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Periostin promotes atrioventricular mesenchyme matrix invasion and remodeling mediated by integrin signaling through Rho/PI 3-kinase

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Abstract

Recent evidence suggests that extracellular matrix components may play a signaling role in embryonic valve development. We have previously identified the spatiotemporal expression patterns of periostin in developing valves, but its function during this process is largely unknown. To evaluate the functional role periostin plays during valvulogenesis, two separate three-dimensional culture assay systems, which model chick atrioventricular cushion development, were employed. These assays demonstrated that cushion mesenchymal cells adhered and spread on purified periostin in a dose-responsive manner, similar to collagen I and fibronectin via $\alpha_v\beta_3$ and β_1 integrin pairs. Periostin overexpression resulted in enhanced mesenchyme invasion through 3D collagen gels and increased matrix compaction. This invasion was dependent on $\alpha_v\beta_3$ more than β_1 integrin signaling, and was mediated differentially by Rho kinase and PI 3-kinase. Both matrix invasion and compaction were associated with a colocalization of periostin and β_1 integrin expression to migratory cell phenotype in both surface and deep cells. The Rho/PI 3-kinase pathway also differentially mediated matrix compaction. Both Rho and PI 3-kinase were involved in normal cushion mesenchyme matrix compaction, but only PI 3-kinase was required for the enhanced matrix compaction due to periostin. Taken together, these results highlight periostin as a mediator of matrix remodeling by cushion mesenchyme towards a mature valve structure.

Keywords: Valvulogenesis; Periostin; Mesenchyme; Integrins; Signal transduction; Embryo; Chick; Virus; Invasion; Migration; Matrix; Compaction; Cushion; Remodeling

Introduction

Embryonic valve formation is a highly coordinated process involving a complex integration of cellular and matrix mediated processes. The initial loci for atrioventricular (AV) valve development are specific subsets of endocardial cells lining the primitive atrioventricular canal, which are adhered to a glycosaminoglycan rich matrix termed the "cardiac jelly" initially secreted by the underlying primary myocardium(Krug et al., 1985; Manasek et al., 1973). These myocardial cells directly subadjacent to the AV endocardial cells begin secreting growth factors, principally bone morphogenetic protein-2 (BMP-2) around Hamburger and Hamilton stage 14 (HH14) (Hamburger and Hamilton, 1992), which initiates a cascade of interrelated signal pathways resulting in an endocardial transformation to mesenchyme (EMT). This is evidenced by the loss of expression of endocardial markers like VE-cadherin, CD31, and NCAM1, and gain of expression of mesenchymal markers such as α-smooth muscle actin (Person et al., 2005). These cells suspend their junctional contacts from neighboring endocardial cells and adopt an activated, migratory phenotype characterized by polarized cell bodies with numerous filamentous membrane extensions. These cells then invade the underlying matrix, secreting conditioning factors such as chondroitin sulfate and heparin sulfate, which both encourage additional mesenchymal invasion and synthesis of fibrillar proteins (Funderburg and Markwald, 1986). By HH25, the cardiac jelly has been remodeled into fully mesenchymalized swellings, dubbed "cushions", which eventually form the valves and septa of the mature 4-chambered heart. The morphogenesis of the AV valves from these cushions involve a process of proliferation, extension, condensation, and delamination (De la Cruz and Markwald, 2000). Beginning at HH26, by

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a process not completely understood, a subendocardial zone of mesenchyme proliferates and migrates, extending the primitive tissue along an AV myocardial substrate (Oosthoek et al., 1998a). The portion of the cushion that interfaces with the myocardium begins to differentiate into a fibroblastic phenotype. This differentiated phenotype then begins to condense the cushion matrix into a thinner, more fibrous tissue. Fenestrations develop in the subadjacent myocardial layers by HH30 through an as yet unknown mechanism, which coalesce and delaminate the primitive leaflet from the myocardial walls. Residual connections to the myocardium are eventually remodeled into the tendinous chords and papillary muscles (De la Cruz and Markwald, 2000; de Lange et al., 2004; Oosthoek et al., 1998b). These processes are largely mediated by the maturing cushion mesenchyme, but the mechanisms behind this matrix remodeling process are largely unknown.

Recent evidence has identified several ECM components that are critical in regulating valvulogenesis. Camenisch et al. (2000, 2002) showed that hyaluronan synthase 2 (Has2) null mouse hearts failed to form cardiac jelly, which inhibited mesenchymal transformation (and ultimately absence of cardiac cushions) through impaired Ras signaling through ErbB2 receptors. The same result (no valves) was noted in hyaluronidase digested rat embryos (Baldwin et al., 1994) and UDP glucose-dehydrogenase (UGDH—an important enzyme in hyaluronic acid processing) deficient zebrafish (Walsh and Stainier, 2001). The proteoglycan versican, which binds collagen and hyaluronan, is also important for cushion formation as versican null mice also do not form cushions (Mjaatvedt et al., 1998). Endocardial transformation to mesenchyme is further characterized by the secretion of extracellular matrix molecules including collagens I, II, III, V, and VI, tenascin, aggrecan, and eventually elastin (Garcia-Martinez et al., 1991; Hurle et al., 1994; Lincoln et al., 2004). Each of these matrix components confers important spatiotemporal signals required for cushion remodeling and may be related to the aforementioned growth factor signaling networks. Lincoln et al. (2006a) determined that FGF stimulation of HH25 AV cushion mesenchyme resulted in the expression of tenascin, while BMP stimulation resulted in aggrecan expression. The effects of these constituents on cushion mesenchymal cells, however, remain to be determined.

We previously identified and characterized the spatiotemporal cardiac expression pattern of periostin, a secreted extracellular matrix protein belonging to the fasciclin gene family. During cardiac development, periostin expression is specifically localized to the subendocardial region of atrioventricular cushions and along the mesenchymal myocardial interface during the period of delamination (Kern et al., 2005; Kruzynska-Frejtag et al., 2001; Norris et al., 2004). This pattern of expression is very similar to the locations of the proliferation/remodeling zones in the AV cushions, suggesting that this ECM protein may play a role in mediating cushion remodeling (De la Cruz and Markwald, 2000).

The objective of this study therefore was to determine if periostin mediates the adhesion, invasion, and matrix condensation of Post-EMT AV cushion mesenchyme, and by what mechanism. To accomplish this, quantitative three-dimensional (3D) assays were developed to measure cell migration, invasion, and condensation independently over time. Data demonstrate that periostin enhances collagen invasion and condensation by cushion mesenchyme, potentially through specific integrin pairs and signal pathway cascades.

Materials and methods

Periostin structure and viral production

Periostin is an 811 amino acid polypeptide comprised of 4 repeating fasciclin domains (Fas1–4), an amino-terminal signal sequence (S.S.) and a putative glycosylation site in the 4th fasciclin domain (Litvin et al., 2005). A full-length mouse periostin clone (kind gift of Dr. Simon Conway, IUPUI) including the start site of translation and kozak sequence was cloned in the sense orientation into the pDNR adenoviral shuttle vector (Clontech). A chicken full-length cDNA clone (Norris et al., 2004) and a LacZ clone were also inserted into separate pDNR shuttle vectors (Supplemental Fig. 1). To make the chicken periostin anti-sense vector, the clone was inserted in reverse. Expression of all three transgenes was driven by the constitutively active cytomegalovirus (CMV) promoter. These clones were inserted directly into the Adeno-X genome through a cre-recombinase reaction, and screened for recombination. Positive recombinants were amplified in HEK293 cells and purified using an adenoviral purification kit (Clontech). To facilitate detection of virally produced periostin, a hemagglutinin (HA) tag was incorporated into the C-terminus of the original parent mouse periostin cDNA clone by PCR.

Immunocytochemistry and Western blotting

For immunocytochemistry, HH25 chick outflow tracts were dissected, pooled, and dispersed (trypsin-EDTA- Sigma). 1×10^4 cells were plated on collagen coated glass slides in M199 media supplemented with 1% insulin selenium transferrin (ITS), 100 U pen/strep, and 1% chick serum. Cells were grown in culture for 4 days, fixed in 4% paraformaldehdye and immunostained for periostin using a rabbit a-chick periostin antibody as described previously (Kern et al., 2005). Western blot analysis was further performed to verify that HH25 AV cushions are capable of being infected by the periostin adenoviruses and to ascertain levels of periostin expression within these tissues. HH25 AV cushions were dissected and placed in hanging drop cultures containing M199+100U pen/strep+1%ITS. The adenoviruses were then added to the droplets at an MOI of 50. After 4 days in culture, cushion explants were removed, spun ($1500\times g$ for 5 min) and resuspended in 30 μl of 1× RIPA buffer containing a 1× protease inhibitor cocktail (Sigma). Samples were loaded onto a 4–15% gradient Tris–HCL protein gel (BIORAD), electrophoresed, blotted onto nitrocellulose and probed for periostin using a rabbit polyclonal α-mouse periostin antibody (1:2000) which reacts with chicken and mouse periostin as previously described in detail (Kruzynska-Frejtag et al., 2004). β-tubulin was used as a normalization control. Densitometric analysis was performed using NIH Image J software. A positive control Western was performed using media and cell lysates from infected and non-infected HEK293 cells. For these Western analyses, a mouse monoclonal HA antibody (HA-7 Sigma) was used at a 1:1000 dilution followed by a goat α-mouse secondary at a 1:10,000 dilution. Detection for all Western analyses used standard ECL detection (Upstate Biologicals).

Generation and purification of full-length periostin

Purified periostin was created by infecting 1×10^6 HEK293 cells with the mouse periostin over-expression (sense) virus (with HA tag) in serum-free conditions. Conditioned media were obtained after 3 days and adjusted to a final 0.5% Triton X-100, to which a cocktail of protease inhibitors was added. The medium was clarified by centrifugation (12,000 rpm; 10 min), then run over immobilized anti-HA beads (Vector Laboratories). The beads were extensively washed with 0.5% Triton X-100/PBS, then with PBS. Finally, bound periostin was eluted using 50 mM diethylamine. Peak levels of periostin eluted in the second bead volume of eluate. This periostin-rich fraction was neutralized by