

Erratum

We apologise to the authors for an error in reproducing some of the figures. The whole article is reprinted in issue no. 9, replacing the one in issue no. 4.

M. Koutsourakis, A. Langeveld, R. Patient, R. Beddington and F. Grosveld. The transcription factor GATA6 is essential for early extraembryonic development.

The transcription factor GATA6 is essential for early extraembryonic development

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SUMMARY

The gene coding for the murine transcription factor GATA6 was inactivated by insertion of a β -galactosidase marker gene. The analysis of heterozygote *GATA6/lacZ* mice shows two inductions of GATA6 expression early in development. It is first expressed at the blastocyst stage in part of the inner cell mass and in the trophoctoderm. The second wave of expression is in parietal endoderm (Reichert's membrane) and the mesoderm and endoderm

that form the heart and gut. Inactivation leads to a lethality shortly after implantation (5.5 days postcoitum). Chimeric experiments show this to be caused by an indirect effect on the epiblast due to a defect in an extraembryonic tissue.

Key words: GATA, Embryonic, Lethality, Extraembryonic, Endoderm, Mouse

INTRODUCTION

The GATA zinc finger transcription factors play a crucial role in the development and differentiation of a number of tissues. These factors bind the basic consensus sequence A/TGATA/G through a conserved Cys-X₂-Cys-X₁₇-Cys-X₂-Cys zinc finger protein motif. Three members of the family, GATA1, GATA2 and GATA3, are all expressed in the haematopoietic system and a number of other (non-overlapping) tissues (Leonard et al., 1993). Each appears to have a different function in the haematopoietic system as the inactivation of each of the genes has shown a different phenotype (Pevny et al., 1991; Simon et al., 1992; Tsai et al., 1994; Weiss et al., 1994; Pandolfi et al., 1995; Ting et al., 1996). Three additional members of the GATA family, GATA4, GATA5 and GATA6, also show a partially overlapping expression pattern in the heart and the intestinal tract (Laverriere et al., 1994; Jiang and Evans, 1996). GATA4 is expressed as early as 7 dpc (days postcoitum) in the prospective cardiac splanchnic mesoderm (Arceci et al., 1993; Kelley et al., 1993; Heikinheimo et al., 1994) and continues to be expressed in the endocardium and the myocardium of the folding heart tube. It is finally expressed in the cardiac myocytes throughout development and adult life. Inactivation of the *GATA4* gene leads to defects in heart tube formation and ventral morphogenesis in vivo (Kuo et al., 1997; Molkenkin et al., 1997) and defective visceral endoderm formation by embryonic stem (ES) cells in vitro (Soudais et al., 1995). The cardiac expression of murine GATA5 is first detected in the precardiac mesoderm. It subsequently appears in the atrial and

ventricular chambers and becomes restricted to the atrial endocardium (Morrisey et al., 1997). The consequences of inactivation of the murine *GATA5* gene have not been fully described but the mice are viable indicating that it may share functions with GATA4 and GATA6 (Molkenkin et al., 1997). Murine GATA6 has been reported to be restricted to precardiac mesoderm, the embryonic heart tube and the primitive gut. It is also expressed in the developing respiratory and urogenital tracts, arterial smooth muscle cells, the bronchi, the urogenital tract and the bladder (Morrisey et al., 1996; Narita et al., 1996; Suzuki et al., 1996). In *Xenopus*, the expression of GATA6 has first been detected at the beginning of gastrulation in the mesoderm and subsequently in the precardiac cells (Gove et al., 1997). Overexpression of GATA6 in the cardiac cells at a time when its expression normally declines (i.e. before the appearance of terminally differentiated markers) results in arrest of cardiomyogenic differentiation, indicating that the *GATA6* gene may act in *Xenopus* to maintain the precursor status (Gove et al., 1997). Thus the available data indicates that GATA6 may be important for heart development. In order to address this question, we first investigated the expression pattern of the murine *GATA6* gene by homologous recombination of a *lacZ* reporter gene into the *GATA6* locus, which also enables us to generate a null mutation. The results show that the expression of the GATA6-driven *lacZ* gene in heterozygote mice is first detected before implantation in the blastocyst, followed by expression in the parietal endoderm just after gastrulation and subsequently in lateral plate mesoderm and the cells that will form the heart and the gut.

However, GATA6-deficient embryos die before heart formation, showing abnormalities at 5.5 dpc, due to an extraembryonic defect. Thus of all the mammalian GATA factors that have been studied to date (GATA1-GATA6), GATA6 appears to be the one that is required earliest in embryonic development.

MATERIALS AND METHODS

Construction of the targeting vectors

The mouse GATA6 cDNA was cloned by screening a mouse 11.5 dpc cDNA library (CLONTECH) with the human GATA6 cDNA as probe. One cosmid containing the mouse genomic locus was isolated from an 129 library using the cDNA probe. For the targeting vector, a 9.5 kb *KpnI* fragment containing the first ATG was subcloned into psp72 containing the PyEn-HSV thymidine kinase (*TK*) gene. The *lacZ*-PMC1NEO cassette (Nuez et al., 1995) was introduced as a *NotI* fragment in the same frame of the GATA6 cDNA into the unique *NotI* site, 79 nt downstream of the ATG (Fig. 1A). To target the second allele, the same construct was used but instead of the *lacZ*-PMC1NEO cassette the *hygromycin*-resistance gene under the PGK promoter was inserted.

ES cells transfection, analysis and differentiation

E14 ES cells were transfected, as described (Hendriks et al., 1996), with 20 µg *KpnI* linearized targeting vector (Fig. 1A). Selection (200 µg/ml G418 or 160 µg/ml hygromycin B and 0.2 mM FIAU) was applied 24 hours after transfection and resistant clones were picked 8 to 10 days later. 560 individual clones were assayed for β-galactosidase activity and the 41 β-gal positives were further analyzed by Southern blotting using 5' external and *lacZ* probe (Fig. 1B). 18 were homologous recombinants and a normal karyotype was confirmed for three of them. Double mutant ES cells were differentiated in vitro along with the parental heterozygous mutant clone according to standard protocols (Doetschman et al., 1985, Rohwedel et al., 1994) using mainly the suspension culture method.

Gel mobility-shift assays

1-week-old embryoid bodies were homogenized mechanically to obtain single-cell suspensions and whole-cell extracts were prepared as has been previously described (Meijer et al., 1990). Gel mobility shift experiments were performed as has been reported (Whyatt et al., 1993) using 1.2 and 1.4 µg of protein from wild-type and mutant cells extracts, respectively. For GATA binding, the high-affinity site of the C31T oligo (Whyatt et al., 1993) was used and for control competition the -200 oligo (deBoer et al., 1988) as well as the Sp1 oligo (Philipsen et al., 1990) were used. The antibody for GATA4 was kindly provided by David Wilson.

Generation of chimeric mice and embryos

Two independent targeted clones were injected into C57BL/6 host blastocysts (Robertson, 1987) and males highly chimeric for coat colour were mated to FVB and C57BL/6 females to generate mice heterozygous for the mutation. Double mutants ES cells were injected into C57BL/6 blastocysts, which were transferred to foster females. Recipients were killed on day 8 or 9 of pregnancy to dissect out the chimeric embryos. Wild-type ES cells were injected into blastocysts derived from heterozygous intercrosses and the chimeric embryos were dissected at 7.5 dpc.

Genotyping of the embryos

Embryos from heterozygous matings were dissected according to standard methods (Hogan et al., 1994) and part or all of the embryo, older or younger than 7.5 dpc, respectively, was used for PCR analysis, as described (Marin et al., 1997), using three primers (Fig.

1A). A sense primer in the *GATA6* gene 150 nt 5' to the *NotI* site (P1: 5'-AGCAAGCTGTTGTGGTCCAC-3'), an antisense 81 nt 3' to *NotI* (P2: 5'-TAACGCCAGGGTTTTCCAG-3'), resulting in a 231 and 375 bp fragment for the wild type and targeted allele, respectively (Fig. 1D).

Whole-mount in situ analysis

β-galactosidase activity was determined in embryonic materials, as described (Marin et al., 1997; Hogan et al., 1994), with fixation time varying from 1 minute for cells, embryoid bodies, blastocysts and blastocyst outgrowths to 3, 5, 12 and 30 minutes for 5.5, 7.5, 8.5 and 9.5 dpc embryos, respectively. After overnight staining at 37°C, the embryos were postfixed in the same fixative overnight at 4°C. For histological analysis, 5-7 µm sections were obtained from stained and postfixed materials, subsequent to paraffin embedding according to standard protocols but using isopropanol instead of xylene.

In vitro culture of blastocyst

Blastocysts were flushed out at day 3.5 of pregnancy in M2 medium and cultured in ES medium without LIF, in 5% CO₂ at 37°C, on gelatinized multichambered glass slides. After 5-9 days in culture, the slides were mounted in PBS for photography and subsequently the cells were scraped off and collected with a mouth pipette for PCR genotyping as described for young embryos.

FISH genotyping

At embryonic day 7.5, chimeric embryos were dissected out of their Reichert's membranes and both embryo and membrane were kept in small volume of PBS on ice. When dissection was completed, the PBS was spun out and the cells were exposed in hypotonic solution for 10 minutes prior to cytospin on glass slides (Haaf and Ward, 1994). The preparations were fixed in 75% methanol/25% acetic acid for 5 minutes and further processed for FISH as has been described (Mulder et al., 1995; Milot et al., 1996).

RESULTS

GATA6 inactivation by *lacZ* insertion

We first studied the pattern of expression of the *GATA6* gene prior to and during heart formation by placing a *lacZ* reporter gene under the control of the endogenous murine *GATA6* gene. This insertion also inactivated the gene coding for GATA6 protein (Fig. 1). The *lacZ*-neomycin resistance cassette was cloned in frame, 79 nucleotides downstream from the ATG start codon of the *GATA6* gene (*NotI* site), creating a cassette with 6.5 and 3 kb of 5' and 3' flanking sequence homologous to the *GATA6* gene and a *HSV-TK* gene for counterselection purposes (Hendriks et al., 1996). The plasmid was transfected into E14 ES cells and 560 clones were isolated after selection and counterselection (see Materials and Methods). It was expected that homologously recombined clones would stain positively for β-gal, since northern blot analysis showed that undifferentiated ES cells expressed GATA6 RNA (data not shown). Thus the 41 clones positive for β-gal staining were selected for further analysis. Southern blot analysis with probes external and internal to the transfected cassette (Fig. 1A,B, probes A and B) showed that 18 clones had undergone homologous recombination placing the *lacZ* gene under *GATA6* control. Homologous recombination results in two restriction fragments after a *ClaI* digest with the external probe A; the original 15 kb fragment and a novel 6.4 kb fragment created by the presence of a novel *ClaI* site in the *lacZ* gene (Fig. 1B, left panel). A *lacZ* probe (Fig. 1B, right panel) detects

the same 6.4 kb restriction fragment, while a non-homologous recombinant (+/+ lane) shows the 4 kb fragment internal to the vector between the *lacZ* *Cla*I site and the plasmid *Cla*I site (Fig. 1A, targeting construct and mutant GATA6). Although all 18 clones contained β -gal-positive cells, not all cells within a clone were stained. This may be because ES cell clones are a mixture of differentiated and undifferentiated cells or more interestingly that undifferentiated ES cells express GATA6 only part of the time or express only one of the two GATA6 alleles.

The *GATA6*^{+/-} embryonic stem cells were retransfected with a second homologous recombination vector. This vector was identical to the first vector with the exception that the *neo* selection marker was replaced by a *hygro* selection gene (Marin et al., 1997). Homologous recombinants were identified as described for the heterozygous knockout above, resulting in 10 clones of homozygous *GATA6*^{-/-} embryonic stem cells out of 96 clones analyzed. We used three different GATA6 antibodies (Nakagawa et al., 1997; Perlman et al., 1998 and Santa Cruz) to show the absence of GATA6 protein in *GATA6*^{-/-} cells. However, none of the antibodies was able to detect specifically GATA6 protein on western blots in wild-type cells. We therefore assayed GATA6-binding activity in vitro using a high-affinity GATA-binding site, C31T oligo (Whyatt et al., 1993) and protein extracts from one week in vitro differentiated ES cells (Fig. 1C). As expected, only one major shift was observed since GATA6-binding activity comigrates with that of GATA4 (Morrisey et al., 1996). In extracts from wild-type cells, this shift decreased significantly by the addition of either 0.5 or 1 μ l of anti-GATA4 antibody and resulted in a supershift (Arceci et al., 1993). The GATA shift was reduced significantly in the double mutant cell extract and disappeared almost completely by the addition of anti-GATA4 antibody while producing

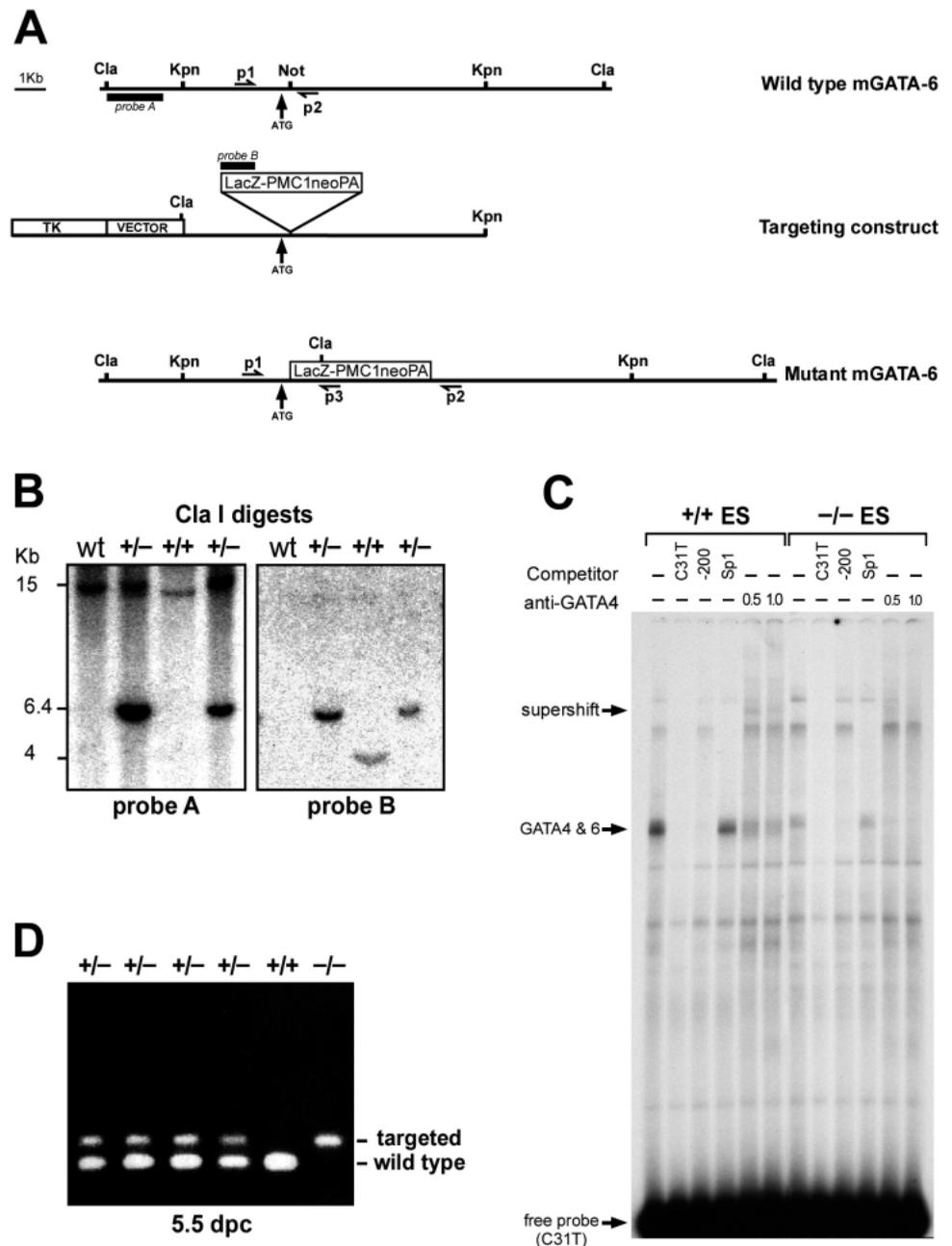
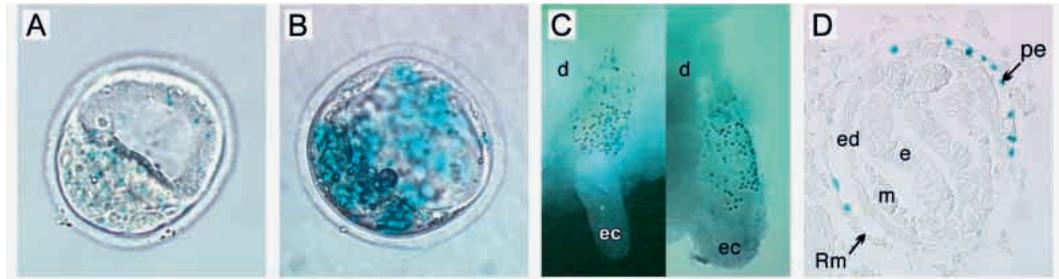


Fig. 1. Targeted disruption of the *GATA6* gene. (A) Schematic representation of the targeting strategy. Top line: Partial restriction map of the murine *GATA6* locus in which the ATG is indicated with the arrowhead. Middle line: Targeting vector containing the polyoma enhancer-herpes simplex virus thymidine kinase (*tk*) gene and the neomycin resistance gene under the mouse phosphoglycerate kinase (PGK) promoter following the *E. coli* β -galactosidase (*lacZ*) gene. Bottom line: The mutant *GATA6* locus resulting from the in frame insertion of the *LacZ*-NEO. (B) Southern blot analysis of *Cla*I-digested genomic DNA from neomycin resistant and β -gal-positive ES clones. A 5' external and a *lacZ* probe were used to distinguish homologous recombination events from random integration. (C) Gel retardation analysis of GATA DNA-binding activity in protein extracts from 1-week-old in vitro differentiated wild-type and mutant ES cells. The specificity of the protein-DNA complex is demonstrated by competition with a 100-fold molar excess of unlabeled oligo (C31T) or another GATA site (-200) or an Sp1-binding site. The GATA complex is inhibited by the addition of GATA4 antibody and results in a supershift. A GATA6 antibody also inhibited the formation of the complex (not shown), however it inhibited the formation of all other shifts including Sp1 and we concluded that this antibody is not specific for GATA6. A second antibody showed no activity in the shifts at all. (D) PCR genotyping of 5.5 dpc embryos from *GATA6*^{+/-} intercrosses using three primers, P1, P2 and P3, to simultaneously amplify both wild-type (231 bp) and targeted (375 bp) allele.

Fig. 2. Expression of *GATA6* gene, as detected by β -galactosidase activity, in early mouse embryos heterozygous for the *lacZ* insertion. (A,B) 3.5 dpc embryos, blastocysts stage, from heterozygote intercrosses with clear difference in the intensity of the staining, presumably corresponding to the presence of one (heterozygote mutant) or two (homozygote mutant) *lacZ* alleles respectively. Expression sites are both in the inner cell mass and in cells lining the blastocoel cavity. (C) A 7.0 dpc embryo exposed from the decidua, just after gastrulation. The only expression site is the parietal endoderm cells of the Reichert's membrane, (d) decidua and (ec) egg cylinder. (D) A 5 μ m transverse section of the embryo in C in which the three germ layers, (e) ectoderm, (m) mesoderm and (ed) endoderm with the surrounding Reichert's membrane (Rm) and the stained parietal endoderm cells (pe) are indicated.



a supershift. Thus *GATA6*-binding activity is absent or reduced drastically in the mutant cells (Fig. 1C). Addition of anti-*GATA1* antibody did not affect any of these complexes and anti-*GATA6* antibodies inhibited any binding (data not shown).

Both the *GATA6*^{-/-} and the *GATA6*^{+/-} ES cells were injected into blastocysts to obtain chimeric embryos and mice, respectively. *GATA6*^{+/-} chimeras were bred to FVB and C57/BL6 mice to obtain germline transfer of the *GATA6*-negative allele. *GATA6*^{+/+} and *GATA6*^{+/-} mice were distinguished by Southern analysis as described above for the ES cell lines, while PCR analysis with three primers (Fig. 1A,D, primers p1, p2 and p3) or in situ hybridization (Fig. 9) was used to genotype embryos.

Expression of *GATA6* prior to day 9.5 of development

First, we analyzed the expression pattern of the *lacZ* marker in heterozygote embryos. *lacZ* expression could be seen as early as the blastocyst stage (3.5 dpc) in agreement with the

expression data of *GATA6* in ES cells. This expression was zygotic since it was observed in crosses between *GATA6*^{+/-} males and wild-type females and also indicative of the genotype since mutant blastocysts showed much higher levels of *lacZ* expression than heterozygotes (Fig. 2B,A, respectively). Staining was evident in only a proportion of the cells of the inner cell mass, which corresponds with the heterogeneous staining seen in ES cells in culture indicating that staining is not uniform in equivalent cells. In addition, cells lining the abembryonic region of the blastocoel cavity were β -gal positive (Fig. 2A,B).

The expression of the *lacZ* reporter just prior to and during heart formation was analyzed in heterozygous embryos from 7.0 to 9.5 dpc. Dissection of the embryos at 7.0 dpc showed no β -gal staining in the embryo proper, but strong staining of the parietal endoderm cells on the inside of the partially or completely removed Reichert's membrane (Fig. 2C,D). At 7.5 dpc, *GATA6*-driven *lacZ* expression was visible in the mesoderm of a late primitive streak embryo (Fig. 3A) and

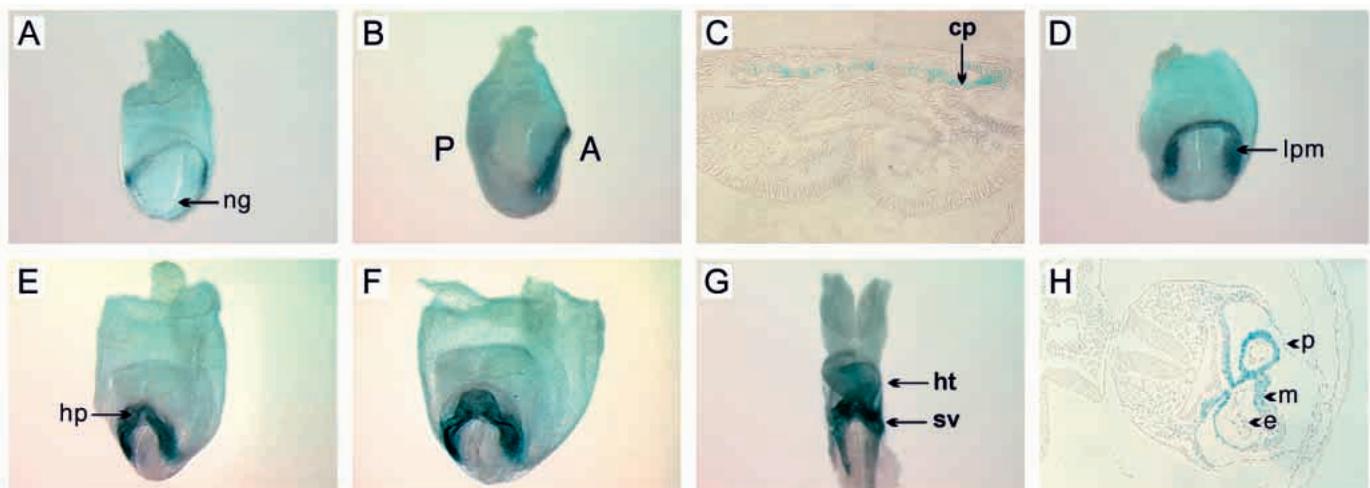


Fig. 3. Embryonic expression of the mouse *GATA6* gene, as demonstrated by β -galactosidase activity in mouse embryos heterozygous for the *lacZ* insertion in the *GATA6* locus. (A) An advanced primitive streak embryo at 7.5 dpc (ng, neural groove) shows mesodermal expression, which is better visualized at 8.0 dpc as part of the lateral plate mesoderm (lpm) (B,D). This mesoderm contains the cardiogenic plate (cp), which is β -gal positive as seen in a transverse section at the level of the head folds of an 8.0 dpc embryo in C. The expression persists as the two heart primordia (hp) are formed (E) and during their fusion in the midline to form the linear heart tube (F). At 8.5 dpc after the looping of the heart tube (ht), *GATA6* is still expressed along the heart tube but staining predominates in the two horns of the sinus venosa (sv) and in the developing foregut (G). Transverse section across the heart tube revealing that expression is restricted to only the myocardium (m) and pericardium (p), and is not evident in the endocardium (e) (H).

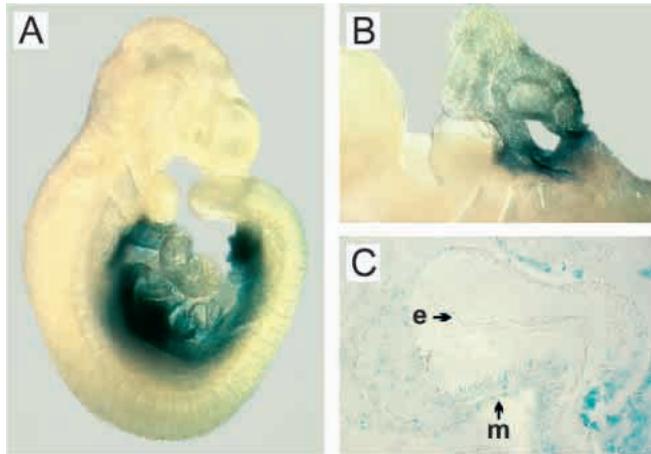


Fig. 4. *lacZ* expression in a *GATA6*^{+/-} embryo at embryonic day 9.5. (A) Whole-mount β -gal staining. Expression persists in the heart tube, mainly in the inflow and outflow tracts, and very strong expression can be seen in the foregut and hindgut as well as in the midgut. (B) Higher magnification of the heart tube demonstrating strong expression in the inflow and outflow tracts, but as shown in C, the myocardium of the whole heart tube is still expressing *GATA6*.

subsequently staining was observed in the lateral plate mesoderm, which contains the cardiogenic plate (Fig. 3B-D). Expression persisted as the two heart primordia formed and fused in the midline to form the heart tube (Fig. 3E,F). After looping of the heart tube (8.5 dpc), *GATA6*-driven *lacZ* was primarily expressed in the two horns of the sinus venosa and in the foregut (Fig. 3G). A transverse section of the heart tube shows that expression is restricted to the myocardium and pericardium, but absent in the endocardium (Fig. 3H). At 9.5 dpc, expression was still observed in the heart tube, particular in the inflow and outflow tracts (Fig. 4A,B). The myocardium, but not the endocardium of the heart tube, was still positive (Fig. 4C). The gene was also strongly expressed in the foregut and hindgut and to a lesser extent in the midgut (Fig. 4A).

The absence of *GATA6* leads to an early lethal defect

Intercrossing *GATA6*^{+/-} mice failed to produce any live born *GATA6*^{-/-} mice in 62 live offspring (mice derived from two different ES cells clones were tested). Genotyping embryos from as early as 6.5 dpc also showed no *GATA6*^{-/-} embryos (Table 1). *GATA6* null embryos could be found at 5.5 dpc (4 out of 20) and earlier (day 4.5 embryos and day 3.5

Table 1. Genotypes resulting from *GATA6* heterozygous mutant matings

	+/-	+/+	Total number
live born	37 (60)	25 (40)	62
9.5 dpc	24 (60)	16 (40)	40
8.5 dpc	64 (58)	47 (42)	111
7.5 dpc	6 (60)	4 (40)	10
6.5 dpc	86 (65)	47 (35)	133*

*Embryos were collected from 11 *GATA6*^{+/-} females and 1-3 empty decidua were found per litter.

blastocysts). We therefore analyzed the expression of the reporter gene during the phase of peri-implantation lethality. At 4.5 dpc just prior to implantation, β -gal staining is restricted to a population of cells on the blastocoelic surface of the inner cell mass adjacent to the trophoctoderm (Fig. 5A). However, we have been unable to confirm that *GATA6* is expressed at 4.5 dpc by whole-mount in situ hybridization. It is therefore possible that the β -gal staining at this stage is a left over from the expression at the blastocyst. After implantation, at 5.5 dpc, β -gal staining was not detectable in heterozygous embryos derived from heterozygous outcrosses. Expression could only be detected at this stage in a subset of embryos obtained from heterozygote intercrosses. In such crosses, a minority of the embryos that were invariably much smaller than their littermates showed a very specific β -gal staining in one or a very few cells (Fig. 5B,C). These retarded embryos were presumed to be *GATA6* null embryos, although we were unable to confirm their genotype by standard PCR after the embryos had been fixed and analyzed for the presence of β -gal staining. Sectioning of 5.5 dpc embryos revealed that a number of them lacked part of the visceral endoderm and showed abnormal development of the embryonic ectoderm that normally underlies this part of the endoderm (Fig. 6).

Differentiation and *lacZ* expression were also investigated in blastocysts flushed from heterozygous intercrosses at 3.5 dpc and cultured under differentiating conditions (Suzuki et al., 1997). After 5-9 days, the cultures were analyzed for cell type and genotyped by PCR. In the early phases of culturing wild-type, heterozygote and homozygote *GATA6* null blastocysts, all developed normally with attachment and outgrowth of the trophoblast cells. However, in the *GATA6* null blastocysts, growth of the inner cell mass was severely impaired and, after 5 days, the ICM remained very small and its cells tended to disperse (Fig. 7). After 9 days in culture, these cells detached and disappeared (data not shown). Development of small,

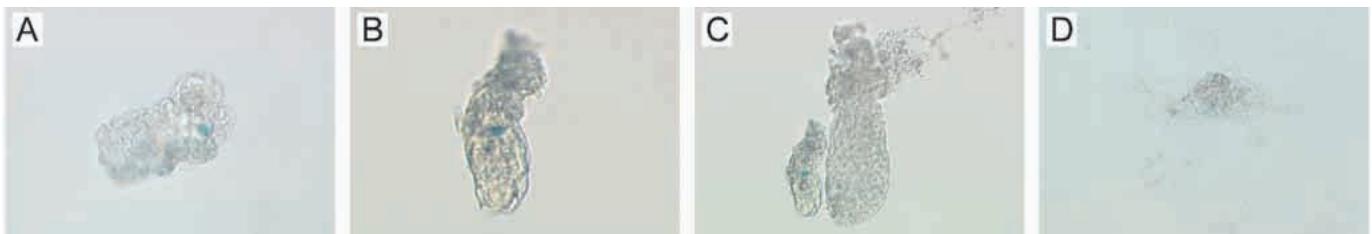


Fig. 5. *GATA6* expression during the phase of peri-implantation lethality. (A) A 4.5 dpc heterozygous embryo in which the β -gal staining is restricted to a few cells on one side of the blastocoelic surface of the inner cell mass adjacent to the trophoctoderm. (B) An implanted 5.5 dpc embryo from heterozygous intercrosses, which contains a few expressing cells and is much smaller than its littermates (C). (D) Heterozygous blastocyst outgrowth, after 7 days in culture, in which expression is restricted to a few cells of the inner cell mass.

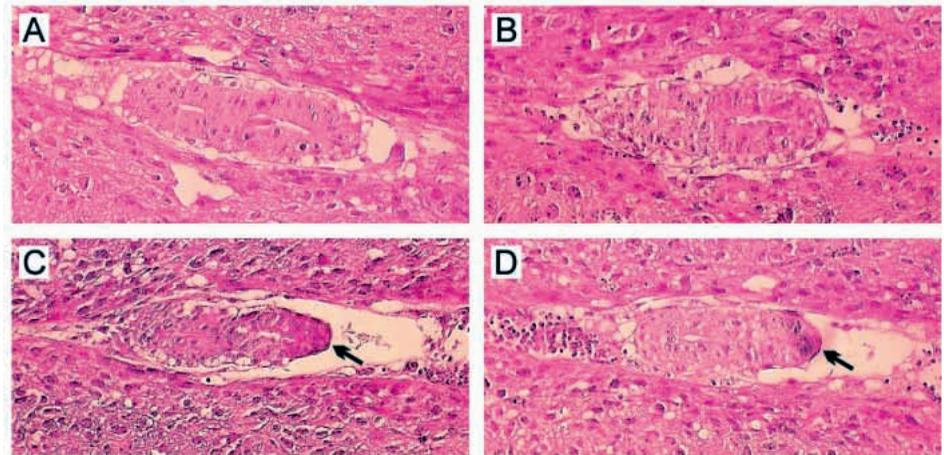


Fig. 6. Haematoxylin and eosin-stained sagittal sections of 5.5 dpc embryos from *GATA6* heterozygous intercrosses. (A,B) Normally developed embryos; (C,D) embryos showing abnormal visceral endoderm surrounding the embryonic ectoderm (arrow).

migrating parietal endoderm cells was evident in all cultured blastocysts. *GATA6* expression was observed by β -gal staining in a small subpopulation of inner cell mass cells in a 1-week-old heterozygous blastocyst outgrowth (Fig. 5D).

These results suggest that the absence of *GATA6* results in lethality at 5.5 dpc, shortly after implantation, due to a defect in cells derived from the inner cell mass. These may be extraembryonic in nature and possibly the primary defect lies in the primitive endoderm lineage.

In order to test this extraembryonic function of *GATA6*, we made two types of chimeric embryos; the first, by injecting wild-type blastocysts with *GATA6* null ES cells to retain wild-type genotype in extraembryonic tissues and, the second, by the reciprocal combination to remove *GATA6* function from extraembryonic tissues (Beddington and Robertson, 1989).

***GATA6* is required for extraembryonic development**

Homozygote *GATA6* null undifferentiated ES cells were injected into C57/BL6 blastocysts and the developing embryos were analyzed at 8.5 and 9.5 dpc. Staining for β -galactosidase activity showed that the *GATA6*^{-/-} ES cells could contribute effectively to the heart tube and sinus venosa and that their progeny were present in both the myocardium and pericardium (Fig. 8C-F). *GATA6*^{-/-} cells also contribute to the gut. We therefore conclude that the absence of *GATA6* does not lead to a cell autonomous defect in the epiblast that is derivative of the inner cell mass giving rise to the embryo proper. In addition, the *GATA6* null cells clearly had not lost

their developmental potential to contribute to the heart and gut. The latter was confirmed by the *in vitro* differentiation of *GATA6* null ES cells. They were differentiated *in vitro* (see Materials and Methods) and analyzed after 23 days in suspension cultures. The *GATA6*^{+/-} and *GATA6*^{-/-} embryoid bodies showed no morphological differences (Fig. 8A,B) and both contained contracting cells, indicating that differentiation of cardiomyocytes was not inhibited in *GATA6*^{-/-} tissues.

Because the extraembryonic tissues in the chimera experiment were derived from the wild-type blastocyst, it leaves the possibility that *GATA6* is essential for the development of the trophoblast or the primitive endoderm, and that it was defects in either or both of these tissues that were responsible for the demise of the embryo. This would be consistent with the abnormal endoderm observed in a number of 5.5 dpc embryos (Fig. 6). In order to perform the reciprocal chimera experiment, it was important to establish that *GATA6* embryos were normally represented among blastocysts selected for injection from heterozygote intercrosses. Blastocysts were collected, selected for injection and then genotyped. Homozygous null blastocysts constituted 20% of the population.

After injecting intercross blastocysts with wild-type ES cells and their implantation in pseudopregnant recipient mice, the resulting embryos were analyzed at 7.5 dpc. The chimeric embryos were dissected from Reichert's membrane and the cells in this membrane were genotyped. Since this tissue receives a low, if any, contribution from the injected ES cells

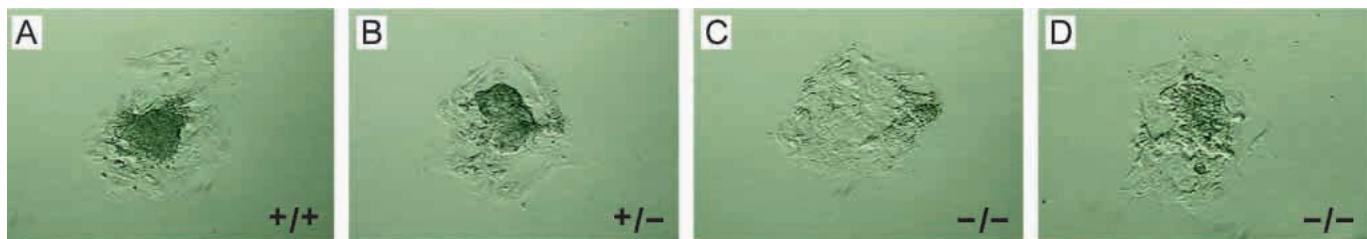


Fig. 7. *In vitro* blastocyst outgrowths after 5 days in culture. (A,B) Wild-type and heterozygous blastocysts, respectively, with the expected outgrowth of trophoblast cells and the formation of an inner cell mass surrounded by the visceral endoderm. (C,D) Homozygous mutants, which exhibit normal outgrowth of the trophoblast but have a small or dispersed inner cell mass. There is no evidence of a visceral endoderm layer enveloping the ICM. In all three genotypes, parietal endoderm cells were observed at the periphery of the outgrowth.

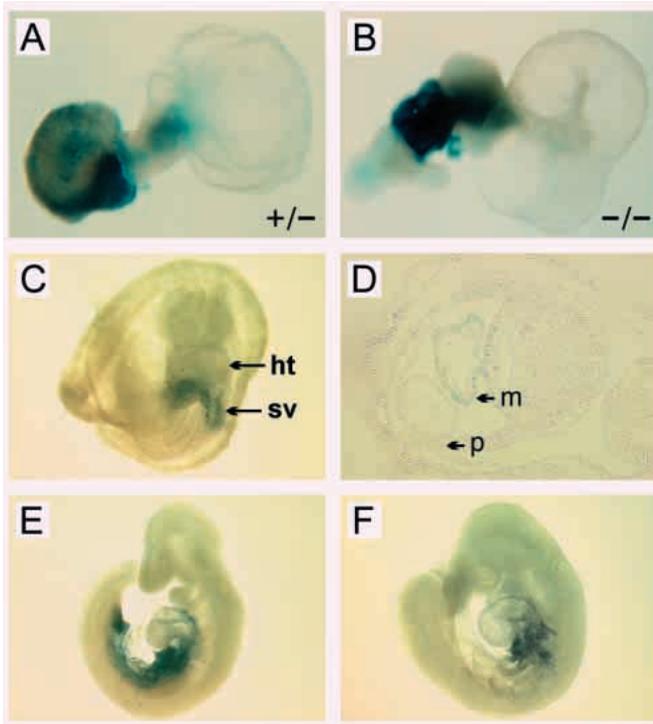


Fig. 8. Developmental potential of the *GATA6*^{-/-} ES cells. (A,B) β -gal-stained embryoid bodies derived from ES cells differentiated in vitro for 23 days in suspension culture. There is no morphological difference between heterozygote and homozygote mutants and they both display contraction activity. When *GATA6*^{-/-} ES cells were injected into wild-type blastocysts their contribution to *GATA6*-expressing tissues was assessed by β -galactosidase activity. (C) 8.5 dpc chimeric embryo demonstrating contribution to both heart tube and sinus venosa and (D) transverse section of the heart tube indicates that both myocardium and pericardium are colonized. (E,F) 9.5 dpc chimeric embryos with contribution sites being all the tissues that express *GATA6* (Fig. 4A).

(Beddington and Robertson, 1989), its genotype would be representative of the host blastocyst. Nuclei were prepared from both the membrane and the embryo proper and subjected to FISH genotyping using a probe specific for the mutant *GATA6* allele (the *lacZ-neo* gene, green) and a probe for a control gene (the *HIRA* gene, red). 73 blastocysts were injected and 58 implantation sites formed, from which 41 conceptuses were recovered. Genotyping showed that 32 of the 41 Reichert's membranes were heterozygote for *lacZ-neo* (two red signals and one green, Fig. 9B), while the remaining 9 membranes were wild type (only two red dots, Fig. 9A). Thus none of the embryos was derived from a *GATA6* null blastocyst, indicating that *GATA6* is required for the development of extraembryonic tissues.

DISCUSSION

Expression and role of *GATA6* in the heart

In this paper, we describe the expression pattern and the role of the transcription factor *GATA6* using homologous recombination to introduce the *E. coli* β -galactosidase (*lacZ*)

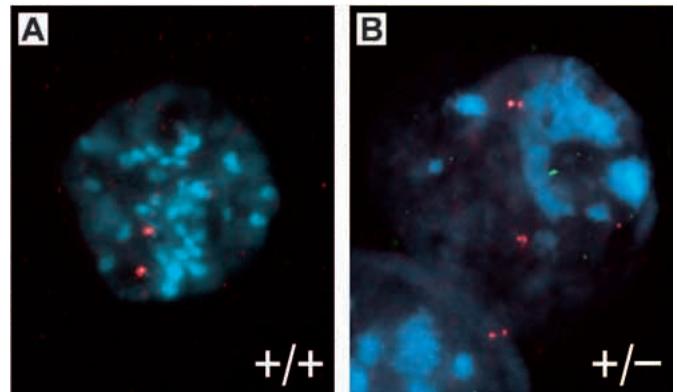


Fig. 9. FISH genotyping of Reichert's membrane nuclei derived from 7.5 dpc chimeric embryos generated by injecting wild-type ES cells into blastocysts from *GATA6*^{+/-} intercrosses. A cocktail probe was used with a cosmid for the *HIRA* locus as a wild-type control (red) and a plasmid with the *lacZ-neo* sequences to detect the *GATA6* mutant allele. (A) A wild-type (two red dots) and (B) a heterozygous (two red and one green dot) mutant embryo.

gene to inactivate *GATA6* and to serve as a reporter for its expression. Previous expression and in vitro binding studies suggested a role of *GATA6* in the development of the heart (Laverriere et al., 1994; Morrisey et al., 1996; Evans, 1997; Gove et al., 1997) and we therefore first analyzed the expression pattern of the reporter gene during embryonic heart formation in heterozygote embryos. As expected (Morrisey et al., 1996), *GATA6*-driven *lacZ* expression is detected in the lateral plate mesoderm (8.0 dpc) containing the cardiogenic plate and persists during the differentiation and migration of cardiomyocytes to form the heart tube. By day 8.5, when the heart has looped, β -gal staining is still evident in both myocardium and pericardium and strong expression is observed in the developing gut.

Since homozygote mutants die well before heart formation, the role of *GATA6* in cardiogenesis could not be assessed directly. Therefore *GATA6*^{-/-} ES cells were generated and their developmental potential tested. The behavior of mutant cells in embryoid bodies in vitro appeared indistinguishable from heterozygous or the wild-type cells with respect to differentiation of functional cardiomyocytes. In vivo when the *GATA6*^{-/-} ES cells were injected into wild-type blastocysts, a number of normal, highly chimeric embryos were obtained and these showed a substantial contribution of the mutant cells to both the heart and the gut. Thus, the absence of *GATA6* protein does not result in a cell autonomous defect in developing cardiomyocytes. This supports the suggestion from the analysis of *GATA4* mutants (Narita et al., 1996; Kuo et al., 1997; Molkentin et al., 1997) that there is a functional redundancy between the two members of the family concerning cardiac development. Unfortunately, elucidation of such a redundant role is not possible through the study of heart development in a double mutant background since *GATA6*^{-/-} lethality is much earlier than the specification of cardiogenic mesoderm.

GATA6 null embryos and early expression

All *GATA6* null embryos fail to develop to gastrulation and die shortly after implantation at 5.5 dpc. Thus we investigated the expression of *GATA6* during peri-implantation development.

Expression can first be seen in blastocysts (3.5 dpc) in a subpopulation of cells in the inner cell mass and in some cells lining the blastocoelic cavity. Interestingly, not all inner cell mass cells stain and such nonuniform staining is also seen in heterozygous *lacZ*-targeted ES clones, where blue staining is evident as a 'salt and pepper' pattern in an otherwise apparently homogeneous population of undifferentiated ES cells. Thus *GATA6(lacZ)* may be cell cycle regulated, monoallelically expressed or reveal some of the heterogeneity in pluripotent cells. Before implantation at 4.5 dpc, when the primitive endoderm has already differentiated as a distinct layer on the surface of the inner cell mass (Nadijcka and Hillman, 1974; Gardner, 1985), *GATA6*-driven *lacZ* is restricted to a localized population of cells on the blastocoelic surface of the inner cell mass adjacent to the trophectoderm. This very restricted expression in a subset of ICM cells can also be seen in heterozygote blastocyst outgrowths in vitro (Fig. 5D). We were unable to confirm this restricted expression pattern at the RNA level by in situ hybridization of *GATA6* RNA. It is therefore possible that *GATA6* is not expressed at that stage. β -gal activity may be very stable and remains detectable for a longer time than *GATA6* RNA. It would seem that it is this first phase of *GATA6* expression in the ICM of the blastocysts that is critical for the survival of the embryo. However, it is not clear whether *GATA6* expression marks ICM cells that will become the epiblast (giving rise to the embryo proper) or cells that will differentiate into primitive endoderm, or a mixture of the two.

At gastrulation, a new site of expression is detected, reflected by the very strong staining in the parietal endoderm cells on the inner surface of the Reichert's membrane. Although these cells are derived from the primitive endoderm, it does not appear that the expression at day 4.5 necessarily corresponds to precursors of the parietal endoderm. At 5.5 dpc, no expression in parietal endoderm could be detected and therefore *GATA6* is not continuously expressed in this lineage from the blastocyst stage. The only expression that could be detected at 5.5 dpc was seen in a few retarded embryos from heterozygote intercrosses. Staining was seen in a few cells and they appeared to be in extraembryonic sites. Since no staining was ever seen in heterozygote outcrosses, these embryos are presumed to be *GATA6* homozygotes and thus indicate that absence of *GATA6* causes abnormalities immediately after implantation, which are manifested as gross retardation and abnormal primitive endoderm differentiation (Fig. 6).

Extraembryonic requirement for *GATA6*

The suggestion, based on expression pattern, that *GATA6* is required for the development of extraembryonic tissue is supported by the generation of chimeric embryos. In a previous study (Beddington and Robertson, 1989), it was shown that ES cells when injected into blastocysts contribute mainly to the epiblast. Occasionally a very low contribution could be observed in trophectoderm and primitive endoderm. Injecting *GATA6*^{-/-} ES cells into wild-type blastocysts generated a number of normal highly chimeric embryos. This endorses the notion that *GATA6* is normally required in extraembryonic lineages. This conclusion is further supported by the opposite experiment that demonstrated the inability of the wild-type ES cells to rescue the *GATA6*^{-/-} phenotype. Moreover, death of the embryo (in vivo) or inner cell mass (in vitro) is not due to a cell autonomous requirement for *GATA6* in the epiblast but

rather to a defect in neighboring supportive tissues. Such a crucial role for visceral endoderm in growth and patterning of the embryo has been demonstrated in the analysis of other knock-out phenotypes such as *HNF4* (Chen et al., 1994), *evx1* (Spryropoulos and Capecchi, 1994), *nodal* (Varlet et al., 1997) and *smad2* (Waldrip et al., 1998). Given the normality of the trophectoderm outgrowth in *GATA6*^{-/-} blastocysts in vitro and the presence of parietal endoderm cells, it is likely that the primary defect lies in the visceral endoderm.

Function of *GATA6*

All *GATA6* null embryos fail to develop to gastrulation and die shortly after implantation at 5.5 dpc. Thus the first wave of *GATA6* expression in the ICM of the blastocyst is critical to the survival of the embryo. The expression of the *GATA* factor with overlapping expression in heart, *GATA4*, has not yet been determined in vivo prior to gastrulation. However, about one third of the embryos with an inactivated *GATA4* gene fail to gastrulate and this is thought to be due to defective formation of visceral endoderm from primitive endoderm at day 4.5 (Molkentin et al., 1997). This is supported by the observation that *GATA4*-deficient ES cells fail to form visceral endoderm in culture (Soudais et al., 1995). Like *GATA6*^{-/-} cells, *GATA4* null ES cells contribute extensively to normal chimeras when injected into wild-type blastocysts (Narita et al., 1997). The majority of *GATA4* null embryos show a defect in the lateral-to-ventral folding of the precardiac splanchnic mesoderm and its underlying endoderm at 7.5 dpc (Molkentin et al., 1997). Both of these tissues also express *GATA6*. *GATA6* expression is increased in the *GATA4*-deficient tissues and this may be responsible for the rescue of the majority of the embryos until after gastrulation (Molkentin et al., 1997). Thus *GATA6* appears to be the more critical factor at the earliest stages where its absence cannot be compensated by *GATA4*. The opposite appears to be the case in the tissues forming the heart and gut. Although the level of *GATA6* is raised, the absence of *GATA4* leads to a lethal defect in folding, while the basic ability of the cells to differentiate appears to be maintained (Molkentin et al., 1997). Interestingly, this lethal folding defect in *GATA4*^{-/-} embryos is rescued in the presence of wild-type visceral endoderm (Narita et al., 1997). Therefore, even here the primary defect appears to be extraembryonic. We were of course unable to determine the effect of a complete absence of *GATA6*-expressing cells during early heart and gut formation because of early lethality. However, *GATA6* null ES cells were able to contribute to all the early heart and gut cells in chimeric embryos, which shows that the absence of *GATA6* does not lead to a cell autonomous defect in these tissues. The cells keep their basic ability to differentiate as was observed in the *GATA4* null embryos. However the chimera experiments do not show that the absence of *GATA6* could have caused similar cell movement problems as observed in the *GATA4* null embryos.

Overexpression of *GATA4* in P19 cells leads to an increase in beating cardiogenic myocytes, while its absence leads to extensive apoptosis and cell death (Grepin et al., 1997). Overexpression of *GATA6* at the time that its expression normally decreases in heart formation in *Xenopus* leads to an excess of cells and thickening of the myocardial muscle (Gove et al., 1997). Similar observations have been made for *GATA1* in the haematopoietic system. The inactivation of *GATA1* leads

to an arrest of erythropoiesis in the foetal liver due to apoptosis of differentiating red cells (Weiss and Orkin, 1995). Overexpression of GATA1 leads to the opposite effect (Whyatt et al., 1997; Whyatt and F. G., unpublished data).

GATA2 and GATA3 are, among other tissues, also expressed in the haematopoietic system (Leonard et al., 1993). In the case of GATA1 and GATA2, they have been shown to be autoregulatory and crossregulatory (Weiss et al., 1994; Tsai et al., 1991) and they can substitute for each other to a considerable extent (Weiss et al., 1994). Nevertheless, they each have a unique role in the development of the haematopoietic system resulting in different phenotypes when the genes have been inactivated in vivo (Pevny et al., 1991; Tsai et al., 1994; Pandolfi et al., 1995; Ting et al., 1996). This suggests that at least part of the function of GATA proteins is not related to the unique properties present in each of the particular GATA proteins, but rather on the conserved central GATA DNA-binding motif (Tsai et al., 1998). In other words, as long as sufficient amounts of any GATA protein are produced at the appropriate time in particular cells they will be functional.

Thus we suggest that GATA6 happens to be the first GATA factor that is required for the growth of a population of cells that contributes to the extraembryonic tissues in the developing blastocyst. Absence of GATA6 would lead to the malfunction of an extraembryonic tissue (probably visceral endodermal as defined by position and chimeras), that is required for the support and growth of the epiblast.

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