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Fibronectin is deposited by injury-activated epicardial cells and is necessary for zebrafish heart regeneration

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ABSTRACT

Unlike adult mammals, adult zebrafish vigorously regenerate lost heart muscle in response to injury. The epicardium, a mesothelial cell layer enveloping the myocardium, is activated to proliferate after cardiac injury and can contribute vascular support cells or provide mitogens to regenerating muscle. Here, we applied proteomics to identify secreted proteins that are associated with heart regeneration. We found that Fibronectin, a main component of the extracellular matrix, is induced and deposited after cardiac damage. In situ hybridization and transgenic reporter analyses indicated that expression of two *fibronectin* paralogues, *fn*1 and *fn*1*b*, are induced by injury in epicardial cells, while the *itgb*3 receptor is induced in cardiomyocytes near the injury site. *fn*1, the more dynamic of these paralogs, is induced chamber-wide within one day of injury before localizing epicardial Fn1 synthesis to the injury site. *fn*1 loss-of-function mutations disrupted zebrafish heart regeneration, as did induced expression of a dominant-negative Fibronectin cassette, defects that were not attributable to direct inhibition of cardiomyocyte proliferation. These findings reveal a new role for the epicardium in establishing an extracellular environment that supports heart regeneration.

Introduction

Fetal and neonatal mice can regenerate lost regions of heart muscle through cardiomyocyte proliferation (Drenckhahn et al., 2008; Porrello et al., 2011), whereas the injured adult mammalian heart has a limited regenerative capacity. By contrast, zebrafish do not significantly lose regenerative potential as they mature, and can regenerate large portions of adult myocardium lost from resection, cryoinjury, or genetic ablation (Gonzalez-Rosa et al., 2011; Poss et al., 2002; Wang et al., 2011). In both zebrafish and mice, the epicardium, a mesothelial cell sheet that covers the heart, is activated to induce embryonic markers after cardiac damage (Lepilina et al., 2006). Epicardial cells have been studied as a source of paracrine signals, a supply of perivascular components or other cell types, and a mediator of inflammation during cardiac repair or regeneration (Huang et al., 2012; Kikuchi et al., 2011a; Smart et al., 2011; Zhou et al., 2011).

The extracellular matrix (ECM) has long been recognized as a key influence on organ formation and repair (Martino et al., 2011). During embryonic heart development, the ECM provides cues for assembly, proliferation and maturation of cardiac cell types (George et al., 1997; Ieda et al., 2009; Magnusson and Mosher, 1998; Trinh and Stainier, 2004). For instance, early cardiomyocytes that shape the zebrafish heart require the core ECM component Fibronectin (Fn) as they migrate toward the midline, a function that also explains observations during murine heart development (George et al., 1997; Trinh and Stainier, 2004). Fn deposition following cardiac damage in adult mammals has been previously documented, where it has been associated with adverse effects like fibrosis (Knowlton et al., 1992; Rysa et al., 2005; Willems et al., 1996; Zhong et al., 2010). Here, we find that Fn is dynamically produced by epicardial cells in response to cardiac injury, and that it is essential for heart regeneration.

Materials and methods

Zebrafish and cardiac injuries

Outbred Ekkwill zebrafish strains (4–10 months of age) were used for ventricular resection surgeries (Poss et al., 2002), or for genetic cardiomyocyte ablation (Wang et al., 2011). Animal density was maintained at approximately 4 fish per liter in all experiments. To ablate cardiomyocytes, animals were treated for 16 h in 0.1 μM 4-hydroxytamoxifen (4-HT) in fish water. Transgenic *tcf*21*:nucEGFP* and zebrafish cardiac genetic ablation strains were previously described (Wang et al., 2011). Progeny from heterozygous matings were raised at a water temperature of 22 °C to 2 months of age, then maintained at 26 °C. *fn*1 homozygous mutants from these clutches were identified by PCR screening, as described

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(Trinh and Stainier, 2004). Heat-shock experiments were performed as described (Kikuchi et al., 2011b). Newly constructed strains are described below. All transgenic strains were analyzed as hemizygotes. All animal procedures were performed in accordance with Duke University guidelines.

fn1:mCherry-NTR

The translational start codon of *fn*1 in the BAC clone CH211-160E15 was replaced with the mCherry-NTR cassette by Red/ET recombineering technology (Gebe Bridges) (Singh et al., 2012). The 5′ and 3′ homologous arms for recombination were a 50-base pair (bp) fragment upstream and downstream of the start codon, and were included in PCR primers to flank the mCherry-NTR cassette. To avoid aberrant recombination between the mCherry-NTR cassette and endogenous *loxp* site in the BAC vector, we replaced the vector-derived *loxp* site with an I-SceI site using the same technology. The final BAC was purified with Nucleobond BAC 100 kit (Clontech), and co-injected with I-SceI into one-cell-stage zebrafish embryos. The full name of this transgenic line is *Tg(fn1:mCherry-NTR)*^{pd65}.

itgb3:EGFP

The translational start codon of *itgb*3 in the BAC clone DKEY-287G12 was replaced with the EGFP cassette by Red/ET recombineering technology (GeneBridges). The procedures of the homologous arm design, the *loxp* replacement strategy and BAC preparation are the same as described above. The full name of this transgenic line is *Tg(itgb3:EGFP)*^{pd66}.

hsp70:dn-fn^{l}*1–9*

A gene cassette encoding human fibronectin^{l}1–9 fragment was PCR amplified with primers containing ClaI/ClaI (plasmid kindly provided by Harold Erickson) (Ohashi and Erickson, 2011). PCR products was gel-purified, digested with restriction enzymes, and ligated into an ClaI digested vector containing the 1.5 kb zebrafish *hsp*70 promoter (Halloran et al., 2000). The plasmid was injected into one-cell zebrafish embryos along with I-SceI to generate transgenic animals. The full name of this transgenic is *Tg(hsp70:fn*^{l}*1–9)*^{pd67}.

Proteomics

Z-CAT zebrafish were treated with EtOH vehicle or 4-HT and ventricles were collected at 7 days post incubation (dpi), 14 dpi, and 30 dpi. Three separate pools of 3 hearts were collected for each time point. Proteomic analysis was performed using a label-free quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) approach after tissue solubilization (Geromanos et al., 2009). The Duke Proteomics Core Facility received snap-frozen extracted zebrafish cardiomyocyte tissue. Each tissue sample was solubilized using a MS-compatible surfactant/burst sonication procedure in which samples were suspended in 50 mM ammonium bicarbonate, pH 8 with 0.25% ALS-1 and subjected to 3 × 10 s probe sonication bursts at 30% power. Samples were spun at 15,000 rpm for 5 min and insoluble material was discarded. A Bradford assay (mini-Bradford, BioRad, Inc.) of all samples was taken after protein isolation to determine protein yield. 25 μg protein from each sample was aliquoted and normalized to 1.0 μg/μL for reduction (10 mM DTT), cysteine alkylation (20 mM iodoacetamide), and trypsin digestion according to a standard protocol (http://www.genome.duke.edu/cores/proteomics/sample-preparation/documents/In-solutionDigestionProtocol_012309.doc). After digestion, all samples were spiked with ADH1_YEAST digest (Massprep standard, Waters Corporation) as a surrogate standard at a concentration of 50 fmol/μg, and acidified to a final concentration of 2% v/v acetonitrile and 1% trifluoroacetic acid. A sample "pool" to be used for column conditioning and QC purposes was generated by removing an equal quantity (5 μg) from each of the samples.

Quantitative LC/MS/MS was performed on 1 μg of protein digest per sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Synapt G2 HDMS high resolution accurate mass tandem mass spectrometer (Waters Corp.) via a nanoelectrospray ionization source. Briefly the sample was first trapped on a Symmetry C18 300 mm × 180 mm trapping column (5 μl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.7 μm Acquity BEH130 C18 75 mm × 250 mm column (Waters Corp.) using a 90-min gradient of 5–40% acetonitrile with 0.1% formic acid at a flow rate of 300 nL/min (nanoliters/minute) with a column temperature of 45 °C. Quantitative data collection for each sample in singlicate and on the sample pool (5 ×) on the Synapt G2 mass spectrometer was performed in data-independent acquisition mode (MS^{E}) using 0.6 second alternating cycle time between low (6 V) and high (15–40 V) collision energy (CE) in the trapping region. Additional qualitative analyses were performed using the pooled sample in both ion-mobility assisted data-independent acquisition (HDMSE) mode or data-dependent acquisition (DDA) mode. Scans performed at low CE measure peptide accurate mass and intensity (abundance), while scans at elevated CE allow for qualitative identification of the resulting peptide fragments via database searching. The total analysis cycle time for each sample injection was approximately 2 h.

The QC pool containing equivalent amounts of all samples was used to condition the UPLC column prior to the study and was run five times throughout the study for quantitative QC and five additional runs for supplementary qualitative identifications (for a total of 34 LC-MS/MS analyses). Treatment groups were evenly distributed across the run queue in a block design, and within each block the sample order was randomized. Following the analyses, data were imported into Rosetta Elucidator v3.3 (Rosetta Biosoftware, Inc), and all LC/LC-MS runs were aligned based on the accurate mass and retention time of detected ions ("features") using PeakTeller algorithm (Elucidator). The relative peptide abundance was calculated based on area-under-the-curve (AUC) of aligned features across all runs. The overall dataset had 204,872 deisotoped features, and high collision energy (peptide fragment) data was collected in 370,834 spectra for sequencing by database searching. This MS/MS data were searched against an NCBI RefSeq database with *Danio rerio* taxonomy (http://www.ncbi.nlm.nih.gov/protein/), which also contained a reversed-sequence "decoy" database for false positive rate determination. After individual peptide scoring using PeptideProphet algorithm (Elucidator), the data were annotated at a $<1\%$ peptide false discovery rate. This analysis yielded identifications for 3897 peptides and 545 proteins across all samples, including 325 proteins with 2 or more peptides quantified. For quantitative processing, the data were first curated to contain only high quality peptides with appropriate chromatographic peak shape and the dataset was intensity scaled to the robust mean across all samples analyzed; the final quantitative dataset for cardiomyocytes was based on 3744 peptides and contains 521 proteins. Protein expression within a sample was determined by summing the intensity of all peptides to a protein, and this sum was compared between groups using standard statistical tools.

Histological methods

In situ hybridization (ISH) was performed on 10 μm cryosections of paraformaldehyde-fixed hearts using digoxygenin-labeled cRNA probes as described (Poss et al., 2002) with the aid of an InSituPro robot (Intavis). Acid Fuchsin-Orange G staining was performed as described (Poss et al., 2002). Primary antibodies used in this study were anti-Myosin heavy chain (MHC; F59,