

## **Stem Cell Reports, Volume 2**

### **Supplemental Information**

#### **Engineered Biomaterials Control Differentiation and Proliferation of Human-Embryonic-Stem-Cell- Derived Cardiomyocytes via Timed Notch Activation**

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Supplemental Information Inventory

Supplemental Figure 1, related to Figure 4: Immunostaining of cardiomyocytes for beta-myosin heavy chain and BrdU incorporation

Supplemental Table 1: List of qPCR primers

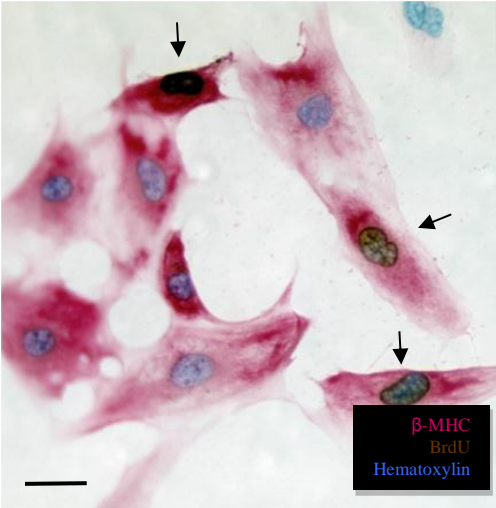
Supplemental Figure Captions

Supplemental Experimental Procedures: Detailed procedures and references

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Supplemental Video 1, related to Figure 3: Video of KDR+ progenitors after 2 weeks of culture on oriented Jagged-1 surface

Supplemental Figure 1, related to Figure 4



Supplemental Table 1, related to Figure 2, 3, 4

Gene	Primer Sequence
<i>OCT4</i>	Forward: GGGTTCTATTTGGGAAGGTAT Reverse: TTCATTGTTGTCAGCTTCCT
<i>BRACHYURYT</i>	Forward: CAAATCCTCATCCTCAGTTTG Reverse: GTCAGAATAGGTTGGAGAATTG
<i>FOXA2</i>	Forward: ATGCACTCGGCTTCCAGTAT Reverse: CCACGTACGACGACATGTTC
<i>SOX1</i>	Forward: GGAATGGGAGGACAGGATTT Reverse: AACAGCCGGAGCAGAAGATA
<i>JAGGED-1</i>	Forward: TCGGGTCAGTTCGAGTTGGA Reverse: AGGCACACTTTGAAGTATGTGTC
<i>HES1</i>	Forward: TGGAAATGACAGTGAAGCACCT Reverse: GTTCATGCACTCGCTGAAGC
<i>HPRT</i>	Forward: TGAACACTGGCAAACAATGCA Reverse: GGTCCTTTTCACCAGCAAGCT

## Supplemental Figure Captions

Supplemental Figure 1, related to Figure 4: Immunostaining demonstrates the presence of BrdU incorporation in proliferating cardiomyocytes. Cardiomyocytes stain pink due to  $\beta$ -MHC expression. Cardiomyocytes induced to proliferate are identified via brown nuclear staining for BrdU incorporation, while non-proliferating cardiomyocytes display a blue nucleus due to hematoxylin counterstaining. Arrows indicate proliferating cardiomyocytes. Scale bar = 20  $\mu$ m.

Supplemental Table 1, related to Figure 2, 3, & 4: qRT-PCR primer sequences

Supplemental Video 1, related to Figure 3: KDR<sup>+</sup> cardiovascular progenitors plated on oriented Jagged-1 surfaces show significant levels of contraction. After 2 weeks in culture, waves of contraction are observed propagating through entire sheets of cells.

## Supplemental Experimental Procedures

### *Preparation of oriented JAG1 biomaterials*

Oriented JAG1 surfaces were fabricated on TCPS surfaces using an indirect immobilization scheme similar to previously described (Beckstead et al., 2006; Beckstead et al., 2008). Briefly, TCPS surfaces were treated overnight with an anti-polyHistidine solution (20 ug/ml in PBS, R&D Systems) at 4°C, blocked for 2 hours with bovine serum albumin (10 mg/ml in PBS, Fraction V, Sigma) to prevent non-specific binding, and finally treated with 6x histidine-tagged recombinant rat JAG1 (0-20 ug/ml in PBS, R&D Systems) for 2 hours. For anti-polyHistidine control surfaces and unoriented JAG1 surfaces, proteins were attached using a direct immobilization scheme (Beckstead et al., 2006; Beckstead et al., 2008). Briefly, TCPS surfaces were treated overnight with an anti-polyHistidine solution (20 ug/ml in PBS) or JAG1 (7.5 ug/ml in PBS) overnight at 4°C, then subsequently blocked for 2 hours with bovine serum albumin (10 mg/ml in PBS). For both indirect and direct immobilization, surfaces were rinsed with PBS between each step.

Notch-signaling fibrin microparticles were fabricated similar to previously described (Gorodetsky et al., 2004). An aqueous solution of bovine fibrinogen (50 mg/mL, Sigma) in phosphate buffered saline (PBS) was emulsified with an Arrow Engineering Electric Stirrer (Model 6000) in mineral oil with 5% Span-80. Various stir rates, homogenizer attachments, and oil:aqueous ratios were used to optimize particle size and shape. Once the emulsion was thoroughly mixed, bovine thrombin (5 U/ml final concentration, Sigma) was added drop-wise to polymerize the suspended fibrinogen microdroplets into fibrin microparticles. Following polymerization, the emulsion was transferred to an Erlenmeyer flask, and heated for 6 hours at 65-70°C while being vigorously stirred. After 6 hours, the reaction was allowed to cool to room

temperature overnight while continuing to stir. The cooled product was washed in hexanes and acetone with the addition of 5% Tween-20 to remove mineral oil. After removal of mineral oil, microparticles were placed in ethanol washes to remove residual surfactant. Following washing, the final product was resuspended in deionized water.

The indirect immobilization procedure of JAG1 onto microparticles was accomplished similar to a previously described method (Osathanon et al., 2009). Terminal carboxyl groups of the fibrin microparticles were activated to amine-reactive sulfo-NHS esters by reaction of the microparticles with a 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.4 mg/ml, Pierce)/*N*-hydroxysuccinimide (NHS, 0.6 mg/ml, Pierce) solution in PBS for 15 minutes at room temperature. The activated microparticles were washed and resuspended in an anti-polyHistidine solution to allow for covalent attachment of the antibody onto microparticles via the formation of an amide bond. After 2 hours, the microparticles were washed and resuspended in varying concentrations of JAG1 overnight at 4°C. Following indirect immobilization, microparticles were washed in PBS and used immediately.

#### *Quantification of ligand immobilization*

To quantify the surface concentration of immobilized proteins, Jagged-1/Fc was first radiolabeled with  $^{125}\text{I}$  using an Iodination Bead (Pierce Biotechnology) Iodination System. Briefly, a 0.1 mg/ml solution of protein was prepared using degassed PBS. In a reaction vessel, one Iodination Bead was incubated with 1 mCi of  $\text{Na}^{125}\text{I}$  (Perkin Elmer) for 5 minutes at room temperature. To initiate the addition of  $^{125}\text{I}$  to tyrosine residues in our proteins of interest via chemical oxidation, prepared protein solutions were added to the reaction vessel and allowed to react for 15 minutes at room temperature. Solution was removed from the reaction vessel to stop

the reaction and subsequently added to BioRad 10DG chromatography columns (BioRad) to purify unreacted  $^{125}\text{I}$  from  $^{125}\text{I}$ -labeled protein. 80 fractions, 0.25 ml each, were collected and their radioactivity measured via 0.1 minute counts on a Cobra II® Series Auto-Gamma® Counting System to identify fractions containing unreacted  $^{125}\text{I}$  and  $^{125}\text{I}$ -labeled protein. Fractions containing  $^{125}\text{I}$ -labeled protein were pooled and added to a second column for further purification. Following radioactivity measurement of fractions obtained from the second column purification, fractions containing purified  $^{125}\text{I}$ -labeled protein were pooled and stored at  $-80^{\circ}\text{C}$  for use in immobilization the following day.

Oriented Jagged-1/Fc surfaces and oriented Jagged-1 microparticles were fabricated via indirect immobilization as previously described. Protein solutions were spiked with the amount of the corresponding  $^{125}\text{I}$ -labeled protein necessary to achieve the minimum signal necessary for detection (50 cpm/ng). BSA-blocked anti-polyHistidine modified TCPS surfaces and anti-polyHistidine-modified microparticles were treated with varying concentrations of these solutions following the protocols described in the previous section. Following washing, 1 minute radioactivity counts were taken. The surface concentrations of immobilized proteins ( $\text{ng}/\text{cm}^2$ ) were determined by correlating the radioactivity of the surfaces with the known concentration of radioactive protein and dividing by the total TCPS surface area or total number of particles used in the reaction. All treatment groups were conducted with  $n=3$ .

#### *Quantification of Notch activation*

Activation of Notch signaling by the biomaterial immobilized ligands was measured using a previously described CBF-1 luciferase assay (Beckstead et al., 2006; Beckstead et al., 2008). For these studies, neonatal human keratinocytes (HEK<sub>n</sub>) were purchase from Cascade Biologics (Portland, OR) and cultured in EpiLife basal medium (Cascade Biologics) and supplemented

with human keratinocyte growth supplement (HKGS, Cascade Biologics) according to manufacturer's instructions. For the luciferase assay, cells were transiently transfected (Fugene 6, Roche Diagnostics, Switzerland) with 900 ng of a firefly luciferase plasmid containing four adjacent CBF-1 binding sequences (gift from L. Liaw, Main Medical Center Research Institute). Included in the transfection was 100 ng of Renilla SV40 construct (Promega Corporation, Madison, WI) as a transfection efficiency control. The following day, cells were passaged and replated on modified surfaces or replated on untreated surfaces and dosed with various microparticle treatments (medium contained 1.5 mM  $\text{Ca}^{2+}$ ). After 24 hours, the protein was collected and luciferase content analyzed using the Promega Dual-Luciferase® Reporter Assay System (Promega). Results are reported as the average firefly to Renilla ratio and normalized to control surfaces. All treatment groups were conducted with n=3.

#### *Human embryonic stem cell differentiation assay*

Female H7 human embryonic stem cells were maintained in mouse embryonic fibroblast-conditioned media (MEF-CM) as previously described (Xu et al., 2001). Undifferentiated hES cells were seeded onto oriented JAG1 surfaces, anti-polyHistidine or Matrigel (Growth Factor Reduced Matrigel, BD Biosciences) control surfaces at a low or high density (100,000 or 200,000 cells/well of a 24-well plate). hES cells were plated in media consisting of 80% Knockout-Dulbecco's Modified Eagle Media, 1 mmol/L L-glutamine, 1% non-essential amino acids, 20% fetal bovine serum, and 0.1 mmol/L  $\beta$ -mercaptoethanol. All treatment groups were conducted with n=3. RNA samples were isolated at days 1, 2, 4, and 8 after plating.

#### *Cardiovascular progenitor cell differentiation assay*



KDR<sup>+</sup> cardiovascular progenitor cells were generated via an embryoid body-based differentiation system (Yang et al., 2008) with cytokines used as follows: days 0-1, BMP4 (0.5ng/ml); days 1-4, BMP4 (10ng/ml), bFGF (5ng/ml), and activin A (6ng/ml) in Stempro 34 media at a 5% CO<sub>2</sub>/5% O<sub>2</sub>/90% N<sub>2</sub> environment. At day 4, embryoid bodies were harvested and dissociated to single KDR<sup>+</sup> progenitor cells with 0.05% trypsin-EDTA. Isolated KDR<sup>+</sup> progenitor cells were plated on either Matrigel, anti-polyHistidine, or oriented JAG1 surfaces in Stempro 34 media and media was changed every 4 days and cells were switched to normoxia at day 12. At day 16 of culture, cells were analyzed via FACS. Treatment groups were conducted with n=3.

#### *qRT-PCR analysis of gene expression*

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to determine gene expression levels in cells of interest. QIAshredders and RNeasy Micro Kit (Qiagen) were used to isolate RNA that was subsequently quantified via spectrophotometry. 0.5 ug of RNA per reaction was reverse transcribed using SuperScript II Reverse Transcriptase. Resultant cDNA was amplified with powerSybr qPCR master mix (ABI) using primers for *OCT4*, *BRACHYURY*, *SOX1*, *FOXA2*, *JAG1*, *HES1*, and *HPRT*. All were annealed at 60°C for 40 cycles. Gene expression was normalized to *HPRT* expression relative to initial cell population.

#### *FACS analysis of cell marker expression*

Cell cultures were dissociated to single cells, fixed, and incubated with primary antibodies for cardiac troponin T (a cardiac lineage marker, ThermoScientific) smooth muscle actin (a smooth muscle lineage marker, Abcam), and PE-conjugated CD31 (an endothelial lineage marker, EBioscience). Following incubation with the corresponding secondary antibodies (goat anti-

mouse IgG-PE and donkey anti-rabbit IgG-APC, Jackson) when necessary, cells were run through FACS along with the following FACS controls: unstained cells, cells + isotype control, cells + only secondary antibody. Analysis was completed using a BD FACSAria II cell sorter and FlowJo software.

#### *Immunofluorescent staining analysis of cardiac differentiation*

Samples were fixed in ice-cold 2% paraformaldehyde for 5 minutes, blocked with a 1.5% solution of horse serum in PBS containing 0.025% Triton-X, incubated with anti-human goat polyclonal NKX2.5 (R&D, 1:400) at 4°C overnight, followed by an Alexa 594-conjugated donkey anti-goat secondary (Invitrogen, 1:100). Samples were then double-stained with anti-alpha-actinin (Sigma, 1:1000) at 4°C overnight, followed by an Alexa 488-conjugated rabbit anti-mouse secondary (Invitrogen, 1:100). Vectashield containing DAPI counterstain was used to visualize nuclei and mount coverslips. Images were collected by a Nikon A1 Confocal System attached to a Nikon Ti-E inverted microscope platform and using water-immersion Nikon 60x CFI Plan Apo objective lens with 1.2 NA. Image acquisition was performed at room temperature using Nikon NIS Elements 3.1 software to capture 12-bit raw files that were then rescaled to 16-bit images for further processing. All images were collected as a single scan with the pinhole adjusted to 1 Airy unit at 1024x1024 pixel density.

#### *Human embryonic stem cell-derived cardiomyocyte proliferation assay*

hES cell-derived MYH7<sup>+</sup> cardiomyocytes were generated from KDR<sup>+</sup> progenitors as described previously using oriented JAG1 surfaces. Cardiomyocytes were maintained in media consisting of 80% Knockout-Dulbecco's Modified Eagle Media, 1 mmol/L L-glutamine, 1% non-essential amino acids, 20% fetal bovine serum, and 0.1 mmol/L  $\beta$ -mercaptoethanol. For serum-starving

media, fetal bovine serum was omitted from the above media and replaced with .16% BSA. For proliferation assays, cardiomyocytes were dissociated into single-cell suspensions in serum-starving media with 5% FBS using TrypLE and re-plated at a density of 40,000 cells / well in four-well tissue culture polystyrene chamber slides (Nunc) with oriented JAG1, unoriented JAG1, anti-polyHistidine, or gelatin surfaces. Cardiomyocytes were cultured for 3 days to allow for cell attachment before cultures were switched to the appropriate treatment conditions. Treatment conditions included serum-starving media alone, or serum-starving media supplemented with .3% DMSO, 5uM  $\gamma$ -secretase inhibitor (Sigma), IGF-1 (10ng/ml, R&D Systems), anti-polyHistidine modified fibrin microparticles, or JAG1-modified fibrin microparticles at a cell:particle ratio of 3:1 or 1:1. After 24 hours of treatment, media was exchanged for the same treatment along with the addition of BrdU (10 umol/l), and cells were cultured for an additional 24 hours after which samples were ready for processing. All treatment groups were conducted with n=7.

#### *Immunostaining analysis of cardiomyocyte proliferation*

Immunostaining was performed as previously described (McDevitt et al., 2005). Samples were fixed in ice-cold methanol for 10 minutes, blocked with a 1.5% solution of goat serum in PBS for 1 hour at room temperature, incubated with a primary antibody against beta-myosin at 4°C overnight, followed by a secondary biotinylated goat anti-mouse antibody at room temperature for 1 hour (1:500, IgG, Vector) and an avidin/biotinylated alkaline phosphatase complex for 30 minutes at room temperature (ABC-AP, Vector). The enzymatic reaction was developed with the addition of the alkaline phosphatase substrate Vector Red (Vector). Antigen retrieval for BrdU was performed by incubating with 1.5N HCl at 37°C for 15 minutes and rinsing twice with 0.1 mol/l Borax buffer (pH 8.5) for 5 minutes. Following blocking with a 1.5% solution of normal goat serum in PBS for 15 minutes at room temperature, samples were incubated with a

peroxidase-conjugated anti-BrdU antibody (1:40, Roche) overnight at 4°C prior to development with 3,3'-diamino benzidine tetrahydrochloride (DAB). Cells were counterstained with hematoxylin, rinsed with acid alcohol, incubated in Scott's blue, and mounted with Aquamount mounting media before coverslipping. A minimum of 800 cardiomyocyte nuclei were counted per well. Only flattened, adherent cardiomyocytes (beta-myosin+) with clearly discernable nuclei were included in cell counts, and small round cells that sometimes attached on top of cardiomyocyte clusters were omitted to ensure confidence in the accuracy of counts. The results are reported as the mean percentage of BrdU+ cardiomyocyte nuclei +/- standard deviation.

#### *AlamarBlue analysis of cardiomyocyte proliferation*

To analyze the effect of multiple microparticle treatments, differentiated cardiomyocytes were generated and plated as previously described. Cardiomyocytes were plated on oriented JAG1, anti-polyHistidine, or gelatin surfaces. Microparticle treatment groups received 1:1 cell:particle treatments for 24 hours on days two and four of treatment, following which they received fresh serum-starving media. All other treatment groups received fresh serum-starving media every 24 hours. An alamarBlue assay (Invitrogen) was used to monitor proliferation levels at days 0, 1, 3 and 5. Briefly, serum-starving media containing 10% alamarBlue was added to wells. After 2 hours, media was removed and chemical reduction of alamarBlue, as measured by changes in fluorescence, was determined. Measurements were compared to a standard curve generated from cardiomyocyte cultures of defined cell number. All treatment groups were conducted with n=3.

## Supplemental References

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