Biomaterials 33 (2012) 6313–6319

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Synergistic effects of hypoxia and extracellular matrix cues in cardiomyogenesis

Renita E. Horton, Debra T. Auguste*

School of Engineering and Applied Sciences, Harvard University, 29, Oxford St., Cambridge, MA 02138, USA

ARTICLE INFO

Article history:
Received 14 May 2012
Accepted 27 May 2012
Available online 18 June 2012

Keywords:
Human embryonic stem cells
Extracellular matrix
Embryoid bodies
Hypoxia
Cardiomyocytes

ABSTRACT

Limited characterization of how the stem cell niche evolves has hindered our ability to mimic the physiological environment. In this paper, we hypothesized that hypoxia-induced extracellular matrix (ECM) cues may facilitate cardiomyogenesis. We evaluated the expression of four ECM proteins – fibronectin, collagen I, collagen IV, and laminin - over a period of 20 days in H1 and H9 human embryonic stem cell-derived embryoid bodies (EBs) under hypoxic (5% oxygen) and normoxic (21% oxygen) conditions. Hypoxic EBs exhibited increased collagen I, collagen IV and fibronectin expression relative to normoxic EBs between days 9–13, which coincided with increased expression of mesoderm genes. The effect of ECM cues was confirmed by plating day 9 EBs on collagen IV, gelatin, and fibronectin-rich substrates for 11 days. Hypoxia/gelatin cultures synergistically increased the cardiomyocyte yield by 1.7 and 5.5 fold relative to normoxia/gelatin and normoxia/collagen IV cultures, respectively. Current differentiation protocols may underestimate the contribution of hypoxia and ECM cues that evolve during EB maturation.

1. Introduction

Standard methods to address human embryonic stem cell (hESC) differentiation have followed a stepwise progression through a series of progenitor cells [1]. This was achieved via the exposure of stem cells to combinations of soluble factors at specific time points and durations. For example, the cardiac differentiation protocol was dependent on the exposure of hESCs to morphogens at specific stages of differentiation [1,2]. Alternatively, it was shown that cardiomyogenesis may be achieved via cues from the ECM in the absence of supplemental growth factors (hESCs [3], mouse embryonic stem cells (mESCs) [4]). Despite the increasing importance of cell–matrix interactions during differentiation, there remains a discontinuity between the temporal introduction of soluble factors and the static presentation of ECM cues as methods for cardiomyocyte differentiation.

ECM proteins play key roles in vasculogenesis signaling cascades; loss of ECM protein or function can alter development. Fibronectins were shown to be essential for blood vessel and heart organization [5]; the absence of fibronectin led to defects in mesoderm and neural development [6]. Laminin γ1 chain-deficient cardiomyocytes had poorly formed basement membranes and defective electrical signal propagation between neighboring cardiomyocytes [7]. The use of collagen inhibitors, cis-4-hydroxy-D-proline and L-2-azetidine carboxylic acid, inhibited cardiac differentiation, which suggested that collagen was essential for cardiomyogenesis [8]. These studies have highlighted the critical role of ECM cues in proper cardiovascular development.

Characterization of the EB niche may help identify ECM cues important in cardiac differentiation. ECM cues were shown to mediate proliferation, participate in signaling cascades required for cardiomyogenesis and provide structural support for myocardium development [9]. The basement membrane surrounding cardiomyocytes was comprised of collagen IV, fibronectin, laminin and proteoglycans [10]. EB-derived cardiomyocytes were reported to be encased in a network of fibronectin, laminin, collagen I, IV and XVIII [11]. ECM remodeling was also important in heart development [6,9] and disease progression (e.g., hypoxia-induced fibrogenesis [12], myocardial infarctions [13]). Cell–matrix interactions have regulated cardiac function and structure from individual cells to tissue level properties.

In conjunction with the ECM, hypoxia plays a central role in embryo development. The native embryo environment was characterized as being between 1% and 10% O_2. Hypoxia was shown to influence cell fate [14–16] and regulate vascular development [17,18]. Hypoxic microenvironments have promoted the differentiation of blood cells, blood vessels and the heart [19]. Hypoxia (4% O_2) increased the cardiomyocyte yield per input embryonic stem cell by 50% relative cells cultured at 20% O_2 [20]. Although, hypoxia has been used to direct differentiation, little is known about the effect of hypoxia on the ECM and how these changes influence differentiation. Deciphering the regulatory role of the EB niche may help identify key factors that regulate cardiomyogenesis.

In this study, we used the model EB system to investigate the effect of hypoxia on the ECM and its effect on cardiomyogenesis. We

* Corresponding author. Tel.: +1 617 384 7980; fax: +1 617 495 9837.
E-mail address: auguste@seas.harvard.edu (D.T. Auguste).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2012.05.063

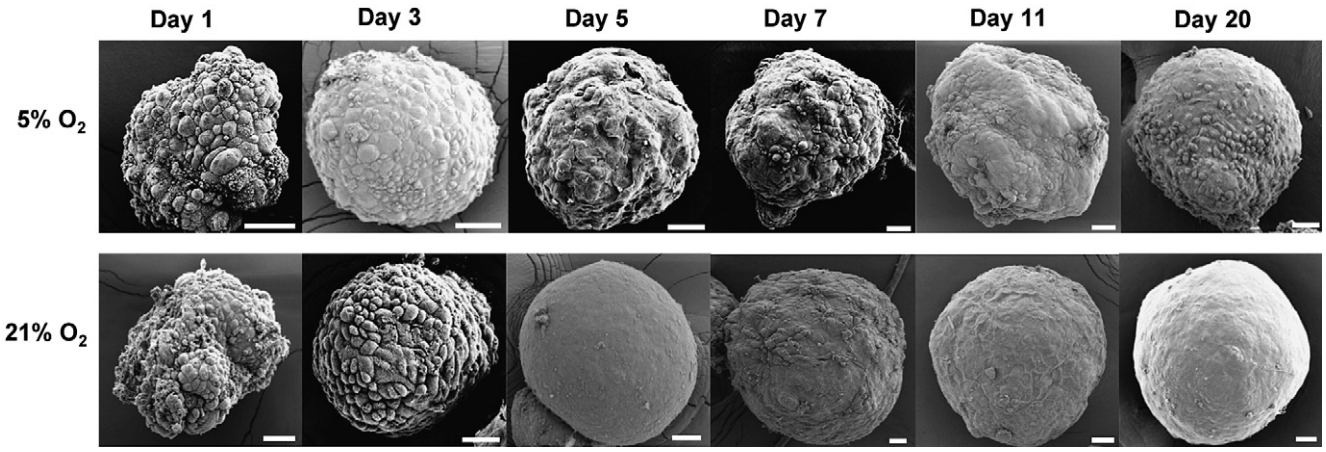

Fig. 1. Topography analysis of EB maturation and ECM deposition. SEM topographical images of EBs at day 1, day 3, day 5, day 7, day 11 and day 20 were analyzed to observe the effect of hypoxia on the EB surface. The EBs evolved from a rough, irregular outer exterior to a smooth structure with less defined cell boundaries due to ECM deposition over time by day 20. Hypoxic EBs exhibited a more heterogeneous ECM deposition in comparison to normoxic EBs, at day 20. Scale bar = 40 μm.

proposed that the temporal regulation of ECM cues played a role in enhancing cardiac lineage commitment observed under hypoxia. Understanding how the ECM evolves and impacts cell differentiation during early EB development may provide a framework for the development of synthetic niches.

2. Materials and methods

2.1. Cell culture

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise specified. hESC culture was carried out at 37 °C in a humidified atmosphere containing 5% CO_2. NIH approved H1 and H9 hESC lines were cultured as described previously [21]. Briefly, hESCs were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblasts (IMEFs) (Global Stem Cells, Rockville, MD) in Dulbecco's modified eagle medium/F-12 supplemented (DMEM/F-12) with 20% Knockout serum replacement (KO-SR), 1 mM L-glutamine, 5–20 ng/ml bFGF(Millipore Billerica, MA), 1% nonessential amino acids and 0.1 mM 2-mercaptoethanol. The medium was changed on a daily basis and cells were passaged every 3–5 days. Stem cell colonies expressed SSEA-4 (Fig. S1a) prior to EB formation, confirming that hESCs were in an undifferentiated state. Furthermore, a decrease in SSEA-4 expression was observed in plated hESC-derived EBs at day 15 (Fig. S1b).

EBs were formed by treating hESCs with collagenase type IV for 30 min. Cells were then sheared off the plate and cultured in suspension on low attachment culture dishes (Sigma–Aldrich, St. Louis, MO). EBs were cultured in Knockout DMEM (KO-DMEM) supplemented with 20% embryonic stem cell qualified fetal bovine serum (ES-FBS), 1 mM L-glutamine, 1% nonessential amino acids and 0.1 mM 2-mercaptoethanol. EBs were then cultured at 37 °C in a humidified incubator with 21% O_2 (normoxia) or 5% O_2 (hypoxia). Cells exposed to hypoxia were cultured in a Secador desiccator (VWR), serving as a hypoxic chamber, constantly flushed with a low O_2 gas mixture containing 5% O_2, 5% CO_2, and 90% N_2 (IGOs, Everett, MA). The gas mixture was humidified by bubbling through a sealed water bottle at 37 °C prior to entering the hypoxic chamber. EB and plated EB media was changed every 2–3 days.

A modified version of the described differentiation protocol was used [22]. Briefly, nine day old normoxic and hypoxic cultured EBs were plated onto 0.1% gelatin (Sigma–Aldrich), 1 mg/ml fibronectin (Sigma–Aldrich), or 0.3 mg/ml collagen IV (Sigma–Aldrich) coated polystyrene plates (VWR, Bridgeport, NJ). Plated EBs were then returned to either 5% or 21% O_2 culture conditions for 6 or 11 days, for a total of 15 or 20 days in culture. For immunostaining, day 15 EBs were dissociated with 0.05–0.25% trypsin for 3–5 min, plated onto 0.1% gelatin (Sigma–Aldrich) coated glass bottom dishes (VWR) and returned to culture as described above.

2.2. Scanning electron microscopy

EBs were fixed in 2.5% v/v glutaraldehyde in phosphate buffered saline (PBS). Serial dehydration was performed by incubating EBs in 200 proof ethanol solutions (50%, 70% and 90%) (Pharmco, Brookfield, CT) in distilled water at room temperature for 1 h followed by an overnight incubation in 100% ethanol. EBs were then critical point dried with liquid CO_2 (IGOs), coated with platinum-palladium (Pt–Pd) using the Cressington sputter coater and imaged with a FESEM Supra55 Variable Pressure (Carl Zeiss, Thornwood, NY) at an accelerating voltage of 3 kV.

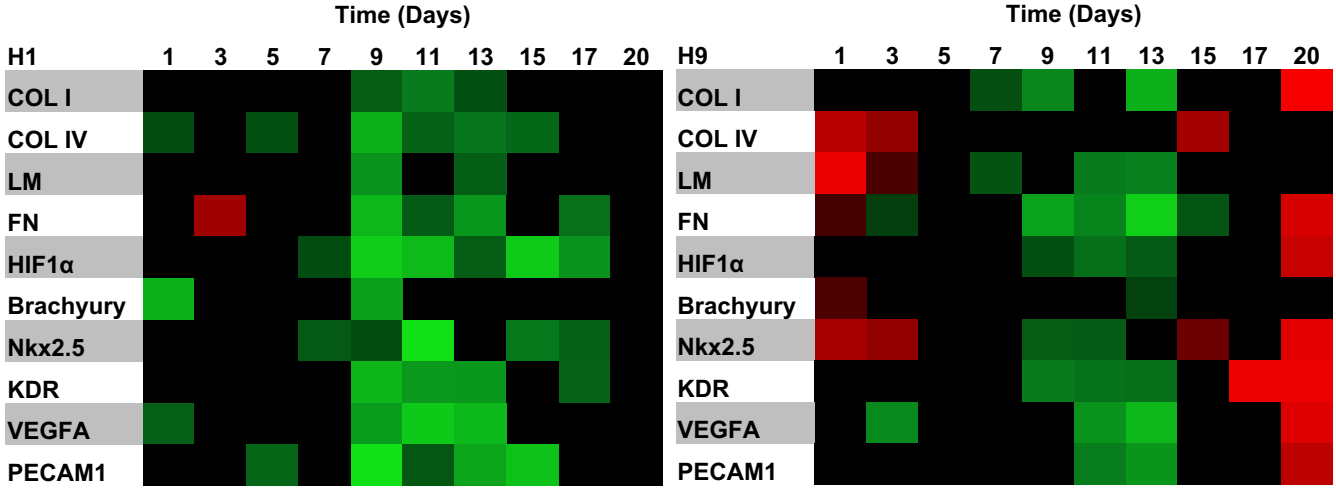

Fig. 2. Heat map analysis of ECM and mesodermal gene expression. A heat map was generated using gene expression profiles of hypoxic relative to normoxic whole EBs. Relative gene expression was reported as statistically downregulated (red) or upregulated (green). Black represents time points where the gene expression ratio was not significant. Middle time points (days 9–13) show overall upregulation of gene expression in hypoxic EBs. Undifferentiated hESCs were used as an internal control. A student *t*-test was used for statistical analysis $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)