A noncoding RNA is a potential marker of cell fate during mammary gland development

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PINC is a large, alternatively spliced, developmentally regulated, noncoding RNA expressed in the regressed terminal ductal lobular unit-like structures of the parous mammary gland. Previous studies have shown that this population of cells possesses not only progenitor-like qualities (the ability to proliferate and repopulate a mammary gland) and the ability to survive developmentally programmed cell death but also the inhibition of carcinogen-induced proliferation. Here we report that PINC expression is temporally and spatially regulated in response to developmental stimuli *in vivo* and that PINC RNA is localized to distinct foci in either the nucleus or the cytoplasm in a cell-cycle-specific manner. Loss-of-function experiments suggest that PINC performs dual roles in cell survival and regulation of cell-cycle progression, suggesting that PINC may contribute to the developmentally mediated changes previously observed in the terminal ductal lobular unit-like structures of the parous gland. This is one of the first reports describing the functional properties of a large, developmentally regulated, mammalian, noncoding RNA.

parity | terminal ductal lobular unit

Noncoding RNAs (ncRNAs) constitute an important and expanding family of regulatory molecules. However, unlike their orthodox cousins, the coding RNAs, which were hitherto thought to comprise the majority of transcriptional output in eukaryotes, their functions have been largely unexplored, in part because until recently their roles in gene regulation have not been fully appreciated. Moreover, ncRNAs are often present in low abundance; thus, their presence has for the large part evaded detection (1).

However, several findings demonstrate that ncRNAs constitute a large and significant proportion of the transcriptional output of complex organisms, thus drawing attention to their importance as potential regulatory molecules (1–8). In many cases, the expression of ncRNAs appears to require finely tuned transcriptional and regulatory events (such as genomic imprinting or transcription from converging promoters), which in numerous cases include the recruitment of spliceosomal machinery and nuclear export (1, 9–12). Moreover, many of these ncRNAs are expressed in a defined temporal–spatial window, thus limiting their activities to a specific stage of development or particular tissue type (see Y. Hayashizaki's response to ref. 13) (1, 14, 15). These observations have led to the hypothesis that ncRNAs act by imposing a higher tier of regulation on a limited pool of structural and catalytic proteins (1, 16). This hypothesis is of specific relevance to the processes of cell and tissue specification and can thus be expected to have a major impact on our understanding of epigenetic processes guiding cell fate.

We previously reported the isolation of GB7, a novel ncRNA identified during a screen for developmentally regulated, differentially expressed genes in the rodent mammary gland (17). We have now designated GB7 as PINC for pregnancy-induced ncRNA, because it is induced by both pregnancy and hormonal stimulation of the mammary gland. Remarkably, PINC remains persistently elevated in cells in the regressed terminal ductal lobular unit-like structures of the mammary gland 4 weeks after withdrawal of hormonal stimuli. Interestingly, this population of cells has also been shown to possess not only progenitor-like qualities but also the ability to survive cell death during mammary gland involution and inhibition of carcinogen-induced proliferation (18–20). The cells of these terminal ductal lobular unit-like structures also retain the signature of a developmentally mediated change in cell fate and partial commitment to secretory differentiation (18). Previously, we proposed that pregnancy causes a change in the molecular pathways governing cell fate in the parous mammary gland (17, 20, 21). One plausible mechanism for how this effect might occur is by epigenetic changes induced during pregnancy affecting the regulatory circuits controlling proliferation, survival, and gene expression (17, 22). Such changes restrict the fate of these cells long after the initial inductive event has been removed (23). The aforementioned developmental observations and the noncoding properties of GB7 (PINC) lead us to speculate that its expression comprises part of an epigenetic memory affecting cell fate decisions during development (20). Herein we characterize in detail PINC expression during mammary gland development and cell-cycle progression in mammary epithelial cells, and we propose a functional role for PINC in the regulation of intracellular pathways affecting proliferation and survival in the mammary gland. To our knowledge, this is one of the first reports describing the functional properties of a large, developmentally regulated mammalian ncRNA.

Results

Comparative Analysis of the PINC Locus. Rat GB7 (PINC) is an alternatively spliced ncRNA with a full-length transcript of 6.3 kb (GenBank accession no. AY035343; PINCA) (17) and several shorter transcripts ranging in size from 1.2 to 4.8 kb (PINCB–PINCG) (Fig. 1A). Sequence analysis revealed multiple stop codons and multiple short predicted ORFs (<200 nt) in all three frames of these additional transcripts (Table 1, which is published as supporting information on the PNAS web site), strongly suggesting that these transcripts are also noncoding. Despite this observation, all seven transcripts are spliced and polyadenylated and contain a conserved polyadenylation signal (AATAAA) located 15–33 nt from the polyA tail. These features, polyadenylation and splicing, provide strong evidence that PINC is the product of RNA polymerase II-mediated transcription and is most likely subject to 5′ capping as well (9).

To determine whether *PINC* contains any conserved features that might provide clues to its function, we performed homology searches against the mouse EST and nucleotide databases. These searches identified two related mouse EST clones (GenBank

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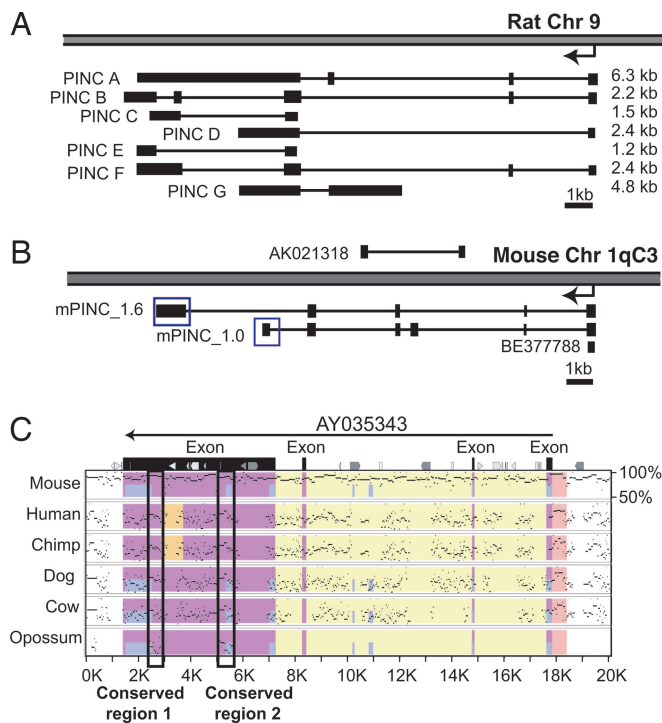
Fig. 1. Overview of the *PINC* genomic region. (*A*) Diagrammatic representation of the rat *PINC* transcriptional locus showing the major 6.3-kb full-length transcript PINC A (GenBank accession no. AY035343) and alternate transcripts B (GenBank accession no. DQ099683), C (GenBank accession no. DQ099682), D (GenBank accession no. DQ105700), E (GenBank accession no. DQ080210), F (GenBank accession no. DQ105701), and G (GenBank accession no. DQ080211). Sizes of the transcripts are indicated. Transcripts initiate from the minus (−) strand and are presented relative to the plus (+) strand of the rat genome. (*B*) Diagrammatic representation of the mouse *PINC* transcriptional locus showing the two transcripts (mPINC_1.6 and mPINC_1.0) identified from the mouse EST database. These transcripts contain unique 3′-terminal exons indicated by two boxed regions. The position of an additional EST clone (GenBank accession no. BE377788) is also indicated. All three transcripts initiate from the minus (−) strand and are presented relative to the plus (+) strand. The position of an independent overlapping transcriptional unit identified as RIKEN clone D530049I02 (GenBank accession no. AK021318) is indicated. AK021318 is transcribed from the plus (+) strand. (*C*) Graphical output from a MULTIPIPMAKER alignment of the *PINC* region and flanking sequences from mouse (assembly May 2004), rat (assembly June 2003), human (assembly May 2004), chimpanzee, dog (assembly July 2004), cow, and opossum genomes using the rat genomic sequence as reference sequence. The transcriptional orientation and position of exons of the main rat transcript (GenBank accession no. AY035343) are indicated above the plot. Genomic features indicated by the colored underlay include conserved exons (light purple), introns (light yellow), alternative exons expressed in the mouse (light blue), and putative proximal promoter (light pink). The exon of an independent, overlapping gene (GenBank accession no. AK024261) is indicated by light orange.

accession nos. DQ059755 and DQ059756), which appear to represent alternatively spliced orthologs of rat *PINC*. The mouse *PINC* gene locus is depicted in Fig. 1*B*. DQ059755 and DQ059756 are 1,630 and 1,017 nt in length, respectively (including polyA+ tails), and are hereafter referred to as mPINC_1.6 and mPINC_1.0. Both transcripts are alternatively spliced and differ from one another by the sequence of their terminal exons and also by the inclusion of an additional exon in mPINC_1.0; however, neither contains a sizeable ORF (≥200 nt; Table 1) and are likely noncoding. Intriguingly, both clones are derived from different mammary gland libraries, thus providing independent evidence for the expression of PINC in the mouse mammary gland. In addition, the mouse *PINC* locus overlaps with an independent transcriptional unit, RIKEN clone

D530049I02 (GenBank accession no. AK021318). mPINC and D530049I02 are encoded on opposite strands but do not share exon sequences, suggesting that they match the criteria of a "nonantisense bidirectional transcriptional locus" and thus are unlikely to regulate one another directly.

BLAST analyses comparing the full-length PINC sequence to genome assemblies and whole-genome sequence databases identified regions in the genomes of the mouse, human, chimpanzee, dog, cow, and opossum that are syntenic with the rat *PINC* locus. The corresponding sequences were obtained through the University of California at Santa Cruz genome browser (http://genome.ucsc.edu) and aligned by using MULTIPIPMAKER (http://pipmaker.bx.psu.edu/pipmaker; Fig. 1*C*). The multispecies alignment showed the presence of two highly conserved regions (boxed regions in Fig. 1*C*) present in the genomes of all seven species. Interestingly, these highly conserved regions correspond, in part, to the unique 3′-terminal regions of mPINC_1.0 and mPINC_1.6, suggesting that conserved functional elements might be encoded in the exons of these two transcripts. In addition, some of this conserved sequence also falls within introns, suggesting that introns may also contain functional sequence elements. A very high level of homology was observed in the 5′ flanking region of *PINC* in most species, suggesting the presence of conserved regulatory elements comprising a proximal promoter. No significant homology was detected between the rat and chicken sequences for the *PINC* locus, although the regions that flank the PINC locus are syntenic and show homologies. In addition, rat *PINC* has no significant homology with any sequences within the fugu, zebrafish, or *Xenopus* genomes, suggesting that *PINC* might be a mammalian-specific gene. To address the question of whether PINC contains a short conserved ORF, we mapped ORFs encoding peptides of ≥50 aa from rat and mouse (Fig. 6, which is published as supporting information on the PNAS web site). However, none of these putative ORFs contained a homologous methionine codon in humans or other species.

To determine whether PINC encodes a microRNA (miRNA), we performed additional alignments with the rat, mouse, and human genomic sequences and identified several areas of extended evolutionary conservation (corresponding to ≥70% homology) over the entire PINC genomic locus (data not shown). Rat sequences comprising these regions were then subjected to secondary structure analysis to identify conserved hairpin structures typical of miRNA precursors. Although two potential precursors with significant similarity ($E < 0.1$ over a >50-nt span of homology) to the corresponding region of the human genome were identified, neither of the corresponding human sequences was predicted to form stable hairpin structures, suggesting that PINC does not encode a miRNA (Craig Burglar and Paul MacDonald, personal communication).

Expression of PINC. PINC was originally identified from a screen for genes that are persistently up-regulated for at least 28 days after pregnancy or after 21 days of treatment with estrogen (E) and progesterone (P) (17). To determine whether PINC is a direct target of E and P or, instead, a marker of alveologenesis, 42-day-old intact rats were treated with E and P for either 2 days (acute treatment) or 7 days (to promote alveologenesis), and PINC RNA levels were examined by quantitative real-time PCR. Mammary glands from 18-day pregnant rats were used as positive control for PINC expression. PINC was not detected after acute treatment with E+P. However, 7-day treatment with either E+P or P alone led to a marked induction of PINC expression, relative to control age-matched virgin glands; treatment with E alone resulted in low but detectable levels of PINC. These data indicate that hormonal treatment (E+P or P alone) sufficient to stimulate modest alveolar budding (Fig. 7, which is published as supporting information on the PNAS web site) induces PINC expression.

We also examined the PINC expression profile at different stages of mammary gland development (Fig. 2*B*). In the mammary gland, PINC expression was induced during pregnancy (18 days pregnant)
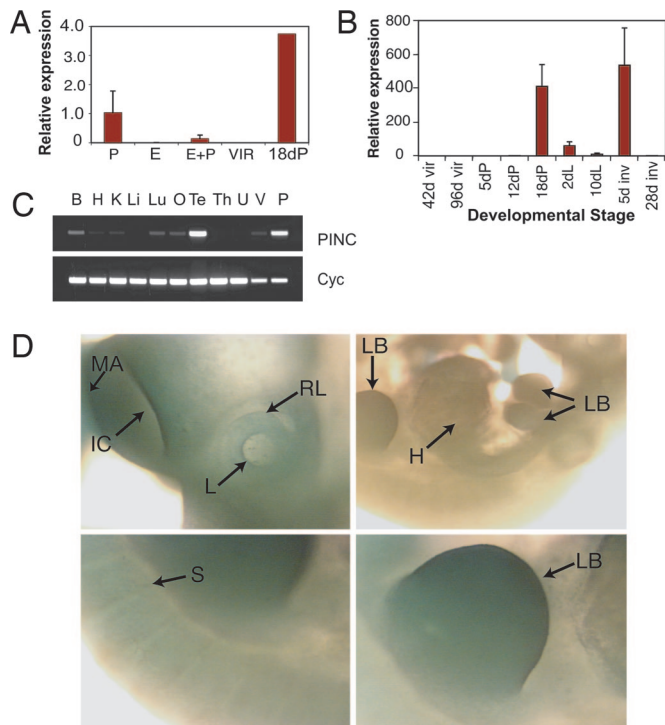
Fig. 2. Developmental and tissue-specific expression of PINC. (*A*) PINC expression in response to the different hormonal regimens described in *Methods*. PINC RNA levels were analyzed by real-time PCR by using primers designed to amplify all seven isoforms of rat PINC and are presented as relative expression when normalized to levels of cytokeratin 8 (a marker of mammary epithelial cells). PINC expression in 18-day-pregnant mammary glands is presented as a positive control (note the use of different scale bars). (*B*) PINC expression at different stages of mammary development in 42-day-old virgin rats (42d vir), 96-day-old virgin rats (96d vir), 5-, 12-, and 18-days-pregnant rats (5dP, 12dP, and 18dP), 2- and 10-days-lactating rats (2dL and 10dL), and rats after 5 and 28 days of involution (5d inv and 28d inv) was assessed by real-time PCR (as described above). (*C*) Tissue-specific expression of PINC. PINC expression was examined by RT-PCR by using RNA prepared from a range of adult tissues: brain (B), heart (H), kidney (K), liver (Li), lung (Lu), ovary (O), testes (Te), Thymus (Th), uterus (U), virgin mammary gland (V), and mid-pregnant mammary gland (P). (*D*) Whole-mount *in situ* hybridization showing PINC expression in a 10.5-day embryo by using a probe that detects both PINC_1.6 and PINC_1.0 transcripts. Expression was detected in the lens (L), heart (H), fore and hind limb buds (LB), intranasal cleft (IC), retinal layer (RL), mandibular arch (MA), and somites (S).

but dropped during lactation, rising again at 5 days of involution. RT-PCR was used to examine the expression of PINC in a number of adult tissues from 96-day-old virgin female rats. PINC was readily detected in 12-days-pregnant mammary gland and testes but could also be detected (at much lower levels) in brain, heart, kidney, ovary, and virgin mammary gland (Fig. 2*C*). Comparable results were obtained from a similar panel of tissues taken from BALB/c mice, the only difference being that PINC was also detected in the uterus (data not shown). *In situ* hybridization experiments showed both transcripts to be abundantly expressed in a range of tissues in mouse embryos. Expression could be detected as early as embryonic day 10.5 and was particularly prominent at the growing margins of the forelimb bud; weaker expression was observed at the retinal layer, developing lens, intranasal cleft, mandibular arch, and heart (Fig. 2*D*). At embryonic days 14.5–16.5 expression was detected in a number of tissues including the hair follicle, whiskers, intestine, heart, lung, developing bone, etc. Thus, PINC expression is abundantly detected during embryogenesis, but high-level expression in adult tissues is restricted to mammary gland and testes.

Because PINC is localized to the lobuloalveolar structures of the
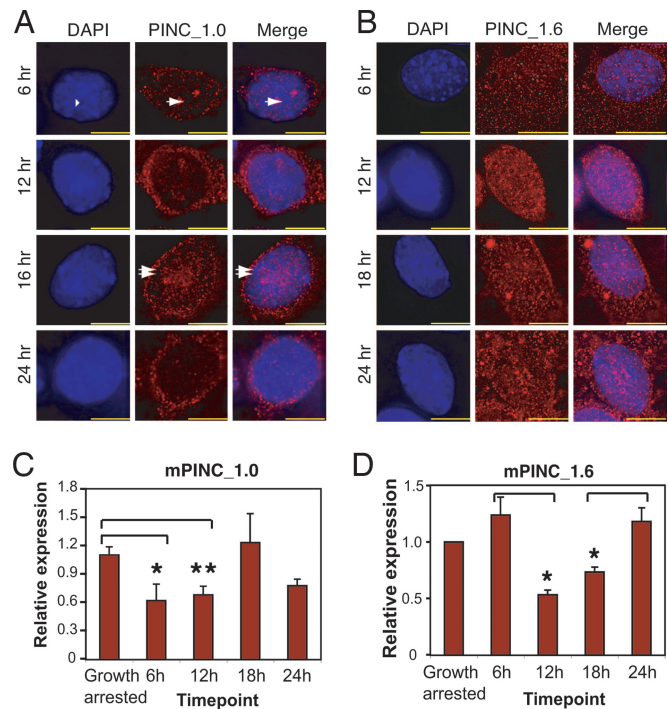
Fig. 3. Expression and intracellular localization of PINC RNA in HC11 cells. (*A* and *B*) FISH showing the localization of mPINC_1.0 (*A*) and mPINC_1.6 (*B*) RNA at different stages of cell cycle in HC11 cells. DAPI-stained images of the same field are shown in parallel. HC11 cells were growth-arrested for 72 h to synchronize cell cycle; normal growth medium was returned, and cells were then fixed on coverslips at various time points over the next 24 h and examined by FISH. Images were obtained by using deconvolution microscopy (10–12 independent fields imaged per time point), and representative captured raw images were deconvolved to produce high-resolution images. (Scale bar: 10 μm.) (*C* and *D*) Expression of mPINC_1.0 (*C*) and mPINC_1.6 (*D*) RNA at different stages of the cell cycle. HC11 cells were growth-arrested for 72 h, and then serum and growth factors were returned. RNA was prepared from cells at various time points after cell-cycle reentry (as indicated), and RNA levels were analyzed by real-time PCR by using primers specific to either mPINC_1.0 or mPINC_1.6. Results were normalized to cyclophilin expression and plotted as relative expression. ∗, $P < 0.05$; ∗∗, $P < 0.005$.

pregnant mammary gland, we predicted that it might be expressed in HC11 cells, a clonal mammary epithelial cell line derived from a mid-pregnant mouse mammary gland (24). Because HC11 cells can be induced to express β-casein, a marker of alveolar differentiation, and have the capacity to form acinar-like structures in three-dimensional culture (25) they appeared to be a suitable *in vitro* model to study the function of PINC. Furthermore, RT-PCR analysis confirmed that mPINC_1.6 and mPINC_1.0 are both expressed in HC11 cells (Fig. 8, which is published as supporting information on the PNAS web site). To examine the intracellular localization of PINC, FISH was performed by using an *in vitro* transcribed RNA probe corresponding to the unique 3′ exon of either mPINC_1.6 or mPINC_1.0. Preliminary experiments comparing the localization pattern of PINC in an asynchronous population of HC11 cells suggested that PINC localization might be regulated by cell cycle (data not shown). Therefore, PINC expression was examined at various time points in synchronized cells after serum and growth factor release from growth arrest. As shown in Fig. 3*A*, mPINC_1.0 was present in a punctate pattern in the nucleus and cytoplasm at 6 h after the readdition of serum and growth factors. Interestingly, nuclear mPINC_1.0 localized to several discrete foci (single arrow), which corresponded to more euchromatic regions (arrowhead); however, during cell-cycle progression (12–16 h) these nuclear foci became more diffuse. At the same time, the

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cytoplasmic mPINC_1.0 signal increased, although mPINC_1.0 remained associated primarily with the perinuclear region (double arrows). By 24 h after reentry into the cell cycle, the nuclear signal diminished and the majority of the signal was localized to the cytoplasm. In contrast, the majority of mPINC_1.6 signal was localized to punctate granular foci within both the nucleus and cytoplasm throughout cell-cycle progression (Fig. 3B). Thus, it appears that localization of each transcript is regulated differentially and that PINC_1.0 localization is regulated in a cell-cycle-specific manner. Last, PINC signal was abrogated by treatment before fixation with Triton X-100-containing CSK buffer, suggesting that PINC is not strongly associated with the nuclear and cytoplasmic matrix (Fig. 9, which is published as supporting information on the PNAS web site).

To determine whether PINC expression varies quantitatively at different stages of the cell cycle, real-time PCR analysis was performed with RNA isolated from HC11 cells at various time points after cell-cycle reentry. In contrast to the FISH analysis, which strongly suggests cell-cycle-dependent regulation of PINC_1.0 localization, total RNA PINC_1.0 levels do not vary significantly in cycling cells. However, they do show a significant decrease as the cells reenter the cell cycle (6-h time point; $P < 0.05$) and continue to remain low at 12 h after cell-cycle reentry ($P < 0.005$). mPINC_1.6 levels, on the other hand, fell significantly ($P < 0.05$) between 6 and 12 h, after readdition of normal growth media, and then slowly recovered by 24 h to the levels observed at the 6-h time point (Fig. 3D). Interestingly, mPINC_1.6 levels do not appear to vary greatly between the 6-h time point and in growth-arrested cells, suggesting that there are no major changes in mPINC_1.6 expression upon the reinitiation of cell cycle. Previous studies using flow cytometry suggest that 53% of HC11 cells have entered S-phase by 14 h after reinitiation of the cell cycle (26); thus, the decrease in PINC expression occurred just before S-phase entry.

Loss-of-Function Experiments. To probe PINC's function in greater detail, we performed knockdown experiments using several *in vitro* transcribed small interfering RNAs (siRNAs) specific to either mPINC_1.6 or mPINC_1.0 (Fig. 4A). Exponentially growing HC11s were transfected with both experimental and control siRNAs, growth-arrested, and analyzed for DNA replication by immunofluorescence detection of BrdU incorporation 6 h after cell-cycle reentry. As expected from previous flow cytometry analyses (26), the number of BrdU-positive cells was extremely low in both the mock-transfected cells and those transfected with an unrelated oligo (Fig. 4B). Similar numbers were observed with cells transfected with an oligo against mPINC_1.0 (1.0A). Surprisingly, cells transfected with an oligo against mPINC_1.6 (1.6A) displayed a 16-fold increase in the number of BrdU-positive cells, suggesting that knocking down mPINC_1.6 facilitated the G_1–S-phase transition (Fig. 4C). Similar results were obtained with two additional independent oligos against mPINC_1.6 ($P < 0.001$) and also by flow cytometry analysis by using the same antibody against BrdU (data not shown).

Because PINC expression was increased during mammary gland involution, when tissue remodeling and apoptosis is occurring, it was of interest to examine the effect of PINC depletion on cell survival. Therefore, HC11s were transfected with siRNA against mPINC_1.6, mPINC_1.0, or the unrelated oligo and then serum-starved for a period of 12 h before TUNEL analysis (Fig. 5A). Normal HC11s are relatively resistant to the effects of serum withdrawal, as exemplified by the limited number of TUNEL-positive cells detected in mock-transfected HC11s. Cells transfected with siRNA against mPINC_1.6 (1.6A) or the unrelated oligo were equally unaffected by serum withdrawal. However, cells transfected with siRNAs against mPINC_1.0 (1.0A, 1.0B, and 1.0C) showed a marked increase in the number of TUNEL-positive cells. This effect was observed with four independent siRNAs against mPINC_1.0 (1.0A, 1.0B, 1.0C, and 1.0D, the results with three of
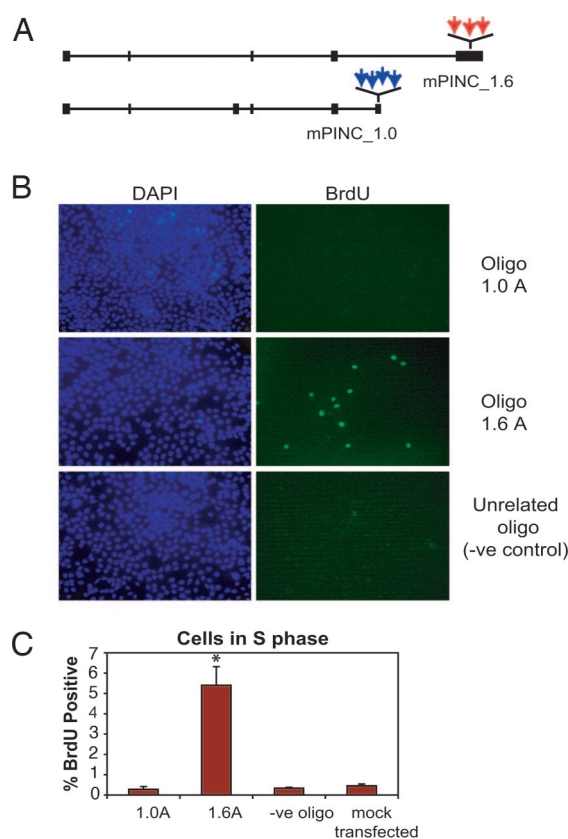

Fig. 4. Knocking down mPINC_1.6 alters cell-cycle progression. (A) siRNA knockdown strategy. siRNAs were designed to target sequences in the unique 3′-terminal exon of mPINC_1.0 and mPINC_1.6 (illustrated diagrammatically) to allow transcript-specific knockdown of each mPINC isoform. (B) Immunofluorescent detection of BrdU incorporation in HC11s transfected with siRNAs against mPINC_1.0 (1.0 A), mPINC_1.6 (1.6 A), or an unrelated negative control oligo and mock-transfected cells. Transfected cells were growth-arrested for 72 h, and then normal growth medium was returned for 6 hours to reinitiate cell cycle. HC11s were labeled with BrdU for 45 min before fixation, and BrdU incorporation was measured by immunofluorescence by using a FITC-conjugated antibody against BrdU. (C) BrdU incorporation was measured by immunofluorescence (as described in B) and calculated as the percentage of BrdU-positive cells relative to total DAPI-stained nuclei averaged over six independent microscope fields. Knocking down mPINC_1.6 (1.6A) resulted in a 16-fold increase in the number of BrdU-positive cells compared with cells transfected with negative control siRNA. The percentage of BrdU-positive cells transfected with siRNA against mPINC_1.0 (1.0A) was similar to that of the control cells. *, $P < 0.002$.

which are shown in Fig. 5A) leading to a 7- to 13-fold increase in TUNEL-positive cells compared with the negative control. Similar results were obtained by immunofluorescence using an antibody against the cleaved form of caspase-3 (Fig. 5B).

Discussion

PINC is an alternatively spliced, polyadenylated, mRNA-like ncRNA persistently expressed in the terminal ductal lobular unit-like structures of the parous rat mammary gland (17). Comparative sequence analysis revealed conserved sequences in the genomes of at least seven mammalian species, leading to the identification of putative orthologs of rat PINC as well as two highly conserved regions with potential functional importance (Fig. 1). Independent evidence from the mouse EST database revealed that at least one of these orthologous genes is expressed, leading to two alternatively spliced isoforms in the mouse (mPINC_1.6 and mPINC_1.0). Although it lacks an apparent protein coding function, its conserved syntenic location and the

A

TUNEL

B

Cleaved caspase-3 activity
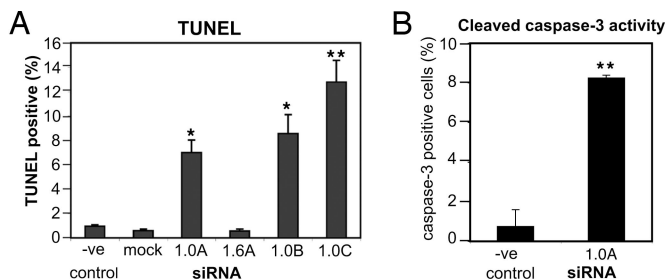
Fig. 5. Knocking down mPINC_1.0 induces apoptosis in serum-free conditions. (*A*) The percentage of TUNEL-positive cells in HC11s transfected with siRNA against mPINC_1.0 (siRNAs 1.0A, 1.0B and 1.0C), mPINC_1.6 (1.6A), negative control siRNA, or mock-transfected siRNA was calculated relative to total number of DAPI-stained nuclei. $**$, $P < 0.0005$; $*$, $P < 0.003$. (*B*) HC11s were transfected with siRNA against mPINC_1.0 (1.0A) or a negative control oligo, maintained under normal growth conditions for 48 h after transfection, and then placed in serum-free medium for 3 h before fixation. Caspase-3 activation was examined by immunofluorescent detection of cleaved caspase-3, and the percentage of cleaved caspase-3-positive cells was calculated relative to the total number of DAPI-stained nuclei. $**$, $P < 0.0005$; $*$, $P < 0.003$. Similar results were obtained with three independent siRNAs against mPINC_1.0 (data not shown).

degree of conservation between these orthologous genes suggests that evolutionary constraints have prevented the divergence of functional elements within this gene. Perhaps the greatest clue to its function lies in the identification of two regions of high evolutionary conservation that encompass sequences specific to the unique 3′-terminal exons of either mPINC _1.0 or mPINC _1.6. RNA interference knockdown experiments using siRNAs specific to either of these exons (and thereby specific to either mPINC_1.0 or mPINC_1.6) point to distinct roles for mPINC_1.0 and mPINC_1.6 in cell survival and regulation of cell-cycle progression, respectively (Figs. 4 and 5), suggesting that an appropriate balance of the two transcripts is important for the maintenance of these cells. Thus, it is tempting to speculate that these conserved elements are central to the function of PINC. These elements perhaps contain binding sites for RNA-binding proteins, transcription factors, or even localization signals that target PINC to a particular cytoplasmic locality.

In HC11 cells intracellular localization of PINC is regulated in a cell-cycle- and transcript-specific manner, with distinct foci observed in both the nucleus and cytoplasm at different time points during the cell cycle (Fig. 3). At the 6-h time point mPINC_1.0 signal was observed as discrete euchromatic nuclear foci. These foci became more diffuse and gradually disappeared as the cell cycle progressed, correlating with a shift to a more cytoplasmic localization. These observations suggest a possible nuclear function for mPINC_1.0, as well as a role in shuttling or signal transduction between the nucleus and cytoplasm. mPINC_1.6, on the other hand, is localized as discrete foci (suggestive of ribonucleoprotein particles) throughout the nucleus and cytoplasm, possibly implying an involvement with intracellular trafficking. Changes in the levels of total PINC RNA suggest that the RNA is relatively unstable or that its stability is regulated in a temporal fashion by its association with RNA-binding proteins.

Based on the known properties of other ncRNAs, what are the most likely functions of PINC? Because PINC does not appear to encode a conserved precursor structure, typical of a "classical" miRNA (27), this type of function seems unlikely. However, recent evidence demonstrating the presence of primate-specific miRNAs (28) suggests that not all miRNAs are conserved; hence, this function cannot be ruled out entirely. PINC does not seem to comprise part of an imprinted locus, or at least is not located in a genomic location referenced by any database of imprinted loci

(www.mgu.har.mrc.ac.uk/research/imprinting/imprin-intro.html). Likewise, PINC does not appear to be part of a sense–antisense pair, but this categorization also depends on the completeness of publicly available databases. Evidence of splicing precludes the possibility that PINC is an expressed pseudogene. Other roles for ncRNAs, based on sequence-directed nucleic acid recognition, have also been proposed (1, 29–33), and it is possible that sequence (or even structural recognition) plays a role in the function of PINC.

The observation that PINC is present in discrete foci in the nucleus (Fig. 3) suggests it might play a role in guiding DNA methylation, transcriptional modulation, or chromatin modification in the nucleus. However, because the PINC RNA signal was abolished by CSK extraction it is likely that it is associated with soluble proteins that can move between the two compartments rather than with the nuclear and cytoplasmic matrix directly. Based on the current evidence and the limited number of known mechanisms for ncRNA function, we foresee a number of potential roles for PINC in transcriptional and/or chromatin modulation as a localized RNA or as part of a cytoplasmic signal transduction pathway. We propose that the conserved domains present in the 3′-terminal exons of mPINC_1.6 and mPINC_1.0 confer discrete functions, and these functions might be mediated by an association with RNA-binding proteins or by sequence-directed nucleic acid recognition.

Function for PINC in the Mammary Gland. We have shown that the levels of mPINC_1.6 decrease before S-phase entry. Conversely, knocking down mPINC_1.6 leads to premature S-phase entry in HC11 cells. These observations suggest that PINC may play a role in regulating cell-cycle progression and that PINC levels may need to be reduced to facilitate S-phase entry. Knocking down mPINC_1.0, on the other hand, predisposes HC11 cells to apoptosis, suggesting that mPINC_1.0 is a cell survival factor.

PINC is up-regulated during alveolar development in the rat mammary gland, but not by short-term treatment with E and P, suggesting that, although it may not be a direct target of these hormones, PINC can serve as a marker of partially committed alveolar cell fate. Previous studies from our laboratory have shown that PINC expression is enriched in a specific population of mammary epithelial cells localized to the terminal ductal lobular unit-like structures of the involuted mammary gland (17). A similar population of cells (20) have also been shown to possess the capability not only to survive involution-mediated cell death (19) but also to resist further proliferative stimuli (such as exposure to chemical carcinogen) (34). These observations and the complementary functions of mPINC_1.0 and mPINC_1.6 (modulating cell survival and cell-cycle regulation) suggest that PINC is more than merely a marker of alveologenesis. We postulate that PINC, therefore, may play a role in modulating cell fate in this specific population of mammary epithelial cells and, as such, forms part of the "functional memory" or "epigenetic imprint" imposed by parity (17, 20, 22). Such epigenetic marks are important for preserving the cell's identity and for preventing cell-lineage aberrations leading to cancer (23). To our knowledge, this is one of the first studies to demonstrate a function for a large, developmentally regulated, mammalian ncRNA.

Methods

Clones and Sequence Analysis. All rat clones were obtained from a rat mammary cDNA library as described previously (17). EST clones (GenBank accession nos. DQ059755 and DQ059756) were obtained from Invitrogen. Sequencing was performed by the Sequencing Core at the Baylor College of Medicine.

The following computational tools were used: REPEATMASKER (www.repeatmasker.org), GENSCAN (http://genes.mit.edu/GENSCAN.html), BLAST (www.ncbi.nlm.nih.gov/blast), and MULTIPIPMAKER. For visual representation of the PINC transcrip-

tional locus, we used the University of California at Santa Cruz genome browser.

Animals. Wistar–Furth rats and BALB/c mice were purchased from Harlan Sprague–Dawley. Animals were housed in an approved facility using Association for Assessment of Laboratory Animal Care guidelines. All experiments were performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* using protocols approved by the Baylor College of Medicine Subcommittee for Animal Use.

Hormonal Manipulations and Developmental Time Points. Forty-two-day-old virgin rats were treated with a priming dose of estradiol benzoate (Sigma) (2.5 μg per animal) as described previously (17), then divided into four groups and treated with 200 μg of E ($n = 3$), 20 mg of P ($n = 3$), 200 μg of E and 20 mg P (E+P; $n = 3$), or vehicle alone [age-matched virgin (AMV); $n = 4$] for a period of 7 days. Hormones were delivered by s.c. implantation of silastic capsule as described (35). For developmental time points, inguinal mammary glands were excised from timed pregnant rats at 6, 12, and 18 days of pregnancy, from 2- and 10-day lactating dams, and from rats after 10 days of lactation and 5 and 28 days of forced involution. In addition, inguinal mammary glands were obtained from 45-day-old (immature) virgin and 96-day-old (mature) virgin rats. In all cases the right gland was flash-frozen for RNA, and the left gland was fixed in 10% neutral-buffered formalin (24 h at 4°C) and stained as described previously (36) to ensure that they displayed morphological features appropriate for each stage of development.

Tissue Culture and RNA Interference Knockdown Experiments. HC11 cells (24), a clonal cell line of COMMA-D, were routinely maintained as previously described (37). For experiments requiring synchronization, normal growth medium was replaced with growth arrest medium (RPMI medium 1640 supplemented with 0.1% bovine calf serum). Double-stranded siRNA against mPINC_1.0 and mPINC_1.6, as well as an unrelated nonspecific control siRNA

(Ambion), were prepared by using the Ambion siRNA Construction kit. Transfection was performed by using 100 nM (final concentration) siRNA and siPORT Amine transfection reagent (Ambion) according to the manufacturer's instructions. Mock transfections were performed by using siPORT Amine alone (no siRNA). Transfection efficiency was monitored by using Cy3-labeled tracer siRNA, and knockdown was monitored by *in situ* hybridization (see Fig. 10, which is published as supporting information on the PNAS web site).

Proliferation and Apoptosis Assays. Proliferation was monitored by BrdU staining as described (38). Apoptosis was detected by TUNEL staining (37) or by immunofluorescence using an antibody specific to the cleaved (active) form of caspase-3 (Santa Cruz Biotechnology).

Real-Time PCR. Quantitative RT-PCR with SYBR green detection (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site) was performed as described (39), and expression levels for rat PINC, mPINC1.6, and mPINC1.0 normalized to either cyclophilin or cytokeratin 8 levels (as indicated in the text).

***In Situ* Hybridization and FISH.** *In situ* hybridization was performed by using digoxigenin-labeled complementary RNA probes and paraformaldehyde-fixed embryos or cells. For embryos, hybridization signals were detected by using an alkaline phosphatase-conjugated antibody against digoxigenin (Roche). For FISH, hybridization signals were detected by fluorescence (as described in *Supporting Materials and Methods*).

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