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Myocardial contraction and hyaluronic acid mechanotransduction in epithelial-to-mesenchymal transformation of endocardial cells

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ABSTRACT

Epithelial-to-mesenchymal transition (EMT) of endocardial cells is a critical initial step in the formation of heart valves. The collagen gel *in vitro* model has provided significant information on the role of growth factors regulating EMT but has not permitted investigation of mechanical factors. Therefore we sought to develop a system to probe the effects of mechanical inputs on endocardial EMT by incorporating hyaluronic acid (HA), the primary component of endocardial cushions in developing heart valves, into the gel assay. This was achieved using a combination collagen and crosslinkable methacrylated HA hydrogel (Coll-MeHA). Avian atrioventricular canal explants on Coll-MeHA gels showed increased numbers of transformed cells. Analysis of the mechanical properties of Coll-MeHA gels shows that stiffness does not directly affect EMT. Hydrogel deformation from the beating myocardium of explants directly led to higher levels of regional gel deformation and larger average strain magnitudes associated with invaded cells on Coll-MeHA gels. Inhibition of this contraction reduced EMT on all gel types, although to a lesser extent on Coll-MeHA gels. Using the system we have developed, which permits the manipulation of mechanical factors, we have demonstrated that active mechanical forces play a role in the regulation of endocardial EMT.

1. Introduction

Understanding how heart valves develop *in utero*, including the complex spatiotemporal regulation of signaling mechanisms and dynamic biomechanical environment, will aid in the creation of viable tissue engineered heart valves and novel treatment strategies for valve diseases. A crucial first step in the formation of heart valves is epithelial-to-mesenchymal transformation (EMT) of specialized endocardial cells, which gives rise to valvular interstitial cells (VICs) which remodel the immature cardiac cushions into mature valve leaflets and maintain adult valves throughout life [1–4]. Additionally, VICs are implicated in disease mechanisms including calcific valve disease and have recently been shown to be responsive to hyaluronic acid (HA) signaling [5]. Understanding the mechanical environment that generates VICs will elucidate cell behavior in both developmental and pathological states and potentially impact the creation of viable tissue engineered heart valve replacements [6].

In humans, mice, and chicks, the developing heart tube consists of a common atrium, ventricle, and outflow tract. An initial step of heart valve formation occurs when regions in the atrioventricular canal (AVC) and outflow tract swell, forming endocardial cushions extracellular matrix (ECM) termed cardiac jelly. EMT occurs when endocardial cells lining the developing heart tube receive a signal to detach from the endocardial cell layer and elongate, before migrating into the cardiac jelly [1,3,6,7]. The cardiac jelly is primarily composed of HA but also contains other ECM and signaling molecules [1–2,8–13]. Over time, the transformed cells that migrate into the endocardial cushions respond to these signals by remodeling the cardiac jelly into the highly structured ECM architecture of mature heart valve leaflets. The spatial and temporal regulation of this process is important for the proper formation of heart valves, and delays or alterations in this signaling can lead to significant impairment of the mature heart valve structure or function.

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In vitro, endocardial EMT is studied via a collagen gel protocol developed nearly 30 years ago [14,15]. Briefly, stage HH16 avian embryos are harvested, and the AVC endocardial cushions are removed before explanting endocardium-side down onto a collagen hydrogel. Over 2 days, endocardial cells migrate out of the explant and onto the surface of the collagen gel, forming an endocardial cell sheet; some of these cells undergo EMT and migrate into the collagen gel. This assay has been performed in both mouse and chicken and demonstrates the high degree of conservation between signaling mechanisms governing endocardial EMT between species, despite the fact that mouse explants do not form endocardial sheets [1,13,16–18]. Also, as the ventricle of the developing heart tube does not undergo EMT, it is frequently used to test the ability for signaling molecules to induce EMT [19]. For a full review on endocardial EMT assays, see Refs. [17,18]. After developing the original collagen gel assay, Bernanke et al. went on to show that soluble HA can affect the level of EMT that occurs *in vitro*, although this study was not pursued further [20]. Studies testing the effects of hyaluronate in embryonic rat hearts demonstrated that HA degradation prevented endocardial cushion formation [21]. Also, Camenish et al. showed that mice lacking HA-synthase 2 ($HAS2^{-/-}$) fail to form heart valves, due to a lack of endocardial cushions, and die at approximately stage E11 with an absence of EMT [16]. However, *in vitro*, EMT can be rescued in $HAS2^{-/-}$ cells by the addition of soluble HA. Based on the role of HA as an important structural and signaling component in EMT, examination of its inclusion in the *in vitro* hydrogel assay to determine effects on EMT is warranted [22–24].

The primary limitation of the collagen gel assay is that it fails to mimic the *in vivo* environment of the developing heart valves in composition (chiefly, lacking HA) which has been recently identified as a key mechanotransduction protein [25]. Further, significant progress has been made in elucidating the signaling mechanisms important for driving EMT, but little work has been done in the biomechanical aspects of this process. Since the biomechanical properties of early and mature heart valve tissues directly relate to valve function, the current lack of knowledge about the relationship between EMT and mechanics needs to be addressed [26,27]. The goal of our study was to create a hydrogel platform incorporating collagen and HA which can be used to study the mechanical context of EMT, in order to test our hypothesis that mechanical factors may play a previously unrecognized role in the regulation of endocardial EMT.

2. Materials and methods

2.1. Gel synthesis

Coll-MeHA gels were synthesized following previously established protocols [24,28,29]. Briefly, HA was modified to contain methacrylate crosslinking groups by reacting HA (#53747, Sigma Aldrich, St. Louis, MO, ~1.6 MDa) with methacrylic anhydride. Coll-MeHA gels were formed by mixing type I collagen (#354249, BD Biosciences, San Jose CA) with MeHA stock solution and neutralizing with 0.1 M NaOH. For crosslinking, 0.1 wt% (to total gel weight) of Irgacure 2529 (#410896, Sigma Aldrich, St. Louis, MO) was mixed into the solution. Gels were crosslinked for 5min under a 365 nm UV wand before incubation at 37 °C and 5% CO_2 to complete collagen gelation. Prior to explant experiments, Coll-MeHA gels were equilibrated overnight in complete media.

2.2. Mechanical analysis of gels

Atomic force microscopy (AFM) was utilized to measure the moduli of Coll-MeHA and collagen only gels. Samples were analyzed using a BioScope Catalyst AFM (Bruker AXS, Santa Barbara CA) operated in Peak Force − Quantitative Nanomechanical Mapping mode in fluid using a calibration protocol developed in our lab. Gels were fully hydrated in PBS prior to AFM measurements. Multiple scans on multiple gels were used to generate average moduli values ($n \geq 8$ per gel composition).

2.3. Gel topography analysis

The fiber structure and overall gel topography were visualized using a Hitachi S-4200 Scanning Electron Microscope (SEM) (Pleasanton, CA). Samples were mounted on SEM posts using conductive tape. Before imaging, scaffolds were coated with gold for 20s using a sputter coater Model-108 (Cressington Scientific, Watford, UK).

2.4. Chick AVC explant harvesting and culture

Stage 16-17 avian endocardial cushions were harvested as described elsewhere [15,30]. Briefly, AVCs were excised from embryos, bisected, and seeded endocardium-side down onto fully hydrated gel surfaces. As controls, 0.12 wt% collagen only gels were utilized; this composition corresponds to the standard collagen gel assay [14,15,20]. Explants were given M199 media with 1% FBS, 1% antibiotic/antimycotic, and 1% insulin–selenium–transferrin solution, on the morning following seeding and fed every 2d after that until 7d total culture time was reached. Explants were imaged using a Nikon-Ti300 inverted microscope (Nikon Inc., Melville NY) equipped with Hoffman Modulated Optics. A minimum of 8 explants were seeded per condition and experiments were repeated with at least 3 different batches of eggs to ensure reproducibility. For mechanical analysis, stage HH16-17 embryos were selected and fresh frozen in OCT according to established protocols [30].

2.5. EMT quantification

The surface of the gels was imaged to measure endocardial sheet size, which directly relates to the number of endocardial cells that have migrated out of the explant. To quantify cell transformation, images were taken every 50 μm throughout the depth of the gel where cell bodies were present. Both endocardial sheet size and number of transformed cells were quantified using ImageJ.

2.6. Proliferation assay

Proliferation of cells from explants on gels was measured using BrdU. Briefly, gels were incubated with 1:1000 BrdU (RPN201V1, Amersham Biosciences, Pittsburgh PA) for 1 h at 37 °C before fixation with 4% paraformaldehyde and stained via a previously established protocol [13]. Images were taken on a Nikon E800 (Nikon Inc., Melville, NY) and processed using ImageJ.

2.7. Deformation measurements

Sequence images of beating explants were taken and a pair of images for each explant were extracted, representing fully relaxed and fully contracted myocardium. These images were digitally subtracted using MATLAB and color coded to show deformation of cells and gels surrounding the explants. The area of this deformation was quantified and normalized to total endocardial sheet area of the relaxed myocardium at the time point of interest, resulting in regional gel deformation for each of the explants. Digital image correlation software was used to generate images of strain fields induced by myocardial beating; these images were overlaid with projections of total invaded cells counts to determine correlations between local deformation magnitude and EMT.

2.8. Inhibition of contractile forces

Explant contraction was inhibited using 1.5 mM ethyl 3-aminobenzoate methanesulfonate salt (A5040, Sigma–Aldrich), otherwise known as the sodium channel inhibitor tricaine [31]. Explants were allowed to adhere overnight to gels before administration of the drug. Physical removal of the myocardium was tested by carefully using forceps to detach the explant from the surface of the gel after the explant had adhered overnight. The explant remained in the well, unattached, for the duration of the experiment. A control group had explants that were removed from the gel surface and then discarded.

2.9. Statistical analysis

All results from chick explant experiments were reported as average value plus or minus standard error of the mean. For mechanical analyses, the median value of each AFM scan was collected and aggregated into an average median value to represent the sample [30]. Weighted average deformations associated with invaded cells were calculated by weighting the local deformation magnitude in a 25 × 25 pixel volume by the total number of invaded cells present in that volume and dividing that value by total invaded cells. The average values of all groups were compared with ANOVA, while pair-wise multiple comparisons were made using the Holm-Sidak post-hoc testing method.

3. Results

3.1. EMT on cross-linked Coll-MeHA hydrogels

Combinations of collagen and HA were tested for AVC explant viability and attachment prior to detailed analyses. For these studies, a modified HA containing methacrylate groups (MeHA) was synthesized [24,28,29]. Most notably, combination crosslinked Coll-MeHA gels that contained only 0.12 wt% collagen (the standard collagen concentration) with any amount of MeHA demonstrated