

# Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor

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## SUMMARY

GATA-4 is a cardiac-specific member of the GATA family of zinc finger transcription factors. During embryogenesis, GATA-4 expression is detected very early in the cardiogenic area and persists later in the developing heart. Studies have shown that GATA-4 is a potent transcriptional activator of several cardiac muscle-specific genes and a key regulator of the cardiomyocyte gene program. Consistent with a role for GATA-4 in cardiomyocyte formation, inhibition of GATA-4 expression by antisense transcripts interferes with expression of cardiac muscle genes and blocks development of beating cardiomyocytes in P19 embryonic stem cells. In order to better define the function of GATA-4 in cardiogenesis, we have carried out molecular analysis of early stages of cardiomyocyte differentiation in GATA-4-deficient P19 cell lines and in P19 cells stably overexpressing GATA-4. The results indicate that GATA-4 is not

required for either endodermal or mesodermal commitment or for initiation of the cardiac pathway. However, in the absence of GATA-4, differentiation is blocked at the precardiac (cardioblasts) stage and cells are lost through extensive apoptosis. In contrast, ectopic expression of GATA-4 in P19 cells accelerates cardiogenesis and markedly increases (over 10-fold) the number of terminally differentiated beating cardiomyocytes following cell aggregation. Together, these findings suggest that, in addition to its role in activation of the cardiac genetic program, GATA-4 may be the nuclear target of inductive and/or survival factors for precardiac cells.

Key words: GATA factors, apoptosis, heart, embryonic stem cells, myocyte differentiation

## INTRODUCTION

A delicate balance between cell differentiation, proliferation and apoptosis is crucial for proper organogenesis (White, 1996; James, 1994), and cell-specific transcription factors, which play pivotal roles in cell fate determination, are critical for understanding the molecular pathways underlying cell behavior during organ formation. One such regulator of cardiac-muscle differentiation is the zinc-finger-containing protein GATA-4. Recently cloned as an activator of the B-type natriuretic peptide (BNP) promoter (Grépin et al., 1994), GATA-4 has since been shown to regulate the expression of many other cardiac genes including atrial natriuretic peptide (Grépin et al., 1994),  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) (Molkentin et al., 1994) and cardiac troponin C (cTpC) (Ip et al., 1994). During embryogenesis, GATA-4 transcripts are detected in cardiac cell progenitors (Heikinheimo et al., 1994; Jiang and Evans, 1996) and, in *Xenopus*, axis disruption experiments showed that expression of the GATA-4 gene is tightly linked to cardiomyocyte specification (Jiang and Evans, 1996). Consistent with a role for GATA-4 in heart development, inhibition of GATA-4 expression by antisense transcripts in the P19 stem cells blocks formation of beating cardiac muscle cells and abolishes transcription of terminal cardiac differentiation

markers (Grépin et al., 1995). Another cardiac muscle restricted transcription factor expressed at early stages of heart development is the homeodomain-containing protein Nkx-2.5 (Lints et al., 1993; Tonissen et al., 1994; Komuro and Izumo, 1993) whose transcriptional properties have been recently characterized (Chen and Schwartz, 1995; Durocher et al., 1996). Unlike GATA-4, Nk2.5/Csx does not appear to be required for cardiomyocyte differentiation but is rather implicated in later morphogenetic stages of heart formation as evidenced by gene inactivation experiments (Lyons et al., 1995). In addition to GATA-4 and Nkx-2.5, a limited number of transcription factors whose expression is enriched in the heart have been isolated. These include the MADS domain-containing proteins MEF2 (Yu et al., 1992; Pollock and Treisman, 1991; Edmondson et al., 1994), the homeobox MHOx (Cserjesi et al., 1992; Martin et al., 1995), the other GATA factors, GATA-5 and GATA-6 (Kelley et al., 1993; Hodgkinson et al., 1993), the basic helix-loop-helix (bHLH) proteins *eHand* and *dHand* (Cserjesi et al., 1995; Srivastava et al., 1995), and the LIM domain protein MLP (Arber et al., 1994). However, the exact function of these factors in the heart and their relationship to or regulation by other known cardiac transcription factors during cardiogenesis remain essentially undefined.

In order to identify the earliest stage at which GATA-4 is required for cardiomyocyte differentiation and to define the hierarchy of transcription factors during cardiac development, we characterized at the cellular and molecular level the cardiac differentiation profile of the wild-type pluripotent P19 cells and that of stable transfectants containing either a GATA-4 sense or antisense vector. Here we show that both endodermal and mesodermal commitments take place in GATA-4-deficient cell cultures as evidenced by the presence of markers of both lineages. However, GATA-4-deficient cells, which are unable to generate terminally differentiated cardiomyocytes (Grépin et al., 1995), contain a high proportion of cells undergoing apoptosis. Molecular analysis of various mesodermal and cardiac transcription factors suggest that, in the absence of GATA-4, cells are blocked at a cardioblast stage and the apoptotic program is activated presumably to eliminate the developmentally arrested cells. In contrast, overexpression of GATA-4 enhances cardiogenesis and greatly stimulates formation of terminally differentiated cardiomyocytes; however, this effect of GATA-4 requires cell aggregation suggesting that transcription factor GATA-4 may be the nuclear target of survival and differentiation signals for premyocardial cells (cardioblast). The data also establish for the first time a hierarchy of cardiac transcription factors during early cardiogenesis.

## MATERIALS AND METHODS

### P19 cell culture and in vitro differentiation

Maintenance and differentiation of wild-type (WT) P19S18 cells and GATA-4-deficient P19 cell lines were conducted in  $\alpha$  minimum essential medium ( $\alpha$ MEM) (Gibco Laboratories, Grand Island NY) supplemented with 7.5% bovine donor serum (Cansera, Canada) and 2.5% fetal bovine serum (Gibco). The detail for the generation of the GATA-4-deficient P19 cell lines have been described previously (Grépin et al., 1995). Briefly, undifferentiated WT P19 cells were stably transfected with a cytomegalovirus (CMV)-driven expression vector expressing the first 330 bp coding sequences of the GATA-4 cDNA in the antisense orientation or the full-length GATA-4 cDNA described previously (Grépin et al., 1994). After selection, several clones were isolated by the standard limiting dilution assay; three that were not expressing the GATA-4 protein were used for detailed analysis. 10 independent clones overexpressing GATA-4 were isolated from three different transfections/selections as well as two pools of 50 independent clones. GATA-4 overexpression in stably transfected clones was verified by gel shift analysis using nuclear extracts prepared from monolayer untreated cells. Clones were grown in the regular complete  $\alpha$ MEM media plus 50  $\mu$ g of G418 per ml to maintain the selection. Cell differentiation was conducted as previously described (Rudnicki and McBurney, 1987).  $10^5$  cells per ml were grown in suspension in bacteriological Petri dishes in medium containing 0.8% dimethyl sulfoxide (DMSO, Sigma, St Louis MO) or 1  $\mu$ M retinoic acid for cardiac or neuronal differentiation, respectively. After 4 days, cells formed aggregates, which were then transferred into tissue culture Petri dishes (Nunc) and maintained without any drug. Days of differentiation were numbered consecutively after the first day of aggregation.

### In situ terminal deoxynucleotidyl transferase assay

In order to detect early endonucleolysis specifically induced during apoptosis, DNA strand breaks in individual cells were labeled with digoxigenin-deoxyuridine triphosphate (dUTP) using exogenous terminal deoxynucleotidyl transferase. Labeled cells are revealed after

incubation with anti-digoxigenin-peroxidase-conjugated antibodies. Briefly, differentiating cells were harvested and fixed in suspension ( $5 \times 10^7$  cells per ml) for 10 minutes at room temperature in 4% neutral buffered formaline. 50-100  $\mu$ l of the cell suspension were dried on a microscope slide and the terminal deoxynucleotidyl transferase were performed according to the instructions of the manufacturer (Apop-toTag™ Oncor, Gaithersburg MD). The percentage of positive cells was obtained after counting at least 15 fields for each cell line.

### Electron microscopy

P19- and GATA-4-deficient cultures were aggregated and treated with 0.8% DMSO for 4 days and then fixed in 1.5% glutaraldehyde in phosphate-buffered buffer (pH 7.4) for 4 hours at room temperature. The cultures were then washed in phosphate buffer, dehydrated in ascending concentration of ethanol and included in Epon. Ultra thin sections were analyzed with a transmission electron microscope.

### RNA extraction and analysis

Total cellular RNA was extracted by the thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Transcripts were detected by semi-quantitative reverse transcription-PCR (RT-PCR) and the specificity of the amplified product was confirmed following Southern blot and hybridization to a corresponding cDNA or internal oligonucleotide probe as described previously (Grépin et al., 1995). The PCR conditions were 94°C for 1 minute, 54°C to 61°C for 1 minute depending on the melting temperature ( $T_m$ ) of the primers and 72°C for 1.5 minutes, repeated for 30 cycles. The quantity of cDNA used in each PCR reaction was chosen in the linear range of the signal according to a dose-response curve carried out for each set of primers. The following pairs of primers were designed, when possible, to encompass an intron to avoid false positive signals due to contamination by genomic DNA: 5' TCA GCT GTC CGA GTC CAA ATC GCT 3' and 5' CTG CAG CTG CTC AAC CAG CTG CAC TGT 3' for goosecoid (Blum et al., 1992); 5' ACT TGG CTC TTC GGT TCT GA 3' and 5' TGA TTC GAG CGG GAA GAG 3' for MHOX (Cserjesi et al., 1992); 5' GCC CTG AGT CTG AGG ACA AG 3' and 5' ATC GTG TTC TTG CTG CCA G 3' for MEF2 (Martin et al., 1993); 5' CCT CTA GAG CAG AGC TGC GCG CGG AGA TG 3' and 5' GGT GGC TTC CGT CGC CGC CGT GC 3' for Nkx-2.5 (Komuro and Izumo, 1993; Lints et al., 1993); 5' TCA GTG CAG CCT ATG ACT GC 3' and 5' CGG CAG ATT GAC TTT GGC 3' for MLP (Arber et al., 1994); 5' CAG CAT GCA CCC TAC GCA A 3' and 5' CCG CTG CTC TGC TCA AAG A 3' for Msx-1 (Kuzuoka et al., 1994); 5' CAA GTT CCG CCA GAA ACA G 3' and 5' ATG GTA GAT GCC ATA TCC AAC C 3' for Msx-2 (Bell et al., 1993). Conditions for GATA-4, tubulin and other cardiac markers have been previously described (Grépin et al., 1995).

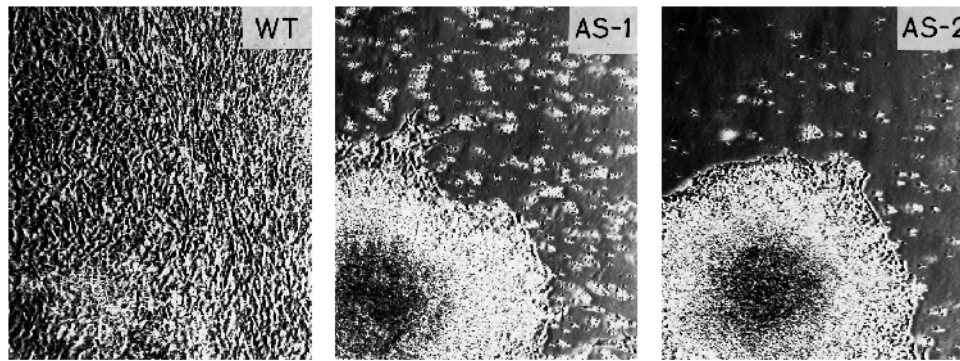
### Immunocytochemistry

P19 cell cultures were dispersed mechanically and plated in four-chamber plastic slides. After cells attached (2 to 3 hours), they were fixed in 4% paraformaldehyde and processed as previously described (Grépin et al., 1995). The rat monoclonal TROMA-1 antibody, which recognizes the endodermal-specific cytokeratin-55 (EndoA) (Vasseur et al., 1985), is a generous gift of Dr McBurney (University of Ottawa, Canada).

## RESULTS

### Programmed cell death occurs specifically after induction of cardiac differentiation in GATA-4-deficient P19 cells

To further characterize the cellular and molecular events occurring during DMSO-induced differentiation of P19 cells lacking functional GATA-4 protein, two independent stable transfectants (AS-1 and AS-2) expressing a GATA-4 antisense



**Fig. 1.** DMSO-treated aggregates of GATA-4-deficient P19 cells show extensive cell death. Phase-contrast photomicrograph of live cultures showing morphologies of WT and two GATA-4 antisense transfectants (AS-1 and AS-2) at day 7 after DMSO-induced differentiation. At this stage, WT P19 aggregates are well spread and contain differentiated cells including beating cardiomyocytes. Note how AS-1 and AS-2 aggregates are poorly spread and display a high proportion of rounded and very refringent cells, some of which are floating. A similar morphology is observed at days 5 and 6. Magnification is  $\times 100$ .

transcript were analyzed. As previously reported (Grépin et al., 1995), these transfectants do not contain GATA-4 protein and are unable to form beating cardiomyocytes expressing terminal differentiation markers. In fact, as shown in Fig. 1, on day 6 following DMSO treatment, when aggregates from WT P19 cells are well spread and contain already beating clusters, aggregates from AS-1 and AS-2 cell lines are poorly spread and show extensive evidence of cell death (about 25%) as quantitated by fluorescence activated cell sorter (FACS) analysis following propidium iodide staining. However, the retinoic acid (RA)-induced differentiation of WT and mutant cell lines into neuroectoderm derivatives is indistinguishable (Grépin et al., 1995). Thus, the dead cells observed in the GATA-4-deficient cultures are characteristic of cells produced upon DMSO treatment. DNA isolated from DMSO-treated WT and GATA-4-deficient P19 cell cultures revealed, in the mutant but not in the WT P19 cultures, evidence of DNA fragmentation typical of apoptotic cells (data not shown). To confirm and quantitate the presence of apoptosis, terminal deoxynucleotidyl transferase assay was performed. As shown in Fig. 2A, black labelled nuclei indicative of cells in which endonucleolytic cleavage has occurred were detected only in DMSO-treated GATA-4-deficient P19 cultures as assayed by the TUNEL method. In fact, by day 5 of differentiation, 25-35% of cells were positive (Fig. 2B) suggesting that most cell death likely occurs by apoptosis. In order to confirm these results, ultrastructural analysis was performed on the same DMSO-treated cultures. Fig. 2C shows that AS-1 and AS-2 cells consist mostly of cells with dark nuclei reflecting clumped dense chromatin and extensive evidence of nuclear and cytoplasmic fragmentation compared to the WT intact cells. Together, these data show that apoptosis specifically occurs in DMSO-treated cultures of GATA-4-deficient P19 cells as soon as day 4 (the earliest time point examined), well before the appearance of markers of terminal cardiomyocyte differentiation.

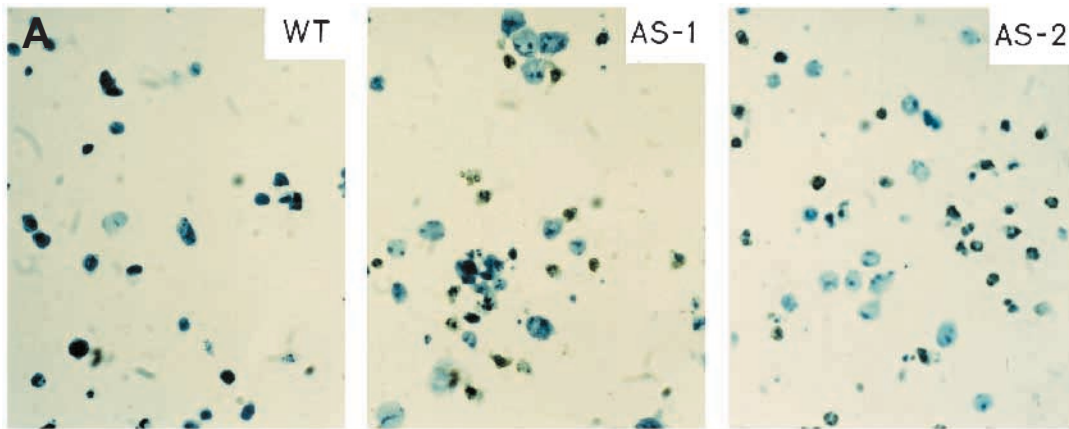
#### **Cardiomyocyte differentiation is specifically arrested in GATA-4-deficient P19 cells**

We then analyzed whether the absence of GATA-4 was affecting the differentiation potential of DMSO-treated P19 cells or whether it was specifically blocking the development

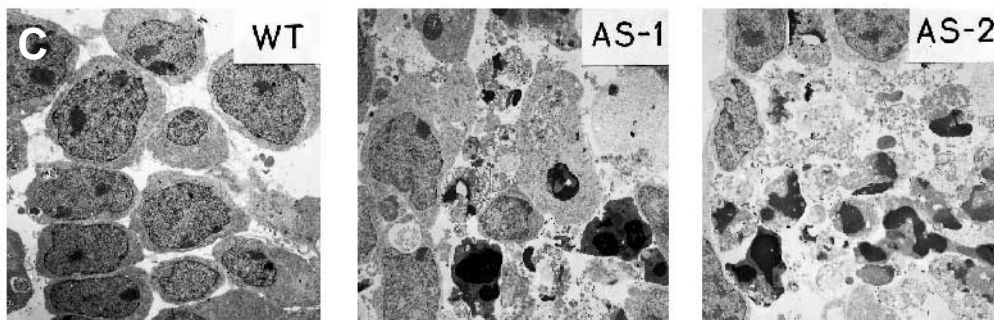
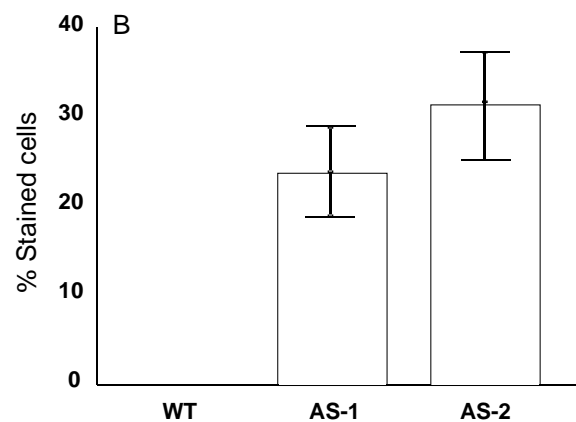
of the mesodermal lineage, which normally gives rise to cardiomyocytes. It has been previously shown that aggregation and treatment of P19 cells with DMSO gives rise to endodermal and mesodermal derivatives whereas RA treatment produces neuroectodermal derivatives (McBurney et al., 1982; Jones-Villeneuve et al., 1982; Edwards et al., 1983). We tested whether lack of GATA-4 affected endodermal differentiation. Fig. 3 shows that both GATA-4-deficient P19 cell lines retain the ability to differentiate into endodermal cells as evidenced by the detection of the endoderm-specific cytokeratine 55 (endoA) (Vasseur et al., 1985) by immunohistochemistry, the number of endoA-positive cells not being significantly different between WT and antisense clones. The absence of cardiomyocyte formation in GATA-4-deficient stem cells has also been reported in ES cells harboring disruptions of both alleles of the *GATA-4* gene (Soudais et al., 1994). However, and in contrast to the present work, the same authors have claimed that absence of GATA-4 disrupts visceral endoderm formation during *in vitro* ES cell differentiation (Soudais et al., 1995) as judged by the absence of the HNF-4 transcription factor. Unfortunately, the presence of either primitive endoderm or parietal endoderm was not tested in this system in part because the culture conditions apparently do not promote formation of parietal endoderm; when culture conditions were changed, endoderm formation was restored in GATA-4 null ES cells (Bielinska and Wilson, 1995.). Thus, and consistent with the role of GATA-4 in ES cell-derived embryoid bodies, lack of GATA-4 expression specifically inhibits cardiomyocyte formation in P19 cells without affecting other parallel (neuronal) or simultaneous (endodermal) differentiation pathways.

#### **GATA-4 absence blocks cardiac differentiation at the premyocardial (cardioblast) stage**

Next, we tested whether mesoderm commitment could still occur in the absence of GATA-4. The genes encoding the transcription factors brachyury T (Kispert et al., 1995) and gooseoid (Blum et al., 1992) have been shown to be transiently expressed in the primitive streak cells and are subsequently restricted to early mesoderm before being extinguished in the differentiated mesoderm-derived cells. Fig. 4A and B



**Fig. 2.** (A) DMSO-treated GATA-4-deficient P19 cultures undergo extensive apoptosis. WT- or GATA-4-deficient transfectants (AS-1 and AS-2) were mechanically dispersed and fixed in solution as described in Materials and Methods for the TUNEL assay. Apoptotic cells are identified by a dense black staining compared to negative blue counterstained cells. The micrographs represent DMSO-treated cells taken at day 5. Magnification is  $\times 400$ . (B) Quantification of apoptotic cells. At least 15 fields were counted for WT and AS-1 and AS-2 lines. The results are expressed as the mean ( $\pm$ s.d.). (C) Ultrastructure of WT- and GATA-4-deficient P19 cells after 4 days of DMSO treatment. Aggregates were fixed and embedded in Epon as described in Materials and Methods before examination with a transmission electron microscope. Note the presence of features of apoptosis such as nuclear condensation and fragmentation only in AS-1 and AS-2 but not in WT cells. Magnification is  $\times 2500$ .



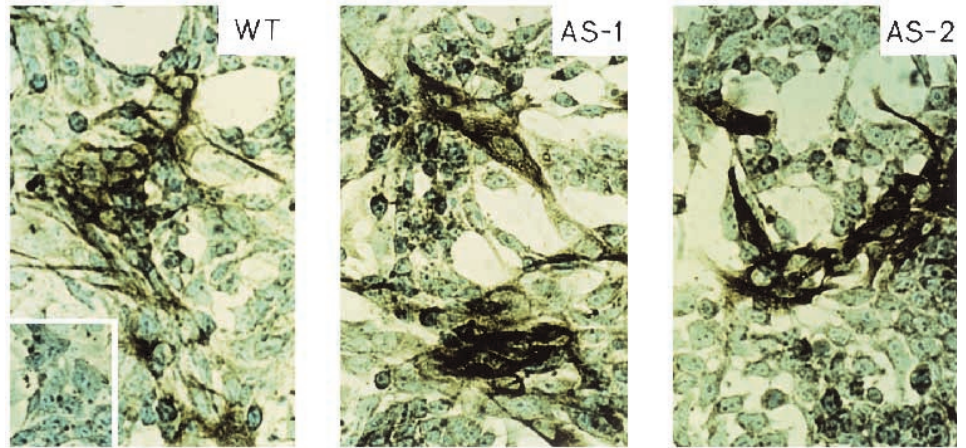
indicate that brachyury and gooseoid are transiently expressed in WT P19 cells induced with DMSO at day 1, 2 and 3 for brachyury, and at day 3 for gooseoid. In agreement with the *in vivo* observations, those two transcription factors are not expressed in undifferentiated P19 cells (EC) but are rapidly induced at the onset of mesoderm induction. Brachyury and gooseoid are both expressed, at least at a similar level, in GATA-4-deficient cultures (AS-1 and AS-2) indicating that the absence of GATA-4 does not interfere with mesoderm commitment. Interestingly, *brachyury* gene expression persisted in the mutant cultures at day 8 instead of being downregulated as in the WT upon terminal differentiation. These results are consistent with a role for GATA-4 in differentiation of committed mesodermal cells.

We then investigated the exact stage at which the GATA-4-deficient cells were blocked along the cardiac differen-

tiation pathway. Genes that are expressed early in the cardiogenic region of the embryo include *MEF2C* (Edmondson et al., 1994; Martin et al., 1993; Lints et al., 1993), *MLP* (mouse LIM protein) (Arber et al., 1994), *MHox* (Cserjesi et al., 1992), *Csx/Nk2.5* (Komuro and Izumo, 1993; Lints et al., 1993) and *GATA-4* (Heikinheimo et al., 1994). Two other homeobox genes, *Msx-1* and *Msx-2*, are also expressed in the developing heart (Chan-Thomas et al., 1993) and *Msx-1* expression is found in the lateral mesoderm at early stages of murine and avian embryogenesis (Hill et al., 1989; Robert et al., 1989). The time course of expression of these genes during normal *in vitro* cardiac differentiation of P19 cells was established using semiquantitative RT-PCR antisense. Fig. 5A shows that *MEF2C* appears to be expressed constitutively during P19 cardiac differentiation although it is upregulated at day 6, a time point that characterizes appearance of



**Fig. 3.** Absence of GATA-4 does not interfere with endoderm differentiation. Immunocytochemical staining of WT and the two GATA-4 antisense transfectants (AS-1 and AS-2) at day 7 of DMSO-induced differentiation with the TROMA-1 monoclonal antibody which recognizes the endodermal-specific cytokeratine 55 (endoA). Aggregates were mechanically dispersed and replated before fixation and staining as described in Materials and Methods. Note the presence of similar number of TROMA-1-positive cells in WT and AS1 and AS-2 cultures. The inset is a negative control. Magnification of all photographs is  $\times 400$ .

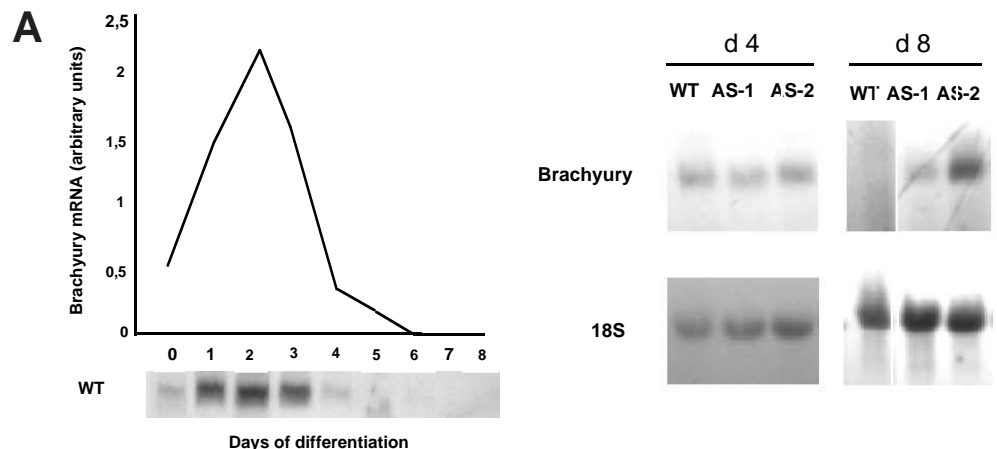


terminal cardiomyocyte markers (Grépin et al., 1995). In contrast, *GATA-4*, *MHox*, *Msx-1* and *Msx-2*, *Nkx-2.5* and *MLP* genes are induced at early stages of differentiation. In order to assign a stage-specific site of GATA-4 action, we analyzed the expression of these early markers in GATA-4-deficient cultures. As shown in Fig. 5B, transcription of *MEF2C*, *Msx-1* and *Nkx-2.5* in the antisense clones is significantly reduced while that of *MHox*, *Msx-2* and *MLP* is undetectable. Thus, in the absence of GATA-4, the stem cells differentiate into mesoderm and initiate the cardiogenic pathway but are unable to proceed beyond a precardiac (cardioblast) stage.

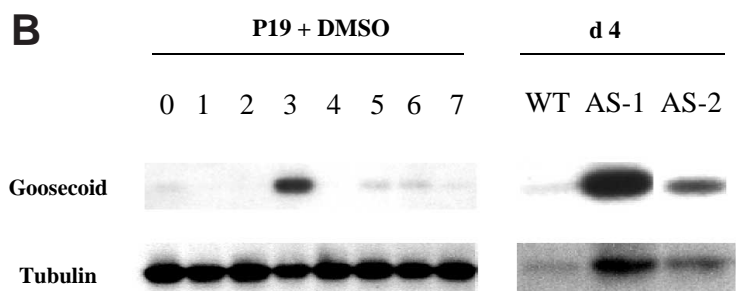
#### Ectopic expression of GATA-4 potentiates cardiomyocyte formation

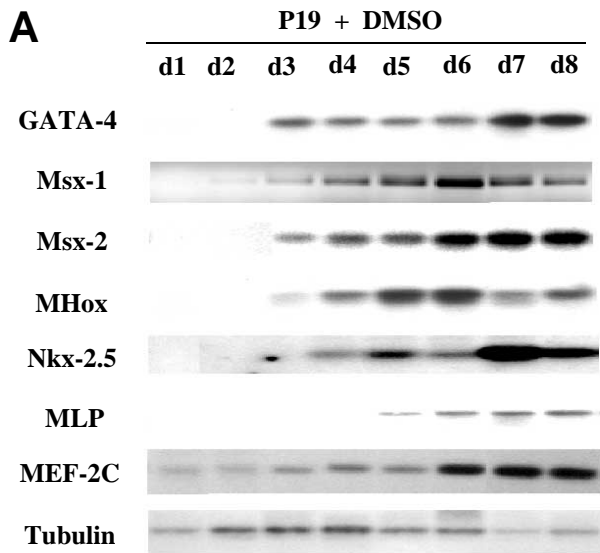
In order to confirm the role of GATA-4 in cardiomyocyte differentiation, we performed gain-of-function experiments by overexpressing GATA-4 in P19 stem cells. Several independent clones as well as a pool of at least 50 clones were analyzed for their differentiation potential. P19-GATA-4-expressing cells did not show morphological or biochemical evidence of cardiomyogenesis when kept in monolayer cultures (data not shown). However, beating cardiomyocytes were evident in these cells upon aggregation in

the absence of any inducer whereas parental P19 cells or P19 cells transfected in RSV-neo did not produce any beating cells (Fig. 6A); moreover, when GATA-4-expressing clones were aggregated and treated with DMSO, beating foci appeared earlier, and their number was markedly increased (Fig. 6B). Ectopic expression of GATA-4 did not markedly alter cell number in these experiments but had a beneficial effect on cell aggregates that was especially obvious in the absence of DMSO. Indeed, when wild-type P19 cells are aggregated without any inducer, they show poor survival and, by day 5-6, most cells are detached and floating in the media. Interestingly, cell viability in the absence of inducer was rescued in P19 clones ectopically expressing GATA-4 (data not shown). The role of GATA-4 in potentiating cardiomyocyte differentiation was confirmed by immunocytochemical staining for muscle-

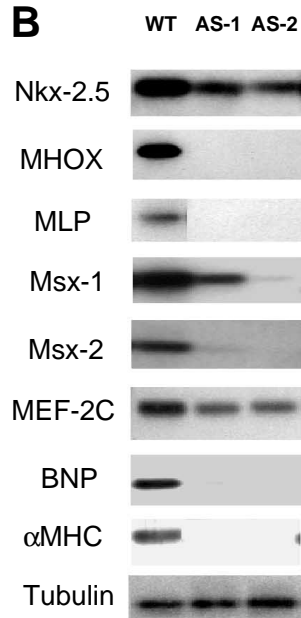


**Fig. 4.** Mesodermal commitment still occurs in the absence of GATA-4. (A) Northern blot analysis of *brachyury* gene expression upon DMSO treatment of WT and GATA-4 antisense clones (AS-1 and AS-2). The left panel shows the time course of brachyury expression by Northern blot analysis in WT P19 DMSO-treated cultures, and the right panel shows its expression at days 4 and 8 of differentiation in WT and antisense clones. (B) Similar analysis was performed for *goosecoid* gene expression except that the transcript levels were assessed by semiquantitative RT-PCR as described in Materials and Methods.

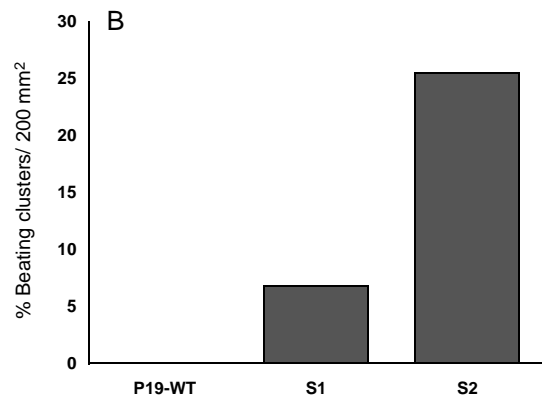
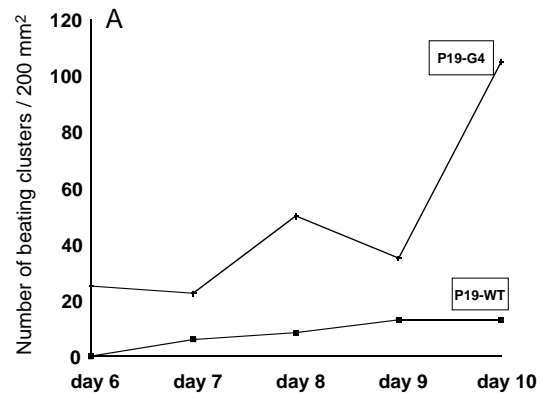




**Fig. 5.** Absence of GATA-4 blocks cardiomyocyte development at the cardioblast stage. (A) Expression of cardiac marker genes during *in vitro* cardiac differentiation of normal P19 cells. Differentiating WT P19 cultures were harvested at the indicated day (days 1 to 7) after DMSO treatment and semiquantitative RT-PCR was performed as described in Materials and Methods. Identical results were obtained for two independent differentiation experiments. (B) WT and GATA-4-deficient P19 cells were induced for cardiac differentiation by aggregation and DMSO treatment. RNA was harvested at day 7 and subject to semiquantitative RT-PCR. Three different batches of RNA from three different differentiation experiments gave the same results shown here.



specific myosin using the MF20 antibody. As shown in Fig. 7, GATA-4-expressing clones contained 13-16 times more MF20-positive cells than the parental P19 cells. Enhanced cardiomyogenesis was also evident by the significant increase of transcripts for several markers of cardiomyocyte differentiation (Fig. 8A) including transcription factors such as Nkx2.5, MLP and MHox, contractile proteins (cTpc,  $\beta$ MHC) and peptide hormones (BNP). Similarly, and consistent with the earlier appearance of beating cells, accelerated cardiogenesis was evidenced at the mRNA level by the earlier appearance of terminal differentiation markers particularly transcripts for the contractile proteins (Fig. 8B). Together, the data is consistent with an important role of GATA-4 in differentiation of mesodermal cells along the cardiogenic pathway.

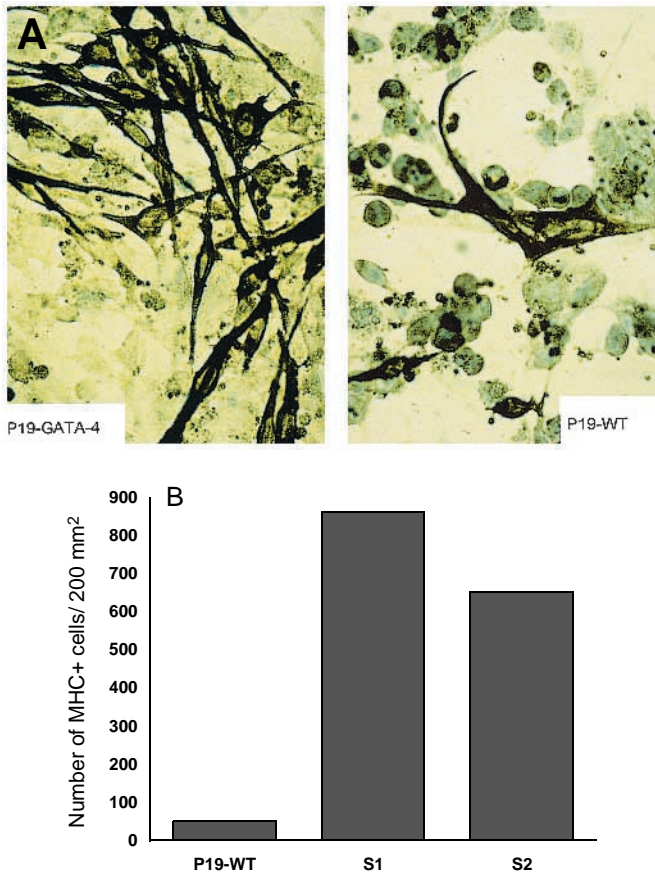


**Fig. 6.** Ectopic expression of GATA-4 in P19 cells increases the number of beating cardiocytes with (A) or without (B) DMSO treatment. In A, note the earlier appearance of beating cells in GATA-4 transfectants. The data shown in B are from one typical experiment using two different P19-GATA-4 clones (S1, S2). Typically 25-50 fields were counted independently by two observers. Similar results were obtained in 2-3 independent experiments using either the individual clones or a pool of about 50 clones.

## DISCUSSION

Heart formation is a crucial event for normal embryonic development and beating cardiac myocytes are one of the first differentiated cell types appearing during embryogenesis. As soon as the gastrulation starts, some of the primitive streak cells evolve into lateral plate mesoderm and migrate to the cardiogenic area of the gastrula. They irreversibly commit to the cardiac lineage to generate premyocardial cells or 'cardioblasts'. Those precardiocytes then terminally differentiate into beating cardiomyocytes, which proliferate and organize themselves to finally give rise to the mature four-chambered heart.

Unfortunately, little is known about the molecular signals that are involved in the specification and survival of cardioblasts. The data presented here indicate that transcription factor GATA-4 is required for survival of cardioblasts and terminal differentiation into mature cardiomyocytes. Moreover, the observation that GATA-4 overexpression potentiates cardiomyogenesis suggests that GATA-4 may be a key nuclear target for inductive and/or differentiation signals in committed cardiomyocyte precursors.

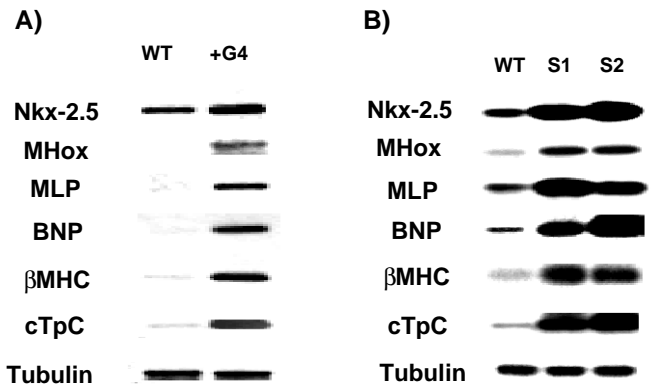


**Fig. 7.** Marked increase in myosin heavy chain (MHC)-positive cells in P19 transfectants overexpressing GATA-4.

(A) Immunohistochemical staining of parental (WT) and P19-GATA-4 clones using the MF-20 monoclonal antibody. The micrograph represents cells stained on day 5 after aggregation and treatment with the DMSO. (B) Quantification of the number of MHC+ cells in parental (P19-WT) and two P19-GATA-4 clones (S1, S2). MHC+ cells were counted by two independent observers and averaged. The results shown are from one typical differentiation experiment.

### Molecular hierarchy during early cardiomyogenesis

The analysis of early cardiac markers has allowed the establishment of a cascade of transcription factor expression which will undoubtedly prove useful in analyzing molecular events underlying cardiac phenotypes. In this respect, it is noteworthy to mention that the P19 system is especially amenable to establishing the time course of appearance of cardiac transcription factors because it is more homogenous than the only other available in vitro model of cardiogenesis consisting of ES-derived embryoid bodies. This is particularly important given that, with the exception of GATA-4 and Nkx-2.5, most transcription factors identified so far in the heart are also present in other muscle cells. The data obtained in this system using both gain and loss of GATA-4 function show that, except for Mef-2C which is constitutively present, GATA-4 is the first cardiac transcription factor to be induced and support its key role in cardiomyocyte differentiation. In particular, the results indicate that expression of GATA-4 precedes that of Nkx-2.5, a result consistent with the phenotype of the Nkx-2.5 null mice and ES cells that implicate this factor in later differentiation



**Fig. 8.** (A) Overexpression of cardiac markers in P19-GATA-4 transfectants. Semiquantitative RT-PCR was carried out as described in Material and Methods using RNA extracted from cells on day 7 after aggregation and DMSO treatment. (B) Consistent with the earlier appearance of beating cells, expression of terminal differentiation markers is also detected earlier in P19-GATA-4 transfectants using semiquantitative RT-PCR on day 4 of aggregation and DMSO treatment.

stages of the heart (Lyons et al., 1995); in fact, inactivation of Nkx-2.5 apparently did not affect expression of GATA-4 (R. Harvey, personal communication) confirming that GATA-4 lies upstream of Nkx-2.5 in cardiac hierarchy. This of course does not imply that GATA-4 is a direct activator of the *Nkx2.5* gene and does not exclude the possibility that GATA-4 and Nkx2.5 may be regulated independently of each other.

Finally, the inability of P19 cells lacking GATA-4 to form beating cardiomyocytes is consistent with the finding that GATA-4 null ES cells are also unable to differentiate into cardiomyocytes (Soudais et al., 1994). However, the same authors reported that GATA-4 null ES cells could contribute to a variety of organs in chimeric mice including the heart (Soudais et al., 1995); given that only whole hearts were analyzed, it is unclear whether GATA-4<sup>-/-</sup> ES cells contributed equally to all the different cell types of the heart. Nevertheless, it is possible that the other GATA factors expressed within the heart, especially GATA-6, could functionally compensate for the absence of GATA-4. This may not be observed in P19 cells that express very low levels of GATA-6 and only at later stages of cardiac differentiation (days 7 and 8). In fact, expression of GATA-6 is not induced in the GATA-4 antisense clones but is upregulated in P19 clones ectopically expressing GATA-4 while GATA-5 transcripts are unaffected by the level of GATA-4 protein (data not shown).

### Transcription factors as nuclear targets for inductive and/or survival signals

The observation that both GATA-4- and MyoD-overexpressing P19 cells still require cellular aggregation for terminal myogenic differentiation and that ectopic expression of C/EBP in NIH3T3 cells leads to adipogenesis only in presence of adipogenic inducers (Wu et al., 1995b), is consistent with a role for transcription factors as targets of inductive and/or differentiation signals. In addition, the apoptotic fate of precardiac P19 cells lacking GATA-4 also suggests a critical role in nuclear signaling of survival factors. In other systems, absence of tissue-specific transcription factors has also been associated

with developmental arrest and apoptosis of committed precursor cells. For example, inactivation of the Ets-1 transcription factor leads to increased rate of apoptosis of immature T cells *in vitro* and a marked decrease in the number of mature thymocytes and peripheral T cells *in vivo* (Muthusamy et al., 1995). Another hematopoietic transcription factor, the basic helix-loop-helix protein SCL/TAL-1, may also be required for survival of T cells since loss of SCL/TAL-1 activity in the Jurkat T cell line induces premature apoptosis (Leroy-Viard et al., 1995). Similarly, disruption of the *HNF-4* gene, which encodes a tissue-restricted transcription factor, causes extensive apoptosis of embryonic ectodermal cells (Chen et al., 1994) while mutation of the *Drosophila* muscle-specific Mef-2 factor leads to loss of myoblasts by apoptosis (Ranganayakulu et al., 1995). Most notably, the erythroid-specific GATA-1 factor, which is essential for terminal erythroid differentiation (Pevny et al., 1991), has been recently shown to be required for survival and maturation of erythroid precursors (Weiss and Orkin, 1995; Blobel and Orkin, 1996). Indeed, *in vitro* differentiation of GATA-1 null embryonic stem cells produces committed erythroid precursors that do not further mature and instead die of apoptosis (Weiss and Orkin, 1995); in addition, estrogen-induced apoptosis of erythroid progenitor cells involves direct inhibition of GATA-1 activity (Blobel and Orkin, 1996). Thus, GATA proteins might represent nuclear targets for survival factors. In *Drosophila*, two gene products have been shown to induce and/or maintain differentiation of precardiac cells; these two secreted molecules are the product of the segment polarity gene *wingless* (Wu et al., 1995a) and Decapentaplegic (*Dpp*), a member of the transforming growth factor  $\beta$  superfamily (Frasch, 1995). In vertebrates, members of the TGF $\beta$  family and other growth factors have been shown to be critical for mesoderm induction (Dawid, 1994; Klein and Melton, 1994; Sugi and Lough, 1995), and hormones and vitamins – such as glucocorticoids and vitamin A which, like estrogens act via nuclear receptors, are known to affect cardiomyocyte differentiation (Gudas, 1994; Hicks et al., 1982). Whether GATA-4 expression and/or activity is altered by these factors merits examination.

Finally, it is noteworthy that, although GATA-4 overexpression greatly enhanced cardiogenesis, cell aggregation was required for development of terminally differentiated cardiomyocytes. In fact, ectopic expression of GATA-4 leads to only subtle changes in untreated monolayer P19 cells; these included downregulation of stem cell markers such as transcription factor Oct3 and the stage-specific embryonic antigen-1 (SSEA-1) but no induction of cardiac markers was noted (data not shown). However, in GATA-4 overexpressing cells, cardiac differentiation could be achieved in presence or absence of DMSO, provided cells were first aggregated suggesting that GATA-4 may relay diffusible and/or cell-contact-generated signals required for cardiomyocyte differentiation. In this respect, it is important to highlight the striking similarity in the apparent mechanism of action of GATA-4 in cardiogenesis with that reported for MyoD in skeletal myogenesis in the P19 system (Skerjanc et al., 1994). Indeed, much like GATA-4, MyoD was ineffective in inducing myogenesis in the absence of cell aggregation and its ectopic expression enhanced terminal differentiation of committed mesodermal cells without altering neuroectoderm differentiation (Skerjanc et al., 1994). These data suggest that, in the

heart, GATA-4 may be a functional homolog of the myogenic basic helix-loop-helix (bHLH) proteins.

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