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REVIEW

The perinucleolar compartment associates with malignancy

Yiping WEN[1,2], Chen WANG[1], Sui HUANG (✉)[1]

[1] *Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, IL 60611, USA*
[2] *College of Veterinary Medicine, Sichuan Agricultural University, Yaan 625014, China*

Abstract **The perinucleolar compartment (PNC) is a unique nuclear substructure, forming predominantly in cancer cells both *in vitro* and *in vivo*. PNC prevalence (percentage of cells containing at least one PNC) has been found to positively correlate with disease progression in several cancers (breast, ovarian, and colon). While there is a clear association between PNCs and cancer, the molecular function of the PNC remains unclear. Here we summarize the current understanding of the association of PNCs with cancer and its possible functions in cancer cells.**

Keywords PNC, cancer, nuclear substructure, gene expression regulation, structure and function

Introduction

Cancers are complex diseases reflecting ever evolving consequences of multi-step complex transformation processes. Along with tremendous molecular changes at genetic and epigenetic levels in a cancer nucleus, the architecture and high order organization of the nucleus undergo alterations correspondingly. For more than a century, the increases in size and number of nucleoli, and other changes in nuclear morphometry have been documented in cancer cells and used as parameters in predicting disease outcomes in cancer patients (Pianese, 1896; Zwerger et al., 2011), demonstrating the levels of changes in nuclear structure directly reflect the levels of transformation of the cancer cells. However, the molecular relevance of the morphological changes in cancer cell nuclei is still not well understood. Whether the structural alterations represent functional requirement of the transformation or merely a consequence of the functional changes remains to be determined. One of the nuclear architecture changes is the formation of the perinucleolar compartment (PNC) in cancer cells. In this review, we summarize the current understanding of the formation and the structure function of perinucleolar compartment in cancer cell.

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Correspondence: Sui HUANG
E-mail: s-huang2@northwestern.edu

PNCs are detected predominantly in cancer cells

The PNC is an irregularly shaped structure with a diameter ranging from 0.25 to 1 μm and is physically associated with the nucleolus. When examined by transmission electron microscopy using conventional UA-lead staining, the PNC is composed of multiple electron-dense strands approximately 80–180 nm in diameter and is structural distinct from the linked nucleolus (Huang et al., 1997). At the light microscopic level, PNCs can be detected either by immunofluorescence using antibodies specifically recognizing PTB, a polypyrimidine tract binding protein, or by in situ hybridization to the RNA components of RNase MRP (Fig. 1) (Matera et al., 1995; Lee et al., 1996; Huang et al., 1997). PNCs are detected predominantly in solid tissue derived cancer cells or cell lines either from patient tumors or transformed *in vitro*. They are rarely present in normal cells including embryonic stem cells from human or mice (Huang et al., 1997; Norton et al., 2008a). PNC structures are stably associated with the nucleoli through interphase in the cell cycle and undergo disassembly and reassembly along with nucleoli during cell divisions (Huang et al., 1997).

PNC prevalence associates with the aggressiveness of cancer *in vivo*

PNC prevalence is highly heterogeneous among various cancer and transformed cell lines (Huang et al., 1997; Norton et al., 2008a). Evaluations of PNC prevalence in the most

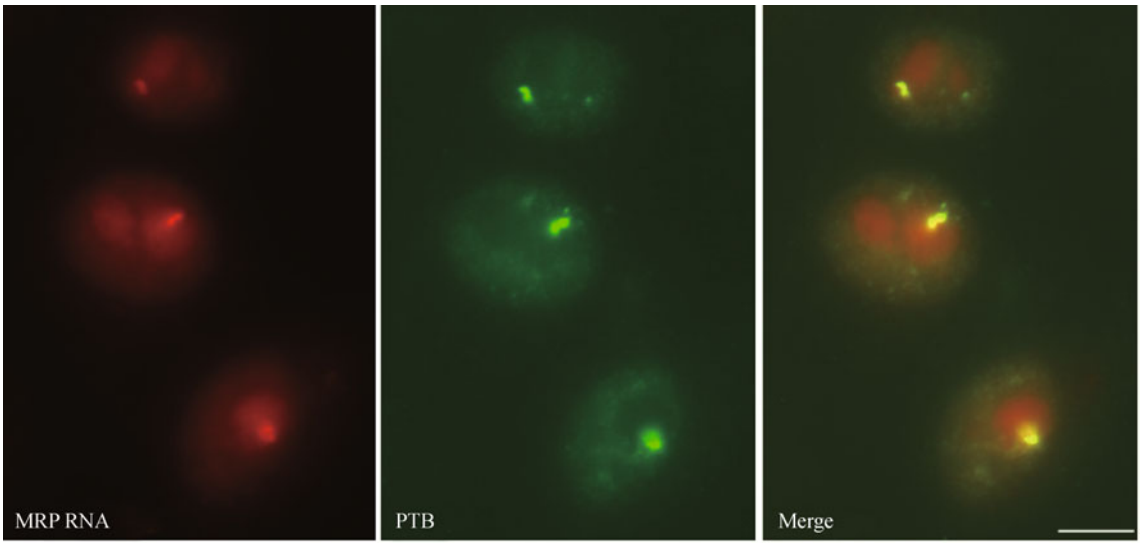

Figure 1 RNase MRP RNA and PTB colocalize in the PNC at the nucleolar periphery. *In situ* hybridizations using specific oligo probe show perinucleolar enrichment of the RNA (left panel, arrows) which is colocalized (right panel, arrows) with a bright PTB immunolabeling signal (middle panel, arrows). The bar = 10 μm.

aggressive regions of primary tumors from breast, ovarian, and colon show consistent positive associations of PNC prevalence with the progression of the diseases (stages and grades) (Kamath et al., 2005; Slusarczyk et al., 2010) (our unpublished data).While PNC prevalence can vary in primary tumor tissues, it reaches near 100% in distant metastases. Univariate analyses using matched cases demonstrate a correlation between high PNC prevalence in primary tumors and poor prognosis of breast cancer patients (Kamath et al., 2005). Multivariate analyses (adding PNC prevalence to tumor size and grade) demonstrate that PNC prevalence contains additional prognostic power in predicting the relapse of early stage infiltrating ductal breast carcinomas (for lymph node negative and 1–3 lymph nodes involved) (Kamath et al., 2005). Similar trends have also been shown in colon and ovarian cancer (Slusarczyk et al., 2010) (our unpublished data). The observations that PNC prevalence increases in parallel with the progression of cancer and reaches nearly 100% in metastases suggest that PNC-containing cancer cells have growth and metastatic advantages over those without detectable PNCs.

PNC prevalence directly reflects the metastatic capability in cells derived from animal tumor models of human prostate cancer

Experimentally, PNC prevalence was evaluated in cancer cell lines enriched either for metastatic capability or for localized growth properties. PC-3 cell line series were designed to isolate populations of cells with varying degrees of malignancy from a single source (Pettaway et al., 1996). PC-3M cells were created by placing a PC-3 xenograft, a human prostate cancer cells grown from resected tumor (PNC prevalence less than 5%), into a nude mouse, allowing the tumor to metastasize, removing a distant metastatic lesion, and then culturing the cells from the metastasis. The one round of enrichment for metastatic capable cells (PC-3M) significantly increased PNC prevalence (87.1%±4.0%) from the parental cells (PC-3) (Pettaway et al., 1996). Subsequently, PC-3M cells were injected into the prostate glands of a nude mouse. Cells were then removed from lymph node metastases and re-injected into the prostate of another nude mouse. This process was iterated four times so as to enrich for a highly metastatic population of cells. The PC-3M LN4 cell line was isolated from a lymph node metastasis removed from the fourth mouse (Pettaway et al., 1996). Nearly all (98.0%± 1.3%) PC-3M LN4 cells contained one or more typical sized or atypically large PNCs (Norton et al., 2008a). In comparison, non-metastatic tumor cells were enriched by isolating PC-3M cells that remained in the prostate of the injected mouse and then by re-implanting these cells into the prostate of another mouse. The PC-3M Pro4 cell line was generated from the prostate of the fourth mouse in the iteration of this process and was therefore enriched with non-metastatic cells (Pettaway et al., 1996). PNC prevalence in PC-3M Pro4 cells (78.0%±4.1%) was significantly less ($p < 0.05$) than the PC-3M LN4 cells. More importantly, the majority of the PNCs in PC-3M Pro4 cell lines were atypically small (Norton et al., 2008a). When PNC prevalence is adjusted to the percentage of cells containing PNCs with diameter larger than 2.2 μm, it shows a close correlation with the ability of the cell population to metastasize (Norton et al., 2008a). These observations are complementary to the findings in tumor tissues where PNC

prevalence showed stepwise increases from primary tumors to lymph nodes and reached nearly 100% in distant metastasis (Kamath et al., 2005), reinforcing the correlation between PNC prevalence and metastatic behavior. Furthermore, the suppression of breast cancer metastasis significantly reduced PNC prevalence in these cells (Norton et al., 2008a). The close association between PNC prevalence and levels of malignancy both *in vitro* and *in vivo* indicate that the formation of the PNC reflects critical changes during transformation that signify metastatic capability of cancer cells.

PNC prevalence can be a cancer specific phenotypic marker

Ideal tumor markers should be those that are associated with the pathophysiology unique to cancer cells (unlimited anchorage independent growth and metastasis) and the reduction of such a marker in cancer cells should then be indicative of improvement in the cellular behavior and be predictive of more favorite patient outcome (Frank and Hargreaves, 2003). This type of marker can be used to screen for specific anti-cancer compounds that disrupt core cellular processes critical for sustaining malignant behavior of cancer cells. These compounds could then be potential candidates for the development of selective and specific anti-cancer chemotherapy. PNCs appear to fit such characteristics. As discussed above, PNCs specifically form in metastatic capable cancer cells. The formation of PNC does not associate with growth traits that are shared by normal and cancer cells, including proliferation rate, glycolysis, differentiation statues (Norton et al., 2008a). Thus, the specific phenotypic representation of metastasis makes the reduction of PNC prevalence an ideal surrogate mark for identifying novel anti-cancer drugs. Compounds that have PNC inhibitory (PNCi) effect may disrupt cellular processes that are required for the maintenance of malignant phenotype as reflected in the loss of PNCs (Fig. 2).

A high-content-put assay screen for PNCi effect using over 150000 small compounds has identified 98 compounds of diverse structures that reduce PNC prevalence by 50% in PC-3M cells (Norton et al., 2009a). Among these PNCi compounds are well-known or clinically used anti-cancer chemotherapeutics including doxorubicin, mitomycin, cisplatin, camptothecin, F-Ara-A, amonafide, and gemcitabine. Additionally, other chemotypes with no known anti-cancer roles were also identified. It is interesting that more than one chemotypes are among the hits (Norton et al., 2009a). One explanation is that the development and maintenance of malignant phenotype is highly complex and involves multiple cellular pathways or mechanisms. Disruption of any one essential mechanism could impact the phenotypes. If PNCs are indeed markers reflecting metastatic capability, it should be disrupted by multiple mechanisms. In fact, two mechanisms, transcription and DNA structure, both are well known targets for the current chemotherapies against cancer are critical for PNC integrity. Crosslinking DNA at the duration without significant impacting pol III transcription (Norton et al., 2009b) or blocking pol III transcription (Wang et al., 2003) is sufficient to disassemble the PNC. Therefore, the structurally diverse hits could serve as molecular probes to help identify key cellular mechanisms that are critical for the maintenance of the PNC and of malignant phenotypes.

A novel analog (MEAN) (Norton et al., 2008b; Liu et al., 2011) of a known anti-cancer compound, amonafide (AMN) has been developed from these hits. MEAN has *in vitro* anti-tumor properties and *in vivo* efficacy in xenograft mouse

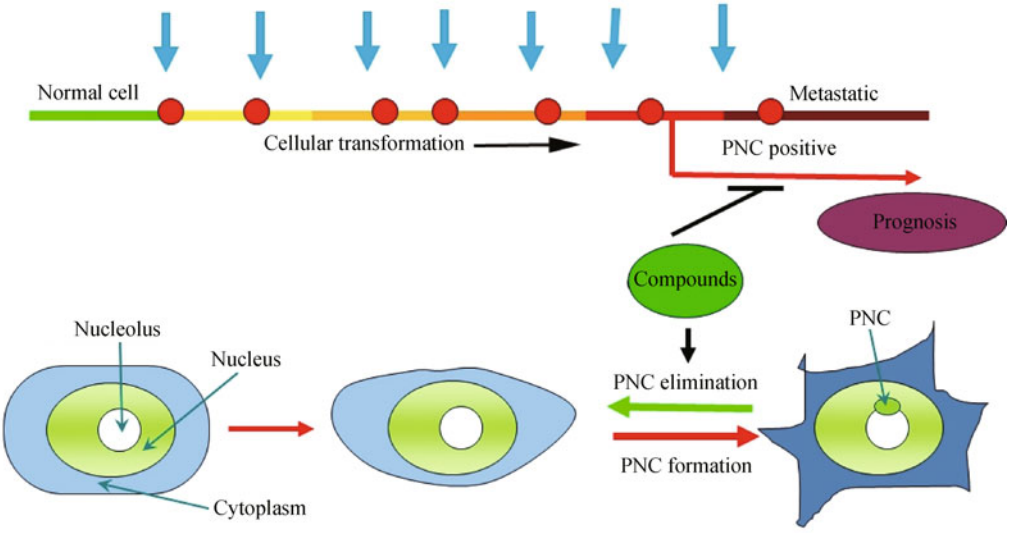

Figure 2 Schematic diagram summarizes that PNCs form as the consequence of transformation. Its formation signifies that cancer cells become capable of metastasis. PNC prevalence, thus, can be used as a cancer marker for anti-cancer drug development.

tumor models of human cancer at doses that elicit little to no toxicity. In comparison to its parental compound, animals tolerate MEAN well through various treatment schedules (Liu et al., 2011). These observations and findings of other PNCi compounds (our unpublished data) further validate that PNC prevalence specifically reflects the malignant behavior of cancer cells and can be used as a selective cancer behavior marker for translational studies.

Molecular function of the PNC

While the link between PNCs and malignancy is increasingly clear, the molecular function of the PNC and its role in cancer remains unresolved. Localization studies identify several non-coding pol III RNAs and RNA binding proteins primarily implicated in pre-mRNA processing to be enriched in the PNC (Matera et al., 1995; Lee et al., 1996; Wang et al., 2003).

The PNC is enriched with RNA binding proteins

The PNC was first described during the characterization of polypyrimidine tract binding protein (PTB) (Ghetti et al., 1992) that binds to the 3′ end of pre-mRNA introns (Garcia-Blanco et al., 1989; Wang and Pederson, 1990). PTB is diffusely distributed in the nucleoplasm in addition to being concentrated in the PNC (Ghetti et al., 1992). PTB is implicated in multiple cellular functions including pre-mRNA splicing (Valcarcel and Gebauer, 1997; Kafasla et al., 2012), splice site selection in alternative pre-mRNA splicing (Perez et al., 1997; Wagner et al., 1999; Wagner and Garcia-Blanco, 2002; Chen et al., 2010). RNA polyadenylation (Lou et al., 1996; Lou et al., 1999; Castelo-Branco et al., 2004; Sawicka et al., 2008), and translational regulation of certain RNA transcripts through IRES (internal ribosome entry site)(Hellen et al., 1994; Kaminski et al., 1995; Witherell et al., 1995; Schneider et al., 2001; Pickering et al., 2003; Sawicka et al., 2008). Furthermore, PTB is a highly dynamic protein that shuttles between the nucleus and the cytoplasm (Kamath et al., 2001), a process that is highly regulated through phosphorylation (Xie et al., 2003). PTB, a multifunctional protein (Sawicka et al., 2008), apparently participates in both nuclear and cytoplasmic functions through the binding of pyrimidine rich-RNA sequences. Thus, PTB may serve as a bridge between RNAs and a variety of cellular factors that fulfill different functions. Many of these functions are altered in cancer cells (Chen et al., 2010).

What could be the functional role of PTB in the PNC? PTB protein shuttles rapidly in and out of the PNC although the exchange dynamics is substantially slower in the PNC than in the nucleoplasm where most of the pre-mRNA processing take place (Pollock et al., 2011). The RNA binding capability is required for PTB to be localized to the PNC (Huang et al., 1997), and immunolabeling of PTB in the PNC structure is insensitive to pol II transcription inhibition, but is sensitive to RNase A treatment (Huang et al., 1997). These findings suggest that association of PTB in the PNC relates RNA binding function that may not be part of pre-mRNA process. Recently, we have shown that PTB interacts with PNC-associated small pol III transcribed RNAs *in vivo*. PTB and RNase MRP RNA colocalize in the PNC as reticulated meshes when examined by super resolution light microscopy of structure illumination mechanisms (Pollock et al., 2011). Interestingly, in PTB knockdown cells, PNC prevalence reduces significantly as measured by *in situ* hybridization to RNase MRP RNA or immunolabeling against CUGBP (Wang et al., 2003), indicating that PTB plays key roles in the localization of RNase MRP RNA and CUGBP to the PNC. These findings together suggest that PTB may be part of novel protein-RNA complexes for these small non-coding RNAs and may play a role in nucleating the RNA-protein complex to the PNC. However, the components of these complexes and their role in function of PNCs and malignancy remain to be investigated.

Other RNA binding proteins in the PNC include CUGBP/hNab50, KSRP (K homology-type splicing regulatory protein) (Hall et al., 2004), Raver 1 (Huttelmaier et al., 2001), ROD1, and nucleolin (Our unpublished data). CUGBP was first isolated in a yeast two-hybrid system because of its interaction with the yeast heterogeneous nuclear ribonucleo-protein (hnRNP) Nab2p (Anderson et al., 1993). CUGBP interacts with polyadenylated RNA and is localized pre-dominantly in the nucleoplasm in addition to being enriched at a perinucleolar site that coincides with the PNC (Timchenko et al., 1996; Huang et al., 1998). CUG-BP also binds the CUG triplet repeats of myotonin protein kinase RNA, which is associated with 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; Ho et al., 2005), as well as deadenylation (Paillard et al., 2003). More recently, CUGBP is also shown to be involved in miRNA and translational regulations (Apponi et al., 2011; Jones et al., 2012; Mahadevan, 2012). Although CUGBP is normally distributed in the nucleus, it becomes predominantly cytoplasmic upon pol II transcription inhibition (Huang et al., 1998), suggesting its nuclear localization requires continuous pol II transcription. However, the CUGBP concentrated in the PNC remains associated with the PNC during transcription inhibition, supporting that CUGBP may have novel interactions in the PNC that are not dependent upon pol II transcription (Huang et al., 1998). However, CUGBP knockdown, unlike PTB knockdown, does not induce the disassembly of PNC (our unpublished data). The role of CUGBP in the PNC remains to be determined.

Other RNA binding proteins that are enriched in the PNC

include KSRP (Gromak et al., 2003; Hall et al. 2004; Apponi et al., 2011), Raver 1, a PTB binding protein (Gromak et al., 2003; Kafasla et al., 2012), and Rod 1 (our unpublished data), all of which are in *in vivo* complexes with PTB, suggesting that they may participate in common processes. Additionally, PSF and p54nrb that are involved in RNA editing and processing (Bond and Fox, 2009; Fox and Lamond, 2010) are also found associated with PNC. It is not clear whether these complexes are mediated by RNA binding in the PNC or they are all in the same complex.

The PNC is enriched with small non-coding RNAs transcribed by pol III

Several small RNAs transcribed by RNA polymerase III (pol III) are enriched in the PNC as detected by FISH combined with immunofluorescence using PTB antibodies. They include RNase MRP and RNase P RNA, SRP RNA, Alu, and some of the Ro autoantigen-associated hY RNAs (Matera et al., 1995; Lee et al., 1996; Wang et al., 2003). However, not all pol III RNAs are detected in the PNC. In situ hybridization to tRNA, U6 and 5S rRNA did not show any enrichment of these RNA in the PNC (Matera et al., 1995). In addition, in situ hybridization to some mRNA (Hall et al., 2004) or rRNAs, 28S and 18S RNAs (our unpublished data) also did not detect these RNAs in the PNC, demonstrating the PNC may be enriched with a subset of pol III RNA.

RNase MRP RNA and RNase P H1 RNA are RNA components of two highly conserved sequence-specific endoribonucleases. The two RNAs share substantial structural homologies (Xiao et al., 2002; Esakova and Krasilnikov, 2010; Perederina et al., 2010; Esakova et al., 2011). Both RNAs are highly conserved through evolution and bind to protein subunits (some of which are shared by both RNA) to form functional RNase particles (Xiao et al., 2002). RNase MRP is involved in pre-rRNA processing and mitochondrial DNA replication (Altman, 1990; Clayton, 1994; Van Eenennaam et al., 2002). RNase P is involved in tRNA and pre-rRNA processing (Jarrous, 2002; Xiao et al., 2002). In situ hybridization and the microinjection of labeled RNase MRP RNA or RNase P H1 RNA show transient associations of these RNA with the nucleolus, suggesting that the functional RNPs assemble in the nucleolus (Clayton, 1994; Jacobson et al., 1995; Matera et al., 1995). Interestingly, RNase MRP or P RNA that are concentrated in the PNC do not appear to be assembled into the known functional RNPs in the PNC since the common protein subunits shared by both RNAs are not enriched in the PNC as evaluated by immunolabeling and tagged expression of these proteins (Wang et al., 2003). These findings suggest that the MRP and RNase P RNA in the PNC are unlikely to be in the same complex with their described protein partners, but rather are in novel RNA-protein complexes. The nature of the complex and the function of the complex in the PNC need to be further explored.

The PNC is enriched with newly synthesized RNA mainly derived from pol III transcription

ABrU incorporation assay was used to determine whether the PNC associated RNAs are newly synthesized. Cells were briefly permeabilized and incubated in a transcription cocktail containing BrU for 5 min using a modified protocol from published work (Jackson et al., 1993; Wansink et al., 1993). Simultaneous detection of the sites of BrU incorporation by immunolabeling with an anti-BrU antibody and of the PNC by anti-PTB antibodies demonstrated heavy incorporation of BrU at the PNC after 5 min pulse labeling (Huang et al., 1998). In fact, the signal of BrU incorporation is frequently more prominantin the PNC than in the remaining majority volume of the nucleoplasm. These results suggest that the PNC is either the site of transcription or the site of the metabolism of newly-synthesized RNA or both (Huang et al., 1998).

What types of RNAs are enriched in the PNC? To address this question, all threeRNA polymerases were inhibited individually and their impacts on PNCs were analyzed. Inhibition of pol I transcription either by siRNA knockdown of upstream binding factor (UBF), a pol I specific transactivator (our unpublished data), or by treatment with cycloheximidethat inhibits pol I transcription in 4 h(O'Keefe et al., 1994) did not alter the maintenance and localization of proteins and RNAs in the PNC (our unpublished data). Together with the lack of detectable rRNAs in the PNC, these findings suggest that that PNC-associated RNA is unlikely derived from pol I transcription. α-amanitin treatment at a concentration that specifically inhibits pol II transcription does not significantly impact the structure of the PNC, or the incorporation of BrU at the PNC (Huang et al., 1998), indicating that the PNC is not enriched with newly synthesized pre-mRNA. In comparison, treatment with α-amanitin at a high concentration that inhibits both pol II and III induces the disassembly of the PNC within 3 h (Huang et al., 1998), indicating that PNC enriched RNA is primarily from pol III transcription.

A direct verification of the role of pol III in the PNC came from the use of a pol III specific inhibition. Three hours after tagetin (Steinberg et al., 1990; Steinberg and Burgess, 1992; Huang et al., 1998) injection into HeLa cells, PNC structures were assayed by both immunolabeling with anti-PTB antibody and in situ hybridization to MRP RNA in the same cells. The results show a loss of the perinucleolar labeling of both PTB and MRP RNA, but not those MRP RNA in the nucleolus serving as functional RNase MRP (Wang et al., 2003). These findings suggest that the newly synthesized RNA in the PNC is mostly derived from pol III transcription and the continuous production of these RNA is important for maintaining PNC structure. However, over-expression of RNase MRP RNA (the most prominent RNA detected in the PNC) with or without simultaneous expression

of PTB and CUG/BP does not induce PNC formation in primary or immortalized cells (our unpublished data), suggesting that the formation of the PNC is a complex process not simply due to the aggregation of overexpressed components of the PNC.

PNCs are associated with chromatins

Through the use of chemical and cell biologic approaches, the PNC is found to nucleate on a DNA locus or multiple loci (Norton et al., 2009). The results of using a panel of compounds with different DNA interactive characteristics show that the DNA base-pairing capability is critical to the PNC structure (Norton et al., 2009). In addition, a CDK1 conditional mutant shows that the number of PNCs at non-permissive temperature increases corresponding to the number of endoreplicationrounds (Norton et al., 2009). The PNC associated locus/loci replicates at early-mid S phase with a peak at 3 h after release from hydroxyurea(Norton et al., 2009). The lack of colocalization of the gene loci that encode PNC enriched RNAs, including RNase MRP, and RNase P RNA, with the PNC demonstrates that the PNC is not the sites of transcription for these RNAs (Matera et al., 1995; Lee et al., 1996; Pollock et al., 2011). Furthermore, high resolution (100 nm) light microscopy (OMX) shows that MRP RNA-PTB-CUGBP labeling at the PNC does not colocalize with newly synthesized RNA (Pollock et al. 2011), suggesting that the RNA is unlikely representing the newly synthesized RNase MRP RNA that are being processed at the PNC. Instead, the RNase MRP RNA containing RNP colocalized to the PNC in a highly fibril and reticulated structures observed by both EM and OMX systems (Huang et al., 1997; Pollock et al., 2011), suggestive of its direct association with chromatin. Furthermore, treatment with TSA, which increases acetylation at H3K9, induces expansion of the PNC locus to a much more fibril structure visible even under lower power light microscopy (Norton et al., 2009). These observations indicate that the nucleation of the RNA-protein complex upon the DNA locus/loci may have direct gene expression regulatory roles in PNC containing cells. The idea needs to be further tested upon the identification of specific DNA loci that nucleate at the PNC.

Summary and future direction

The PNC is a unique nuclear substructure that forms during oncogenic transformation in solid tumor cells. The formation of PNCs reflects changes in cancer cells that confer their ability to metastasize. A high PNC prevalence in primary tumors of breast, colon, and ovary associates with poor patient outcome. PNC prevalence reduction can be a phenotypic marker for chemotherapeutic development of selective and effective anti-cancer agents. Molecularly, PNCs are enriched with newly synthesized non-coding RNA primary transcribed by RNA polymerase III, some of which form novel RNA-protein complexes in the PNC. RNA polymerase III transcription and DNA structural integrity are required to maintain the PNC structure. PNCs are nucleated upon unknown DNA loci. These clues lead to a working model, in which the formation of PNC may play roles in regulating specific pol III RNA expression in cancer cells and the function of these RNA may relate to nucleolar activities that are significantly altered during carcinogenesis. Future studies need to identify the molecular components of the PNC, including the novel RNA-protein complexes and the gene loci that nucleated on the PNC. The structural understanding of the PNC will allow for further investigations to tease out their functional roles in PNC, nucleoli, and malignancy.

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Compliance with ethics guidelines

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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