell lines. These results suggest that this phenomenon is not particularly rare in transformed cells. However, the percentage of cells displaying nestin-positive nuclei was approximately 10% in these cell lines, suggesting that these cells show no great advantage in clonal selection.[85] A role for nestin in the nucleus is not yet clear, although previous cross-linking experiments revealed that nestin binds to DNA in N-myc-amplified N-type neuroblastoma cell lines.[79]

Recently, nestin was found on the cell surface of 14 glioma stem cell lines, and the positivity for nestin at the cell surface ranged from 1.4% to 70%. Isolated nestin-positive cells showed increased sphere-forming capacity and sphere size. The authors proposed that surface nestin underwent post-translational modification by γ-secretase and that, after plasma membrane exposure, nestin may be used as a marker for the isolation and characterization of CSCs in glioblastoma.[86] Surface nestin was originally identified in murine glioma stem cells (GSCs) and it was reported as a ~60-kDa N-terminal isotype of nestin. Surface nestin was detectable with GSC-targeting peptide with selective binding affinity to undifferentiated GSCs. Glioma stem cell-targeting peptide conjugated with fluorochrome is able to undergo internalization and its colocalization with nestin-positive GSCs was observed both in a glioma cell population maintained *in vitro* as well as in a murine glioblastoma model *in vivo*.[87] Surface nestin thus seems to be a suitable target molecule for identification of CSCs in some types of brain tumors, especially in gliomas. In addition to the detection methods described above, the application of specific mAb against the N-terminal part of the nestin molecule or of GSC-targeting peptide could also be used for eradication of these cells because peptide- and antibody-based radiopharmaceuticals or cytotoxic conjugates are discussed as very promising strategies in cancer treatment.[88–90]

In conclusion, nestin is undoubtedly a putative CSC marker of both neurogenic tumors and tumors of epithelial or mesenchymal origin. The coexpression of nestin with other CSC markers, particularly CD133, Oct3/4, and Sox-2, should be considered as a specific CSC phenotype; however, the verification of this phenotype using functional assays is required for each tumor type. Some experimental results suggest that nestin expression is closely associated with self-renewal capacity, although the detailed mechanisms underlying this relationship remain unclear.

Disclosure Statement

The authors have no conflict of interests.

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