Depolymerized Hyaluronan Induces Vascular Endothelial Growth Factor, a Negative Regulator of Developmental Epithelial-to-Mesenchymal Transformation

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Abstract—Cardiac malformations constitute the most common birth defects, of which heart septal and valve defects are the most frequent forms diagnosed in infancy. These cardiac structures arise from the endocardial cushions through dynamic interactions between cells and the extracellular matrix (cardiac jelly). Targeted deletion of the hyaluronan synthase-2 (Has2) gene in mice results in an absence of hyaluronan (HA), cardiac jelly, and endocardial cushions, a loss of vascular integrity, and death at embryonic day 9.5. Despite the requirements for Has2 and its product, HA, in the developing heart, little is known about the normal processing and removal of HA during development. Cell culture studies show that HA obtains new bioactivity after depolymerization into small oligosaccharides. We previously showed reduction in Has2 expression and diminished presence of HA at later stages of heart development as tissue remodeling formed the leaflets of the cardiac valves. Here we show that small oligosaccharide forms of HA (o-HA) act antagonistically to developmental epithelial-to-mesenchymal transformation (EMT), which is required to generate the progenitor cells that populate the endocardial cushions. We further show that o-HA induces vascular endothelial growth factor (VEGF), which acts as a negative regulator of EMT. This is the first report illustrating a functional link between oligosaccharide HA and VEGF. Collectively, our data indicate that following endocardial cell EMT, native HA is likely processed to o-HA, which stimulates VEGF activity to attenuate cardiac developmental EMT. (Circ Res. 2006;99:583-589.)

Key Words: epithelial-to-mesenchymal transformation • VEGF • hyaluronan • endocardial cushion

The glycosaminoglycan hyaluronan (HA) participates in creating a water-enriched macromolecular environment and is believed to regulate embryonic development by establishing a hydrated, low-resistance matrix. This matrix promotes loss of cell contact inhibition, facilitating cell motility. Although very high concentrations of HA sterically hinder cell contacts attenuating cell motility,1 most physiological levels of HA promote migration, differentiation, and cell aggregation.2-4 In general, during rapid expansion of extracellular space, there is temporal elevation in the deposition of HA by HA syntheses (Has) to support organogenesis.5,6

A prime example of HA activity is observed in endocardial cushions of the forming heart. HA mediates the rapid expansion of these cushions by organizing the extracellular matrix (ECM) and hydrating the cardiac jelly where primitive cushions begin to promote unilateral blood flow.6 Epithelial-to-mesenchymal cell transformation (EMT) occurs in cardiac cushions to produce progenitor valve cells that both remodel primitive tissue into functioning valve structures and contribute to the inferior aspect of the atrial septum and membranous portion of the ventricular septum. This is a crucial developmental step as the heart is the first organ to form, the function of which is required to support the rapidly growing embryo. This complex developmental process involves many cell types and molecules, including matrix-associated proteoglycans, growth factors, and transcription factors. The HA synthase-2 (Has2)-dependent production of HA is critical for morphogenesis of the cardiac valves and maturation of the 4-chambered heart.7,8 Although the activity of Has2 and deposition of HA are known to support cardiac development, effects of depolymerized native high-molecular-weight HA (HMW-HA) into small oligosaccharide fragments (o-HA) are not understood. HA can exhibit new bioactivity once processed into o-HA. For example, in vitro studies using cell lines demonstrate that o-HA (3 to 10 length) induces migration and proliferation of endothelial cells.9,10 In addition, it has been previously noted that, during cardiac cushion development, hyaluronidase activity is significantly elevated in the myocardial regions of increased mesenchymal migration.11 Small o-HA induces tyrosine phosphorylation and protein kinase C activity concomitant with proliferation of BAECs.12 In contrast, HMW-HA acts antagonistically to this...
activity and subsequent angiogenic events. The mechanisms responsible for the disparate roles of the different forms of HA are not yet known.

The role of removing HMW-HA by degrading it into o-HA in physiological development is not understood but is speculated to have a substantial function during organogenesis. We believe that during cardiogenesis, HA serves distinct bioactive roles linked to each polymeric form of HA. We have previously shown native HMW-HA obligate for both structural formation of the heart chambers and developmental EMT within cardiac cushions to create the cells that form valves and septa. Here, we use both an in vivo and an in vitro endocardial cushion morphogenesis model system to show that depolymerized HA (o-HA) is antagonistic to developmental EMT. Furthermore, o-HA induces vascular endothelial growth factor (VEGF) mRNA and protein in primary endocardial cushion explants. We also detect increased VEGF expression following introduction of o-HA into cardiac cushions of AV canals (AVCs). This results in abnormal endocardial cushion morphogenesis with a concomitant block in production of mesenchymal cells. This phenotype is reversed in vitro by soluble VEGF receptor-1 molecule (sFlt). sFlt acts as an extracellular sink to sequester o-HA--induced VEGF, restoring the production of cardiac mesenchyme. These data define a functional link between o-HA and VEGF attenuation of developmental EMT. These novel findings also define a molecular mechanism whereby o-HA is able to promote angiogenic activity through a VEGF-mediated mechanism. Thus, o-HA and VEGF mark a transition during heart valve development where they cause cessation of EMT and promote angiogenetic developmental processes required for proper cardiovascular development.

Materials and Methods

Primary Explant Cultures and Analysis

Timed pregnant Swiss Webster mice were purchased from Taconic (Hudson, NY). Pregnant female mice were euthanized under a protocol approved by the Institutional Animal Care and Use Committee, and embryos were collected at embryonic day 9.5 (E9.5). Atrioventricular (AV) canals were microdissected away from the embryo bodies in sterile 1× Tyrode's salt buffer (GIBCO/Invitrogen, Eugene, Ore). Endocardial cushions were explanted and cultured on hydrated type I collagen (BD Biosciences, Bedford, Mass) gels as previously described. Twenty hours after establishing the cultures, explants were supplemented with a 150 μL total volume of the indicated treatments: medium 199 (M-199) (Invitrogen), M-199 with 0.5 mg/mL HMW-HA (Genzyme, Cambridge, Mass), M-199 with 1 mg/mL oligosaccharide HA (Seikagaku Corp, East Falmouth, Mass), or M-199 with 1 mg/mL o-HA plus 20.0 ng/mL sFlt (Fitzgerald, Concord, Mass). HA and sFlt concentrations were consistent with the use of these reagents, as previously reported. Myocardial tissue removed by rinsing the gels 48 hours after treatment, and explant samples were fixed with 2% paraformaldehyde/PBS for 20 minutes. Differential interference contrast microscopy image documentation was performed on an inverted Olympus microscope (Leeds, Minneapolis, Minn) with Hoffman optics using an Olympus LHS50 video camera and ImageJ software (NIH). Fluorescent images were taken on a Leica microscope using Image-Pro Plus software (Media Cybernetics, Inc). Mesenchymal cells were enumerated by 3 observers naive to the culture conditions based on established morphometric parameters, α-smooth muscle actin staining, and migration and invasion into the collagen gels. Total cell numbers were determined through use of Image-Pro Plus software using a macroprogram based on nuclear staining with bis-benzamide (Invitrogen). Mesenchymal cell production is shown as a percentage, representing final averaged scores of mesenchyme over total cell numbers. Statistical determination calculated by 2-sample Students t test with 99% confidence interval.

Reverse-Transcription PCR

Total RNA was extracted from the explant gel cultures using TRIzol (Invitrogen), according to the protocol of the manufacture, except for the use of 1 mL of TRIzol per gel. The cDNA was synthesized using the Ambion Retroscript kit (Austin, Tex) with 2 μg of RNA as template. The target amplicons were generated with 1 U of Taq polymerase (Promega, Madison, Wis) in conjunction with 0.25 μL of 100 mmol/L dNTP mix (Invitrogen), 12 μL of diethyl pyrocarbonate (DEPC) H2O, 2.0 μL of 10× Taq DNA polymerase buffer with MgCl2 (Promega), and 200 ng of oligonucleotide primers designed to generate a 92-bp product for VEGFA and a 341-bp product for 28S rRNA (rRNA forward primer: 5'-TGGAAGCCCCTGGAATTTAACAC-3'; rRNA reverse primer: 5'-GGTATCGGTCCTGACCC-3'; VEGF forward primer: 5'-GACGCTTGATGTTAAGAAACGGC-3'; VEGF reverse primer: 5'-GGTGTCACCTGAGG-3'). Thermocycling conditions were as follows: 95°C for 3 minutes; then 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute; followed by 72°C for 5 minutes. Amplicons were separated on 2.0% Tris-acetate-EDTA (TAE) agarose gels and visualized by ethidium bromide staining and imaged using a Gel Logic 100 Image system (Kodak).

Immunofluorescence

Immunofluorescent staining of cells was performed as previously described. Briefly, gels were rinsed with 1× PBS and permeabilized with 0.5% Triton X-100 (Fisher Biotech, Fair Lawn, NJ) in Pipes solution (Sigma). After blocking (3% BSA, 0.05% Tween in PBS), gels were incubated overnight with primary antibodies at 4°C with mild orbital rotation. Nonspecific binding of primary antibodies was reduced by a series of washes (×10) with 1× PBS containing 0.2% BSA/0.005% Tween-20, followed by incubation with the secondary antibodies at 4°C overnight. Washes were performed as above, and gels were mounted on slides using aqueous polycrylamide (Polyscience Inc, Warrington, Pa). Antibody to α-smooth muscle actin was purchased from Sigma Chemical Co and used at a 1:100 dilution. Goat anti-mouse conjugated to Alexa-546 (Invitrogen) was used as the secondary antibody at a 1:500 dilution (Invitrogen) during the wash procedures for the secondary antibody.

Enzyme-Linked Immunosorbent Assay

VEGF protein detection was performed using a specific VEGF ELISA kit (R&D Systems, Minneapolis, Minn) per the protocol of the manufacturer and as previously reported.

In Vivo, Chick Cushion Injections and VEGF mRNA Detection

Fertilized chicken eggs (obtained from Hy-Line International, Spencer, Iowa) were incubated at 37°C for 55 to 60 hours to stage 14 to 15, according to the developmental staging series of Hamburger and Hamilton (HH). Embryos were dissected from the egg and placed in EC (early chick) culture on agar-albumin substratum as described by Chapman et al. Embryos were cooled to room temperature to slow the heart, then cardiac cushions were injected with 20 to 25 μL of 1 mg/mL o-HA, 0.5 mg/mL HMW-HA, or PBS as a control. Solutions were prepared with 0.2% fast green dye (1:250 dilution; Sigma) for easy visualization of injections (see the online data supplement, available at http://circres.ahajournals.org). Injections were performed under a Leica MZ APO dissecting microscope, and images captured with Leica FireCam 1.6 software.

Following injection, embryos were covered with high glucose DMEM (GIBCO/Invitrogen, Carlsbad, Calif) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and glutamine (0.29 mg/mL) solution (GIBCO/Invitrogen), placed in a humid
chamber within a sealed bag containing 95% O₂ and 5% CO₂, and incubated at 37°C for 15 to 18 hours until embryos developed to stage HH18.

Stage HH18 embryos were carefully removed from the vitelline membrane, rinsed with PBS, then fixed with 4% paraformaldehyde/PBS overnight at 4°C. Embryos were rinsed with PBS, and then dehydrated through a graded methanol series. Embryos were then either immediately prepared for sectioning and staining with alcian blue or hematoxylin and eosin; or processed for whole-mount in situ hybridization according to the methods described by Nieto et al.40 Plasmid DNA encoding a 843-bp fragment corresponding to the *gallus gallus* VEGF cDNA sequence (GenBank accession no. BU269995) was obtained from the Biotechnology and Biological Sciences Research Council ChickEST database (Clone ChEST820k21; Geneservice Ltd, Cambridge, UK). RNA probe was prepared from DNA linearized with NotI using T3 RNA polymerase (Roche) and were detected by color reaction with nitroblue tetrazolium (NBT) (338 μg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (115 μg/mL). Digoxigenin (DIG)-labeled riboprobes were generated according to the suggestions of the manufacturer (Roche). Whole-mount embryos were postfixed and processed for serial sectioning. VEGF mRNA was detected and imaged using a Leica DM2500 microscope and Image-Pro Plus software.

**Results**

**Small o-HA Attenuates Developmental EMT**

Progress in understanding EMT during development has been facilitated by an in vitro 3D collagen gel culture system. This assay has been used as a model of cardiac mesenchyme formation using chick embryonic tissues on hydrated type I collagen gels.14 Runyan and Markwald15 modified this assay to show temporal and regional specification for EMT in the endocardial cushions and a dependence of this process on inductive signals from the adjacent myocardium. We previously established the parameters of this assay for recapitulating EMT events using endocardial cushions derived from mouse embryos.16 Normal developmental EMT and mesenchymal cell production is observed in naïve endocardial cushion explants derived from the AVCs of E9.5 embryos (Figure 1A and 1B). HMW-HA supports this production of mesenchyme (Figure 1C).8 In contrast, culturing AVC explants in the presence of o-HA results in a dramatic block in EMT and an uncharacteristic outgrowth of endocardial cells (Figure 1C). This reduction in mesenchymal cells is highly significant when compared with control explant cultures ($P=0.018 \times 10^{-4}$; see Figure 4). This observation suggests that depolymerized forms of HA are antagonistic to developmental EMT.

**Upregulation of VEGF by o-HA**

Following treatment of primary explant cultures with o-HA, VEGF mRNA increases compared with control cultures (Figure 2A). In multiple experiments, naïve endocardial cushion cultures show little to no expression of VEGF message (Figure 2A, lanes 2, 5, and 8). In contrast, cultures

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** o-HA attenuates endocardial cushion EMT. A, Schematic representation of the endocardial cushion EMT assay. B and C, Cardiac cushion explants derived from the AVCs of E9.5 embryos show normal EMT in naïve conditions (B) as well as in the presence of HMW-HA (C). D, EMT is attenuated in cultures treated with o-HA. Arrows indicate areas of mesenchymal cells; e, endocardial outgrowth; dashed lines outline the explant center (footprint where myocardium was removed).

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Regulation of VEGF by o-HA. A, Three representative experiments shown for RT-PCR detection of VEGF transcripts from RNA isolated from naïve (controls in lanes 2, 5, and 8). HMW-HA treatment (lanes 3, 6, and 9) or o-HA treatment (lanes 4, 7, and 10). Relative levels are compared with rRNA transcripts (bottom panels). C indicates naïve controls; -RT, no reverse transcriptase in first strand cDNA synthesis. B, Levels of VEGF protein detected by ELISA. There is a significant increase in VEGF production in the o-HA group over controls ($P=0.007$; $n=10$ for o-HA group; $n=8$ for naïve; $n=8$ for HMW-HA).
treated with o-HA exhibit a substantial increase in mRNA for VEGF (Figure 2A, lanes 4, 7, and 10). We did detect elevated levels of VEGF mRNA in HMW-HA samples. However, this less substantial increase in mRNA may reflect endogenous hyaluronidase activity in the explant cultures leading to a slight elevation in VEGF message from de novo o-HA production. In support of the effect of o-HA on VEGF mRNA, we also detected significantly elevated levels of VEGF protein in the media of o-HA–treated cultures compared with naïve and HMW-HA–treated explants (Figure 2B, P=0.007). o-HA–treated cultures appear to phenocopy AVC explants derived from E10.5 embryos or cultures treated with VEGF.17,18 We previously defined the physiological role of VEGF as a negative regulator of developmental EMT during endocardial cushion morphogenesis. Specifically, VEGF expression is suppressed in the myocardium lining the endocardial cushions until E10.5 in mouse embryos. At this point, VEGF becomes enriched and provides signals for angiogenic activity concomitant with cessation of EMT.17 Experimental conditions of hypoxia in cushion explant cultures or tetracycline-induced ectopic VEGF production by the myocardium in vivo supports the ability of VEGF to block EMT within the endocardial cushions.17 Our current observations suggest that o-HA may attenuate EMT through a VEGF-mediated pathway.

**o-HA–Induced Phenotype Rescued by sFlt**

To confirm that the o-HA–stimulated VEGF activity is the central mechanism mediating reduced levels of developmental EMT, o-HA–treated explants were cultured in the presence of sFlt. sFlt sequesters VEGF, acting as an effective dominant negative inhibitor of VEGF signaling.17,19 In the presence of sFlt, the o-HA–induced phenotype was fully reversed, displaying complete restoration of EMT (Figure 3D and 3H). Primary explant cultures treated with o-HA and sFlt exhibit normal production of mesenchymal cells, with characteristic stellate appendages consistent with typical cushion EMT,18 as shown in the control cultures (Figure 3A through 3F). These cells express α-smooth muscle actin comparable to mesenchymal cells in control cultures (Figure 3, compare 3E and 3H). The decrease in overall percentage of mesenchyme present in o-HA cultures (Figure 3C and 3G) returned to normal levels in the presence of sFlt (Figure 4; P=0.042×10−4). This result indicates that VEGF is the key regulator of o-HA-mediated attenuation of developmental EMT in endocardial cushions. The ability of sFlt to negate the inhibitory effect of o-HA on EMT strongly supports VEGF as a principle negative regulator of cardiac developmental EMT.

**Ectopic o-HA Effects VEGF Expression and Endocardial Cushion Morphogenesis In Vivo**

To determine whether the in vitro effects of HA are maintained in vivo, endocardial cushions of HH14 to HH15 chick embryos were injected with either o-HA, HMW-HA, or PBS as a control (see the online data supplement). Embryos were maintained in culture to stage HH18, at which time we compared the expression of VEGF and development of the cardiac cushions. Histological analyses showed reduced expansion of the AV cushions and altered morphology of the AVCs in o-HA–treated samples compared with those treated with HMW-HA or PBS (Figure 5). Alcian blue staining highlighted the reduced cushion volume in o-HA embryos (Figure 5D) compared with HMW-HA and PBS control cushions (Figure 5A and 5G). Hematoxylin and eosin staining showed mesenchymal cells present in the endocardial cushions of HMW-HA– and PBS–treated cushions (Figure 5B and 5H). In contrast, there was a drastic reduction in mesenchymal cells present in o-HA–treated cushions (Figure 5E). Thus, o-HA–injected endocardial cushions contained fewer overall cells and showed disrupted cushion morphology compared with those injected with HMW-HA or PBS controls. Collectively, this indicates a disruption in EMT and reinforces the observations from the in vitro experiments that o-HA is antagonistic to EMT.

In addition, we detected changes in VEGF expression in the AVC endocardial cushions injected with o-HA (Figure 5). o-HA injected into the cushions at stage HH14 markedly increased levels of VEGF in the myocardium lining the cushions by stage HH18 of development (Figure 5F).
was little to no detection of VEGF in the myocardial layer lining the endocardial cushions in HMW-HA– or PBS-treated samples (Figure 5C and 5I). In contrast, VEGF was detected in an extended pattern throughout the myocardium of the endocardial cushions in o-HA–injected embryos (Figure 5, compare 5F with 5C and 5I). These data provide evidence that the role of HA fragments in our explant cultures is similar to those found within in vivo conditions. Collectively, these findings suggest that premature induction of VEGF in the AVC inhibits endocardial cushion formation.

**Discussion**

Diverse biological roles have been attributed to HA, including hydration and expansion of extracellular spaces as well as binding to cell surface receptors to induce cell signaling.\(^1\),\(^2\) Studies by Baldwin et al.\(^21\) show that degradation of HA with hyaluronidase treatment of whole conceptus cultures results in abnormal endocardial cushion formation. The requirement for native HMW-HA in endocardial cushion morphogenesis and developmental EMT was shown in the Has2-deficient mouse line.\(^8\) Homozygote embryos die at E9.5 from cardiac failure caused by malformed hearts as a result of a complete absence of HA and defective EMT.\(^8\),\(^22\) HA has previously been shown in in vitro studies to take on new biological roles once broken down into small oligosaccharide fragments (o-HA).\(^13\) These o-HA forms have proinflammatory effects, primarily through monovalent interactions with its primary receptor, CD44, thereby enhancing nuclear factor \(\kappa\)B DNA-binding activity.\(^13\) Although the association of HMW-HA to CD44 is inhibited by o-HA between 6 and 18 sugars, there is no apparent function for CD44 in cardiovascular development.\(^20\),\(^22\),\(^23\) In this report, we use both in vitro and in vivo assays to study developmental EMT and present evidence that o-HA induces increased levels of VEGF, which is inhibitory to EMT. In murine in vitro explant assays, VEGF levels are markedly increased at both the mRNA and protein

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**Figure 4.** Significant overall reduction in formation of mesenchymal cells by o-HA–stimulated VEGF activity. o-HA treatment reduces the percentage of mesenchymal cells by 6-fold (compare naive with o-HA). Explants treated with o-HA and sFlt have a nearly 5-fold increase in percentage of mesenchymal cells relative to o-HA–treated explants. Percentage mesenchymal cells calculated as described in Material and Methods. The average total and mesenchymal cells are as follows (mesenchymal/total): naive: 64/584; naive+sFlt: 47/386; HMW-HA: 22/294; o-HA: 20/1047; o-HA+sFlt: 96/969.

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**Figure 5.** o-HA increases VEGF expression, disrupting endocardial cushion morphogenesis in vivo. HMW-HA–injected AVCs have an abundance of Alcian blue–positive staining ECM as well as an abundance of cushion mesenchyme (A and B). In contrast, o-HA–injected cushions (D and E) have reduced levels of extracellular matrix and mesenchymal cells compared with PBS controls (G and H). No VEGF expression was detected in the myocardium of HMW-HA (C) or PBS (I) samples. In contrast, the myocardium expresses VEGF in o-HA–injected cardiac cushions (F). Images A, D, and G are stained with Alcian blue and eosin counterstain (red); images B, E, and H are stained with hematoxylin and eosin. VEGF in situ images appear in C, F, and I. a indicates atrium; v, ventricle; ec, endocardial cushion; e, endocardium; m, myocardium
levels following treatment with o-HA compared with controls. Production of mesenchyme is significantly decreased as a result of a block in EMT following treatment with o-HA. This phenotype is reversed by treatment with a soluble VEGF receptor, sFlt, with complete restoration of EMT. Our in vitro studies are supported by in vivo avian embryo experiments shown in Figure 5. This is the first report, to our knowledge, of VEGF expression in chick embryonic heart and shows exclusion of VEGF mRNA in the AVC, thereby marking the boundaries of the chambers. This supports the notion that the pattern of VEGF expression indicates a role in heart partitioning. In addition, injected o-HA not only increases and extends the pattern of VEGF expression, but also decreases EMT within the endocardial cushion regions compared with controls. Our data provide a direct functional link between o-HA and VEGF in the regulation of developmental EMT. Furthermore, cushion endocardial cells retain the competency to execute transformation as the effect of o-HA is reversible in this system through the neutralization of VEGF by sFlt.

The ability of o-HA to displace activity of native HMW-HA suggests that an unidentified HA receptor may mediate this o-HA function. Alternatively, morphogen gradients may be dramatically altered with disruption of HA-enriched extracellular matrices in the cushions, causing attenuation of inductive signals for EMT. In this regard, large polymeric forms of HA are reported to potentiate morphogen gradients as seen with the activation of ErbB3/ErbB2 receptors following HMW-HA stimulation. Similarly, changes in HA-matrices may facilitate liberating matrix-bound forms of VEGF. Heparan sulfated proteoglycans (HSPG) can potentiate VEGF activity by neutralizing VEGF and promoting morphogen gradients as seen with the activation of ErbB3/ErbB2 receptors following HMW-HA stimulation. Similarly, changes in HA-matrices may facilitate liberating matrix-bound forms of VEGF. HSPG can potentiate VEGF activity by neutralizing VEGF and promoting morphogen gradients. It is appreciated that VEGF depends on HSPG for full biological activity.

Thus, a change from HMW-HA to o-HA may alter HSPG in this system through the neutralization of VEGF by sFlt. Our observations emphasize the importance of strict temporal and spatial control of developmental EMT in cardiac cushion regions. Tight regulation of VEGF and production of progenitor valve cells is dynamically dependent on deposition and processing of HA. The importance of appropriate timing and dosage of VEGF during development is highlighted by shared cardiovascular developmental defects, including abnormalities in cushion regions, by both mice heterozygous for 1 VEGF allele and transgenic mice, which have a 2- to 3-fold increase in VEGF. Recently, Chang et al, demonstrated that VEGF is repressed by a calcineurin/NFAT mechanism to prevent VEGF production in the myocardium of AV cushions until E11 or after EMT occurs. This finding is consistent with our previous work defining the expression of VEGF in endocardial cushions. Our new data indicate o-HA acts as a stimulus to induce VEGF expression that then limits EMT. Alterations in endocardial cushion morphogenesis can lead to deficits in valvular cell formation or, conversely, an overabundance of cells resulting in stenotic valves. In vivo VEGF becomes enriched in the myocardium that underlies the forming endocardial cushions at the time when EMT ceases. In light of the present data, we propose a model (Figure 6) that regulation of EMT during cushion development is sequentially regulated by specific forms of HA. Initially, establishment of HMW-HA provides a hydrated matrix for cellular migration and supports morphogen gradients required for EMT. HA becomes degraded into small oligosaccharide fragments as cardiac cushion volume is reduced during the remodeling phase of valve development. Depolymerized HA stimulates production of VEGF. This regional VEGF drives an epithelial phenotype within the endocardium, thereby terminating endocardial competence for EMT. This o-HA–VEGF activity limits the extent of endocardial cell delamination and subsequent formation of mesenchymal cells. This molecular control over the magnitude of valve mesenchyme production is crucial because unabated EMT can result in hyperproliferative and stenotic valves. This is manifested in Noonan syndrome, in which inductive signals through mitogen-activated protein kinase mechanisms are unchecked because of mutations in SHP-2 tyrosine phosphatase, leading to pulmonary stenosis. Thus, our observations provide the basis for consideration of an o-HA–VEGF mechanism as a means for novel repair strategies to ameliorate hyperproliferative or stenotic heart valves.

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Disclosures
None.

References


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Supplemental Fig. 1. Avian cushion injections. (A) A blue dye indicates location of AV cushion injection. (B) Injected solution clearly visible within AV cushion following injection.