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Research Article

Parietal endoderm secreted SPARC promotes early cardiomyogenesis in vitro

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Abstract

Cardiomyogenesis proceeds in the presence of signals emanating from extra-embryonic lineages emerging before and during early eutherian gastrulation. In embryonic stem cell derived embryoid bodies, primitive endoderm gives rise to visceral and parietal endoderm. Parietal endoderm undergoes an epithelial to mesenchymal transition shortly before first cardiomyocytes start to contract rhythmically. Here, we demonstrate that Secreted Protein, Acidic, Rich in Cysteine, SPARC, predominantly secreted by mesenchymal parietal endoderm specifically promotes early myocardial cell differentiation in embryoid bodies. SPARC enhanced the expression of *bmp2* and *nkx2.5* in embryoid bodies and fetal cardiomyocytes. Inhibition of either SPARC or Bmp2 attenuated in both cases cardiomyogenesis and downregulated *nkx2.5* expression. Thus, SPARC directly affects cardiomyogenesis, modulates Bmp2 signaling, and contributes to a positive autoregulatory loop of Bmp2 and Nkx2.5 in cardiomyocytes.

Keywords: SPARC; Nkx2.5; Bmp2; Parietal endoderm; Extra-embryonic endoderm; Early cardiomyogenesis; Embryoid body

Introduction

Cardiomyogenesis is regulated by intercellular morphogenetic signaling factors of the Tgf-β/Bmp and Fgf families, by hedgehog, wingless, and notch proteins [1–5], and by the interleukin-6 family members leukemia inhibitory factor (Lif) [6,7] and cardiotrophin-1 (Ct1) [8]. Several of these factors are expressed by extra-embryonic lineages and contribute to mesoderm formation and to its commitment to the cardiomyogenic lineage [9]. Induction of cardiomyogenesis is evident from the expression of transcription factors such as Gata4, Gata6, Nkx2.5, and Mef2C [10,11]. These factors initiate the program for myocardial gene expression and drive morphogenic events involved in the formation of the four-chambered heart [12–14]. First cardiomyocytes emerge from the craniolateral splanchnic mesoderm, which extends laterally to the junction between the embryonic proper and extra-embryonic endoderm [9]. In vivo extra-embryonic visceral and parietal endoderm (PE) formation precedes cardioblast formation, as in vitro. Increased numbers of PE cells correlate with substantial larger numbers of cardiomyocytes in embryoid bodies (EBs) [7]. PE positively influences cardiomyogenesis in EBs in a paracrine manner, independent of LIF and its receptor LIFR [7]. Factors secreted by extra-embryonic endoderm lineages are sufficient to drive embryonic stem cell (ESC) derived primitive ectoderm into the cardiomyogenic lineage in EBs [7]. This suggests an influence of extra-embryonic endodermal lineages on early mesoderm formation.

EBs undergo a differentiation program reminiscent of early murine embryogenesis mimicking faithfully pre-

Abbreviations: EB, embryoid body; EMT, epithelial to mesenchymal transition; ESC, embryonic stem cell; PE, parietal endoderm; PE-S, parietal endoderm-conditioned medium.

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gastrulation lineage commitment [15]. Within 2 days after ESC aggregation, corresponding to day 4.5 of murine embryogenesis, primitive endoderm develops, covers the remaining ESCs, and provides an inductive signal for ESCs to become primitive ectoderm [7,16,17]. Primitive endoderm engulfing the EB by day 4 differentiates to visceral endoderm. Continuous culture in suspension promotes differentiation to epithelial visceral endoderm building large bubbles on top of the primitive ectoderm. Contact of primitive endoderm to a collagen containing extracellular matrix induces their commitment to the PE lineage [18,19]. Finally, PE migrates and undergoes an epithelial to mesenchymal transition (EMT) [7,20–22].

In vivo, other endodermal lineages such as the visceral endoderm [23,24], the pharyngeal endoderm [25], and anterior endoderm strongly promote cardiomyogenesis in embryonic tissue explants and EBs, respectively [26,27]. These endodermal lineages secrete a variety of growth factors, including Bmp2, activin A, Fgfs, Lif, insulin, and Igfs. They all influence proliferation, survival, or differentiation of mesodermal precursors to cardiomyocytes under certain conditions [4,28–30].

To specify the source of factors influencing cardiomyogenesis, we investigated the development of extra-embryonic endoderm in EBs. We isolated and characterized primitive, visceral, and parietal endoderm and demonstrated that mesenchymal PE-conditioned medium positively influences cardiomyogenesis. Secreted Protein Acidic and Rich in Cysteine (SPARC) also named osteonectin was identified as a novel and potent modulator of early cardiomyogenesis.

SPARC is a matricellular glycoprotein with various functions in developmental processes [31], involved in angiogenesis [32], differentiation of keratinocytes [33], and in vitro differentiation of skeletal myoblasts [34]. SPARC binds Ca^{++}-ions and modulates the interaction of cells with the extracellular matrix [35]. It also binds to growth factors such as PDGF and VEGF [35], interacts with the Tgf-β1 receptor complex [36], and affects the expression of secreted proteins [37]. During embryogenesis, SPARC is highly expressed in PE and the developing heart [38]; however, in $sparc^{-/-}$ mice, no defects in heart formation have been observed [39]. In addition to its extracellular function in cell adhesion, cytoplasmic SPARC has been demonstrated to migrate into nuclei of embryonic chicken [40] and murine lens epithelial cells [41].

Here, we demonstrate that SPARC is upregulated during EMT of EB derived PE, that it promotes cardiomyocyte differentiation at the very beginning of in vitro cardiomyogenesis, and that it specifically induces the upregulation of *bmp2* and *nkx2.5* in cardiomyocytes. SPARC specifically requires Bmp2 and Bmp2 requires SPARC for promotion of cardiomyogenesis in upregulation of *nkx2.5*.

Materials and methods

Generation of embryoid bodies, extra-embryonic endodermal cells, and cardiomyocytes

Culture of AB2.2 ESCs, EB formation, and PE isolation has been described previously [6,7]. Primitive endoderm was generated from ESCs cultured in suspension in the absence of LIF for 3 days. Visceral endoderm was isolated from epithelial bubbles of EBs. In this particular case, EBs were generated from only 200 ESCs to suppress ectoderm and mesoderm formation [7] and maintained in suspension for 10–14 days. To obtain PE cultures with an epithelial phenotype and mesenchymal phenotype, respectively, we took advantage of the fact that LIF inhibits PE development in a concentration-dependent manner [7] by reversing EMT [42]. An ESC line constitutively expressing low levels of LIF was generated by stable transfection of $lif^{-/-}$ ESCs [43] with a *lif* transgene under the control of the CMV promoter [44]. EBs generated from these cells gave rise to PE cultures with an epithelial phenotype at high cell density and a mesenchymal phenotype at low cell density.

EBs were cultured in the presence of PE-conditioned medium, mixed 1:1 with fresh EB differentiation medium [6] starting day 4, 5, 6, 7, or 8. Day 7 EBs were cultured with conditioned medium from primitive, visceral, parietal, mesenchymal–parietal, and epithelial–parietal endoderm cells, respectively. Cell culture supernatants were obtained from cultures with equal cell numbers to ensure comparable concentrations of growth factors. Primitive endoderm was removed from EBs on day 6 as described [7], and cultured with conditioned media from primitive, visceral, and mesenchymal–parietal endoderm cells, respectively, from day 7 on. EBs were treated with 3 μg/ml PYS-2 derived SPARC (Sigma, S5174) or goat polyclonal anti SPARC antibody (Santa Cruz, sc13326) starting day 7, at dilutions 1:100 or 1:500, and with mesenchymal-PE-conditioned medium and anti SPARC antibody, 1:500, respectively. Controls containing the same concentrations NaN_3 (0.001%) as medium with the diluted antibody had no visible negative effect on cardiomyogenesis in EBs. EBs were treated with 100 ng/ml Bmp2 (R&D systems, # 355-BEC) starting day 7, or 1.5 μg/ml anti-Bmp2 antibody (R&D systems, #MAB 3552). Cardiomyogenesis was compared by counting EBs with beating cardiomyocytes daily. Percentage of EBs with beating cardiomyocytes were normalized to control experiments. The surface of beating EBs was determined using digital camera (Canon Powershot G5) generated videos of beating EBs and subsequent analysis of beating area with Adobe Photoshop 7.0 software.

Primary cardiomyocytes were isolated from E14 129Sv mouse embryos. Heart tissue was digested in a mixture of 5 mg collagenase, 0.4 ml pancreatin, 9.6 ml PBS at 37°C for 2 × 15 min. Cardiomyocytes were enriched by adsorption of cardiac fibroblasts at 37°C in M4 [DMEM supplemented with 4% fetal calf serum (Sigma), 2 mmol/l glutamine, 0.05