Title: Periostin induces intracellular cross talk between kinases and hyaluronan in atrioventricular valvulogenesis‡

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‡ Running title: Periostin promotes valvulogenesis by intracellular signals

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CAPSULE

Background: Periostin-null mice exhibit a myxomatous atrioventricular valve phenotype. We propose two mechanisms: periostin binds to collagen and links it to cell-surface receptors; periostin/β-integrin signalling promotes valve morphogenesis.

Results: Periostin/β-integrins/focal-adhesion-kinase/PI3K/Erk signal promote hyaluronan-synthase-2 activation, matrix-remodeling and valve-progenitor cell survival/differentiation.

Conclusion: The phenotype of periostin-null valves is consistent with a role for PN-cell signaling through integrin receptors.

Significance: Periostin is a valvulogenic signaling morphogen.

ABSTRACT

Periostin (PN), a novel fasciclin-related, matricellular protein, has been implicated in cardiac development and postnatal remodeling, but the mechanism remains unknown. We examined the role of PN in mediating intracellular kinase activation for atrioventricular valve morphogenesis using well defined explant cultures, gene transfection systems and Western blotting. The results show that valve progenitor (cushion) cells secrete PN into the extracellular matrix (ECM), where it can bind to integrins and activate integrin/FAK signaling pathways and downstream kinases, PI3K/Akt and Erk. Functional assays with prevalvular progenitor cells showed that activating these signaling pathways promoted adhesion, migration and anti-apoptosis. Through activation of PI3K/Erk, PN directly enhanced collagen expression. Comparing PN null to WT mice also revealed that expression of hyaluronan (HA), and activation of hyaluronan-synthase-2 (Has2) is also enhanced upon PN/integrin/focal-adhesion-kinase (FAK)-mediated activation of PI3K, and/or Erk; an effect confirmed by the reduction of HA synthase 2 in PN null mice. We also identified in valve progenitor cells a potential autocrine signaling feedback loop between PN and HA through PI3K, and/or Erk. Finally, in a 3D assay to simulate normal valve maturation in vitro, PN promoted collagen compaction in a kinase dependent fashion. In summary, this study provides the first, direct evidence that PN can act to stimulate a valvulogenic signaling pathway.
INTRODUCTION:

Periostin (PN) is a matricellular protein containing fascin gene motifs that encode an ancient family of adhesion proteins (1,2). Like most matricellular proteins, PN can function as a bridge connecting the cell surface to structural extracellular matrix (ECM) glycoproteins, thereby potentially modulating cell–matrix interactions related to adhesion, migration, tissue formation and growth (3,4). In normal heart development, PN promotes collagen fibrillogenesis and matrix organization. Developmental profiles of PN expression peak immediately following birth when valve primordia complete their morphogenetic transformation from mesenchymal swellings, called “cushions”, into mature, sculpted leaflets. During this period of peak PN secretion, the prevalvular ECM is progressively compacted and organized into specific layers or zones. Thereafter, when remodeling is completed, PN falls to baseline levels and does not increase again unless an injury occurs that promotes fibrosis (5,6).

At all developmental time points, PN is expressed in valve or ventricular fibroblasts or their mesenchymal (“cushion”) progenitor cells, which are derived by the transformation of epicardium or endocardium cells (7-9). In PN null mice, the atrioventricular (AV) valves of those that survive into early adult life have a myxomatous-like phenotype characterized by interspersed “pockets” of undifferentiated, cushion-like tissue, diminished collagen expression and poorly organized regions of non-compacted ECM (10,11). These alterations in AV valve matrix organization and collagen secretion correlate with a significant loss of biomechanical properties (12,13). Thus, based on the phenotype of the AV valves in PN null mice (14,15), we propose that PN has an important developmental role in the differentiation and maturation/compaction of prevalvular cushion cells into valve fibroblasts and their subsequent remodeling into sculpted leaflets. The question is - how does periostin do this? Is it by directly binding to extracellular structural proteins like collagen or elastin (promoting cross-linking), and/or is it through signaling mechanisms triggered by the binding of PN to cell surface receptors such as αβ3 and αβ5 integrins (16,17). The valve phenotype of the periostin null mouse does not allow us to distinguish between these two candidate mechanisms.

To address this question, we isolated prevalvular cushion cells from embryonic chicks and mice and determined whether PN, normally secreted by prevalvular cushion cells or added to culture medium, activates intracellular signaling kinases through binding to cell surface receptors, and, if so, whether these signaling changes affect cell behaviors related to normal valvulogenesis. Receptor candidates tested were specific β-integrins (αβ1, αβ3 and αβ5). Our findings provide evidence that in cultured valve progenitor cells, PN can directly activate intracellular kinases through integrin binding including those downstream of focal adhesion kinase (FAK), PI3K, Akt and Erk. In vivo and in vitro data collectively indicate that PN can promote activation of Has2 by promoting phosphor-serine and this increase in p-serine level is correlated with increase in hyaluronan synthesis, and the survival of prevalvular progenitor cells. Similarly PN can promote phospho-threonine and this activation in p-threonine-Has2 is correlated to downregulation in HA synthesis. We have also linked PN-induced integrin/FAK-mediated PI3K and MAPK signaling to changes in morphogenesis of prevalvular cushion cells (adhesion, migration, survival), and to their differentiation into a valve fibroblastic lineage as reflected by enhanced collagen I(COL1α1) synthesis and the generation of contractile forces sufficient to compact and align collagen fibrils as occurs in normal valve maturation.

MATERIALS AND METHODS

Animals and cell culture: Wild type (WT) mice (C57BL/6 strain) were obtained from Jackson laboratories, ME. PN deficient mice on a C57BL/6 genetic background were provided by Dr. Simon Conway (IUPUI, IN). Mice at 8–10 weeks of age were used in experiments as previously described (10). All animal care and experimentation were done in accordance with the institutional guidelines. Adult sheep valve cells were provided by Dr. Norris, and Dr. Bischoff (18).
After removing the mitral valves from mice, and HH40 chickens, the valves were minced and digested with 2 µg/ml collagenase for 30 min at 37°C. The cellular digests were seeded on 0.5% gelatin coated tissue culture plates using Medium 199 (M199, Life Technology) containing 5% fetal bovine serum (FBS) containing 0.5 ng/ml EGF, 5 µg/ml insulin, 2 ng/ml bFGF, 100 U/ml Penicillin, and 100 µg/ml Streptomycin, and incubated at 37°C with 5% CO2/95% air. Experiments were done with mouse and chick valve cells from passages 1–4. FBS was from Atlanta Biological (GA), and L-glutamine, Gentamicin sulfate and Ampthericin B were from Hyclone. Nonidet P-40, ethylene glycol tetraacetic acid (EGTA), sodium orthovanadate, glycerol, phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, aprotinin, and hydroxyethyl piperazine ethane sulphate (HEPES), were purchased from Sigma (St. Louis, MO). The antibodies against PN, collagen-1, HSP47, p-Erk, Erk, p-Akt, Akt, β3-actin, β1- and β5-integrins, and the horse radish peroxidase linked anti rabbit and anti-mouse antibodies, and Luminol reagent were purchased from commercial sources (Santa Cruz Biotechnology, Abcam, Ebioscience, Sigma, Thermo Fisher, and Southwest Technologies, Inc.). PN antibody for immunohistochemistry was provided by Dr. Hoffman (10,11). PN expression vector was provided by Dr. Akira Kudo (Yokohama, Japan). Monoclonal Has2 antibody for immunoprecipitation is from Santa Cruz Biotechnology (C-5, sc-365263), and anti-phospho serine, and anti-phospho threonine antibodies are from Life science (or Zymed Corp.)

**Cell lysis and immunoblotting:** Prevalvalvar mesenchymal cells were cultured until they were confluent. Cells were washed twice at 4°C with PBS, harvested with 0.05% Versene, and then washed in cold PBS again as described previously (19-27). The cells were pelleted by centrifugation at 5000 x g for 2 min at 4°C. The pellets were treated with the lysis buffer containing 1% Nonidet P-40, 0.5 mM EGTA, 5 mM sodium orthovanadate, 10% (v/v) glycerol, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 µg/ml aprotinin, and 50 mM HEPES, pH 7.5. The lysates were clarified by centrifugation at 12,000 x g for 10 min at 4°C and then stored at -80°C as described previously.

For SDS-PAGE, the denatured cell lysates were loaded onto a 4–12% gradient polyacrylamide gel at 15–30 µg of protein per lane in an Invitrogen mini gel apparatus. Proteins were transferred to nitrocellulose membranes and blocked for 1 h with 5% nonfat dry milk in Tris buffered saline containing 0.1% Tween-20 followed by washing in the same Tris-Tween buffer. The membranes were probed with the appropriate antibody diluted in Tris-buffered saline containing 5% bovine serum albumin (for polyclonal antibodies) or 5% nonfat dry milk (for monoclonal antibodies) followed by treatment with peroxidase-linked secondary antibodies and luminol reagents. The proteins on the blots were detected with antibodies for PN, β3-, β1-, β5-integrins, HSP47, p-Erk, Erk, p-Akt, and Akt (19-24). β-tubulin and β-actin were used as internal standards. Sizes of proteins were estimated from prestained molecular weight standards electrophoresed in the same gel as the samples. Immunoreactive bands were quantified by densitometry. Each protein sample was analyzed from at least 3 independent experiments from each set of fibroblasts.

**Immunoprecipitation with anti-Has2 antibody and western blot analyses:** For immunoprecipitation, all procedures were done at 4°C unless otherwise mentioned. Cell lysates were diluted to 1 µg protein/µl using the lysis buffer above. Aliquots of 500 µl of lysate were mixed with 5µl of anti-Has2-antibody, or nonimmune IgG as described previously by our group (20,27). The immune complex was captured by adding 80 µl of 1:1 (v/v) protein A-Sepharose 4B suspension and incubated for another 1 h. The Sepharose 4B beads were collected by brief centrifugation followed by washing three times in ice-cold lysis buffer, three times with lithium chloride buffer (5 mM LiCl, 0.1 mM sodium orthovanadate, 0.1 M Tris-HCl, pH 7.4), and three times with buffer containing 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM Tris-HCl, pH 7.4. Finally, the immune complexes on the beads were recovered with 500 µl of SDS-containing denaturing buffer and heated to 65°C for 5 min. Cell lysates (normalized for protein
concentration) were analyzed by immunoblotting with anti-phospho-serine or reblotting with anti-Has2 as a loading control using SDS-PAGE (4–12% gradient gels) as described previously (22–25).

For SDS-PAGE, the denatured immunoprecipitates were loaded onto a 10% polyacrylamide gel at 15–30 µg of protein per lane as described in the “Cell lysis and immunoblotting” section (20,27).

RNA-silencing: ControlsiRNA (scrambled siRNA); β1- β3- and β5-integrin siRNAs (sc-35675; sc-35677; sc-35681 respectively) and PNsiRNA (sc-61325), FAKsiRNA (sc-35353) were from Santa Cruz Biotechnology. All the treatments and transfection experiments were done with cells that were serum starved for 24 h.

Control shRNA (scrambled shRNA) and PN shRNA cloning in pSicoR vectors: Double stranded oligonucleotide cassettes for control shRNA and PN shRNA were prepared. The linearized pSicoR vectors were ligated to the double-stranded oligonucleotide cassettes (22). The resulting pSicoR-PNshRNA (PNshRNA) transfectants constitutively silence PN genes in the cells. PSicoR-scrambledshRNA (controlshRNA) transfectants were used as controls as described previously (19,22,25,28).

Cell adhesion assay (25): Antibodies against integrins were purchased from EMD Millipore. Ninety-six well plates were coated with or without PN (1 µg/ml), at 4° C overnight. Plates were blocked with 10 µg/ml heat-denatured fatty acid free bovine serum albumin for 30 min. Chick cushion valve cells (10^5/ml in M199 with 0.1% fatty acid free bovine serum albumin) were incubated with function inhibiting anti-integrin antibodies (β1-, β3- and β5-integrins) for 10 min before plating. Then 100 µl of the cell suspension/well was added onto plates and incubated for 30 min at 37° C under 5% CO₂ with the lid off. After incubation, unattached cells were removed by rinsing with PBS. Attached cells were fixed in 5% gluteraldehyde for 20 min and stained with 0.1% crystal violet. After washing, stains were dissolved in 200 µl of 10% acetic acid, and color was read at 575 nm in a Bio-Tek plate reader.

Cell migration assay: Cell migration assays were done using 24-well, chemotaxis chambers (Nalgene) as described previously (25). Polycarbonate filters with a pore size of 8 µm (Nalgene) were coated with 5 µg/ml fibronectin (Life Technologies) at 4° C overnight in phosphate-buffered saline (PBS) and dried under sterile air as described previously (25). FGF was diluted to 10 ng/ml in M199 supplemented with 0.5% fatty acid free bovine serum albumin (Sigma), and 600 µl of the final dilution was placed in the lower chamber of a modified Boyden chamber.

PN was over-expressed by PNcDNA expression vector (empty vector was used as a control) in valve cells followed by treatment for 18 h with control antibody (Cont-Ab), or with PN-Ab, or with 10 µM LY294002 (PI3K/Akt inhibitor), or with 10 µM U0126 (Erk inhibitor). In a companion experiment, cells after PN overexpression were also transfected with control (Cont) siRNA, or FAKsiRNA alone for 48 h, or they were first transfected with FAKsiRNA followed by incubation with PN-Ab. After the treatments, cells were washed and trypsinized for the minimum time required to achieve cell detachment. Approximately 5x10⁴ cells suspended in 100 µl of M199 with 0.5% bovine serum albumin were placed in the upper compartment. The cells were allowed to migrate for 3 h at 37° C in a humidified chamber with 5% CO₂. After the incubation period, the filter was removed, and the cells persisting on the upper side of the filter were removed with a cotton swab. The cells on the filters were fixed with 4% formaldehyde and stained with hematoxylin. Migration was quantified by counting cells in three random fields (100 x) for each filter. Alternatively, filters were stained in 0.1% crystal violet, washed, and eluted with 10% acetic acid in 96-well plates. Quantification was done based on absorbance at 575nm in a Bio-Tek plate reader.

Caspase-3 assay: Caspase-3 activity was measured in a plate reader at 405 nm by using the kit from Millipore following the manufacturer’s instruction. The assay is based on detection of the chromophore p-nitroaniline (p-NA) after cleavage from the substrate DEVD-p-NA. The absorbance of released p-NA is
measured at 405 nm. Difference in absorbance between control and treated samples determined the increase in caspase-3 activity. The assay was done in 96 well plates. Cytosolic extracts (1.0 x 10^6 cells in 300 µl in cell lysis buffer) were mixed with 10 µl of substrate (3 mg/ml of DEVD-p-NA). Buffer blanks (without substrate and cell extract) and substrate blanks (with substrate but without cell extract) were included in the assay. The plates were incubated for 1 h at 37° C, using Bio-Tek plate reader at 405 nm.

**Immunohistochemical staining in the heart sections:** Heart sections from WT and PN null mice were deparaffinized using standard procedures and permeabilized with 0.1% Triton X-100 in PBS. PN, HA and Ki67 were localized in sections by immunohistochemical staining using PN antibody (10,11), HA by HA binding protein (HABP from Saikagaku corporation) and a Ki67 antibody (Millipore) following standard protocols. As a negative control, the primary antibody was replaced with non-immune rabbit IgG, or sections were treated with Streptomyces hyaluronidase followed by treatment with HABP (in both cases, no staining was observed).

**Quantitative real-time polymerase chain reaction (qRT-PCR):** Total RNA from mouse valve tissues or cultured fibroblast cell pellets was isolated with RNeasy spin columns (Qiagen, Valencia, CA) following the manufacturer’s instructions. The integrity of the RNA was verified by BioAnalyzer. Total RNA (100 ng) was reverse-transcribed into first-strand complementary DNA (cDNA) (QuantiTect Reverse Transcription Kit, Qiagen). Real-time PCR amplification reaction mixture (25 µl) contained 12.5 µl of 2 x SYBR Green PCR Master Mix following the Bio-Rad protocol, 5 µl of diluted RT product (1:20), and 0.5 µM sense and antisense primer sets.

Has1/2/3 primers: sequence of Calve S and colleagues(29)
COL1α1 and GAPDH primer: sequence of Yang and colleagues(30).

The primer sequences were as follows (30):
**COL1α1 (m):**
Forward (F)-5’-gagccctcgcttccgtactc-3’,
Reverse (R)-5’-tgttccctactcagccgtctgt-3’,
Has1 (m):
F- 5’-gagccctgtacaaccaaag-3’
R- 5’-ctcaaccaacgaaggaaggag -3’
Has2 (m):
F- 5’-gagccaccaagttctgcttc -3’
R- 5’-cttcctcaeggegagagtc-3’
Has3 (m):
F- 5’-tgaccagctgctgcaccttg-3’
R- 5’-cccgctcagttgaagccat-3’
GAPDH (m):
F- 5’-tgtcatcatactggcaggttctc-3’,
R-5’-catggcctcegtgttctca-3’.

The qRT-PCR assays were done in 3 individual experiments with triplicate samples using standard conditions. After sequential incubations at 50° C for 2 min and 95° C for 10 min, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95° C for 15 s, annealing and extension at 60° C for 60 s. The standard curve was made from a series dilution of template cDNA. Expression levels of COL1α1, and Ha-synthases mRNA were calculated after normalization with the housekeeping gene GAPDH. Variation within samples was less than 7%. Statistical analysis was done with the Student’s paired “t” test.

**Transient transfection using cushion prevalvular mesenchyme:** All transfections were done using Lipofectamine (Invitrogen) in cultures at ~75% confluence. After each transfection, the cultures were grown for another 48 h for further transfection, or treatment, and analyzed for each experimental design.

**Floating collagen gel cultures and quantitation of gel contraction:** Experiments were done essentially as previously described (31). Briefly, 24-well tissue culture plates were pre-coated with BSA. For PN-induced effects, cells were pre-transfected with vector control or PncDNA expression vector, and transfectants were grown for 48 h. For PN inhibition, the cells were first transfected with control shRNA (scrambled shRNA), or PNshRNA followed by co-transfection with Pn cDNA expression vector. These transfectants were then grown for another 48 h. In a parallel set of experiments to inhibit β1-integrin, or FAK, or PI3K, cells were pre-incubated in the presence of 10 µM of LY294002 (PI3K/Akt inhibitor), 10 µM of PF573228 (FAK inhibitor), or 5 µg/ml of blocking antibody against β1-integrin, or 5 µg/ml of blocking antibody against CD44 for 2
h, prior to PN overexpression. Finally, these transfected and treated HH40 chick cushion mesenchyme cells were suspended in Medium 199 with 1% FBS, 100 U/ml Penicillin, and 100 μg/ml Streptomycin, and mixed with a collagen solution (1 part HEPES, pH 8.0; 4 parts collagen 3 mg/ml (Vitrogen 100, Cohesion Technologies, Palo Alto, CA), and 5 parts medium [2x M199]), which yielded a final concentration of ~80,000 cells/ml and 1.2 mg/ml of collagen. A collagen cell suspension (1 ml) was added to each of four wells (Nunc). After polymerization at 37° C for 2 h, the gels were detached from the wells followed by addition of 1 ml of the medium. For inhibition experiments, after polymerization, lattices were detached from tissue culture plates and treated further with or without 10 μM inhibitor, or 5 μg/ml of blocking antibody against β1-integrin for 24 h. Contractions of the gels were quantified by measuring the decrease in gel diameter over a 24-48 h period.

**Statistical analysis.** Each experiment was repeated three times for each set of fibroblasts, and pooled for statistical analysis. Western blot analyses, mRNA analyses, caspase-3, migration, collagen gel contraction experiments for each separate experiment were repeated between 3 and 4 times, depending upon the particular experiment. Data are expressed as ± SD. The Student’s two tailed t-test (Microsoft Excel software) was used for comparison between two groups. Statistical analysis of the Western blots was done using *t* test with Mann-Whitney modification or analysis of variance (ANOVA) as applicable. When analysis included more than two groups, one-way analysis of variance was used. P-values (*P*) ≤ .05 were considered statistically significant.
RESULTS:

Technically, it is difficult to obtain enough mesenchyme cells from mouse embryos. This inherent problem of small cell numbers is a historic problem for developmental biological studies. Therefore, we did most of the proposed experiments with embryonic chick to optimize the isolation method and yields. In addition to chick, we also included fibroblasts isolated from adult sheep mitral valves to obtain sufficient numbers of fully differentiated valve cells to do the relevant kinase signaling experiments prior to using cells isolated from WT and PN null mice. The aims of this study were: first, to use biochemical analyses of prevallvular mesenchyme cells isolated as primary cultures from embryonic or adult valves to assess whether endogenous (i.e. cell secreted) PN or exogenously added PN can activate intracellular signaling pathways; Second, to assess whether integrin/FAK-induced PI3K/Akt, and/or Erk signals, modify prevallvular mesenchymal cell morphogenetic behaviors (e.g., adhesion, migration, anti-apoptosis, and synthesis of hyaluronan) necessary for valvulogenesis. Third, to determine whether PN-integrin interaction promotes kinase-mediated differentiation of valve progenitor cells into fibroblasts as evidenced by increasing collagen synthesis and the potential to compact (align) collagen in 3D gel assays.

PN promotes β3-integrin/FAK/Akt/Erk1/2 activation in chick and mouse embryonic AV valve cells, and adult sheep valve cells.

We and others have shown that PN mediates the formation and remodeling of the inlet valves of the ativoventricular (AV) junction, most likely through an integrin dependent process (5,32). However, candidate mechanisms linking PN and integrin interaction to the regulation of valvulogenesis are still poorly understood and often attributed to PN acting as an adaptor protein crosslinking ECM fibrils or linking ECM to the cell surface. Here we test the hypothesis that PN can signal intracellular changes in valvulogenesis through the autophosphorylation of the cytoplasmic tyrosine kinase domain of FAK following PN-integrin engagement, which serves to recruit signaling proteins such as Src, and the subsequent activation of multiple downstream signaling pathways, e.g. PI3K/Akt and Erk kinases. These kinases have been associated with migration, survival and phenotype stability of many cell types (33,34) and thus were logical candidates for testing putative PN signaling in normal valvular morphogenesis.

Figures 1-3 show the effects of PN on the phosphorylation of FAK and its downstream target proteins, PI3K/Akt and Erk1/2, in chick and mouse valve progenitor cells, and in adult sheep valve fibroblasts. Figure 1 shows western blots of cell lysates of cultured embryonic (HH40) chick valve cells that were isolated during the period of active differentiation and remodeling. The cells were either untreated or incubated with PN followed by transfection with either control siRNA or β3-integrin siRNA. Lysates of cultured cells that were incubated with PN and with control siRNA significantly increased phosphorylation of p-FAK, p-Akt and p-Erk1/2 (42/44) compared with lysates from untreated cultures (Figure 1A; lane 2 compared to lane 1). Quantitation of the lysate bands normalized to β-actin bands is shown in figure 1B, which also demonstrates that PN indeed stimulates the activation of FAK, Akt, and Erk (Figure 1A; lanes 2, 4, and 5 of panel 2 vs. panel 1). Lysates of cultured cells that were incubated with PN after siRNA treatment to inhibit β3-integrin expression significantly decreased phosphorylation of p-FAK, p-Akt and p-Erk1/2 (42/44) compared with lysates from untreated cultures or with lysates of cultured cells that were incubated with PN and control siRNA (Figure 1A; lane 3 compared to lane 2). Figure 1B also demonstrates the effectiveness of the β3-integrin siRNA on β3-integrin protein expression. Silencing β3-integrin in the presence of PN blocked phosphorylation of FAK, Akt, and Erk without altering the level of PN (Figure 1B; lanes 2, 4, and 5 of panel 3 vs. panel 2). These results indicate that PN can stimulate FAK and Akt phosphorylation through β3-integrin.

Figure 2 shows the results of western blots of lysates from fully differentiated valve interstitial fibroblasts harvested from adult sheep. The sheep valve (SV) cell cultures were treated with PN after inhibiting endogenous PN expression...
by siRNA treatment. The addition of PN in the presence of control siRNA in both sheep and chick embryonic valve mesenchymal cells (Figure 2A, lane 3, and Figure 1A, lane 2) gave essentially identical results as observed for that of addition of PN only (data not shown), indicating that fully differentiated valve cells still retain responsiveness to PN. Interestingly, by silencing PN gene expression in differentiated SV fibroblasts, the activation of the FAK, PI3K/Akt and Erk kinases was abolished (Figure 2A, lane 4 vs. lane 3). It is still noteworthy that at least over the short term, exogenously added PN can interact with cell surface integrins to elicit intracellular changes in kinase activation (Figures 1 and 2).

Figure 3 shows the results of western blots using lysates from cultures of embryonic valve interstitial cells harvested from E15.5 mouse mitral valves that were treated using the same protocol as used for the embryonic chick valve cells (Figure 1). As with the chick valve cells, overexpression of PN in mouse AV cushion cells also induced activation of p-FAK (Figure 3A,B), and of p-Akt and p-Erk without altering the total levels of Akt and Erk (Figure 3C,D). However, overexpression of PN only had a small inductive effect on β3-integrin expression Figure 3A, B). These results (Figures 1-3) indicate that mouse prevalvular cushion cells, like those of the embryonic chick cushion mesenchyme, exhibit sensitivity to PN sufficient to activate p-FAK and retain that responsiveness even when fully differentiated.

To further examine the role of FAK signaling network in valvulogenesis, we examined the activation of PI3K/Akt and MAPK signaling pathways in WT E16.5 mouse valve cells (Figure 3E) in the presence or absence of PF573228, a selective inhibitor of FAK. PF573228 treatment potently suppressed the activation of PI3K/Akt, Erk1/2, and FAK on Tyr925 without affecting total levels of FAK (data not shown) and β-tubulin (Figure. 3E). This observation suggests that FAK lies upstream of PI3K/Akt and Erk1/2 proteins.

Once phosphorylated on Tyr925, FAK serves as a docking site for p85 regulatory subunit of PI3K(35-38) through recruitment of growth factor receptor-bound protein 2 (Grb2) (36), which is known to be involved in Raf/MEK/ERK signalling (35,36). We then focussed on the activation of PI3K/Akt and MAPK/ERK in response to exogenous addition of PN at various time points. In cultured E16.5 WT mouse valve cells, stimulation with PN results in immediate activation of PI3K and MAPK/Erk signaling (Figure 3F). An important distinction from the results of the experiment in figure 3F is that MAPK/ERK activation in cultured cells is transient (typically lasting at a high level for only a few hours (Figure 3F 4h), after which a sustained small increase in Erk activation persists). In contrast, persistent detectable levels of activated Akt are observed over several hours (Figure 3F, 24 h) in WT mouse valve cells. Such dramatic differences in signalling kinetics may have important functional implications when comparing biological responses in valve morphogenesis.

Next, to examine the role of PI3K and MAPK signalling networks in valve morphogenesis, we examined the activation of PI3K/Akt and MAPK in the presence and/or absence of a PI3K inhibitor (LY294002) and an Erk inhibitor U0126. The LY294002 treatment potently suppressed the activation of PI3K/Akt and Erk1/2 (Figure 3G) without affecting total levels of p-FAK (data not shown), whereas U0126 treatment did not have any effect on Akt activation but significantly suppressed Erk activation (Figure 3G). Thus, these results suggest that FAK accounts for the ERK1/2 responses through PI3K/Akt-dependent, or -independent mechanisms, and that PI3K regulates Erk activation (Figure 3G) as has been seen in other cell types (39). Thus, to our knowledge, these results are the first to indicate that PN can activate specific intracellular kinases that are known to be associated with adhesion, migration and survival, and that PN activation of these kinases was mediated through binding to β3-integrin. As noted below, PN signaling is not restricted to this particular integrin receptor alone as other signaling responses were observed for β1- and β5-integrins.

Of potential importance for future studies, as shown in figures 1 and 2 we did not detect similar changes in PN or integrin expression after a one time treatment of valve progenitor.
cells with exogenous addition of PN compared to transfecting PN expression vectors which require endogenous protein synthetic mechanisms to make and secrete PN. This suggests to us that adding exogenous PN in this system does not always replicate the cellular distribution or molecular interactions that result from increased endogenous synthesis of PN. One explanation for this is that PN interacts with transmembrane integrins whose kinase active binding sites are situated on the cytoplasmic side of the plasma membrane. Upon activation, integrins can interact with PN or other matricellular proteins or assemble into extracellular matrices. Simple addition of exogenous PN cannot duplicate all of these configurations. This is reflected in results in activation of intracellular kinases in figures 1, 2, and 3 for p-FAK, p-Akt and p-Erk1/2 signals. Thus, in the subsequent experiments, we used PN expression vectors to promote overexpression of endogenously synthesized PN to promote a more accurate, physiologically relevant representation of signaling than a simple addition to the culture medium.

PN-integrin interactions promote cell behaviors associated with valvular morphogenesis.

Valve morphogenesis involves an active elongation phase in which the primitive preavalvular cushions extend from the inner margins of the myocardial walls into the lumen of the AV junction to form the mesenchymal models of the future mitral and tricuspid valve leaflets (40-42). These phases begin at E15.5 and continue into early postnatal life and are accompanied by changes in cell growth (cell number and size), adhesion, migration, matrix synthesis and alignment into linear histological arrays (36).

**Adhesion:** As indicated in figures 1 and 2, exogenously added and endogenously secreted PN activated integrin-mediated FAK, a kinase generally recognized for its potential to promote adhesion and migration in a number of cell systems including metastatic tumor cells (43). To determine if PN can affect preavalvular cushion cell adhesion (as a potential first step towards migration) through an integrin dependent mechanism, we cultured chick HH40 valve cells during their period of active elongation with blocking antibodies for β1-, β3- or β5-integrins prior to plating on culture wells coated with either BSA or PN (Figure 4). The cells exhibited robust adhesion to culture wells coated with PN (panel A), which was substantially inhibited by blocking antibodies against β3-integrin, and to a lesser extent with β1-integrin, but not with β5-integrin (panel B, lanes 2 and 3 vs. lanes 1). An isotype-matched control antibody did not inhibit the adhesion of these cells at this concentration (panel B, lane 5 vs. lane 1). Adhesion to BSA-coated wells was less than for PN and essentially invariant among the antibody treatments (panel B).

In a companion experiment, the effect of PN on adhesion of these cells was variably blocked by all 3 integrin antibodies in a dose-dependent manner with β3-integrin antibodies having the most effect (data not shown). The optimum concentration of β3-integrin antibody for inhibition of adhesion of these cells to PN coated plates was 5 µg/ml.

**Cell growth:** To determine whether PN promoted growth of cushion primordia through an effect upon increased cell growth, we assessed the expression of Ki67 in three sections of AV cushions from WT and PN null 16.5 ED mouse hearts. Only a two-fold difference in the number of Ki67+ cells was observed in WT cushions compared to those of PN nulls (Figure 5A). These in vivo cell proliferation data were consistent with BrdU incorporation assays using in vitro WT mouse cushion cell cultures with or without silencing of PN, which indicated a modest decrease in cell proliferation when endogenous PN is silenced (Figure 5B, lane 4 vs lane 3). To what extent proliferation drives cushion growth and elongation remains an open question. While growth factors, such as BMP, TGFβ, and EGF certainly may come into play (42,44), based on our findings, PN signaling would seem to contribute partly to the answer.

**Apoptosis:** An alternative mechanism to proliferation to promote growth would be to inhibit apoptosis. To determine if PN potentially affects apoptosis, E16.5 mouse AV cushion cell cultures were transfected for 48 h with a PN silencing vector (PNshRNA), a control vector,
or full length sense PNC DNA expression vector to over-express PN, or treated with integrin blocking antibodies. The treated cultures were subsequently assayed by ELISA for caspase-3 levels, an active apoptotic marker. The knockdown of PN substantially increased the caspase-3 level compared to cells transfected with the vector alone (Figure 6, lane 4 vs. lane 3). In contrast, over-expression of PNC DNA expression vector essentially kept the level of the apoptotic marker at a basal level (Figure 6, lane 2 versus lane 1). Treatment with blocking antibody to $\beta_3$-integrin also increased the level of caspase-3 as did blocking antibody to $\beta_1$-integrin, but to a lesser extent (Figure 6, lanes 5,6 vs. lane 2), indicating that interactions of PN with $\beta_3$-integrin or $\beta_1$-integrin in mouse valve cells can protect them from apoptosis and thereby promote growth by inhibiting cell death.

**Migration**: The initial expression of PN in prevalvular cushion cells occurs immediately after their transformation from the AV endocardial epithelium as they migrate to colonize the acellular cardiac jelly (consists primarily of hyaluronan (45)) of the primitive AV cushion swellings. Expression of PN continues as waves of prevalvular mesenchyme extend distally into the cushions as they elongate into the lumen to form the primitive leaflets. These findings suggest that PN may promote the movements of prevalvular mesenchyme cells as they progressively colonize the elongating cushions during their morphogenesis. To determine if PN affected migratory behavior through an integrin-linked signaling mechanism, we used explant cultures from E15.5 mouse valves, the period of active elongation. Figure 7 shows that treatment of explant cultures with PNC DNA expression vector stimulated migration ~4-fold, which was prevented by: (i) a PN-blocking antibody (lane 5), (ii) silencing FAK with FAKsiRNA alone (lane 6), or with PN-Ab treatment (lane 7), and (iii) LY294002, a PI3K/Akt inhibitor (lanes 8). However, the Erk specific inhibitor U0126 (10 µM) did not affect migration (lane 9). These results indicate that PN/ $\beta_3$-integrin/FAK/PI3K/Akt signaling pathway promotes AV cushion cell migration. The lack of engagement of Erk is consistent with recent findings that Erk pathways may be involved in other biological functions, e.g. proliferation or collagen synthesis (46-49).

**PN can induce collagen 1α1 (COL1α1) gene expression in mouse cushion cells by both PI3K/Akt and Erk pathways.**

There is evidence that PN elevates collagen synthesis several fold in adult remodeling diseases that lead to fibrosis (30). Whether PN can also directly upregulate type-1 collagen (collagen 1) expression during development has not been demonstrated for prevalvular mesenchyme, even though collagen becomes the key structural protein of the inlet valves. Because PN supported and enhanced cushion migration through a $\beta_3$-integrin/FAK interaction (Figure 7), we asked whether PN signaling could also induce or promote collagen synthesis. Expression of collagen is normally associated with differentiation of valve progenitor cells into interstitial fibroblasts. Figure 8A shows that COL1α1 mRNA expression in PN null cushion cells is significantly lower than for WT cushion cells. Figure 8B western blots show that overexpression of PN significantly increases COL1α1 protein expression in the lysates and medium of cultured WT cushion mesenchyme cells treated with PNC DNA expression vector and a control shRNA compared to PNC DNA expression vector with PNshRNA (lane 2 compared with lane 3). To determine if PN induced COL1α1 expression through an integrin-dependent signaling mechanism, we analyzed overexpression of PN in the presence of inhibitors of specific intracellular kinases. Optimal concentrations of the PI3K/Akt kinase inhibitor (LY294002 (10 µM)), and of an Erk inhibitor (U0126 (10 µM)) were first determined with dose experiments (data not shown). Mouse prevalvular cushion cells were then pre-treated for 12 h with or without these optimal doses of the inhibitors, or with a blocking antibody for $\beta_3$-integrin ($\beta_3$-integrin Ab) with an appropriate control antibody (Control-Ab). The treated cells were then stimulated with PNC DNA expression vector for 24 h, and COL1α1mRNA expression was measured by qRT-PCR. Figure 8C shows that each of these treatments limited or restricted the anticipated increase in COL1α1 expression in the following rank order: LY294002 (lane 5) > U0126 (lane 4) > $\beta_3$-integrin Ab (lanes 3).
versus control antibody (lane 2). Since PN activated both Akt and Erk in chicken and sheep valve fibroblasts (Figures 1 and 2), the direct activation of the PI3K/Akt and Erk pathways appears, at least in part, to directly increase COL1α1 expression in differentiating mouse prevalvular cushion cells by PN/β3-integrin signaling.

**Cross-talk between PN/β1-integrin/PI3K, and HA-CD44 signaling regulates compaction of collagen matrix.**

One of the most important morphogenetic steps in valvulogenesis is compaction of the valve matrix into attenuated mature leaflets with highly ordered, linear arrays of collagenous ECM. In figure 4, we indicated that both PN-mediated β1- and β3-integrins are responsible for valve progenitor cell adhesion and are largely redundant, whereas PN-mediated β5-integrin has a modest effect in regulating FAK and MAPK signaling. We have found that these two β1- and β3-integrins are the redundant receptors that play key role PN induced cell survival/migration functions, but β1-integrins signaling is more responsible for PN induced contractile function compared to that of β3-integrins. That is why in this paper the cushion cell survival functions were shown with β3-integrins whereas cushion cell contraction functions were shown with β1-integrins. We have focused on β1-integrin for contractile function of cushion cells because this specific integrin also regulates the actin-binding protein, filamin-A (FLNA)(50). Studies have identified point mutations in FLNA in patients with non-syndromic, myxomatous valvular diseases like mitral valve prolapse (51,52). We have found similarity both in the expression patterns of PN and FLNA, and in the phenotypes of PN and FLNA knockout mice, each of which have inhibited ECM remodeling of AV valve primordia (10,52-55). FLNA is an actin binding protein that anchors various transmembrane proteins to the cytoskeleton, and it provides a scaffold for many cytoplasmic signaling proteins involved in actin cytoskeleton remodeling. Thus, we hypothesize that during valve progenitor cell growth/cell migration, β1-integrin may have a non-redundant role for regulating valve maturation/differentiation, and work is in progress to determine if FLNA may be the downstream target of PN/β1-integrin signaling. In the present study, we tested whether PN and its interaction with β1-integrin receptors affect the potential of cushion prevalvular cells to compact and organize collagenous ECM using 3-D collagen contraction assays. In this assay, the collective contractile force of cells embedded within a collagen gel lattice was determined by measuring changes in the diameter of the gel over time (the smaller the diameters, the greater the contractile forces). Figure 9 shows that control cell cultures contracted the diameter of the gel ~50% after 2 days in culture, whereas cultures overexpressing PN contracted the diameter of the gel ~80-90%. The ability of the seeded cells to contract collagen gels was abolished by: (i) silencing PN using PNshRNA (lane 3), (ii) treatment with blocking antibodies to β1-integrin (lane 4), and (iii) pharmacological inhibitors for FAK (PF573228) (lane 5) and PI3K/Akt (LY294002) (lane 6) prior to PN overexpression. Because HA can signal through its receptor CD44 (56), we determined if the cross-talk between PN/β1-integrin/PI3K signaling and HA-CD44 signaling regulates 3-D collagen contraction by treating the cushion cells with blocking antibody against CD44 prior to PN overexpression (Figure 9, lane 7 compared to lane 1 and lane 2). These treatments inhibited compaction even when PN was being over expressed (Figure 9, lane 3-7 compared to lane 2). These results provide strong evidence that cross-talk between PN signaling mediated through the downstream targets of integrins (FAK and PI3K) can induce contractile forces in target cushion cells sufficient to contract (compact) collagen in a manner consistent with homeostatic valve maturation as indicated by the progressive attenuation and histological zonation of leaflets. In addition, HA/CD44 signaling plays a key role in cushion cell differentiation and maturation (Figure 9). Since HA/CD44 signaling, like that of PN/integrin signaling, has been linked to promoting cell survival and migration (57), PN/integrin signals are amplified by a stimulatory effect on HA/CD44 signaling through an upregulation of Has2 expression/activity. The putative cross-talk between these
two signaling pathways might serve to enhance signaling effects of both PN and HA.

**Comparison of HA in developing AV cushions of WT and PN null mice indicates that HA synthesis is a target of PN signaling.**

One of the most abundant ECM components in developing AV cushions is hyaluronan (HA) (45,58). HA is an extracellular glycosaminoglycan that, along with chondroitin sulfate proteoglycans like versican, has osmotic properties that can expand extracellular spaces in developing AV cushion growth. This potentially facilitates cushion cell migration during the normal period of cushion growth and elongation (ED14.5–ED18.5). However, as shown in Figure 10A, HA is greatly reduced when the PN gene is knocked out, which correlates with failure of the cushions to grow, fully elongate and mature. These findings led us to determine whether HA synthesis is a direct binding target of PN signaling in prevalvular AV cushions.

To do so, cushion cell cultures were prepared from E16.5 embryonic heart AV valves microdissected from wild type and PN-null mice and examined for HA synthase 2 (Has2) and secretion of HA (Figure 11 A-G). Cells from PN-null cushions were transfected with PNcDNA expression vector as part of a “rescue” experiment. Immunoprecipitates were prepared from the lysates using monoclonal anti-Has2 antibodies and analyzed for phospho (p)-serine, p-threonine residue(s) within the native Has2 protein (59). This procedure will determine the extent of Has2 protein phosphorylation at serine/threonine sites of Has2, which play critical regulatory roles in the activation of Has2 and its potential to secrete HA. The synthesis of HA is an energy-consuming process where the ratio of ATP/AMP-activated kinase (AMPK) controls the synthesis of HA (59,60). During energy stress, the ratio of AMP/ATP increases with consequent activation of AMP kinase. Activated AMP kinase phosphorylates threonine in HAS2, which inactivates the anabolic process to synthesize HA (59,60). Inhibiting anabolic processes and inducing catabolic pathways to restore ATP levels back to normal would be needed to synthesize the UDP-GlcNAc substrate (61,62). On the other hand, phosphorylation of a serine residue would be critical for the normal activation of Has2 to maintain HA synthesis (59–62).

In figure 11A, western blots demonstrate that p-serine-Has2 was reduced in the PN-null cells, but restored or increased when PN-null valve were transfected in culture with PNcDNA expression vector to the same extent as seen in WT cells. Interestingly, the p-threonine level is high in the PN-null primary cell cultures, and the level was decreased in PN-null valve cell cultures transfected with PNcDNA expression vector (Figure 11D). These results indicate that threonine phosphorylation of Has2 correlates with reduction in the HA synthetic activity in PN-null cell cultures, and that serine phosphorylation of Has2 correlates with higher synthesis of HA in PN/WT mouse cushion progenitor cell cultures. From the results in Figure 11D we cannot rule out the presence of other proteins in the Has2 immunoprecipitate. In order to know which of the HA synthases in the primordia and cells are affected by PN, we therefore investigated Has1, Has2, and Has3 transcript levels in WT and PN-null cushion progenitor cells. Results in Fig.11E shows that HA synthase isoforms are differentially localized in post-EMT cushion cells. The endogenous mRNA level of Has2 remains significantly increased, whereas HAS3 only modestly increased while Has1 level remains very low (Fig.11E). Because Has2 null mice die early in cushion development (prior to EMT) (45,63), the observed high level of Has2 (Figure 11E) may play a major role in the post-EMT stage when the level of PN remains high (Figure 10).

A previous study reported that ERK-mediated-serine phosphorylation of all three HA synthases increased their specific activity for HA synthesis in cancer cells (64). In figure 3, we showed that PN/integrin/FAK-dependent ERK1/2 responses could be modulated by the PI3K/Akt signalling. We next explored the possibility that PN-dependent Has2 activation and synthesis could be modulated by the PI3K/Akt, and/or Erk signalling. Results in figures 11D-11E show that both PI3K and Erk inhibitors attenuated the p-serine–Has2 level and decreased HA synthesis in WT mouse valve cell cultures. In addition, since PI3K in this mouse
valve primary cell culture regulates the Erk activation (figure 3), the effect of PI3K on inhibition on p-serine-Has2 level and HA synthesis is higher compared to that of Erk inhibition alone (figures 11D-11E).

While these in vivo and in vitro findings support the hypothesis that PN can promote activation of Has2 and thereby increase synthesis of HA, they did not necessarily prove that PN directly induced signalling for these events. Thus, we determined whether activation of Has2 (and subsequent HA synthesis) is mediated by PI3K, a downstream kinase target of PN-integrin interaction as shown in figures 1-3. We focused on PI3K because our studies with cancer cells show that the HA synthesis is highly dependent on PI3K signalling (20,21), and these results can be comparable to our findings in figure 11D, and in figure 11E. Cultures of chick prevavular cushion cells were pretreated with the PI3K inhibitor LY 294002 (10 µM) for 45 min, and then transfected with expression vectors of full length PN cDNA to promote PN expression, or with plasmids that constitutively activated PI3K (CA-PI3K)(20) and cultured for an additional 48 h. Figure 12A shows that the effect of overexpressing PI3K on the induction of PN synthesis is greater than the response to PNCDNA expression vector with CA-PI3K (Figure 12A, lane 2 compared to lane 4). Pretreatment of the cells with the PI3K inhibitor LY294002 almost overrode the effects of PN overexpression and PI3K overexpression on both Akt phosphorylation and PN expression (Figure 12A, lane 3 and lane 5 compared to lane 2 and lane 4). In a parallel set of experiments, the ELISA-like assay for HA (Figure 12B) also showed that the effect of overexpressing PI3K on the induction of HA synthesis is greater than the response to PNCDNA expression vector with or without CA-PI3K (Figure 12B, lane 2, lane 3 and lane 4 compared to lane 1). HA synthesis also reverted to control levels (lane 1) when cushion cells were treated with LY294002 (Figure 12B, lane 5, lane 6 and lane 7 compared to lane 2, lane 3, and lane 4 respectively). These results further emphasize that PN stimulates Has2 expression/activation (Figure 11) and HA synthesis (Figures 10 and 12B), and that this stimulation is induced by PN/β3-integrin/FAK-signalling mediated through PI3K. In contrast MEK1/2 inhibitors U-0126 (20 µM) did not alter the combined stimulatory effect of CA-PI3K and PNCDNA overexpression (Figure 12B, lane 8 compared to lane 4). Taken together, these data indicate that the PN induced intracellular signalling pathways directly stimulate HA synthesis and secretion in a time frame that correlates with the elongation of AV cushions into the future mitral or tricuspid leaflets (Figure 10). Interestingly, an unexpected finding was that by inhibiting PI3K, the expression of PN itself was inhibited (Figure 12A, lane 3 compared with lane 2) indicating that a positive feedback loop may exist between PN and PI3K and/or Erk and HA to sustain PN expression, and increase p-serine-Has2 expression and HA production, and its downstream signalling potential through integrins.

**DISCUSSION:**

Coordinated regulation of valve progenitor cell migration, increased cell growth, adhesion with secretion, assembly and alignment of ECM is essential for remodeling of the AV mesenchymal primordia (cushions) into mature valve leaflets. The process begins at midgestation (about E14.5) when PN expression begins to increase, and finishes postnatally as PN expression declines to a low, barely detectable baseline level (51, 52). This suggested a potential role for PN in valvulogenesis that was confirmed by globally deleting the PN gene (10,65). The PN null AV valves exhibited a non-compacted, myxomatous-like phenotype, which indicated that differentiation and remodeling had been adversely affected (7,10,11). At least two not necessarily mutually exclusive mechanisms could account for this abnormal phenotype. First, PN, like other matricellular proteins, could directly bind to collagen fibrils, cross-linking them or linking them to binding sites of cell surface (15). The second mechanism, and the hypothesis of this study, is that interaction of PN with cell surface receptors induces downstream signalling activities in prevalvular mesenchymal cells that regulate their differentiation and morphogenesis. Our study tested this mechanism using well-defined explant culture and gene transfection models.
The results shown in figures 1-3 demonstrate that PN in embryonic mouse AV cushion cells increased activation of p-FAK, p-Akt and p-Erk. However, the regulation of ERK signalling in a PI3K-dependent manner via FAK (figure 3E and 3G) has not been previously described. Although our data suggest that ERK is one key downstream effector of PI3K-dependent cell survival activity in these cells, we cannot exclude the possibility that PN-β1/3-integrin may also regulate signalling pathways in addition to ERK that contribute to the survival of these cells.

Silencing RNA against β3-integrin inhibited these responses, indicating that activation of these kinases was mediated through binding of PN to β-integrin heterodimeric receptors (Figures 4, 5, 6), which are well known for their binding to ECM proteins (53). Using blocking antibodies against β1-integrin, β3-integrin, and β5-integrins, our data in figure 4 show that β3-integrin and β1-integrin are necessary to attach and spread prevallvalar cushion cells on a migratory surface. Under these conditions, PN-induced cushion cell migration was mediated through downstream FAK and PI3kinase/Akt signalling (Figure 7). These data strongly suggest that PN interaction with integrin receptors cooperates with FAK and PI3K/Akt signalling to promote cellular activities required for normal valvulogenesis. Interestingly, blocking Erk has little or no effect on migration (Figure 7). However, PN activation of Erk appears to correlate with expression of collagen (Figures 1, 2). While our data do not indicate whether PN directly regulates collagen mRNA and protein expression in valve progenitor cells, we observed reduced COL1α1 mRNA production in cultured PN null cushion cells (Figure 8A), which is consistent with the reduction in collagen expression observed in vivo in PN null valves (15). Also, enhanced expression of COL1α1 mRNA was observed following overexpression of PN in cultured WT cushion cells, which was prevented by inhibiting β3-integrin, PI3K, or Erk (Figure 8C), indicating that PN most probably induces COL1α1 expression directly through the β3-integrin/FAK/PI3K/Akt and/or Erk pathways. Since PN can also bind to β5- and β1-integrins, it is possible that these integrins also might induce COL1α1 expression through these same pathways.

Our data in figure 9 suggest the interaction of PN and β-integrin can also generate forces that compact collagen in 3D gel assays, which we used to simulate the morphogenetic role of PN in compacting prevallvalar cushions into attenuated valve leaflets. How PN-integrin binding generates this contractile force is not known, but the data in figure 9 suggest that it is likely related to kinase activation of a downstream effector mechanism such as the actin cytoskeleton through activation of FAK/PI3K. Changes in the actin cytoskeletal organization are well known to occur at sites of FAK/PI3K expression (57,66). While our compaction assays strongly link a PN-integrin/FAK/PI3K signalling pathway with generating contractile forces sufficient to compact collagen, there is no clear downstream molecular target that might link a PN-induced signalling pathway with the actin-cytoskeleton. One possible candidate is FLNA. Additionally, since the cross-talk between PN and HA-CD44 signalling that plays important role in cushion cell contraction (Figure 9), HA-CD44 signalling may also target FLNA to activate contractility as has been seen in other cell types (67). We have found similarity both in the expression patterns of PN and FLNA, and in the phenotypes of PN and FLNA knockout mice, each of which have inhibited ECM remodeling of AV valve primordial (10,53). This phenotype is also characteristically seen in patients with mitral valve prolapse, including a non-syndromic subset of patients with point mutations in FLNA (52,54,55). Filamin A is an actin binding protein that anchors various transmembrane proteins to the cytoskeleton, and it provides a scaffold for many cytoplasmic signalling proteins involved in actin cytoskeleton remodeling. We recently demonstrated that the loss-of-function mutations in FLNA that are associated with human mitral valve prolapse, can reduce cell spreading and migratory potential and disrupt a signalling network that balances RhoA and Rac1 GTPases that regulate FLNA binding capabilities(68). Future studies are needed to determine if FLNA is a downstream target of PN signalling...
pathways and, if so, whether PN/FLNA are part of a central regulatory network that generates contractile forces required to compact and maturate cushions into leaflets.

Finally, a link was found between PN signalling pathways and the activation of HA synthesis (through HA synthase2 (Has2)) and HA secretion. Has2 is expressed by most, if not all, cells and is essential for life. While the HAS1 and 3 null mice are developmentally normal, the HAS2 null mouse dies at an early embryonic stage prior to the period when endocardial endothelial cells normally undergo epithelial-mesenchymal transition (EMT) to form prevalvular mesenchyme. This suggest that Has 2 expression is important for EMT, however the level of Has2 continues to remain high after EMT when cushion mesenchyme normally expands and elongates distally into the lumen of the heart beginning at E15.5 and continuing into early postnatal life (36). Organ cultures of the wild type heart tube endothelial cells undergo EMT to form cardiac cushion mesenchyme at the initiation of HA synthesis by Has2. In contrast, organ cultures of the Has2 null heart endothelium do not undergo any EMT, but do so if hyaluronan is added to the culture medium (45,63,69). In the initial experiment using the cell lysate we found that Has2 antibody recognizes two bands associated between ~50-60 kD (Figure 11A). We also found that the slow moving band of the doublets of Has2 changes with over expression of PN and with silencing of PN (Figure 11A). The faster moving band close to ~50 kD remains fairly constant (Figure 11A), indicating that Has2 must remain inactive (likely the ~50 kDa band) until it is inserted into the plasma membrane where it is activated to synthesize and extrude the hyaluronan chains. Moreover, as shown in the figure 11B, stimulation of hyaluronan synthesis correlated with both an increase and a decrease of the expression of the slow moving band close to ~60 kD of the doublet Has2 expression in E16.5 WT cushion cells. These data indicate that Has2 phosphorylation may occur for the activation of Has2 protein and this activation may be correlated to hyaluronan synthesis. Within a protein, phosphorylations can occur on several amino acids. Phosphorylation on serine is the most common, followed by threonine. Tyrosine phosphorylation is relatively rare in most of the eukaryotes. The correlative evidence in figure 11A and 11B led us to perform the immunoprecipitation experiment with anti-Has2 antibody and then to probe the Has2-immunnoprecipitates with p-serine and p-threonine antibodies to provide further evidence that p-serine and p-threonine on Has2 are involved in regulation of enzyme activity in responses to PN stimulation and inhibition. Our findings demonstrate that in the absence of PN (e.g., in null mice), there is a change in the balance of serine vs. threonine phosphorylation of Has2. In PN null prevalvular cells, p-serine-Has2 is low while p-threonine-Has2 is high (Figures 11C, and 11D). Such a ratio correlates with low ATP/AMP ratios(70) that are known to inhibit HA synthesis by enhancing phosphorylation of Has2 at threonine sites that decreases its HA synthetic activity as indicated in figure 10. In figure 11, we show that the levels of Has2-serine-phosphorylation (fig.11C) and the levels of Has2-threonine-phosphorylation (Figure. 11D) parallel the changes observed in HA expression/synthesis in WT vs. null mouse valves (Figure 10, and Figure 11E). In addition, our study found that Has2 mRNA had highest expression in WT cushion tissues and had significant statistical difference when compared with that of PN-Null (Figure 11E). The expression of HA-synthases in ex vivo cushion cultures from both WT mouse and PN-null mouse, and the expression of HA and PN in WT and PN-null valve sections (figure 10) indicate that these two molecules are closely associated. Moreover, the expression of Has2 and its activation in response to PNcDNA in PN-null cells (Figures 11C- E) also indicates that PN is one of the stimulator of expression/and activation of Has2 and HA secretion. However, it is unlikely that any effect of PN on promoting HA secretion is related to the EMT process as mesenchyme formation is not affected by deletion of the PN gene. Thus, whatever role PN has in promoting HA synthesis, it is likely to be a post-EMT function and this process requires PI3K, and PI3k regulated Erk (Figures 11F, 11G and 12A-B).

While our findings indicate that ERK activation is also enhanced by a PI3K-dependent
mechanism through Tyr925-FAK (Figure 3F), we do not have an explanation as to the mechanism. Normally Tyr925-FAK serves as a docking site for the p85 regulatory subunit of PI3K that strongly activates PI3K (35-38). Overall, our studies reveal a unique mechanism of the dramatic differences in signalling kinetics of PI3K over ERK (Figure 3F), and PI3K-dependent regulation of ERK activation (Figure 3F) may provide some insight as to why prevalvular cells when induced to overexpress PI3K (using CA-PI3K) have less sensitivity to a single-agent Erk inhibitor U0126 for HA production (figure 12B, lane 8 compared to lane 4).

**Summary:** The results presented in this study provide a more comprehensive picture of the role of PN in valve morphogenesis by testing the hypothesis that PN secreted by developing valve progenitor cells induces signalling pathways that change biological functions related to morphogenesis via binding to cell surface receptors, β3- and β1-integrins. Interaction between PN and β-integrins was found to activate FAK and downstream PI3K and MAPK pathways that lead to the phosphorylation of AKT and Erk, factors that are well known for their effects upon survival, adhesion/migration, and collagen synthesis. Because PN can promote HA synthesis and HA, in turn, can activate intracellular signaling pathways through CD44 receptors, it is possible that one consequence of PN/integrin activation of Has2 is to indirectly amplify its own signaling potential. HA/CD44 signaling, like that of PN/integrin signaling, has also been linked to promoting cell survival and migration (57). Thus, a putative positive feedback loop between these two signaling pathways might enhance activation of PI3K/cell survival signaling. This would also be consistent with our previous study (71,72) where PI3K, and its downstream signaling component Akt (72), control HAS2 expression at the molecular level. Furthermore, this study demonstrates for the first time that signaling pathways activated by PN/β-integrin interaction in developing valve cells can also stimulate HA synthesis and activation of Has2 protein at a p-serine site. There is increasing evidence that phosphorylation of serine and threonine residues in Has2 to control hyaluronan synthesis whether or not it is activated (59,73). The immunoprecipitates in figure 11 C, D and F correlate well with the HA synthesis results in figure 11 B, i.e. phospho-serine increases when HA synthesis increases and phospho-threonine increases when HA synthesis decreases, as is expected from the data discussed by Hascall’s group(73). Thus, our results provides the evidence that stimulation (and inhibition) of hyaluronan synthesis may be correlated with an increase (and a decrease) of the expression of the slow moving band of the doublet of Has2 expression in E16.5 WT cushion cells in response to stimulation, (or inhibition) of periostin. A detailed characterization of the serine and threonine sites of phosphorylation may lead to the understanding of the role of serine/threonine kinases in the regulation of cellular functions in response to PN. Work is in progress to determine if PI3K/Akt, as well as Erk regulated feedback stimulation of hyaluronan, involves transcription or other posttranslational modifications of Has2. Our findings in Figures 1-12 lead to the hypothesis that PN-integrin signaling pathways might be coordinately linked to HA signaling pathways through its adhesion receptor, CD44 for valve cell differentiatation/ maturation through collagen compaction, and may be for collagen production as has been seen in our recent study in fibrogenic lung fibroblasts (19). This hypothesis is included in figure 13, which shows a model summarizing our findings in which we propose that by binding to specific integrins, PN activates intracellular kinase signalling pathways (e.g. FAK/PI3K/MAPK) and this signalling co-operatively works with HA-CD44 signalling to promote fibrogenic differentiation and maturation of AV prevalvular mesenchyme into mitral and tricuspid valve leaflets.
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**FOOTNOTES**

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3The abbreviations used are: PN, periostin; HA, Hyaluronan; ECM, extracellular matrix; AV, atrioventricular; collagen-1α1, collagen type I; PI3K, PI3-kinase; Erk, Extracellular signal–regulated kinase; controlshRNA, pSicoR-scrambledshRNA; PNshRNA, pSicoR-PNshRNA.
FIGURE LEGENDS

Figure 1. PN induces β3-integrin/FAK/AKT/Erk1/2 activation in embryonic chick AV prevalvular cushion cells (HH40). A: Chick AV cells were either treated with PN (1 µg/ml) for 12 h followed by transfection with control siRNA, or with β3-integrin siRNA. The cells were then cultured for 48 h. Cell lysates were prepared for immunoblot analyses for integrin-β3, p-FAK, PN, p-Akt, p-Erk1/2, and β-actin. B: Densitometric quantification of the western blot analyses of “A” from three different experiments is shown (expressed as % expression of β-actin). Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. P ≤ .05 (*) was considered statistically significant.

Figure 2. PN induces β3-integrin/FAK/AKT/Erk1/2 activation in adult sheep valve cells. A: Mitral valve cells from adult sheep were either treated or not with PN (1 µg/ml) for 12 h followed by transfection with control shRNA or PN shRNA. After the treatments cells were then cultured for 48 h. Cell lysates were prepared for immunoblot analyses for integrin-β3, p-FAK, PN, p-Akt, p-Erk1/2 and β-actin. (B-C): Densitometric quantification of the western blot analyses of “A” from three different experiments (expressed as % expression of β-actin). Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. P ≤ .05 (*) was considered statistically significant.

Figure 3. PN induces β3-integrin/FAK activation in embryonic (E15.5) mouse AV cells. (A), (C). E16.5 WT mouse mitral prevalvular cushion cells (mouse cushion cells) were either transfected with PNcDNA expression vector, or with vector control. Cells were then grown for 48 h, and cell lysates were analyzed by immunoblots for β3-integrin, p-FAK, PN and β-tubulin in one experiment (Figure 3A), and for p-Akt, Akt, p-Erk [1(42)/2(44)], Erk, and β-tubulin in another experiment (Figure 3C). (B). Densitometric quantification of the western blot analyses of “A” from three different experiments is shown (expressed as % expression of β-tubulin). (D). Densitometric quantification of the western blot analyses of “C” from three different experiments is shown (expressed as % expression of β-tubulin). Erk and Akt are not shown in the bar graph “D”. Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. P ≤ .05 (*) was considered statistically significant. (E) E16.5 WT mouse cushion cells were treated with or without 5 µM FAK inhibitor PF573228 for 1 h. Cells were then grown for 24 h, and cell lysates were analyzed by immunoblots for p-Tyr925 FAK, p-Akt, p-p44/42 MAPK p-Erk1/2 p-Thr202/Tyr204, and β-tubulin antibodies. (F) E16.5 WT mouse cushion cells were treated with or without 1µg/ml of PN for various times (as shown in the figure). Cell lysates were prepared after harvesting the cells and immunoblotted for p-Akt, p-Erk1/2, and β-tubulin. The ratio of fold increase in p-Akt and p-Erk1/2 in PN-treated cells were quantified by densitometry and are expressed as percent of control of the corresponding phosphoprotein. Data represent the mean±SD of three independent experiments. (G) E16.5 WT mouse cushion cells were treated with or without 10 µM PI3K inhibitor LY294002, or 10 µM Erk inhibitor U0126 for 2 h. Cells were then grown for 24 h, and cell lysates were analyzed by immunoblots for p-Akt, p-p44/42 MAPK pErk1/2 pThr202/Tyr204, and β-tubulin antibody

Figure 4. PN supports embryonic AV chick valve cell adhesion through β1- and β3-integrins. A: WT chick prevalvular cushion cells (HH40) were incubated without or with the indicated antibodies (5 µg/ml, 60 min, 4° C), washed, and seeded in 24-well plates coated with BSA or with PN. Cells from this experiment were stained with crystal violet (A), and the dye was extracted with 33% acetic acid and quantified by measuring absorbance at 630 nm (B). *(P<0.05) for statistical significance of β1- and β3-integrins vs. BSA in PN treated cultures.

Figure 5. PN-mediated cell survival in vivo in embryonic (E15.5) mouse AV sections (A) and proliferation in vitro in embryonic (E15.5) mouse AV cells (B). A: Percent of cushion mesenchymal cells expressing Ki67 (red) in sections of E 16.5 AV cushions in wild type (WT) and PN null mice. At E16.5, left AV prevalvular cushion leaflets have a higher expression of the Ki67 proliferation marker
compared with PN null mice at a similar plane of section (A). Results show the percent of cells expressing Ki67. Data represent an average of 3 sections ± SE. B: Mouse mitral preavral cushion cells were either transfected with PNC expression vector, or with vector control, or with control shRNA (scrambled shRNA), or with PN-shRNA. Cells were then grown for 48 h. Cell proliferation levels were measured using the BrdU Cellular ELISA Kit and are expressed as percent of untransfected control cells. Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. *P ≤ .05 (*) was considered statistically significant.

Figure 6. Interaction of PN with β1- and β3-integrins inhibits expression of caspase-3, an apoptotic marker. Mouse AV valve cells were transfected with empty vector control, or with PNC expression vector followed by transfection with either Control shRNA, or with PN-shRNA, or with treatments with antibodies to β1- or β3-integrin as described in Methods. Presumptive apoptotic activity was then measured by processing the cultures for caspase 3 activity by an ELISA kit and was expressed as percent of untransfected control cells.

Figure 7. PN induces cell migration through a FAK/PI3K/Akt dependent pathway in primary cultures of E15.5 mouse AV cushion fibroblasts. PN was over-expressed by PNC expression vector (empty vector was used as a control) in valve cells followed by 18 h treatment with control antibody (Cont-Ab), or with PN-Ab, or with 10 µM LY294002 (PI3K/Akt inhibitor), or with 10 µM U0126 (Erk inhibitor) (lanes 1-6). In the same experiment, cells after PN overexpression were also transfected with Control (Cont) siRNA, or FAKsiRNA alone for 48 h, or they are first transfected with FAKsiRNA followed by incubation with PN-Ab after PN overexpression. After the treatment, cells were trypsinized and analyzed for migration assays as described in Methods. Migration was quantified by counting cells in 3 random high power fields for each filter. Data are expressed as the number of cells per field from four independent fields and are expressed as the mean ± S.D. of 4 independent experiments. Statistical analyses were done using t test with Mann-Whitney modification as applicable. *P ≤ .05 (*) was considered statistically significant.

Figure 8. PN upregulates the expression of collagen1α1 (COL1α1) via PN-integrin mediated PI3K/Akt and Erk signaling pathways in vitro. (A) Total RNAs were extracted from the valve cells isolated from PN null and WT mice. Real-time quantitative PCR (qRT-PCR) for COL1α1 was done to determine relative mRNA levels of COL1α1 as described in the Methods. The data are representative of 3 independent experiments for each set of cultures. The data are represented as mean+/– SD (n = 3 independent experiments for each set of cultures) relative mRNA levels after normalization with GAPDH levels. *P ≤ .05 (*) was considered statistically significant. (B) Cultures of WT mouse valve (E15.5) fibroblasts were transfected with vector control, or with PNC expression vector co-transfection with control shRNA, or with PN-shRNA. After 48 h of second transfection, cell lysates and media were analyzed by western blots for COL1α1, and lysates analyzed for HSP47 and β-actin as described in Methods. The data are representative of 3 independent experiments for each set of cultures. (C) Cultures of WT mouse valve (E15.5) fibroblasts were transfected with vector control, or PNC expression vector followed by 12 h treatment with 5 µg/ml ControlAb, or with 5 µg/ml β3-integrin Ab, or with PI3K inhibitor LY294002 (10 µM), or Erk inhibitor U0126 (10 µM). Total RNAs were extracted and analyzed by real time quantitative PCR (qRT-PCR) for COL1 to determine relative mRNA levels as described in Methods. The data are represented as mean+/– SD of 3 independent experiments for each set of cultures. *P ≤ .05 (*) was considered statistically significant.

Figure 9. Collagen compaction of HH40 chick mitral valve cells. Chick cushion fibroblasts were placed within collagen gel lattices as described in Methods. Contraction was monitored by measuring gel weights. Upper panel, Mean and SD of gel diameters are shown as a measure of contraction. *P<0.05 versus untreated control cells. Lower Panel, Photograph shows the contraction obtained in each gel.
Figure 10. PN and HA expression during AV cushion differentiation and maturation.  (A) Immunohistochemical (IHC) expressions of PN (red) and HA (green) at E16.5 after fusion show predominant endocardial staining of cushions in WT mitral valves (MV) while PN null MV show minimal HA staining. Both PN and HA images for MV sections of WT and PN-Null are at the same magnification (scale bars 100 µm). (B) IHC of PN and HA expressions merged at E16.5 WT and PN null mouse. Left panel: Predominant staining for PN and HA merged expressions (yellow) is shown in tricuspid valves (TV) and mitral valves (MV) of total heart sections of WT mouse while PN null TV and MV show minimal HA staining. The mouse heart sections of WT and PN-Null mouse is at the 5x magnification (scale bars 100 µm). Right panel: Similarly predominant staining for PN and HA merged expressions (yellow) is shown in MV sections of WT mouse while MV sections of PN null show minimal HA staining. The mouse MV section with merged image is at the 10x magnification (scale bars 100 µm). (C) Quantification of HA in WT and PN null valve cell cultures. Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. P ≤ .05 (*) was considered statistically significant.

Figure 11. PN regulates hyaluronan synthase 2 (Has2) phosphorylation and HA synthesis in mouse valve cells.  (A) WT cushion cells were transfected with empty vector, or with PNCeDNA expression vector, or with control siRNA, or with PN siRNA. Cell lysates from cells which were grown in presence of 5% serum were directly probed with antibody against Has2 and β-actin. (B) HA secreted in the medium of the experiment in “A” was measured by an ELISA-like assay and was expressed as percent of untransfected control cells. (C-D) WT cushion cells and PN null cushion cells were transfected with empty vector, or with PNCeDNA expression vector. After 48h of transfection, the cells were kept in optimem media for 16h to bring all the cells to G0 phase to avoid unnecessary phosphorylation due to serum, then lysed in presence of protease and phosphates inhibitor (from Sigma). The cell lysate were then immunoprecipitated with Has2 antibody and the blot was prepared from the immunoprecipitate fraction, washed with Lithium-chloride/PBS and probed first with anti p-Serine-Ab (Poly-Z-PS1, Invitrogen) or anti p-Threonine-Ab (Z-PT1, Invitrogen) (to detect proteins containing p-Ser, or p-Threonine residues) or reblotting with anti-HAS2-Ab (C-5, sc-365263) as a loading control. Data are representative of three experiments. (E) Total RNAs were extracted from the valve cells isolated from PN null, WT mice, and from serum starved WT control cushion cells. Real-time quantitative PCR (qRT-PCR) for Has1, Has2, Has3, and GAPDH were done to determine relative mRNA levels of Has1, Has2 and Has3 as described in the Methods. The data are represented as mean+/− SD (n = 3 independent experiments for each set of cultures) after normalization with GAPDH levels and expressed as relative mRNA levels compared with serum starved WT control cushion cells. P ≤ .05 (*) was considered statistically significant. (F) p-serine-Has2 was detected in Has2-immunoprecipitates of cell lysates from E16.5 WT mouse mitral prevavalvular cushion cells treated with or without 10 µM LY294002, or 10 µM U0126 for 1 h. Data are representative of three experiments. (G) HA secreted in the medium of the experiment in “F” was measured by an ELISA-like assay. Data are expressed as ± SD. Statistical analyses were done using t test with Mann-Whitney modification as applicable. P ≤ .05 (*) was considered statistically significant.

Figure 12. PI3K regulates HA synthesis and PN expression in chick AV valve cells. (A) Cells were either transfected with vector control (cont) cDNA, or CAP3KcDNA, or PNCeDNA expression vector followed by treatment with PI3K inhibitor LY294002 (10 µM for 2 h at 37°C), or treatment with Erk inhibitor U0126 (10 µM for 2 h at 37°C), and the transfectants were cultured for 48 h. Cell lysates were prepared and immunobLOTS analyzed for PN, pAkt, and β-actin. (B) Hyaluronan released into the medium was measured by an ELISA-like method and expressed as % control (vector control transfectant).

Figure 13. Signaling model for the matricellular protein, periostin, in AV valvulogenesis. Results of our signalling studies are summarized in the proposed model. PN binding to β1 or β3-integrin activates (phosphorylates) FAK, which activates downstream MAPK/Erk and PI3K/Akt to regulate
prevalvular cell growth, survival, differentiation into fibroblasts and matrix organization (maturation). PN binding to β3-integrin also activates Has2 mRNA expression, Has2 phosphorylation and HA synthesis. The interaction of HA with CD44, may, in turn, amplify the downstream effects of PN on cushion cells differentiation/maturation process.
Figure 1

A. Chicken valve cell lysate

WB:

- β3-Integrin
- p-FAK
- PN
- p-Akt
- p-Erk1/2
- β-actin

|       | 1 | 2 | 3 |
|-------|---|---|---|
| PN    | - | + | + |
| Cont sRNA | - | + | - |
| β3-Integrin sRNA | - | - | + |

B. Chicken valve cell lysate

Panel 1

[Graph showing fold change (% β-actin) for Untreated, PN + Cont sRNA, PN + β3-Integrin sRNA]

Panel 2

[Graph showing fold change (% β-actin) for Untreated, PN + Cont sRNA, PN + β3-Integrin sRNA]

Panel 3

[Graph showing fold change (% β-actin) for Untreated, PN + Cont sRNA, PN + β3-Integrin sRNA]

Figure 2

A. Sheep valve cell lysate

WB:

- β3-Integrin
- p-FAK
- PN
- p-Akt
- p-Erk1/2(42/44)
- β-actin

|       | 1 | 2 | 3 |
|-------|---|---|---|
| PN    | - | + | + |
| Cont sRNA | - | + | - |
| PN sRNA | - | - | + |

B. Sheep valve cell lysate

Panel 1

[Graph showing fold change (% β-actin) for Untreated, PN]

Panel 2

[Graph showing fold change (% β-actin) for Untreated, PN]

C. Sheep valve cell lysate

Panel 1

[Graph showing fold change (% β-actin) for Untreated, PN + Cont sRNA, PN + PN sRNA]

Panel 2

[Graph showing fold change (% β-actin) for Untreated, PN + Cont sRNA, PN + PN sRNA]
Figure 3

A. 
- β3-integrin
- PN
- p-Fak
- β-tubulin

Vector control - +
PN cDNA - +

B. 
- Fold change (% tubulin)

1. Vec control
2. PN cDNA

C. 
- p-Akt
- Akt
- p-Erk1/2
- Erk
- β-tubulin

Vector control - +
PN cDNA - +

D. 
- Fold change (% tubulin)

1. p-Akt
2. p-Erk (42)
3. p-Erk (44)

E. 
- p-FAK
- p-Akt
- p-Erk1/2
- β-tubulin

PF573228 - +

F. 
- Protein activation (% untreated control)

30 min, 1h, 2h, 4h, 16h, 24h

G. 
- Akt
- p-Erk1/2
- Erk1/2
- LY294002
- U0126
Figure. 4

A. Treatment with blocking antibodies

B. Absorbance at 630 nm

Figure. 5

A. Percent of cells expressing K5/7 in WT and PN/Null mice

B. Fold increase in mouse cushion formation (BrdU ELISA assay)
Figure 12.
Figure 13.
Periostin induces intracellular cross talk between kinases and hyaluronan in atrioventricular valvulogenesis
Shibnath Ghatak, Suniti Misra, Russell A. Norris, Ricardo Moreno Rodriguez, Stanley Hoffman, Robert A. Levine, Vincent C. Hascall and Roger R. Markwald

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