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## *Review*

# Nestin structure and predicted function in cellular cytoskeletal organisation

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**Summary.** Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development in the CNS, PNS and in myogenic and other tissues. Upon differentiation, nestin becomes downregulated and is replaced by tissue-specific intermediate filament proteins. Interestingly, nestin expression is reinduced in the adult during pathological situations, such as the formation of the glial scar after CNS injury and during regeneration of injured muscle tissue. Although it is utilised as a marker of proliferating and migrating cells very little is known about its functions or regulation. In depth studies on the distribution and expression of nestin in mitotically active cells indicate a complex role in regulation of the assembly and disassembly of intermediate filaments which together with other structural proteins, participate in remodeling of the cell. The role of nestin in dynamic cells, particularly structural organisation of the cell, appears strictly regulated by phosphorylation, especially its integration into heterogeneous intermediate filaments together with vimentin or α-internexin.

**Key words:** Nestin, Intermediate filaments, Cytoskeleton

### Introduction

Nestin is an intermediate filament protein expressed predominantly in rapidly dividing progenitor cells of developing and regenerating tissues. Cell division requires that cytoplasmic and nuclear compartments be disassembled, reorganized and partitioned into daughter cells. These processes of extensive remodeling are orchestrated by components of the cytoskeleton, a composite of microtubules (20 nm in diameter), intermediate filaments (8-12 nm in diameter) and actin microfilaments (6 nm in diameter) (Geisler et al., 1989; Klymkowsky, 1996; Ku et al., 1996; Fuchs and Cleveland, 1998; Goldman et al., 1999).

Intermediate filaments, of which nestin is a member, comprise more than forty individual proteins that can be divided into six main classes (I-VI) based on their molecular structure (Lendahl et al., 1990; Steinert and Liem, 1990). Class I and class II are basic and acidic keratins of epithelial cells; class III proteins include desmin, GFAP, peripherin and vimentin; class IV consists of neurofilaments and α–internexin and class V are nuclear lamins. Nestin comprises a novel class VI intermediate filament protein (Lendahl et al., 1990). Intermediate filament proteins are differentially expressed in tissues and depending on the cell type may comprise from 1 to 85% of total protein, where they are arranged as homogenous or heterogeneous polymers (Zehner, 1991; Goldman, 2001).

Changes within the spatial and temporal expression of intermediate filament proteins regulate remodeling of the cell cytoskeleton during development. This is particularly striking in the CNS where intermediate filaments exhibit sequential expression; preimplantation embryos express cytokeratins (Classes I and II); following neurulation, multipotent CNS cells express nestin (class VI) and vimentin (class III). Finally terminal differentiation involves down-regulation of nestin and induction of neurofilaments (class IV) in neurons or GFAP (class III) in astrocytes (Steinert and Liem, 1990).

Identified in 1985 (Hockfield and McKay, 1985), nestin is expressed in the majority of mitotically active CNS and PNS progenitors that give rise to both neurons and glia (Cattaneo and McKay, 1990; Lendahl et al., 1990; Lendahl, 1997; Mujtaba et al., 1998). Nestin is also found in myogenic precursors of skeletal muscle and heart (Lendahl et al., 1990; Sejersen and Lendhal, 1993; Kachinsky et al., 1994, 1995), as well as in the developing tooth bud (Terling et al., 1995), testis (Fröjdman et al., 1997) and hair follicle sheath progenitor cells of the skin (Li et al., 2003).

Nestin is downregulated in all cells upon differentiation (Zimmerman et al., 1994; Lothian and Lendahl, 1997), but reappears transiently after injury to muscle or the CNS where it has been found in reactive

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astroglia of the brain and in ependymal cells of the rat spinal cord after injury (Lendahl, 1997; Krum and Rosenstein, 1999; Namiki and Tator, 1999; Pekny et al., 1999; Vaittinen et al., 2001). Moreover, adult tissues such as CNS and skin contain small populations of nestin positive stem/progenitor cells (Johansson et al., 2002; Li et al., 2003). In fact, nestin is now wildly used as a marker for stem cells that characteristically display features such as multipotency, self renewal and regeneration, yet little is known about nestin function. In this review the basic biological properties of nestin are described and possible functional roles in cell remodeling during mitosis are explored.

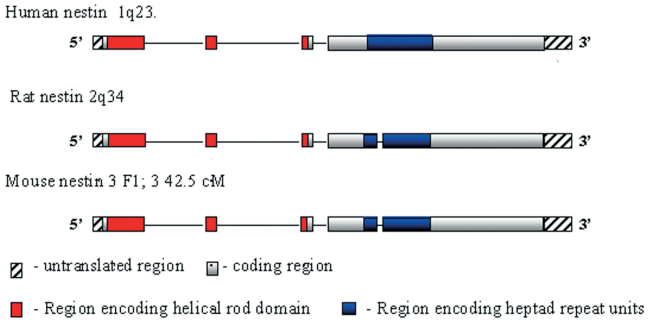


**Fig. 1.** A diagram depicting the exon/intron structure of the nestin gene; the intron pattern is conserved between the human, rat and mouse nestin genes. The first three introns are in identical positions however the fourth intron has not been reported for the human gene. The regions encoding the α-helices and the 11 amino acid repeats have been shaded.

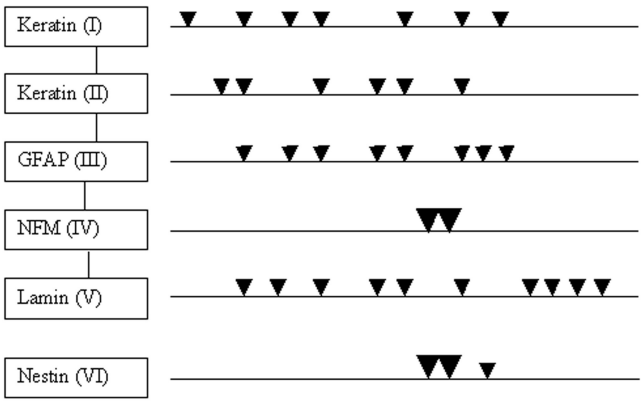


**Fig. 2.** A schematic representation of intron positions in the genes encoding the six different classes of intermediate filament proteins. Filled triangles indicate intronic positions. Identical intronic positions in NF and nestin are indicated by larger triangles. Keratins (Ichinose et al., 1988; Krauss and Franke, 1990); GFAP (Balcarek and Cowan, 1985); NFM (Myers et al., 1987); Lamin (Döring and Stick, 1990); nestin (Lendahl et al., 1990). Note: the positions of the first two introns of nestin are perfectly conserved relative to those of neurofilament. The introns, situated in the fourth coil of the a-helical region, are spliced at exactly the same nucleotide position (From Dahlstrand et al., 1992).

## The *nestin* gene and its evolution

The grouping and nomenclature of nestin as a distinct intermediate filament protein is based on the exon/intron structure of the gene; the positions and lengths of its introns are highly conserved (Fig. 1). Moreover two of the introns are in identical positions to those of *neurofilament* genes (Fig. 2). Taken together with their similarity in sequence (20% in regions encoding the conserved protein rod domains) (Fig. 3), it is reasonable to argue that *nestin* and the three *neurofilament* genes arose from a common ancestor by gene duplication (Dahlstrand et al., 1992) (Fig. 2).

## The promoter region of the *nestin* gene controls spatial and temporal expression

Expression of the *nestin* gene is driven by a minimal promoter, present between residues -11 and +183 in the 5'-non-coding region. The promoter contains two adjacent Sp1-binding sites necessary for promoter activity, but lacks a functional TATA box (Cheng et al., 2004). Enhancer elements that specifically regulate expression in myogenic and neural precursors are in the first and second intron respectively (Zimmerman et al., 1994).

Within the second intron of nestin reside two separate enhancer elements; a 204-bp midbrain specific enhancer between bases 1068 and 1271, and a 206-bp pan-CNS enhancer between bases 1272 and 1477 (Lothian and Lendahl, 1997; Lothian et al., 1999). These two enhancers function independently of each other and contain at least two distinct regulatory sites. In the midbrain enhancer, there is a midbrain-specifying element, between bases 1068 and 1199 and a general transcriptional potentiator element, between bases 1200 and 1255. The midbrain element reproducibly restricts expression to the ventral midbrain yet in its absence, gene expression is nonspecific (Lothian et al., 1999; Kappen and Yaworsky, 2003). Similarly, the CNS enhancer contains two critical sites between bases 1272 and 1400 and between bases 1401 and 1477. These sites interact to enhance activity throughout the developing nervous system (Yaworsky and Kappen, 1999; Lothian et al., 1999). Such multiple regulatory elements are presumably required to ensure *nestin* expression in CNS

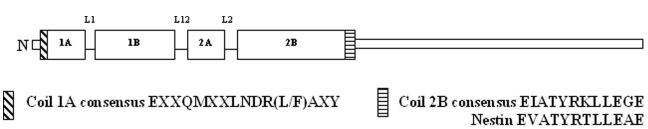


**Fig. 3.** A diagram depicting intermediate filament structure, showing conserved sequences and rod subdomains. Coils 1A and 1B are separated by linker L1, coils 1B and 2A are separated by L12, and coils 2A and 2B are separated by L2. Nestin has a short N terminal region and no linker L2 region. N and C designate amino and carboxyl termini, respectively.

progenitor cells at specific locations along the anterior-posterior and dorsal-ventral axes of the embryo.

The CNS and midbrain specific enhancer elements are thought to be regulated by nuclear hormone receptors (TRs, RXR, RAR, COUP-TF) and POU-domain transcription factors. These transcription factors regulate early embryonic patterning, cell migration and proliferation (Lothian and Lendahl, 1997; Jaworsky and Kappen, 1999; Lothian et al., 1999).

Remarkably, in the adult CNS, nestin expression is regulated by upstream specific enhancer elements which differ from elements that direct expression in embryos and cultured adult CNS cells (Johansson et al., 2002). Detailed characterization of nestin enhancers may be important for future studies aimed at repairing the adult CNS.

## The nestin protein

Nestin is a large protein (>1600 amino acids), structurally similar to other intermediate filaments, with a highly conserved α-helical core domain of 300-330 amino-acids flanked by amino- and carboxy-terminal domains. The common core, referred to as the rod domain, is composed of several α-helical coils, coils 1A and 1B separated by linker L1, coils 1B and 2A separated by L12, and coils 2A and 2B (Fig. 3). The coils are essential for the creation of coiled-coil dimers that associate in an antiparallel fashion to form tetramers and protofilaments, which then combine laterally to form filaments (Fuchs and Weber, 1994; Marvin et al., 1998).

The C-terminal region of nestin contains 1306 amino-acids and a conserved heptad repeat unit. By contrast, the amino terminus of nestin is much shorter than that of other intermediate filament proteins (Dahlstrand et al., 1992). Variation in the amino and carboxy terminal ends of intermediate filament proteins allows for complex binding to an array of structural proteins.

The deduced human nestin protein is shorter than the rat and mouse proteins by 187 and 203 amino acids respectively (Dahlstrand et al., 1992; Yang et al., 2001). The difference in length is due to variation in the number of C-terminal heptad repeat units; human nestin has 18 repeats of the heptad S/PLEK/EEN/DQES/PLR, whereas there are 41 repeats of an almost identical motif (SLEK/EENQEXLR) in rat and mouse. While the carboxy-terminal region is only 55% conserved, the rod α-helical region is highly conserved (82%) between man, rat and mouse (Dahlstrand et al., 1992; Yang et al., 2001). Thus the carboxy-terminal region and the α-helical domain of human nestin have evolved at quite different rates which highlights the importance of the helical rod domain in copolymer formation. Similarly, other filament proteins, including neurofilament, have conserved rod domains while the lengths of their C-terminal repeat units vary (Julien et al., 1988; Lees et al., 1988) (Table 1, Fig. 3).

Mutational analysis has identified conserved sequences within the rod domain specifically within coils 1A and 2B that are essential for normal intermediate filament assembly (Hatzfeld and Weber, 1992; Kouklis et al., 1992; Fuchs and Weber, 1994). Proteins containing mutations in rod-end sequences were found to disrupt assembly of normal intermediate filament subunits *in vivo* and *in vitro* and to result in accumulation of nestin aggregates in the cytoplasm of CNS precursors and radial glia *in vivo* (Letai et al., 1992; Marvin et al., 1998).

**Table 1.** Rates of conservation of intermediate filament protein domains.

| INTERMEDIATE FILAMENT | α-HELICAL REGION | C-TERMINUS |
|---|---|---|
| GFAP | 93 | 96 |
| NFH | 97 | 74 |
| NFM | 99 | 84 |
| NFL | 98 | 89 |
| Lam C | 99 | 92 |
| Nestin | 82 | 55 |

The percentage of amino acid conservation between man and mouse (for nestin, man and rat) for the α-helical domain and the carboxy terminus of several intermediate filament proteins. Values denote amino acid conservation for GFAP (Balcarek and Cowan, 1985; Reeves et al., 1989), NFH (Julien et al., 1988; Lees et al., 1988), NFM (Levy et al., 1987; Myers et al., 1987), NFL (Lewis and Cowan, 1986; Julien et al., 1987), lamin C (Fisher et al.,1986; Riedel and Werner, 1989; Dahlstrand et al., 1992), nestin (Lendahl et al., 1990; Dahlstrand et al., 1992).

*Note: For most intermediate filament proteins the α-helical region is more highly conserved than the C-terminal region, indicating different rates of conservation (Julien et al., 1988; Lees et al., 1988).

**Note: Nestin is the least conserved particularly at the carboxy-terminal end. The amino acid sequence in the N terminal α-helical region is 82% conserved whereas the carboxy-terminal region is only 55% conserved between man and rat.

## Nestin assembles into polymers with other intermediate filaments

Nestin preferentially forms intermediate filaments by assembly with a variety of intermediate filament proteins, particularly type III vimentin and type IV α-internexin (Marvin et al., 1998; Eliasson et al., 1999; Steinert et al., 1999). The formation of filaments composed of heterodimers and heterotetramers rather than homodimers is presumably because nestin contains a very short N-terminus, a domain known to be essential for filament protein assembly (Fuchs and Weber, 1994; Herrmann and Aebi, 2000). This possibility is supported by *in vitro* studies of nestin-vimentin coassembly, which demonstrate that nestin inhibits filament formation in a concentration dependant manner when present at concentrations greater than 50% (Steinert et al., 1999). Moreover, composites of nestin-vimentin and nestin-α-internexin heterodimers are more stable than nestin homodimers but less stable than vimentin and α-internexin homopolymers when subjected to increasing concentrations of urea *in vitro* (Steinert et al., 1999).

To explain the multiple roles of nestin in regulation of cellular structure, a model has been proposed in which nestin-vimentin heteropolymers would attach to a core of vimentin homopolymers (Fig. 4). The long carboxy-terminal of nestin would protrude from this filament body and could function as a linker or cross-bridge between intermediate filaments, microfilaments and microtubules (Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988). Thus nestin may play a role in connecting the three components of the cytoskeleton and coordinate changes in cell dynamics (Herrmann and Aebi, 2000) (Fig. 5).

The prescribed role for nestin in the organization of intermediate filaments during mitosis is supported by *in vitro* studies showing that, nestin transfected into cultured cells colocalises with vimentin consistently throughout the cell cycle (Chou et al., 2003; Sahlgren et al., 2003). During interphase, filamentous networks consisting of nestin and vimentin polymers were observed extending from the perinuclear region to the cell surface; during the transition from late prophase to metaphase, nestin and vimentin copolymer networks simultaneously reorganized into cage-like structures surrounding the nucleus; during telophase, nestin and vimentin networks were disassembled and vimentin and nestin were extensively colocalised in punctate and diffuse structures (Chou et al., 2003; Sahlgren et al., 2003).

## Phosphorylation of nestin is associated with disassembly of filaments

To bring about the changes in morphology required within rapidly dividing and migrating cells, structural proteins need to be assembled and disassembled in a strictly regulated spatial and temporal manner. Several lines of evidence indicate that nestin is involved in this process during mitosis; nestin forms stable polymers with several intermediate filament proteins. Moreover, paradoxically, its expression is also associated with requisite intermediate filament breakdown. The mechanisms that control this diversity in function are still not fully understood, but phosphorylation is thought to be an important factor (Eriksson et al., 1992; Skalli et al., 1992; Ku et al., 1996; Inagaki et al., 1997).

Low levels of phosphorylation are associated with filament assembly. By contrast, during mitosis, a three-fold increase in the phosphorylation of nestin and a six-fold increase in the phosphorylation of vimentin coincide with dramatic reorganisation of filament networks (Sahlgren et al., 2001). In particular, phosphorylation of nestin at Threonine$^{316}$ by cdc2 kinase causes partial disassembly of nestin-containing intermediate filaments (Sahlgren et al., 2001). Notably, Threonine$^{316}$ is located in the highly conserved carboxyl terminus near the end of the rod domain, a region important for filament assembly. Furthermore, mutations in this region are associated with intermediate filament-related diseases, including severe cases of epidermolysis bullosa simplex (Letai et al., 1992; Zimmerman et al., 1994; Sahlgren et al., 2001).

Phosphorylation of nestin may regulate not only its polymerisation with type III and type IV intermediate filament proteins, but may alter also, its links with other cytoskeletal components (Steinert et al., 1999; Herrmann and Aebi, 2000; Sahlgren et al., 2001). The long C terminal repeats contain serine residues that are predicted to serve as additional phosphorylation sites. Their phosphorylation would change the configuration of the side arms and affect the formation of cross-bridges between nestin intermediate filaments and other cytoskeletal components. In summary, phosphorylation

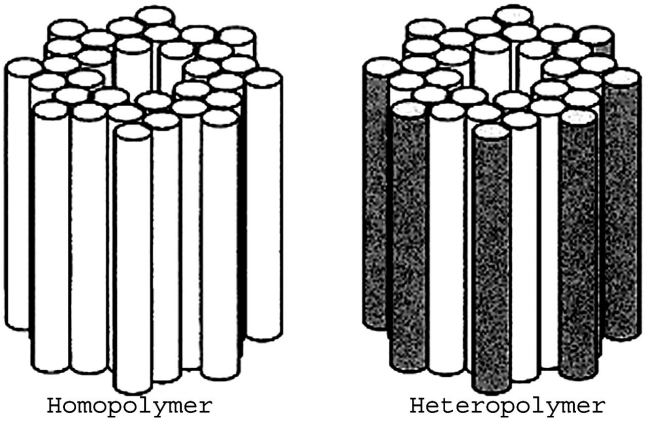


**Fig. 4.** Cross-sectional representation of individual proteins in an intermediate filament. **a.** In the homopolymeric situation, tetramers are integrated within a symmetrical filament with minimal hollow core (Steinert et al., 1999). **b.** Mixed polymers of vimentin-nestin or α-internexin-nestin may form such that the nestin (shaded subunits) reside on the filament periphery (adapted from Herrman and Aebit, 2000).

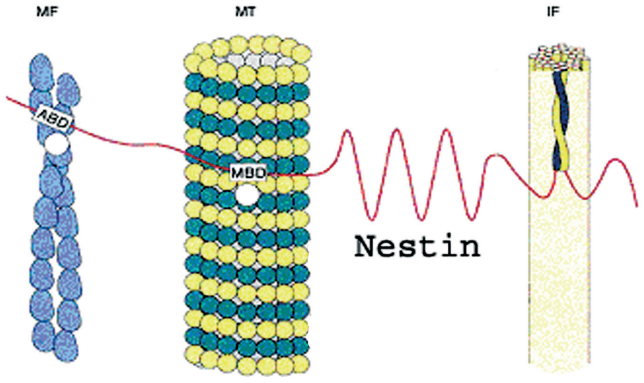


**Fig. 5.** Schematic representation of the interaction between microfilaments (MF), microtubules (MT) and intermediate filaments (IF). The intermediate filament protein consists of a vimentin (yellow)/nestin (blue) heterodimer. Nestin has a long non-α-helical carboxy-terminal end that may stably link it to MTs and MFs. (adapted from Herrman and Aebit, 2000).

and dephosphorylation of nestin may modulate respectively, disassembly and assembly of intermediate filaments within supramolecular structures and thus control dynamic changes in cell ultastructure (Hirokawa et al., 1984; Steinert et al., 1999).

Similarly, phosphorylation has been shown to regulate the spatial organization of intermediate filament proteins such as vimentin (Chou et al., 1989, 1990). In fact, during mitosis, the disassembly of vimentin intermediate filaments requires the presence and phosphorylation of both nestin and vimentin (Chou et al., 2003). To induce the disassembly of vimentin polymers during mitosis, nestin works in concert with the ubiquitous mitotic kinase, maturation/M-phase-promoting factor, MPF, which phosphorylates vimentin at Ser-55 in the amino-terminal head domain, the region required for dimerisation (Aubin et al., 1980; Jones et al., 1985; Chou et al., 1996, 2003; Sahlgren et al., 2003). The role of nestin in vimentin disassembly was recently confirmed by downregulation of nestin with specific siRNAs *in vitro*, which blocked the disassembly of vimentin intermediate filaments in mitotic cells (Chou et al., 2003).

The disassembly of vimentin intermediate filaments is not a feature of all cells – in fact, many cell types do not express nestin. In dividing cells in which nestin is absent and there is no apparent disassembly of vimentin filaments, there is alternately, a restricted local disassembly of intermediate filaments at the cleavage furrow in late cytokinesis (Yasui et al., 2001).

## Nestin association with cytoplasmic trafficking in rapidly dividing progenitor cells

The advantage of mitotic disassembly of vimentin filaments for cells expressing nestin remains unknown. Mitotic and spreading interphase cells, containing nonfilamentous keratin or vimentin, are able to move protein particles at high speeds along microtubules with molecular motors kinesin and dynein (Prahlad et al., 1998; Windoffer and Leube, 1999; Helfand et al., 2002). It is possible that this ensures the rapid transport of precursor molecules between various cytoplasmic compartments. Therefore, nestin expression may be associated with increased cytoplasmic trafficking in progenitor cells undergoing rapid rounds of division, interspersed with active interphase migration. Such characteristics are common features of cells in early developing nerve and muscle tissues (Lendahl et al., 1990; Sejersen and Lendahl, 1993; Kachinsky et al., 1995; Vaittinen et al., 1999) and in regenerating adult tissues (Frisen et al., 1995; Vaittinen et al., 1999).

Nestin may also play a role in the asymmetric allocation of cytoskeletal and other cellular factors to daughter cells. For example, within the developing neural tube, ventricular cells continue to proliferate whereas differentiated cells migrate towards the pial surface. Polarized distribution of material between dividing and differentiating daughter cells within the neuroepithelium may be caused by nestin-mediated disassembly and uneven partitioning of motile vimentin particles during mitosis (Frederiksen and McKay, 1988; Rakic, 1988; Chou et al., 2003).

## Conclusion

Intermediate filaments represent the least understood part of the cytoskeleton. Although many parameters are known, the reasons for the existing diversity of intermediate proteins as well as their individual functions remain unknown. Because nestin is expressed in the majority of mitotically active CNS and PNS progenitors, it is currently widely used as a marker for neural stem cells yet its apparent diversity of roles in heterogeneous cells is still not completely understood. Several researchers have performed complex experiments and detailed analyses to ascertain its functions. While these have aided in the understanding of the complexity of cell dynamics, the intricate role of nestin in cellular proliferation during development and regeneration remains to be conclusively defined.

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