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Cardiac arterial pole alignment is sensitive to FGF8 signaling in the pharynx

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Abstract

Morphogenesis of the cardiac arterial pole is dependent on addition of myocardium and smooth muscle from the secondary heart field and septation by cardiac neural crest cells. Cardiac neural crest ablation results in persistent truncus arteriosus and failure of addition of myocardium from the secondary heart field leading to malalignment of the arterial pole with the ventricles. Previously, we have shown that elevated FGF signaling after neural crest ablation causes depressed Ca^{2+} transients in the primary heart tube. We hypothesized that neural crest ablation results in elevated FGF8 signaling in the caudal pharynx that disrupts secondary heart field development. In this study, we show that FGF8 signaling is elevated in the caudal pharynx after cardiac neural crest ablation. In addition, treatment of cardiac neural crest-ablated embryos with FGF8b blocking antibody or an FGF receptor blocker rescues secondary heart field myocardial development in a time- and dose-dependent manner. Interestingly, reduction of FGF8 signaling in normal embryos disrupts myocardial secondary heart field development, resulting in arterial pole malalignment. These results indicate that the secondary heart field myocardium is particularly sensitive to FGF8 signaling for normal conotruncal development, and further, that cardiac neural crest cells modulate FGF8 signaling in the caudal pharynx.

Keywords: Heart development; FGF8, outflow tract; Arterial pole; Cardiac neural crest; Secondary heart field; Double outlet right ventricle; Persistent truncus arteriosus; Calcium transient; mkp3

Introduction

Development of the arterial pole of the 4-chambered heart requires complex morphogenetic movements to establish a single vascular channel with a myocardial-to-vascular smooth muscle interface. This single vascular channel is later divided into two outflow vessels by the cardiac neural crest cells (CNC). Finally, the two outflow vessels are remodeled to align them with the right and left ventricles.

The secondary heart field (SHF) has recently been shown to provide the myocardial–smooth muscle interface at the arterial pole (Waldo et al., 2005a). The SHF-derived myocardium gives rise to the conotruncal myocardium and development of this myocardium is critical for normal alignment of the two outflow vessels with respect to the ventricles (Yelbuz et al., 2002; Ward et al., 2005). Clinically, malalignment of the arterial pole occurs in the conotruncal defects, as seen in double outlet right ventricle (DORV) and tetralogy of Fallot. In both of these defects, the aorta overrides the ventricular septum. Recently, we have shown that CNC are needed for normal development of the myocardial component of the SHF prior to the time they begin septation of the outflow vessels (Waldo et al., 2005b). Ablation of the pre-migratory CNC results in both the absence of septation, as well as, malalignment of the arterial pole because of the failure of the SHF myocardium to develop normally. It is not known how CNC affect SHF development because the two cell populations are not in proximity when defective development of the SHF occurs.

In chick embryos, the CNC begin their migration into the caudal pharynx (arches 3–6) at stage 10 (Hamburger–Hamilton stages). At stage 12, the CNC pause in the circumpharyngeal

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ridge (Le Douarin et al., 1992; Kuratani and Kirby, 1992), and beginning at late stage 13, they populate the dorsal third pharyngeal arch by migrating between the pharyngeal ectoderm and endoderm. This is repeated as the succeeding arches 4 and 6 develop. It is during this period that the myocardium is added to the outflow tract from the SHF. At stage 22, almost 4 days after leaving the dorsal neural tube, CNC finally migrate into the cardiac outflow tract where they will form the aorticopulmonary septation complex that will divide the outflow into the aorta and pulmonary trunk.

The SHF is the splanchnic mesoderm located in the floor of the caudal pharynx behind the outflow attachment to the pharynx. Beginning at stage 14, the SHF migrates into the outflow providing truncal myocardium and allowing lengthening of the looping primary heart tube until stage 18 (Waldo et al., 2001, 2005a; Mjaatvedt et al., 2001; Kelly et al., 2001). Recently, we have shown that the heart tube is shortened and abnormally looped after neural crest ablation (NCA) because of the failure of the SHF-derived myocardium to migrate into the outflow and ultimately leads to malalignment of the outflow vessel (Yelbuz et al., 2002; Waldo et al., 2005b). Because abnormal looping occurs well before the CNC reach the outflow, the SHF-related defects appear to be caused indirectly by NCA (Hutson and Kirby, 2003). This suggests that CNC may modulate signaling factors in the pharynx.

The first evidence that fibroblast growth factor (FGF) 8 signaling was disrupted in the CNC-deficient pharynx came from a study examining the primary myocardial dysfunction also observed after NCA. We showed in culture that conditioned media from the NCA pharynx could reproduce the depressed myocardial Ca^{2+} transient of the primary heart tube and that the transient could be rescued with an FGF8b-neutralizing antibody (Farrell et al., 2001). This suggested that FGF signaling was elevated after NCA.

FGF8 provides a variety of mitogenic, survival, pro- or anti-differentiation signals to pattern tissues and is required for normal craniofacial, cardiac and limb development (Schneider et al., 2001; Alsan and Schultheiss, 2002; Crossley and Martin, 1995; Crossley et al., 1996). Although there are at least 4 to 8 alternatively spliced *FGF8* isoforms in humans and mouse, the chick only produces 2 isoforms: *fgf8a* and *fgf8b* (Basilico and Moscatelli, 1992; Haworth et al., 2005). FGF8b isoform has been shown in the literature to be more biologically potent than FGF8a isoform. Most of the in vivo embryological studies comparing the biological activity of the isoforms have been done by the Nakamura and colleagues working in the chick hindbrain. They have shown that FGF8b isoform is 100 times more potent than FGF8a (Sato et al., 2001). The FGF8a and FGF8b isoforms differ by only 11 amino acids. A recent publication elucidating the crystal structure of FGF8b, FGF8a and the FGF receptors has shown that these 11 amino acids greatly enhance the binding affinity of the FGF8b isoform to the FGF receptors whereas the FGF8a isoform has very low binding affinity and this accounts for the potent biological activity of the FGF8b isoform (Olsen et al., 2006). FGF signaling is mediated via tyrosine kinase receptors (FGFRs) that act through a number of transduction pathways. While FGF8-null mice die during gastrulation (Meyers et al., 1998; Sun et al., 1999; Moon and Capecchi, 2000), hypomorphic mice have cardiac outflow, pharyngeal arch artery and pharyngeal gland defects reminiscent of the NCA phenotype (Abu-Issa et al., 2002; Frank et al., 2002).

Fgf8 is expressed in the lateral pharyngeal ectoderm and endoderm at the time CNC begin to populate the caudal pharyngeal arches and as the myocardium is added to the looping heart from the SHF (Farrell et al., 2001). Because FGF8b antibody rescued the myocardial calcium transient in culture, we hypothesized that FGF8 signaling is overabundant in the pharynx in the absence of the CNC. This excessive signaling prevents addition of myocardium from the SHF causing a shortened outflow tract that ultimately results in cardiac arterial pole alignment defects.

In this study, we demonstrate by quantitative PCR that total *fgf8* and *fgf8b* message and downstream targets are elevated in the caudal pharynx in NCA embryos between stages12 and 14, coinciding with the time the CNC should enter the pharynx. Using an FGF reporter in transiently transfected cells, we determined that the FGF8b isoform has much more potent signaling properties than FGF8a. Also using this reporter, we were able to show that FGF8 signaling was increased in the pharynx after NCA. All of the indirect effects of NCA, i.e. looping, addition of myocardium from the SHF, myocardial function and arterial pole alignment, could be rescued in ovo by treating the embryos with either anti-FGF8b or the FGFR blocker, SU5402. This rescue was time and dose-dependent. Conversely, reducing FGF8 signaling below normal levels in sham-operated embryos resulted in failure of myocardial addition from the SHF, abnormal looping and arterial pole alignment defects.

Materials and methods

Embryo preparation

NCA and sham-operated embryos were prepared as described previously (Waldo et al., 1996). At Hamburger and Hamilton stage 10, the vitelline membrane was torn. FGF8b function blocking antibody (varying concentrations between 40 and 400 μM, R&D Systems), 10–20 μM of the FGFR1 blocking compound SU5402 (Calbiochem, La Jolla, CA), PBS (control for FGF8b antibody) or DMSO/PBS (1:1000 in PBS, SU5402 control) was dropped onto the embryos. The embryos were incubated for 24 h (stages 14–16), assessed for heart looping and collected for either immunohistochemistry or myocardial calcium transient measurements (see below). Some embryos were incubated until day 9 and examined histologically for arterial pole alignment. In a second series of experiments, embryos were treated with the anti-FGF8b 24 h after NCA and assessed for arterial pole alignment at day 9. In all experiments, no differences were observed between PBS and DMSO/PBS treated control embryos, and therefore are reported as PBS controls. For cell tracing experiments, SHFs were injected at stage 14 with Tetramethylrhodamine-succinyl ester (TAMRA-SE, Molecular Probes) as previously described (Ward et al., 2005) and examined for migration into the outflow at stage 18.

Quantitative RT-PCR

RNA was isolated from caudal pharynges of sham or NCA embryos between stages 12- and 15 using RNAeasy minicolumn (Qiagen) and reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). A minimum of 11 pools of RNA was isolated for each stage and condition. Real-time quantitative PCR analysis was performed with primers specific for *hprt*, and *18S*, *fgf8t*, *fgf8b*, as well as the FGF8 downstream target genes, *Pea3*, *Erm*, *Er81* and *mkp3* using IcQ Supermix containing SYBR Green (Bio-Rad) with a Bio-Rad quantitative