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Differential regulation of Tbx5 protein expression and sub-cellular localization during heart development

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Abstract

The T-box transcription factor Tbx5 can interact with Nkx2.5 and Gata4 transcription factors to synergistically regulate heart-specific genes in the nucleus. While a nuclear role for Tbx5 is clearly defined, we have previously shown that Tbx5 shuttles from nuclear to cytoplasmic sites, forming a complex with the PDZ-LIM protein LMP4 on the actin cytoskeleton. In this study, using a developmental series of chicken hearts, we provide the first evidence for differential Tbx5 protein expression and sub-cellular localization during cardiogenesis. At the tissue level, we show temporally and spatially restricted Tbx5 co-expression with LMP4. In cells co-expressing LMP4 and Tbx5 we demonstrate dynamic Tbx5 re-localization from exclusively nuclear to nuclear and cytoplasmic expression in the atrio-ventricular cushion. Furthermore, in coronary vessel development we show exclusive cytoplasmic localization of Tbx5, indicating a function for Tbx5 in the cytoplasm. In addition, we discover unknown regulation of Tbx5 and LMP4 expression in epicardial tissue, suggesting a specific role for Tbx5 in epicardial formation. These studies provide *in vivo* significance of the LMP4/Tbx5 protein interaction, suggesting both nuclear and cytoplasmic roles for Tbx5. The shuttling between nuclear and cytoplasmic sites reveals a novel mechanism for Tbx transcription factor regulation in chicken heart development allowing new insights for a better understanding of the molecular basis of hand/heart birth defects associated with TBX5 mutations.

Keywords: Tbx5; LMP4; Myocardium; Epicardium; Coronary artery; Cardiac myocyte; Cytoskeleton; Heart development

Introduction

Mutations in the transcription factor TBX5 cause the congenital disease Holt–Oram syndrome, which is characterized by truncations of the upper limbs and heart malformations (Basson et al., 1999; Li et al., 1997). The heart malformations primarily include septal and conduction defects although valvular abnormalities have also been reported (Basson et al., 1999; Mori and Bruneau, 2004). While Tbx5 is critical for forelimb and heart development in all vertebrates, little is known about its regulation (Agarwal et al., 2003; Bruneau et al., 2001). In a protein–protein interaction screen we identified a novel Tbx5 binding protein, LMP4, a member of the PDZ-LIM protein family. When co-expressed with Tbx5 in the cell, the transcription factor relocates from the nucleus to cytoplasmic sites and binds LMP4 along the actin cytoskeleton (Krause et al., 2004). This sequestration of the transcription factor at actin filaments has a negative regulatory effect on Tbx5 target genes (Camarata et al., 2006).

Initial insights into *Tbx5* cardiac function during development have come from its mRNA expression profile. In chick, zebrafish and mouse *Tbx5* expression is initiated very early in heart development and revealed expression throughout the cardiac crescent and the early heart tube (Begemann and Ingham, 2000; Bruneau et al., 1999; Liberatore et al., 2000). As the heart tube develops, *Tbx5* mRNA becomes restricted to the more posterior regions that will later form the sinus venosus, atria, and left ventricle of the four-chambered heart (Bruneau et al., 1999; Liberatore et al., 2000). Whole mount mRNA *in situ* hybridizations in the chicken revealed ubiquitous and dynamic *LMP4* expression in every heart chamber, as well as the outflow tract (Krause et al., 2004). These studies have suggested domains of *Tbx5* and *LMP4* co-expression as well as domains where *LMP4* is expressed in the absence of *Tbx5*.

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Both knock-out and over-expression studies in the mouse and chicken have provided insight into the function of *Tbx5* in heart development. Despite expression early in development, knock-out *Tbx5*$^{del/del}$ mice have demonstrated that *Tbx5* is not essential for heart tube formation or normal initiation of endocardial, myocardial and epicardial layers (Bruneau et al., 2001). However, *Tbx5* is important for proper heart chamber patterning. Heterozygous mutant *Tbx5*$^{del/+}$ mice exhibit enlarged hearts with abnormal chamber size, including a dilated right atrium and ventricle as well as atrial septal defects (Bruneau et al., 2001). In contrast, in both chicken and mouse, *Tbx5* over-expression in the myocardium results in a reduction of overall heart size and reduced trabeculation of the ventricles (Hatcher et al., 2001; Liberatore et al., 2000). Mis-expression of *Tbx5* in the left or right ventricle of the mouse and chicken revealed that the *Tbx5* expression gradient between left and right ventricles is critical for patterning of the ventricles and position of the inter-ventricular septum (Takeuchi et al., 2003).

In order to interpret the phenotypes caused by *Tbx5* manipulation, an appreciation of the step wise development of the heart and its formation from multiple cell lineages is essential. From the cardiac crescent, a linear tube forms, consisting of myocardium, lined on the inside by endocardium. The posterior portions of the tube will form the sinus venosus, atria and left ventricle while the anterior portions will form the right ventricle and outflow tract. During further development the heart tube undergoes looping, followed by septation to form the mature, four chambered heart. As looping occurs, a third layer, the epicardium, is added, covering the outer surface of the myocardium. The epicardium originates predominantly from the proepicardial organ (PEO), an outgrowth of cells near the septum transversum (Männer, 1992; Viragh and Challice, 1981). Following attachment of the PEO to the myocardium along the inner curvature of the heart, the proepicardial cells migrate to cover the surface of the myocardium. Recent work has demonstrated that cells derived from the aortic sac also contribute to the epicardium; however, these cells cover only the distal portion of the outflow tract (Perez-Pomares et al., 2003).

The heart consists of myocardium, which forms cardiac muscle, sandwiched between epicardial and endocardial layers (Viragh et al., 1989). In addition to forming the endothelial lining of the myocardium, endocardial cells undergo epithelial-to-mesenchymal transition (EMT) to form the atrio-ventricular and outflow tract cushions, which in turn contribute to the formation of the heart valves and septum (De la Cruz et al., 1983; Markwald et al., 1996). Epicardial cells have multiple functions as well. In addition to covering the surface of the myocardium, epicardial cells undergo EMT, giving rise to mesenchymal cells which will migrate into the sub-epicardial space and myocardium. Epicardially derived cells (EPDCs) will form cardiac fibroblasts, the coronary arteries and even contribute to the atrio-ventricular cushion and intraventricular septum (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 2002; Vrancken Peeters et al., 1999).

As an inroad to understand *Tbx5* function, many studies have relied largely on *Tbx5* mRNA distribution and mis-expression of RNA levels during heart development. Our previous work in biochemical and cell culture experiments demonstrated a regulatory interaction between LMP4 and Tbx5 sub-cellular localization and function (Camarata et al., 2006). To extend the *in vitro* findings and to provide *in vivo* validation, this study examines LMP4 and Tbx5 protein co-expression and localization in different cell populations in the developing chicken heart including coronary vessels.

Materials and methods

Tissue sectioning

Chicken embryos at specified developmental stages were dissected free of membranes and placed into cold PBS. Samples were embedded in Tissue Tek OCT (Sakura Finetek) and frozen in a dry ice/methanol bath. Sections were sliced at 10–12 μm on a Leica CM3050S cryostat (Leica Microsystems) and processed for immunohistochemistry as described.

Immunohistochemistry and imaging

Samples were fixed in 4% paraformaldehyde (PFA) followed by 1% Triton X-100 permeabilization, blocking with 20% normal goat serum (NGS; Invitrogen) and sequential incubation with primary and secondary antibodies in PBS with 0.2% bovine serum albumin (BSA)+10% NGS. Affinity purified rabbit polyclonal anti-LMP4 (Camarata et al., 2006) and anti-Tbx5 (Khan et al., 2002) were used at a 1:500 dilution. In cases where tissue was double labeled for Tbx5 and LMP4, LMP4 antibodies were directly coupled to rhodamine using the EZ-Label protein labeling kit (Pierce Biotechnology). Anti-sarcomeric myosin (MF20), developed by D.A. Fischman; anti-vimentin (AMF-17B), developed by A.B. Fulton; and quail marker (QCPN), developed by B. Carlson and J. Carlson, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa) and diluted at 1:500. Anti-caldesmon (CALD5; Sigma) was diluted 1:1000. Primary antibodies were detected using Alexa 488- and Alexa 546-conjugated secondary antibodies at 1:1000 dilutions (Molecular Probes). Filamentous actin was detected using Alexa Fluor 488 Phalloidin at 1:300 dilution (Molecular Probes). Nuclei were stained using DAPI (Roche) at 1:1000 dilution. To verify antibody specificity, the Tbx5 and LMP4 antibodies were blocked by pre-incubation with 2 mg/ml of the respective peptides against which they were raised, followed by immunohistochemistry as described (Fig. S1). In addition, specificity of the secondary antibodies was confirmed by omitting the Tbx5 or LMP4 antibodies in the reaction (Fig. S1Y-AA). Confocal microscopy was performed using a Zeiss 510 META system (Zeiss, Inc) equipped with a Plan Apochromat 63x/1.4 Oil DIC lens. Overview images were created using the tiling feature, with a Plan Neofluar 25x/0.80 Imm DIC objective. Images were processed in Adobe Photoshop CS2.

Generation of chimeras

Quail to chick chimeras were generated as follows. PEOs were harvested from HH16 or HH17 Japanese quails (*Cotournix, cotournix japonicum*) and immediately transferred into windowed and inked HH16 or HH17 chick hosts. Grafted quail PEOs were positioned underneath the dorsal side of the heart tube next to the chicken proepicardium and 50 μl of a 10× mixture of penicillin, streptomycin and fungizone (Gibco) was pipetted over the yolk sac. Windows were covered with parafilm and embryos allowed to mature until HH29 at 38°C. Following incubation, chimeric embryos were processed for tissue sectioning as described above.

Results

Tbx5 and LMP4 protein expression during chicken heart development

Tbx5 and LMP4 have revealed a dynamic mRNA expression profile throughout heart development (Bruneau et