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Hyaluronan: Genetic insights into the complex biology of a simple polysaccharide

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It is appropriate that this review should appear in a volume dedicated to Mert Bernfield. Much of my interest in the cell biology of the extracellular matrix, particularly during development, echoes Mert's pioneering studies. His kind but provocative questioning during meetings is especially missed. The glycosaminoglycan hyaluronan is ubiquitous, and is especially abundant during embryogenesis. Hydrated matrices rich in hyaluronan expand the extracellular space, facilitating cell migration. The viscoelastic properties of hyaluronan are also essential for proper function of cartilage and joints. Recent understanding of hyaluronan biology has benefited from the identification of genes encoding hyaluronan synthases and hyaluronidases, genetic analysis of the roles of hyaluronan during development, elucidation of the biochemical mechanisms of hyaluronan synthesis, and by studies of human genetics and tumors. This review focuses on recent studies utilizing hyaluronan-deficient, gene targeted mice with null alleles for the principal source of hyaluronan during mid-gestation, hyaluronan synthase-2 (has-2).
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Hyaluronan is synthesized by three enzymes encoded on separate chromosomes

Molecular cloning of mammalian hyaluronan synthases was accomplished using expression cloning [1,2] and RT-PCR using degenerate oligonucleotides based on the sequence homology amongst *S. pyogenes* hyaluronan synthase and other glycosyltransferases [3–5]. Expression of cDNAs encoding each of the hyaluronan synthases reconstitutes hyaluronan biosynthesis in cultured cells [6,7]. Moreover, cell free synthesis of hyaluronan by the recombinant protein demonstrates that, as is true for prokaryotic hyaluronan synthases, a single polypeptide is capable of synthesizing hyaluronan [6,8,9]. Mouse and human hyaluronan synthase genes are located on different chromosomes, suggesting that gene duplication and divergent evolution has resulted in differing gene regulation and kinetic properties of the encoded synthases [4,6,10].

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Genetic analysis reveals that hyaluronan is required during mammalian embryogenesis

Gene targeting reveals an essential role for hyaluronan synthase-2 (has-2), the most widely expressed hyaluronan synthase during mid-gestation in the mouse [11]. This is undoubtedly due to the fact that the has-2 enzyme is the major source for hyaluronan during development. Mid-gestational mouse embryos lacking functional *has-2* genes (but still possessing functional *has-1* and *has-3* genes) have 3% of wild-type levels of hyaluronan as measured by flurophore-assisted carbohydrate electrophoresis (FACE) analysis. Thus, neither has-1 nor has-3 suffice to replace the requirement for hyaluronan, nor are they upregulated in response to the absence of hyaluronan.

Grossly, *has-2* null embryos exhibit growth retardation evident by ~E9.0, often with marked pericardial edema consistent with a cardiovascular defect. On dissection, the yolk sac is seen to be abnormal with a characteristic punctuate appearance. Red cells are present but not confined to vessels (Figure 1A and B). Cross-sections (insets in Figure 1A and B) reveal absence of the normal vitelline vessels in the has-2 null embryo. Grossly, the *has-2* null embryo is smaller than wild-type (or heterozygous) littermates at E9.5, with numerous malformations (Figure 1C and D). Strikingly, the highly organized vascular bed revealed

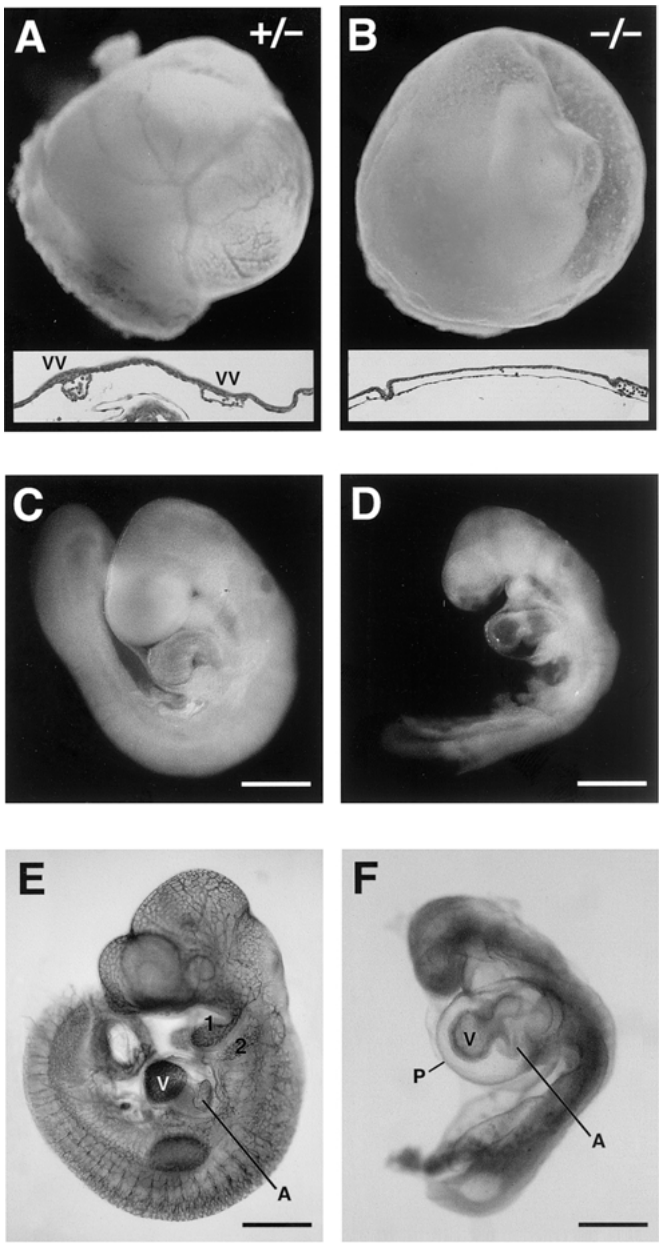

Figure 1. Multiple abnormalities in *has-2*$^{-/-}$ deficient embryos (see [12] for details). A, B: Wild-type (A) and *has-2*$^{-/-}$ (B) yolk sacs at E9.5. Compared with wild type, *has-2*$^{-/-}$ embryos exhibit abnormalities in yolk sac vasculature including separation of the endoderm from mesoderm obliterating the vitteline vessels (VV) and giving them a characteristic punctate appearance (B). C, D: E9.5 wild-type (C) and *has-2*$^{-/-}$ (D) embryos. Grossly, the *has-2*$^{-/-}$ embryo (D) is smaller than its wild-type littermate and has distorted somites. Note that the heart of the *has-2*$^{-/-}$ embryo is relatively bloodless. E, F: PECAM (CD31) staining of E9.5 wild-type (E) and *has-2*$^{-/-}$ (F) embryos. The wild-type littermate exhibits a characteristic highly developed vasculature, whereas the *has-2*$^{-/-}$ lacks an organized vasculature, and has a collection of fluid in the pericardial space (P). Branchial arches 1 and 2 are indicated by numerals. V, ventricle; A, atrium.

with whole mount staining for CD31 (PECAM) present at E9.5 in wild type embryos is absent in *has-2* null embryo (Figure 1E and F).

Histologically, the *has-2* null embryo exhibits complete absence of the expanded extracellular spaces so common in mid-gestation embryos. Figure 2 shows an expanded, hyaluronan-rich matrix ("cardiac jelly") in the developing heart stained with alcian blue to localize sulfated glycosaminoglycans (Figure 2A) or using biotinylated cartilage link module to detect hyaluronan (Figure 2C). The *has-2* null heart completely lacks cardiac jelly, and is devoid of staining for sulfated glycosaminoglycans (Figure 2B) and hyaluronan (Figure 2D).

Composite matrices containing hyaluronan are necessary to expand the extracellular space

The *has-2* null phenotype is remarkably similar to that of the heart defect (*hdf*) mouse, which in the homozygous state also results in embryonic lethality at ~E9.5. The *hdf* mutation results from a random insertion into the *Cspg2* (versican) gene [12,13]. Versican is a hyaluronan binding proteoglycan. Accordingly, we evaluated the distribution of hyaluronan, versican, and their respective mRNAs in the E9.5–10.5 heart. Their expression is highly similar during mid-gestation [11]. Figure 3 shows representative results in the E9.5 heart. Panel A is a hematoxylin and eosin stained section through the heart showing the common atrium, atrioventricular cushions and the common ventricle. The largely acellular nature of the cushions at E9.5 is apparent, as is their interposition between the common atrium and ventricle. Panel B is a similar section stained using biotinylated link protein to detect hyaluronan, a major component of the cushion matrix. The inset shows a small cluster of transforming endothelial cells surrounded with a dense hyaluronan coat. Panel C shows a composite of a Nomarski DIC image superimposed on a pseudocolored red immunofluorescence stain for versican. Finally, a day later mesenchymal cells are proliferating and migrating actively to populate the cushions of the atrioventricular canal (C) and outflow tract (O) and has-2 mRNA is very abundant (Figure 3D).

Collectively, the similar domains of expression of *versican* and *has-2* and the common phenotypes of *has-2* and *versican* null mice (with one important exception, epithelial to mesenchymal transformation discussed below) indicate that the expanded extracellular matrix present in the developing heart requires both versican and hyaluronan for stabilization. This resembles the requirement for bikunin in expansion of the hyaluronan-rich matrix in the cumulus oophorus [14]. Clearly, intermolecular interactions between proteins with hyaluronan binding motifs [15] are essential in forming and stabilizing hyaluronan-rich matrices. Many other hyaluronan binding molecules are present in hyaluronan-rich matrices in the developing heart, e.g., fibulin and it is not known if they also participate in matrix deposition or stabilization.

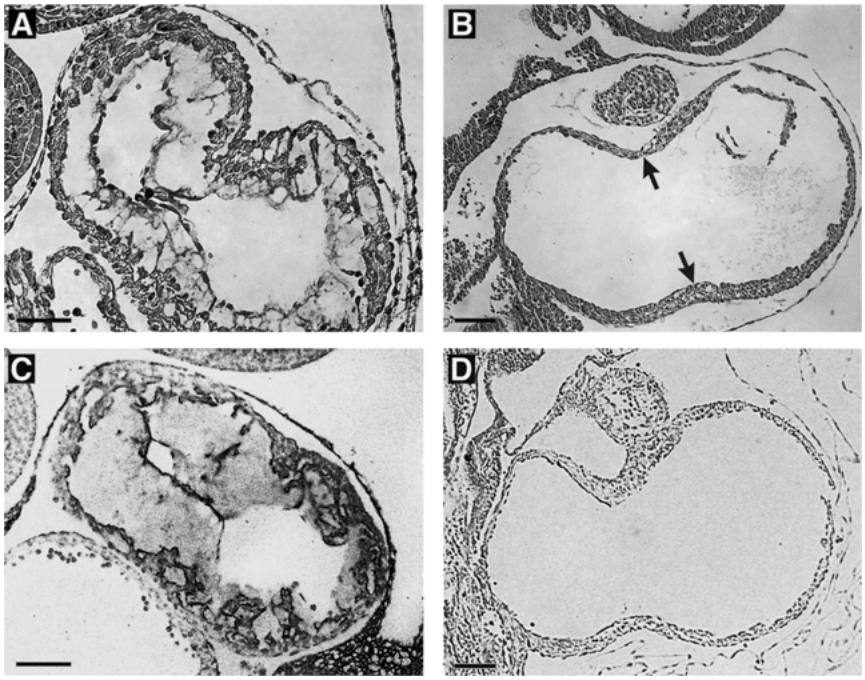

Figure 2. *has-2*$^{-/-}$ deficient embryos lack an expanded extracellular matrix containing hyaluronan or acidic proteoglycans. A histological section through the atrioventricular canal of the heart taken from E9.5 wild-type (A, C), or *has-2*$^{-/-}$ deficient embryos (B, D) was stained with a biotinylated probe for hyaluronan (C, D) or alcian blue to detect sulfated glycosaminoglycans (A, B). Note the virtual absence of the hyaluronan-rich, largely acellular cardiac jelly in the *has-2*$^{-/-}$ deficient embryo.

Genetic dissection of the role of hyaluronan in stimulation of intracellular signaling pathways resulting in cell migration and invasion in the developing heart

Genetics has been essential in dissecting the physiological role of hyaluronan. Previously, understanding its role was complicated by its ubiquitous distribution in tissues and in reagents used in tissue culture, e.g., serum. While hyaluronidases have been used to remove hyaluronan, in some cells its degradation products exhibit *de novo* proinflammatory active mediated in part via toll-like receptor activation of nuclear factor, NF-κb [16]. Thus, studies of tissues genetically deficient in hyaluronan derived from the has-2 null mouse embryo have yielded particularly informative and unambiguous insights into the role of hyaluronan in cell migration and transformation. Cells in hyaluronan-rich matrices (e.g., embryonic tissues) often exhibit marked migratory activity. This was historically attributed to the ease of penetrating a hydrated, hyaluronan-rich matrix. However, a complementary mechanism is suggested by the ability of hyaluronan to activate intracellular signaling pathways stimulating cell migration and invasion [17].

Heart development provides a physiological paradigm for studying programmed cell migration and invasion. The embryonic heart is extensively invaded and remodeled by migrating cells. Indeed, exogenous hyaluronan stimulates cell migration during cardiac development [18,19]. Exogenous hyaluronidase affects ventricular function *ex vivo* [20]. As shown above, hyaluronan is a major component of the acellular cardiac jelly. Rapid expansion of the cardiac jelly occurs in the atrioventricular canal and cardiac outflow tract forming the structures termed cardiac cushions (Figure 3A). These cushions function as primitive valves preventing regurgitant blood flow. Beginning at day E9.5 in the mouse, the endothelial cells overlying the atrioventricular cushions undergo programmed transformation into mesenchyme and migrate into the underlying cushions (Figure 3B). These mesenchymal cells contribute to the atrioventricular septum, aortic, pulmonary, and mitral and tricuspid valves. Because atrioventricular septal defects are the most common sporadic congenital heart defect [21], considerable interest has focused on the molecular mechanisms of cushion morphogenesis [22].

Cell migration and invasion during atrioventricular canal morphogenesis requires hyaluronan

To our surprise, the programmed transformation of endothelial cells to mesenchyme in atrioventricular canal explants was totally deficient in *has-2* null embryos (Figure 4). This was not attributable to developmental arrest or other abnormalities, as co-culture with normal explants (not shown), exogenous hyaluronan or adenovirus-mediated transfection of *has-2* cDNA completely restored normal morphogenesis *in vitro* (Figure 4E–G). Epithelial to mesenchymal transformation is

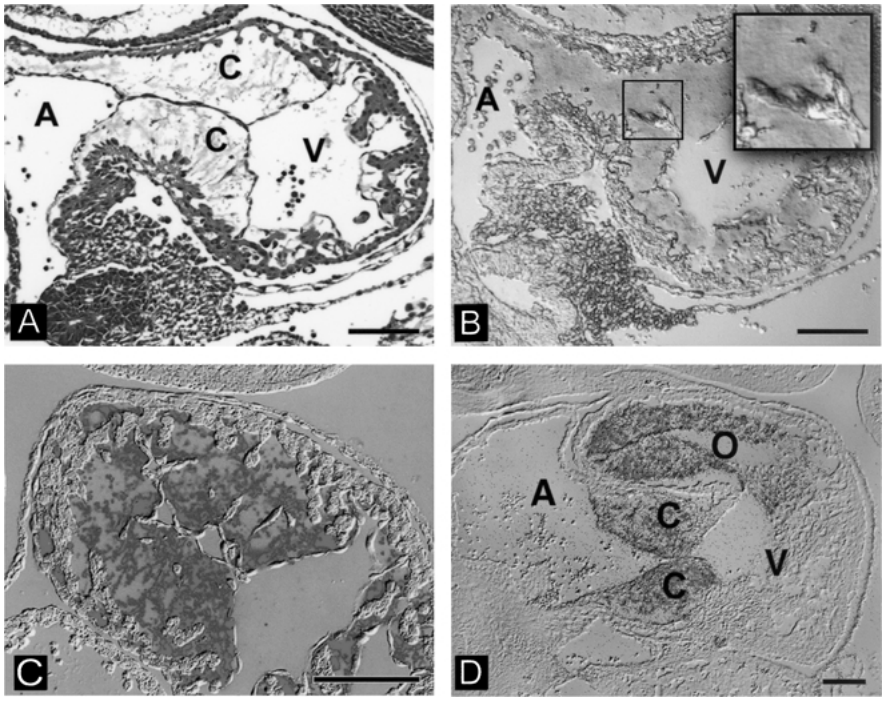

Figure 3. Hyaluronan, has-2 mRNA and versican are expressed in similar domains during cardiac development. Panel A shows a hematoxylin and eosin stained section thought the heart at E9.5 showing the common atrium (A), separated from the ventricle (V) by the atrioventricular cushions (C). Outgrowths of ventricular myocytes into the lumen of the ventricule are evident. Panel B shows a section cut at a similar stage and stained for hyaluronan using biotinylated link protein. The inset shows a small cluster of transformed endothelial cells with hyaluronan concentrated on their surface invading the cardiac jelly. Panel C is a pseudocolored (the fluorescent signal is red in the color image) imposition of a Nomarski DIC and immunofluorescent image showing high concentrations of versican in cardiac jelly. Panel D is a pseudocolored montage of dark field *in situ* hybridization for has-2 mRNA (red in the color image) and Nomarski DIC. The mesenchymal cell population in the atrioventricular cushions (C) and in the outflow tract (O) massively express massive has-2 mRNA.

reportedly normal in the *hdf* mouse [12], so this defect is specific to hyaluronan. Thus, hyaluronan is required for two pivotal developmental events in the heart: matrix expansion and initiation of cell migration (Figure 5) [11,23].

Because activity of the G protein, Ras, is implicated in atrioventricular transformation [24], we reasoned that signaling pathways resulting in Ras activation normally triggered by hyaluronan might be deficient in the *has-2* null embryo. Indeed, as shown in the lower panel of Figure 4, adenovirally-mediated transfection with a cDNA encoding a dominant-negative form of Ras-inhibited transformation, whereas constitutively active Ras rescues the *has-2* null phenotype. In addition, hyaluronan-mediated rescue of the *has-2* null is inhibited by expression of dominant-negative Ras. One well characterized signaling pathway resulting in Ras activation and involving hyaluronan occurs via a family of receptor tyrosine kinases (ErbB) [17] (Figure 6). In carcinoma cells, binding of hyaluronan to the cell surface hyaluronan receptor CD44 transactivates ErbB2 stimulating Rac and Ras signaling [25]. Hyaluronan binding to CD44 also results in c-Src kinase-mediated tumor cell migration [26] and, via interactions with Rho-kinase (ROK) activates insositol 1,4,5-triphophate (IP3) receptor-mediated calcium signaling inducing endothelial cell migration. Similarly, CD44 enhances neuregulin signaling by Schwann cells [27]. If a similar interaction occurs during cardiac development, it seems likely that null mutations in ErbB receptors or cognate ligands might also result in atrioventricular canal developmental defects mimicking the *has-2* null mutation. Provocatively, null mutations in *ErbB1, ErbB2, Erb3*, and *Erb4*, as well as a ligand for ErbB3, neuregulin-1 all exhibit cardiovascular phenotypes [11,23].

Hyaluronan and neuregulin-1 signaling via ErbB2-ErbB3 are required for normal epithelial-mesenchymal transformation during cardiac morphogenesis

To answer this question, we utilized genetics and biochemistry to dissect the role of hyaluronan and ErbB receptors and ligands during cardiac morphogenesis. Mouse mutants in *ErbB2, ErbB3, ErbB4* and *neuregulin-1* were obtained from collaborators. We used these existing mutant lines to document atrioventricular canal morphogenesis *in vivo,* and to confirm biochemically the localization of the receptors [23]. Immunohistochemistry and protein expression profiling revealed that ErbB receptors were present and activated (i.e., tyrosine

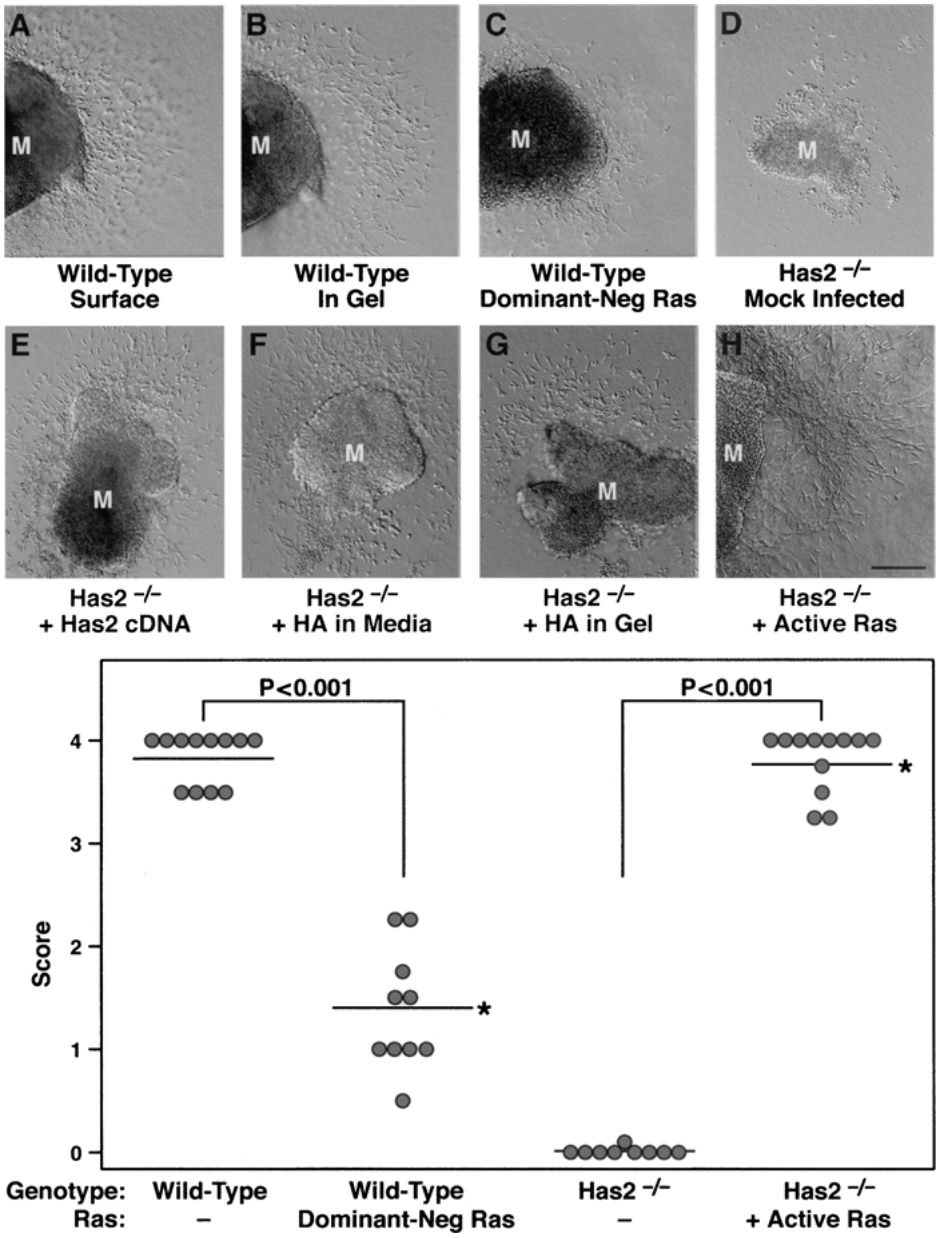

Figure 4. Hyaluronan is required for cell migration and transformation during cardiac development. The upper panel (A–H) are Nomarski DIC images of atrioventricular canal explants cultured on a type I collagen gel for 72 hr. In this model, myocytes and endothelial cells sort and organize into homogeneous tissues and recapitulate their programmed *in vivo* behavior. Myocytes (labeled "M") form a contractile unit, whereas the endothelial cells migrate over the surface of the collagen gel and transform into mesenchyme. This is shown by comparing Panel A (taken at the surface) with B (focused within the gel). In contrast, endothelial cells from the *has-2*$^{-/-}$ mouse neither migrate over nor invade the gel (Panel D). Normal cell migratory and invasive activity is completely restored by exogenous hyaluronan (F, G) or by transfection with a cDNA encoding has-2 (E). Finally, adenovirally-mediated transfection with a cDNA encoding a constitutively active form of Ras also restores migration and invasion (H). The lower panel shows results from a quantitative assay for transformation. Dominant-negative Ras inhibits transformation in wild-type atrioventricular canal explants, and constitutively active Ras rescues the defective transformation by *has-2*$^{-/-}$ explants.

phosphorylated) both *in situ* and in the well characterized organotypic culture model of atrioventricular canal morphogenesis shown in Figure 6 [11]. Using cultured atrioventricular canal explants, we showed that ErbB2 (an orphan receptor with no known ligand) is activated in both myocardium and mesenchyme. ErbB3 (that lacks a tyrosine kinase domain) is activated primarily in mesenchyme. This suggests that ErbB3 binds ligand, heterodimerizes with ErbB2, and becomes activated by trans-phosphorylation (Figure 7). The gene for neuregulin-1, an ErbB3 ligand, is expressed primarily in the

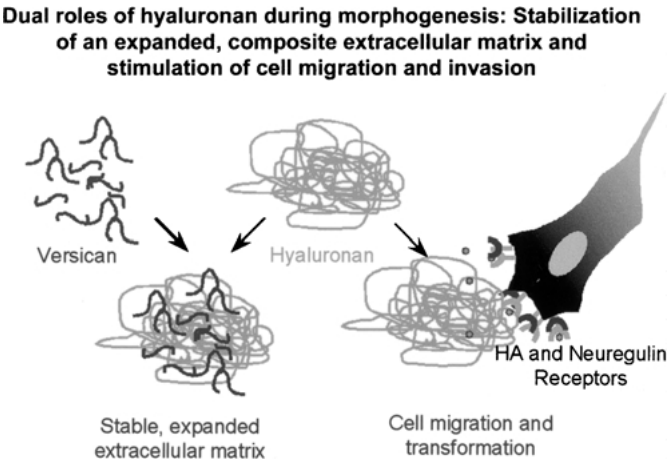

Figure 5. Dual roles of hyaluronan during cardiac development. Left: A composite matrix is required for a stability, expanded extracellular matrix. Genetic analysis shows that such matrices are absent in animals lacking either hyaluronan (*has-2*$^{-/-}$ mouse) or the hyaluronan binding proteoglycan versican (*hdf* mouse). Right: Hyaluronan is required for activation of intracellular signaling pathways culminating in cell migration and transformation.

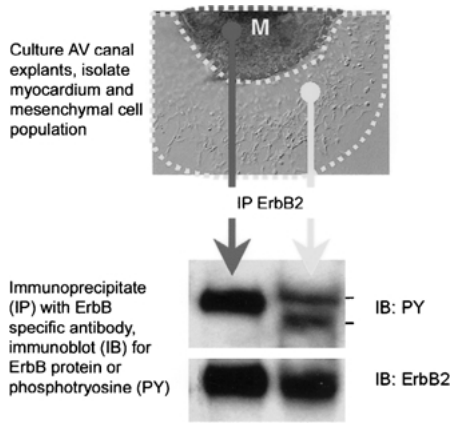

Figure 6. Method used to provide biochemical insights into atrioventricular canal morphogenesis. Atrioventricular canal explants are cultured for 72 hr, and the myocardium (M) and transforming endothelial cell population isolated by microdissection. Cells are extracted using lysis buffer preserving phosphorylated proteins, and immunoprecipitated. Immunoblotting is then performed to identify components of signaling pathways. This example shows the presence of activated (tyrosine phosphorylated) ErbB2 in both myocardial and mesenchymal cell populations.

endothelium of the atrioventricular canal [28]. Neuregulin-1 is cleaved and released from the cell surface in soluble form, and can stimulate mesenchymal cells by interacting with ErbB3 receptors. To further test the involvement of ErbB receptors, we used organotypic cultures from mice bearing null alleles of *ErbB2*, *ErbB3*, *ErbB4* and *neuregulin-1*. Quantification of epithelial-mesenchymal transformation revealed significant reduction in *ErbB2* homozygous and heterozygous null explants, and in *ErbB3* homozygous null explants. Atrioventricular explants from *neuregulin-1* null mice revealed normal morphogenesis, probably because of the multiple redundant ErbB3 ligands. Thus, ErbB2 and ErbB3 play important, non-redundant roles in atrioventricular canal morphogenesis.

Recombinant, soluble receptors were used as another probe for ErbB function in organotypic culture. Soluble ErbB3 (sErbB3, p85) is a potent competitive inhibitor of neuregulin, inhibiting ErbB2, ErbB3, and ErbB4 [29]. Atrioventricular canal explants from wild-type mice cultured in the presence of sErbB3 exhibited a characteristic, well-organized endothelial sheet surrounding the myocardium, quite distinct from the normal immediate transformation into mesenchyme of the E10.5 mouse atrioventricular canal explant [30]. Herstatin, or soluble ErbB2 is another inhibitor of ErbB function [31,32]. Adenoviral-mediated transfection of wild-type atrioventricular canal explants with a cDNA expressing herstatin similarly profoundly inhibited atrioventricular canal morphogenesis. Herstatin inhibited tyosine phosphorylation of ErbB2, and sErbB3 inhibited tyrosine phosphorylation of ErbB3.

Exogenous hyaluronan restores signaling via ErbB2-ErbB3

Finally, if hyaluronan is engaging signaling pathways via activation of ErbB receptors, exogenous soluble hyaluronan should restore phosphorylation of ErbB2 and ErbB3, which were lacking in the *has-2* null embryo. Addition of hyaluronan restored fully atrioventricular canal morphogenesis and phosphorylation of ErbB2 and ErbB3 (Figure 8). Interestingly, addition of soluble neuregulin-1 also rescued atrioventricular canal morphogenesis of the *has-2* null embryos. In these experiments we could not exclude up-regulation of has-1 or has-3 expression resulting in increased endogenous hyaluronan synthesis. Collectively, these results suggest the role of hyaluronan in atrioventricular canal morphogenesis detailed in Figure 9.

In the presence of hyaluronan and sErbB3, endothelial organization and migration are intact. This places ErbB and Ras activation at the stage of epithelial-mesenchymal transformation termed "activation" (characterized by cellular shape change and loss of cadherin-mediated junctions) and "invasion", when the activated endothelial cells adopt a mesenchymal phenotype, invade the subjacent cardiac jelly, and activate a mesenchymal gene expression profile. From these data we cannot determine if hyaluronan is required solely for initiation of epithelial morphogenesis, or for activation and invasion of endothelium as well. The remarkable expression of has-2 by activated and migrating mesenchymal cells, and their hyaluronan-rich pericellular matrices suggests the latter.

Hyaluronan receptors involved in epithelial-mesenchymal transformation

Restoration of epithelial-mesenchymal transformation in *has-2* null embryos requires only ng/ml quantities of hyaluronan.

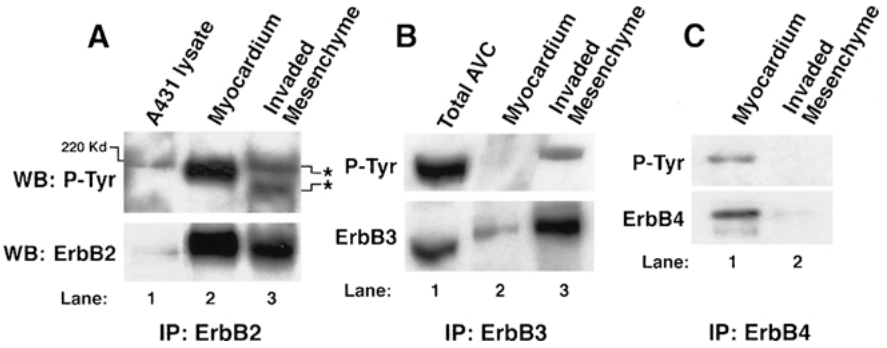

Figure 7. Repertoire of ErbB receptors expressed in atrioventricular canal tissues. All experiments were carried out using cells isolated from a 72 hr culture of a E9.5 atrioventricular canal on collagen gels. Panel A: Immunoprecipitation of ErbB2 and immunoblotting for phosphotyrosine. Lane 1 is A431 cell line lysate (a positive control for ErbB2 activation), lane 2 isolated myocardium and lane 3, isolated mesenchymal cells. Both myocardium and mesenchyme contain activated ErbB2. Panel B: Immunoprecipitation of ErbB3 and immunoblotting for phosphotyrosine. Lane 1 is the entire atrioventricular canal tissue, lane 2 the myocardium and lane 3, mesenchymal cells. Activated ErbB3 is present primarily in the mesenchymal cell population. Panel C: Immunoprecipitation of ErbB4 and immunoblotting for phosphotyrosine. Lane 1 is the myocardium and lane 2, mesenchymal cells. Activated ErbB4 is present primarily in the myocardial cell population.

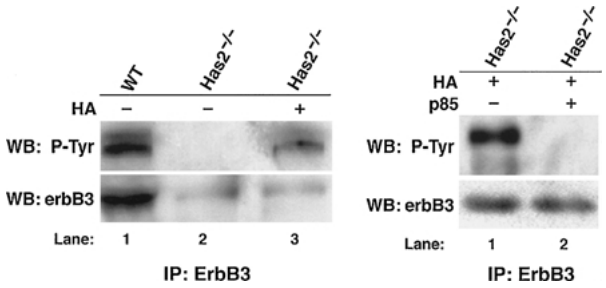

Figure 8. Exogenous hyaluronan restores activation of ErbB2/ErbB3 during atrioventricular canal morphogenesis. Left Panel: In wild-type explants (lane 1), ErbB3 is phosphorylated but not in has-2$^{-/-}$ explants (lane 2). Addition of exogenous hyaluronan (HA) restores ErbB3 phosphorylation (lane 3). Right Panel: To demonstrate a requirement for ErbB2 activation in ErbB3 phosphorylation, the soluble inhibitor p85 (soluble ErbB2) is used. In contrast to the control (lane 1), p85 prevents the hyaluronan dependent phosphorylation of ErbB3.

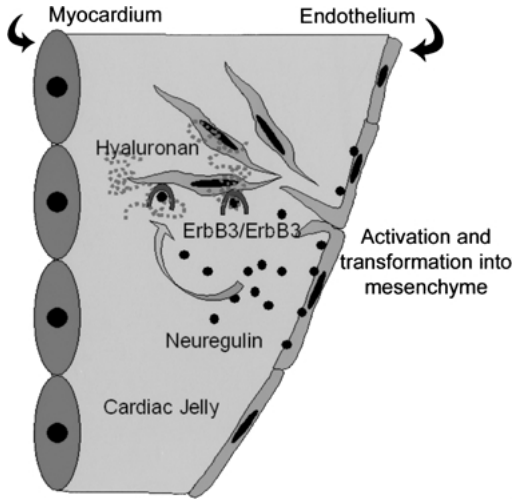

Figure 9. Model for the role of hyaluronan during cardiac morphogenesis revealed by genetic analysis. In the absence of hyaluronan, normally provided via the activity of the hyaluronan synthase-2 enzyme, endothelial cell organization and migration are defective. Exogenous hyaluronan fully restores the normal phenotype. However, if hyaluronan is present and ErbB receptors (or the downstream effector Ras) inhibited, a highly organized endothelial monolayer forms without activation or invasion. Thus, hyaluronan is required for epithelial organization and migration, and appears to act synergistically with ErbB receptors and ligands in initiating transformation to the mesenchymal phenotype.

This concentration is consistent with an interaction of hyaluronan with an effective molecular weight of ∼1,800 (based on the interaction of hyaluronan with link module) to a binding partner with an avidity of nM to μM (10^{-9} to 10^{-6}). We have used mice lacking CD44 [33] as well as antibodies inhibiting CD44 binding to hyaluronan to elucidate its role in atrioventricular canal morphogenesis. Mice lacking CD44 have no known cardiac defects. Moreover, organotypic cultures of atrioventricular canals exhibit normal morphogenesis *in vivo*. Finally, KM201, a monoclonal blocking binding of mouse CD44 to hyaluronan [34] had no effect on morphogenesis. Alternative candidates including the receptor for hyaluronate mediated mobility or receptor for hyaluronan-mediated motility (RHAMM) remain to be investigated [17].

Hyaluronan and cancer

The involvement of hyaluronan in epithelial-mesenchymal transformation has obvious implications in normal embryonic development, but also in carcinogenesis. Many carcinomas are associated with increased deposition of hyaluronan in the

surrounding stroma, as well as CD44 or RHAMM expression [35–39]. Manipulation of hyaluronan biosynthesis in tumor cell lines has yielded variable results, in some cases increasing migration and metastasis, and in others diminishing cell migration. Hyaluronan synthase-2 is one of seven candidate genes in the minimally over-represented 8q24 region in prostate cancer associated with progression [40]. Over-expression of has-2 increases adhesion of human prostate tumor cells to endothelium [41].

Human genetic disorders with potential links to hyaluronan

Ramsden reported a patient with extreme cutaneous thickening and folding, associated with marked elevations of serum hyaluronan (3100 μg/L) [42]. Hyaluronan biosynthesis by dermal fibroblasts was increased, and plasma hyaluronidase activity normal. Similar elevations in plasma hyaluronan were found in the Chinese Shar-Pei dog, leading the authors to speculate that a common abnormality in regulation of hyaluronan synthesis might underlie this novel phenotype.

Natowicz described a female patient with a deficiency of serum hyaluronidase designated as mucopolysaccharidosis IX [43]. This patient exhibited periarticular soft tissue masses, short stature, no evidence of neurological or visceral involvement, and histological and ultrastructural evidence of a lysosomal storage disease. The proband was subsequently found to be a compound heterozygote, with a nonconserved substitution in a putative active site residue and a premature termination codon [44]. Three hyaluronidase genes (*HYAL1*, *HYAL2* and *HYAL3*) are clustered on human chromosome 3p21.3, and another two genes (*HYAL4* and *PH-20/SPAM1*) and a pseudogene (*HYALP1*) are similarly clustered on chromosome 7q31.3 [45]. The complex and overlapping expression patterns of hyaluronidases may explain the relatively mild phenotype of this patient reported by Natowicz to lack *HYAL1* [4,43]. Mice with genetic abnormalities in the *PH-20* exhibit reduced fertility associated with diminished ability of sperm to penetrate hyaluronan [46].

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