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ETS-dependent regulation of a distal *Gata4* cardiac enhancer

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ABSTRACT

The developing heart contains an inner tube of specialized endothelium known as endocardium, which performs multiple essential functions. In spite of the essential role of the endocardium in heart development and function, the transcriptional pathways that regulate its development remain largely undefined. GATA4 is a zinc finger transcription factor that is expressed in multiple cardiovascular lineages and is required for endocardial cushion development and embryonic viability, but the transcriptional pathways upstream of *Gata4* in the endocardium and its derivatives in the endocardial cushions are unknown. Here, we describe a distal enhancer from the mouse *Gata4* gene that is briefly active in multiple cardiac lineages early in cardiac development but restricts to the endocardium where it remains active through cardiogenesis. The activity of this *Gata4* cardiac enhancer in transgenic embryos and in cultured aortic endothelial cells is dependent on four ETS sites. To identify which ETS transcription factors might be involved in *Gata4* regulation via the ETS sites in the enhancer, we determined the expression profile of 24 distinct ETS factors in embryonic mouse hearts. Among multiple ETS transcripts present, ETS1, FLI1, ETV1, ETV5, ERG, and ETV6 were the most abundant in the early embryonic heart. We found that ETS1, FLI1, and ERG were strongly expressed in the heart at embryonic day 8.5 and that ETS1 and ERG bound to the endogenous *Gata4* enhancer in cultured endothelial cells. Thus, these studies define the ETS expression profile in the early embryonic heart and identify an ETS-dependent enhancer from the *Gata4* locus.

Introduction

The embryonic cardiovascular system begins to assemble after gastrulation around embryonic day (E) 7.5 in the mouse. Bilaterally symmetrical mesodermal progenitors in the anterior lateral mesoderm fuse at the midline to fashion a linear heart tube made up of an outer layer of myocardial cells that surround an inner layer of endothelial cells known as the endocardium (Brand, 2003). As the linear heart tube forms, angioblasts in the lateral mesoderm differentiate into endothelial cells and form the first lumenized vessels in the embryo (Flamme et al., 1997). As development proceeds, the heart undergoes extensive morphogenesis, which ultimately separates the right and left sides and inflow and outflow regions (Brand, 2003). In parallel, the vasculature is elaborated, forming a complex network of vessels to deliver blood and nutrients, pumped by the heart, throughout the developing embryo (Drake and Fleming, 2000).

The endocardium is continuous with the vascular system but is composed of specialized endothelial cells (Harris and Black, 2010). Endocardial cells have distinct cytoskeletal and connective characteristics compared to other types of endothelial cells (Andries and Brutsaert, 1993; Brutsaert and Andries, 1992; Melax and Leeson, 1967). During embryonic development, endocardial cells perform unique roles in the developing heart: they induce some myocardial cells to form essential trabeculae, ridges of myocardium within the ventricles (Stankunas et al., 2008; Wagner and Siddiqui, 2007), and a subset of specialized cells from the endocardium undergo endocardial-to-mensenchymal transformation (EMT) to populate the endocardial cushions, which give rise to the cardiac valves and portions of the interventricular and atrial septa (Hutson and Kirby, 2007; Person et al., 2005; Snarr et al., 2008). The unique nature of the endocardium may arise, at least in part, from signals received by the endocardium during cardiogenesis, which are different than signals in other areas of endothelial specification in the embryo (Misfeldt et al., 2009). Importantly, malformations in the endocardium and its derivatives underlie numerous pediatric and adult cardiac diseases (Bruneau, 2008; Markwald et al., 2010). In spite of its role in development and disease, molecular and genetic characteristics that distinguish the endocardium from other endothelial cells remain incompletely defined, and the cell-autonomous transcriptional pathways important for endocardial development are poorly understood (Harris and Black, 2010).

The ETS family of transcription factors is widely appreciated for its role in endothelial cell development and function (De Val and Black, 2009; Hollenhorst et al., 2011; Sato, 2001). The ETS family is composed of thirty factors in mammals. ETS proteins are defined by the presence of a conserved DNA-binding domain and a common consensus target sequence, GGA(A/T) (Hollenhorst et al., 2011). Human

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endothelial cells express 19 ETS factors (Hollenhorst et al., 2004). Among them, ETS1, ETS2, FLI1, ETV2, and ETV6 are required for normal vascular development in mice (Barton et al., 1998; Lee et al., 2008; Spyropoulos et al., 2000; Wang et al., 1997; Wei et al., 2009). ETS factors regulate vascular development by binding consensus target sequences in many endothelial genes, such as *Cdh5*, *Tie2*, and *Flk1*, which are important in differentiated endothelial cell function (De Val and Black, 2009). In addition, ETS factors directly regulate other transcription factor genes, suggesting ETS factors are important nodes in transcriptional pathways that govern endothelial specification (De Val and Black, 2009; De Val et al., 2008; Pimanda et al., 2007, 2008).

The GATA family of transcription factors participates in the transcriptional pathways that regulate the development of several cardiovascular lineages, including the myocardium, epicardium, and endocardial cushions (Lepore et al., 2006; Lugus et al., 2007; Rivera-Feliciano et al., 2006; Rojas et al., 2008; Song et al., 2009; Xin et al., 2006; Zeisberg et al., 2005; Zhao et al., 2008). The GATA family consists of six factors that share a zinc-finger DNA-binding domain that binds to the GATA consensus sequence, (A/T)GATA(A/G), which is found in numerous cardiovascular gene regulatory regions (Charron and Nemer, 1999; De Val and Black, 2009; Molkentin, 2000; Patient and McGhee, 2002). GATA4 is a GATA transcription factor that is required for myocardial and endocardial development (Oka et al., 2006; Rivera-Feliciano et al., 2006; Rojas et al., 2008; Zeisberg et al., 2005). *Gata4*-null mice have defective endoderm development leading to cardia bifida (Kuo et al., 1997; Molkentin et al., 1997), and mice lacking GATA4 in the myocardium display defective cardiomyocyte proliferation and embryonic lethality (Rojas et al., 2008; Zeisberg et al., 2005). Mice lacking *Gata4* in the endothelium have defective endocardial cushion development and die around E12.5 of apparent heart failure (Rivera-Feliciano et al., 2006). In spite of its importance in heart development, the transcriptional pathways upstream of *Gata4* in cardiovascular development are unknown.

In this study, we identify a distal enhancer of the mouse *Gata4* gene. This enhancer, referred to as *Gata4* G9, is active in the cardiac crescent at E7.5 and in the linear heart at E8.5. As the heart undergoes looping morphogenesis, enhancer activity remains strong in the endocardial layer but rapidly diminishes in the myocardial layer. At E11.5 and later stages, the enhancer is active only in the endocardium and its derivatives in the endocardial cushions. We define a minimal region of the enhancer that is necessary and sufficient for endocardial activity in transgenic mouse embryos and in cultured aortic endothelial cells. We show that the enhancer is dependent on four consensus ETS binding sites, since mutation of the sites completely abrogates enhancer activity *in vivo*. To determine which ETS factors regulate *Gata4* via the ETS sites in the enhancer, we assessed the expression of twenty-four ETS factors in the embryonic heart. Among these factors, transcripts for FLI1, ETV5, ETV6, ETS1 ERG, ELF1, ETV1, and ETS2 were the most enriched, and ETS1 and ERG bound to the endogenous *Gata4* G9 enhancer in cultured aortic endothelial cells as determined by chromatin immunoprecipitation. Thus, these studies define the ETS expression profile in the early embryonic heart, and identify a cardiac enhancer from the *Gata4* locus that is active predominately in the endocardium and is directly regulated by ETS proteins.

Materials and methods

Bioinformatic analyses, cloning, and mutagenesis

Mouse, human, and cow sequences were compared using BLAST and VISTA (Altschul et al., 1990; Mayor et al., 2000). The 1945-bp *Gata4* G9 fragment was generated by PCR from mouse genomic DNA using the following primers: 5′-gctggacctgtctcgagcacacttgttat-3′ and 5′-tctcagaataacccgggatgactattt-3′. The 404-bp *Gata4* G9 [902–1305] fragment was generated from *Gata4* G9 using the following primers: 5′-ggcgtttctcgagtagtccttggatgccagaa-3′ and 5′-ataatcacccgggcctgttgccctccgccct-3′. The deletion construct *Gata4* G9 [Δ902–1305] was generated by PCR from *Gata4* G9 using the following mutagenic primer and its reverse complement: 5′-gctctgagggacg-caggggatccagtgagc-3′. All *Gata4* G9 fragments were cloned into the XhoI–XmaI sites of the transgenic reporter plasmid *hsp68-lacZ* (Kothary et al., 1989). The 4× ETS mutant was generated by PCR using the following mutagenic primers and their reverse complements: ETS-E mutant, 5′-ttctgataaatgggttgctccacccctccc-3′, ETS-H mutant, 5′-ctcagactgatgggcaataccattgtctg-3′, and ETS-I/J mutant, 5′-ccagggcgacaggcattgcagaggcgtgggg-3′.

Generation of transgenic mice

Transgenic *lacZ* reporter fragments were generated by gel purifying Xho–SalI fragments from parental *hsp68-lacZ* plasmids. Pronuclear injection of transgene fragments was performed as described previously (Dodou et al., 2004). The presence of *lacZ* transgenes in embryo yolks sacs or tail biopsies was detected using PCR and primers specific for the transgene or by Southern blot using a probe specific for *lacZ*. All experiments using animals were reviewed and approved by the UCSF Institutional Animal Care and Use Committee and complied with all institutional and federal guidelines.

X-gal staining, immunofluorescence, and immunohistochemistry

β-galactosidase activity in transgene-positive embryos was detected by X-gal staining, as described previously (Anderson et al., 2004). Transverse sections from X-gal stained embryos were prepared and counterstained with Neutral Fast Red as described previously (Dodou et al., 2003). For section immunofluorescence and immunohistochemistry, embryos were sectioned, de-waxed, boiled in antigen retrieval solution (Biogenex), and blocked in PBS containing 10% sheep serum and 0.1% Triton X-100. For whole mount immunohistochemistry, embryos were boiled in antigen retrieval solution (Biogenex), treated with 5% H_2O_2, and blocked in PBS with 5% milk, 5% sheep serum, and 0.1% Triton X-100. The following primary antibodies were used at 1:100 dilutions in blocking serum: rabbit anti-ETS1 (Santa Cruz; sc-350), mouse anti-GABPA (Santa Cruz; sc-28311), rabbit anti-FLI1 (Santa Cruz; sc-356), rabbit anti-ERG1/2/3 (Santa Cruz; sc-353), rabbit anti-ETV6 (Santa Cruz; sc-11382), mouse anti-myosin heavy chain (DHSB; MF20), mouse anti-GATA4 (Santa Cruz; sc-25310), chicken anti-β-galactosidase (Abcam; Ab9361), and rat anti-endomucin (eBioscience; 14-5851-82). The following secondary antibodies were used at 1:300 dilutions in blocking serum: biotinylated goat anti-rabbit IgG (Vector Laboratories; BA-1000), biotinylated goat anti-mouse IgG (Vector Laboratories; BA-9200), Alexa Fluor 594 goat anti-chicken IgG (Invitrogen; A11042), streptavidin Alexa Fluor 488 (Invitrogen; S32354), and Oregon Green anti-mouse IgG (Invitrogen; O-6383). For immunofluorescence, slides were mounted and photographed as described previously (Rojas et al., 2009). For immunohistochemistry, immunoperoxidase staining and photography were performed as described previously (Rojas et al., 2009).

Cell Culture, transfections, and reporter assays

For transfection experiments, full-length *Gata4* G9, G9[902–1305], and mutant constructs were subcloned from *hsp68-lacZ* as KpnI fragments into the KpnI site of a modified pGL2-Basic vector (Promega) that contains the thymidine kinase (TK) minimal promoter. Bovine aortic endothelial cells (BAEC) were purchased from Cambrex Bio and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% Penicillin/Streptomycin. Transient transfections were performed in 24-well plates using Lipofectamine LTX (Invitrogen) following the manufacturer's recommendations. Transfection mixtures contained 0.4 μg of reporter