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Original research article

S1pr2/Gα$_{13}$ signaling regulates the migration of endocardial precursors by controlling endoderm convergence

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ABSTRACT

Formation of the heart tube requires synchronized migration of endocardial and myocardial precursors. Our previous studies indicated that in S1pr2/Gα$_{13}$-deficient embryos, impaired endoderm convergence disrupted the medial migration of myocardial precursors, resulting in the formation of two myocardial populations. Here we show that endoderm convergence also regulates endocardial migration. In embryos defective for S1pr2/Gα$_{13}$ signaling, endocardial precursors failed to migrate towards the midline, and the presumptive endocardium surrounded the bilaterally-located myocardial cells rather than being encompassed by them. *In vivo* imaging of control embryos revealed that, like their myocardial counterparts, endocardial precursors migrated with the converging endoderm, though from a more anterior point, then moved from the dorsal to the ventral side of the endoderm (subduction), and finally migrated posteriorly towards myocardial precursors, ultimately forming the inner layer of the heart tube. In embryos defective for endoderm convergence due to an S1pr2/Gα$_{13}$ deficiency, both the medial migration and the subduction of endocardial precursors were impaired, and their posterior migration towards the myocardial precursors was premature. This placed them medial to the myocardial populations, physically blocking the medial migration of the myocardial precursors. Furthermore, contact between the endocardial and myocardial precursor populations disrupted the epithelial architecture of the myocardial precursors, and thus their medial migration; in embryos depleted of endocardial cells, the myocardial migration defect was partially rescued. Our data indicate that endoderm convergence regulates the medial migration of endocardial precursors, and that premature association of the endocardial and myocardial populations contributes to myocardial migration defects observed in S1pr2/Gα$_{13}$-deficient embryos. The demonstration that endoderm convergence regulates the synchronized migration of endocardial and myocardial precursors reveals a new role of the endoderm in heart development.

1. Introduction

A key process during vertebrate heart development is formation of the primitive heart tube, which is comprised of an inner endocardial and an outer myocardial layer (Glickman and Yelon, 2002; Stainier, 2001). Precursors of each layer originate from distinct bilateral populations (Keegan et al., 2004; Lee et al., 1994; Schoenebeck et al., 2007) and migrate medially, in a synchronized manner, to the embryonic midline. Once there, they coalesce to form a single population, with the endocardial precursors clustering at the center (Bussmann et al., 2007; Evans et al., 2010; Trinh and Stainier, 2004). Subsequently, the cardiac precursor populations undergo complex morphogenetic movements to form a heart tube with proper structures and dimensions (Bussmann et al., 2007; Holtzman et al., 2007; Moreno-Rodriguez et al., 2006).

The migration of endocardial and myocardial precursors must be coordinated to ensure that both cell types reach specific locations along the midline at the correct time (Bussmann et al., 2007; Holtzman et al., 2007). In zebrafish, the timing and routes of migration for these cell types differ: the endocardial precursors, which lie anterior to the myocardial populations within the anterior lateral-plate mesoderm, migrate both medially and posteriorly at around the 6-somite stage (6 s), arriving at the midline region at ~16 s; in contrast, myocardial precursors on each side begin to migrate medially at 14 s, fuse into a single population, and join the endocardial precursors at the midline at 18 s (Bussmann et al., 2007; Holtzman et al., 2007; Trinh and Stainier, 2004; Ye

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et al., 2015). In *clo* mutant embryos, in which endothelial (and thus, endocardial) precursors are absent, the myocardial cells undergo relatively normal medial migration and form a single cluster (Holtzman et al., 2007). Thus myocardial migration appears to be independent of endocardial precursors. However, after arriving in the midline region, myocardial cells in wild-type embryos initiate angular migration towards endocardial cells, in a manner dependent on the endocardial precursors (Holtzman et al., 2007). A recent study showed that endocardial precursors likewise do not rely on the medial migration of myocardial precursors, but that they do depend on the latter for signals governing their proper differentiation (Palencia-Desai et al., 2015). Notwithstanding this independence of the myocardial and endocardial precursors with respect to their own migration, their coordinated migration, as well as proper development of the myocardium and endocardium, are required for formation of several heart structures: the atrioventricular canal, cardiac valves, and cardiac septa (Armstrong and Bischoff, 2004; Bussmann et al., 2007; Hinton and Yutzey, 2011; Totong et al., 2011).

Studies in a number of animal models have shown that the migration of myocardial cells depends not only on their intrinsic potentials, but also the extracellular environment. In particular, the endoderm (which is adjacent to the cardiac mesoderm) is critical for myocardial migration, as demonstrated by the fact that in embryos lacking endoderm the myocardial cells fail to migrate towards the midline (David and Rosa, 2001; Narita et al., 1997; Reiter et al., 1999; Yelon et al., 1999). Not only the presence, but also the movements, of endodermal tissue and myocardial precursors are tightly associated (Madabhushi and Lacy, 2011; Maretto et al., 2008; Varner and Taber, 2012). We recently found that the medial migration of myocardial precursors is controlled by convergent movement of the endodermal sheet, which is itself regulated by S1pr2/Gα_{13} signaling (Ye and Lin, 2013), and our follow-up studies revealed strikingly complex and dynamic associations between the endoderm and myocardial precursors during their medial migration (Ye et al., 2015). Similar associations between the movement of endoderm and myocardial cells during heart tube assembly have been observed in quail (Aleksandrova et al., 2015). These data support the notion that endoderm movement is important for myocardial migration.

Like the myocardium, the endocardium requires the endoderm for its formation and morphogenesis. In chick, endocardial precursors attach to the endoderm (Viragh et al., 1989) and this interaction is required for their proper differentiation (Sugi and Markwald, 1996, 2003). As in the case of myocardial cells, absence of the endoderm stalls medial migration of the endocardial cells (Wong et al., 2012). Additional studies in quail suggest that endocardial cells undergo only minimal active migration, with their movement dictated largely by the surrounding tissues (Aleksandrova et al., 2012). Nevertheless, how the endoderm influences endocardial migration during development is not well understood. Moreover, how endocardial and myocardial precursors interact during heart-tube formation remains unclear. Notably, in zebrafish *mil/s1pr2* mutants, in which myocardial migration and endoderm convergence are impaired (Kawahara et al., 2009; Kupperman et al., 2000; Osborne et al., 2008; Ye and Lin, 2013), the endocardial precursors failed to migrate medially, remaining in lateral locations adjacent to the myocardial cells (Holtzman et al., 2007). This observation raises the possibility that, like myocardial precursors, their endocardial counterparts depend on endodermal movement to migrate towards the midline.

Here we employ transgenic lines in which the myocardial precursors, endocardial precursors, and endoderm are labeled to monitor the relative movements of these cell types during heart-tube formation. We find that, like myocardial precursors, endocardial precursors associate dynamically with the endoderm during their medial migration. Strikingly, in S1pr2/Gα_{13}-defective embryos, the endocardial cells fail to migrate towards the midline, instead moving prematurely in the posterior direction, towards the myocardial cells, and this outcome results in the formation of two disorganized cardiac populations and in misplacement of the endocardium that was formed outside, rather than inside of cardiac populations. Thus, our findings reveal that endoderm convergence regulates the synchronized migration of endocardial and myocardial precursors.

2. Materials and methods

2.1. Zebrafish strains and husbandry

Zebrafish were maintained as described previously (Xu et al., 2011). Animal protocols were approved by the University of Iowa Animal Care and Use Committee. Embryos were obtained by natural mating staged according to morphology, or hours post fertilization (hpf) at 28.5 °C, as described previously (Kimmel et al., 1995). For those fixed after 24 h post fertilization (hpf), embryos were raised at 28.5 °C; for those fixed and mounted for live imaging at segmentation, embryos were raised at 25 °C. The following lines were used: wildtype AB and Tuebingen; *mil*^{m93} (Kupperman et al., 2000); *Tg(fli: EGFP)* (Lawson and Weinstein, 2002); *Tg(myl7: memGFP)*, *Tg(sox17: memCherry)*, *Tg(sox17: mCherry-2A-gna13a)* (Ye et al., 2015); *Tg(kdrl: moesin-EGFP)* (Wang et al., 2010); *Tg(kdrl: mCherry)* (Wong et al., 2012); *Tg(nkx2.5: ZsYellow)* (Zhou et al., 2011), *Tg(nkx2.5: Kaede)* (Guner-Ataman et al., 2013).

2.2. RNA and Morpholino injections

mRNA and MOs were injected into embryos at the one-cell stage. Capped message human Gα_{13} (GNA13) RNA synthesized by the mMessage mMachine kit (Ambion) was injected at 100 pg (Lin et al., 2005). The previously validated MOs targeting the following genes were used: *gna13a* and *gna13b* (2–3 ng each) (Lin et al., 2005), *s1pr2/mil* (15 ng) (Kawahara et al., 2009), *sox32* (4 ng) (Wong et al., 2012), *etv2* (12.5 ng) (Sumanas and Lin, 2006).

2.3. Whole-mount in situ hybridization (ISH) and immunofluorescence (IF)

ISH was performed as described previously (Lin et al., 2005; Thisse and Thisse, 2008). The following antisense RNA probes were used: *nfatc1* (Palencia-Desai et al., 2011), *cdh5* (Bussmann et al., 2007), *myl7* (Yelon and Stainier, 1999), *fli* (Lawson and Weinstein, 2002). Whole-mount IF staining was performed as described previously (Ye et al., 2015). The following antibodies were used: anti-ZsYellow (1:200, Origene); anti-Kaede (1:200, MBLI); anti-ZO1 (1:200; Invitrogen); anti-Fibronectin (1:400, F3648, Sigma); anti-MF20 and S46 (1:200, DSHB).

2.4. Kaede photoconversion

Tg(nkx2.5: Kaede) embryos were photoconverted using a Leica DMI 6000 fluorescence microscope with a 20 × /NA 0.7 objective and a UV filter. At 10 s, embryos were mounted in 0.8% low melting point agarose in a custom-made glass bottom dish and imaged using a GFP filter, followed by continuous exposure to UV light for 60 s. Subsequently, embryos were imaged using an RFP filter to confirm the conversion.

2.5. Microscopy, time-lapse imaging, and image processing

For still epifluorescence images, live or fixed embryos were