

Implications of nestin in breast cancer pathogenesis (Review)

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Abstract. The aim of the present review was to summarize the current knowledge of the involvement of nestin in breast cancer (BC) pathogenesis. Nestin is a member of the class VI family of intermediate filament proteins, originally identified as a marker of neural stem cells and subsequently demonstrated to be expressed in BC and other cancer types. In normal breast tissue, nestin is expressed in the basal/myoepithelial cells of the mammary gland. In BC, nestin identifies basal-like tumours and predicts aggressive behaviour and poor prognosis. Nestin expression has also been detected in BC stem cells and newly-formed tumour vessels, being a factor in promoting invasion and metastasis. The present review provides an up-to-date overview of the involvement of nestin in processes facilitating BC pathogenesis and progression.

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1. Introduction

According to Globocan, breast cancer (BC) is the most common cancer in women, with an estimated 1.67 million new cancer

cases being diagnosed in 2012 (1). Globocan estimates that BC is the 5th most common cause of mortality among the overall cancer mortalities (522,000 mortalities) and is considered the principal cause of cancer-associated mortality in women in less developed regions (324,000 mortalities). The recent decline in mortality rates of BC in developed regions has been associated with more effective screening programmes, improved chemotherapeutic options and targeted therapy (2-4).

For a number of years, pathologists have recognized the biological heterogeneity among BCs. BCs are diversified into a few biologically distinct subtypes with specific pathological features and different clinical behaviours (5-7). Initially, breast tumours were classified according to histological type, grade and the expression of steroid receptors (8,9). However, pioneering gene expression profile studies demonstrated that the morphological heterogeneity of BC is also reflected at the gene expression level (10-13). These studies allowed for the classification of BCs into five intrinsic molecular subtypes: Luminal A and B; normal breast-like; with overexpression of erb-b2 receptor tyrosine kinase 2/human epidermal growth factor receptor 2 (HER2); and basal-like carcinomas (10-13). However, gene expression profiling-based techniques are not very common in daily clinical practice due to their relatively high cost. Routine histopathological subclassification of BC is primarily accomplished by immunohistochemical (IHC) detection of oestrogen receptor (ER), progesterone receptor (PR), HER2 and marker of proliferation Ki-67 (Ki-67) (14). Different expression patterns of these biomarkers provide a simple clinical classification system for BCs and allow for the determination of therapeutic approaches. According to The St. Gallen surrogate classification, BCs may be classified by IHC into four subtypes: Luminal A (ER⁺, PR⁺, HER2⁻ and low Ki-67 index); luminal B (ER⁺, PR⁺ and HER2⁺ or high Ki-67 index); HER2-positive (ER⁻, PR⁻, HER2⁺); and triple negative breast cancer (TNBC; ER⁻, PR⁻, HER2⁻) (15). TNBC is one of the most challenging subtypes among BC due to the lack of targeted therapies and its highly aggressive clinical behaviour (16-18). TNBCs may also be clustered into at least two distinct molecular classes according to their different behaviours: The basal phenotype and non-basal-like TNBCs (5). This distinction within TNBCs is important from a clinical perspective due to the different responses to chemotherapeutic treatment of each subtype (19). The basal-like subtype originates in the myoepithelial/basal cells that line the outer layer of mammary ducts, and is characterized by a high proliferation rate, aggressive pathological features and an extremely

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poor clinical outcome (20-22). Although the identification of basal-like cancer is optimally performed by gene expression profiling, attempts have been made to establish a reliable IHC panel for its identification. According to Nielsen *et al* (23), basal-like BCs are characterized as ER, PR and HER2-negative tumours with overexpression of basal cytokeratins 5/6 or epidermal growth factor receptor (EGFR). This IHC panel, termed 'Core Basal', has been validated and widely applied for basal-like subtype identification (23,24). However, to depict the heterogeneity of BCs, the evidence from a number of studies demonstrates that certain basal-like by gene expression tumours weakly express steroid receptors, which results in the misclassification of the majority of them as luminal tumours by IHC (25-30). According to the current standards, the luminal subtype is reported when <1% of the tumour cells express ER by IHC. Tumours weakly expressing ER comprise a relatively rare subgroup characterized by positive nuclear staining of 1-10% cells (29,31), with an incidence ranging between 1 and 6.7% (32,33). A number of studies demonstrated that approximately one-half of weakly ER-positive cases exhibit different gene expression patterns compared with luminal tumours, overlapping with the basal-like gene profile and predicting poor prognosis (29,31). Currently, patients in this subgroup are scheduled to receive endocrine therapy, which in that case may not only be ineffective, but may also expose them to unnecessary side effects, thus limiting their opportunities to benefit from chemotherapy-based treatments. To date, a single panel of IHC markers for the specific identification of basal-like tumours remains to be validated. However, considering recent findings, nestin, a marker of stem cells, appears to be a sensitive IHC marker for basal-like subtype identification, regardless of ER status (34,35).

Nestin (a neural stem cell protein) is a member of the class VI family of intermediate filament (IF) proteins, originally identified as a marker of neural progenitors and subsequently observed to be expressed in a wide range of other cell types (36-38). Nestin was initially identified as a protein expressed in the developing central nervous system (CNS) in neuroepithelial stem cells that give rise to neurons and glia (39,40). The expression of nestin in adults is uncommon; it is restricted to very specific cell types and may be induced only during particular processes i.e., development and regeneration (41-44). In tissues, nestin-positive cells are limited to defined locations where they may function as quiescent reserve cells, which are capable of proliferation, differentiation and migration when reactivated (37). During cell differentiation, nestin expression is subsequently downregulated and replaced by tissue-specific IF proteins (45). Except for the expression in the CNS, nestin has additionally been identified in stem and progenitor cells of the muscles (46-48), teeth (49), testes (50), pancreas (51), intestines (52), bone marrow (53), hair follicles (54), endothelium (55,56) and other tissues. Nestin expression in tumour cells was initially reported in neuroectodermal tumours of the CNS (57,58). Further studies on nestin expression demonstrated that it is also present in epithelial cancer, including breast (59), prostate (60), pancreatic (61), lung (62), ovarian (63) and other cancer types. Additionally, in a number of tumours the expression of nestin is considered a factor indicating poor prognosis (64-67). Furthermore, nestin expression in tumours is not limited to cancer cells, and is also

present in newly-forming tumour vessels and cancer stem cells (CSCs) (56,68).

The present review discusses the role of nestin as a significant agent involved in the mechanisms underlying BC progression. Being involved in proliferation, angiogenesis and self-renewal, nestin may be hypothesized to be an important factor in BC pathogenesis.

2. Nestin protein structure

The human nestin protein consists of 1,621 amino acids with a predicted molecular weight of 177.4 kDa. Nestin was classified as new type (type VI) of IF as it did not fall clearly into any of the previously described types (69). As in all IFs, nestin has a highly conserved α -helical core domain of 300-330 amino acids flanked by N- and C-terminal domains (36). However, nestin has two intrinsic features that determine its two unique properties. A short N-terminal head domain prevents nestin self-assembly and, as a result, nestin co-polymerizes forming heterodimers with other IFs, most favourably with vimentin (70,71). Additionally, nestin has an unconventionally long C-tail domain that protrudes from the filament structure, being accessible to post-translational modifications and protein interactions (36).

3. Role of nestin in cell cycle regulation and survival

Although the exact mechanisms of action of nestin in BC development remain unknown, there have been certain studies elucidating its role in the cell cycle, proliferation and survival. Accumulating data suggests that IF protein phosphorylation markedly alters the structure/dynamics of IFs in cells (72,73). Nestin has multiple phosphorylation sites and has been demonstrated to be phosphorylated by kinases involved in cell cycle regulation, for example cyclin-dependent kinase 1 [additionally termed cell division control protein 2 homolog (cdc2)] and cyclin-dependent kinase 5 (Cdk5) (74,75). In rat cells, cdc2 kinase is involved in the phosphorylation of nestin in the highly conserved rod domain, regulating its intracellular organization during mitosis (4). Recently, it was reported that nestin phosphorylation directly affects the proliferation of cancer cells (76). Nestin also modulates Cdk5 activity, a kinase that has recently been demonstrated to be involved in tumorigenesis, with functions ranging from cell proliferation to invasion and angiogenesis (74,77-79). In BC, the overexpression of the Cdk5/p35 complex is associated with markers of poor prognosis, TNBC, Her2⁺ expression and high grade of malignancy (79). In normal cells, nestin serves as a potent survival determinant acting via cytoplasmic sequestration of the Cdk5/p35 complex, which protects cells from oxidative stress-induced cell death (80). In the nucleus, Cdk5 forms a complex with p25, which is generated as a product of the proteolytic cleavage of p35. Nestin, acting as a protein scaffold, stabilizes p35 and prevents the translocation of Cdk5/p25 to the nucleus, which is a required step for the induction of cell death (80). Nestin was also reported to drive protein hedgehog (Hh)-dependent tumorigenesis by binding to transcriptional activator Gli3, a zinc finger transcription factor that negatively regulates Hh signalling (81). The Hh pathway has been recognized as one of the key signalling pathways involved in

the pathogenesis of TNBC (82). Lines of evidence suggest that nestin is directly involved in the mechanisms underlying tumour growth and progression. However, further studies are required to clarify its oncogenic role in BC.

4. Nestin expression in the normal breast and in BC

The first evidence of nestin expression in human BC tissues was demonstrated by Li *et al* (59). In that study, nestin expression was detected in the regenerative compartment of the normal human mammary gland in addition to BC tumour cells. The study demonstrated that in mammary ducts and lobules, nestin was expressed in the subluminal compartment in two morphologically distinct cell types: In columnar basal cells expressing tumour protein p63 and normal cytokeratin-14; and in filamentous myoepithelial cells expressing desmin (59). Additionally, the study demonstrated that during murine pregnancy, nestin coordinately colocalized with p63 in columnar basal cells and was involved in the regenerative cycle of the mammary gland. On the other hand, nestin expression in myoepithelial cells appeared to be stable throughout the course of pregnancy. Nestin-expressing cells have also been isolated from human breast milk, which suggests that it may be a novel source of putative stem cells (83-85). A further study indicated that these nestin-expressing cells were able to differentiate into neural stem cells and neurons (86). The role of nestin in the process of self-renewal in BC has been confirmed by additional studies (87-89).

5. Nestin as a marker of basal-like BC

The study by Li *et al* (59) demonstrated that nestin expression is increased among BRCA1-associated tumours and suggested that nestin may be a selective marker of basal-like BCs. However, the study had important limitations. Specifically, the classification of basal-like tumours was not based on the results from gene expression profiling, and rather on the IHC expression patterns of ER, PR and HER2. Furthermore, the number of analysed samples was relatively low (16 cases classified as TNBCs, 16 cases as luminal and 16 cases as HER2⁺), thus the results require verification in further large population-based studies. However, the high number of nestin-expressing cases (14/16) among the TNBCs and the lack of expression reported in luminal (0/16) and HER2-positive (0/16) tumours suggested that nestin may be an important marker of BC differentiation. Further studies confirmed the clinical implications of nestin expression in TNBC and basal-like tumours (34,55,90-97). Parry *et al* (90), in an IHC study of 245 human invasive BC cases, demonstrated that nestin expression in tumour cells was predominantly observed in basal-like tumours, as defined by the IHC panel proposed by Nielsen *et al* (23). Notably, the researchers reported that 2.6% of luminal cancer cases also expressed high levels of nestin and a few other basal markers. The study also demonstrated that nestin expression was positively correlated with grade III tumours, a high Ki-67 proliferation index and nuclear expression of p53 (90). Although nestin expression was associated with poor survival rates among patients with nodal metastasis, nestin was not an independent prognostic factor in multivariate analysis (90). Similar results were

obtained by Liu *et al* (91), who demonstrated an association of nestin with the TN phenotype, lymph node metastasis and p53 expression. In that study, it was also reported that nestin expression was a poor prognostic factor among patients with lymph node metastasis; however, in general, nestin was not an independent prognostic factor for BC. Finally, Liu *et al* (91) demonstrated that an increased expression level of nestin was noted in invasive tumours when compared with *in situ* tumours. Another study conducted on locally advanced T4 tumours demonstrated that nestin was associated with the TN phenotype, poor prognosis and inflammatory breast cancer (IBC) (92). IBC is a rare and very aggressive subtype of BC characterized by rapid progression, poor prognosis and enhanced lymphovascular invasion (98,99). A total of ~80% of highly aggressive inflammatory tumours were reported to express nestin (92). Nestin-expressing cells were also detected in the lymphovascular emboli of IBC, which may suggest that these clusters of cells expressing nestin and other stemness markers may be CSCs (100). Notably, the study by Piras *et al* (92) reported that nestin expression was present in cancer cells and also in peritumoural stromal cells with a fibroblast-like morphology, although the analysis did not reveal any association with TNBC or IBC. Rogelsperger *et al* (101) reported that nestin co-localized with the melatonin receptor (MT1) in tumour cells among BC patients with higher disease stages (II and III) and with a higher risk of relapse.

Although gene expression profiling is the gold standard facilitating accurate classification of basal-like tumours, its application is expensive and impractical in everyday practice. Thus, surrogate IHC panels are the first-choice strategy in basal-like subtype identification, even with their lower sensitivity and specificity. In order to identify the best individual IHC marker, Won *et al* (93) tested 46 biomarkers associated with the basal-like subtype, against a gene expression profile gold standard. On the basis of the highest odds ratio, nestin was the best positive biomarker identifying the basal-like intrinsic subtype among all of the investigated biomarkers. Furthermore, a combined IHC panel composed of nestin positivity and/or inositol polyphosphate-4-phosphatase negativity (nestin⁺/INPP4B⁻) had a better sensitivity and specificity compared with any other proposed IHC panel for basal-like subtype identification (93). In their further study, application of the nestin⁺/INPP4B⁻ panel enabled the classification of the majority of weakly ER-positive cases as basal-like, in accordance with the results obtained by the gold standard assay (35). To validate these findings, the researchers evaluated the prognostic value of nestin as a single positive biomarker of basal-like cancer in a large cohort of 3,641 early-stage invasive BC cases (34). Currently, all established IHC panels for basal-like cancer identification require ER negativity (23,102,103), therefore any new IHC marker that may be interpreted independently of ER may be a valuable diagnostic tool. Therefore, nestin, as an abundantly and stably expressed cytoskeletal protein, may be helpful for classifying clinically problematic cases with weak expression of ER. In a cohort comprising 2,323 ER⁺ cases as defined by IHC, nestin⁺ expression was noted in 5% of ER⁺ tumours (120 patients) and was an independent poor prognostic factor for patients within this subgroup (34). The same trend for nestin was observed in ER⁺ patients receiving endocrine therapy only; however, due

to the small number of cases, the results were not statistically significant. To support the findings that nestin expression may reliably identify basal-like tumours within the ER⁺ subgroup, Asleh *et al* (34) analysed 672 nestin⁺ cases against a gene expression profiling assay. As in their previous study (35), nestin expression was strongly associated with the intrinsic basal-like subtype, established by the gold standard assay. These results contributed to the improved understanding of the issue of nestin⁺ ER⁺ cases reported by Parry *et al* (90), which according to the standards were classified as luminal by IHC. In the study by Asleh *et al* (34), nestin expression was observed in 371 (10%) out of all the investigated cases and was associated with high-risk clinicopathological factors (younger age, higher grade and high proliferation index). In contrast to Parry *et al* (90) and Liu *et al* (91), the analysis of long-term survival data demonstrated that nestin positivity was an independent poor prognostic factor in BC. However, the previous studies examining the prognostic value of nestin have certain limitations which may clarify such an inconsistency in the results. For instance, in the studies by Liu *et al* (91) and Parry *et al* (90), the number of nestin⁺ cases included in the analyses was relatively low, comprising 24 and 20 cases respectively. Additionally, the study by Asleh *et al* (34) analysed early-stage BC cases of which 42% of the patients had not received any systemic treatment, whereas all the patients studied by Parry *et al* (90) received anthracycline-based adjuvant chemotherapy, which may have influenced the survival rates. Recent data from a second large cohort by Kruger *et al* (96) also support the finding that nestin is associated with aggressive tumour characteristics (size, grade, proliferation, p53 expression and blood vessel invasion) and the basal-like BC phenotype. Notably, in contrast to Liu *et al* (91), Kruger *et al* noted nestin expression more frequently in patients with negative lymph node status. In fact, it had been proposed in previous studies that basal-like BCs preferentially spread haematogenously, giving rise to metastatic deposits in the brain and lungs (104). Similar to Asleh *et al* (34), the researchers confirmed the predictive value of nestin as an independent prognostic factor associated with poor prognosis (96). Additionally, the study by Kruger *et al* (96) demonstrated that nestin protein, in addition to nestin mRNA expression, was associated with DNA repair associated (BRCA1) germline mutations, particularly among young women. Nestin appeared to be a stronger predictor of BRCA1 germline mutation compared with any other investigated core marker [including cytokeratin 5 (CK-5), EGFR, P-cadherin and intrinsic basal-like subtype]. Moreover, nestin mRNA expression levels and signature scores varied among TNBC subgroups, as described by Lehmann *et al* (105), being highest in the basal-like 1, mesenchymal and mesenchymal stem-like subgroups (96). Recently, the potential of treatment of BRCA-associated cancer with poly-(ADP ribose) polymerase inhibitors (PARPi) has been recognised, with a number of molecules being under clinical trials (106). As a strong predictor of BRCA1 mutation, nestin may be a valuable tool for identifying patients eligible for PARPi targeted therapy, although the validity of its predictive value requires further study.

An additional study of 2,930 cases investigated whether the protein expression pattern in primary tumours may influence the first site of distant metastases (107). It was revealed

that the expression of nestin, prominin-1 (CD133) or CK-5 in the primary tumour were significantly associated with brain metastases as the first site of distant recurrence. Since nestin and CD133 are proteins expressed by neural stem cells, that previous study appeared to be in accordance with Paget's 'seed and soil' theory, postulating that metastasis depends on the interaction between cancer cells and the specific organ microenvironment (108). Hypothetically, BC cells expressing neural stem cells markers may also share the same features and may thus be particularly well adapted to the brain microenvironment to initiate brain metastases. However, the biological mechanisms associated with these proteins and BC brain metastases remain unknown. According to prognostic prediction, the 1-year survival rate of patients with BC brain metastases is <20% (109). Meisen *et al* (110) proposed nestin-targeted therapy for breast cancer metastases in two murine models. Targeting BC brain metastases with an oncolytic virus programmed to kill nestin-expressing cells, and to deliver the extracellular domain of brain angiogenesis inhibitor 1, significantly enhanced the survival of mice with established metastatic BC brain tumours. Meisen *et al* (110) also investigated the cytotoxic effect of the same nestin-targeting virus on four human BC cell lines corresponding to the different biological types of BC. According to Neve *et al* (111), BC cell lines may be clustered into three major subsets (luminal, basal A and basal B), which reflect the molecular subtypes of primary tumours. Using the publicly accessible 'Neve breast cancer dataset', Meisen *et al* (110) compared nestin gene expression in 50 BC cell lines included in the analysis. Although nestin mRNA was detected in all investigated cell lines, increased expression levels were observed in the basal A cluster, which matched the Perou basal-like signature of primary tumours (10,11). The *in vitro* cell viability assay on the cell lines representing the luminal (MCF7 and SKBR3), Basal A (MDA-MB-468) and Basal B (MDA-MB-231) clusters indicated that the same oncolytic virus exerted its cytotoxic effect on all the investigated cell lines (110). Predictably, the most significant cytotoxicity was observed in the MDA-MB-436 cells expressing the highest levels of nestin.

6. Nestin as a marker of BC stem cells

CSCs are defined as a small subpopulation of undifferentiated tumour cells that are characterized by their capacity for self-renewal and ability to differentiate into multiple tumour cell types (68,112,113). The ability of CSCs to reproduce a continuously growing tumour, described as 'tumorigenicity', is responsible for therapeutic resistance and metastasis (68,114). The first study demonstrating the involvement of nestin in the biology of CSCs analysed nestin expression in CSCs isolated from different types of human CNS tumours (115,116). Further studies have confirmed the utility of nestin as a potent marker of tumour cells exhibiting a stem-like phenotype. In BCs, cells with a stem-like phenotype may be identified based on the expression patterns of cell surface markers, and are described as CD44 antigen (CD44)⁺ and signal transducer CD24 (CD24)^{-/low} (117). Further studies demonstrated that a high number of CD44⁺/CD24^{-/low} cells may be particularly observed in TN tumours and cell lines, being a predictor of poor prognosis and aggressive behaviour (118,119). Liu *et al* (120)

demonstrated that these CD44⁺/CD24^{-low} cells, which highly expressed nestin and another relevant stem cell marker [POU domain, class 5, transcription factor 1 (Oct-4)], had a greater ability to form mammospheres *in vitro* (119). Furthermore, it was demonstrated that nestin and Oct-4 co-expression was significantly associated with younger age, higher histological grade, lymph node metastasis and TN phenotype. Furthermore, multivariate analysis of survival indicated that the co-expression of these two markers was associated with shorter survival independent of age, lymph node metastasis and TN phenotype. In IBC, nestin expression was detected in cells forming tumour emboli within lymphatic vessels (100). These nestin⁺ cells formed spheroids expressing other CSC markers (including CD44⁺/CD24^{-low}, aldehyde dehydrogenase cytosolic 1 and CD133) and stemness-associated transcription factors [Oct-4, homeobox protein NANOG (Nanog) and transcription factor (SOX-2)]. Furthermore, it was identified that nestin⁺ spheroids were resistant to radiotherapy and chemotherapy and strongly contributed to distant metastasis (121). The study by Apostolou *et al* (89) confirmed the co-expression of nestin and other stemness markers (including Oct3/4, Nanog and SOX-2) in circulating tumour cells (CTCs) isolated from clinical patients. Notably, their results indicated a marked association between the expression of these markers in CTCs and the stage of the disease. The mechanism underlying the involvement of nestin in the biology of CSCs was described by Zhao *et al* (88). In that study, the researchers investigated the role of nestin in primary CSCs isolated from BC tumours. First, they studied the behaviour of naturally occurring nestin^{high} and nestin^{low} populations of CSCs. Subsequently, the authors examined the behaviour of primary CSCs with genetically modified nestin expression by overexpressing or silencing the *NES* gene. Cells with nestin^{high} and nestin-overexpressing cells had potent tumorigenicity, displayed by rapid mammosphere formation *in vitro* and the induction of solid tumours *in vivo*. Silencing of nestin expression induced cell cycle arrest at the G2/M phase, promoted apoptosis and reduced the expression of epithelial-mesenchymal transition (EMT) markers (including N-cadherin, vimentin and α -smooth muscle actin). EMT is a dynamic process that appears to facilitate tumour metastasis by switching the cell phenotype from epithelial to mesenchymal (122,123). Furthermore, nestin silencing significantly upregulated the expression of proteins involved in the inhibition of the Wnt/ β -catenin pathway, which is crucial for the regulation of the proliferation and tumorigenicity of stem cells (124-127). In addition, the inhibition of the Wnt/ β -catenin pathway in nestin^{high} CSCs significantly limited their tumorigenicity.

To date, a number of natural compounds have been investigated to target breast CSCs. The inhibitory effect of Huaier aqueous extract and nitidine chloride (a natural polyphenolic compound isolated from the root of *Zanthoxylum nitidum*) on CSCs has been tested in BC cell lines (128,129). The two compounds significantly reduced the number of CD44⁺/CD24^{-low} cells and decreased the expression of nestin and other pluripotency markers (including Nanog and Oct-4). It was suggested that the inhibition of nestin, Nanog and Oct-4 occurred via the inhibition of the Hh pathway (128,129). The Hh signalling pathway is associated with normal mammary gland development and its activation, either by mutation or

aberrant expression of pathway components, leads to BC development (130). A recent study reported that in TNBC, nestin expression may be regulated by the SOX-10 transcription factor, which directly binds to the nestin promoter and enhances its expression (131). SOX-10 is specifically expressed in mammary cells exhibiting the highest levels of stem/progenitor activity, and in breast tumours with stem-like behaviour (132). *SOX10* knockdown significantly inhibits nestin expression and decreases the stem-like features of TNBC, in terms of the CD44⁺/CD24^{-low} cell ratio and tumour sphere-forming abilities (131). Importantly, the effect of *SOX10* knockdown on the properties of CSCs is partly counteracted by enforcing nestin expression.

7. Role of nestin in BC angiogenesis

Angiogenesis is the process of new blood vessel formation, which involves numerous mechanisms mediating the growth and modification of a capillary network (133,134). The process was first described in breast tumours by Folkman (135) and became the basis for further research, demonstrating that sustained angiogenesis is an integral part of the progression of the majority of cancer types (135-137). Studies on nestin expression in human tumours have demonstrated that nestin is expressed in tumour cells and also in tumour vessels (55,138). These observations led to further research examining the role of nestin in the process of angiogenesis in breast and other cancer types.

The first immunohistochemical study on the nestin-expressing microvasculature in BC clinical samples was conducted by Kruger *et al* (139). The study aimed to evaluate angiogenesis via the assessment of immature (nestin⁺) and proliferating (Ki-67⁺) endothelial cells in breast tumour tissue. The study demonstrated that nestin and Ki-67 co-expression in tumour vessels, in addition to nestin⁺ microvessel density (nestin⁺MVD), were significantly associated with the basal-like phenotype and a negative ER and PR status. The study also assessed the prognostic value of the vascular proliferation index (VPI), which was calculated as the ratio between the number of nestin⁺Ki-67⁺ microvessels and the total number of nestin⁺ microvessels expressed as a percentage. It was demonstrated that the VPI was increased in invasive ductal carcinoma (IDC) compared with invasive lobular carcinoma (ILC). Furthermore, an increased VPI was significantly associated with a shorter overall survival in univariate and multivariate analyses. However, the prognostic value of nestin⁺MVD was not confirmed in the study (139). The prognostic value of nestin⁺MVD was demonstrated in our recent study (56). It was observed that the number of nestin-expressing microvessels was noticeably increased in invasive tumours compared with pre-invasive lesions. Additionally, a high value of nestin⁺MVD was associated with disease progression, stage, lymph node metastasis, high tumour grade and the TN phenotype. Our study demonstrated that a high value of nestin⁺MVD was an independent factor for poor prognosis in BC. Notably, it was also reported that the number of nestin-expressing vessels was significantly correlated with immature CD34⁺, although not with mature platelet endothelial cell adhesion molecule (CD31)⁺ vessels (56). To confirm these results, nestin expression was analysed in endothelial cell lines isolated from

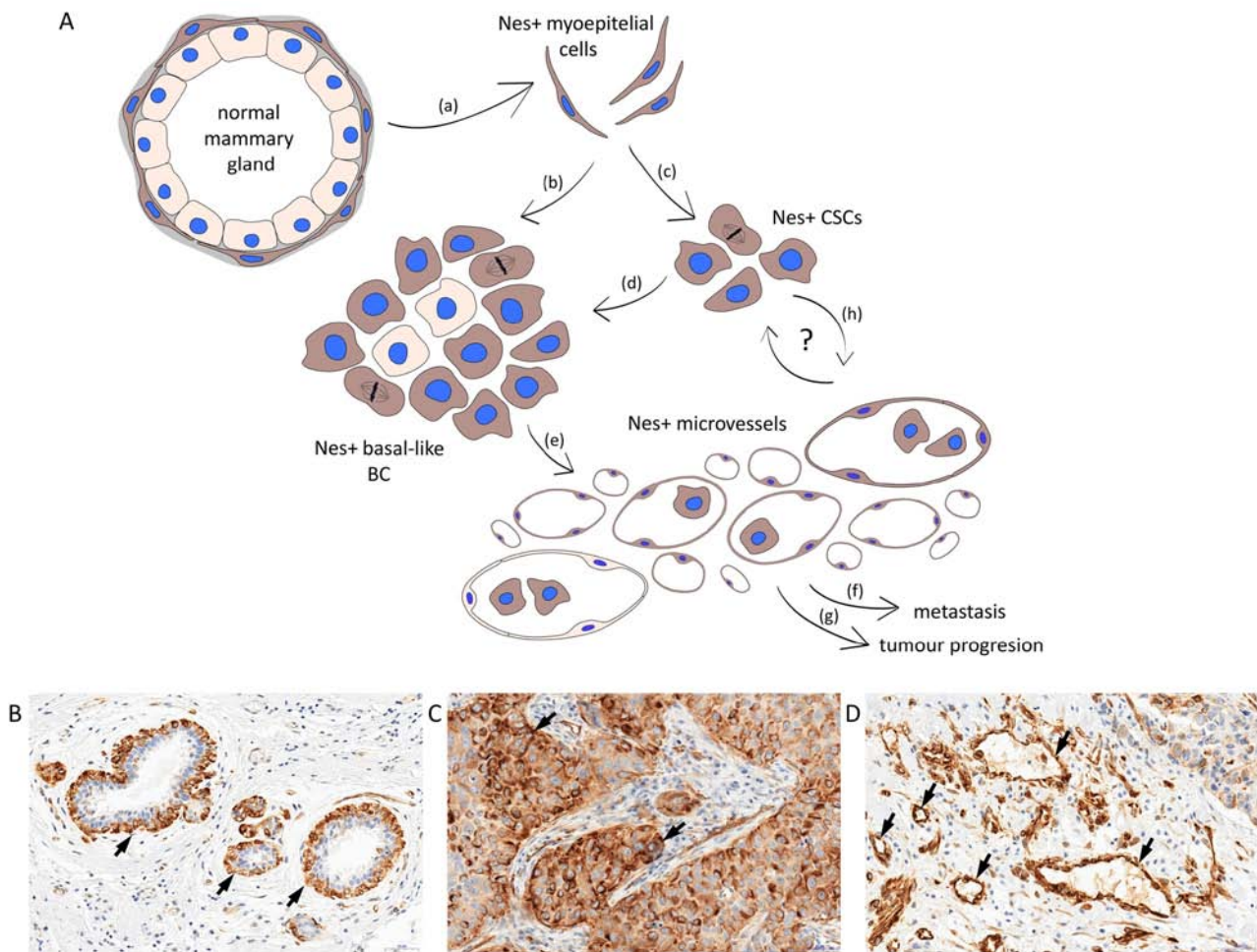


Figure 1. Role of nestin in the pathogenesis of basal-like BC. (A) Nestin expression (brown) is detected in the basal/myoepithelial layer of the normal mammary gland. (a) Nestin⁺ myoepithelial cells give rise to (b) nestin⁺ basal-like tumours and (c) nestin⁺ CSCs that are able to (d) repopulate the tumour. Nestin expression is also observed in newly-formed tumour vessels. The number of nestin-expressing vessels correlates with (e) nestin expression in tumour cells, (f) metastasis and (g) tumour progression. (h) Some evidence indicates that cancer stem cells are also able to differentiate into endothelial cells and/or form vessel-like structures through a process termed vasculogenic mimicry. Immunohistochemically detected nestin expression in the basal/myoepithelial layer of (B) the normal mammary gland, (C) the basal-like BC and (D) the BC associated-vessels. Magnification, x300. Nes, nestin; BC, breast cancer; CSCs, cancer stem cells.

various types of human vessels. Different patterns of nestin expression were noted in the human endothelial cells according to their maturity. It was observed that particularly high nestin expression occurred in an early endothelial progenitor cell line, originating from human umbilical cord blood (140), compared with endothelial cells from dermal microvessels and the umbilical vein (56). Taken together, these results suggested that nestin expression may reflect the progenitor nature of vessels, and that it is primarily limited to undifferentiated and newly forming vessels.

Based on the knowledge that nestin is also detected in pericytes (141), Nakagawa *et al* (142) evaluated the differences in vascular density (CD31⁺MVD), vascular proliferation (vasohibin-1⁺/CD31⁺ ratio) and microvessel maturation (nestin⁺/vasohibin-1⁺ ratio) between IDC and ILC. The maturation of blood vessels may be marked by the presence of pericytes surrounding the microvessels. It has been demonstrated that tumour microvessels are characterized by abnormal pericyte coverage in addition to disturbed interactions between pericytes and endothelial cells, the two of which may contribute to metastatic dissemination (143). Notably, Nakagawa *et al* (142)

reported nestin expression only in pericytes; however, they did not mention the presence of nestin in the endothelium. The study demonstrated that the CD31⁺MVD and the vasohibin-1⁺/CD31⁺ ratio were significantly higher in ILC compared with IDC. However, nestin immunoreactivity in intratumoural microvessels in ILC was significantly lower. It was suggested that the proliferation of microvessels was enhanced in ILC, although these vessels were less mature compared with those in IDC (142).

In another study, it was demonstrated that nestin expression in BC cells was positively correlated with the area and number of vessels expressing different endothelial antigens, including nestin, CD31, CD34 and transcription factor SOX-18 (97). Another recent study also confirmed that nestin expression in tumour cells was associated with higher VPI and blood vessel, although not lymphatic vessel, invasion (96). Furthermore, a positive correlation was observed between the area and number of nestin-expressing vessels and vessels expressing the transcription factor SOX-18, which is an important regulator of vascular development contributing to the differentiation of mesenchymal stem cells into endothelial cells (144-146).

Notably, it was demonstrated that nestin expression in tumour cells correlated with the area and the number of nestin⁺ vessels, which may support hypotheses on vascular mimicry and/or the trans-differentiation of CSCs into endothelial cells (147,148). In BC, numerous studies have reported the presence of dysfunctional and disorganized vessels with a defective endothelium, in addition to the presence of vascular-like channels formed of tumour cells (149-151). Bussolati *et al* (87) demonstrated that nestin-expressing breast tumour CSCs were able to differentiate into epithelial cells and also into endothelial cells, *in vitro* and *in vivo*. Culturing nestin⁺ CSCs in the presence of serum promoted their differentiation towards the epithelial lineage. On the other hand, culturing nestin⁺ CSCs in the presence of VEGF resulted in their differentiation into endothelial cells. Differentiated endothelial cells were able to express endothelial markers and organize into capillary-like structures in Matrigel. Furthermore, implantation of nestin⁺ CSCs in severe combined immunodeficient (SCID) mice led to tumour development in which some of the intratumoural vessels were of human origin, suggesting the *in vivo* endothelial differentiation of CSCs. Notably, the study also demonstrated that endothelial-differentiated cells derived from nestin⁺ CSCs were able to form an *in vivo* human vessel network and eventually an epithelial tumour when implanted in Matrigel in SCID mice. This may suggest that a non-differentiated population of tumorigenic nestin⁺ CSCs is maintained among endothelial-differentiated breast CSCs (87).

8. Nestin in BC-targeted therapy

Undoubtedly, nestin is a valuable predictive marker which has the potential to be implemented in clinical practice, in order to achieve improved and more accurate diagnosis of BC. Since the use of nestin-targeted treatment in the mouse model of BC-related brain metastasis has resulted in a notable inhibition of disease progression, further research on possible applications in clinical patients is worthy of attention (110). A number of studies on different types of neoplasms have confirmed that inhibition of nestin results in the decreased proliferation, migration and invasion of tumour cells (61,88,152-154). Notably, nestin expression is primarily restricted to a few types of normal cells, while it is abundantly expressed by tumour cells, newly-formed vessels and CSCs. Therefore, the therapy targeting nestin may be a potent and multidirectional therapeutic strategy with limited side effects. Improved understanding of the mechanisms through which nestin facilitates BC may aid the development of a powerful novel therapeutic agent.

9. Conclusion

The emerging evidence suggests that nestin is undeniably an important factor involved in BC progression (Fig. 1A). In the breast, nestin expression is detected in the basal/myoepithelial layer of normal mammary glands (Fig. 1B) in basal-like BC (Fig. 1C) and BC-associated vessels (Fig. 1D). As a specific marker of basal-like tumours and a marker of poor prognosis, it has the potential to become a biomarker used in daily clinical practice. Numerous studies have confirmed the role of nestin in important processes in tumour progression,

including self-renewal and angiogenesis; however, the molecular mechanisms underlying these phenomena require further clarification. In conclusion, in BC, nestin may serve as a promising prognostic factor and a potential therapeutic target for tumour suppression and angiogenesis inhibition.

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Authors' contributions

AN made substantial contributions to the conception of the article and created the major draft of the manuscript; PD contributed to the conception of the article and revised the manuscript critically for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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