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Proteins Secreted By Embryonic Stem Cells Activate Cardiomyocytes Through Ligand Binding Pathways

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Abstract

Human embryonic stem cells (hESC) underly embryogenesis but paracrine signals associated with the process are unknown. This study was designed to 1) profile native proteins secreted by undifferentiated hESC and 2) determine their biological effects on primary neonatal cardiomyocytes. We utilized multi-analyte, immunochemical assays to characterize media conditioned by undifferentiated hESC versus unconditioned media. Expression profiling was performed on cardiomyocytes subjected to these different media conditions and altered transcripts were mapped to critical pathways. Thirty-two of 109 proteins were significantly elevated in conditioned media ranging in concentration from thrombospondin (57.2 ± 5.0 ng/ml) to nerve growth factor (7.4 ± 1.2 pg/ml) and comprising chemokines, cytokines, growth factors, and proteins involved in cell adhesion and extracellular matrix remodeling. Conditioned media induced karyokinesis, cytokinesis and proliferation in mono- and binucleate cardiomyocytes. Pathway analysis revealed comprehensive activation of the ROCK 1 and 2 G-protein coupled receptor (GPCR) pathway associated with cytokinesis, and the RAS/RAF/MEK/ERK receptor tyrosine kinase (RTK) and JAK/STAT-cytokine pathway involved in cell cycle progression. These results provide a partial database of proteins secreted by pluripotent hESC that potentiate cell division in cardiomyocytes via a paracrine mechanism suggesting a potential role for these stem cell factors in cardiogenesis and cardiac repair.

Keywords

Embryonic stem cells; secreted proteins; pluripotent stem cells; cardiomyocytes; Intercellular signaling peptides and proteins; paracrine signaling

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Introduction

Pluripotent stem cells serve as source and target of signals during embryogenesis that result in both autoactivation and stimulation of surrounding cells to undergo histogenesis [1,2]. These complex interactions suggest that stem cells create a microenvironment comprising mitogens, growth factors, cytokines and chemokines to produce the intricate morphogenic processes associated with organ and tissue formation. Recent efforts have been directed at delineating the underlying signal molecules that sustain pluripotency and/or control differentiation fate within stem cells as well as those factors that influence cells of the surrounding microenvironment. This interest has been fueled by recognition that damage to mature tissues often triggers a regeneration process recapitulating characteristics of the developmental process including recruitment of stem cells either from within or extrinsic to the injured tissue [3,4]. Understanding the stem cell microenvironment may help explain why successful regeneration does not occur after damage to tissues where intrinsic stem cells are known to be present e.g. those within the central nervous system and the ventricular myocardium [5,6]. Therefore, defining the biological factors secreted by embryonic stem cells may provide insight into clinical therapies for regeneration and repair of damaged tissues by activation of intrinsic progenitor cells, recruitment of extrinsic stem cells to the injury site or by improving the efficacy of extrinsic stem cell grafts.

Previous efforts to define the proteome of embryonic stem cells and their derivatives have interrogated stem cell lysates using mass spectrometry to identify large numbers of proteins including many that could be classified based on their potential to affect other cells in the extracellular microenvironment [7,8]. Recent “bottom up” discovery methods have refined this approach by focusing on factors specifically found in media conditioned by embryonic stem cells [9,10]. The present study was designed to extend this paradigm by combining both 1) a quantitative analysis of soluble, native proteins in media conditioned specifically by undifferentiated, pluripotent human embryonic stem cells (hESC) and 2) sensitive immunocytochemical and gene expression assays delineating the response of primary cardiomyocytes to the hESC conditioned media. Under the appropriate culture conditions, neonatal cardiomyocytes exhibit a prototypical cardiac muscle phenotype including rhythmic beating and a limited capacity to proliferate [11]. Therefore we sought to gain insight into the underlying mechanisms whereby stem cell factors affect prospective target cells such as cardiomyocytes by defining the signal pathways and potential factors capable of initiating a response. An important part of this approach was the use of media conditioned by undifferentiated hESC where potentially confounding factors produced by feeder cells were eliminated followed by analysis without the complicating effects of multiple freeze-thaw cycles, media fractionation, depletion and proteolytic digestion of the native protein targets.

Materials and Methods

Embryonic Stem cell culture

The human embryonic stem cell line, Hsf1 (NIH:UCO1), was obtained from UCSF (University of California, San Francisco) according to regulations for embryonic stem cell use at UCSF and the University of Pittsburgh. The hESC were initially expanded on CF-1 feeder cells mitotically inactivated by treatment with 20µg/ml Mitomycin C (Sigma, St. Louis, MO) for 2 hours at 37°C. Individual stem cell colonies were manually transferred to growth factor reduced Matrigel™ (BD Biosciences, San Jose, CA) diluted to 1mg/ml with high glucose DMEM (Dulbecco's Modified Eagle Medium) basal media (Gibco, Invitrogen, Carlsbad, CA) in the absence of other cells. These colonies were expanded in media comprising equal amounts of 1) high glucose DMEM + 20% KOSR (Gibco Knockout™ serum replacer) + 20ng/ml bFGF (human recombinant, Invitrogen) (hESC media) that was conditioned by inactivated CF-1 cells, and 2) 50% hESC media alone. The CF-1 conditioned media enhanced attachment of the stem

cells to the Matrigel substrate but they were gradually weaned over a three day period to 80% DMEM + 20% KOSR + 40ng/ml bFGF + 0.5µg/ml Noggin (Fc mouse chimera-derived, R&D Systems, Minneapolis, MI) to eliminate factors from CF-1 cells. Stem cell conditioned media was collected every 48 hours from wells containing undifferentiated stem cells only. The media was filtered (0.22µm) for testing on purified primary rat neonatal cardiomyocytes cultured for 3 to 7 days prior to initiation of these studies. Control media was treated identically but no cells were present in the wells.

Targeted Proteomics Assay

Fluorokine multi-analyte profiling (xMAP, Luminex 100 platform) (Luminex, Austin, TX) was employed to measure levels of defined proteins in conditioned and unconditioned media samples. The technology incorporated polystyrene microsphere sets, each with unique spectral signatures conjugated with a biotinylated capture antibody specific for a unique human target. The assays utilized a 96-well microplate format and were processed according to the manufacturer's protocol, including generation of a standard curve for each target prepared in background medium diluent (DMEM). The Bio-Plex suspension array system and Bio-Plex Manager software 4.0 (Bio-Rad Laboratories, Hercules, CA) were employed to calculate analyte levels by comparison to standard curves obtained for each analyte repeated in triplicate at 2 dilutions. An ELISA was performed to assay the presence of TGF-β in unconditioned and conditioned media both before and after acid activation of the latent form via a quantitative sandwich immunotechnique as previously described [11]. We also utilized a chemiluminescent ELISA to analyze media for the presence of neuregulin 1 (R&D Systems) and a sandwich ELISA to assay for amphiregulin and cyclooxygenase 2 (COX2) (Aushon Biosystems, Billerica, MA). A total of 109 specific proteins were analyzed by these assays. Statistical significance was determined by the Student's T-test corrected for multiple testing [12].

Cardiomyocyte purification and culture

Cardiac cells were obtained from ventricles of hearts removed aseptically from neonatal (postnatal day 2 or 3) Sprague-Dawley rats at Zivic Labs (Zelienople, PA) under Institutional Animal Care and Use Committee approval (SVM03.00) using CO₂ for euthanasia. Twenty to 25 ventricles were processed simultaneously for each experiment using sequential Percoll gradient centrifugation [11]. The purification protocol involved serial digestion of finely minced ventricular tissue in pancreatin/collagenase type 2 followed by Percoll gradient centrifugation. The process provided highly enriched cardiomyocytes with <5% fibroblasts present upon initial plating on gelatin coated 100mm dishes (0.1%) in DMEM:M199 (4:1) with 10% horse serum (Hyclone Lot APH21830) and 5% FBS (Hyclone Lot APH21746). If fibroblasts were present (>5%) 12 hours after initial plating, cells were subjected to differential adhesion preplating to remove contaminating fibroblasts and endothelial cells prior to final seeding. Studies were performed in 60mm dishes or 6-well plates at equivalent cell plating concentrations (~400 cells/plate) for six days [11]. Studies performed at clonal densities comprised plates seeded with cardiomyocytes at ~100 cells/plate. Complete media changes were performed every 24 hours with plates assigned randomly to receive either standard cardiomyocyte media, or cardiomyocyte media mixed 1:1 with unconditioned or hESC conditioned media. Horse serum and fetal bovine serum are a source of growth factors (e.g. IGF1:100-300ng/ml, TGFβ1:2-20ng/ml and FGF2:1-25pg/ml; Hyclone Application note:SC0801) so serum concentrations were adjusted to a constant level under all conditions to maintain these values. Control plates received 50% unconditioned media, with the same adjustments of HS and FBS. Parallel dishes were fixed in methanol for immunostaining.

Data were obtained from separate culture plates at sequential 24 hour time-points under each media condition. Each data point per individual study represented the average of results obtained from 2 to 5 culture plates depending on the overall yield after cardiomyocyte

purification. These studies were repeated in 5 independent cardiomyocyte harvests. Immunocytochemical analysis of myosin and vimentin expression confirmed that fibroblasts represented <5% of the cells in all plates included in the analysis.

Immunocytochemical analysis

Cardiomyocytes and hESC were fixed in ice cold methanol, nuclei were permeabilized with 0.1% Triton X-100 and cells were blocked in 5% goat serum + 0.3% BSA in PBS (Goat Block). Cardiomyocytes were incubated in MF20 (Iowa Studies Developmental Hybridoma Bank, Iowa City, IA) (1:1 in Goat Block), a hybridoma supernatant that recognizes myosin heavy chain protein [13] exclusionary to cardiomyocytes versus other cardiac cells followed by goat anti mouse Alexa Fluor 488 (Invitrogen). Fibroblasts were identified by vimentin expression (ab:V9, Santa Cruz Biotechnologies, Santa Cruz, CA). Cardiomyocytes were incubated in MPM2 (1:200; Millipore, Billerica, MA), an antibody that recognizes phosphorylated epitopes of proteins involved in mitosis [14], followed by a secondary antibody conjugated to Alexa Fluor 546 (Invitrogen). Nuclei were stained using Hoescht 33342 (1:1000 in PBS). Both undifferentiated (5 days culture) and differentiated embryonic stem cell colonies (12 days culture at high density) were incubated with a monoclonal antibody to OCT4 (1:200; Santa Cruz Biotechnologies), a transcription factor/protein present in undifferentiated embryonic stem cells. The secondary antibody was goat anti mouse IgG conjugated to Alexa Fluor 488 (Invitrogen). Images were acquired using a Nikon TE200 microscope (10× or 20× Plan Fluor phase contrast objectives) with a Qimaging Micropublisher 5.0 RTV camera and QCapture Pro software (Surrey, BC). Original images were 28.1MB with pixel dimensions of 2560 × 1920 and final cardiomyocyte images were identically cropped (780 × 585 pixels). Hsf1 images were scaled down without cropping (810 × 608 pixels). OCT4 fluorescence staining of both undifferentiated and differentiated Hsf1 cells was captured at 4 seconds exposure.

The differentiation assay for hESC colonies utilized antibodies specific to germinal layers associated with embryogenesis. Cells were fixed, permeabilized and blocked as described previously. The stem cell colonies were incubated with antibodies to α -fetoprotein (AFP) (1:200, rabbit polyclonal; Dako, Carpinteria, CA), a cytoplasmic endodermal marker of early liver parenchyma; nestin (1:500, human-specific rabbit polyclonal; Millipore) an intermediate filament of early neuroectoderm; or desmin (1:500, mouse monoclonal; Millipore, Billerica, MA) an intermediate filament of early mesoderm. Secondary antibodies were goat anti rabbit IgG-Alexa Fluor 488 for AFP, goat anti rabbit IgG-Alexa Fluor 546 for nestin and goat anti mouse IgG Alexa Fluor 488 for desmin. Images were scaled down without cropping (810 × 608 pixels). Immunofluorescence staining of each germ layer was captured at an exposure time of 1 second.

RNA isolation, hybridization and expression profiling

Cardiomyocytes were harvested 24 or 48 hours after initiation of the test media experiments for RNA expression profiling studies. These studies were performed in duplicate and in parallel to the other culture studies using higher densities of cardiomyocytes (2.5×10^5) to obtain sufficient RNA for a direct comparison of transcript expression profiles between cells in unconditioned or conditioned media. Biological replicates were pooled and 3 microarray replicates were processed and analyzed for each condition. RNA purification was performed using TRIzol reagent (Invitrogen) as previously described [11]. Briefly, TRIzol was added to culture plates (5mls/100mm dish) after they were rinsed with ice cold PBS. The cells were harvested with a cell scraper followed by homogenization. The aqueous layer was collected after organic phase separation and RNA was precipitated, washed (80% ethanol) and resuspended in nuclease-free water. Criteria for inclusion in array studies was spectrophotometric absorption ratio 260/280 > 1.8 (NanoDrop, Wilmington, DE) and RIN value > 8.0 via electrophoretic analysis (Agilent Bioanalyzer 2100; Agilent Technologies, Santa

Clara, CA). In vitro transcription (IVT) and hybridization were performed using the CodeLink™ iExpress Assay Reagent Kit and CodeLink Expression Bioarray Hybridization Protocols. RNA (200ng) was reverse transcribed including bacterial controls followed by 2nd strand synthesis and IVT in the presence of biotinylated nucleotides to produce biotin-11-UTP labeled cRNA. Ten micrograms of cRNA was purified, fragmented and hybridized to Codelink Bioarrays (rat whole genome or rat Uniset 1) for 18 hours followed by washing and staining of bound target cRNA with Alexa Fluor 647 (Invitrogen). The arrays were rinsed, dried and then scanned using the Genepix 4000B scanner (Axon Instruments, Foster City, CA) and CodeLink Expression Analysis software v. 5.0. Final analysis was performed on the raw data normalized to bacterial spike-ins and corrected for the dynamic range by median normalization within each array. Significant transcripts were defined using the Significance Analysis of Microarrays software (v. 3.0) [15] for paired comparisons ($q < 0.05$) and overrepresentation of transcripts for biological and functional pathways was performed using Ingenuity Pathways Analysis (IPA v. 6.5, Ingenuity Systems, Redwood City, CA).

Results

Undifferentiated hESC as a Source of Secreted Factors

The embryonic stem cell colonies grew in a monolayer constituting approximately 10,000 cells per grouping and 30 to 35 groups/well on matrigel in 6-well plates (Fig. 1, panel A). Parallel wells of stem cells were subjected to immunocytochemical analysis for markers of pluripotency (OCT4⁺) and differentiation to confirm that they remained undifferentiated during media conditioning (Fig. 1, panel A) rather than adopting a differentiated cell lineage phenotype (Fig. 1, panel B, Fig. 2) [16]. To confirm that these cells retained properties of embryonic stem cells, colonies were subjected to a differentiation paradigm triggered by administration of serum and/or growth factors. These cells no longer expressed OCT4 as a marker of pluripotency (Figure 1, panel B) but exhibited positive staining for α -fetoprotein representing cells of endodermal origin (Fig. 2, panel A); nestin indicating cells of ectodermal origin (Fig. 2, panel B); or desmin demonstrating their ability to form cell types of mesodermal origin (Fig. 2, panel C). These parallel assays confirmed that the conditioned media utilized for the proteomics analysis and biological testing was derived from predominantly undifferentiated stem cell colonies and that these cells retained the ability to form tissues of the three primary germ layers consistent with their identification as pluripotent stem cells.

Analysis of hESC Secreted Proteins

High-resolution, multi-analyte profiling of unconditioned versus conditioned media revealed a rich chemokine environment spanning a broad dynamic range (0.1 picogram to 100 nanograms per milliliter). Thirty-two of 109 proteins subjected to analysis were detected at significantly higher levels in the pooled stem cell conditioned media (3 expansions) versus the unconditioned media (Fig. 3; Supplemental Table S1). Basic FGF was added separately to both unconditioned and conditioned media and decreased significantly after exposure to the stem cell colonies. Results obtained from replicate assays of pooled media were confirmed in comparisons of individual media expansions to matched control media.

A contingent of proteins significantly elevated in conditioned media was associated with extracellular matrix (ECM) remodeling and cell–cell interactions. Thrombospondin, a secreted glycoprotein that modulates cell adhesion, was expressed at the highest level (TSP-1; 57.0 ± 0.70 ng/ml; Fig. 3A). Thrombospondin is thought to play a role in preventing angiogenesis and is known to activate latent TGF β which also was present at relatively high levels in the conditioned media (Fig. 3B) [17]. Endostatin, a collagen proteolytic cleavage product that binds to basement membrane targets, was also detected at high levels in the conditioned media (ENDO; 4.5 ± 3.0 ng/ml; Fig. 3A). Like thrombospondin, endostatin has anti-angiogenic

properties but is essential for ocular development and maintenance of vision [18]. Multiple matrix metalloproteinases (MMP: 1, 2, 9: Fig. 3A) and tissue inhibitors of metalloproteinases (TIMP: 1, 4: Fig. 3A, B) were expressed at high levels in conditioned media (1.5 ng/ml to 25 ng/ml) indicating a significant presence of these cell surface molecules. Both total and active forms of plasminogen activator inhibitors that play key roles in ECM remodelling increased to high levels in the conditioned media with tPAI-1 exceeding 12 ng/ml (Fig. 3A). Finally, soluble cellular adhesion molecules, sVCAM-1 (vascular) and sICAM-1 (intercellular) and mesothelin (MESO), a surface protein involved in cell adhesion, were present at significantly elevated levels in conditioned media (Fig. 3B).

Several growth factors were produced by the hESC. Hepatocyte growth factor was present at low levels in the unconditioned media (HGF; 15.2 ± 9.9 pg/ml: Fig. 3B) but markedly increased (278.3 ± 93.7 pg/ml) in the conditioned media. Nerve growth factor was absent in unconditioned media but was detected in the stem cell conditioned media (NGF; 7.4 ± 1.2 pg/ml: Fig. 3B). Insulin growth factor binding protein-1 and 2 were significantly elevated in the conditioned media with IGFBP-2 among the highest levels (42.1 ± 0.5 ng/ml) of all proteins (IGFBP-2: Fig. 3A, IGFBP-1: Fig. 3B). ERBB2 (v-erb-b2 avian erythroblastic leukemia viral oncogene) was present in conditioned media at low but statistically significant levels (ErbB2: 19.2 ± 10.6 pg/ml: Fig. 3B). ERBB2 does not bind growth factor receptors directly but forms stabilizing heterodimers with EGF family members bound to their receptor sites thereby enhancing downstream signaling processes [19].

Four members of the chemokine family (cysteine-cysteine motif) were present at significantly elevated levels in the conditioned media. These included monocyte chemoattractant protein, (MCP-1: Fig. 3A), macrophage inflammatory protein α and β (MIP1 α : Fig. 3B, MIP1 β : Table S1) and eotaxin (Table S1). The concentrations of these molecules spanned the dynamic range of the assay with MCP-1 expressed at relatively high levels (8.8 ± 8.4 ng/ml) while eotaxin levels were at the lowest range of detection (approximately 2 pg/ml). Macrophage inhibitory factor (MIF) interacts with C-X-C chemokine receptors and was also expressed at significantly elevated levels in the conditioned media (MIF; 292.7 ± 26.8 pg/ml: Fig. 3B) further emphasizing the importance of these chemokines among the repertoire of proteins produced by the stem cells.

Three members of the tumor necrosis factor receptor super family (TNFRSF) were present at elevated levels in conditioned media including TNFRSF6 (sFAS) and TNFRSF1A (TNF-R1). Both exhibited picogram levels in the conditioned media (99.3 ± 19.5 and 30.9 ± 9.0 pg/ml respectively: Fig. 3B). TNFRSF1B (TNF-R2) was higher in the conditioned media based on significantly elevated intensity values but was at the lower limit of the calibration curve (~ 2.0 pg/ml) (Table S1). These pleiotropic cytokines have been implicated in a broad range of functions extending from host defense to apoptosis to organogenesis. Other important cytokines detected in conditioned media were interleukins 6 and 8 (IL-6 and IL-8: 43 and 46 pg/ml: Fig. 3B) along with increased IL-1R α (IL-1Ra; 18.0 ± 0.5 pg/ml: Fig. 3B). Both IL-2 and IL-12 p40/p70 also were significantly increased based on intensity values and correlated with levels at or below 10 pg/ml i.e. the lower limit of the calibration standards for these targets (Table S1). Interferon- α was secreted by the stem cells and was significantly elevated in the conditioned media (IFN α ; 38.9 ± 4.5 pg/ml: Fig. 3B).

Four factors were present in the conditioned media in classifications distinct from the others or with as yet to be defined functions. Kallikrein 10 (KLK 10: Fig. 3A) is a secreted serine protease with growth and tumor suppressor function that was present at 25.0 ± 0.04 ng/ml in conditioned media [20]. Whey acidic protein domain-4 disulphide 2 (WFDC2), also known as human epididymis 4 (HE4: Fig. 3A), was detected at relatively high levels in the conditioned media (1.0 ± 0.4 ng/ml). This secreted protein is involved in development of oronasal,

pulmonary, and reproductive tissues and has been proposed as a biomarker for lung cancer [21]. Alpha-fetoprotein is a fetal serum protein involved in the processes of differentiation and growth in the fetus and was detected at levels in the conditioned media comparable to IGFBP2 and HGF (AFP: 27.4 ± 2.2 ng/ml; Fig. 3A) [22]. Finally, heat shock protein-70, a ubiquitous, extracellular membrane bound protein with multiple functions was expressed at relatively high levels in the conditioned media but was not present in the unconditioned media (HSP-70: 299.6 ± 11.2 pg/ml; Fig. 3B). The entire list of 109 targets comprising the proteomics assay are provided in Supplemental Table 1 including the raw intensity values (mean $\pm$ S.D. p value) obtained for each analyte in unconditioned and conditioned media.

Effect of hESC Conditioned Media on Cardiomyocytes

Neonatal cardiomyocytes displayed rhythmic contractions within 24 hours after plating and for the study duration of six days. The proliferation rate of cardiomyocytes maintained in cardiac media diluted 1:1 with unconditioned media was indistinguishable from those grown in cardiac media alone (Figure 4) while the number of cardiomyocytes grown in hESC conditioned media was consistently higher. Media effects on proliferation were significant via 2-Way ANOVA and post-hoc analysis indicated that the greatest change in cardiomyocyte number occurred in the first 24 hours (Figure 4). Since population assessment was obtained from replicate mass culture plates at each time-point, the proliferative effect on cardiomyocyte numbers was independently validated in each of the plates for each of the test conditions.

Results obtained in mass culture were validated in clonal density studies where cardiomyocytes were seeded (~ 100 cells per 60 mm dish) in standard cardiomyocyte media or cardiomyocyte media mixed 1:1 with either unconditioned or hESC conditioned media. The number of two-cell cardiomyocyte colonies (indicative of a single cell division) present 24 hours after plating was significantly higher in conditioned media than either unconditioned or standard cardiac media (conditioned media= 16.0 ± 5.5 vs. unconditioned media= 5.5 ± 6.2 two-cell colonies/plate, $n=4$, $p < 0.03$; standard media= 1.8 ± 0.9 clusters). This finding extended to 48 hours as well (conditioned media= 39.4 ± 9.9 vs. unconditioned media= 32.0 ± 13.5 two-cell colonies/plate). The conditioned media applied to both high and low density cultures was obtained from 3 different embryonic stem cell expansions and one study in which the 3 expansions were pooled.

Microscopic review of the cultures after immunocytochemical assays revealed no obvious morphological differences in the cardiomyocytes treated with unconditioned or embryonic stem cell conditioned media. However, a subpopulation of cardiomyocytes positive for cell division ($V9^+$, $MF20^+$, $MPM2^+$) was evident among cells maintained in conditioned media but were rarely seen in the control media. MPM2 immunofluorescence was focused within the nucleus during prophase, increased in cytoplasmic localization during anaphase and metaphase, and diminished during telophase and upon completion of cytokinesis (Figure 5A-E). It was interesting to note that the myosin contractile structure of the cardiomyocytes was at least partially retained during the replication process as indicated by continuous positive MF20 staining of both parental and daughter cells. Mitosis was evident among cardiomyocytes with a single nucleus as well as with binucleate cardiomyocytes. In fact, karyokinesis in some binucleate cells occurred and was restricted to only one of the two parental cell nuclei as indicated by MPM2 staining suggesting extrinsic mitotic stimuli were sequestered to one nucleus despite the presence of another nucleus in the same cytoplasm (Figure 5E). Nevertheless, these cells completed the process of cytokinesis and no cardiomyocytes with more than 2 nuclei were detected in the cultures under any conditions other than those undergoing cell division.

Transcripts Associated with Proliferation are Detected by Expression Profiling

Analysis of the transcripts from cardiomyocytes grown in conditioned media revealed significant increases spanning a wide range of intensities based on Significance Analysis of Microarrays software (SAM: v.3.0; false detection rate $\leq 5\%$) [15]. (The entire dataset is deposited in GEO under accession number GSE13708). Analysis of these transcripts using Ingenuity Pathways Analysis (IPA v.6.5) revealed that expression patterns in conditioned media at both 24 and 48 hours significantly overrepresented the molecular functions: “cellular growth and proliferation” and “cellular movement” based both on p value ($p < 1.06 \times 10^{-6}$) and number of molecules (24 hr.=204 transcripts, 48 hr.=131 transcripts). In contrast, cardiomyocytes cultured in unconditioned media yielded only 12 transcripts associated with “cellular growth and proliferation” at 24 hours while none were detected at 48 hours.

The data obtained via SAM analysis designating significantly increased transcripts were crossed with genes subserving functions of mitosis, cytokinesis, cell cycling and proliferation derived from the Gene Ontology Database, the Protein Lounge Pathways Database and manual curation of the literature. Multiple cyclin dependent kinases (6,9), cyclins (E1,D1), cell division cycle transcripts (A2,A7,A8) and Polo-like Kinase 1 were significantly elevated consistent with cell cycling and replication (Figure 6A). Conditioned media also induced significant elevation of cell division cycle gene 42 and effector proteins (CDC42, CDC42EP2/3), RAS Homolog Gene Family, Member A (RHOA) and RAS-Related C3 Botulinum Toxin Substrate (RAC1) which play pivotal roles in cytokinesis (Figure 6B) [23]. Multiple septins including septin 2 plus RHO-Associated Coiled-Coil Forming Kinase 2 (ROCK2) and cofilin 1 (CFL1) were elevated further reinforcing a pattern of gene expression consistent with active cell division (Figure 6B). At the same time, there were no significant increases in transcripts associated with apoptosis detected under any of the conditions. Since only a small component of the cardiomyocyte cell population was undergoing cell division in these studies, the ability to detect these differences among plates was indicative of the sensitivity of the array methodology which relied on linear mRNA amplification (50 to 100-fold) and validated 30 mer probes [24].

Several signal transduction pathways associated with cardiomyogenesis, development and cytokinesis emerged from these data. Specifically, cardiomyocytes cultured in stem cell conditioned media exhibited increased transcripts associated with 1) Janus kinases (2 and 3) that interact with Stat1 and Stat3 transcription factors which were concomitantly elevated, 2) with transcript activation of the RTK pathway comprising RAS/RAF/MEK/ERK and 3) increased transcripts for multiple members of the ROCK 1 and 2 GPCR pathways which play a role in centromere positioning during mitosis and actin cytoskeleton assembly (Figure 7). Thus the findings obtained via expression profiling of separate cultures corroborated those obtained via immunocytochemical analysis on parallel cultures indicating cell cycle progression in the conditioned media. These significant changes were detected across the entire signal transduction pathway despite the fact that transcripts from actively dividing cells were diluted against the transcriptional background of non-proliferating cells. The microarray intensity data representing the compendium of transcripts associated with these signal transduction pathways as determined by SAM and IPA analysis are provided in Supplemental Table 2.

Discussion

The current study established that human embryonic stem cells maintained in the pluripotent state are a fertile source of diverse chemokines, cytokines, growth factors and molecules involved in remodeling of the extracellular matrix. These results are the first to delineate a direct effect of proteins secreted by undifferentiated embryonic stem cells on purified cultures of neonatal cardiomyocytes. Furthermore, they represent the first analysis of media conditioned

by Hsf1 embryonic stem cells to our knowledge and included an experimental paradigm eliminating any factors produced by feeder cells. Previous studies in our laboratory using fibroblast-conditioned media confirmed that neonatal cardiomyocytes undergo minimal proliferation in culture [11]. A surprising observation of the present study was that only a few colonies of hESC (~10) generated concentrations of proteins comparable to that from confluent plates of fibroblasts in previous studies. Thus, these data demonstrate the ability of undifferentiated hESC to influence their microenvironment via paracrine mechanisms despite their intrinsically small cytoplasmic volume and limited morphological complexity.

We used cells from a precisely characterized human embryonic stem cell line (Hsf1, NIH:UCO1), expanded them in the absence of other cells and maintained them in an undifferentiated state to generate conditioned media without confounding factors produced by feeder cells. The proteomics assay compared targets in the conditioned media to media treated identically except for exposure to the stem cells. Procedural advantages of this analysis of the conditioned media included minimizing the number of preparatory freeze-thaw cycles, elimination of proteolytic digestion, dispensing with depletion of dominant constituents such as albumin, and eliminating complex filtration, fractionation and enrichment procedures. Furthermore, the concentration of each protein per sample was determined using calibrated recombinant standards specific to each immunochemical probe thereby revealing absolute differences among individual proteins across media types. The similarity of results obtained by analyzing hESC conditioned media across individual and pooled specimens indicated that these cells behaved in a consistent manner with each separate expansion.

The hESC conditioned media induced a significant, transient proliferation of cardiomyocytes confirmed by immunocytochemical analysis demonstrating karyokinesis and cytokinesis in mono- and binucleate contractile cells. Previously, cardiomyocyte proliferation has been experimentally induced in vitro and in vivo by manipulation of transgenes or administration of exogenous factors e.g. FGF1, periostin, and neuregulin1 [25-27]. Whether these factors play a role in the intrinsic biology of cardiogenesis and cardiac physiology remains to be determined. Neuregulin 1 was not detected in the hESC conditioned media. However, some of the factors we identified e.g. HGF and insulin like growth factor binding protein 1, have demonstrated cardioprotective effects when applied to cardiomyocyte cultures either through a reduction in apoptotic activity or enhancement of metabolism [28,29]. Other proteins identified in this study including kallikrein 10 [30], thrombospondin [31], and MCP1 [32] have been implicated as factors important in successful recovery from myocardial infarct. Thus our findings reinforce previous studies suggesting a role for these factors in cardiomyocyte function. It is interesting to speculate that the transient success of hESC grafts in treating experimental models of myocardial infarct in previous studies may have been, in part, attributable to the presence of soluble factors produced by the undifferentiated stem cells prior to adopting a cell fate [33, 34].

Combinatorial analysis of the proteomics and array data revealed distinct activation of GPCR, RTK and cytokine pathways involved in cell cycling, cytokinesis, and cardiomyocyte differentiation consistent with previous studies [35,36]. Specification of these pathways was based on detection of comprehensive activation of gene transcripts across the entire pathway---from prospective ligands identified in the media to transcription factors activated in the cardiomyocytes---rather than extrapolating from a single or small number of genes. These data indicate that undifferentiated stem cells are a unique resource of secreted factors with the potential to activate otherwise quiescent cardiomyocytes through established biological pathways. If so, hESC media may represent a useful resource for the identification of biologically relevant factors that stimulate cardiac cell proliferation or serve as a therapeutic adjuvant in future studies. Rigorous studies beyond the scope of the present experiments will be necessary to define the specific factors in the hESC media responsible for the proliferation

response with the realization that the proteins may be outside of those assayed in the present study. At the same time, we cannot rule out that the Hsf1 cells consumed or altered molecules in the media during conditioning thereby inducing a cardiomyocyte proliferative response by their absence or modification.

Thirty-two chemokines, cytokines and growth factors were significantly elevated in the hESC conditioned media from among the 109 screened targets. Several of these factors or their precursors (ENDO, sFAS, TSP-1, latent TGF- β , PAI-1, MIF, IGFBP-2, MMP1, MMP9, TIMP1 and AFP) have also been detected in media conditioned by either undifferentiated H1 and/or H9 embryonic stem cell lines using mass spectrometry after extraction, digestion, fractionation, and repeated injection [10]. Perhaps equally important was the concurrence between these studies regarding targets that were not detected in either case. TIMP-3 was the only contradictory factor inasmuch as it was detected at low levels in media conditioned by the H1 or H9 stem cell lines but was not detected in the media of the present study using Hsf1 cells. These findings suggest that undifferentiated, pluripotent stem cells may secrete a common signature of proteins associated with their paracrine role in embryogenesis. Furthermore, the interaction of human ESC secreted factors with rat cardiomyocytes is consistent with the role of mouse fibroblast secreted factors in the maintenance of hESC pluripotency suggesting an evolutionarily conserved signal-receptor pattern between hESC and rodent cells [37]. Finally, the data suggest that stem cells may play a supportive role in tissue repair and regeneration based on the rich compendium of mitogenic factors they secrete into the local microenvironment.

Conclusion

Undifferentiated Hsf1 human embryonic stem cells secrete a diverse profile of proteins over a broad range of concentrations despite their small size and high nucleus to cytoplasm ratio. Proteins detected in media conditioned by these cells overlapped with previously published profiles for separately derived embryonic stem cells, H1 and H9, indicative of a common proteomic secretory signature among these distinct stem cell lines. Hsf1 conditioned media induced proliferation in cultured mono- and binucleate cardiomyocytes via canonical GPCR, RTK and cytokine pathways indicating the presence of biologically active ligands consistent with a role in cardiac development and potentially useful in cardiac repair strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Human embryonic stem cells (hESC) cultured in an undifferentiated state and after onset of colony differentiation

Panel A: An Hsf1 colony on growth factor reduced Matrigel for 5 days. The phase contrast image is on the left, DNA specific Hoescht 33342 stain highlighting the nuclei in the middle, and the right panel is an immunofluorescence image of the same colony after binding of an antibody specific to OCT4 indicative of pluripotency. Images captured at 20 $\times$ magnification. Panel B: An hESC colony after 12 days in culture conditions designed to induce differentiation. The phase contrast image depicts changes in colony morphology with loss of OCT4 expression in the right panel. The images were captured at 10 $\times$ magnification.

Figure 2. hESC differentiate into cells derived from all three germ layers

Left and center panels comprise phase contrast and Hoescht images as in Figure 1. Panel A demonstrates a differentiated colony yielding cells of endodermal phenotype indicated by immunofluorescence for α -fetoprotein. The colony in panel B exhibits immunofluorescence for an antibody specific to nestin indicative of differentiation into neuroectodermal cells. Images of panel A and B were acquired at 10 $\times$ magnification. Panel C demonstrates a colony with a subset of cells positive for an antibody to desmin indicating differentiation into a mesodermal cell type. The images in panel C were acquired at 20 $\times$ magnification.

Figure 3. Proteins elevated in hESC conditioned media versus unconditioned media

Panel A depicts proteins from 1ng to 100ng/milliliter concentration and Panel B from 1pg to 1000pg/ml on a log scale to across the broad dynamic range of the assays (n=3 expansions). TSP-1: thrombospondin-1, IGFBP: insulin growth factor binding protein (1,2); AFP: α -fetoprotein; MMP: matrix metalloproteinase (1,2,9); TIMP: tissue inhibitor of metalloproteinases (1,4); tPAI-1: plasminogen activator inhibitor-1 (t:tissue); MCP-1: monocyte chemoattractant protein 1; ENDO: endostatin; KLK 10: kallikrein 10; HE4: human epididymis factor 4; MESO: mesothelin; TGfb-lat: latent transforming growth factor beta; HSP-70: heat shock protein 70; MIF: macrophage inhibitory factor; HGF: hepatocyte growth factor; CAM: cell adhesion molecule (s: soluble, v:vascular, I:intercellular); TNFRSF: tumor

necrosis factor receptor super family (6, 1A); IL: interleukin (8,6,1Ra: 1 receptor α); MIP-1A: macrophage inflammatory protein-1a; IFN α : interferon- α ; ErbB2: erb-b2 avian erythroblastic leukemia viral oncogene; NGF: nerve growth factor. The entire data set is provided in Supplementary Table 1 (Table S1).

Figure 4. Proliferation assay of cardiomyocytes in hESC conditioned media

Average cardiomyocyte number per plate determined over a 6-days in standard cardiac media (standard), cardiac media diluted 1:1 with ES cell conditioned media (conditioned) or diluted 1:1 with unconditioned media (unconditioned). Five separate experiments were performed with ≥ 2 plates for each timepoint in each media condition and initial cardiomyocyte seeding at 400 cells/plate. The values are mean $\pm$ standard deviations for $n=5$ in each condition at each time point. Stars indicate a statistically significant difference in cell numbers among the groups at each timepoint. The number of cells present in standard cardiomyocyte media and unconditioned media were not different at any timepoint.

Figure 5. Cardiomyocytes undergoing cell division 24 hours after exposure to hESC conditioned media

Panel A: A cardiomyocyte in late telophase with persistence of the cleavage furrow. The center panel indicates the presence of myosin heavy chains as determined by immunofluorescence specific to the MF20 antibody while anti-MPM2 delineated phosphorylated proteins associated with onset of mitosis as demonstrated in the right panel. Panel B: Cells undergoing metaphase are present with spindle fibers evident in the MPM2 panel. Panel C: A cardiomyocyte in anaphase is depicted including the presence of centrosomes distinguishable in the MPM2 panel. Panel D: A centrally located cardiomyocyte in prophase is indicated by the enlarged immunofluorescent nucleus undergoing chromatin replication as indicated by the nuclear focus

of MPM2 in the right panel. Panel E: A binucleate cardiomyocyte is depicted in which only one of the two nuclei is undergoing mitotic division and in anaphase based on Hoescht and MPM2 staining. See methods for immunocytochemical protocol.

Figure 6. Expression of transcripts associated with proliferation in cardiomyocytes cultured in conditioned media

Fold-change is compared to the level of expression at the beginning of each of the studies (n=3) but statistical comparisons were based on intensity values provided with each set of bars for 48 hour conditioned samples presented as mean $\pm$ SD. Each transcripts was significantly increased in conditioned media at 24 or 48 hours but was not increased in unconditioned media. Panel A: CDK: Cyclin Dependent Kinases 6 and 9; CCN: Cyclin E1 and D1; CDCA: Cell Division Cycle 2, 7, and 8; PLK1: Polo-like Kinase 1. Panel B: CDC: Cell Division Cycle 42 and Cell Division Cycle 42 Effector Protein 2 and 3; RHOA: Ras Homolog Gene Family, Member A; RAC1: RAS-Related C3 Botulinum Toxin Substrate 1; ROCK2: RHO-Associated Coiled-Coil Forming Kinase 2; CFL1: Cofilin 1.

Figure 7. Model of activation/transduction of cardiomyocyte proliferation in hESC cell conditioned media

All transcripts from ligands through transcription factors (except LimK) depicted in the model exhibited a statistically significant increase in conditioned media at 24 or 48 hours compared to the level at the beginning of the study. None were significantly increased in unconditioned media. The boxes contain proteins significantly elevated in the conditioned media which could act as ligands for the specified pathways---nomenclature provided in Figure 3. Transcripts associated with the GPCR (G-Protein-Coupled Receptor) pathway and the process of cytokinesis are depicted. Transcripts associated with the RTK (Receptor Tyrosine kinase) pathway are also shown including transcription factors (FOS, FOSL1, JUNB, JUND and MYCN) associated with cyclin activation and proliferation. A separate pathway associated with cardiomyocyte differentiation exhibited significant elevation of transcription factors AP2a, MEF2D, STAT1 and 3. Transcripts associated with cytokine activation of the JAK-STAT pathway included multiple JAKs and elevation of STAT3 and STAT1 associated with

cell cycle progression. The microarray intensity data representing nomenclature of all transcripts associated with these signal transduction pathways are provided in Supplementary Table 2 (Table S2).