

The Roles and Molecular Mechanisms of Nestin Expression in Cancer with a Focus on Pancreatic Cancer

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Abstract

Nestin is a class VI intermediate filament protein that is expressed in a variety of stem and progenitor cells, including primitive neuroepithelial cells and pancreatic exocrine progenitor cells. Nestin expression is closely associated with rapidly proliferating progenitor cells during development and repair processes, and higher nestin expression levels correlate with poor prognosis in some cancers. Nestin regulates many molecules during cell processes in both normal and neoplastic tissues, and its transcription is regulated by enhancer regions in the second intron of the nestin gene that bind to SOX, Class III POU, and N-Myc. Nestin forms heterodimers with other intermediate filaments, with the class III intermediate filament protein vimentin being its main partner. Nestin/vimentin copolymers provide an anchor for glucocorticoid receptors, regulate insulin-degrading enzyme, and promote the phosphorylation-dependent disassembly of vimentin during mitosis. Nestin regulates cell proliferation, the cell cycle, cell survival, and apoptosis by regulating cyclin-dependent kinase-5 (Cdk5), phosphoinositide 3-kinase, and AKT. We reported previously that nestin regulates cell motility, invasiveness, and cell morphology in a pancreatic cancer cell line via regulation of F-actin and E-cadherin. This review focuses on the roles of nestin in cancer stem cells and in tumor-initiating cells in the pancreas. In the pancreas, nestin plays important roles in the proliferation, differentiation, and survival of both progenitor cells and cancer cells.

Keywords: Pancreatic cancer; Nestin; Intermediate filament; Migration

Introduction

Microfilaments, intermediate filaments, and microtubules comprise the three major groups of cytoskeletal proteins in non-muscle cells. Intermediate filaments, which are 10-nm in diameter, form an interconnected scaffolding-like cytoplasmic network. There are six major classes of intermediate filaments, each with restricted expression that depends on cell type, developmental stage, and functional status [1]. Intermediate filaments help organize the morphology of the cytoskeleton, and they have also been implicated in cell signaling, organogenesis, and cell metabolism [2].

Nestin is a class VI intermediate filament protein and neuroepithelial stem cell marker [3,4]. Although nestin is expressed in primitive neuroepithelial cells in early stages of human embryogenesis, it is not generally expressed in differentiated Central Nervous System (CNS) cells [5]. Nestin is downregulated as neuroepithelial stem cells cease dividing and differentiate along their respective neural or glial lineages [6]. Homozygous deletion of exon 1 of the mouse nestin gene causes incomplete embryonic lethality, with around 90% penetrance and increased apoptosis of neural stem cells. Accordingly, nestin is considered essential for neural stem cell self-renewal [7]. Notably, in the 10% of nestin-knockout mice that survive until birth, the only evident anatomical abnormality is that the mice have smaller organs. These results indicate that nestin is important not only in progenitor cells, but also during embryonic differentiation and organ formation. Furthermore, nestin is expressed in adult neural stem cells during tissue repair processes [8]. Specifically, nestin is re-expressed in reactive astrocytes during the repair process in damaged rat brains [9,10]. In addition to expression in neuroepithelial cells, nestin is expressed in pancreatic stem/progenitor cells [11] and in heart, skin, testis [12], blood vessel [13], and muscle cells. Generally speaking, nestin is expressed in rapidly proliferating progenitor cells and regenerating tissues [14]. Furthermore, nestin is expressed in malignant tumors [15], including pancreatic cancer [16,17], prostate cancer [18], breast cancer

[19], glioblastoma [20,21,22], gastrointestinal stromal tumor [23], trichoblastoma [24], squamous cell carcinoma of the skin [25], lung cancer [26,27,28], angiosarcoma [23], dermatofibrosarcoma [29,30], and malignant melanoma [31,32]. In addition, higher expression levels of nestin correlate with poorer prognosis in breast cancer [33,34] and lung cancer [26,27], in highly advanced-stage melanoma [31] and glioma [20,21], and in highly invasive pancreatic cancer [16].

Nestin is incapable of self-assembly due to its very short N-terminal head domain and instead co-polymerizes to form heterodimers with other intermediate filament proteins, including desmin [35], vimentin, and α -internexin; notably, keratin does not form heterodimers with nestin [36]. Nestin can form homodimers and homotetramers *in vitro*, but these filaments cannot form by themselves [36]. Copolymers of nestin and other intermediate filaments serve as scaffolds for cytoplasmic proteins [37] and modulate the phosphorylation and activation via direct binding [38]. In the past, nestin was thought to localize only to the cytoplasm, but recent studies revealed that nestin localizes to the nucleus as well [39]. Furthermore, nestin interacts with nuclear DNA in neuroblastoma cells, thereby modulating the aggressiveness of the tumor [40].

Taken together, these data show that nestin regulates not only protein function, but gene function as well via direct binding to DNA. Nestin may thus be involved in many cellular processes in normal

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and neoplastic tissues. In this review, we summarize the molecular mechanisms that regulate nestin expression and the function of nestin in malignant tumors, with a focus on pancreatic cancer.

Regulation of the Expression and Structure of Nestin

Nestin expression is regulated by enhancer regions in the first [41] and second introns [42,43] of the nestin gene (Table 1, Figure 1). A 714-bp evolutionarily conserved 3' portion of the second intron is reported to be sufficient to control nestin expression in rat and human CNS progenitor cells [45]. Enhancer of human nestin gene in the second intron is divided into two separate domains: one more 3' region required for pan-CNS progenitor cell expression and a second that controls midbrain expression [44]. A 120-bp region in the second intron of the human nestin gene contains putative binding sites for nuclear hormone receptors including Retinoic Acid Receptor (RAR), Retinoid X Receptor (RXR), and Thyroid Hormone Receptors (TR). Binding of SOX, Class III POU [46,47], and N-Myc [40] to the second intron of the nestin gene is also reported to transcriptionally regulate nestin expression levels. In human umbilical vein endothelial cells (HUVECs), nestin expression is regulated by an element in the first intron [41].

As noted above, nestin co-polymerizes with other intermediate filament proteins in cells. The class III intermediate filament protein vimentin is the main partner of nestin, and these intermediate filaments form fibers [48] and non-fibrous particles [38]. In astrocytes with reduced vimentin levels, nestin fails to polymerize, thereby suggesting that nestin polymerization requires vimentin [48]. Nestin/vimentin copolymers provide an anchor for the glucocorticoid receptor, which is a key regulator of growth and differentiation in embryonic development [37]. Disassembled vimentin/nestin complexes bind to insulin-degrading enzyme and regulate the activity of the enzyme [38]. Furthermore, direct binding of nestin to vimentin regulates vimentin phosphorylation [49].

Roles of Nestin in Cell Proliferation and Cell Death

Nestin is a multi-functional protein that regulates many different types of molecules (Table 2, Figure 2). Previous reports have shown that nestin is essential for cell proliferation, cell cycle regulation, and

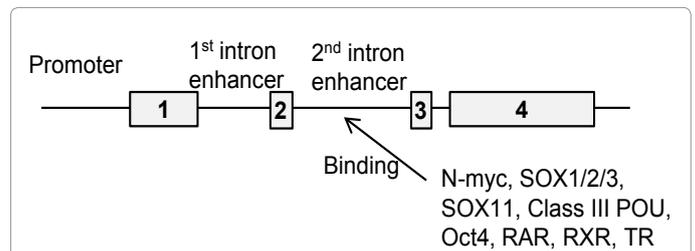


Figure 1: Nestin structure and transcriptional regulation of the nestin gene. The nestin gene, localized to the long arm of chromosome 1, consists of 4 exons and 3 introns: Exon 1, bases 1-916; Intron 1, bases 917-2058; exon 2, bases 2058-2182; Intron 2, bases 2183-3870; Exon 3, bases 3871-3944; intron 3, bases 3945-4192; exon 4, bases 4193-8635. Octamer-binding transcription factor 4 (Oct4); retinoic acid receptor (RAR); retinoid X receptor (RXR); and Thyroid Hormone Receptor (TR).

Interacting protein	Mechanisms	Function	Reference
DNA	Binding	Unknown	[40]
Vimentin	Binding and phosphorylation	Reorganization, disassembly, and phosphorylation of vimentin during mitosis	[49]
Glucocorticoid receptor	Binding and supply Cytoplasmic anchoring	Cell proliferation and cell cycle control	[37]
Cdk5	Binding to the alpha-helical domain of nestin	Oxidant-induced apoptosis	[54]
p35	Ubiquitination and turnover	Oxidant-induced apoptosis	[54]
Cdk5/p35	Binding	Activity and turnover	[56]
PI3K	Expression level regulation	Proliferation and differentiation	[52]
AKT	Phosphorylation	Proliferation	[32]
E-cadherin	Expression level	Cell adhesion	[17]
Insulin-degrading enzyme	Binding	Enzyme activity	[38]

Table 2: Molecules that are functionally regulated by nestin.

Interacting protein	Mechanism	Function	Reference
N-myc	Binds to intron 2	Regulates transcription of nestin	[40]
SOX1/2/3	Binds to intron 2	Regulates transcription of nestin	[46]
SOX11	Binds to intron 2	Regulates transcription of nestin	[46]
Class III POU	Binds to intron 2	Regulates transcription of nestin	[46]
Oct4 (Class V POU)	Binds to intron 2	Regulates transcription of nestin	[47]
Vimentin	Binds nestin	Copolymerization	[48]
α-internexin	Binds nestin	Copolymerization	[36]
Cdk5	Binds and phosphorylates Thr316 and Thr1495 of rat nestin	Regulates the interaction of nestin and p35	[54]
Cdk5	Not determined	Regulates the expression of the nestin protein	[55]
cdc2 kinase	Phosphorylates Thr316 of rat nestin	Reorganization during mitosis	[56]

Table 1: Molecules that regulate the expression and function of nestin.

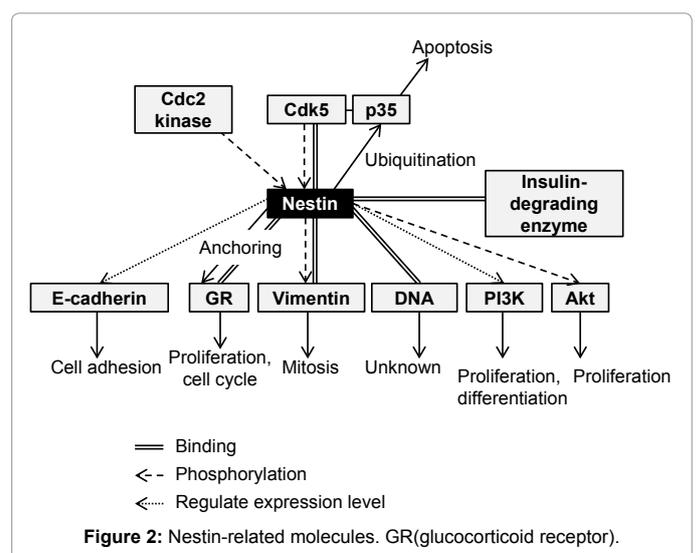


Figure 2: Nestin-related molecules. GR(glucocorticoid receptor).

cell survival. Changes in the organization states of microtubules and microfilaments are highly conserved both during the assembly of the mitotic spindle and during the formation of the contractile ring in cytokinesis, but the structural changes in intermediate filament networks appear to be cell-type and intermediate filament-type specific

[50]. Nestin promotes the phosphorylation-dependent disassembly of vimentin during cell mitosis and regulates the cell cycle [49].

Nestin expression is closely associated with rapidly proliferating progenitor cells during development and repair processes [51]. In neuroblastoma cell lines, inhibition of nestin causes a decrease in the cell growth rate and in anchor-independent cell growth [40]. Nestin expression is also a key determinant in suppressing the anti-proliferative activity of the glucocorticoid receptor, and knockdown of nestin induces cell cycle arrest (G1 arrest) in human glioblastoma cell lines [37]. In neural stem cells derived from nestin-knockout mice, the number of apoptotic cells was significantly increased [7]. Nestin downregulation markedly suppresses activation of the Phosphoinositide 3-Kinase (PI3K) pathway, but not the Mitogen-Activated Protein Kinase (MAPK) pathway in neural progenitor cells [52]. Nestin promotes the activation of PI3K, and is therefore essential for the proliferation of neural progenitor cells.

Lu et al. showed that glioblastoma cells that had high nestin expression levels formed larger tumors than glioblastoma cells with low nestin expression levels [53]. In our study, short hairpin RNA (shRNA) that targeted nestin suppressed glioblastoma [22] and melanoma cell growth *in vitro* [32]. In addition, we found that phosphorylation of AKT was inhibited by the downregulation of nestin in melanoma [32].

Nestin serves as a scaffold for cyclin-dependent kinase 5 (Cdk5), in which binding is restricted to a specific region adjacent to the alpha-helical domain of nestin. Furthermore, nestin regulates turnover of Cdk5/p35, resulting in oxidant-induced apoptosis in neural progenitor cells [54]. Cdk5-knockout mice show failed neuronal differentiation and cell cycle arrest along with increased nestin expression in the cortex [55]. During mitosis, nestin reorganization is regulated by cdc2 kinase [56]. Cdk5, which is regulated by nestin, is overexpressed in pancreatic cancer cells and is activated by mutant K-ras [57]. These lines of evidence suggest a relationship between nestin and cell proliferation, but the nature of this relationship remains controversial. In particular, suppression of nestin expression in a glioblastoma cell line, A172, elicits an irreversible G1/S cell cycle arrest [37]. We found that nestin shRNA suppressed cell growth in the A172 glioblastoma cell line [22] and the A375 melanoma cell line [32], but not in PANC-1 and PK-45H, which are pancreatic cancer cell lines [60]. Inhibition of nestin using Small Interfering RNA (siRNA) suppresses cell growth in PANC-1 and PK-45H cells via induction of apoptosis, but did not result in G1/S arrest. These seemingly conflicting results regarding the role of nestin in cell proliferation may be due to differences between cell lines or between the use of shRNA and siRNA. Further studies are needed to clarify the roles of nestin in cell proliferation.

Nestin in Pancreatic Cancer

Nestin and pancreatic cancer cell motility

Nestin has been proposed to play a role in tumor invasion in melanoma [32], prostate cancer [18], glioma [22], and pancreatic cancer [16,17]. Nestin expression correlates with nerve invasion and the invasion of peripancreatic tissue margins in pancreatic cancer, but does not correlate with prognosis in pancreatic cancer [16]. Similarly, in brain tumors, nestin seems to correlate with tumor grade, but does not support its prognostic value in glioma [20,21]. Nestin immunostaining delineates glioma invasion into adjacent gray and white matter [58]. In neuroblastoma cell lines, nestin regulates cell proliferation and cell motility, but not cell invasiveness [40]. Inhibition of nestin expression in pancreatic cancer reduces migration and invasion *in vitro* and also

reduces liver metastases in immunodeficient mice [17]. These reports indicate that nestin's function in cell motility is restricted to certain types of cells, including pancreatic cancer cells; however, the underlying mechanisms have not yet been clarified.

We reported previously that the expression pattern of F-actin is altered by nestin inhibition in pancreatic cancer [17] and melanoma cells [32]. In melanoma cells, nestin and F-actin are partially co-expressed. F-actin plays important roles in cell motility [59]; therefore, direct binding of nestin and F-actin may have implications for cell motility. In pancreatic cancer cells, decreased nestin expression increases the expression and alters the localization of E-cadherin to the periphery of the cells; in addition, it induces an epithelial phenotype in the cells [60]. E-cadherin suppresses cell motility; therefore, the roles of nestin in pancreatic cancer may be related to alterations in the expression levels and intracellular localization of E-cadherin.

Many lines of evidence indicate that vimentin is a key regulator of cell motility, and vimentin is considered a mesenchymal marker during the Epithelial Mesenchymal Transition (EMT) [60]. Vimentin regulates cell motility via Extracellular Signal-regulated Kinase (ERK) [61], AKT1 [62], Axl [63], and Scrib [64]. In migrating astrocytes, vimentin reorganizes to form a polarized network that is coextensive with microtubules in cell protrusions, and Adenomatous Polyposis Coli (APC) is a crucial regulator of intermediate filament organization including vimentin [65]. Nestin forms heterodimers, mainly with vimentin, and nestin regulates the phosphorylation of vimentin [49]; therefore, the roles of nestin in cell motility may be related to its interactions with vimentin. There are no therapies that target vimentin, and such a therapy might be expected to have side effects because vimentin is such a widely expressed protein. In contrast, expression of nestin is more specific than that of vimentin and is restricted to certain types of cells. Hypothetically, nestin-targeting cancer therapy for pancreatic cancer might be expected to have fewer side effects than therapy that targets vimentin. Nestin also regulates the phosphorylation of Cdk5, and Cdk5 is reported to regulate cell motility and invasiveness in pancreatic cancer cells [57]. Thus, the interaction of nestin and Cdk5 may be involved in cell motility in pancreatic cancer.

Nestin in pancreatic cancer morphology

Cell morphology is controlled to a large extent by the coordinated organization of the extracellular matrix, plasma membrane, and the cytoskeleton. In pancreatic cancer tissues, nestin expression correlates with invasiveness but not with histological grading [16]. Notably, pancreatic cancer cells with decreased expression levels of nestin appear sheet-like, with tight cell adhesion due to increased expression of E-cadherin in the cell membrane at cell-cell junctions [17]. These cells also have increased F-actin expression at the periphery of the cells, along with obvious stress fiber formation. In glioblastoma, nestin shRNA-transfected cells show increased cell attachment to extracellular matrices [22]. In 3-dimensional cell culture plates with square-shaped grids on the base, glioblastoma colonies normally have an asteroid-like appearance, but colonies of the nestin shRNA-transfected glioblastoma cells appear nearly round. In addition, the nestin shRNA-transfected glioblastoma cells show markedly higher F-actin expression levels, particularly at the periphery of the cell colonies near the sites of cell attachment.

Nestin in pancreatic cancer stemness

Recently, a relationship between the EMT and cell stemness was reported [66]. Certain types of cancer stem cells, termed migrating

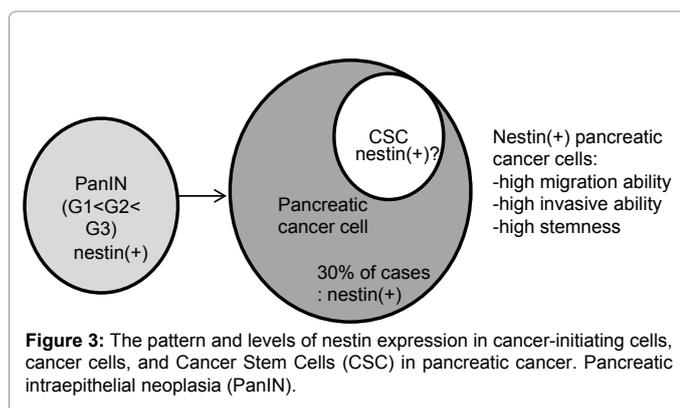
cancer stem cells, possess high migration ability [67]. It is possible that the roles of nestin in cell motility may be related to cell stemness. The definitions of cancer stem cells and cancer stem-like cells are still controversial, but the tumor formation rate in mice using a serial dilution method is considered a standard way to identify cancer stem cells. Previous studies have shown a relationship between nestin and cancer stem cells in glioblastoma [53] and melanoma [32]. In addition, CD24-positive ovarian cancer stem cells show high expression levels of nestin [68]. In pancreatic cancer, sphere cells cultured in stem cell culture medium that contain abundant cancer stem cells show high expression of CD133/CD44/octamer-binding transcription factor 4 (Oct4)/nestin [69]. Inhibition of nestin in pancreatic cancer cells does not alter cell differentiation [17]. We reported previously that shRNA that targets nestin suppresses sphere formation by melanoma cells [32], while overexpression of nestin induces increased sphere formation by cervical cancer cells [70]. We observed the same results using pancreatic cancer cell lines, but there have been no reports describing correlations between nestin expression levels and the tumor formation ratio using the dilution method in immunodeficient animals. Further studies are needed to determine the roles of nestin in cancer stem cells in pancreatic cancer.

Nestin in pancreatic cancer carcinogenesis

In our previous study, immunohistochemical analysis revealed that nestin and other stem cell markers, including CD24, CD44, C-X-C Chemokine Receptor Type 4 (CXCR4), and Epithelial Specific Antigen (ESA), are expressed in pancreatic intraepithelial neoplasias (PanINs) and that increased expression levels correspond to increased PanIN grade [71]. In addition, targeting of oncogenic K-ras to a population of pancreatic exocrine progenitors, which are characterized by nestin expression, is sufficient to induce the formation of PanINs, which are putative precursors to pancreatic cancer [72,73]. Furthermore, acute pancreatitis accelerates the initiation and progression of pancreatic cancer in mice expressing oncogenic Kras in the nestin cell lineage [73]. These findings suggest that nestin-positive pancreatic cells are tumor-initiating cells in pancreatic cancer (Figure 3). Accordingly, we suggest that nestin may have important roles in carcinogenesis in pancreatic cancer.

Conclusion

In summary, nestin is essential for progenitor cell and cancer cell proliferation, differentiation, and survival. In pancreatic cancer, nestin expression is closely correlated with cell morphology, proliferation, invasion, and metastasis; therefore, nestin immunostaining of pancreatic cancer tissues may reflect the biological behavior of pancreatic cancer.



Nestin may play important roles in carcinogenesis, and in cancer stem cells in particular, by activating or reorganizing other proteins involved in pancreatic cancer. Further studies that include more pancreatic cancer cases are needed to clarify the molecular mechanisms involved in nestin expression and function and to explore the potential of anti-nestin therapies for treating pancreatic cancer.

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