Loss of Pnn expression results in mouse early embryonic lethality and cellular apoptosis through SRSF1-mediated alternative expression of Bcl-xS and ICAD**

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Running title: Pnn depletion induces cellular apoptosis

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Summary

Pinin (Pnn), a serine/arginine-rich (SR)-related protein, has been shown to play multiple roles within eukaryotic cells including in cell-cell adhesion, cell migration, regulation of gene transcription, mRNA export, and alternative splicing. In this study, an attempt to generate mice homozygously deficient in Pnn failed due to early embryonic lethality. To evaluate the effects of loss of Pnn expression on cell survival, RNA interference experiments were performed in MCF-7 cells. With Pnn-depletion, cellular apoptosis and nuclear condensation were observed. In addition, nuclear speckles were disrupted, and expression levels of SR proteins were diminished. RT-PCR analysis showed that alternative splicing patterns of SRSF1 as well as of apoptosis-related genes Bcl-x and ICAD were altered and expression levels of Bim isoforms were modulated in Pnn-depleted cells. Cellular apoptosis induced by Pnn depletion was rescued by overexpression of SRSF1 which also restored generation of Bcl-xL and functionless ICAD. Pnn expression is, therefore, essential for survival of mouse embryos and the breast carcinoma cell line MCF-7. Moreover, Pnn-depletion, modulated by SRSF1, determines cellular apoptosis through activation of expression of pro-apoptotic Bcl-xS transcripts.

Key words: Pnn, pinin, apoptosis, SRSF1 (ASF/SF2), Bcl-xS, ICAD
**Introduction**

Sequencing of the human genome and genomes from other organisms has underlined the importance of alternative pre-mRNA splicing for the expression of a myriad proteins from an unexpectedly small set of genes (Waterston et al., 2002; International Human Genome Sequencing Consortium, 2004). Intron removal is regulated by a mechanism involving RNA *cis*-elements and their interaction with a complex of splicing factors. Among these splicing factors, serum/arginine-rich (SR) proteins and SR-related proteins have been shown to play an important role through their modulation of alternative splicing which generates different isoforms of proteins in a tissue-specific manner. Impairment in alternative splicing mechanisms usually causes disease (Solis et al., 2008).

Pinin (Pnn), a SR-related protein, was originally characterized as a desmosome-associated protein found at the cytoplasmic face of the desmosome plaque at the convergence of intermediate filaments (Ouyang and Sugrue, 1992; Ouyang and Sugrue, 1996). Subsequent studies have demonstrated that Pnn not only exists at epithelial cell-cell contact sites, but also localizes in the nuclear “speckle” domain, a subnuclear region for assembly and storage of splicing factors (Brandner et al., 1997; Ouyang, 1999). Closer examination of the amino acid sequences of Pnn indicated that Pnn is a SR family-related protein because it contains an Arg-Ser (RS) domain in its carboxyl terminus but not the regular RRM (RNA recognition motif) structure found in genuine SR proteins. Pnn is multi-functional within the nucleus. Its roles include regulation of alternative splicing, mRNA export, and transcriptional activity regulation (Wang et al., 2002; Li et al., 2003;
Alpatov et al., 2004). Yeast two hybrid assay and co-immunoprecipitation experiments demonstrated that Pnn interacts with several splicing-related SR family proteins, including SRp75, SRm300, SRp130, SR-cyclophilin and RNPS1 and binds preferentially to spliced-mRNA (Li et al., 2003; Lin et al., 2004; Zimowska et al., 2003). Apart from its roles within the nucleus, Pnn has also been shown to be involved in other cellular functions including the enhancement of cell-cell adhesion (Ouyang and Sugrue, 1996) and corneal cell migration (Shi et al., 2000b). Abnormal expression of Pnn in tumor cells has also been reported (Shi et al., 2000a). Recent studies have further indicated that depletion of Pnn in vitro affects cell-cell adhesion (Joo et al., 2005) and attenuates expression levels of SR proteins (Chiu and Ouyang, 2006).

An increasing number of SR and SR-related proteins and functions are being identified (Long and Caceres, 2009; Zhong et al., 2009). Most of the functions of the SR and SR-related proteins identified so far have been revealed in cell culture experiments; in contrast, only a few knockout approaches have been applied (Moroy and Heyd, 2007; Mende et al., 2010). Targeted disruption of Sfrs10 (Mende et al., 2010), SRp20, SRSF2 (also known as SC35), and SRSF1 (also known as ASF/SF2) resulted in early embryonic lethality (Moroy and Heyd, 2007). Recently, using a tissue-specific mouse model with deletion of exons 3 to 8 in pnn gene, investigators showed that Pnn plays important roles in development of the heart, cornea, and intestine (Joo et al., 2007; Joo et al., 2010a; Joo et al., 2010b). Although all of these loss-of-function mouse models demonstrated that SR proteins are
important for embryonic development, the underlying mechanism relating loss of SR protein expression to embryonic lethality or cellular apoptosis has not been clarified.

To explore the biological function of Pnn in mammals, we generated a targeted Pnn disruption mouse model by replacing exon 1 and exon 2 of pnn with a β-geo (β-galactosidase/neo fusion) selection cassette. Since homozygous Pnn deficiency was found lethal at an early embryonic stage, we further used RNAi to deplete Pnn expression in vitro and explored the mechanism underlying the in vivo observations. Results from RNAi-treated culture cells showed that loss of Pnn expression not only altered SRSF1 alternative splicing but induced apoptosis in MCF-7 cells through alternative expression of Bcl-xS. We further found that overexpression of SRSF1 rescued cellular apoptosis and reversed expression of the pro-apoptotic Bcl-xS isoform in Pnn-depleted MCF-7 cells.

Materials and Methods

Construction of shRNA plasmids

Pnn-shRNA-811 was directly purchased from Open BioSystem (Huntsville, AL); it is a microRNA based vector, containing a stretch of 19 bp against mouse Pnn starting from nucleotide 811. Other RNAi vectors were constructed based on pSuper system (Oligoengine, WA). Construction of pSuper-based shRNA vectors was performed according to the manufacturer’s instructions. Sense and antisense oligonucleotides were annealed and ligated with Bgl II and Hind III-digested pSuper-Neo-GFP vectors. The constructs were confirmed by restriction enzyme digestion and sequencing. The sequences used in Pnn-shRNA
constructs were as follows; Pnn-shRNA-411: 5′-GCAAAGGAA CCGACGAATA-3′; Pnn-shRNA-925: 5′-GGTAAGGTGGCTCAGCGAG-3′. The numbers used in each construct indicate the first nucleotides of each pSuper-based shRNA sequence relative to the murine Pnn translation-starting site. SiRNA duplexes against Pnn (Cat no. 4392420, siRNA ID: 10757 and 10759) were purchased from Ambion (Austin, TX) and used to knockdown Pnn expression with lipofectamine transfection. For transfection, 5 × 10⁴ cells were cultured 24 hours prior to transfection and siRNA duplex/lipofectamine mixture was added to the medium and incubated for 48 hours.

Immunofluorescence staining

Purified plasmids were transfected into MCF-7 cells with lipofectamine 2000 (1μg DNA and 2 μl lipofectamine/5 × 10⁴ cells, Invitrogen, Carlsbad, CA) followed by seeding into cover slips. Thirty-six hours after transfection, cells were fixed with 4% paraformaldehyde and permeated with 0.5% Triton X-100 and incubated with either Pnn-specific rabbit anti-serum P3A (1:1000 dilution), mouse monoclonal anti-ASF/SF2 antibody (1:200, Chemicon, CA), mouse monoclonal anti-SC35 antibody (ATCC CRL-2031, 1:1000 dilution), rabbit polyclonal anti-PARP antibody (1:1000, Santa Cruz Biotech, California), rabbit polyclonal anti-cleaved PARP antibody (1:100, Chemicon, CA), mouse monoclonal anti-SR protein antibody (ATCC CRL-2067, clone 1H4, 1:100 dilution), or rabbit polyclonal anti-U1A antibody (1:200, Abcam, MA) at 4°C overnight. Rhodamine or AMCA-conjugated goat anti-rabbit or mouse IgG (Jackson Immunoresearch Laboratories, PA) were then used to
localize signals and cover slips were observed with a fluorescence microscope equipped with epifluorescence (Axioscope II plus, Zeiss, Germany).

**Construction and preparation of AAV-ASF**

Full-length cDNA of human ASF/SF2 was amplified with the primer pair set:

GGGTCGAC-GTACGAGAGCGAGATCTGCTA and

GGAAGCTTCCATGTCGGGAGGTGGTGTG-AT and then subcloned to

pDsRed-Monomer-C1 (Clontech, CA) to generate the DsRed-ASF fusion protein. The DsRed-ASF cDNA was further subcloned to pAAV-MCS (Stratagene) to construct the vector AAV-ASF to express DsRed-ASF fusion protein in the adeno-associated virus (AAV) system. To prepare AAV, the AAV-293 cell line (Stratagene) was cultured in DMEM growth medium 48 hours prior to transfection. Cells were co-transfected with AAV-ASF, pAAV-Helper (Stratagene), and pAAV-RC (Stratagene) and incubated for 72 hours. To obtain viral stocks, transfected AAV-293 cells were scraped into 15 ml tubes and then subjected to four rounds of freeze/thaw by alternating the tube between a dry ice-ethanol bath and a 37°C water bath vortexing briefly after each thaw. Cellular debris was collected by centrifugation at 10,000 × g for 10 min at room temperature. Supernatants containing viral stock were transferred into a fresh tube and stored at -80°C.

**Minigene construction and RT-PCR**

Bcl-x minigene (Bcl-x-g) was constructed following Massiello et al. (Massiello et al., 2004). In brief, two fragments of Bcl-x genomic DNA were amplified by PCR and genomic DNA
extracted from MCF-7 was used as a template. One fragment containing exon 1, intron 1, exon 2, and partial intron 2 was amplified with following primer pairs:

**GCATTCCTCGAGGGCGGA TTTGAATGTAGGT and**

**GCATTTCGGCCGAGAGAAGCCCAGGATAGGA; another primer set:**

**GCATTCCGGCCCGCCCTGACCCCTGCCCCATCTCGTT and**

**GCATTCAAGCTTACCAGCGGTTGAAGCGTTCCCT was used to amplify the DNA**

fragment containing partial intron 2 and exon 3. DNA fragments were then subcloned to pcDNA 3.1-myc-His. For minigene isoform expression assay, minigenes were co-transfected with siRNA duplex and/or infected with AAV-ASF. RT-PCR was then performed 48 hours after transfection. To detect the mRNA isoform expression of genes of interest by RT-PCR, total RNA was extracted from cells and then reversed-transcribed with oligo-dT primer and MMLV (Epicentre Biotechnologies, Madison, WI) to synthesize cDNA. PCR was carried out for 35 cycles (94°C, 30 sec; 58°C, 30 sec; 72°C, 2 min). Specific primer sets are listed in Supplementary Table 1. Results of PCR were examined in 1.5% agarose gel electrophoresis.

**Nuclear fractionation and western blot**

Whole cell lysates were subjected to western blot with anti-Pnn, anti-β-actin, anti-SC35, or anti-ASF/SF2 antibodies, while nuclear fractions extracted with Nuclear Extract Kit (Active Motif, CA) were used for PARP western blot. For nuclear fractionation, in brief, cells were washed with PBS and incubated with detergent-containing hypotonic buffer. After incubation on ice for 15 min and centrifugation at 850 × g at 4°C, supernatant (cytoplasmic
fraction) was removed, while insoluble pellets containing nuclei were further resuspended with complete lysis buffer and incubated at 4°C for 30 min with orbital shaking. After vigorous vortexing and centrifugation, supernatants (nuclear fraction) were transferred to pre-chilled tubes and stored at -80°C. Nuclear fractions and whole cell lysates were separated by SDS-PAGE and electro-transferred to PVDF membrane followed by blocking with 5% milk in TPBS (0.05% Tween 20 in PBS pH 7.0). Blots were then incubated with either P3A (1:6000), mouse monoclonal anti-SRSF1(ASF/SF2) antibody (1:1000, Chemicon, CA), mouse anti-SRSF2 (SC35) (1:1000, Sigma, MO), mouse monoclonal anti-β-actin antibody (1:1000, Sigma, MO), or rabbit polyclonal anti-PARP antibody (1:1000, Santa Cruz Biotech, CA) at room temperature for 1 hour. Signals were detected with HRP-conjugated goat anti-mouse, goat anti-rabbit, or rabbit anti-goat IgG with ECL (Perkin Elmer, MA).

Results

Depletion of Pnn induces apoptosis in vitro

To gain insight into the in vivo functions of pnn, we employed ES cell gene-targeting and homologous recombination to generate Pnn-deficient mice (Supplementary Fig. 1). However, no homozygote offspring or embryos earlier than blastocyst were found from heterozygote crossing (Supplementary Table 2), we therefore concluded that deficiency of Pnn leads to early embryonic lethality.
To explore the mechanism responsible for Pnn deficiency-induced embryo lethality, we employed RNA interference (RNAi) to knockdown Pnn expression in cultured cell lines. To achieve this, a pSuper-based shRNA, Pnn-shRNA-411, was transfected into MCF-7 cells. Immunofluorescence microscopy showed that Pnn immunostaining was almost absent in Pnn-shRNA-411-transfected cells (Fig. 1A, a-c). The specificity of this shRNA in depleting Pnn expression was confirmed by transfection with pSuper null vector (Fig. 1B, d-f) or a pSuper-based shRNA against p53 (p53-shRNA, Fig. 1B, g-i), neither of which reduced Pnn immunostaining (Fig. 1B, e, h). The nuclei of Pnn-shRNA-411-transfected cells, shown by DAPI staining, were reduced in size and displayed a condensed pattern (Fig. 1A, arrows in c, f, i, o). Since the condensed nucleus is one of the hallmarks of apoptotic cells, we went on to examine whether loss of Pnn triggers cell apoptosis by immunostaining Pnn-shRNA-411-transfected cells with an antibody against poly (ADP-ribose) polymerase (PARP). In apoptotic cells, PARP is cleaved by caspase-3 into two fragments that can be identified by specific antibodies. In Pnn-shRNA-411-transfected cells, PARP immunostaining was totally abolished (Fig. 1A, d-f) with prominent cleaved-PARP staining (C-PARP, Fig. 1A, g-i), suggesting that Pnn deficiency induced apoptosis. Other nuclear protein, like p53, was not affected or reduced in amount (Fig. 1A, j-l), while expression levels of cell proliferation marker, ki-67, was diminished remarkably in Pnn-depleted cells (Fig. 1A, m-o). Complete abolition of Pnn expression seems to be necessary for cell apoptosis since knockdown of Pnn by another Pnn sequence-specific shRNA (Pnn-shRNA-925), which reduces Pnn expression in MCF-7 cells to a lesser degree, did not cause apoptosis (Fig. 1B, a-c). Finally, we also calculated the apoptotic events and
percentage of loss of Pnn expression upon Pnn-depletion in shRNA-treated MCF-7 cells. Diminished or loss of Pnn expression as well as apoptotic events occurring in Pnn-depleted cells were significantly higher than those transfected with control pSuper-EGFP vector or Pnn-shRNA-925 (Fig. 1C and 1D), demonstrating complete abolition of Pnn is required and necessary for cellular apoptosis in MCF-7 cells.

As our results suggested that Pnn deficiency causes mouse embryo early lethality, we further tested whether Pnn depletion-induced apoptosis occurred in embryonic stem (ES) cells. A pluripotent ES cell line derived from E3.5 mouse embryos with characteristics of early developing embryos, E14, was used for RNAi experiments. Since Pnn-shRNA-411 failed to work on E14 cells, a construct, Pnn-shRNA-811, driven by mouse U6 promoter, was used to reduce Pnn expression in mouse ES cells. Following Pnn-shRNA-811 transfection, the expression level of Pnn in ES cells was significantly reduced (Fig. 2, a-b). Consistent with the knockdown of Pnn in MCF-7 cells, transfected cells also displayed condensed nuclei in a Pnn depletion-dependent manner (Fig. 2, arrows in c, f, i). Furthermore, full-length PARP also disappeared in Pnn-shRNA-811-transfected ES cells (Fig. 2, g-h), indicating that apoptosis indeed occurs in the absence of Pnn expression in ES cells. These results may explain the mechanism by which targeted disruption of pnn in mouse embryos elicited early embryonic lethality.

In addition to immunofluorescence staining, we also performed western blot to quantify the expression levels of Pnn and cleaved-PARP in cells lacking Pnn expression. To improve
transfection efficiency and quantification, a siRNA duplex mixture was used to knockdown Pnn expression in MCF-7 cells and the expression levels of Pnn displayed a dose-dependent reduction (Fig. 3). In addition, the apoptotic marker, cleaved-PARP (Fig. 3) was found only in the Pnn-siRNA-transfected cells. To eliminate the off-target effect of siRNA duplex that may cause cellular apoptosis with elevated cleaved-PARP, we performed Western blot to confirm the knock down effect of Pnn on cleaved-PARP (c-PARP) and full-length PARP (f-PARP) expression with another set of Pnn-shRNA (Supplementary Fig. 2). The results showed that following Pnn depletion, c-PARP expression levels gradually elevated with concomitant diminishing f-PARP expression. Taken together, the combined results from the in vivo gene targeting and the in vitro RNAi-treated cultured cells suggest that Pnn expression is essential for the survival of cultured MCF-7 cells and the growth of early embryos.

**Loss of Pnn abolishes expression of nuclear speckle-associated SR proteins**

Depletion of SR protein expression, such as those of SRSF1 and SRSF2, has been reported to arrest the cell cycle and induce apoptosis (Li et al., 2005; Xiao et al., 2007). Our previous study also demonstrated a positive correlation between Pnn expression level and that of nuclear speckle proteins (Chiu and Ouyang, 2006). To elucidate whether apoptosis induced by Pnn depletion is related to loss of expression of certain SR proteins, we examined the expression patterns of SR proteins in Pnn-depleted MCF-7 cells. In Pnn-shRNA-411 transfected cells, apparent absence of immunostaining for SRSF2 (Fig. 4e), SRSF1 (Fig. 4h), and general SR proteins (Fig. 4b, demonstrated by 1H4 antibody staining) was identified.
Significant higher numbers of cells with reduced SRSF1 immunostaining was also apparent when calculating cells transfected with shRNA-411 versus those with pSuper-control vector or shRNA-925 (Fig. 1E). Consistently, depletion of Pnn in ES cells also reduced SR protein expression (Fig. 2, d-f, demonstrated by 1H4 antibody staining). Furthermore, cells devoid of SR protein immunostaining again displayed condensed nuclei (Fig. 4, arrows in c, f, i).

Western blot showed attenuated SRSF1 and SRSF2 expression levels following Pnn depletion in MCF-7 cells (Fig. 3). Despite the apparent absence of SR proteins in nuclear speckles, unlike SRSF1 and SRSF2, U1A, a component of snRNPs, displayed a diffuse staining pattern throughout the nucleoplasm in cells lacking Pnn expression (Fig. 4, j-l), indicating that Pnn absence specifically compromises SR protein family expression rather than other components of the splicing machinery.

**Loss of Pnn promotes pre-apoptotic Bcl-xS alternative expression and alters SRSF1 splicing patterns**

Pnn has been identified in pre-mRNA splicing functional complexes in several genome-wide proteomic analyses (Chen et al., 2007) and also demonstrated to be a splicing regulator (Wang et al., 2002). Since numerous apoptotic factors are modulated via alternative splicing (Schwerk and Schulze-Osthoff, 2005), it is conceivable that cellular apoptosis triggered by Pnn depletion may be through differential isoform expression of certain apoptotic factors. To identify the putative apoptotic factors whose mRNA isoform expression was modulated by Pnn depletion, RT-PCR was employed to measure the expression level of each mRNA isoform. The results showed that Bcl-x and Bim displayed altered isoform expression.
following Pnn depletion (Fig. 5A, lanes 1-3), while other apoptotic factors displayed little or no change in isoform expression ratio (Fig. 5A, lanes 1-3). Short mRNA isoform of Bcl-x (Bcl-xS) was found in MCF-7 cells transfected with Pnn-shRNA-411, thereby increasing the expression ratio of short form to long form (S/L) but was absent in cells transfected with pSuper control vectors (Fig. 5A, Bcl-X panel, compare lanes 2 and 3 vs. 1). Bcl-xS has been demonstrated to be involved in the apoptosis pathway, and overexpression of Bcl-xS resulted in apoptosis in MCF-7 cells and tumors in nude mice (Clarke et al., 1995; Ealovega et al., 1996). To confirm the results of the RT-PCR analysis of endogenous Bcl-x isoform expression, we constructed a Bcl-x minigene (Bcl-x-g) which can be spliced to generate Bcl-x short form and long form (Fig. 5B) for in vivo splicing assay. Consistent with the result shown for endogenous Bcl-x mRNA isoform expression, the Bcl-xS to Bcl-xL (S/L) ratio was increased along with increment of Pnn siRNA duplex dosage, while Bcl-xL was diminished in Pnn-depleted cells (Fig. 5B, lanes 1-3). Therefore, using RT-PCR analysis of mRNA isoform profiles of apoptotic factors, we found that Pnn depletion could activate expression of pro-apoptotic Bcl-xS. In addition to Bcl-x, we also noted that expression levels of Bim were increased in Pnn-depleted MCF-7 cells (Fig. 5A, Bim panel). Several isoforms of Bim, a member of Bcl-2 family with known pro-apoptotic activity via a Bcl-2 related pathway, have been found and each of them has been reported to be capable of promoting apoptosis (O’Connor et al., 1998). Although the expression levels of Bim transcripts were increased, the ratio among different isoforms appeared similar in control and shRNA-transfected MCF-7 cells.
As Pnn modulated Bcl-x alternative splicing, we tried to determine whether the attenuated expression of SRSF1 at the protein level (Fig. 3) was due to alternative splicing of transcripts modulated by Pnn depletion. Two SRSF1 mRNA isoforms, a short and long form, are found in MCF-7 cells. The major difference between the short and long form is the insertion of intron 3 into the long form, which generates a function-less mRNA isoform with frame shift mutation. We noted that in control MCF-7 cells the major product of SRSF1 gene was the functional short form, while in Pnn-depleted cells, the function-less isoform (the long form) of SRSF1 increased concomitant with decreasing short form (Fig. 5A, SRSF1 panel, lanes 1-3). Over-expressing SRSF1 in Pnn-depleted cells by infection with SRSF1-containing adenovirus-associated virus (AAV-SRSF1), however, increased the functional SRSF1 mRNA (Fig.5A, SRSF1 panel, lane 4). This phenomenon suggests that reduction in SRSF1 protein level in Pnn-depleted cells (Fig.3) may be through splicing inhibition of functional SRSF1 mRNA isoform. Collectively, these data indicate that Pnn depletion in MCF-7 cells not only induces splicing activation of certain pro-apoptotic factors but also decrease SRSF1 protein expression through altered splicing of SRSF1 at transcript levels.

**Overexpression of SRSF1 rescues apoptosis induced by Pnn depletion**

Previous studies have demonstrated that SRSF1 is essential for the proliferation and survival of cells and that Bcl-x isoform expression is regulated by SRSF1 (Li et al., 2005; Paronetto et al., 2007). Diminished SRSF1 expression in Pnn-depleted cells suggested that apoptosis triggered by Pnn depletion may be through decreased expression of SRSF1. To clarify
whether apoptosis is a direct consequence of Pnn depletion or indirect via SRSF1 downregulation, we overexpressed SRSF1 to see whether it was possible to rescue Pnn-depleted MCF-7 cells.

To monitor overexpression of SRSF1, we used HA-tagged SRSF1 (SRSF1-HA) for transfection and AAV-SRSF1 for infection. Pnn-shRNA-411-transfected MCF-7 cells were either co-transfected with SRSF1-HA (Fig. 6, d-l) or infected with AAV-SRSF1 (Fig. 6, a-c) before being analyzed by immunostaining. Although Pnn expression was abolished by RNAi (Fig. 6, d-f), with SRSF1-HA or AAV-SRSF1 expression, apoptotic morphology was not found. Both nuclear condensation (Fig. 6, a-c) and cleaved-PARP (Fig. 6, g-l) were absent in SRSF1 overexpressed Pnn-depleted MCF-7 cells. Overexpression of SRSF2 or RNPS1, however, did not restore cellular apoptosis elicited by pnn depletion (data not shown), demonstrating the specificity of SRSF1 rescue. To confirm the immunostaining results, western blot was used to monitor the reduction in Pnn expression and the expression of AAV-SRSF1 and cleaved-PARP (Fig. 3, lane 4). Compared to those in cells without AAV-SRSF1 infection (Fig. 3, lane 3), the expression levels of cleaved-PARP were reduced (Fig. 3) and the diminished expression of SRSF2 was slightly restored in AAV-SRSF1-infected Pnn-depleted cells (Fig. 3). The combined results of immunostaining and Western blot suggest that overexpression of SRSF1 could restore to a great extent cellular apoptosis induced by Pnn depletion and that cellular apoptosis triggered by loss of Pnn is an indirect consequence mediated by SRSF1 downregulation.
Furthermore, AAV-SRSF1 was used to infect MCF-7 cells transfected with either Pnn-shRNA-411 or siRNA duplex before RT-PCR analysis for monitoring Bcl-xS expression (Fig. 5A and 5B). Compared to those in cells transfected only with Pnn-shRNA-411, expression levels of Bcl-xS mRNA were decreased in cells with additional SRSF1 overexpression (Fig. 5A, Bcl-X panel, lanes 3 and 4). A similar result, not only a decrease in Bcl-xS but also an increase in Bcl-xL, was observed in Bcl-x-g minigene assay following overexpression of SRSF1 in Pnn-depleted cells (Fig. 5B). Taken together, overexpression of SRSF1 in Pnn-depleted cells was able to revert the Bcl-x alternative splicing pattern by decreasing activated expression of Bcl-xS, thereby explaining the loss of the apoptotic phenotype elicited by SRSF1 overexpression in Pnn-depleted MCF-7 cells.

In addition to Bcl-x, regulation of alternative splicing of inhibitor of CAD (ICAD) also occurs via SRSF1 expression (Li et al., 2005). Overexpression of ICAD enhanced resistance to apoptosis in renal carcinoma cells treated with chemotherapy (Hara et al., 2001). In this study, since the functional isoform of ICAD (long form, ICAD-L) was decreased along with Pnn depletion, RT-PCR analysis was used to investigate whether mRNA isoform expression of ICAD was mediated through SRSF1 overexpression in Pnn-depleted MCF-7 cells. Our results showed that functional isoform of ICAD mRNA was decreased with Pnn-shRNA-411 transfection but recovered upon SRSF1 co-expression (Fig. 5A, ICAD panel, lanes 3 and 4). These data along with the Bcl-x splicing pattern modulation data not only confirm the role of SRSF1 in inducing cellular apoptosis triggered by Pnn depletion, but also demonstrate that
SRSF1 mediated activation of splicing of Bcl-x towards the xS mRNA isoform and decrease in ICAD-L in Pnn-depleted cells.

Discussion

In our previous study, we demonstrated that Pnn is constitutively expressed during mouse development from the two-cell stage embryo (Leu and Ouyang, 2006). Additionally, a recent proteomic study also reported that Pnn is expressed in pluripotent ES cells (Gundry et al., 2010). We thus hypothesized that Pnn may play an important role in early murine embryogenesis. In this study, we found that loss of Pnn expression through in vivo gene targeting resulted in early embryo lethality prior to blastocyst development. Furthermore, by means of embryonic stem cells and breast carcinoma cells, we observed Pnn deficiency-related cellular and biochemical changes and found that loss of Pnn expression not only perturbed distribution patterns and expression level of speckle-associated proteins but also resulted in apoptosis in both cell types. Based on the known role of Pnn in the regulation of mRNA alternative splicing (Wang et al., 2002; Chiu and Ouyang, 2006), we analyzed the alteration of isoform expression of several apoptosis-related genes and revealed that mRNA splicing was actively driven toward the pro-apoptotic isoforms of Bim, Bcl-x, and ICAD in Pnn-depleted MCF-7 cells. Pnn depletion also reduced functional SRSF1 messages through alternative splicing. Finally, we showed that the apoptotic and altered mRNA splicing events associated with loss of Pnn expression can be rescued with overexpression of SRSF1.
It is interesting to note that different cell types are susceptible to Pnn depletion to different extents. We found that, like ES and MCF-7 cells, HUVEC also displayed an apoptotic phenotype when Pnn expression was abolished (Supplementary Fig. 3). Nevertheless, cell lines of MDCK and COS-7 did not undergo apoptosis following Pnn depletion, although reduced expression of SR family proteins could be observed (Chiu and Ouyang, 2006; and this study). Recently, using a conditional knockout mouse model (Joo et al., 2010a; Joo et al., 2010b), Joo et al. reported tissue-specific Pnn inactivation in embryonic corneal and intestinal epithelial cells and showed that loss of Pnn expression impairs cellular differentiation but does not result in apoptosis. Their results, along with ours revealing that Pnn deficiency results in early embryonic lethality, correlate with previous studies demonstrating that complete loss of SR proteins leads to embryonic lethality (Moroy and Heyd, 2007; Mende et al., 2010), but development of distinct embryonic organs are not impaired in tissue-specific SR protein knockout mice (Ding et al., 2004; Xu et al., 2005). These findings may point to a cell type-dependent role for Pnn in cellular apoptosis.

To gain insight into the mechanism responsible for Pnn depletion-induced apoptosis, we investigated a series of pro-apoptosis genes whose expressions are regulated by alternative mRNA splicing. First, we found that Bcl-xS mRNA increased along with depletion of Pnn in MCF-7 cells (Fig. 5, A and B). In addition, enhanced expression of Bim isoforms, indicative of cellular apoptosis, was also observed (Fig. 5A). To confirm that expression of Bcl-xS, a hallmark of apoptotic signal expression, was indeed associated with loss of Pnn expression, we generated Bcl-x minigene (Bcl-x-g) to examine the Pnn depletion-induced generation of
Bcl-xS in an in vivo splicing assay (Fig. 5B). Consistent with the RT-PCR results for endogenous mRNA in MCF-7 cells, reporter splicing assay reinforced our assertion that Pnn depletion-induced apoptosis was through a Bcl-xS-related pathway. Second, in MCF-7 cells, Pnn depletion was accompanied by a decrease in functional ICAD. Therefore, ICAD, which has been shown to be involved in resistance to apoptosis (Hara et al., 2001), seems to play a crucial role in Pnn depletion-induced apoptosis. Because both Bcl-x and ICAD alternative splicing has been reported to be regulated by SRSF1 (Li et al., 2005; Paronetto et al., 2007), the means by which SRSF1 expression is regulated by pnn is crucial in Pnn depletion-induced apoptosis. Our data reveals SRSF1 transcripts can be modulated by Pnn depletion at transcript level through alternative splicing of functional SRSF1 (Fig. 5A), thus may hint that SRSF1 functions as a mediator of regulation of apoptosis via alternative generation of pro-apoptotic genes. The fact that SRSF1 splicing is regulated by Pnn expression level also points to the important role of Pnn in modulating mRNA alternative splicing, which has been demonstrated previously by in vivo reporter gene splicing assay (Wang et al., 2002; Chiu and Ouyang, 2006), but whether the effect of Pnn on splicing of SRSF1 transcripts is direct or mediated by some other factors remains to be studied. The reduced expression of SRSF1 in Pnn-depleted MCF-7 cells, together with the resistance to apoptosis and reversal of alternative splicing events through overexpression of SRSF1 (Fig. 5), further suggest that SRSF1-mediated alternative splicing of Bcl-xS and ICAD may play critical roles in Pnn depletion-induced apoptosis.
In our present and previous study (Chiu and Ouyang, 2006), we observed loss of nuclear speckle-associated SR family protein expression upon immunofluorescent staining of Pnn RNAi-treated cells, regardless of the cell type (Fig. 4). Nuclear speckles are subnuclear domains enriched with mRNA splicing factors, including members of the SR protein family and snRNPs (Lamond and Spector, 2003; Kiss, 2004). To investigate whether Pnn depletion affects only SR protein expression or even disrupts nuclear speckle structure, we used immunostaining to observe the expression patterns of U1A, a component of snRNPs, in Pnn-depleted MCF-7 cells. In contrast to normal speckle-associated manner in un-transfected cells, U1A, unlike SRSF1 and SRSF2, displayed a diffuse staining pattern throughout the nucleoplasm in cells devoid of Pnn expression (Fig. 5, k, l). In addition, the distribution pattern of exogenously expressed SRSF1 in Pnn-depleted cells was altered to a peri-nucleolus pattern (Fig. 6, e, h, k). The abnormal distribution pattern of both U1A and exogenously expressed SRSF1 along with the disappearance of certain SR proteins may indicate functional and/or structural disruption of nuclear speckles in Pnn-depleted MCF-7 cells. The relationship between disruption of nuclear speckles and apoptosis in Pnn-depleted cells is in agreement with a recent study that indicated that cell cycle arrest is caused by loss of organization of nuclear speckles (Sharma et al., 2010). Although it remains unclear why loss of pnn expression alters nuclear speckle structure and expression of its associated proteins, our data imply that Pnn plays a pivotal role in the splicing regulation of SRSF1 at transcript level (Fig. 5A) and its expression levels may thus be tightly involved in the maintenance of speckle structure.
Based on our results, it seems that alternative expression of Bcl-xS isoform is indispensable for SRSF1-mediated Pnn depletion-induced apoptosis. Recent studies, however, suggest a complex array of protein interaction may be related to Pnn-associated apoptosis. In addition to a Bcl-xS-related pathway, a complex named apoptosis-and splicing-associated protein (ASAP) consisting of SAP18, RNPS1, and different isoforms of Acinus has been isolated and demonstrated to be involved in the apoptosis of HeLa cells (Schwerk et al., 2003). Since Pnn has been reported to interact with RNPS1 in human cells (Li et al., 2003) and with SAP18 in Drosophila cells (Costa et al., 2006), it is plausible that apoptotic events induced by loss of Pnn might be through this ASAP-related pathway. Further experiments are needed to examine this alternative.

In conclusion, the present study demonstrated that apoptosis elicited by loss of Pnn was through ASF/SF2-mediated alternative expression of Bcl-xS and ICAD in certain tumor cells and embryos. The results of this study provide significant insight into the biological roles of Pnn, and also suggest potential clinical application.

**Conflict of interest**

The authors declare no conflict of interest.

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References


Figure Legends

Figure 1

**Depletion of pnn induces cellular apoptosis in MCF-7 cells.** (A) MCF-7 cells transfected with pnn-shRNA-411, which contains both shRNA expression cassette against Pnn and EGFP reporter, were immunostained 36 hours post-transfection. Cultured cells transfected with pnn-shRNA-411 (a, d, g, j, m) were identified by EGFP expression (arrows). Pnn-shRNA-411 transfected cells displayed little or no pnn staining (b) and exhibited
condensed chromatin which was recognized by DAPI staining (c, f, i, l, o). In addition to condensed nuclei, Pnn-shRNA-411-containing cells did not stain for full-length PARP (e) but stained positively for cleaved PARP (h). Nuclear protein p53 (k) was not affected by Pnn-depletion, but proliferation marker Ki-67 was absent in Pnn-depleted cells (n). (B) pSuper-based shRNA constructs other than Pnn-shRNA-411 could not reduce Pnn-expression or induce nuclear condensation. Pnn-shRNA-925 (sipnn-925), pSuper control, p53-shRNA (sip53) were transfected into MCF-7 cells and recognized by EGFP expression (arrows in a, d, g). Little or no change in Pnn expression was observed following immunostaining with polyclonal anti-Pnn antibody (b, e, h), and no nuclear condensation was found with DAPI staining in EGFP-expressed MCF-7 cells (c, f, i). (C) Calculation of shRNA treated-cells with different expression levels of Pnn. 48 hours after transfection, cells were fixed and stained with antibodies against Pnn for determination of the efficacy of shRNA on reducing Pnn expression. For calculation, only EGFP+ cells were counted. Expression levels of Pnn were classified to 3 levels (normal, reduced, and loss of expression). In cells transfected with pSuper, normal expression of Pnn were found in 96% EGFP+ cells. In cells transfected with shRNA-925, 81% cells with normal Pnn expression, 16% cells with reduced Pnn expression, while 3% cells were loss of Pnn. In cells transfected with shRNA-411, 13% cells with normal Pnn expression, 38% cells with reduced Pnn expression, while 49% cells were loss of Pnn expression. (D) Calculation of shRNA treated-cells with apoptotic nuclei. The apoptotic cells were recognized by condensed nuclei. Only EGFP+ cells were counted. Most cells transfected with pSuper did not show condensed nuclei, while 6% of shRNA-925 and 41% of shRNA-411 transfected
cells showed condensed nuclei. (E) Calculation of shRNA treated-cells with reduced SRSF1 expression.

48 hours after transfection, cells were fixed and stained with specific antibodies to determine whether loss of Pnn resulted in reducing SRSF1 expression. For calculation, only EGFP+ cells were counted. Most cells transfected with either pSuper or shRNA-925 did not show reduced SRSF1 expression, while 34% cells transfected with shRNA-411 were with reduced expression of SRSF1. Each experiment was performed three times and each time one hundred cells were counted. Asterisks denote $P$ values as indicated in the graph.

**Figure 2**

**Loss of pnn induces apoptotic morphology in ES cells.** ES cells of E14 origin co-transfected with pnn-shRNA-811 and pSuper null vector (for EGFP expression) expressed EGFP (arrows in a, d, g), but had reduced expression of Pnn (b), SR proteins (e), and PARP (h). Condensed nuclei were also found in pnn-shRNA-811 transfected cells (c, f, i).

**Figure 3**

**Western blot analysis of SR proteins and PARP in Pnn-depleted MCF-7 cells.**

MCF-7 cells were transfected with siRNA duplex and then subjected to western blot 36 hours after transfection. Lane 1 shows cells transfected with 5 nM control oligo, and lane 2 and 3 show cells transfected with increasing amounts (5 nM and 10 nM) of siRNA duplex against Pnn. Infection of AAV-SRSF1 in the presence of siRNA duplex is shown in lane 4.
With siRNA duplex transfection, expression levels of Pnn, SRSF1, and SRSF2 were reduced compared to the oligo control (lanes 1-3). In apoptotic cells, PARP was cleaved by caspase-3. The cleaved-PARP increased along with transfection of siRNA duplex (lanes 1-3), but decreased with AAV-SRSF1 infection (lane 4). The recombination Dsred-SRSF1 protein was only detected in cells infected with AAV-SRSF1 (arrowhead in lane 4). Numbers below each lane represent expression levels of pnn, SRSF1, and SRSF2 after normalization with β-actin loading control.

Figure 4

**Diminished expression of SR proteins and altered distribution of snRNP in Pnn-depleted MCF-7 cells.** Cells transfected with Pnn-shRNA-411 were fixed 36 hours after transfection and immunostained with anti-SR proteins antibody 1H4 (b), anti-SRSF2 monoclonal antibody (e), anti-SRSF1 monoclonal antibody (h), monoclonal anti-Pnn antibody (l), and polyclonal anti-U1A antibody (k). Transfected cells were recognized by EGFP signals (arrows in a, d, g, j) and nuclei were counter-stained with DAPI (c, f, i). Not only Pnn, but also speckle-associated SR proteins were depleted following pnn-shRNA-411 transfection. In contrast to the disappearance of SR proteins, snRNP U1A showed a diffused nuclear staining pattern throughout nucleoplasm (k) in MCF-7 cells with Pnn depletion (l).

Figure 5

**Altered isoform expression of SRSF1, ICAD, Bcl-x, and Bim in Pnn-depleted MCF-7**
(A) RT-PCR analysis of apoptosis-related genes in Pnn-depleted MCF-7 cells. Total RNA was extracted from MCF-7 cells transfected with pnn-shRNA-411 and then subjected to RT-PCR 24 or 48 hours after transfection. Lane 1 shows results for cells transfected with pSuper control plasmid, and lanes 2 and 3 show results for cells transfected with Pnn-shRNA-411 for 24 or 48 hours. Lane 4 shows results from cells transfected with Pnn-shRNA-411 in the presence of AAV-SRSF1. Apoptosis related genes whose mRNA isoforms may be altered during apoptosis processes are indicated on the left. mRNA expression levels of Bim isoforms (indicated by asterisks) increased in pnn-depleted cells but decreased following SRSF1 overexpression. Functional isoform (short form) of SRSF1 decreased with concomitant increase in function-less long form upon Pnn depletion, but restored after SRSF1 overexpression. Bcl-xS, a short mRNA isoform of Bcl-x, was increased in Pnn-depleted cells but diminished with SRSF1 overexpression. Decreased expression of the functional form of ICAD (long form, ICAD-L), an inhibitor of CAD, was also observed in Pnn-depleted MCF-7 cells. (B) Bcl-x reporter gene (minigene) analysis in pnn-depletion MCF-7 cells. Bcl-x-g was constructed with PCR-amplified Bcl-x genomic DNA fragments consisting of sequences from exon 1 to exon 3 of bcl-x. Two mRNA isoforms (Bcl-xS and Bcl-xL) can be derived from Bcl-x-g and detected by RT-PCR with specific primers as indicated by the arrowhead. Results in lane 1 are for cells transfected with 5 nM control oligo, and those in lane 2 and 3 are for cells transfected with 5 nM and 10 nM siRNA duplex against Pnn. Infection of AAV-SRSF1 in the presence of siRNA duplex is shown in lane 4. With siRNA duplex transfection, isoform expression of Bcl-xL was reduced compared to the oligo control. AAV-SRSF1 infection, however, restored the
expression levels of Bcl-xL. The expression of β-actin is showed as the control.

**Figure 6**

**Overexpression of SRSF1 rescues apoptosis induced by pnn depletion.**

MCF-7 cells co-transfected with pnn-shRNA-411 and SRSF1-HA or DsRed-SRSF1 were fixed and immunostained 48 hours post-transfection. Cultured cells transfected with pnn-shRNA-411 (a, d, g, j) were identified by EGFP expression (arrows). Overexpression of SRSF1 was recognized with an anti-HA antibody (e, h, k) or DsRed fluorescence (b). With SRSF1 overexpression, lack of Pnn expression elicited by pnn-shRNA-411 was observed (f), while apoptosis-related nuclear condensation was partially restore to a regular chromatin configuration. PARP (l), but not the apoptotic marker cleaved-PARP (i), was found in SRSF1 overexpression Pnn-depleted cells.