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PROSPECTS

Perinucleolar Compartment and Transformation

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Abstract The perinucleolar compartment (PNC) is a sub-nuclear structure that preferentially localizes to the nucleolar periphery. The PNC is found predominantly in transformed cells both in vitro and in vivo. PNC prevalence (the percentage of cells containing at least one PNC) positively correlates with the progression of breast cancer and patient survival. PNCs are highly enriched with newly synthesized RNA polymerase III transcripts and RNA-binding proteins. The structural integrity of the PNC is dependent upon the transcription of these RNAs and a critical level of the polypyrimidine tract binding (PTB) protein, as assayed by the localization of other PNC-associated proteins. These observations suggest a model in which the PNC is a dynamic, functional organelle that forms under specific physiological conditions favoring cellular transformation and might be involved in the metabolism of RNA polymerase III transcripts. J. Cell. Biochem. 95: 217–225, 2005.

Key words: perinucleolar compartment (PNC); nuclear sub-structure; RNA metabolism; transformation

Cellular compartmentalization has long been understood as a means of organizing processes and regulating function. Considerable advances have been made in the understanding of the specific localization of factors within the non-membrane bound structures and sub-structures of the nucleus [Lamond and Spector, 2003; Taddei et al., 2004; Zink et al., 2004]. Nuclear speckles, Cajal bodies, promyelocytic leukaemia (PML) bodies, and PNCs are among several nuclear structures that are known for their unique nuclear localization patterns, the factors that are associated with them and their involvement in nuclear functions. The appearance and/or mislocalization of some nuclear sub-structures, such as PNCs and PML bodies have been shown to be relevant to malignancy [Kamath et al., 2004; Zink et al., 2004]. In this study, we will summarize the current understanding of PNC structure and function as well as its association with the malignant phenotype.

STRUCTURE OF THE PNC

The perinucleolar compartment (PNC) was first described through the localization of the polypyrimidine tract binding (hnRNP I/PTB) protein using immunolabeling during the characterization of the protein [Ghetti et al., 1992]. The PNC was subsequently characterized as an irregularly shaped structure (ranging from 0.25 to 1 μm in diameter) that is associated with, but structurally distinct from the nucleolus (Fig. 1) [Huang et al., 1997]. Electron microscopic analyses of optimally fixed HeLa cells indicate that the PNC consists of multiple thick, electron dense strands, each measuring approximately 80–180 nm in diameter [Huang et al., 1998]. Three-dimensional computer reconstruction of electron microscopic images from serially thin-sectioned HeLa cell nuclei demonstrate that the PNC forms a reticulated meshwork on the nucleolar surface [Huang et al., 1998]. Time-lapse microscopic analyses of living cells that transiently express green-fluorescent protein tagged PTB (GFP-PTB) to demarcate the PNC reveal small, distinct movements of the PNC along the nucleolar periphery over time [Huang et al., 1997]. The PNC is clearly detectable throughout

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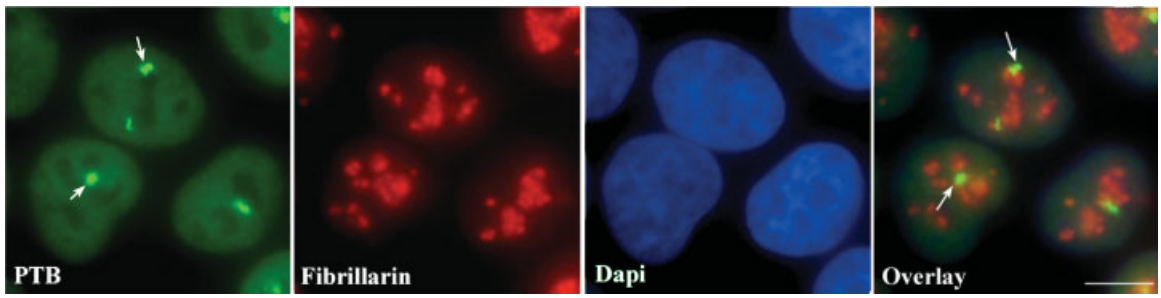

Fig. 1. The localization of PNC in HeLa cells. Monoclonal antibody SH54, specific for the PTB protein, immunolabels the perinucleolar compartment (PNC) in green (marked with arrows), at the periphery of the nucleolus. Nucleoli are marked by the immunolabeling of fibrillarin in red. The bar, 10 μm.

interphase, disassembles at the beginning of mitosis in parallel with the disassociation of nucleoli, and reassembles adjacent to nascent nucleoli at late telophase in daughter nuclei [Huang et al., 1998; Huang, 2000].

The PNC is associated with an insoluble fraction of the nucleus. The structure can be detected at the nucleolar periphery after cellular extraction with detergent and up to 500 mM salt. Even though the PNC cannot be isolated, it can be co-purified with nucleoli using a standard nucleoli purification protocol (our unpublished data). Treatment of permeabilized cells with RNase A but not DNase I prior to fixation abolishes the PNC as assayed by immunolabeling of PNC-associated proteins [Huang et al., 1998]. These findings suggest that the PNC is a RNA dependent sub-nuclear structure that is associated with less soluble nuclear remnants.

KNOWN COMPONENTS OF THE PNC

A number of RNAs and RNA-binding proteins have been found to be concentrated in the PNC. The RNAs identified thus far are non-coding RNAs transcribed by pol III. The RNA-binding proteins localized to the PNC are primarily shown to be involved in the metabolism of pol II transcripts. Some, but not all, of the known protein components of the PNC have been shown to interact with one another and some of the PNC-associated pol III transcripts. For example, PTB is reported to interact with Raver1 [Huttelmaier et al., 2001] and nucleolin [Singh et al., 2004], while some of the hY RNAs are known to complex with PTB [Fabini et al., 2001] and nucleolin [Fouraux et al., 2002]. Additionally, it is not known whether these interactions take place in the PNC since these proteins also co-localize in other regions of the cell [Huttelmaier et al., 2001; Singh et al., 2004]. The possibility that these RNA-binding proteins may interact with a subset of pol III transcripts in the PNC is currently under investigation.

RNA Components

RNase P H1 RNA is the RNA component of a sequence-specific endoribonuclease complex. RNase P is highly conserved throughout evolution, and is the universal enzyme responsible for the maturation of the 5′ end of tRNA [van Eenennaam et al., 2000; Jarrous, 2002; Xiao et al., 2002]. RNase P H1 RNA also localizes within the nucleolus where the functional RNase assembles and participates in pre-ribosomal RNA (pre-rRNA) processing. In addition to being distributed throughout the cells, the RNA is highly enriched in the PNC [Matera et al., 1995].

Mitochondrial RNA processing (MRP) RNase is another highly conserved sequence-specific endoribonuclease. RNase MRP functions in both, pre-ribosomal RNA processing and mitochondrial DNA replication [van Eenennaam et al., 2000]. Mutations in the MRP RNA (*RMRP*) gene located on the short arm of chromosome 9 are reported to be responsible for the disease cartilage hair hypoplasia (CHH) [Ridanpaa et al., 2001]. Fluorescence in situ hybridization experiments localize RMRP to the PNC in addition to its concentration in the nucleolus and diffuse distribution throughout the nucleus and cytoplasm [Matera et al., 1995; Lee et al., 1996; Wang et al., 2003]. Interestingly, RNase RMRP is structurally related to the RNase P H1 RNA, and both endoribonuclease complexes share a number of protein subunits, indicating an evolutionary link between the two functional complexes [van Eenennaam

et al., 2000]. Whether this relationship also influences the localization and/or function of these RNAs in the PNC has yet to be explored.

The hY RNAs are small (69–112 nucleotides) RNAs that interact with the 60-kD Ro protein along with other proteins to form evolutionarily conserved Ro ribonucleoproteins (RNPs). Ro RNPs are abundant in the cytoplasm at approximately 1% the level of ribosomes [Wolin and Steitz, 1984], but their function remains unclear. Although hY1, hY3, and hY5 RNAs are present in the PNC, the core components of the Ro RNPs, Ro [Matera et al., 1995] and La (our unpublished data), have not been detected in the PNC. Interestingly, hY1 and hY3, but not hY5, have been reported to interact with PTB and nucleolin which are also components of the PNC [Fabini et al., 2001; Fouraux et al., 2002]. It is not known whether all of these components exist in one complex, nor whether these interactions are specific to the PNC. It has been hypothesized that newly transcribed hY RNAs (excluding hY5 RNA, which remains in the nucleus) may first move to and accumulate in the PNCs before being transported into the cytoplasm [Fouraux et al., 2002].

Other pol III transcripts that have been localized to the PNC include Alu RNAs and the RNA component of the signal recognition particle (SRP) [Wang et al., 2003]. However, not all pol III transcripts are detected in the PNC. In situ hybridization to other pol III RNAs such as tRNA, U6, and 5S rRNA, fail to colocalize with the PNC [Matera et al., 1995; our unpublished data]. Thus far, pol II transcripts do not appear to be specifically associated with the structure [Hall et al., 2004; our unpublished data], nor are ribosomal RNAs, including the 28S and 18S RNAs (our unpublished data).

Protein Components

A number of RNA-binding proteins with diverse functions are found to be concentrated in the PNC. The first protein identified in the PNC, PTB, is a 57 kD RNA-binding protein that preferentially binds pyrimidine rich sequences [Ghetti et al., 1992]. PTB is implicated in multiple RNA related functions including pre-mRNA splicing, splice site selection in alternative pre-mRNA splicing [Wagner and Garcia-Blanco, 2001], regulation of RNA polyadenylation [Lou et al., 1996; Lou et al., 1999; Castelo-Branco et al., 2004], and translational regulation of certain RNA transcripts through internal ribosome entry sites (IRES) [Kozak, 2003; Stoneley and Willis, 2004]. These findings demonstrate that PTB functions in both nuclear and cytoplasmic processes, probably through the binding of pyrimidine rich-RNA sequences. Thus, PTB may serve as a bridge between RNAs and a variety of cellular factors that fulfil different functions. We have shown that PTB shuttles between the PNC and the nucleoplasm [Huang et al., 1998], as well as between the nucleus and the cytoplasm [Kamath et al., 2001]. The inter-compartmental shuttling dynamics of the protein may reflect its functional engagement in various cellular processes. Previous studies indicate that three RNA recognition motifs (RRMs) of PTB are necessary and sufficient for its RNA-binding [Kaminski et al., 1995]. Likewise, we found that at least three RRMs at either the C- or N-terminus of PTB are required for the enrichment of the protein in the PNC [Huang et al., 1997]. In addition, localization of PTB to the PNC is sensitive to RNase A treatment [Huang et al., 1998]. More recently, our lab has shown that the maintenance of a critical level of PTB is required for the structural integrity of the PNC [Wang et al., 2003]. Altogether these results suggest that the function of PTB in the PNC is related to its RNA-binding activity.

Another PNC enriched RNA-binding protein, CUG-BP/hNab50, was first isolated in a yeast two-hybrid system due to its interaction with the yeast heterogeneous nuclear ribonucleoprotein (hnRNP) Nab2p [Anderson et al., 1993]. CUG-BP/hNab50 interacts with polyadenylated RNA and is localized predominantly in the nucleoplasm in addition to being enriched at a site at the periphery of the nucleolus [Timchenko et al., 1996]. Double labeling experiments show that the perinucleolar localization of this protein coincides with the localization of the PNC [Huang et al., 1998]. CUG-BP/hNab50 binds to the CUG triplet repeats of myotonin protein kinase RNA, which is implicated in the autosomal dominant neuromuscular disease myotonic dystrophy [Timchenko et al., 1996]. Accumulating evidence suggests that CUG-BP is also involved in alternative splicing [Savkur et al., 2001; Charlet et al., 2002; Zhang et al., 2002], as well as deadenylation [Paillard et al., 2003].

Raver1 is an 80 kD multidomain protein that contains three RRMs, two nuclear localization

signals (NLS), and one Rev-like nuclear export sequence (NES) [Huttelmaier et al., 2001]. Although, the presence of the RRMs is suggestive, the RNA-binding specificity of Raver1 is not clear. Raver1 also complexes with microfilament-associated proteins, including vinculin, metavinculin, α-actinin, and tropomyosin at cytoplasmic microfilament attachment sites [Huttelmaier et al., 2001; Gromak et al., 2003]. In addition, Raver1 interacts with PTB and localizes to the PNC [Huttelmaier et al., 2001], though whether these interactions are restricted to the PNC remains to be investigated.

KSRP (K homology-type splicing regulatory protein) is highly expressed in neural cells and is required for neural-specific splicing of the N1 exon of c-src mRNA [Min et al., 1997; Chou et al., 1999; Markovtsov et al., 2000]. KSRP is found in all PNCs of neuroblastoma cells, however, the PNCs in the neuroblastoma cells do not appear to be involved in the processing of the c-src mRNA [Hall et al., 2004]. In contrast, KSRP is localized only to a subset of PNCs in HeLa (human cervical carcinoma) cells [Hall et al., 2004; our unpublished data]. The functional role of KSRP in the PNCs of HeLa cells has not yet been evaluated.

The RNA-binding protein ROD1 was first described as the mammalian homolog of a negative regulator of differentiation ($nrd1^+$) in the yeast *Schizosaccharomyces pombe*. The human *ROD1* gene encodes a 56 kD protein and contains four predicted RRMs [Yamamoto et al., 1999], which show remarkable homology to those of PTB. Overexpression of ROD1 effectively blocks both 12-O-tetradecanoyl phorbol-13-acetate-induced megakaryocytic and sodium butyrate-induced erythroid differentiation of K562 human leukemia cells without affecting their proliferative ability [Yamamoto et al., 1999]. In addition, human homologs, such as those in the PTB protein family, have also been found to act as negative regulators of differentiation [Ichikawa et al., 2002]. We have found that ROD1 localizes within the PNCs (our unpublished data).

Nucleolin is a 92 kD abundant nucleolar protein that contains four consensus RNA-binding domains (CS-RBD) [Bugler et al., 1987], each of which consists of approximately 80 amino acid residues with two highly conserved regions, the RNP motifs [Query et al., 1989]. Nucleolin is a multifunctional protein that has been shown to be involved in rDNA transcription, pre-rRNA processing, ribosome assembly, and pre-mRNA processing [Tuteja and Tuteja, 1998; Ginisty et al., 1999; Srivastava and Pollard, 1999]. Although nucleolin is a major component of the nucleolus, it has been shown to shuttle between nucleus and cytoplasm [Borer et al., 1989], supporting its apparent role as a cell surface receptor involved in extracellular matrix attachment [Dickinson and Kohwi-Shigematsu, 1995]. Recently, we found that nucleolin is also enriched in the PNC. Thus far, nucleolin is the only protein component of the PNC that also localizes in the nucleolus. Whether the interactions between nucleolin and PTB and the hY RNAs [Fouraux et al., 2002; Singh et al., 2004] occur in the PNC is a question that is currently being addressed.

Similar to other nuclear compartments, the list of known components of the PNC is far from complete. Investigations are currently underway to identify additional components of the PNC and to analyze molecular interactions that occur in the PNC. Revelation of a complete list of PNC components will help elucidate the function of the PNC and its role in transformation.

PNC AND TRANSFORMATION

The cell nucleus is a complex organelle where DNA replication, RNA transcription, processing, and transport play critical roles in regulating gene expression. During malignant transformation, these nuclear processes undergo significant alterations, which result in abnormalities in gene expression and ultimately cell behavior. Alterations in nuclear functions are often reflected in detectable changes in nuclear structure [Kamath et al., 2004; Zink et al., 2004]. Nuclear morphometry has long been one of the key factors in tumor grading [Kamath et al., 2004]. Our earliest studies demonstrate that the presence of PNC positively correlates with transformation in cultured cells [Huang et al., 1997].

To evaluate the relationship between the PNC and malignancy in vivo, we recently examined PNC prevalence (the percentage of cells containing at least one PNC) in a large number of normal and cancerous paraffin-embedded breast tissue samples. Breast cancer was chosen for the initial evaluation because of its high prevalence and its significant morbidity and mortality. PNC were detected in tissue samples using immunohistochemistry that labels PTB, a

protein highly concentrated in the PNC. At least 500 nuclei in the most active area of each sample were scored for PNC prevalence. Systematic evaluation of PNC prevalence in normal, benign, and malignant breast tissues in triple blinded studies (independent investigators from three laboratories each had knowledge of either patient information, PNC prevalence scoring, or statistical analyses, but not all three) demonstrate that PNC prevalence significantly correlates with the progression of breast cancer (by the criteria of staging and grading) [Kamath et al., 2005]. PNC prevalence in primary tumors, lymph nodes, and distant metastases show a step-wise increase from a median of 23% in primary tumors to nearly 100% in distant metastases. In addition, univariate and multivariate (controlling for tumor size and grade) analyses demonstrate that early stage patients with invasive ductal carcinomas containing a higher PNC prevalence have a poor prognosis [Kamath et al., 2005]. These findings link PNC prevalence to the progression of breast cancer in vivo and suggest that PNC containing cells have metastatic advantages. These findings also demonstrate the potential for PNC prevalence to be used as a prognostic marker for breast cancer, particularly for stage I patients to whom limited prognosis is available. Consequentially, approximately 70% of patients from this group are over treated with unnecessary adjuvant chemotherapy that negatively impacts their quality of life. Larger scale studies are underway to further confirm whether PNC prevalence can be a useful tumor marker for breast cancer. Other studies are also in progress to evaluate the association between PNC prevalence and cancers from other tissue types.

FUNCTION OF THE PNC

Although the sub-nuclear localization of the PNC has been described for over a decade and its association with malignancy is becoming more clear, little is understood regarding the function of the PNC. The concentration of a subset pol III RNA transcripts and RNA-binding proteins in the PNC is suggestive of a role in RNA metabolism. Despite the fact that most of the RNA-binding proteins in the PNC are not known to interact with the RNAs with which they co-localize in the PNC, several lines of evidence support an RNA-related function in the PNC.

First, several observations indicate that RNA is an essential component of the PNC: (A) RNase, but not DNase treatment of permeablized cells prior to fixation eliminates PNCs [Huang et al., 1998]; (B) the PNC actively incorporates Br-UTP and FITC-CTP in a DNA dependent manner after a short pulse labeling using permeablized cells, suggesting that the incorporation of labeled nucleotides is derived from transcription [Huang et al., 1998]. Analysis of the source of RNA indicates that the nascent RNA in the PNC is probably not synthesized by pol I since inhibition of pol I either by addition of actinomycin D in the transcription cocktail or by pre-treatment of cells with cycloheximide does not affect Br-UTP incorporation [Huang et al., 1998]. In addition, neither 28S rRNA [Matera et al., 1995], 18S rRNA (our unpublished data) nor fibrillarin [a pre-rRNA processing factor; Huang et al., 1997] are detected in the PNC. It also appears that pol II transcripts are not concentrated in the PNC since in situ hybridization specific to several different pol II RNAs did not show enrichment in the PNC [Hall et al., 2004; our unpublished data], and the PNC is not sensitive to pol II transcription inhibition [Huang et al., 1998]; and (C) our recent studies indicate that the structural integrity of the PNC is dependent upon continuous pol III transcription. Injection of tagetin, a pol III specific inhibitor, into HeLa cells results in a loss of perinucleolar labeling of both PNC-associated RNAs and RNA-binding proteins [Wang et al., 2003] within 2 h. This finding also suggests that PNC-associated RNAs are mostly derived from pol III transcription. Overexpression of one of the PNC-associated RNAs, RMRP, from a pol II promoter followed by injection of tagetin partially blocks the tagetin-induced PNC disassembly [Wang et al., 2003], demonstrating that it is the RNA rather than activity of the polymerase that is important for the integrity of the PNC.

Secondly, the RNA-binding protein, PTB, is also important for the structural maintenance of the PNC. Deletion mutagenesis analysis shows that at least three RRMs at either the C- or N-terminus of the PTB protein are required for it to be targeted to the PNC [Huang et al., 1997]. Since at least three RRMs at either terminus are necessary for effective RNA-binding [Kaminski et al., 1995], this finding suggests that RNA-binding capability is crucial

for PTB to be localized in the PNC. Additionally, when PTB protein levels are knocked out by siRNA, there is a reduction in the size of PNC and the number of cells that contain the structure [Wang et al., 2003]. These findings suggest that the maintenance of PNCs requires a minimal level of PTB protein that is able to bind RNA. However, overexpression of either PNC-associated RNAs or PNC-associated proteins, PTB or CUG/BP, or expression of a combination of both RNA and proteins does not induce PNC formation in primary or immortalized cells, suggesting that other key factors, possibly those yet to be identified, are essential to the function of the structure.

Since the PNC is sensitive to pol III transcription inhibition and contains newly synthesized RNA, we and others considered it possible that the PNC might be the site of transcription for these RNAs. However, in situ hybridization to four genes that encode RNAs detected in the PNC did not show any meaningful association with the PNC [Matera et al., 1995; our unpublished data], suggesting that the PNC is unlikely to be the site of transcription for these RNAs. Another possible explanation for the enrichment of the RNAs in the PNC is that the PNC could be where the functional RNPs such as RNase MRP or P or Ro RNP are assembled. However, localization studies using immunolabeling to some of the protein components [Matera et al., 1995] or expression of GFP-tagged proteins of the RNP sub-units (our unpublished data) did not show any enrichment of the mature RNP components in the PNC. These findings deem the possibility unlikely as well. Recently, we found that the inhibition of pol III transcription disassembles the PNC within a short period of time, but does not disrupt the localization of RMRP in its functional RNPs in the nucleolus [Wang et al., 2003]. Together with the fact that the PNC is highly enriched with newly synthesized RNA, we suggest that the RNAs are concentrated in the PNC prior to be assembled into mature or functional RNPs in the nucleolus or other cellular destinations. Since live cell analyses showed that PTB shuttles in and out of PNCs rapidly [Huang et al., 1997], the association of these RNAs with the PNC is likely to be dynamic rather than long-term storage or simple aggregation. Based on all these findings, we propose a working model that the PNC is involved in the trafficking of a subset of newly synthesized pol III RNA transcripts in transformed cells. There are several possibilities one can envision for the role of PNC in the metabolism of these RNAs (Fig. 2): (1) the PNC could be the site of RNA processing,

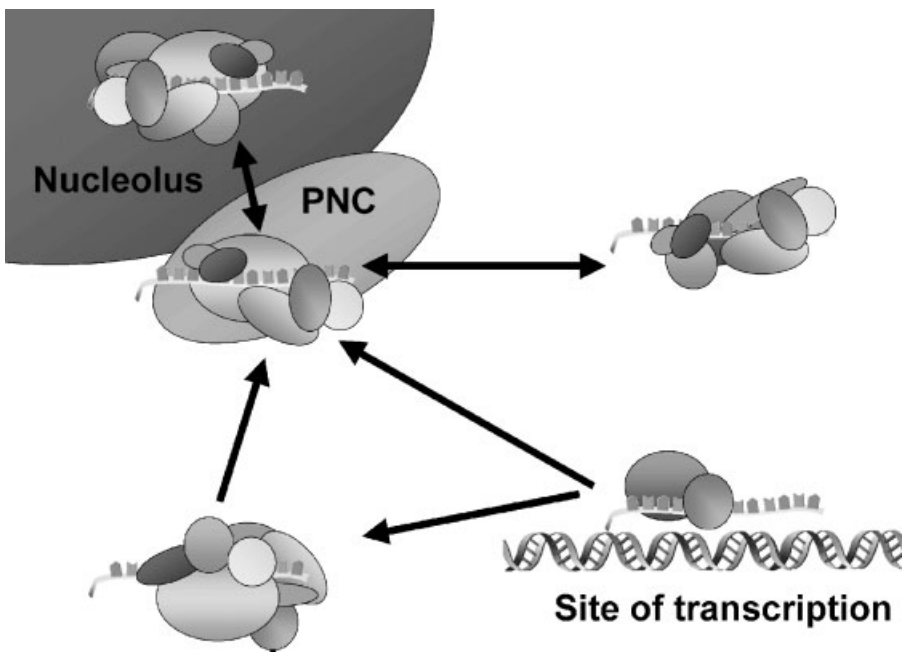

Fig. 2. A working model proposes that the PNC could be involved in RNA trafficking for a subset of newly synthesized RNA. RNAs are rapidly transported either directly from the sites of transcription or via other RNPs to transiently associate with the PNC.

although the processing of this group of RNAs is currently unclear; (2) the RNAs could be sequestered in the PNC prior to being assembled into functional complexes; (3) the PNC may act as the site of assembly for RNA export complexes for the cytoplasmic translocation of some of the PNC-associated RNAs; and (4) it could also be the site for degradation of excess RNAs. Studies are currently underway to test these ideas.

The close association between PNCs and transformed phenotype raises the question regarding their functional link. The PNC is highly enriched with a subset of pol III RNA transcripts and its integrity is dependent upon the continuous transcription of pol III. However, overexpression of these PNC-associated RNAs and/or proteins is not sufficient to induce the formation of the structure. These findings lead to speculation that the formation of the PNC might be triggered by two possible mechanisms that are not mutually exclusive; (1) formation of the PNC could partially be due to increased expression levels of the PNC-associated components in transformed cells. Existing literature and our unpublished studies showed that pol III transcription is significantly increased during transformation [White, 2004]. This may contribute to the increases in the level of RNPs associated with the PNC, and therefore allow the visualization of the PNC and (2) factors that have yet to be identified in the PNC are altered either at the expression levels or functional capabilities in transformed cells in such a manner that they are responsible for the nucleation of the RNAs and RNA-binding proteins in the PNC. Following this logic, the PNC could be part of the manifestation of transformation from normal to abnormal expression levels of RNA, RNA-binding proteins as well as abnormal nucleation factors of the PNC.

On the other hand, the PNC may not only be a consequence of transformation, but it may function in maintaining the transformed phenotype. For example, ribosome synthesis and nucleolar structure are grossly altered during transformation, which is probably due to the high demand for protein synthesis in tumor cells [Kamath et al., 2004; Zink et al., 2004]. Interestingly, some of the components of the PNC are involved in rDNA transcription, pre-rRNA processing, ribosome assembly, and translation including, nucleolin, PTB, RNase MRP, and P RNAs [Tuteja and Tuteja, 1998; Ginisty et al., 1999; Srivastava and Pollard, 1999; van Eenennaam et al., 2000; Jarrous, 2002; Xiao et al., 2002; Kozak, 2003; Stoneley and Willis, 2004]. Since the PNC is physically associated with the nucleolus, it is possible that these close spatial links allow for a rapid translocation of these components to their functional destinations. Studies are underway to test these possibilities.

SUMMARY

The PNC is a dynamic nuclear sub-structure highly concentrated with newly synthesized RNAs (primarily pol III transcripts) and RNA-binding proteins. The formation of the PNC is closely linked to malignant transformation as demonstrated both in vitro and in vivo. Although the function of the PNC remains unclear, findings from our group and others suggest that the PNC is involved in trafficking of a subset of newly synthesized pol III RNA. Studies are underway to identify the macromolecular composition of the PNC and its functional role in malignancy.

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