Developmental Biology 368 (2012) 345–357

Contents lists available at SciVerse ScienceDirect

Developmental Biology

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

Pod1/Tcf21 is regulated by retinoic acid signaling and inhibits differentiation of epicardium-derived cells into smooth muscle in the developing heart

Caitlin M. Braitsch [a], Michelle D. Combs [a], Susan E. Quaggin [b], Katherine E. Yutzey [a,*]

[a] Division of Molecular Cardiovascular Biology, Cincinnati Children's Hospital Medical Center, ML 7020, 240 Albert Sabin Way, Cincinnati, OH 45229, USA
[b] The Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Room 871Q, 600 University Avenue, University of Toronto, Toronto, Ontario, Canada M5G 1X5

ARTICLE INFO

Article history:
Received 11 January 2012
Received in revised form
31 May 2012
Accepted 1 June 2012
Available online 9 June 2012

Keywords:
Heart development
EPDC
Pod1
Tcf21
Chicken
Mouse

ABSTRACT

Epicardium-derived cells (EPDCs) invade the myocardium and differentiate into fibroblasts and vascular smooth muscle (SM) cells, which support the coronary vessels. The transcription factor Pod1 (Tcf21) is expressed in subpopulations of the epicardium and EPDCs in chicken and mouse embryonic hearts, and the transcription factors WT1, NFATC1, and Tbx18 are expressed in overlapping and distinct subsets of Pod1-expressing cells. Expression of *Pod1* and *WT1*, but not *Tbx18* or *NFATC1*, is activated with all-*trans*-retinoic acid (RA) treatment of isolated chick EPDCs in culture. In intact chicken hearts, RA inhibition leads to decreased Pod1 expression while RA treatment inhibits SM differentiation. The requirements for Pod1 in differentiation of EPDCs in the developing heart were examined in mice lacking Pod1. Loss of Pod1 in mice leads to epicardial blistering, increased SM differentiation on the surface of the heart, and a paucity of interstitial fibroblasts, with neonatal lethality. Epicardial epithelial-to-mesenchymal transition (EMT) and endothelial differentiation of coronary vessels are relatively unaffected. On the surface of the myocardium, expression of multiple SM markers is increased in Pod1-deficient EPDCs, demonstrating premature SM differentiation. Increased SM differentiation also is observed in Pod1-deficient lung mesenchyme. Together, these data demonstrate a critical role for Pod1 in controlling mesenchymal progenitor cell differentiation into SM and fibroblast lineages during cardiac development.

Introduction

During vertebrate embryonic development, epithelial cells from the proepicardium (PE), located at the venous pole of the primitive looped heart, migrate to the surface of the myocardium and form the epicardium (Gittenberger-de Groot et al., 2010). The mechanisms of epicardial formation and derivation of the coronary vasculature are conserved in vertebrates, including chicken and mouse embryos (Reese et al., 2002). A subset of epicardial cells undergoes EMT and invades the subepicardial space, thereby becoming EPDCs (Wessels and Perez-Pomares, 2004). Subepicardial EPDCs proliferate, invade the myocardium, and contribute to the fibrous matrix and coronary vasculature of the mature heart (Gittenberger-de Groot et al., 2010; Mikawa and Gourdie, 1996). EPDCs represent a multipotent progenitor population with the potential to differentiate into fibroblast and SM cell lineages, and also contribute to coronary endothelial cells and possibly cardiac myocytes at lower frequencies (Cai et al., 2008; Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Katz et al., 2012; Smart et al., 2011; Smart et al., 2009; Zhou et al., 2008). Multiple transcription factors (TFs), including Pod1 (Tcf21/Capsulin/Epicardin), Wilms' Tumor 1 (WT1), NFATC1, and Tbx18, as well as signaling molecules, such as retinoic acid (RA), have been implicated in EPDC lineage development (Gittenberger-de Groot et al., 2010). The molecular interactions among specific signaling pathways and TFs in regulation of EPDC differentiation into particular cell lineages, including SM, are not well characterized.

Pod1 is a bHLH transcription factor expressed in the PE, epicardium, and EPDCs of embryonic chick and mouse hearts (Armstrong et al., 1993; Combs et al., 2011; Kraus et al., 2001; Quaggin et al., 1998; von Scheven et al., 2006). Loss of Pod1 leads to neonatal lethality with lung, kidney, and spleen defects, and mesenchymal Pod1 expression regulates lung and kidney epithelial morphogenesis (Lu et al., 2000; Quaggin et al., 1999). In kidney mesenchymal progenitors, Pod1 promotes myofibroblast differentiation (Plotkin and Mudunuri, 2008). However, the role of Pod1 in the heart has not been investigated in detail. In addition to Pod1, WT1, NFATC1, and Tbx18 also are expressed in the PE, epicardium, and EPDCs in chick and mouse embryos, and are required during heart morphogenesis (Armstrong et al., 1993; Combs et al., 2011; Kraus et al., 2001). WT1 regulates epicardial EMT, while epicardial NFATC1 promotes EPDC invasion of the

* Corresponding author. Fax: +1 513 636 5958.
E-mail address: katherine.yutzey@cchmc.org (K.E. Yutzey).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.06.002

myocardium (Combs et al., 2011; Kirschner et al., 2006; Moore et al., 1999; von Gise et al., 2011). Tbx18 contributes to formation of the sinus horn myocardium from pericardial mesenchyme (Bussen et al., 2004; Christoffels et al., 2006). However, the specific cellular functions and upstream regulatory mechanisms of these TFs, in particular Pod1, during EPDC development are relatively unknown.

Diverse signaling pathways regulate PE and EPDC development (Merki et al., 2005; Morabito et al., 2001; Schlueter et al., 2006; Zamora et al., 2007). In the epicardium and subepicardial EPDCs, retinaldehyde dehydrogenase-2 (RALDH2) is broadly expressed (Moss et al., 1998; Perez-Pomares et al., 2002). Upon invasion of the myocardium, EPDC expression of RALDH2 is downregulated, concurrent with EPDC differentiation into fibroblasts and SM cells. RA signaling in EPDCs is required to promote myocardial proliferation and to control coronary vasculogenesis, as determined by analysis of Retinoid X Receptor-α (RXRα) and RALDH2 null mouse models (Jenkins et al., 2005; Lin et al., 2010; Merki et al., 2005; Sucov et al., 1990). RA function in EPDCs, however, remains poorly understood.

In this study, we examine Pod1 expression relative to the expression patterns of other TFs in epicardium and EPDCs, as well as differential upstream regulation of Pod1 and other EPDC TFs. Pod1 function in EPDC differentiation in vivo also was examined in mice. Studies in chicken primary cell cultures and isolated hearts demonstrate that RA promotes *Pod1* and *WT1* expression, while also inhibiting SM differentiation, in EPDCs. Loss of Pod1 in mice leads to increased EPDC differentiation into SM and also to reduced numbers of interstitial fibroblasts in the developing heart.

Materials and methods

Chick and mouse embryo collection

Fertilized white leghorn chicken eggs (Charles River Laboratories) were incubated at 38 °C under high humidity, and embryos were sacrificed at E4 and E7. Pod1 heterozygous (*Pod1*$^{+/-}$) mice, harboring one *Pod1/LacZ* knock-in allele, were obtained (Quaggin et al., 1999). The *Pod1/LacZ* loss-of-function allele contains a *LacZ* expression cassette in lieu of the *Pod1* transcription initiation codon and basic helix-loop-helix (bHLH) domain; thus β-galactosidase (βGal) is expressed instead of Pod1 from this locus (Quaggin et al., 1999). *Pod1*$^{-/-}$ mouse embryos were produced by timed mating of *Pod1*$^{+/-}$ animals with the presence of a copulation plug defined as embryonic day 0.5 (E0.5). Mouse embryos were collected from E11.5-E18.5. Wild type and *Pod1*$^{+/-}$ littermate embryos also were analyzed. Genotyping for the *Pod1/LacZ* allele was performed as previously described (Quaggin et al., 1999). All animal procedures were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee and performed following institutional guidelines.

Immunolocalization

Chick and mouse embryos were collected, fixed, dehydrated, and paraffin-embedded as previously described (Lincoln et al., 2004). Antibody labeling for immunofluorescence (IF), immunocytochemistry (ICC), and immunohistochemistry (IHC) was performed as previously described with modifications (Combs and Yutzey, 2009). Antigen retrieval was performed in boiling citric acid based antigen unmasking solution (1:100, Vector Laboratories) for 3–7 min under pressure. The following primary antibodies were used: Pod1 (1:100, Santa Cruz Biotechnology), Tbx18 (1:250, Santa Cruz), NFATC1 (1:100, Santa Cruz), WT1 (1:50, MyBioSource.com), NFATC1 (1:100, BD Pharmingen), ALDH1A2 (RALDH2) (1:100, Sigma Aldrich), WT1 (1:100, EMD Bioscience), Smooth Muscle Myosin (Myh11) (1:300, Biomedical Technologies), Calponin (1:100, Abcam), α-Smooth Muscle Actin (αSMA) (1:100, Sigma), Endomucin (Emcn) (1:250, eBioscience), E-Cadherin (1:150, Santa Cruz), SM22α (Transgelin) (1:100, Abcam), βGal (1:2000, Abcam), and Collagen Type I (Col1a1) (1:100, Millipore). Corresponding Alexa-donkey anti-rabbit-488, Alexa-donkey anti-mouse-568, Alexa-donkey anti-mouse-488, Alexa-donkey anti-rabbit-568, Alexa-goat anti-rabbit-488, Alexa-goat anti-mouse-555, Alexa-goat anti-mouse-488 (Invitrogen), or donkey anti-chicken-FITC (Abcam) conjugated secondary antibodies were applied as previously described (Combs and Yutzey, 2009). Alternatively, Renaissance Tyramide Signal Amplification Plus Fluorescein and Tetramethylrhodamine kits (Perkin Elmer) were used as described previously (Combs et al., 2011). For double IF experiments using two rabbit primary antibodies, Zenon Rabbit IgG Labeling Kit (Invitrogen) was used as per manufacturer's instructions. Nuclei were stained using 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1:10,000, Invitrogen).

For ICC, cultured EPDCs were fixed in 4% paraformaldehyde or cold 100% methanol (MeOH) for 1 h at 4 °C. Cells were washed in PBS and treated with 0.3% hydrogen peroxide for 30 min. ICC and IHC were performed using ImmunoPure ABC Ultra-Sensitive Peroxidase IgG Staining Kits (Fisher) or ImmunoCruz LSAB Staining Systems (Santa Cruz) per manufacturers' instructions. After incubation, horseradish peroxidase detection with 3,3-Diaminobenzidine (DAB) Enhanced Metal Substrate Kit (Fisher) was performed per manufacturer's instructions. Whole mouse heart IHC using anti-SM22α antibody was performed as previously described (Lincoln et al., 2004).

IF was detected using a Zeiss LSM 510 confocal microscope, and images were captured with Zeiss LSM version 3.2 SP2 software in parallel using identical confocal laser settings with constant PMT filters and integration levels. Alternatively, IF was detected using a Nikon A1-R LSM confocal microscope, and images were captured with NIS-Elements D 3.2 software in parallel using identical confocal laser settings, with constant PMT filters and integration levels.

Pictomicrographs of ICC and IHC tissue were obtained using either an Olympus BX51 microscope using NIS-Elements D 3.2 software, or using a Nikon SMZ1500 microscope, DXM1200F digital camera, and ACT-1 2.70 software.

Quantification of protein expression and colocalization

Images obtained by IF were used to quantify TF expression and colocalization in chick and mouse heart sections. The number of cells expressing each TF was quantified using Image J64 software. Single-channel images were converted to binary, a specific threshold value was set, and expression above this threshold value was used to quantify the number of cells expressing each TF, including Pod1, WT1, NFATC1, and Tbx18. Positive nuclei were counted in the epicardium and EPDCs. A Pod1 index was calculated by dividing the number of TF-positive (TF$^+$) Pod1$^+$ cells by the total number of Pod1$^+$ cells, multiplied by 100%. Data were collected from three independent embryos ($n=3$) for each antibody combination, and approximately 730 cells were counted from 4 to 6 sections per embryo. Three independent experiments were performed in biological duplicate ($n=3$).

Quantification of the number of SM22α$^+$ cells in the subepicardium and shallow myocardium, detected by IHC, per heart section was performed on E17.5 *Pod1*$^{+/-}$ and *Pod1*$^{-/-}$ tissue. SM22α expression was analyzed using pictomicrographs obtained at 600× magnification in comparable heart sections for each genotype. The number of SM22α-expressing cells was quantified