Development 129, 4045-4055 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV2902

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

Georges Nemer and Mona Nemer*

Laboratoire de Développement et Différenciation Cardiaques, Institut de Recherches Cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, Québec H2W 1R7, and Département de Pharmacologie, Université de Montréal, Canada
*Author for correspondence (e-mail: nemerm@ircm.qc.ca)

Accepted 4 June 2002

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., 1999; 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

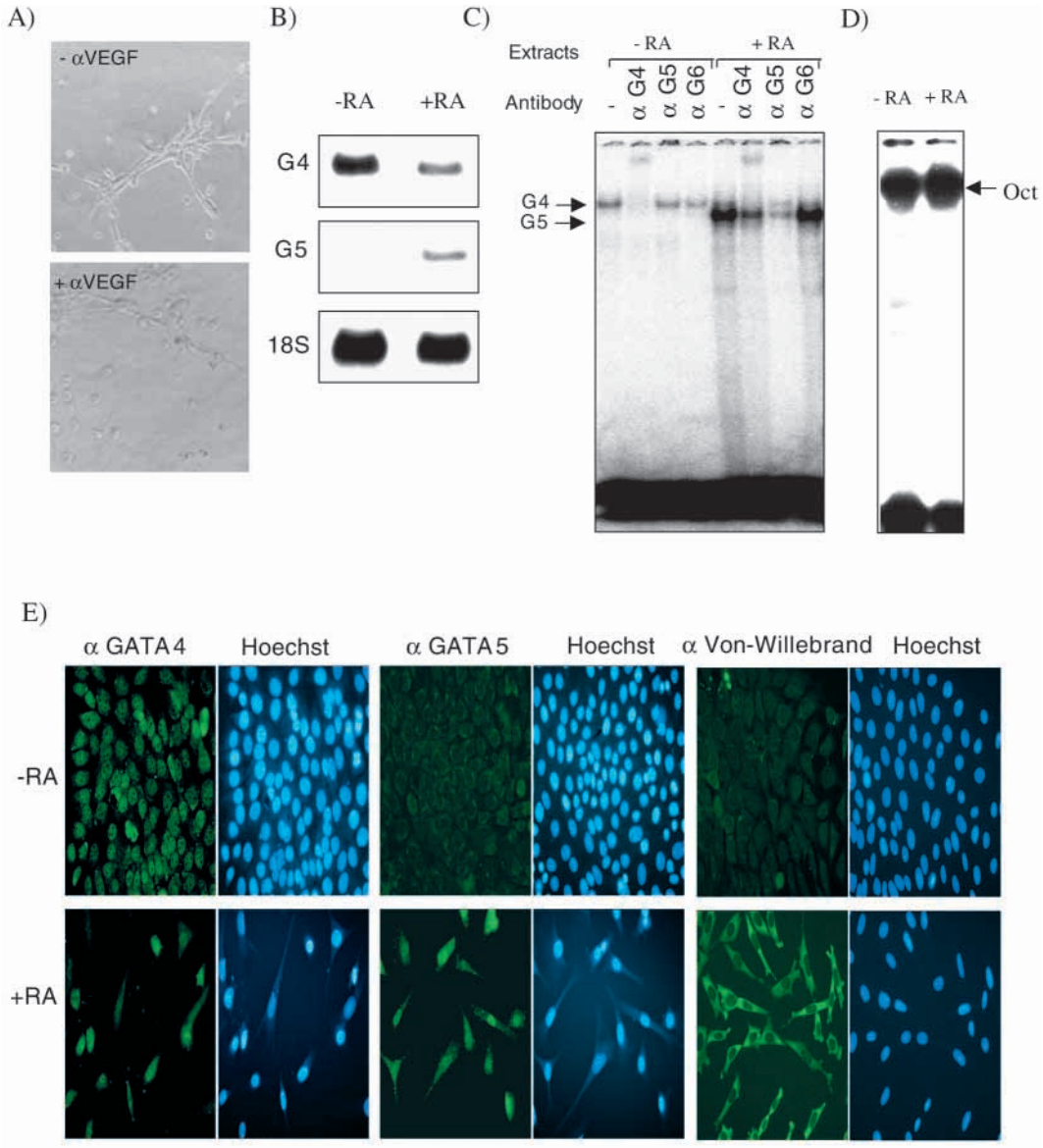

Fig. 1. Characterization of the TC-13 cells. (A) When grown on matrigels, TC13 cells form row-like structures reminiscent of angiogenesis in vitro (–αVEGF). When treated with an antibody against VEGF (+αVEGF), row formation is inhibited and the cells stay rounded. (B) GATA5 transcripts are present only 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.

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

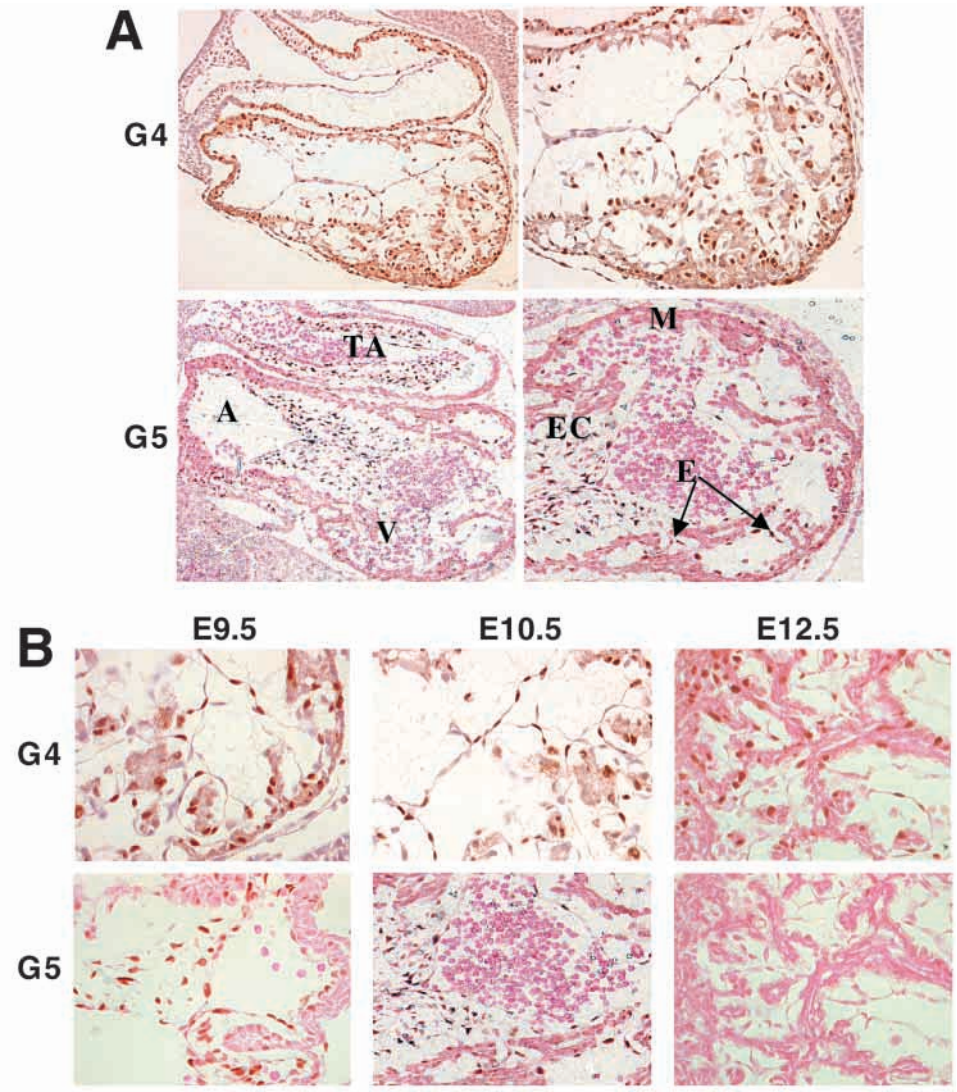

Fig. 2. Endocardial expression of GATA4 and GATA5 in the developing heart. Immunocytochemical staining of staged mouse embryo sections with anti-GATA4 and -GATA5 polyclonal antibodies. (A) Low magnification of E10.5 stained embryos reveals abundant expression of GATA4 in atrial (A) and ventricular (V) myocytes, in endocardial cells, and in some cells of the endocardial cushion (EC). GATA5 labeling is restricted to endocardial (E) and endocardial cushion cells both in the atrio-ventricular cushion and in the truncus arteriosis (TA), which will give rise to the outflow tract. Staining is absent in the myocardium (M). (B) Expression of GATA5 is transient in endocardial cells. Note how GATA5 staining is undetectable at E12.5 whereas GATA4 is still present both in endocardial and myocardial cells. Counterstaining with Eosin and Hematoxylin.

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 NF-ATc activation blocks endocardial differentiation at a pre-endocardial stage. Moreover, GATA5 and NF-ATc, 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

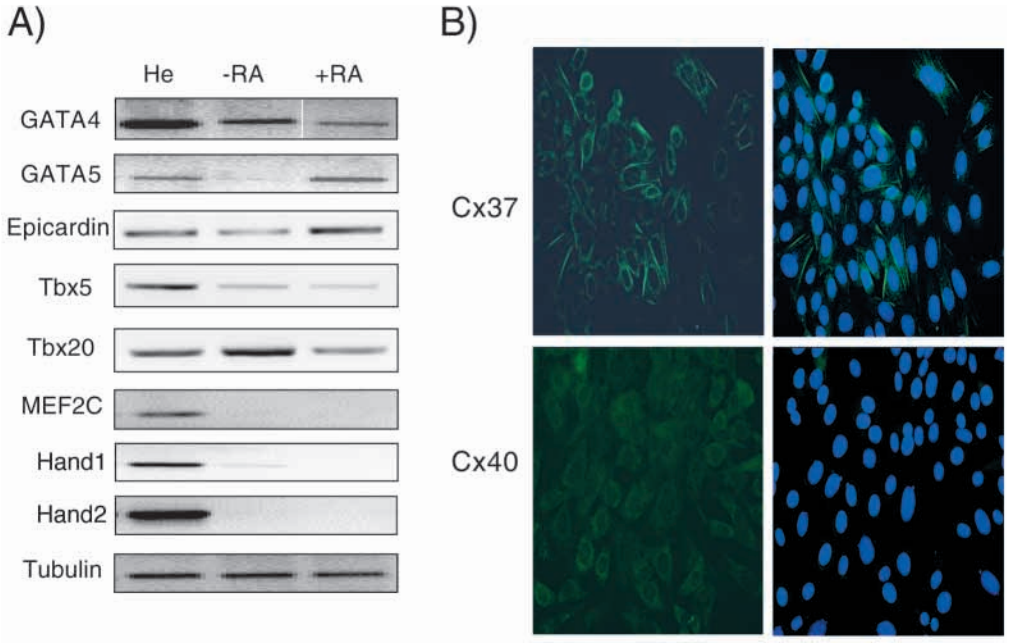

Fig. 3. Endocardial identity of differentiated TC13 cells. (A) Semi-quantitative RT-PCR analysis was carried out on total RNA isolated from undifferentiated (–RA) and differentiated (+RA) cells following 4 days of RA treatment, as well as from hearts (He) of neonatal Sprague Dawley rats. Tubulin transcript amplification was used as control. The oligonucleotides and conditions used are listed in Table 1. The results shown are from one representative experiment. (B) Immunocytochemical detection of the Cx37 and Cx40 gap-junction proteins. Note the specific green fluorescent labeling of the cell membrane with the anti-Cx37 antibody but not with the anti-Cx40 antibody. RNA analysis also confirmed the presence of Cx37 but not Cx40 transcripts in these cells (not shown). Nuclei are stained with the Hoechst dye.

previously (Nemer et al., 1999). The NF-ATc 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 μg of reporter gene was used per well and total DNA was kept constant at 3 μg. For synergy assays, 25 ng of each expression vector (GATA5 and NF-ATc) 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′ CAGGAATGTGTCT**GATA**AATCAGA **GATA**ACCCA 3′). For NFAT, the probe used was the –927 BNP (CTATCC-T**TTTGTTTTCCAT**CCTG) that was shown to interact with NFAT3 (Molkentin et al., 1998). In the mutant NFAT probe, the binding site was altered as follows: TTTGAATTGGA. 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 $dT_{12\text{-}18}$ 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. The Cx37 and Cx40 antibodies were purchased from Alpha Diagnostic, and used at a dilution of 1/200. 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

Table 1. Oligonucleotides used for RT-PCR

Gene	Species	Oligonucleotide sequences	Annealing temp. (°C)	Length (bp)
Msx1	Mouse	CAGCATGCACCCTACGCAA CCGCTGCTCTGCTCAAAGA	59	502
BMP2	Mouse	AGCAAGGGGAAAAGGACA CCGGGAACAGATACAGGAAG	59	541
Gata5	Rat	GAACCAGTGTGCAACGCCTG CGCAGGGCCAGGGCACACCA	58	409
Gata4	Rat	GACACCCCAATCTCGATATG TTACGCGGTGATTATGTCCC	58	730
Gata6	Mouse	CAAACCAGGAAACGAAAAC AACAAAGGCACAGAAATCAC	55	457
Nkx2-5	Mouse	CCTCTAGAGCAGAGCTGCGCGCGGAGATG GGTGGCTTCCGTCGCCGCCGTGC	61	278
Hand2	Mouse	TACCAGCTACATCGCCTAC TCTTTCTTCCTCTTCTCCTC	55	120
Hand1	Mouse	AACCTCAACCCCAAAAGCC GGAAGGGAAAGGAAGGGAAAG	59	278
ET-1	Mouse	ATGGATTATTTTCCCGTGAT TCAATGTGCTCGGTTGTGCG	54	608
ErbB3	Mouse	GGAGGCTTGTCTGGATTCTG ATTGCCATCCTCTTCCTCTA	58	283
Gata2	Mouse	ACCCGATACCCACCTATCCC AGTCACCATGCTGGACGGGT	60	300
Cx37	Mouse	TTCCTCTTCGTCAGCACACC TCTTACACAGCACGCTGACC	59	243
Cx40	Mouse	ATGGGTGACTGGAGCTTCC CACAAAGATGATCTGCAGTACCC	53	255
T (brachyury)	Mouse	CAATGCCATGTACTCTTTC GGCTGTAATCTCCTCATTC	57	364
Epas1	Mouse	AGTTGGCTCATGAGTTGCCC GATGGGTGCTGGATTGGCTC	59	595
Nfatc	Mouse	AGGTGCAGCCCAAGTCTCAC GTGGCCATCTGGAGCCTTCT	60	620
Mef2C	Mouse	GCCCTGAGTCTGAGGACAAG ATCGTGTTCTTGCTGCCAG	58	472
Epicardin	Mouse	CCTAGGCAAGAGGAGGAAGG ATAAAGGGCCACGTCAGGTT	60	300
Tie2	Mouse	CCATCCAAACATCATCAATCTC CCTCCTAAGCTAACAATCTCCC	58	464
Tenascin X	Mouse	GGCTCAGGTGCCCCTGGTGG TTCTACAGTGCCCAGCTGCGGG	59	752
Flt1	Mouse	CCCCTCTTTCAACATCTTC CCACACCTGCTTCAAAAC	55	620
Tbx5	Mouse	CAAACTCACCAACAACCACC GCCAGAGACACCATTCTCAC	59	423
Tbx20	Mouse	GCAGCTCCTCAAACAGATGG TCAGTGAGCCTGGAGGAGTC	59	300
Tubulin	Rat	TCCATCCACGTCGGCCAGGCT GTAGGGCTCAACCACAGCAGT	61	537

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

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 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; Edn1) 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 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.

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-

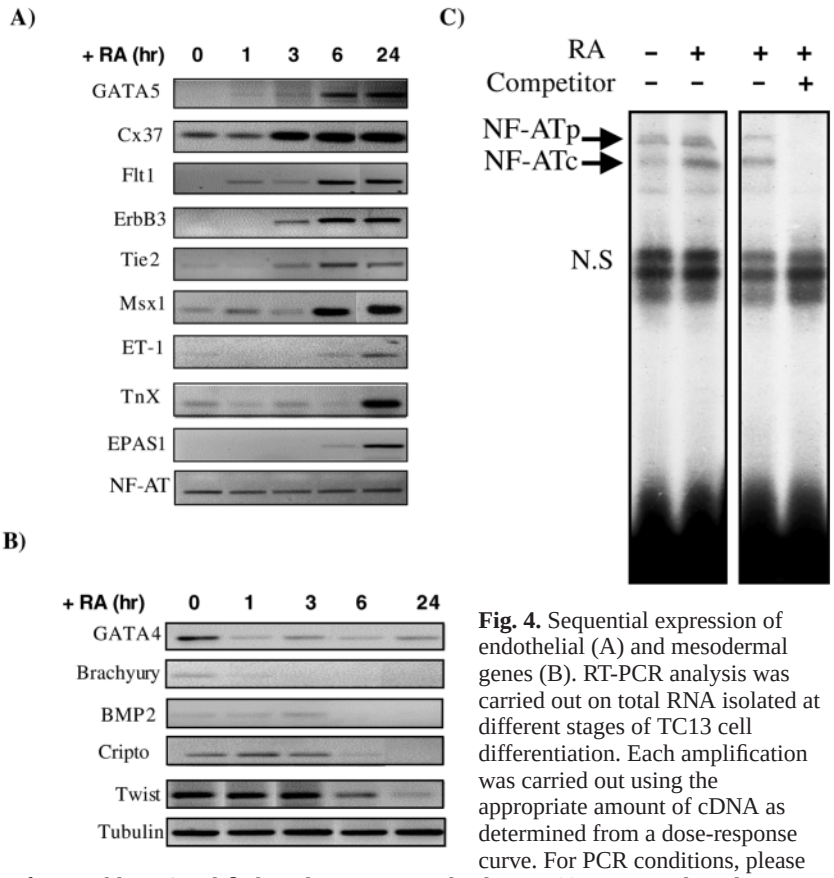

Fig. 4. Sequential expression of endothelial (A) and mesodermal genes (B). RT-PCR analysis was carried out on total RNA isolated at different stages of TC13 cell differentiation. Each amplification was carried out using the appropriate amount of cDNA as determined from a dose-response curve. For PCR 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.

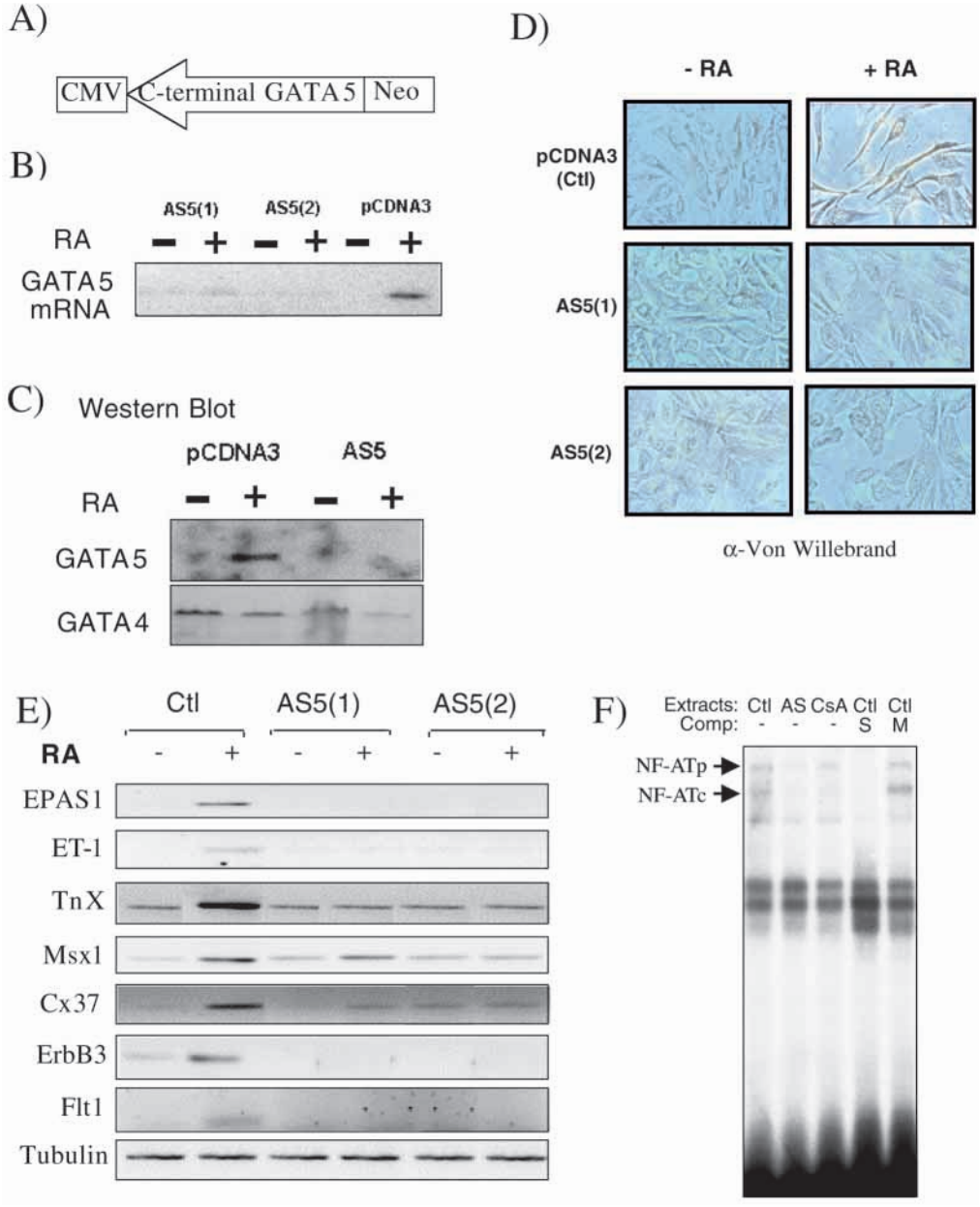

Fig. 5. Inhibition of endocardial differentiation in GATA5 antisense transfectants. (A) Schematic representation of the GATA5 antisense construct. The cDNA fragment spanned from 850 bp to 1200 bp where 1 is the A of the initiating methionine. (B) RT-PCR analysis was conducted on RNA isolated from two different TC13 clones expressing antisense GATA5 (AS5) as well as TC13 cells transfected with the control pCDNA3 vector. The GATA5 primers used detect only the endogenous transcripts. Note how the antisense inhibits accumulation of GATA5 transcripts following RA treatment. (C) Western blot analysis confirms that antisense clones do not express GATA5 protein. GATA4 levels are only slightly decreased in the GATA5 antisense but are further decreased in response to RA, as in control cells. 20 μg of nuclear extracts were resolved on a 15% SDS-PAGE and blotted on a nylon membrane with the respective antibodies. (D) GATA5-deficient TC13 cells do not elongate and remain negative for Von Willebrand factor staining even after 5 days of treatment with RA. No arrest in cell proliferation was observed in these cells compared to control cells. The data shown are from two independent clones, AS5(1) and AS5(2). (E) RT-PCR analyses show that terminal endocardial markers (EPAS-1, ET-1, Tn-X, ErbB3) are not induced in the antisense GATA5 transfectants after 4 days of RA treatment. (F) Gel shift analysis using extracts from RA-treated GATA5 antisense transfectants (AS) or RA-treated control (Ctl) in presence of cyclosporine A (CsA). NF-AT binding is decreased in AS- and in CsA-treated cells. Note that in both cases, the decrease is greater for NF-ATc than NF-ATp. Binding was competed by 100× self probe (S) but not a mutant probe (M).

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, NF-ATc binding was no longer induced (Fig. 5F) although mRNA levels were unchanged (data not shown). This suggests that GATA5 is not

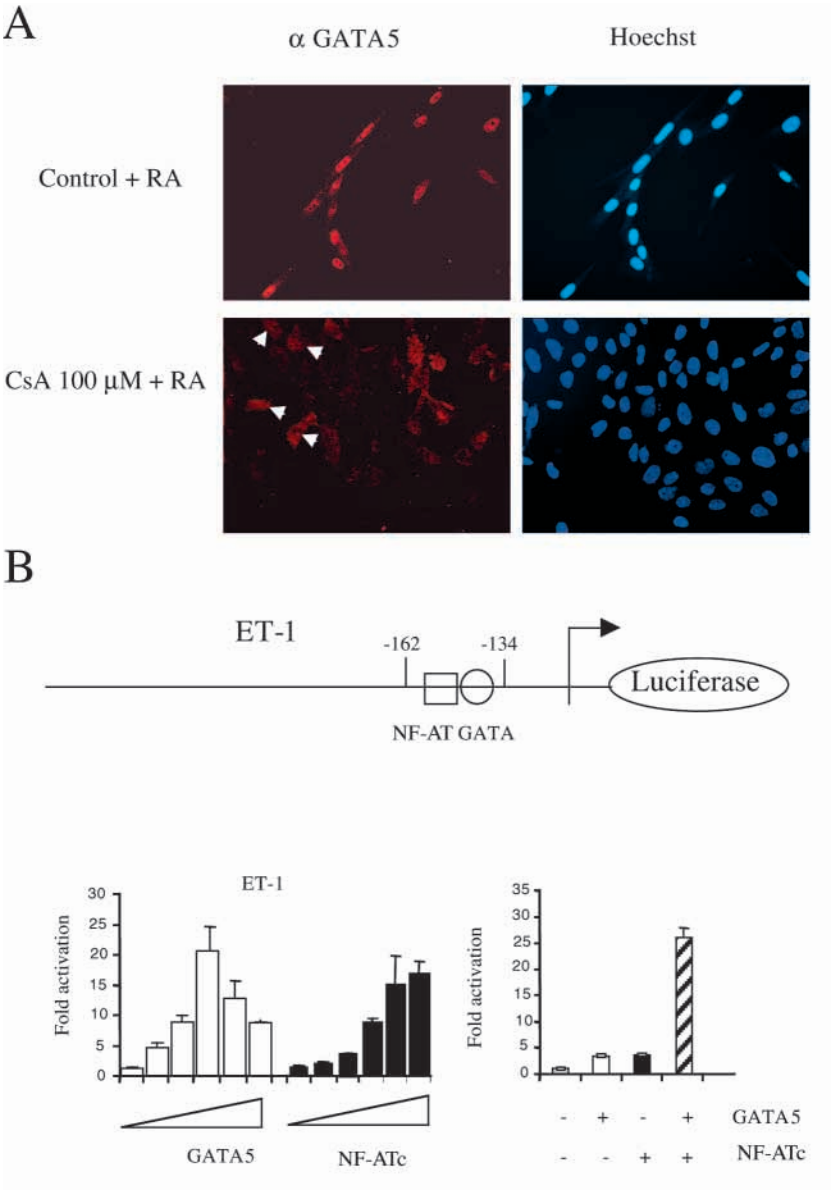

Fig. 6. GATA5 and NF-ATc collaborate in endocardial differentiation. (A) Blocking NF-ATc activation using cyclosporine A (CsA) inhibits TC13 differentiation and reduces GATA5 induction in response to retinoic acid (RA). Immunocytochemistry using the GATA5 antibody was carried out as described in Materials and Methods. Note how cells treated with CsA do not elongate or align (arrowheads) and show reduced but persistant staining for GATA5 (red nuclei) suggesting that GATA5 induction precedes NF-ATc activation in endocardial differentiation. (B) GATA5 and NF-ATc synergistically activate the endothelin 1 (ET-1) promoter. (Top) A schematic representation of the ET-1 promoter showing the close proximity of the NF-ATc and GATA binding sites. (Bottom) A 1.4 kbp endothelial-specific ET-1 promoter is transcriptionally activated by GATA5 and NF-ATc as evidenced in cotransfection with increasing amounts (0.25, 50, 100, 250, 500 ng) of either GATA5 or NF-ATc expression vectors in TC13 cells (left panel). At limiting amount of expression vectors (25 ng) GATA5 and NF-ATc synergistically activate the ET-1 promoter (right panel). The empty pCDNA3 and pCD-SRα backbone vectors of GATA5 and NF-ATc, respectively, are used as controls. The results are expressed as fold activation and represent the mean of three independent experiments, each done in duplicate.

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-AT$_3$ 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

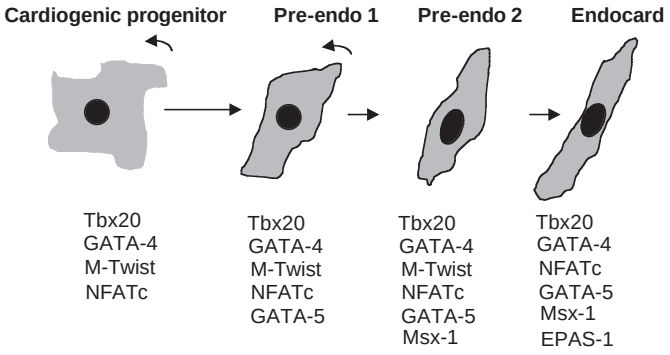

Fig. 7. Hierarchy of transcription factors during in vitro endocardial differentiation. Only transcription factors present at the different stages are indicated. Note that GATA5 is the earliest factor that is transcriptionally induced followed by Msx1 and EPAS1. Expression of GATA4 and Tbx20 is maintained, though at a reduced level. Reduced expression of GATA5 following cyclosporine A treatment suggests that calcium signaling may be an upstream GATA5 activator in endocardial cells. The results obtained suggest that GATA5 is required at an early stage of endocardial differentiation. Consistent with in vivo data, NF-ATc transcripts are present from the earliest stages of endocardial commitment, but NF-ATc is activated and required at later stages that remain undefined (Ranger et al., 1998). 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.

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 (Morrisey et al., 1997; Laverriere et al., 1994; Kelley et al., 1993; MacNeill et al., 2000; Reiter et al., 1999).

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 *Gata*5 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.,

1994), and ET-1, which was shown to be a preferential GATA5 target (Nemer et al., 1999). It is also possible that GATA5 may be the nuclear target of inductive signals required for endocardial differentiation, such as TGFβ (Ramsdell and Markwald, 1997). In this regard, it is noteworthy that, in myocardial cells, GATA4 is targeted by cardiogenic factors of the TGFβ family (Schultheiss et al., 1997; Monzen et al., 1999).

In addition to the myocardium, GATA4 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 GATA4 in valve development and heart morphogenesis as revealed from a knock-in mutation affecting GATA4 interaction with its cofactor FOG2 (Crispino et al., 2001). Whether this reflects a requirement for GATA4 in the endocardium or in the myocardium (or both) remains uncertain given that the role of GATA4 in the endocardium is undefined. Interestingly, other GATA factors, including GATA5, which is coexpressed with GATA4 in the endocardium, did not compensate for GATA4 absence. Our finding that GATA4 expression precedes GATA5 in endocardial differentiation may explain why GATA5 could not compensate for GATA4 in the mutant mice and suggest that GATA4 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 GATA4 and GATA5 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.

We are grateful to Dr L. Chalifour for generously providing the TC13 cell line, to Lise Laroche for secretarial assistance, and to members of the Nemer laboratory for discussions and suggestions. This work was supported by a grant from the Canadian Institutes of Health Research (MOP-36382). GN was recipient of a GIBCO-IRCM and a Boehringer Ingelheim Canada studentship; MN holds a Canada Research Chair in Molecular Biology.

REFERENCES

al Moustafa, A. E. and Chalifour, L. E. (1993). Immortal cell lines isolated from heart differentiate to an endothelial cell lineage in the presence of retinoic acid. *Cell Growth Diff.* **4,** 841-847.

Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Soults, J., Grayzel, D., Kroumpouzou, E., Traill, T. A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J. G. and Seidman, C. E. (1997). Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat. Genet.* **15,** 30-35.

Beals, C. R., Clipstone, N. A., Ho, S. N. and Crabtree, G. R. (1997). Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin- sensitive intramolecular interaction. *Genes Dev.* **11,** 824-834.

Biben, C., Weber, R., Kesteven, S., Stanley, E., McDonald, L., Elliott, D. A., Barnett, L., Koentgen, F., Robb, L., Feneley, M. and Harvey, R. P. (2000). Cardiac septal and valvular dysmorphogenesis in mice heterozygous for mutations in the homeobox gene Nkx2-5. *Circ. Res.* **87,** 888-895.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E. and Seidman, J. G. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106,** 709-721.

Burch, G. H., Bedolli, M. A., McDonough, S., Rosenthal, S. M. and Bristow, J. (1995). Embryonic expression of tenascin-X suggests a role in limb, muscle, and heart development. *Dev. Dyn.* **203,** 491-504.

Calligaris, R., Bottardi, S., Cogoi, S., Apezteguia, I. and Santoro, C. (1995). Alternative translation initiation site usage results in two functionally distinct forms of the GATA1 transcription factor. *Proc. Natl. Acad. Sci. USA* **92,** 11598-11602.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435-439.

Chan-Thomas, P. S., Thompson, R. P., Robert, B., Yacoub, M. H. and Barton, P. J. (1993). Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. *Dev. Dynam.* **197,** 203-216.

Charron, F. and Nemer, M. (1999). GATA transcription factors and cardiac development. *Sem. Cell Dev. Biol.* **10,** 85-91.

Chen, Y. and Solursh, M. (1995). Mirror-image duplication of the primary axis and heart in Xenopus embryos by the overexpression of Msx-1 gene. *J. Exp. Zool.* **273,** 170-174.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D. and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA4 with FOG cofactors. *Genes Dev.* **15,** 839-844.

de la Pompa, J. L., Timmerman, L. A., Takimoto, H., Yoshida, H., Elia, A. J., Samper, E., Potter, J., Wakeham, A., Marengere, L., Langille, B. L., Crabtree, G. R. and Mak, T. W. (1998). Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* **392,** 182-186.

Delorme, B., Dahl, E., Jarry-Guichard, T., Briand, J. P., Willecke, K., Gros, D. and Theveniau-Ruissy, M. (1997). Expression pattern of connexin gene products at the early developmental stages of the mouse cardiovascular system. *Circ. Res.* **81,** 423-437.

Dorfman, D. M., Wilson, D. B., Bruns, G. A. and Orkin, S. H. (1992). Human transcription factor GATA2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J. Biol. Chem.* **267,** 1279-1285.

Eisenberg, L. M. and Markwald, R. R. (1995). Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ. Res.* **77,** 1-6.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J. and Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380,** 439-442.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D. and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat. Genet.* **18,** 266-270.

Grépin, C., Dagnino, L., Robitaille, L., Haberstroh, L., Antakly, T. and Nemer, M. (1994). A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell. Biol.* **14,** 3115-3129.

Grépin, C., Nemer, G. and Nemer, M. (1997). Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA4 transcription factor. *Development* **124,** 2387-2395.

Harada, M., Itoh, H., Nakagawa, O., Ogawa, Y., Miyamoto, Y., Kuwahara, K., Ogawa, E., Igaki, T., Yamashita, J., Masuda, I., Yoshimasa, T., Tanaka, I., Saito, Y. and Nakao, K. (1997). Significance of ventricular myocytes and nonmyocytes interaction during cardiocyte hypertrophy: evidence for endothelin-1 as a paracrine hypertrophic factor from cardiac nonmyocytes. *Circulation* **96,** 3737-3744.

Iio, A., Koide, M., Hidaka, K. and Morisaki, T. (2001). Expression pattern of novel chick T-box gene, Tbx20. *Dev. Genes Evol.* **211,** 559-562.

Ito, E., Toki, T., Ishihara, H., Ohtani, H., Gu, L., Yokoyama, M., Engel, J. D. and Yamamoto, M. (1993). Erythroid transcription factor GATA1 is abundantly transcribed in mouse testis. *Nature* **362,** 466-468.

Kasahara, H., Lee, B., Schott, J. J., Benson, D. W., Seidman, J. G., Seidman, C. E. and Izumo, S. (2000). Loss of function and inhibitory effects of human CSX/NKX2.5 homeoprotein mutations associated with congenital heart disease. *J. Clin. Invest.* **106,** 299-308.

Kelley, C., Blumberg, H., Zon, L. I. and Evans, T. (1993). GATA4 is a novel transcription factor expressed in endocardium of the developing heart. *Development* **118,** 817-827.

Kraus, F., Haenig, B. and Kispert, A. (2001). Cloning and expression analysis of the mouse T-box gene tbx20. *Mech. Dev.* **100,** 87-91.

Kurihara, Y., Kurihara, H., Oda, H., Maemura, K., Nagai, R., Ishikawa,

T. and Yazaki, Y. (1995). Aortic arch malformations and ventricular septal defect in mice deficient in endothelin-1. *J. Clin. Invest.* **96,** 293-300.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. and Evans, T. (1994). GATA4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269,** 23177-23184.

Lee, M. E., Temizer, D. H., Clifford, J. A. and Quertermous, T. (1991). Cloning of the GATAbinding protein that regulates endothelin-1 gene expression in endothelial cells. *J. Biol. Chem.* **266,** 16188-16192.

Lee, R. K., Stainier, D. Y., Weinstein, B. M. and Fishman, M. C. (1994). Cardiovascular development in the zebrafish. II. Endocardial progenitors are sequestered within the heart field. *Development* **120,** 3361-3366.

Liao, W., Bisgrove, B. W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D. J. and Stainier, D. Y. (1997). The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. *Development* **124,** 381-389.

Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor mef2c. *Science* **276,** 1404-1407.

Lough, J. and Sugi, Y. (2000). Endoderm and heart development. *Dev. Dyn.* **217,** 327-342.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. *Genes Dev.* **9,** 1654-1666.

MacNeill, C., Ayres, B., Laverriere, A. C. and Burch, J. B. (1997). Transcripts for functionally distinct isoforms of chicken GATA5 are differentially expressed from alternative first exons. *J. Biol. Chem.* **272,** 8396-8401.

MacNeill, C., French, R., Evans, T., Wessels, A. and Burch, J. B. (2000). Modular regulation of cGATA5 gene expression in the developing heart and gut. *Dev. Biol.* **217,** 62-76.

Matsumoto, K., Saga, Y., Ikemura, T., Sakakura, T. and Chiquet-Ehrismann, R. (1994). The distribution of tenascin-X is distinct and often reciprocal to that of tenascin-C. *J. Cell Biol.* **125,** 483-493.

Mikawa, T., Cohen-Gould, L. and Fischman, D. A. (1992). Clonal analysis of cardiac morphogenesis in the chicken embryo using a replication-defective retrovirus. III: Polyclonal origin of adjacent ventricular myocytes. *Dev. Dynam.* **195,** 133-141.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R. and Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93,** 215-228.

Molkentin, J. D., Tymitz, K. M., Richardson, J. A. and Olson, E. N. (2000). Abnormalities of the genitourinary tract in female mice lacking GATA5. *Mol. Cell Biol.* **20,** 5256-5260.

Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Habara-Ohkubo, A., Nakaoka, T., Fujita, T., Yazaki, Y. and Komuro, I. (1999). Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA4. *Mol. Cell. Biol.* **19,** 7096-7105.

Morrisey, E. E., Ip, H. S., Tang, Z. H., Lu, M. M. and Parmacek, M. S. (1997). GATA5 – a transcriptional activator expressed in a novel temporally and spatially-restricted pattern during embryonic development. *Dev. Biol.* **183,** 21-36.

Nemer, G., Qureshi, S. A., Malo, D. and Nemer, M. (1999). Functional analysis and chromosomal mapping of *GATA5*, a gene encoding a zinc finger DNA-binding protein. *Mamm. Genome* **10,** 993-999.

Pan, J. and McEver, R. P. (1993). Characterization of the promoter for the human P-selectin gene. *J. Biol. Chem.* **268,** 22600-22608.

Pehlivan, T., Pober, B. R., Brueckner, M., Garrett, S., Slaugh, R., van Rheeden, R., Wilson, D. B., Watson, M. S. and Hing, A. V. (1999). GATA4 haploinsufficiency in patients with interstitial deletion of chromosome region 8p23.1 and congenital heart disease. *Am. J. Med. Genet.* **83,** 201-206.

Peng, J., Zhang, L., Drysdale, L. and Fong, G. H. (2000). The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. *Proc. Natl. Acad. Sci. USA* **97,** 8386-8391.

Puri, M. C., Partanen, J., Rossant, J. and Bernstein, A. (1999). Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. *Development* **126,** 4569-4580.

Ramsdell, A. F. and Markwald, R. R. (1997). Induction of endocardial cushion tissue in the avian heart is regulated, in part, by TGFbeta-3-mediated autocrine signaling. *Dev. Biol.* **188,** 64-74.

Ranger, A. M., Grusby, M. J., Hodge, M. R., de la Brousse, F. C., Gravallese, E. M., Hoey, T., Mickanin, C., Baldwin, H. S. and Glimcher, L. H. (1998). The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* **392,** 186-190.

Reiter, J. F., Alexander, J., Rodaway, A., Yelon, D., Patient, R., Holder, N. and Stainier, D. Y. (1999). Gata5 is required for the development of the heart and endoderm in zebrafish. *Genes Dev.* **13,** 2983-2995.

Robb, L., Mifsud, L., Hartley, L., Biben, C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Harvey, R. P. (1998). epicardin: A novel basic helix-loop-helix transcription factor gene expressed in epicardium, branchial arch myoblasts, and mesenchyme of developing lung, gut, kidney, and gonads. *Dev. Dyn.* **213,** 105-113.

Schattemann, G. C., Loushin, C., Li, T. and Hart, C. E. (1996). PDGF-A is required for normal murine cardiovascular development. *Dev. Biol.* **176,** 133-142.

Schott, J. J., Benson, D. W., Basson, C. T., Pease, W., Silberbach, G. M., Moak, J. P., Maron, B. J., Seidman, C. E. and Seidman, J. G. (1998). Congenital heart disease caused by mutations in the transcription factor NKX2-5. *Science* **281,** 108-111.

Schultheiss, T. M., Burch, J. B. and Lassar, A. B. (1997). A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev.* **11,** 451-462.

Shimizu, T., Kinugawa, K., Yao, A., Sugishita, Y., Sugishita, K., Harada, K., Matsui, H., Kohmoto, O., Serizawa, T. and Takahashi, T. (1999). Platelet-derived growth factor induces cellular growth in cultured chick ventricular myocytes. *Cardiovasc. Res.* **41,** 641-653.

Shivdasani, R. A., Fujiwara, Y., McDevitt, M. A. and Orkin, S. H. (1997). A lineage-selective knockout establishes the critical role of transcription factor GATA1 in megakaryocyte growth and platelet development. *EMBO J.* **16,** 3965-3973.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270,** 1995-1999.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M. L., Brown, D. and Olson, E. N. (1997). Regulation of cardiac mesodermal and neural crest development by the bhlh transcription factor, dhand. *Nat. Genet.* **16,** 154-160.

Stainier, D. Y., Weinstein, B. M., Detrich, H. W., III, Zon, L. I. and Fishman, M. C. (1995). Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* **121,** 3141-3150.

Tanaka, M., Chen, Z., Bartunkova, S., Yamasaki, N. and Izumo, S. (1999). The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. *Development* **126,** 1269-1280.

Tian, H., McKnight, S. L. and Russell, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11,** 72-82.

Timmerman, L. A., Healy, J. I., Ho, S. N., Chen, L., Goodnow, C. C. and Crabtree, G. R. (1997). Redundant expression but selective utilization of nuclear factor of activated T cells family members. *J. Immunol.* **159,** 2735-2740.

Ting, C. N., Olson, M. C., Barton, K. P. and Leiden, J. M. (1996). Transcription factor GATA3 is required for development of the T-cell lineage. *Nature* **384,** 474-478.

Weber, H., Symes, C. E., Walmsley, M. E., Rodaway, A. R. and Patient, R. K. (2000). A role for GATA5 in Xenopus endoderm specification. *Development* **127,** 4345-4360.

Wunsch, A. M., Little, C. D. and Markwald, R. R. (1994). Cardiac endothelial heterogeneity defines valvular development as demonstrated by the diverse expression of JB3, an antigen of the endocardial cushion tissue. *Dev. Biol.* **165,** 585-601.

Zhao, Y. Y., Sawyer, D. R., Baliga, R. R., Opel, D. J., Han, X., Marchionni, M. A. and Kelly, R. A. (1998). Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J. Biol. Chem.* **273,** 10261-10269.