Cooperative interaction between GATA5 and NF-ATc regulates endothelial-endocardial differentiation of cardiogenic cells

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SUMMARY

In vertebrates, heart development is a complex process requiring proper differentiation and interaction between myocardial and endocardial cells. Significant progress has been made in elucidating the molecular events underlying myocardial cell differentiation. In contrast, little is known about the development of the endocardial lineage that gives rise to cardiac valves and septa. We have used a novel in vitro model to identify the molecular hierarchy of endocardial differentiation and the role of transcription factor GATA5 in endocardial development. The results indicate that GATA5 is induced at an early stage of endothelial-endocardial differentiation prior to expression of such early endocardial markers as Tie2 and ErbB3.

Inhibition of either GATA5 expression or NF-ATc activation, blocks terminal differentiation at a pre-endocardial stage and GATA5 and NF-ATc synergistically activate endocardial transcription. The data reveal that transcription factor GATA5 is required for differentiation of cardiogenic precursors into endothelial endocardial cells. This, in turn, suggests that the GATA5 pathway may be relevant to early stages of valvuloseptal development, defects of which account for the majority of human birth malformations.

Key words: Endocardium, GATA5, Heart Development, Transcription Factors, NF-AT

INTRODUCTION

In human, cardiac septal and valvular abnormalities are the most frequent birth defects, yet few regulators of these important developmental events are known. Valvuloseptal tissues arise from endocardial cells that undergo a mesenchymal transformation, a process regulated by the myocardium (Eisenberg and Markwald, 1995). Thus, as cardiac development progresses, differentiation and cell-cell interaction between cardiomyocytes and endocardial cells are critical for normal heart morphogenesis and function. Disruption of genes essential for these processes perturbs normal heart development as best exemplified by mutations in two myocyte-specific transcription factors, Nkx2-5 and Tbx5, which have been linked to human congenital cardiac septal defects (Basson et al., 1997; Schott et al., 1998). Mutations in Nkx2-5 are also associated with valvular abnormalities but the molecular basis for these is undefined (Kasahara et al., 2000). In mice, inactivation of one Tbx5 allele recapitulates the cardiac defects observed in Holt-Oram patients (Bruneau et al., 2001) and indicates that Tbx5 is a key regulator of cardiomyocyte differentiation. Similarly, inactivation of the Nkx2-5 gene in mice reveals an essential role for Nkx2-5 in the expression of several cardiac genes (Tanaka et al., 1999) and in heart morphogenesis (Biben et al., 2000).

Commitment and differentiation of the myocardial and endocardial lineages are among the earliest events of cardiogenesis as the primitive heart tube is formed of an outer myocardial and an inner endocardial layer which will give rise to the valves and septa. In recent years, significant progress has been made towards elucidating the molecular pathways underlying patterning of the myocardium and differentiation of cardiomyocytes. Indeed, several cardiomyocyte transcription factors have been identified and shown to be required for various stages of cardiomyocyte development and heart morphogenesis. This includes the zinc finger protein GATA4 (Grépin et al., 1997; Crispino et al., 2001), the homeodomain containing protein Nkx2-5 (Lyons et al., 1995; Tanaka et al., 1999), the T-box factor Tbx5 (Bruneau et al., 2001), the MADS protein Mef2C (Lin et al., 1997), and the basic helix-loop-helix proteins Hand1 and Hand2 (Srivastava et al., 1995; Firulli et al., 1998; Srivastava et al., 1997). In contrast, the molecular events and transcription factors underlying endocardial differentiation remain largely undefined. In fact, the embryonic origin of endocardial cells is still being debated. Evidence for distinct origin of endocardial and vascular endothelial cells was only recently provided from analysis of the zebrafish mutants faust and cloche – which lack endocardial but not vascular endothelial cells (Reiter et al., 1997; Liao et al., 1997). In mice, the Tie2 (Tek- Mouse Genome Informatics) receptor tyrosine kinase was found to be essential for endocardial development but dispensable for vascular endothelia (Puri et al., 1999) and expression of NF-ATc marks endocardial but not vascular endothelial cells (de la Pompa et al., 1998) indicating that the two endothelial subtypes are biochemically distinct. At present, NF-ATc is the only
transcription factor shown to be essential for endocardial development (de la Pompa et al., 1998; Ranger et al., 1998).

Retroviral labeling studies in chick and quail embryos (Schultheiss et al., 1997; Mikawa et al., 1992) as well as cell lineage tracing in zebrafish embryos (Lee et al., 1994) suggest that endocardial and myocardial precursors are present in the heart-forming regions, but, whether they share a common progenitor remains uncertain (Lough and Sugi, 2000). The establishment of the QCE-6 cell line, which originates from MCA-treated tissue explants of HH stage-4 Japanese quail embryos, and which can be differentiated into both endocardial and myocardial cells (Schultheiss et al., 1997), supports the existence of a common bipotent cardiogenic precursor. However, the cloche mutation in zebrafish results in a heart that is deficient only in endocardial but not myocardial cells (Stainier et al., 1995); whether this reflects specific dependence on cloche for endocardial differentiation of a bipotent precursor or the existence of distinct myocardial and endocardial precursors cannot be resolved at this stage. Finally, in birds, the characterization of the JB3 antibody, which recognizes a fibrillin-like protein, suggests that there are at least two endocardial subpopulations, a JB3+ one originating within the precardiac mesoderm field, which gives rise to endocardial cells of the cushion and valves, and a JB3− population originating from the nearby heart field mesoderm, which gives rise to the remaining endocardial cells of the heart (Wunsch et al., 1994). Thus, at present, the spatial and temporal appearance of endocardial progenitors as well as the signaling pathways underlying the various stages of endocardial differentiation remain major unanswered questions. The

Fig. 1. Characterization of the TC-13 cells. (A) When grown on matrigels, TC13 cells form row-like structures reminiscent of angiogenesis in vitro (−αVEGF). When treated with an antibody against VEGF (+αVEGF), row formation is inhibited and the cells stay rounded. (B) GATA5 transcripts are present only in differentiated cells. Northern blot analyses using 20 μg of total RNA isolated from undifferentiated (−RA) or differentiated (+RA) TC13 cells were used to detect Gata4 or Gata5 mRNA as described in Materials and Methods. (C) Identification of GATA binding activity. Gel Shift assays were carried out using 5 μg of nuclear extracts and a probe corresponding to the −90 bp GATA element of the BNP promoter as detailed in Materials and Methods. Note that GATA4-containing complexes have a higher mobility than GATA5 complexes. The GATA4 antibody totally supershifted the GATA binding in the undifferentiated cell extracts. GATA5 binding was present only in extracts from RA-treated cells and it was blocked by the GATA5 antibody. GATA4 binding was still detected after RA treatment but GATA5 represented the majority of GATA binding. (D) Control Oct1/2 binding using the same extracts as C. (E) Immunocytochemical staining of untreated (−RA) and treated (+RA) TC13 cells. Cells were fixed in methanol and incubation with the different antibodies was carried out overnight at 4°C as described in Materials and Methods. Staining was revealed by an FITC-avidin D conjugate antibody. Green fluorescent nuclear staining for GATA proteins and cytoplasmic labeling for Von Willebrand factor are observed. Note that only endothelial cells (elongated shape) are positive for GATA5 and Von Willebrand factor.
Identification of stage-specific molecular markers and the development of in vitro models of endocardial differentiation will help greatly to identify key regulators of endocardial development and heart morphogenesis.

We report the characterization and use of such an in vitro model consisting of a mesodermal cell line derived from the hearts of polyomavirus large T-antigen (PVLT) transgenic mice which can be differentiated into endothelial cells upon retinoic acid (RA) treatment (al Moustafa and Chalifour, 1993). Differentiation with RA leads to down-regulation of early cardiac mesoderm markers, including GATA4, Twist and Tbx20, and activation of an endocardial endothelial phenotype characterized by the sequential appearance of various molecular markers. In this system, downregulation of GATA5 expression or inhibition of NFATc activation blocks endocardial differentiation at a pre-endocardial stage. Moreover, GATA5 and NFATc, which are presently the only known transcription factors required for endocardial differentiation, synergistically activate endocardial transcription, suggesting that they cooperate in endocardial differentiation. The results pave the way for the identification of upstream regulators and downstream targets of GATA5 that may be relevant to endocardial development and heart morphogenesis.

MATERIALS AND METHODS

TC13 cell culture and differentiation

The TC13 cell line was obtained from Dr Lorraine Chalifour (al Moustafa and Chalifour, 1993). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% horse serum (GibcoBrl Great Island, USA). For endothelial differentiation, cells were treated with 10−5 M RA in presence or not of serum. GATA5-deficient TC13 cell lines were obtained using the pCDNA3 expression vector harboring a 350 bp GATA5 cDNA in the antisense orientation. To ensure specificity, the cDNA used corresponded to the C-terminal portion of GATA5, which is the least conserved among the GATA4, 5, and 6 family and did not include the highly conserved zinc finger sequence. Briefly, undifferentiated TC13 cells were transfected with the GATA5 antisense or the backbone vector using the calcium-phosphate precipitation method. Selections were carried out using 125 μg of G418 (GibcoBRL) per ml of culture medium. A pool of at least 20 clones was analyzed and 5 antisense clones were isolated by the conventional limiting dilution assay and further characterized.

Plasmids and transfections

The ANF 0.7 kbp and the ET-1 1.4 kbp promoters fused to luciferase as well as the GATA5 full length expression vector were described.
previously (Nemer et al., 1999). The NF-A Tc expression vector was a kind gift from Dr G. Crabtree (Stanford University, USA) and was described previously (Beals et al., 1997). TC13 cells were transfected using the calcium phosphate precipitation method. Briefly, 30,000 cells per well were plated on a 12-wells plate. 1 mg of reporter gene was used per well and total DNA was kept constant at 3 mg. For synergy assays, 25 ng of each expression vector (GA TA5 and NF-A Tc) were used. Luciferase activity was measured 36 hours after transfection by an LKB luminometer. The results are the mean of 3 independent experiments, each done in duplicate.

Gel shift assays

Nuclear extracts of undifferentiated and differentiated TC13 cells were obtained as described previously. Each binding mixture contained 3-5 µg of nuclear extracts. The probe used for GATA binding corresponded to the –90 BNP promoter GATA site (5’ CAGGAATGTTGCTGATAAATCAGA GATAACCCA 3’). For NFAT, the probe used was the –927 BNP (CTATCC-TTTGTTTCCATCTCG) that was shown to interact with NFAT3 (Molkentin et al., 1998). In the mutant NFAT probe, the binding site was altered as follows: TTTGAAATTGG. The octamer probe and conditions for octamer and GATA binding were described previously (Grépin et al., 1994). NFAT binding was carried out according to Timmerman et al. (Timmerman et al., 1997).

RNA extraction and PCR analysis

Total cellular RNA was extracted according to the thiocyanate-phenol-chloroforme method. cDNAs were generated from 5 µg of total RNA using an oligonucleotide dT12-18 in the presence of AMV-RT (Promega). Semi-quantitative PCR was conducted using specific oligonucleotides for each gene and a dose-response assay was carried out to determine the optimal amount of cDNA to be used for PCR amplification using the following: 3 minutes at 94°C, 30 seconds at 94°C, 30 seconds annealing temperature for each oligonucleotide pair, and 1 minute/kb at 72°C, repeated for 29 cycles. Amplification of tubulin was used as an internal control. PCR products were resolved on 1.2% agarose gels. The analysis was carried out in duplicate with RNA isolated from at least two different experiments.

Western blots

Nuclear extracts (20 µg) of TC13 cells were boiled in Laemmli buffer and resolved on SDS-PAGE. Proteins were transferred on Hybond-PVDF membranes and immunoblotted using the Renaissance Chemiluminescence system (NEN Life Sciences, Boston). Rabbit GATA4 and GATA5 antibodies were used at a dilution of 1/500, and revealed with an anti-rabbit horseradish peroxydase antibody (Sigma) at a dilution of 1/10,000.

Immunocytochemistry

TC13 cells were plated on 35 mm Petri dishes and fixed with 100% methanol. The GATA5 antibody was produced in rabbits by injecting a GATA5 truncated protein (corresponding to the C-terminal domain) fused to GST. The purified GATA5 antibody was used at a dilution of 1/50 and revealed by an anti-avidin D FITC or rhodamine or peroxydase conjugate. The anti-Von-Willebrand and anti-GATA4 antibodies were purchased from Santa Cruz Biotechnology and used at a 1/200 dilution. An avidin-D fluorescein-coupled antibody was used to visualize the staining.

Staged mouse embryos at E9.5, 10.5 and 12.5 were dissected, fixed in 4% paraformaldehyde and paraffin embedded. GATA5 staining was carried out as described above. Counterstaining was done with 1% Eosin.

RESULTS

GATA5 expression is induced in differentiated TC13 cells

When treated with 10–5 M RA, TC13 cells fully differentiate into endothelial-like cells expressing the Von Willebrand factor (al Moustafa and Chalifour, 1993). TC13 cells can also grow on matrigels in serum-free medium and form tubular and vesicle-like structures reminiscent of in vitro angiogenesis (Fig. 1A). This is inhibited when the cells are incubated with
Endocardial differentiation requires GATA5 4049

Table 1. Oligonucleotides used for RT-PCR

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anti-VEGF antibody, consistent with the key role of VEGF in regulating angiogenesis and vasculogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). Because TC13 cells were derived from the heart, we hypothesized that they may represent cardiogenic progenitors that differentiate into endothelial endocardial cells. To test this hypothesis, we analyzed expression of various molecular markers, including the cardiac subfamily of zinc finger GATA proteins, GATA4, and GATA5. GATA4 is an early marker of the precardiac mesoderm; within the heart, GATA4 transcripts are present in myocardial and endocardial cells whereas GATA5 mRNA is largely restricted to endocardial cells (Morrisey et al., 1997; Kelley et al., 1993). The presence and identity of GATA factors in undifferentiated and differentiated TC13 cells was assessed using Northern blot analysis (Fig. 1B), gel shift assays (Fig. 1C) and immunocytochemistry (Fig. 1E). GATA4 transcripts were abundant in undifferentiated cells and downregulated in cells treated with RA (Fig. 1B). In contrast, GATA5 transcripts and protein were detected only in differentiated cells (Fig. 1B,C) and only nuclei of differentiated TC13 cells stained positive for
GATA5 expression is required for endocardial differentiation

The upregulation of GATA5 at early stages of TC13 cell differentiation and its highly specific temporal expression in the endocardium in vivo, which closely resembles that of NF-

factor EPAS1 (Tian et al., 1997) is also consistent with the in vivo temporal expression pattern (Tian et al., 1997; Burch et al., 1995; Kurihara et al., 1995). TC13 cells also express the gap junction protein connexin 37 (Cx37) found in all endothelia but do not express Cx40 (Fig. 3B), which marks the vascular endothelium but not the endocardium (Delorme et al., 1997). Finally, the constitutive presence of NF-ATc transcripts is consistent with the in vivo detection of NF-ATc mRNA in precardiac cells of D7.5 mouse embryos and throughout endocardial differentiation although NF-ATc is activated at later stages and in specific cells through nuclear translocation (de la Pompa et al., 1998). Similarly, while NF-ATc mRNA levels remained unchanged following RA-induced differentiation (Fig. 3A), NF-ATc DNA binding activity was upregulated in nuclear extracts of differentiated TC13 cells (Fig. 3C). Together, these results are consistent with the hypothesis that TC13 cells represent an early cardiogenic progenitor capable of differentiating into endocardial endothelial cells.

A time-course analysis of cardiac and endothelial genes present before and after treatment with RA was carried out. As shown in Fig. 4, undifferentiated TC13 cells express early cardiac mesoderm markers that are downregulated upon RA-dependent differentiation; they include, in addition to GATA4, the transcription factors Brachyury, Twist, and the growth factor BMP2. In contrast, endothelial markers like Flt1, Tie2 and endothelin 1 (ET-1; Etn1) are all induced upon treatment with RA. Interestingly, GATA2, which is present in hematopoietic and vascular endothelial cells but not in endocardial cells (Lee et al., 1991; Dorfman et al., 1992) is absent in both untreated and RA-treated TC13 cells (data not shown), whereas GATA5 is upregulated at early stages of differentiation, coincident with expression of Flt1 and prior to induction of such early endothelial markers as Tie2, ErbB3 and Cx37 (Gja4). The induction of the genes coding these proteins before other markers of terminal endothelial differentiation like ET-1, tenascin X (TnX) and the endothelial-specific transcription

an anti-GATA5 specific antibody (Fig. 1E). Gel shift analysis using anti-GATA4- and GATA5-specific antibodies identified GATA4 as the exclusive GATA binding activity in undifferentiated cells and GATA5 as the major GATA binding activity in RA-treated cells, which also contained GATA4 (Fig. 1B). The presence of both GATA4 and GATA5 in differentiated TC13 cells accurately reflects the in vivo distribution of the proteins within endocardial and endocardial cushion cells (Fig. 2). Nevertheless, the temporal expression pattern of the two proteins is distinct with GATA4 labeling of endocardial cells being more persistent while GATA5 transcripts and protein become undetectable in the heart by E12.5 (Fig. 2B). The transient expression of GATA5 in endocardial cells is reminiscent of the cardiac expression pattern of transcription factor NF-ATc which is essential for progression of endocardial cell differentiation and valve development (Ranger et al., 1998; de la Pompa et al., 1998). In order to further define the molecular identity of the TC13 cells, we carried out semi-quantitative RT-PCR to assess the presence of other known cardiac transcription factors. The results show that factors whose expression is downstream of GATA4 in cardiomyocyte differentiation, like MEF2C, Nkx2-5 and the Hand proteins are not detected in TC13 cells. However, two cardiac-enriched T-box proteins, Tbx5 and, to a greater extent, Tbx20, are present in undifferentiated cells, with Tbx20 levels decreasing upon differentiation. The presence of Tbx5 and 20 in TC13 cells is noteworthy, given that Tbx20 expression in the lateral plate mesoderm precedes that of Nkx2-5 (Kraus et al., 2001; Iio et al., 2001) and that Tbx5 was recently suggested to be upstream of Nkx2-5 and GATA4 (Bruneau et al., 2001). In addition to GATA5, expression of epicardin, a basic helix-loop-helix transcription factor present in epicardial and endocardial cushion cells of the embryonic heart (Robb et al., 1998) was upregulated in differentiated cells (Fig. 3A and Table 1).

A sequential expression of endothelial (A) and mesodermal genes (B). RT-PCR analysis was carried out on total RNA isolated at different stages of TC13 cell differentiation. Each amplification was carried out using the appropriate amount of cDNA as determined from a dose-response curve. For PCR conditions, please refer to Table 1. Amplified products were resolved on 1.2% agarose gels and visualized under UV light. (C) Gel shift analysis of NF-AT proteins in extracts from TC13 cells treated with RA for 0 (−) or 24 (+) hours. Specific complexes corresponding to NF-ATp and NF-ATc (Timmerman et al., 1997) are displaced by 100-fold excess of cold probe (competitor). Note how NF-ATc complexes are increased in RA-treated extracts. N.S, nonspecific.
Endocardial differentiation requires GATA5

ATc, suggest a role for GATA5 in endocardial differentiation. To test the effect of loss of GATA5 function on endocardial differentiation, stable TC13 transfectants expressing an antisense GATA5 cDNA were generated. Four independent neomycin-resistant clones were fully analyzed and the results were identical in all cases. As shown in Fig. 5, GATA5 antisense specifically targeted GATA5, blocking both mRNA and protein accumulation in response to RA. In contrast, GATA4 protein levels were not significantly altered in undifferentiated cells and were similarly decreased in response to RA treatment as control cells, indicating that antisense GATA5 transfectants are still responsive to RA (Fig. 5C). The expression of the GATA5 antisense cDNA had no effect on the morphology or proliferation of undifferentiated cells (Fig. 5D and data not shown). However, in the absence of GATA5 protein, treatment with RA did not lead to the appearance of endothelial-like morphological and biochemical changes nor to a decrease in cell proliferation as in control cells, even with increasing concentration (10^{-4} M) and time (5 days) of RA treatment (Fig. 5D). Furthermore, unlike control cells, endothelial markers like ET-1, Flt1, and EPAS1 were not induced in cell lines lacking GATA5 indicating that GATA5 expression is required for endocardial differentiation (Fig. 5E). In order to molecularly define the stage at which differentiation was arrested, RT-PCR analyses were carried out. As shown in Fig. 5E, early endothelial markers like Msx1 and Cx37 were induced, albeit at a relatively lower level than in control cells. However, in the absence of GATA5 protein, treatment with RA did not lead to the appearance of endothelial-like morphological and biochemical changes nor to a decrease in cell proliferation as in control cells, even with increasing concentration (10^{-4} M) and time (5 days) of RA treatment (Fig. 5D). Furthermore, unlike control cells, endothelial markers like ET-1, Flt1, and EPAS1 were not induced in cell lines lacking GATA5 indicating that GATA5 expression is required for endocardial differentiation (Fig. 5E). In order to molecularly define the stage at which differentiation was arrested, RT-PCR analyses were carried out. As shown in Fig. 5E, early endothelial markers like Msx1 and Cx37 were induced, albeit at a relatively lower level than in control cells. However, in the absence of GATA5 protein, treatment with RA did not lead to the appearance of endothelial-like morphological and biochemical changes nor to a decrease in cell proliferation as in control cells, even with increasing concentration (10^{-4} M) and time (5 days) of RA treatment (Fig. 5D). Furthermore, unlike control cells, endothelial markers like ET-1, Flt1, and EPAS1 were not induced in cell lines lacking GATA5 indicating that GATA5 expression is required for endocardial differentiation (Fig. 5E).
required for transcriptional activation of NF-ATc but that GATA5 induced signals are necessary for NF-ATc activation.

In mice, NF-ATc is required for proper differentiation of endocardial cells into valves and septa. Although the exact function of NF-ATc and its molecular targets are undefined, NF-ATc appears to be required at a post-endocardial cushion stage. We used the in vitro model to test the transcriptional hierarchy between GATA5 and NF-ATc. Treatment of TC13 cells with cyclosporine A (CsA), an inhibitor of NF-ATc activation (Fig. 5F), blocked RA-dependent endothelial differentiation as judged by the morphologic appearance of elongated (Fig. 6A) and von Willebrand-positive cells (data not shown). Consistent with a role for NF-ATc at a later stage of endocardial development, induction of GATA5 was reduced though not abolished in cells treated with CsA. In cardiomyocytes, GATA-4 and NF-AT3 have been shown to physically and functionally interact to regulate gene expression in the setting of cardiac hypertrophy (Molkentin et al., 1998). We therefore tested whether GATA5 and NF-ATc collaborate in regulating endocardial transcription thereby affecting cell differentiation. For this, we used the GATA-dependent ET-1 promoter, which is preferentially regulated by GATA5 (Nemer et al., 1999). As shown in Fig. 6B, the ET-1 promoter is synergistically activated in TC13 cells by co-expression of GATA5 and NF-ATc suggesting that these two transcription factors act cooperatively to induce endocardial gene expression. Together, the results identify a molecular hierarchy for endocardial differentiation and suggest that GATA5 cooperates with NF-ATc for terminal endocardial cell differentiation.

DISCUSSION

Cell commitment, migration, and a balance between cell proliferation and differentiation of the different cell populations of the heart are crucial for normal cardiogenesis. Abnormalities in the expression of any factor involved in these events contribute to congenital heart disease, which represents the largest class of birth defects in human. Mutations in cardiomyocyte-specific transcription factors such as GATA4, Nkx2-5 and Tbx5 have already been implicated in congenital human cardiac disease (Pehlivan et al., 1999; Schott et al., 1998; Basson et al., 1997). The data presented above indicate that GATA5 is required for endothelial endocardial cell differentiation. Given that these cells give rise to the cardiac cushion and valves, the results implicate GATA5, or its effectors, in valve formation and possibly in cardiac malformations.

GATA5 is a member of the zinc finger family of GATA proteins, which play critical roles in cell differentiation and organogenesis (reviewed by Charron and Nemer, 1999). The
Gata5 gene is expressed during early embryogenesis in the primitive endoderm and in the precardiac mesoderm; within the heart, GATA5 is largely restricted to the endocardial cells where it is present until mid gestation when it is switched off in the heart but persists in other organs like lung, gut epithelium and the urogenital ridge. This pattern of expression as well as the primary GATA5 sequence are conserved across species (Morrissey et al., 1997; Lavrierve et al., 1994; Kelley et al., 1993; MacNeill et al., 2000; Reiter et al., 1999). The role of Msx1 and EPAS1 in endocardial differentiation is still undefined; Msx1 is present in endocardial cushion cells but not in the endocardium, consistent with a role in epithelial-mesenchymal transformation (Chan-Thomas et al., 1993). EPAS-1 is required at terminal stages of endothelial maturation and remodeling (Peng et al., 2000). The appearance of both factors after GATA5 during in vitro endocardial differentiation is consistent with their in vivo distribution and function, at later stages of endocardial differentiation.

A critical role for GATA5 in endoderm and heart development was demonstrated by the finding that the zebrafish faust mutant, characterized by defects in endocardial and myocardial differentiation and migration, maps to Gata5 (Reiter et al., 1999). The sequence of the Gata5 gene in faust mutant reveals a splicing defect resulting in a 31 bp insertion that introduces a frame shift disrupting the entire C-terminal domain of the protein or a deletion within the second zinc finger (Reiter et al., 1999). These mutations would result in the production of a transcriptionally inactive protein unable to bind DNA or activate transcription; moreover, the most frequently isolated cDNA encodes the C-terminal frame shift mutant which, based on structure:function studies (Nemer et al., 1999) may act as a dominant-negative GATA protein. faust mutants lack endocardial cells and have reduced number of myocytes; since GATA5 expression is predominantly in endocardial cells, the defect in myocytes may be the result of defective endocardial-myocardial signaling including decreased levels of paracrine factors like neuregulins, ET-1 and PDGF that act on myocyte growth (Zhao et al., 1998; Harada et al., 1997; Schattemann et al., 1996; Shimizu et al., 1999). Additionally, since GATA5 is present in a few myocytes, a cell autonomous role in the myocardium cannot be excluded. Experiments in Xenopus embryos also revealed a critical role for GATA5 in endoderm differentiation (Weber et al., 2000) but did not address its role in the heart.

In contrast to the situation in zebrafish and in Xenopus, the role of GATA5 in mammalian development remains uncertain largely because of the phenotype of mice in which the Gata5 gene was mutated. These mice were viable and fertile but females displayed genitourinary abnormalities, raising the possibility that other GATA factors may compensate for GATA5 in endoderm and heart development (Molkentin et al., 2000). While this possibility cannot be excluded, it is worth noting that the strategy used targeted the first coding exon, resulting in deletion of the first 157 aa; a truncated protein containing both zinc fingers and the C-terminal activation domain could still be produced and would be transcriptionally active (Nemer et al., 1999). In fact, characterization of the Gata5 locus and cDNA analysis has already revealed the presence of two alternate non-coding first exons (MacNeill et al., 1997) resulting in two distinct GATA5 transcripts, one of which lacking the entire exon 2 (which was targeted in the mouse model). Such N-terminal truncated protein – which is found in embryonic but not adult heart (MacNeill et al., 1997) – retains DNA binding and transcriptional activation properties (Nemer et al., 1999). Given that alternate splicing and alternate translation initiation have also been reported for GATA1 (Ito et al., 1993; Calligaris et al., 1995; Ito et al., 1993), the possible presence of truncated GATA5 protein in the GATA5 ‘null’ mice cannot be ruled out. Consequently, the role of GATA5 in mammalian development and more specifically its conserved role in the heart cannot be unequivocally determined based on the mouse model.

The work presented here, using a novel in vitro cell system, indicates that GATA5 is essential for differentiation of committed cardiogenic precursors into endothelial endocardial cells, suggesting that GATA5 function in heart development is indeed conserved across species. The results also provide further evidence for an autonomous role of GATA5 in cardiac cells as suggested by analysis of the zebrafish faust mutant (Reiter et al., 1999). Based on the time course expression of the different endothelial markers, it is also suggested that GATA5 is not essential for initiation of endocardial endothelial differentiation but rather it appears to be required for progression of the differentiation program. This is consistent with the in vivo expression pattern of GATA5 and the findings in the faust mutant. Interestingly, the role of GATA5 in terminal endocardial differentiation is reminiscent of the role of GATA4 in myocyte differentiation (Grépin et al., 1997) of GATA1 in terminal erythroid differentiation (Shivdasani et al., 1997) and GATA3 in T-cell differentiation (Ting et al., 1996).

The molecular basis underlying the role of GATA5 in endocardial differentiation is not defined yet but the in vitro system described here will allow identification of GATA5 target genes as well as GATA5 collaborators. It is noteworthy that many endocardial genes may well be direct GATA5 targets as they contain conserved GATA binding sites in their promoter. These include Msx1 (Chen and Solursh, 1995), P-selectin (Pan and McEver, 1993), TnX (Matsumoto et al., 1993), Gata4 (Nemer et al., 1999). Given that alternate splicing and alternate translation initiation have also been reported for GATA1 (Ito et al., 1993; Calligaris et al., 1995; Ito et al., 1993), the possible presence of truncated GATA5 protein in the GATA5 ‘null’ mice cannot be ruled out. Consequently, the role of GATA5 in mammalian development and more specifically its conserved role in the heart cannot be unequivocally determined based on the mouse model.

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1994), and ET-1, which was shown to be a preferential GA Tα4 factor. In addition to the myocardium, GA Tα4 is also prominently expressed in endocardial cells in vivo and in TC13 cells throughout differentiation (Figs 1-5). Recent genetic evidence suggests an important role for GA Tα4 in valve development and heart morphogenesis as revealed from a knock-in mutation affecting GA Tα4 interaction with its cofactor FOG2 (Crispino et al., 2001). Whether this reflects a requirement for GA Tα4 in the endocardium or in the myocardium (or both) remains uncertain given that the role of GA Tα4 in the endocardium is undefined. Interestingly, other GA Tα factors, including GA Tα5, which is coexpressed with GA Tα4 in the endocardium, did not compensate for GA Tα4 absence. Our finding that GA Tα4 expression precedes GA Tα5 in endocardial differentiation may explain why GA Tα5 could not compensate for GA Tα4 in the mutant mice and suggest that GA Tα4 may be essential for survival and/or proliferation of endocardial progenitors. The characterization of an in vitro model of endocardial differentiation will greatly help elucidate the molecular pathways and genes involved in commitment and differentiation of the endocardial lineage, including upstream GA Tα4 and GA Tα5 regulators and downstream targets. This in turn might provide much needed insight into valvuloseptal morphogenesis, defects of which account for the majority of congenital heart defects.

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