# The role of the transcription factor GATA-6 in mouse embryonic development

(De rol van de transcriptie factor GATA-6 tijdens de embryonale ontwikkeling van de muis)

**Proefschrift**

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*Σα βγεις στον πηγαιμό για την Ιθάκη,*
*να εύχεσαι νάναι μακρύς ο δρόμος,*
*γεμάτος περιπέτιες, γεμάτος γνώσεις.*
*Τους Λαιστρυγόνας και τους Κύκλωπας,*
*τον θυμωμένο Ποσειδώνα μη φοβάσαι,*
*τέτοια στον δρόμο σου ποτέ σου δεν θα βρεις,*
*αν μεν' η σκέψις σου υψηλή, αν εκλεκτή*
*συγκίνησις το πνεύμα και το σώμα σου αγγίζει.*
*Τους Λαιστρυγόνας και τους Κύκλωπας,*
*τον άγριο Ποσειδώνα δεν θα συναντήσεις,*
*αν δεν τους κουβανείς μες στην ψυχή σου,*
*αν η ψυχή σου δεν τους στήνει εμπρός σου.*

*Να εύχεσαι νάναι μακρύς ο δρόμος.*
*Πολλά τα καλοκαιρινά πρωΐα να είναι*
*που με τι ευχαρίστησι, με τι χαρά*
*θα μπαίνεις σε λιμένας πρωτοειδωμένους*
*να σταματήσεις σ' εμπορεία Φοινικικά,*
*και τες καλές πραγμάτιες ν' αποκτήσεις,*
*σεντέφια και κοράλλια, κεχριμπάρια κ' έβενους,*
*και ηδονικά μυρωδικά κάθε λογής,*
*όσο μπορείς πιο άφθονα ηδονικά μυρωδικά*
*σε πόλεις Αιγυπτιακές να πας,*
*να μάθεις και να μάθεις απ' τους σπουδασμένους.*

*Πάντα στον νου σου νάχεις την Ιθάκη.*
*Το φθάσιμον εκεί ειν' ο προορισμός σου.*
*Αλλά μη βιάζεις το ταξείδι διόλου.*
*Καλλίτερα χρόνια πολλά να διαρκέσει*
*και γέρος πιά ν' αράξεις στο νησί,*
*πλούσιος με όσα κέρδισες στον δρόμο,*
*μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.*

*Η Ιθάκη σ' έδωσε τ' ωραίο ταξείδι.*
*Χωρίς αυτήν δεν θάβγαινες στον δρόμο.*
*Άλλα δεν έχει να σε δώσει πιά.*

*Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.*
*Έτσι σοφός που έγινες, με τόση πείρα,*
*ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν.*

*«ΙΘΑΚΗ»*
*Κωνσταντίνος Καβάφης (1863-1933)*

*As you set out for Ithaka*
*Hope the voyage is a long one,*
*Full of adventure, full of discovery.*
*Laistrygonians and Cyclops,*
*Angry Poseidon-don't be afraid of them:*
*you'll never find things like that on your way*
*as long as you keep your thoughts raised high,*
*as long as a rare excitement*
*stirs your spirit and your body.*
*Laistrygonians and Cyclops,*
*wild Poseidon-you won't encounter them*
*unless you bring them along inside your soul,*
*unless your soul sets them up in front of you.*

*Hope the voyage is a long one.*
*May there be many a summer morning when,*
*with what pleasure, what joy,*
*you come into harbors seen for the first time;*
*may you stop at Phoenician trading stations*
*to buy fine things,*
*mother of pearl and coral, amber and ebony,*
*sensual perfume of every kind-*
*as many sensual perfumes as you can;*
*and may you visit many Egyptian cities*
*to gather stores of knowledge from their scholars.*

*Keep Ithaka always in your mind.*
*Arriving there is what you are destined for.*
*But do not hurry the journey at all.*
*Better if it lasts for years,*
*So you are old by the time you reach the island,*
*wealthy with all you have gained on the way,*
*not expecting Ithaka to make you rich.*

*Ithaka gave you the marvelous journey.*
*Without her you would not have set out.*
*She has nothing left to give you now.*

*And if you find her poor, Ithaka won't have fooled you.*
*Wise as you will have become, so full of experience,*
*you will have understood by then what these Ithakas mean.*

*"ITHAKA"*
*Constantinos Cavafy (1863-1933)*
*(translated by E. Keeley & P. Sherrard)*

## CONTENTS

**Aim and outline of this Thesis**

Different members of the GATA family of transcription factors have been studied extensively in our lab. The role of GATA-1 in the differentiation of erythroid blood cells and of GATA-3 during T-lymphocyte development are two typical examples. GATA-6 is the most recently characterized member of the family. Based on its expression pattern during mouse embryonic developmentfor a role for GATA-6 in cardiogenesis had been speculated.

To investigate what role GATA-6 may play during embryogenesis we used targeted inactivation of the gene in Embryonic Stem (ES) cells (**chapter 2**). Unexpectedly, homozygote mutant embryos die just after implantation at embryonic day 5.5. Generation of chimeric embryos in which the *GATA-6* mutant cell population was confined in either the embryo or to the extraembryonic tissues revealed that the primary defect in *GATA-6* null embryos lies in an extraembryonic cell lineage. Further *in vivo* and *in vitro* analysis of the mutant embryos suggested that the affected lineage is the visceral yolk sac endoderm, a derivative of the primitive endoderm.

Cardiogenesis could not be directly studied since mutant embryos die well before heart development starts (embryonic day 8.5). However, in chimeric embryos, *GATA-6 -/-* ES cells give rise to cardiomyocytes in apparently normal hearts, possibly due to redundant functions with the co-expressed *GATA-4* and *-5*. In contrast, *GATA-6* is the only member of the family that is expressed in the lung endoderm. Following on a published observation showing no contribution of *GATA-6* null ES cells to the lung epithelium, we decided to generate more highly chimeric embryos to analyze the development of the lung, which is a derivative of another endoderm lineage, the definitive endoderm (**chapter 3**). Surprisingly, we found that lung endoderm can be formed from *GATA-6* mutant cells. However, this mutant endoderm has subsequent morphogenetic and differentiation defects.

The importance of GATA-6 protein levels during lung development was confirmed by a different approach. The gene was overexpressed in transgenic mice with a pulmonary epithelium specific promoter (**chapter 4**). High levels of the protein resulted in branching defects and more interestingly in a block of lung endoderm differentiation to distal alveolar epithelium.

**Chapter 1** is a general introduction. Since we have found that GATA-6 has unique functions in both primitive and definitive endoderm, the endoderm lineages are introduced first. Their origin, development and current knowledge of their molecular control is presented. Then, the function of GATA proteins during mouse embryogenesis is briefly described.

# Chapter 1

# Introduction

## *THE ENDODERM IN THE MOUSE EMBRYO*

During development of the mouse embryo, *endoderm* characterizes two cell lineages with distinct origin and developmental fate. First, just before implantation, the primitive endoderm is formed. This is considered to be an extraembryonic lineage since it contributes to the membranes surrounding the developing embryo. Following gastrulation, the definitive endoderm is established as one of the three embryonic germ layers that give rise to all embryonic tissues. The endoderm layer forms the gut tube that further differentiates to generate a number of thoracic and abdominal organs.

### Primitive Endoderm

*Origin*

Primitive endoderm is first evident at embryonic day 4.5 (E4.5) when the hatched elongated blastocyst is about to implant. It is formed as an epithelial layer of cells on the free surface of the inner cell mass (ICM) facing the blastocoelic cavity and the distal mural trophectoderm (Figure 1A) [1]. Subsequent to this differentiation event, the remaining population of cells in the ICM is referred to as the primitive ectoderm or epiblast. It is not quite clear how this endodermal cell layer arises, but transmission electron microscopy illustrated that cytological evidence for primitive endoderm differentiation is correlated with reorientation and loosening of the inner cell mass [2]. The differentiated character of primitive endoderm was demonstrated by cell lineage tracing studies in chimeric embryos, which showed that primitive endoderm cells do not colonize any other embryonic tissue except the primitive endoderm [3]. Likewise, primitive ectoderm cells colonize different parts of the embryo but not the primitive endoderm [4].

Following the demarcation of this lineage, further differentiation results in the formation of the two derivatives of the primitive endoderm, the parietal and the visceral endoderm. Originally, based on *in vitro* experiments, it was thought that there is a consecutive differentiation first to parietal and then to visceral endoderm [5]. However, cell tracing studies did not support this hypothesis and they actually revealed that primitive endoderm consists of bipotential precursor cells [3, 6].

*Parietal endoderm; Reichert's membrane*

From the primitive endoderm layer, a number of cells start to dissociate and migrate onto the inner surface of the trophectoderm (TE) (Figure 1A). This detachment is thought to be the primary force for their further differentiation that will define them as the parietal endoderm (PE) lineage, a population of specialized cells synthesizing a thick basement membrane known as Reichert's membrane [2] (Figure 1B). Initially, there is already a basement membrane secreted by the TE itself, which fosters adhesion and migration of the developing PE [7]. The Reichert's membrane acts as a filtrative layer between the conceptus and the

mother and during development there is a continuous recruitment of cells onto the membrane by migration of individual parietal endoderm cells from the margins of the epithelial layer bordering the visceral endoderm [8].

The process of PE differentiation can be mimicked *in vitro* by using F9 embryonal carcinome cells (EC) and from such experiments it was revealed that trophectoderm can actually induce PE [9] and that both extracellular matrix and molecules like parathyroid hormone-related peptide and TGF-β regulate this differentiation [10-12]. *In vivo*, differentiation of PE is defined by the expression of a set of molecular markers, including the extracellular matrix component *laminin* (*LN*) [13], the *secreted protein acidic and rich in cysteine* (*SPARC*) [14] and the intermediate filament *vimentin* [8]. These markers distinguish PE cells from other primitive or visceral endoderm cells.

*Visceral endoderm*

The primitive endoderm cells that remain in contact with the epiblast form a continuous, simple epithelium specialized for secretion and absorption, known as the visceral endoderm (VE) or visceral yolk sac endoderm [2] (Figure 1B). The constant contact of the primitive endoderm cells with the epiblast is believed to be necessary for the further differentiation of the VE lineage [15]. This view is supported by emerging molecular data from *in vitro* studies implicating BMP and Hedgehog signaling. *BMP-4* is expressed in the core of the epiblast (or embryoid body in *in vitro* experiments) and the newly differentiated primitive endoderm surrounding the epiblast expresses *BMP-2* and *Indian Hedgehog*. The latter two signals are transduced to the core of the epiblast and newly induced, as yet unknown molecules, together with BMP-4 act on the primitive endoderm to promote VE differentiation. In the absence of these signals, PE is formed instead [16, 17]. The exact molecular cascade in this interaction is far from clear but the important role of members of the TGF-β family, such as BMPs, is further supported by the mutant phenotype of downstream transducers of this pathway. ActRIA, a Type I receptor of TGF-β ligands, and the downstream transducer Smad4 are both required for differentiation and further function of the VE [18, 19]. In addition, other molecules, like GATA-4 [20-22], GATA-6 (chapter 2) [23], hepatocyte nuclear factor-4 (HNF-4) [24] and variant hepatocyte nuclear factor-1 (vHNF-1) [25, 26] were shown to be important regulators of VE differentiation by loss of function studies in the mouse.

Once the VE is formed, it has an influence on the further development of the underlying epiblast. Such effect is first evident in the formation of the proamniotic cavity just after implantation (Figure 1C). The process involved is known as cavitation and results in the organization of the compact epithelial mass of the epiblast into a pseudostratified columnar epithelium that by day 6.0 lines a cavity formed in the core of the epiblast. Cavitation starts near the periphery of the epiblast (embryoid body) and proceeds inward. *In vitro* studies suggest that it is the result of the interaction of two signals. One signal depends on the presence of the

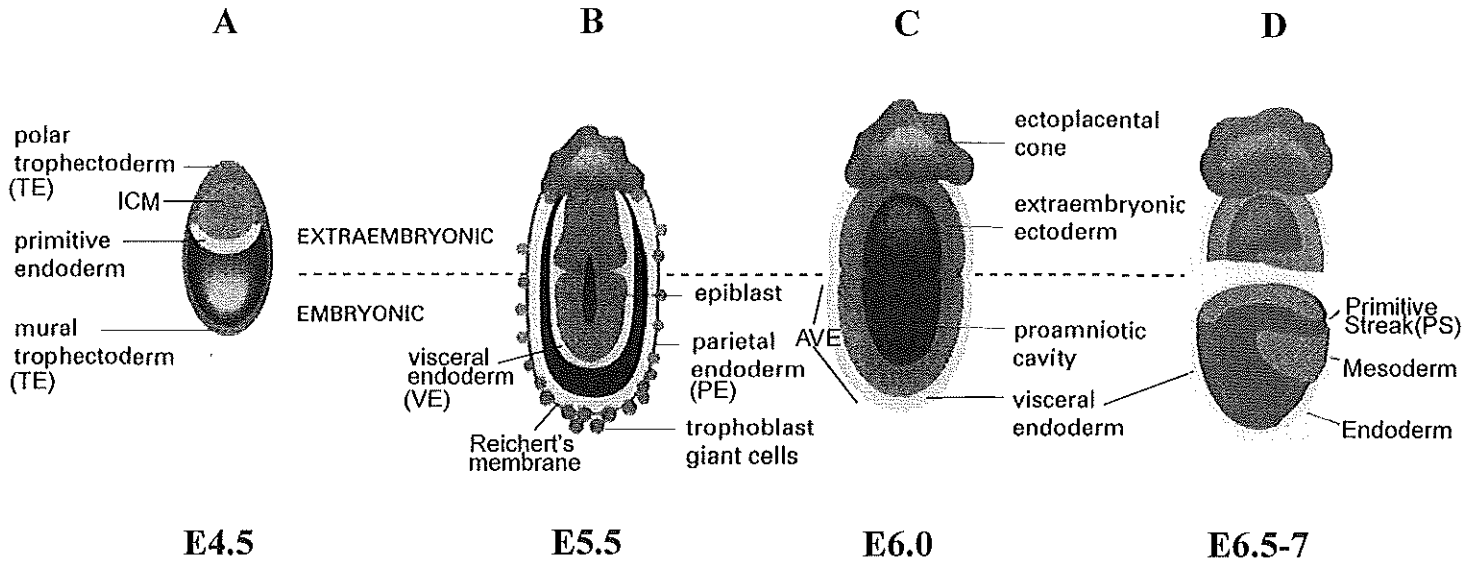


**Figure 1.** Schematic representation of mouse development from implantation (E4.5) up to gastrulation (E6.5). (**A**) At the time of implantation, the embryo is composed of three distinct tissue lineages: trophectoderm (TE), primitive endoderm and epiblast (or inner cell mass, ICM). (**B**) The primitive endoderm subsequently differentiates into parietal (PE) and visceral endoderm (VE). Visceral endoderm surrounds the epiblast and it subsequently forms the yolk sac, while parietal endoderm forms the Reichert's membrane that is the outer most membrane surrounding the embryo. (**C**) Before gastrulation, anterior visceral endoderm (AVE) is a molecularly distinct part of the visceral endoderm that marks the anterior site of the embryo. (**D**) At gastrulation, the primitive streak (PS) is formed at the posterior site of the embryo, through which epiblast cells migrate and form either the mesoderm or the definitive endoderm. The latter emerges from the anterior-most primitive streak. In C and D the parietal endoderm of the Reichert's membrane is not shown. The figure is adapted from *Hogan et al.* [1].

outer endodermal layer and causes the death of the inner ectodermal cells, and another promotes survival of the single layer of columnar epithelial cells lining the cavity [27]. In a more recent study it was shown that BMP signaling is involved in this process and specifically, BMP-2 from the VE, alone or with BMP-4 from the epiblast, promotes apoptosis of the inner cells and differentiation of the outer layer of columnar cells [16]. Since inactivation of individual *BMPs* has not resulted in a cavitation-specific defect, BMP-2 and –4 are considered as functionally redundant in this model [28, 29].

Studies of different targeted mutations in the mouse have shown that the function of a number of genes that are expressed in the visceral endoderm is crucial for the further survival and development of the underlining epiblast. Two members of the hepatocyte nuclear factor (HNF) family of transcription factors represent a well-studied example. *HNF-4* is expressed exclusively in the VE before gastrulation and when inactivated, the maturation of VE as a tissue is affected and the epiblast undergoes apoptosis. Although primitive streak and mesoderm are initially formed, embryonic structures are severely affected and the embryo dies [24, 30]. Targeted mutation of *HNF-3*, also expressed in VE, results in abnormally formed derivatives of the primitive streak, like the node and the notochord [31]. A chimeric study, confining the *HNF-3* mutation exclusively to the VE or to the epiblast, demonstrated that the gene function is required in the VE for the proper morphogenesis of the primitive streak [32]. Similar effects have been described for other genes, like the homeobox *even-skipped evx1* [33] and *msd* [34] genes, establishing a crucial role of the VE in the normal progression of gastrulation.

The influence of the VE on derivatives of the epiblast, has also been well documented. The first example came from the *in vitro* study of erythropoiesis and vasculogenesis in *GATA-4* mutant embryoid bodies, which lack visceral yolk sac endoderm [22]. Although, primitive erythrocytes and endothelial cells were differentiated, the absence of VE resulted in the lack of organized yolk sac blood islands and vascular channels [35]. A subsequent study using an explant culture system demonstrated that VE is necessary for specifying posterior fate in the mesoderm, such as the formation of haemotopoietic and endothelial cell progenitors. During normal development, the extraembryonic mesoderm that migrates beneath the VE in the exocoelomic cavity differentiates and forms blood islands and a mesothelial layer. In the absence of VE this differentiation is blocked. Furthermore, when epiblast cells that are destined to form anterior structures are exposed to the same part of the VE, they are respecified to form blood and endothelial cells [36]. Such an instructive role of the VE in a specific derivative of the epiblast is not a sole case. Anterior neural patterning has been attributed to gene function in the VE and *nodal*, a member of the TGF-β family, is an example of VE influence on the development of rostral neural structures [37]. *HNF-3* mutants also have impaired anterior patterning [31]. Additionally, and more interestingly, a part of the VE which is called anterior visceral endoderm (AVE) marks the anterior

site of the embryo before gastrulation begins and it has been shown to be crucial for anterior neural patterning [38].

*AVE (anterior visceral endoderm)*

AVE is a medial strip of visceral endoderm that underlies approximately the anterior third of the embryo at a stage when no primitive streak has formed (E6.0, Figure 1C). The identification of this anterior center came as a contradiction to the widely accepted idea that the formation of the primitive streak at the beginning of gastrulation (E6.5) marks the posterior side of the embryo and therefore establishes the anterior-posterior (A-P) axis of the conceptus [39]. In the mouse, AVE is not morphologically different from the rest of the VE. However, it is distinct in the rabbit embryo AVE where it is more columnar in character than the rest of the VE [40]. Cell lineage studies showed that the origin of the AVE is a handful of endoderm cells situated at the distal tip of the E5.5 embryo [41]. These cells then migrate to one side, opposite to where the primitive streak will be formed, and they give rise to the stripe of the VE that marks the anterior side of the embryo. Although this anisotropic cell movement has not been explained so far, it is well-documented from the expression pattern of a number of genes that mark this tissue, like the homeobox gene *Hex* [41]. Furthermore, in embryos mutant for *Otx-2*, which is also expressed in AVE, there is a failure of such migration and *Otx-2* mutant endodermal cells accumulate at the tip of the embryo. As a result AVE is not formed [42, 43]. TGF-β signaling seems to be important for the formation of the AVE since the downsream transducer Smad-2 is required in VE for its A-P polarity. In the absence of the gene product AVE is not formed and the epiblast gives rise to posterior derivatives only [44].

There are more genes that molecularly identify the AVE in the mouse. *VE-1 antigen* [45], *Lim-1*, *goosecoid* and *cerberus-related 1* [46, 47] specific expression in the VE precedes the onset of gastrulation. At the time of primitive streak formation, the AVE is further patterned and it is subdivided at least in two distinct domains. The most anterior domain, which corresponds to the site of future heart development, is marked by the onset of *Mrg-1* expression [48]. Immediately posteriorly, *Hesx-1* is expressed in a population of AVE, which overlies epiblast destined to form anterior CNS [49, 50]. Most of these genes that are expressed in the early AVE are also expressed in the node (anterior most end of the streak), which is necessary for expression of CNS markers in the anterior ectoderm. Removal of the AVE during early stages of gastrulation results in the compromise of subsequent expression of anterior CNS markers [49]. Additionally, in the *Cripto* mouse mutant there is no primitive streak or node formed, yet the distally localized AVE in these embryos is sufficient for the adjacent epiblast to assume an anterior character [51]. These observations were suggestive of a potential role of the AVE in anterior patterning. Further functional proofs came from the studies of targeted inactivation of genes that are expressed in the AVE.

One of the important questions was whether gene expression in the AVE itself is critical for anterior patterning or the subsequent gene expression in the node. The most valuable experiment to address this question has been the generation of chimeric embryos with different combinations of genotypes between the host blastocyst and the injected ES cells. This experiment enables the specific loss of function of a gene either in the extraembryonic endoderm (blastocyst derivative) or in the epiblast (ES cells derivative). In that way any defect present in the embryo could be attributed to the function of the gene in a particular tissue, either extraembryonic VE or embryonic node. Inactivation of *Otx-2* and *Lim-1* resulted in embryos that had a compromised anterior development exhibiting varying degrees of holoprosencephaly or more extensive foreshortening of the CNS [52, 53]. When mutant ES cells were used to generate chimeric embryos in which the VE was derived from the wild type host blastocysts the anterior ectoderm defects where rescued [43, 54]. In the converse experiment, when *Otx-2* or *Lim-1* mutant blastocysts were injected with wild type ES cells, chimeric embryos had the same anterior defect as seen in the null embryos. Thus, regardless of the presence of wild type embryonic tissues, including the node, the mutant VE provided by the injected blastocyst was causing the same malformations [43, 54, 55]. These experiments establish that in the null embryos the primary defect for anterior neural patterning lies in the VE and more specifically in the AVE where *Otx-2* and *Lim-1* are expressed.

Although the chimeric studies prove that the defect is in the AVE, they do not actually explain how this induction is achieved. It seems that one of the action of the AVE onto the underlying epiblast is to induce expression of the same molecules that pattern the AVE itself and thereby transferring this pattern across to the epiblast. This is evident for *Otx-2* and *Hesx-1* in the prospective neurectoderm [49, 50, 56] and for *Mrg-1* in the cardiac mesoderm [48]. Therefore it is thought that AVE is initially responsible for establishing anterior-most identity and that other signals including the ones from the node and its derivatives serve to maintain and further establish this pattern [38, 39]. This combinatorial interaction among the embryonic layers has been demonstrated in transplantation studies of axis induction in the mouse embryo. AVE alone was not able to induce ectopic neural tissue and only when combined with both anterior epiblast and node tissue the ectopic axis exhibited full induction of anterior neural genes [57]. Additionally, germ-layer explant assays suggested that AVE may actually suppress the induction of posterior character, indirectly allowing generation of anterior identity. In *Otx-2* mutant embryos AVE is not able to prevent posteriorizing signals and as a result there is no anterior induction [42]. Furthermore, the BMP antagonists *Chordin* and *Noggin*, which are produced by the node, are required for forebrain development [58], suggesting that in the presence of a normal AVE there are other molecules in the epiblast required for subsequent elaboration of anterior patterning. This is also supported by the phenotype of *Wnt-3* mutant embryos. Although AVE is formed

and patterned properly, there is no anterior-posterior neural patterning as a result of the absence of the primitive streak and its derivatives [59].

**Definitive Endoderm**

*Origin*

The definitive endoderm derives from the epiblast or primitive ectoderm as one of the three germ layers that are formed during gastrulation (E6.5). Gastrulation follows the formation of the primitive streak (PS) at the posterior site of the egg cylinder (Figure 1D) and is the process by which the totipotent epiblast cells are partitioned into three germ layers [60]. The ectoderm forms skin and CNS; the mesoderm forms blood, bone and muscles; and the endoderm contributes mainly to respiratory and digestive tracts. This elaborate cell differentiation and rearrangement is achieved by the migration of epiblast cells through the streak with both mesoderm and endoderm emerging out of the PS. This process of specification is not yet clear and may happen before the cells enter the streak or as they are passing through it. There is even the possibility that as the cells exit they remain multipotent and by entering the right environment their fate is specified. From cell lineage tracing studies it has been shown that the first endoderm originates from the most anterior site of the streak [61]. However, a well-defined progenitor pool that gives rise to this cell lineage has not been identified and single-cell labeling experiments suggest that sharp boundaries in cell fate do not exist in the early streak stage embryos [62]. In fact 75% of cells labeled in the region of the anterior streak contribute to multiple germ layers.

Endoderm has not been studied as extensively as the other two germ layers, largely because of a lack of specific molecular markers. However, a better understanding of the molecular control of its formation has been obtained in lower vertebrates [63]. In Xenopus, although specification is mediated by maternally provided molecules (vegT), the subsequent maintenance relies on cell-cell interaction and the importance of signaling pathways, like TGF-β, and WNT, is well documented. Recently, GATA-5 has been implicated in Xenopus endoderm specification by its early endoderm specific expression and mainly by its ability to respecify mesoderm towards an endodermal fate [64]. In the mouse embryo there is little knowledge of the molecular control of endoderm formation and only loss of function studies have implicated some genes in this process.

As mentioned earlier, embryos mutant for *Smad-2*, one of the downstream transducers of TGF-β signaling, have a primary defect in the visceral endoderm resulting in the lack of proper anterior patterning [44]. When this primary defect was rescued in chimeric embryos by wild type extraembryonic tissues, a requirement of Smad-2 function was revealed for the formation of definitive endoderm [65]. Mutant cells never became endoderm and they could only be detected as mesoderm or ectoderm. Furthermore, in highly chimeric embryos the identification of a wild type endoderm sheet next to almost exclusively *Smad-2*

mutant ectoderm and mesoderm implies that only a small number of precursor cells give rise to the definitive endoderm. Another TGF-β related activity, *nodal*, is a possible candidate for endoderm induction. In addition to its function in the VE to induce anterior identity [37], the gene is expressed in the posterior epiblast at the onset of gastrulation and it is essential for proper PS formation [66]. Specific defects in the formation and migration of endoderm cells have been demonstrated in embryos lacking *Furin/SPC1*, a protease controlling proteolytic maturation of TGF-β and related molecules including Nodal and BMP-4 [67]. This is consistent with the idea that a balance of TGF-β/activin and BMP signals regulate definitive endoderm formation. However, specific mutations of *BMPs* do not directly prove this since BMPs, like BMP-4, have a more general function during gastrulation [28]. A more indirect support is the phenotype of embryos mutant for the BMP antagonists *Noggin* and *Chordin*, which are expressed in the node and its derivatives. Their function is crucial for anterior patterning but in their absence there is a marked reduction of definitive endoderm leading to a later reduction in pharyngeal endoderm [58].

*Early endoderm patterning*

Anterior-posterior identity of the endoderm seems to be coupled with its initial generation since the first endoderm to emerge out of the streak becomes anterior endoderm. However, a molecular A-P patterning along the endoderm sheet has not been established yet and there is only a handful of molecules that are known to be differentially expressed along the A-P axis. The initial induction of their expression is not fully understood but the involvement of adjacent tissue layers has been suggested by the nature of the molecules themselves and by explant recombination assays.

Most of the molecules expressed in the anterior endoderm are the same as can be found earlier in the anterior ectoderm and even earlier in the AVE. Therefore, it is likely that the pattern in the anterior ectoderm is relayed back to the gut endoderm. A typical example is the expression of *Hesx-1* that is first detected in the AVE and then in the underlying ectoderm. At the end of gastrulation, *Hesx-1* expression is maintained in anterior head ectoderm and is induced in the adjacent gut endoderm as it displaces visceral endoderm [49]. Expression of *HNF-3*, *goosecoid*, *cerberus-like*, *Hex*, and *Otx-2* [43, 49] has also been reported in the anterior endoderm, while *intestinal fatty acid binding protein* (*IFABP*) [68] and *Cdx-2* [69] are expressed in the posterior endoderm. The significance of this pattern has not been elucidated but gene targeting studies have revealed that some of these genes function in subsequent differentiation of gut tube and in organogenesis. Anterior endoderm gives rise to foregut and in mice lacking the transcription factor HNF-3β in the embryonic lineages, both foregut and midgut were absent [32]. The function of Hex in anterior endoderm is required for proper liver formation while in the thyroid it is necessary for specification [70]. *Otx-2* null embryos die early during gastrulation [53]. However, in embryos highly chimeric

with *Otx-2* mutant cells, the rostral foregut fails to form (Ang, S-L unpublished observation).

An *in vitro* germ layer explant study with mouse embryos has recently suggested that during gastrulation both mesoderm and ectoderm direct the differentiation of the adjacent endoderm [71, 72]. Furthermore, it has been demonstrated that soluble factors are potential mediators of this interaction and that they act in a concentration dependent manner. Specifically, FGF-4 was implicated as one of the inductive signals of the mesoderm, which was able to induce specific gene expression in the endoderm. Higher levels of the factor could induce *somatostatin* (*SS*) expression, a later marker of posterior gut endoderm that give rise to the pancreas and small intestines. *NeuroD* is expressed more anterior in the gut tube, marking the domain of stomach and pancreas and is induced by lower levels of FGF-4. These two observations support a role of FGF-4 as a posterior morphogen since the *in vivo* source of the protein is the PS [73], which is more proximal to the posterior *SS* expressing endoderm than to the endoderm expressing *NeuroD*. In support of a role of FGF signaling in endoderm patterning is the study of ES cells mutant for the receptor *FGFR-1*. These cells accumulate in the PS and although they can form neural structures, they were never detected in endoderm derivatives [74].

These data suggest that, although initial endoderm formation could be cell autonomous or maternally controlled like in Xenopus, the subsequent specification and possible early A-P patterning of the endodermal sheet depends on signals from adjacent embryonic layers. The major issue that is not fully clarified yet is whether these signals are permissive or instructive. In other words, is the mesectoderm only reinforcing a pre-pattern within the endoderm or is it actively specifying the endoderm? In agreement to previous studies in chick embryos, the explant recombination study in mouse [72] suggests that the signals from the other layers are instructive since they are able to induce gene expression patterns in a naïve endoderm and to some extent to respecify fate in the endoderm. However, some anterior markers were already present in the endoderm at the time of its isolation before culturing. Additionally, the respecification activity of the mesenchyme was not equally affecting all endoderm markers. Therefore, the possibility of a pre-existing pattern still remains. It rather seems that there is a combination of both instructive and permissive signals from the mesectoderm, which supports the further differentiation and specification of distinct domains within the endoderm.

*Gut tube morphogenesis*

Subsequent to its formation, endoderm undergoes a complex and well-co-ordinated morphogenesis that results in the formation of the gut tube. According to cell lineage-tracing studies [61, 75], the first endoderm originates from the most anterior site of the streak and intercalates into the overlying visceral endoderm (E6.5). The newly formed endoderm eventually displaces the primitive visceral endoderm as it migrates in an anterior direction to form the anterior endoderm.

Cells that emerge later from the streak form more posterior endoderm together resulting in a sheet of endoderm cells that extends from the anterior head folds to the posterior primitive streak (E7.5) (Figure 2). The anterior- and posterior-most endoderm invaginates into the embryo, forming the foregut and hindgut pockets respectively (E8.5). These pockets, known also as anterior and caudal intestinal portal (AIP and CIP, respectively), extend towards the midgut and eventually fuse to form the gut tube (E9). The ends of this tube are initially closed but they open by the fusion of the ectoderm and endoderm at the level of the oral and urogenital/anal plates. Most of the mouth as well as the rectum derive from the ectoderm. From the three different parts of the gut tube a number of organs originate: the foregut is the progenitor of thyroid, lung, liver, pancreas and stomach; the midgut of the small intestines and the hindgut of the large intestines.

The whole process of gut tube formation is not understood either at a cell-cell interaction or at a molecular level. Targeted mutations in the mouse have implicated, thus far, very few molecules in gut folding. As explained earlier, *GATA-4* mutation results in abnormal ventral closure of the gut tube and chimeric studies have shown this to be an effect of the overlying visceral endoderm [76]. Members of the BMP (-2, -4, -5 and -7) signaling family can be added to this list since in their absence similar folding defects were observed [28, 77, 78].

*Patterning of the gut tube*

With the formation of the gut tube and the aforementioned A-P patterning in the early endoderm a question emerges. Do specific regions of the endodermal sheet or the early gut tube correlate with organ formation? And furthermore, is there a pattern in the gut tube that directly links gene expression domains and specific organ territories?

The first question has been addressed in different vertebrates by fate mapping experiments that trace the fate of endoderm cells in definitive digestive organs. Although organisms like the chick have been predominantly used due to accessibility and ease of embryo culturing [79], similar conclusions were drawn by Lawson et al. [61] in a study of mouse endoderm cell fate. The labeling of single mouse endoderm cells after gastrulation and their tracing later, following foregut and hindgut formation, resulted in the definition of four regions (I-IV) along the gut endoderm that are fated to generate specific organs, as illustrated in Figure 2. However, in agreement with the chick studies, there are no sharp boundaries of domains of contribution and the organs have rather overlapping presumptive territories. The data from the explant studies is consistent with these observations and taken together, they strengthen the notion that the A-P nature of gastrulation stage endoderm is quite plastic. In fact, it is the interaction with the mesoderm at this stage that renders the endoderm in a more determined state, resulting gradually in a gut tube endoderm that is irreversibly specified. The significance of this initial specification is more apparent later, during organ formation, when new inductive signals can only be effective on an already specified endoderm (see later). This

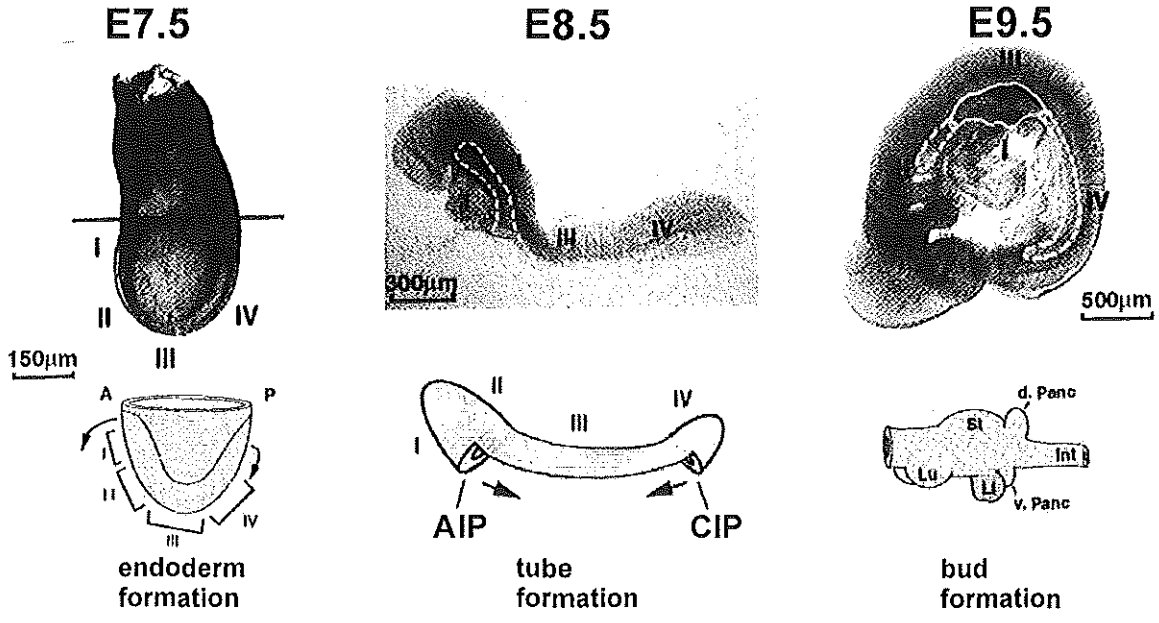


**Figure 2.** Development of the definitive endoderm and formation of the gut tube. On the upper panel, embryonic development is shown subsequent to gastrulation up to the initiation of organogenesis. The gut tube is outlined by white dotted line. Below each embryonic stage, a schematic representation of the endodermal sheet or the developing gut tube is depicted. At the end of gastrulation the endoderm is a sheet of cells surrounding the embryo proper. At E8.0, gut tube formation begins at the anterior foregut by the folding of region I over region II, which results in the formation of the anterior intestinal portal (AIP) that then migrates in a posterior direction. The same time, region IV in the hindgut folds over to form the caudal intestinal portal (CIP) that migrates in an anterior direction. These migrations, in combination with embryonic turning, close the midgut and form the primitive gut tube by E9.0. The roman numerals I-IV represent regions of E7.5 endoderm that fate map to regions I-IV of E8.5 gut, as it was demonstrated by *Lawson and Pedersen* using single endoderm cell-tracing experiments [75]. **Region I** contributes to the ventral foregut and derivative organs such as thyroids, lungs, liver and ventral pancreas. **Region II and III** give rise to dorsal foregut and midgut, which contribute to dorsal pancreas, stomach, duodenum and part of the intestines. **Region IV** forms the hindgut, which contributes to the large intestine and colon. The lower right schematic representation depicts the initiation of organogenesis, which does not necessarily correspond to its above embryonic stage of E9.5. The figure is adapted from *Wells & Melton* [72, 86].

process of sequential induction allows for broad regions of endoderm to become progressively determined towards the establishment of organ domains.

An interesting mechanism of gut endoderm determination has been put forward recently from studies on presumptive liver endoderm. Specification of hepatocytes is associated with the expression of liver specific genes like serum albumin. The investigation of the liver-specific enhancer of this gene revealed that prior to gene expression, binding sites for both GATA and hepatocyte nuclear factor (HNF-3β) proteins are occupied [80]. This occupancy was suggested as a mechanism of rendering the endoderm competent for further specification into liver primordia. The necessary subsequent signals would provide the additional factors that are needed for the actual transcriptional activation of the liver specific genes. Such a way of enabling competence of a gene to be activated during development is described as genetic potentiation [81]. The involvement of both of these families of transcription factors in differentiation of endodermal lineages [20, 21, 32, 82] and their characterized interactions with other cofactors [83, 84] make this mechanism a plausible way of specification at a molecular level. In addition, this mechanism was subsequently coupled to the mesodermal role on gut endoderm determination. Initially, the occupancy of binding sites is not restricted to the prospective liver endoderm and it is the posterior mesoderm that actually prevents albumin expression and allows further specification in the prospective intestinal endoderm. This correlates with the pluripotency of the gut endoderm at that early stage. However, two days later the posterior mesoderm mediates the loss of binding site occupancy and the posterior endoderm is then irreversibly specified as intestinal [85].

A better understanding of genetic potentiation would require the identification of more of the molecules involved and their regulated expression along the different territories of the gut tube. Although a variety of gene expression patterns have been described along the gut tube, they do not necessarily imply a well-defined A-P pattern [86, 87]. Logical candidates would be the *Hox* genes since they provide positional information along the A-P axis in other germ layers. However, it is unlikely that they do so in the endoderm. Only a few *Hox* genes are expressed in the endoderm and their expression limits do not always correlate with boundaries between the organs. Furthermore, mutations in these genes do not cause A-P transformations of the organs in the gut but only some tissue malformations [87]. In fact, gut mesoderm has a more defined *Hox* expression pattern [88], which could have an indirect influence on the patterning of the gut tube. Studies in the chick gut suggest that the endoderm itself might dictate mesodermal *Hox* expression and that signaling molecules like those of the Hedgehog family could be involved in this induction [89]. Additionally, Sonic hedgehog from the endoderm seems to influence the differentiation of the mesoderm and induction of BMPs could be one of the mechanisms [90, 91]. The importance of Hedgehog and TGF-β signaling in the development of the gut has been confirmed in mice mutant for *Sonic* and *Indian hedgehog* [92] and for the *Type II* receptors of the TGF-β related

ligand, activin [93]. Overall it seems that boundaries of gene expression do not strictly correlate with domains of specific organs. Even in the case of the ParaHox gene *pancreatic-duodenum-homeobox 1* (*Pdx-1*) that is necessary for pancreatic outgrowth and development [94], its expression pattern is wider than the pancreatic bud. In figure 3, a schematic representation is depicted with the expression of a number of transcription factors in different domains of the gut tube, according to Wells and Melton [86]. Some organ-specific gene functions that were revealed by targeted inactivation in the mouse are also indicated.

*Organogenesis*

Morphogenesis of the gut tube is coupled to the initiation of organ formation so that by the time the tube is fully folded (E9.5) differentiation of organ specific tissue is evident (Figure3). Apart from the digestive tract, the rest of the organs are branching out from the main tube by the formation of an initial bud. Although it is not known how overlapping gene expression patterns may dictate where organ buds will arise, it is established that organ budding and subsequent morphogenesis involve reciprocal interactions between gut epithelium and the adjacent condensed mesoderm, referred to as mesenchyme. So, at this stage gut endoderm that is already specified by combination of possible intrinsic signals and early mesodermal induction (discussed earlier) is receiving new signals for further organ specific differentiation. There is no longer plasticity and the signals from the neighborhood can only be permissive now. Thus, mesenchymal signals cannot instruct endoderm fate anymore, they can only be effective on endoderm pre-determined for the formation of a specific organ. Such interactions have been described in both chick and mouse during the formation of liver, pancreas and lung.

The liver primordium is first evident as a thickening of the ventral endodermal epithelium and then as a bud of cells that proliferate and migrate into the surrounding septum transversum mesenchyme. Classical transplantation experiments in the chick by LeDouarin [95] showed that it is actually the cardiac mesoderm that induces liver formation in its adjacent ventral endoderm. Further studies in the mouse confirmed this interaction and showed that cardiac mesoderm induces both hepatic gene expression and outgrowth of endodermal epithelium [96]. At the molecular level members of the fibroblast growth factors family (FGFs) were implicated by their dynamic expression pattern in the cardiac mesoderm and their ability to substitute for cardiac mesoderm in *in vitro* endoderm differentiation into liver [97]. While this substitution was complete for the induction of liver specific gene expression, the morphogenetic action of the cardiac mesoderm seemed to require additional, yet unknown signals. Subsequent to the formation of the liver bud there are more signals required, as revealed by loss of function studies of different mouse genes. The homeobox protein Hex was recently characterized as providing a critical function for early migration of hepatic endoderm into the septum transversum [70]. Earlier studies have highlighted the

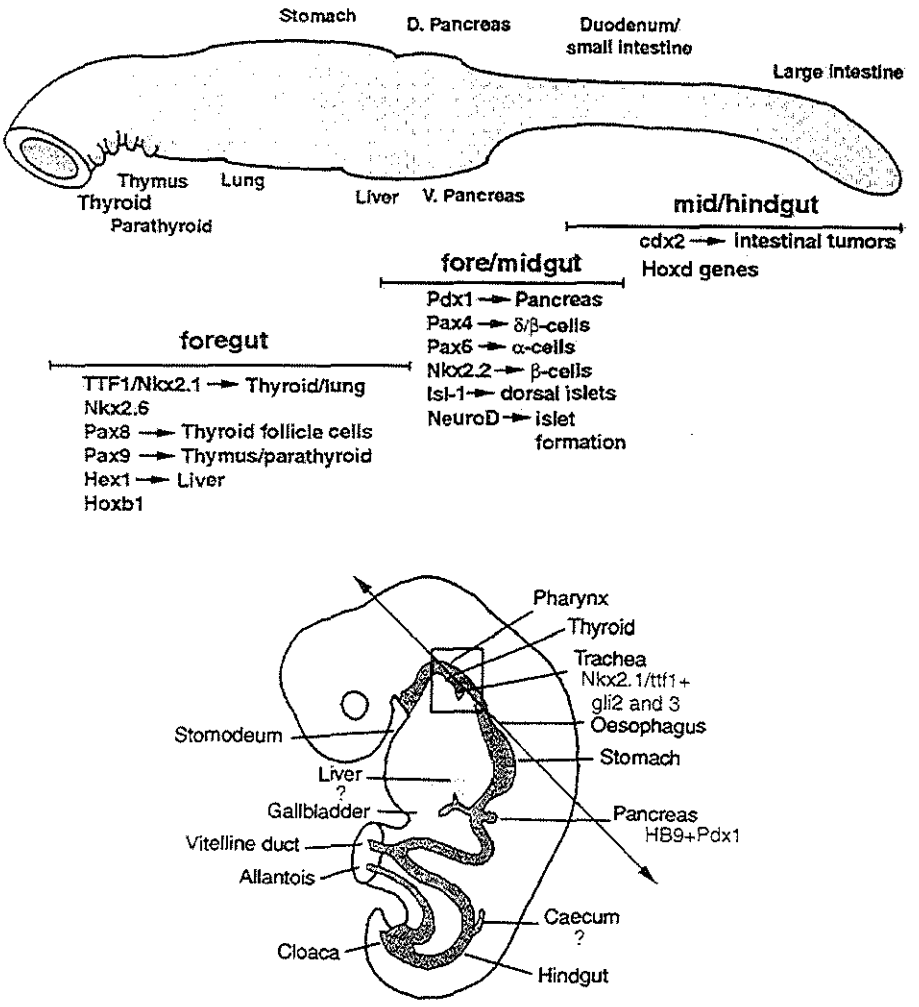


**Figure 3.** The upper part is a schematic representation of the E9.5 gut tube showing expression of several transcription factors along the tube, and regions of the gut that contribute to specific organs. Transcription factors have been grouped according to their relative A-P expression at this time of development, but any D-V expression differences that some of them have are not shown. The transcription factors that have been genetically disrupted in mice show a corresponding phenotype, indicated by the arrows. In the lower part a scheme of mouse-chicken embryo shows the position of the glands that bud out from the gut tube. Genes that are participating in the initiation of these organs are indicated, while question marks imply that there is no knowledge of budding genes. The figure is adapted from *Wells & Melton* [86] and from *Grapin-Botton & Melton* [87].

orchestrating role at that stage of the septum transversum mesenchyme and have identified a number of different signaling molecules [98, 99]. For example, hepatocyte growth factor (HGF) initiates a well-defined molecular pathway crutial for hepatic growth that involves c-met and c-jun. Another molecule from the mesenchyme, the homeobox gene *Hlx*, seems to control a distinct signaling cascade. Although the phenotype of mutant embryos is related to defective haematopoiesis, the mutant cells are able to support haemotopoiesis in a different environment [100]. This would suggest that proliferation and integrity of hepatocytes as maintained by mesenchymal signals are required not only for the proper formation of the organ but also for the development of some of its functions.

Tissue interaction is critical for pancreas development as well. The formation of this organ begins with a dorsal and a ventral protrusion of the gut tube. The ventral component derives from the ventral foregut that is immediately adjacent to the hepatic endoderm and its initiation and further differentiation is less well defined. For the dorsal component, the notochord is initially in immediate contact during pancreatic differentiation. When notochord was deleted in chick embryos, expression of dorsal pancreatic genes was lost [101] and instead, shh was induced. As it was known that only the presumptive pancreatic endoderm does not express shh in the gut tube, this observation implied that notochord promotes pancreatic differentiation by inhibiting shh expression. In accordance with this, cyclopamine mediated inhibition of shh in the gut tube induced ectopic pancreatic gene expression [102]. Molecular analysis identified FGF-2 and activin-betaB as the notochordal signaling molecules responsible for endodermal *shh* repression [103]. For further differentiation of the organ, a number of different gene functions are required (Figure 3) and the Notch signaling pathway has been implicated in the decision between the endocrine and exocrine fate [104].

## Lung Development

*Lung endoderm specification*

Lungs derive from the anterior ventral foregut, just anterior to the stomach and liver, as illustrated in Figure 2. The onset of lung development coincides with the appearance of the dorsal pancreatic bud, but occurs later than thyroid and liver bud outgrowth. A number of transcription factors, as shown in Figure 3, and signaling molecules, like shh, are expressed along the anterior endoderm that give rise to the lung primordium. However, to date there is no correlation between a specific gene expression domain and a presumptive lung territory on the primitive gut tube. Additionally, the absence of a defined structure that is in close contact with this territory, like notochord in the pancreatic endoderm, has made the study of pulmonary endoderm specification more elusive.

One of the molecules that is considered important for such specification is HNF-3β. Inactivation of the gene does not affect definitive endoderm formation but only subsequent invagination and gut tube formation [31]. Chimeric rescue of the primary defects revealed that the HNF-3β function is essential in the definitive endoderm for the formation of both foregut and midgut [32]. This implies that the role of HNF-3β in lung endoderm specification is not direct since in its absence the foregut itself is not formed. Another molecule that has been implicated is GATA-6. Its function is considered essential for pulmonary endoderm specification on the basis of the observation that *GATA-6 -/-* ES cells do not contribute to the lung epithelium in chimeric embryos [23]. However, highly chimeric embryos with *GATA-6 -/-* ES cells have lung epithelium consisting of *GATA-6* mutant cells in extensive areas (chapter 3). Although the mutant epithelium has subsequent morphogenetic and differentiation defects, this study implies that GATA-6 is not essential for pulmonary endoderm specification, at least in a chimeric context.

*Initial morphogenetic events*

At E9.5, the first step in the development of the lungs is the protrusion of the two primary lung buds on the ventrolateral wall of the foregut (Figure 2). Concomitantly, the foregut is divided by a longitudinal septum into two tubes. The dorsal one will become the esophagus and the ventral one with the two buds forms the trachea (Figure 4A). In this way the alimentary and the respiratory systems are permanently separated. Further morphogenesis is distinct for the trachea and the lung buds. While the trachea remains as an endodermal tube surrounded by mesenchyme, the two endodermal buds undergo extensive branching morphogenesis to generate the two main bronchi and the respiratory tree. Each bud has a left-right (L-R) identity that is retained even if the organ develops isolated in culture and it follows a distinct branching pattern. The left primary bud grows out as the future left bronchus and sprouts several secondary buds along its lateral side in a precise spatiotemporal sequence (Figure 4A). The right bronchus also

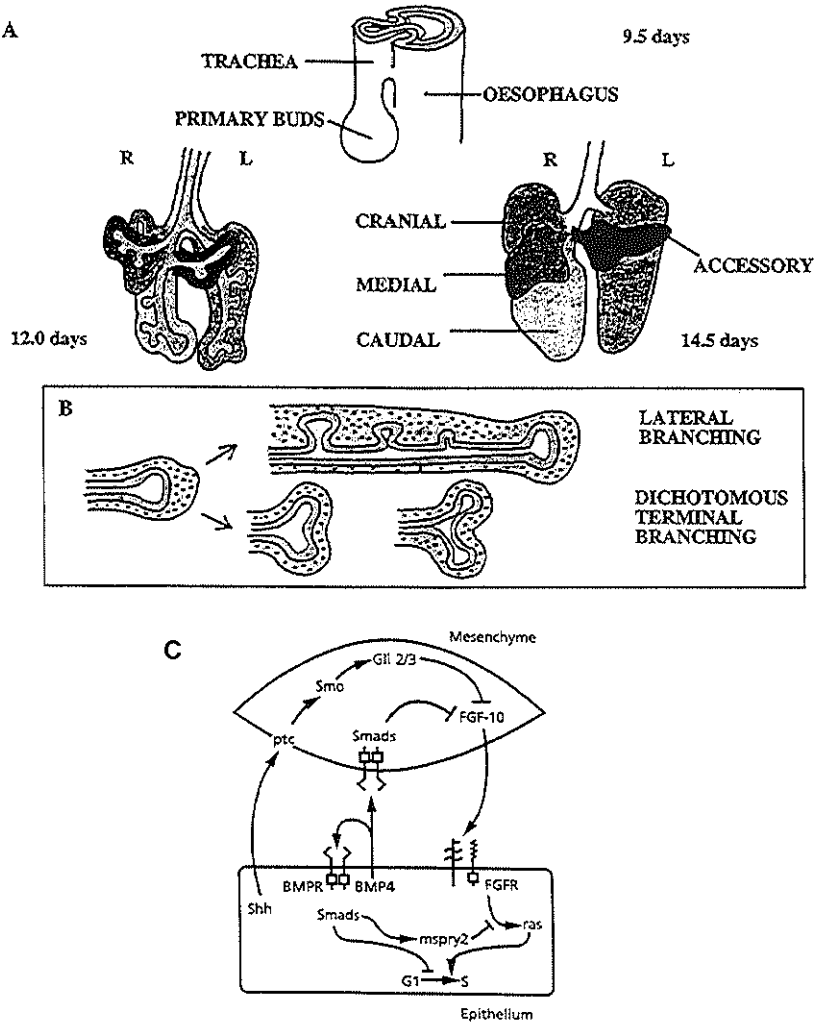


**Figure 4.** Schematic representation of mouse lung development and molecular pathways involved in epithelial-mesenchymal interactions. (**A**) Around E9.5, the trachea and primary buds develop from the ventral foregut. The tracheal-oesophageal groove eventually closes and separates the alimentary and pulmonary systems. By E12.5, the precursors of the five primary lobes have been established and the regular branching pattern of the epithelial endoderm can be clearly followed. L-R asymmetry is well established. At E14.5, the five lobes (right cranial, medial, caudal and accessory lobes and left lobe) are well developed. (**B**) Two different branching processes seen in early lung development, lateral and dichotomous branching. The mesenchyme surrounding the epithelial layer is distinctly marked by a stippled pattern. (**C**) A representation of key inductive and suppressive signalling mechanisms that mediate epithelial-mesenchymal interactions during branching morphogenesis and epithelial differentiation. For more details see Branching Morphogenesis in text. The figure is adapted from *Hogan et al.* [105] and from *Warburton et al.* [165].

generates an asymmetric series of secondary buds, but the pattern is quite different. The first bud to arise projects dorsally, followed closely in time and space by a lateral and a ventral bud, and then by several lateral buds. This highly organized branching pattern is of great significance. The first three secondary buds on the right eventually give rise to separate lung lobes, resulting in a right lung that is composed of four distinct lobes, compared with only one on the left (Figure 4A) [105, 106]. In humans there are three lobes on the right and two on the left lung.

*Molecular control of early morphogenesis*

Although the mechanisms controlling the site of lung bud initiation are not yet known, epithelial-mesenchymal interactions seem to be critical for primary bud outgrowth. The importance of the mesenchyme has been demonstrated long ago by transplantation experiments. The inability of the trachea endoderm to bud was reversed when trachea mesenchyme was exchanged for distal mesenchyme. The result was the formation of ectopic buds that grow, branch and differentiate like normal pulmonary epithelium. In the converse experiment, proximal mesenchyme from around the trachea was able to inhibit outgrowth and branching in the distal endoderm [107, 108]. The nature of the molecules involved in such an interaction had already been suggested by these experiments since the mesenchymal influence could be exerted even in the absence of direct contact. Further genetic studies have now directly implicated FGF and Shh signaling in early pulmonary morphogenesis. Retinoids and the homeobox transcription factor TTF-1 are also involved in these early morphogenetic events.

FGF signaling during lung bud initiation and outgrowth is mediated by the local expression of *FGF-10* (the homologue of the Drosophila *branchless*) in the immediate mesoderm [109] and its receptor *FGFR2-IIIb* in the foregut endoderm [110]. In mice with a targeted inactivation of *FGF-10,* the trachea is normally formed, but there is no initiation of lung bud formation [111, 112]. The same phenotype was observed in mice with different mutations in the *FGFR-2* gene [113]. Further use of *in vitro* explant systems gave a better insight into the function of FGF signaling in lung bud outgrowth. FGF-10 in the mesenchyme seems to act as chemoattractant for the adjacent endoderm and induces and promotes its budding [114]. Additionally, FGF-10 alone is able to induce budding from trachea endoderm denuded from proximal mesenchyme [115]. The FGF signaling is critical throughout branching morphogenesis and the mechanism of its action will be discussed in more detail later. However, the initial induction and localization of *FGF-10* in the mesenchyme remains elusive.

The secreted molecule Shh binds to its receptor patched (Ptc) resulting in the activation of the segment polarity gene *smoothened* (*Smo*) and the Gli zinc finger proteins that start to function as transcription factors. *Shh* is expressed in the foregut endoderm and persists during the formation of the lung endoderm [116]. The receptor *Ptc* and the three identified *Glis* (*Gli1-3*) are all expressed in the lung splanchnic mesoderm [117, 118]. Failure to initiate signaling in *shh-/-* embryos

results in a respiratory system that has no separation between trachea and esophagus (tracheoesophageal fistula) and primary buds that give rise to sac-like lungs without any lobes or endoderm branches [119]. Inactivation of both *Gli2* and *Gli3* genes results in a number of overlapping respiratory defects. *Gli3* mutant embryos have lungs that are smaller and show localized shape changes [117], while lack of Gli2 results in stenosis of the esophagus and trachea and hypoplastic, abnormally lobulated lungs. When Gli3 was reduced by heterozygous deletion in a complete *Gli2* deficient background the lung phenotype is more severe and the embryos show tracheoesophageal fistula. Remarkably, compound null mutant embryos, deficient for both Gli2 and Gli3, do not form oesophagus, trachea or lungs [120]. Thus, Glis have specific and overlapping functions in transducing shh signaling during the early morphogenetic stages. Furthermore, the differences in *shh* and *Gli* mutant phenotypes suggest that Gli may be a common mediator of different signaling pathways or that Shh can also act independent of Gli. Currently, this is not clear and downstream targets of the Gli transcription factors are not known.

Malformations similar to *shh* null lungs are observed in embryonic lungs mutant for the homeobox transcription factor *TTF-1*. The gene is expressed in the ventral foregut and subsequently in the respiratory endoderm. When *TTF-1* is deleted the abnormal sac-like lungs originate directly from an unseparated tracheo-esophageal tube [121]. Tracheoesophageal fistula with a variety of lung defects, including lung agenesis, is observed in embryos deprived for vitamin A [122]. Accordingly, similar phenotypes are observed in the lungs of embryos double mutant for the *RAR* and *RAR* receptors [123]. The important role of retinoids (RA) in early pulmonary development is further demonstrated by the expression of the RA-synthesizing enzyme (*RALDH-2*) in the ventral foregut [124], including the area where the lung buds form. The use of a mouse with a RA responsive element in a reporter gene also showed that by E9.5, RA and its receptors are activated in the foregut [124]. These findings suggest that there are several signaling pathways involved in early morphogenesis of the respiratory system and that initial trachea and lung morphogenesis share most of them. However, the argument that trachea and lung morphogenesis are coordinated by independent events is supported by the normal development of the trachea in the absence of FGF signaling. Both *FGF-10* and *FGFR-2* mutant embryos have lung agenesis but formation of the trachea proceeds normally [111-113].

The left-right (L-R) identity of the two initial lung buds is determined by the L-R asymmetry in the gut tube [39]. Expression of the bicoid class of homeobox gene *Pitx2* marks the left side of the gut and subsequently the left bud [125]. *Pitx2* expression starts in the lateral plate mesoderm (LPM) where it is induced by lefty-1, lefty-2 and nodal, all members of the TGFβ family that are expressed in the left side of the embryo [126]. Inactivation of *Pitx2* affects asymmetry in the lungs and results in right isomerism [127, 128]. In accordance with this, bilateral expression of the gene, as a result of bilateral *lefty-2* and *nodal*

expression in *lefty-1* mutant embryos, leads to left isomerism [129]. Random L-R laterality in the lungs is observed in embryos mutant for *HNF-4* (also known as *HFH-4*). This laterality defect is caused by the absence of cilia due to downregulation of the *left-right dynein* (*lrd*) gene, coding for a microtubule-based motor protein [130].

*Branching morphogenesis*

In each one of the five lobes the branching pattern can be traced up to about E12.5 and it is highly reproducible from embryo to embryo. This highly ordered sequence of patterning events referred to as branching morphogenesis and results in the establishment of the bronchial tree by the end of the pseudoglandural stage of lung development (E16.5). There are mainly two branching processes, the lateral and the dichotomous branching (Figure 4). The former is particularly important in establishing the length and overall shape of the lobes. It involves a single bud growing out for an extended distance accompanied by the sprouting of secondary lateral buds at specific distances from the leading tip. In the latter, a terminal bud splits into two branches, each of which continues to grow along a different axis, but not necessarily to the same extent [105].

As for the primary bud outgrowth, epithelial-mesenchymal interactions are also crucial for branching morphogenesis. Initially, it was thought that this interaction is mediated exclusively by the interaction of the extracellular matrix of both tissues. Components of the basement membrane regulate cell proliferation and absence or inhibition of the interaction of epithelial cells with basement membrane has a direct effect in lung development [131, 132]. Branching morphogenesis was considered to be the result of continued assembly and degradation of extracellular matrix [133]. According to this hypothesis the proximal epithelium is constrained by a well-organized, sheath-like extracellular matrix. In contrast, the rapid growth and expansion of the distal epithelium is associated with the matrix degrading activity of the adjacent distal mesenchyme. This activity and the growth of the epithelium leads to disruption of the basal lamina, which cannot be assembled fast enough to keep pace with epithelial proliferation. This results in the local collapse of the epithelial sheet and the formation of a cleft in which assembly of an intact basal lamina can be restored and the growth is restrained. Bilateral to this cleft, proliferating epithelial cells can expand resulting in dichotomous branching. Lateral sprouting is less well served by this model. However, it can be seen as the result of a localized degradation of basal lamina by the mesenchyme that allows the bud to grow out through the site of disruption (Figure 4B, page 17). The molecular study of different components of the extracellular matrix confirms its important role during branching morphogenesis. Gene inactivation of the *laminin 5* chain isoform leads to abnormal lobe septation and bronchiolar branching [134]. In lung explant cultures blocking expression of *fibrillin-2* and *SPARC* (*secreted protein acidic and rich in cystein*) result in mesenchymal expansion and branching defects, respectively [135, 136]. Nevertheless, extracellular matrix cannot be the sole

regulator of branching morphogenesis. Recent molecular studies have implicated different secreted signaling molecules as regulators of the epithelial-mesenchymal interactions that underlie the branching process (Figure 4C, page 17) [105, 137].

FGF signaling, mentioned already in primary bud outgrowth, is an important player during branching morphogenesis. In fact the branching pattern seems to be controlled by FGF signaling. For the very first branching event, the outgrowth of the primary bud, expression of *FGF-10* in the splanchnic lung mesenchyme is essential. In the absence of the ligand lung buds are not formed [111, 112]. The expression pattern of the gene subsequent to this initial event (E10.5-E12.5) correlates with positions in the mesenchyme towards which endoderm will bud and grow. That includes the mesenchyme surrounding the distal tips as well as mesenchymal domains adjacent to the lateral buds. *FGF-10* expression in these domains precedes the formation of lateral buds and it actually marks the endodermal sites from which lateral buds will initiate [109]. This mechanism is evolutionary conserved since the *Drosophila* homologue of *FGF-10*, *branchless*, is expressed in an analogous manner during trachea budding [138].

The significance of this dynamic expression pattern and the mechanism with which FGF-10 induces budding have been studied in mouse lung explant cultures. Implantation of an FGF-10 bead in whole lung cultures results in deviation of epithelial growth towards the bead, distorting the architecture of the branching tubules [114]. This effect is evident only in the distal epithelium. The proximal mesenchyme seems to inhibit the action of FGF-10 and proximal epithelium is not responsive. However, both proximal and distal epithelium denuded from mesenchyme respond to the FGF-10 source. When isolated endoderm is placed on a Matrigel (a mixture of basal lamina components) opposite to a source of FGF-10 (bead), the result is expansion and migration of the endoderm towards the bead [114, 115]. Thus, FGF-10 exerts a proliferative and chemoattractant effect on the endoderm and it induces local budding. The absence of mesenchyme in these experiments indicates that FGF-10 alone is able to induce budding and that its action is in the endoderm rather in the mesenchyme where it is expressed. This is mediated by the FGFR-2 receptor, which is expressed in the epithelium throughout early lung development [110]. However, the precise mechanism of how the epithelial cells sense the FGF-10 gradient and move in the Matrigel is not yet known.

A member of the TGF-β family, *BMP-4* is expressed in the growing tips of the distal epithelium [139]. The *in vitro* bud induction system with the FGF-10 source revealed that local expression of BMP-4 in the growing tip is the result of the response to the FGF-10 gradient. During bud initiation there is no BMP-4 expression and the response to FGF-10 results in the induction of BMP-4 during the bud extension [115, 140]. But what is the function of BMP-4 in the epithelium? Exogenous BMP-4 in the culture system and transgenic overexpression in the distal lung epithelium affects cell proliferation. Blocking endogenous BMP-4 with its antagonist Noggin promotes budding and outgrowth [115, 139]. Thus, the

induction of *BMP-4* expression in the growing tips might serve to control epithelial proliferation and restrain the further growth of the bud. This would imply that BMP-4 counteracts the action of its inducer FGF-10. In transgenic lungs overexpressing *BMP-4* branching is severely affected suggesting that absence of BMP-4 expression is needed for bud induction and that epithelium with constant high levels of the protein cannot respond to FGF-10 from the mesenchyme [139]. Expression of *FGF-10* in these lungs has not been studied and therefore any conclusion about the involvement of BMP-4 in the dowregulation of *FGF-10* that occurs subsequent to bud outgrowth cannot be drawn.

Downregulation of *FGF-10* expression in the mesenchyme seems to be influenced by endoderm since lung mesenchymal cells grown alone in culture show a marked increase in the levels of *FGF-10* mRNA [141]. It therefore seems likely that soluble factors produced by the epithelium have an inhibitory effect on *FGF-10* expression. A potential mediator for such interaction is *shh*, which is expressed in the epithelium. Its downstream effectors *Ptc*, *smo* and *Gli* are all expressed in the lung mesenchyme. Although shh can be detected throughout the lung epithelium, the branching distal tips express higher levels of the protein [139]. In the lungs of *shh-/-* embryos, *FGF-10* expression is no longer focal as in wild type, but it is rather diffuse and branching morphogenesis is impaired [142]. Addition of shh recombinant protein in lung cultures results in inhibition of *FGF-10* expression in the mesenchyme [141]. *In vivo* transgenic overexpressing of shh leads to increased proliferation in the mesenchyme and *FGF-10* downregulation [143].

The above mentioned molecular observations led to a model for branching morphogenesis that is based on the localized expression of *FGF-10* in the lung mesenchyme. The ligand acts on the endoderm via its receptor FGFR-2. The result of this signaling is an increase in epithelial cell proliferation and a chemotactic movement of the epithelial sheet towards the high levels of FGF-10. Following initiation, during the expansion of the bud, FGF signaling induces *BMP-4* expression in the growing epithelium. At certain BMP-4 levels growth of the tip is arrested, probably due to decrease in cell proliferation. The endoderm is no longer responsive to FGF-10 and initiation of additional buds from the same point is prevented. At the same time shh activity at the distal tip extinguishes expression of *FGF-10* in the adjacent mesenchyme. As the *FGF-10* expression domains have now become lateral and symmetrical, initiation of two buds is induced on either sides of the grown tip where *BMP-4* expression is lower. This is dichotomous branching. In a similar fashion, if subsequent to tip arrest the FGF-10 domain moves to one lateral site it can induce a bud in the endoderm that expresses lower level of *BMP-4*. The result is lateral branching [106, 115].

This model explains branching morphogenesis as continues cycles of dichotomous and lateral branching, which are directed by the dynamic expression pattern of *FGF-10* in the mesenchyme. Considering the reproducibility of the early branching, *FGF-10* expression has to be highly patterned. At present, there is no

knowledge of the molecular control of this patterning. It has been speculated that hox genes, which are expressed in the splanchnic mesenchyme may provide the patterning for *FGF-10* expression, at least in the very early stages [144, 145]. However, there is no direct evidences for that. It is also not known whether the FGF signaling in this process is mediated exclusively by FGF-10. FGF-1 is also chemotactic for lung endoderm in vitro, although weaker than FGF-10 [114]. It can bind with high affinity to FGFR-2, like FGF-10, but it is expressed later during lung development in a pattern distinct to *FGF-10* and its role in lung morphogenesis is still elusive [109]. *FGF-7* expression is also detected in lung mesenchyme although at a lower level and at stages when branching is well under way [146, 147]. FGF-7 promotes proliferation of the endoderm in the *in vitro* system, but it does not significantly induce bud outgrowth [144]. The targeted inactivation of *FGF-7* does not affect normal lung development *in vivo* [148] but overexpression of the ligand results in some pulmonary malformations (dilation of epithelial tubules) [149].

It is also not known how FGF-10 induces BMP-4 expression in the endoderm and whether it is the only inducer. The *in vitro* bud outgrowth experiments clearly indicate that BMP-4 induction is not direct since a considerable time interval is required [115]. The transcription factor *TTF-1* is also expressed at the bud tip epithelium [150] and in the abnormally branched *TTF-1-/-* lungs *BMP-4* expression is dramatically reduced [121]. Although expression of FGF-10 has not been studied in these lungs, an independent regulation of *BMP-4* by TTF-1 cannot be excluded. A good example of FGF independent regulation of *BMP-4* is seen in the lungs that lack the basic helix-loop-helix factor Pod1 that is normally expressed in the mesenchyme. In these lungs expression of *FGF-10* remains normal but epithelial BMP-4 is markedly reduced [151]. Thus, *BMP-4* induction requires at least one more distinct regulatory pathway initiated in the mesenchyme.

It is also not clear whether shh is the only signal from the endoderm that in turn downregulates *FGF-10* expression in the mesenchyme. TGFβ-1 negatively regulates branching in cultured lungs *in vitro*. It downregulates *FGF-10* expression and presumably interferes with FGF-10 mediated chemoattraction [141]. It also affects epithelial cell proliferation [152]. Inhibition of its type II receptor or the intracellular effectors *Smad2, 3* and *4* block the influence of TGFβ and results in enhanced branching [153, 154]. *TGF-1* is expressed in the subepithelial mesenchyme in areas where *FGF-10* is not expressed [141]. It was shown that it stimulates synthesis of matrix components that are deposited in the epithelial-mesenchymal interface and are crucial in stabilizing clefts and preventing local budding [155]. Such action would counteract the influence of FGF-10 from the mesenchyme. However, *in vivo* gene targeting of *TGF-1* does not affect lung development [156, 157] while targeting of *TGF-2* and *–3* does [158, 159]. Recently a more defined negative feed back loop has been identified based on Drosophila branching where the *sprouty* gene is induced by FGFs and the encoded

cysteine-rich protein antagonizes the action of FGFs [160]. In mice, there are three sprouty-related genes and two of them are expressed in the lung, *Spry2* in the epithelium and *Spry4* in the mesenchyme [161]. *In vitro* antisense oligonucleotide inactivation of *Spry2* expression leads to significant enhancement in branching morphogenesis [162]. Conversely, in transgenic lungs overexpressing *Spry2* the epithelium is hypoblastic (D. Warburton, unpublished).

A number of other transcription factors and signaling molecules are expressed during branching morphogenesis, either in the epithelium or in the mesenchyme of the lung [163-165]. Some, like GATA-6, are required for normal branching (chapter 3), although it is not clear how they interact with other factors. The current model of branching morphogenesis is probably incomplete as it is based on a very few signaling pathways. Moreover, the upstream signals that control these pathways and the downstream effector molecules are not yet known. Clarification of more gene functions and molecular interactions during lung development will result in a more comprehensive model for branching and ultimately, in the integration of the molecular and the extracellular matrix models.

*Lung epithelium*

The initial branching of the two primary lung buds (E9.5) results in the formation of a tubular system, which is called the primordial system. This tubular structure is lined by columnar epithelium that is considered as primordial epithelium up to around E14.5. Then, differentiation results in two sharply demarcated parts, a proximal part with columnar epithelium and a distal part with more cuboidal epithelium. The former constitutes the bronchial, air conducting part of the lung while the latter is restricted to the acinar tubules in the respiratory portion of the lung. The similarity in the shape of primordial and proximal epithelium led initially to the conclusion that the bronchial system is established first and that subsequent branching at its distal tips generates the distal respiratory epithelium. However, a more extensive histological analysis does not support this hypothesis [166] and it is also contradicted by recent molecular data [165].

Epithelial differentiation begins in an extensively branched epithelium. The sharp demarcation of the two different epithelia simply suggests that the bronchial and respiratory systems each originate from a separate part of the primordial system. Branching morphogenesis is however, not completed by E14.5. Even in the bronchial system the number of generations of bronchial branches may not be the final. New branches can only arise by lateral branching of primordial epithelium that is still present in the bronchial part of the lung. In the distal part, the acinar tubules undergo a more extensive morphogenesis, which occurs during the developmental stages that follow the pseudograndular stage, after E16.5 until puberty (P30). It involves transitional structures with a duct-, sac-, or pouch-like shape that all constitute the prospective pulmonary acinus, which is the distal respiratory unit of the developing lung [166]. The definitive pulmonary acinus is established when growth has stopped, after puberty. Scanning electron microscopy

revealed that it consists of one or two generations of rather short respiratory bronchioles and about three generations of alveolar ducts opening into alveolar sacs [167]. The abrupt transition of the epithelium can be seen in the respiratory bronchioles where the distal part consists of cuboidal/squamous epithelium while the more proximal part contains columnar epithelium [168].

In the developing lung the columnar epithelium consists of tall cells studded with microvilli. Around E17 there is an increasing number of ciliated columnar cells, which are interspersed with protruding nonciliated cells. The latter are known as Clara cells and can be recognized after E18 by their apical cytoplasmic protrusion, their tall shape, oblong nuclei, and the fact that they rise higher than the adjacent ciliated columnar cells. Molecularly they are marked by the expression of the *Clara Cell marker-10* (*CC10*) protein [169]. The ciliated cells of the lung, like other ciliated cells, are molecularly identified by expression of the winged-helix protein *HFH-4* (*hepatocyte nuclear factor-3/forkhead homologue-4*) [170].

The cuboidal epithelium in the prospective pulmonary acinus is exclusively of the alveolar type. By E15 the embryonic Type II alveolar cells can be identified. They have large, fairly round nuclei and distinct ultrastructural features with the most typical being large apical and basal glycogen fields [171]. In general, glycogen may constitute a source of energy and in these particular cells it may serve as a substrate for surfactant phospholipid biosynthesis, which is their main subsequent function. Before birth the glycogen fields disintegrate and normally their presence is considered as a distinct sign of cell differentiation in prenatal Type II cells. Ultrastructural studies revealed that endoplasmic reticulum is regularly found in the glycogen fields and it constitutes the envelope of glycogen containing cytoplasmic inclusions. These inclusion bodies show a widespread distribution and they are extremely variable in appearance. In fact, five main types of inclusion bodies have been identified during the course of Type II cell maturation and they occur with intermediate and composite forms [172]. Morphological similarities and differences in electron density suggest that these inclusion bodies are primary stages in the formation of multilamellar bodies, which are structures typical for the mature Type II alveolar cells [168, 172].

The embryonic Type II cells have a primary morphogenetic function in pulmonary acinus formation since they are the only dividing cells. Furthermore, they are the stem cells of the complete alveolar epithelium. In addition to the mature Type II alveolar cells, which are mainly involved in surfactant production, they also give rise to the more flat Type I alveolar cells that appear before birth in the derivative structures of the acinar tubules (ducts, sacs and pouches). The function of Type I cells is to mediate gas exchange and they are always in close proximity with the capillaries. This proximity is thought to be the main driving force of their differentiation [172]. However, the transformation process of the embryonic Type II cells into Type I cells is not understood. It has been even suggested that Type I cells are the result of further differentiation of mature Type II

cells. Support for this hypothesis comes from in vitro transdifferentiation experiments [173, 174]. From the moment of epithelial differentiation (E14.5), the precursor-Type II cells can be molecularly identified by the expression of different surfactant proteins, like *Surfactant Protein-C* (*SP-C*) [175]. The mature Type II cells sustain surfactant protein expression, while the differentiation to Type I cells is associated with diminishing surfactant expression.

The aforementioned cell types are only a few, out of the many, present in the lung epithelium. Some of their functions, like mucus secretion, production of surfactant and gas exchange are among the essentials for normal respiration. However, the other cell types may influence their development and differentiation. The pulmonary endocrine cells (PNE) could be such an example. These clusters of cells (or individual cells) are among the first to differentiate from the primitive lung epithelium and it is thought that they may regulate further epithelial differentiation by expressing a number of cytokines [165, 176, 177].

The current knowledge of the molecular control of pulmonary epithelial differentiation is very limited. Gene targeting studies have implicated some gene functions as essentials for this process. In most of the cases the differentiation defect is coupled to an earlier branching morphogenesis defect, making evaluation more difficult. However, for others, the clear involvement in branching only implicates that the gene function is dispensable for normal differentiation. The shh pathway appears to be a typical example. Mutant lungs fail to branch and they form two bilateral sacs subsequent to the formation of the primary buds. Nonetheless, proximo-distal (P-D) differentiation appears to take place and both proximal and distal epithelial markers were detected in the mutant lungs [142]. The function of TTF-1, GATA-6, HNF3-β and TGFβ-1 seem to be important for both branching and epithelial differentiation. Gene inactivation of *TTF-1* results in a phenotype similar to shh mutant lungs with the difference that distal epithelial differentiation is also impaired [142]. The generation of highly chimeric lungs with *GATA-6-/-* ES cells resulted in abnormally branched lungs that lack any P-D differentiation (chapter 3). Furthermore, transgenic embryos overexpressing the protein under the control of the *human surfactant protein-C* promoter [178], have an epithelium that is less extensively branched but more interestingly its differentiation to distal alveolar epithelium is blocked. None of the mature forms of the alveolar epithelium (Type I and II) are detected, while proximal epithelium is fully differentiated (chapter 4). The same promoter was used to generate transgenic lungs overexpressing HNF3-β or TGFβ-1. In addition to morphogenetic arrest, epithelial differentiation in these transgenic lungs was blocked at the pseudoglandunal stage of lung development [179, 180].

Proximal cell fate has been associated with HFH-4, which is specifically expressed in ciliated proximal epithelium [170]. Mutant mice lack cilia and they have random left-right laterality of the lung [130]. Interestingly, transgenic lungs with ectopic expression of the gene in the distal epithelium have an altered epithelial differentiation in addition to branching defects. Instead of alveolar

epithelium, the pulmonary acinus is lined with ciliated cells, which are typical for proximal epithelium [170]. Thus, HFH-4 seems to be required for developing the proximal phenotype and the protein is able to induce this phenotype in epithelium that is destined to be distal [181, 182].

BMP-4 is considered as an important determinant for distal epithelial fate. Transgenic overexpression of *BMP-4* resulted in markedly decreased distal differentiation, monitored as *SP-C* expression. However, ultrastructural analysis suggested that Type I cell fate was promoted instead, although molecular confirmation could not be obtained due to a lack in Type I specific markers [139]. A subsequent transgenic study in which BMP-4 was blocked, either by a dominant negative BMP-4 receptor (*BMPRIB*) or by the secreted BMP-4 antagonist *Noggin,* introduced the idea of signaling centers regulating P-D cell fates in the lung [140]. Inhibition of BMP-4 activity resulted in a severe reduction in distal epithelial cell types and in a concurrent increase in proximal cell types. Both morphology (presence of cilia) and expression of markers, like *HFH-4* and *CC10,* confirm the latter. According to the proposed model, BMP-4 is part of an apical signaling center that influences epithelial cell fate in its close proximity. Cells exposed to high levels of BMP-4 would maintain or adopt a distal character, while cells receiving little or no BMP-4 signal would initiate a proximal differentiation program. FGF-10 is anticipated to be part of such a signaling center since it is one of the inducers of *BMP-4* expression in the epithelial tip of the growing bud [115]. However, endoderm that buds towards an FGF-10 bead does not show a particular distal character, at least with regard to surfactant protein expression [114]. In the trachea transdifferentiation experiments, FGF-7 in combination with other factors was able to induce distal fate, but FGF-10 was unable to do so [183]. Nevertheless, the proposed role of BMP-4 concentration in pulmonary cell fate acquisition is analogous to the mechanism of cell fate determination in the limb. The progressive zone that is induced in the mesenchyme by FGF signaling from the apical ectodermal ridge (AER) shows high levels of *BMP-4* expression. Cells leaving this zone differentiate as proximal cell types, while cells remaining in the zone continue to proliferate and acquire a more distal cell character [184, 185]. It therefore seems likely that combinatorial FGF-10 and BMP-4 signaling may generate an apical signaling center in lung endoderm, which coordinates both branching morphogenesis and P-D epithelial differentiation.

## *GATA FAMILY OF TRANSCRIPTION FACTORS*

The GATA family of transcription factors consists of proteins that are able to regulate transcription by binding to DNA elements in cis containing the consensus sequence A/TGATAA/G. This activity is mediated by a highly conserved Cys-$X_2$-Cys-$X_{17}$-Cys-$X_2$-Cys zinc finger motif which is present in the carboxy-terminus of each of the members of the family. A second zinc finger motif, more proximal to the amino-terminus of the protein, modulates DNA binding and interacts with other proteins-cofactors. GATA factors have been characterised in a number of different species ranging from yeasts to humans. In fungi they regulate nitrogen metabolism, light induction, siderophore biosynthesis and mating-type switching [186]. The Caenorhabditis elegans END1 GATA factor controls gut endoderm specification [187] and in Drosophila, the *serpent* gene is involved in haematopoiesis and endodermal midgut specification [188, 189] while *pannier* functions during specification of cardiac cell fate [190]. In higher vertebrates there are six members identified to date and they are subdivided into two subfamilies. The first three members consist the haematopoietic subfamily since they all have unique functions during the development of different haematopoietic lineages although they are also expressed in other tissues [191, 192]. GATA-4, -5, –6 are the heart and gut related members [84, 193].

### *GATA-1*

*Expression and mutant phenotype*

GATA-1 is the founder and the best studied member of the GATA family of transcription factors. In the haematopoietic system, it is expressed predominantly in the erythrocyte lineage but also in megakaryocytes and mast cells [194-196]. It is also expressed in the Sertoli cells of the testis [197]. Loss of function studies in mice revealed that GATA-1 protein is required for survival and maturation of both primitive and definitive erythroid precursors [198-202]. GATA-1 promotes erythropoiesis not only by the induction of lineage specific gene expression but also by controling cell proliferation [203] and apoptosis [192]. When *GATA-1* expression is elevated the precursor cells proliferate at the expense of differentiation, resulting in the block of erythropoiesis [205, 206]. In the megakaryocyte lineage, GATA-1 controls proliferation and growth of precursor cells affecting platelet development [207, 208]. In Sertoli cells there is no *in vivo* data demonstrating a unique function of GATA-1 and only expression and *in vitro* studies have implicated the protein in the repression of anti-Mullerian hormone expression after birth [209].

*Regulations and interactions*

Within the haematopoietic system, GATA-1 exerts its function by binding to the consensus sites in the DNA of target genes and by interacting with other

proteins. This dual function is important not only for developing a lineage-specific phenotype but also in determining cell fates for lineage specification. Of the two zinc fingers, the carboxy-terminal one is both necessary and sufficient for binding to the DNA, while the amino terminal one shows no independent DNA binding activity [210]. However, the amino terminal zinc finger was shown to bind to a more diverse site, with potential implication in transcriptional repression [211]. Furthermore, the amino finger influences the specificity of the binding of the carboxy terminal finger to the DNA. Depending on the binding site(s), it can stabilize, disrupt or modify binding to the DNA resulting in diverse transactivation properties of the GATA-1 protein [212]. By this mechanism GATA-1 (and probably other GATAs) can discriminate between genes and elicit distinct responses from them.

The amino terminal zinc finger mediates interaction with other proteins. The best example is the Friend of GATA-1 (FOG-1) zinc finger protein, which can enhance or repress GATA-1-mediated activation [213, 214]. The significance of this interaction is demonstrated by the phenotype of mice mutant for *FOG-1*, which is similar to that of *GATA-1* mutants but with a more enhanced defect in the megakaryocyte lineage [215]. Furthermore, humans carrying a mutation in the amino terminal finger of *GATA-1*, which is shown to affect interaction with FOG-1, have dyserythropoietic anemia and thrombocytopenia [216]. GATA-1 interaction with the widely expressed molecules CBP and p300 could extend the function of the protein beyond the erythroid lineage. These transcription cofactors were shown to promote GATA-1 activity and although acetylation could be one of the mechanisms, the nature and significance of the interaction is not yet clear [217-219]. There are better-understood interactions that demonstrate the role of GATA-1 outside the erythroid and megakaryocyte lineages. Generally, GATA-1 together with FOG-1 is thought to maintain the multipotency of the haematopoietic precursors. Modulating GATA-1 activity by removing FOG (C/EBP mediated downregulation) leads to the differentiation of eosinophils [220]. Inhibition of GATA-1 binding to the DNA by the myeloid specific transcription factor PU.1 allows the differentiation of the myeloid lineage [221, 222].

Thus, the crucial role of GATA-1 during haematopoiesis is mediated by the DNA binding ability of the carboxy-terminal finger and by the crucial function of the amino-terminal finger in modulating this binding and furthermore in interacting with other tissue specific or general transcription cofactors. However, the two zinc fingers of GATA-1 alone are not sufficient to recapitulate the full function of the protein. The recombination of a *GATA-3* cDNA into the *GATA-1* locus resulted in a partial rescue of the survival of the erythroid precursors due to the insufficient accumulation of GATA-3 protein [223]. When different GATAs were expressed under a *GATA-1* locus control construct, which is able to dictate proper protein accumulation, embryonic haematopoiesis was rescued but adult erythropoiesis was impaired [224]. This suggests that the almost identical zing

fingers possessed by the haematopoietic GATAs do not make them functionally equivalent and that GATA-1 has a unique function during haematopoiesis.

### *GATA-2*

*Expression and mutant phenotype*

*GATA-2* is highly expressed in haematopoietic precursors and at a lower level in early erythroid cells, mast cells and megakaryocytes [225]. Outside the hematopoietic system, *GATA-2* expression overlaps with that of *GATA-3* in many sites of the developing central nervous system [226] and in the placenta [227]. Lack of *GATA-2* in mice results in early embryonic lethality due to defective haematopoiesis, and virtually all lineages are affected [228]. This phenotype pointed to a crucial role of GATA-2 in the survival and expansion of early haematopoietic precursors or stem cells [229]. During differentiation of pluripotent haematopoietic cells, *GATA-2* expression declines and in the erythroid lineage this is necessary for the further development of the erythroid phenotype [230-232]. The erythroid specific GATA-1 protein mediates this *GATA-2* downregulation, which does not occur in embryos lacking GATA-1 [200]. When *GATA-2* expression is sustained in the haematopoietic progenitors, there is a block in their amplification and differentiation, implying that GATA-2 protein is a crucial determinant in influencing self-renewal versus differentiation [232]. In the placenta, GATA-2 together with GATA-3 regulates the synthesis of placental hormones. Placentas lacking *GATA-2* secrete less angiogenic activity and the decidual tissue adjacent to these placentas displays markedly reduced neovascularization [233].

*GATA-2 and Central Nervous System*

During development of the central nervous system, *GATA-2* is expressed as early as embryonic day 9 in the developing hindbrain. More specifically, the protein is detected in ventral rhombomere 4 and transiently in rhombomere 2 [226]. In rhombomere 4, expression of *GATA-2* is induced by Hoxb1 and it is necessary for further induction of *GATA-3* expression. Studying null mutant embryos proved this hierarchy and furthermore, it was shown to be critical for the correct projection and migration of neuron originating from rhombomere 4 (vestibuloacoustic efferent, facial branchiomotor) [234]. After embryonic day 9, *GATA-2* is expressed in many different sites of neuronal differentiation and it has been suggested that, like in haematopoiesis, GATA-2 is involved in the maintenance of the pool of ventral neuronal progenitors [226]. This was clearly demonstrated for the V2 interneurons, which were drastically diminished in *GATA-2* null mutant embryos [235]. When all three critical functions of GATA-2 mentioned above were rescued by a YAC transgene, another unexpected role of the protein was revealed. Rescued pups died of hydroureternephrosis caused by severely deformed kidneys and ureters and they also displayed a number of other genitourinary abnormalities [236].

*GATA-3*

*Expression and mutant phenotype*

Although GATA-3 is considered the third haematopoietic GATA factor, its only site of expression in the haematopoietic system is in T-lymphocytes of the thymus. Outside the haematopoietic system, it is expressed in a number of different tissues and organs like ectoplacental cone, central and peripheral nervous system, otic and optic vesicles, kidneys, liver, adrenal glad, endothelial cells [237, 238]. Loss of function of *GATA-3* in mice results in an early lethality around embryonic day 12 due to extensive internal bleeding and severe deformities of the brain and the spinal cord [239]. *In vitro* culturing of *GATA-3* mutant fetal liver cells suggested that the protein is required for definitive fetal liver haematopoiesis although no lineage specificity was demonstrated. Only recently, expression of *GATA-3* was described in intraembryonic hemogenic sites, like the floor of the dorsal aorta and some mesodermal aggregates beneath the aorta, suggesting a potential role in intraembryonic stem cell generation [240].

*GATA-3 and T-cell development*

*GATA-3* mutant ES cells are able to contribute *in vivo* to all different haematopoietic lineages except thymocytes and mature peripheral T-cells [241, 242]. In this lineage GATA-3 is required for the development of the early T-cell progenitors and protein expression is associated with distinct differentiation stages [241]. It is involved in the induction of all the Th2 cells specific cytokines, thereby playing a key regulatory role as mediator of the humoral immune response [243-245]. Increased levels of the protein have been associated with allergic response [246] and a dominant negative form of GATA-3 inhibits allergic inflammation in a murine model of asthma [247]. This dominant negative form revealed that acetylation is an important modification of the protein and that this alone affects T-cells survival and homing to secondary lymphoid organs [248]. Interaction with other proteins (ROG-repressor of GATA) is emerging as one of the mechanism through which GATA-3 regulates expression of cytokine genes and thereby differentiation to Th2 versus Th1 lineage [249]. The crucial function of GATA-3 in the T-cell lineage and more specifically in the Th2 cells, renders the protein as a key regulator in this haematopoietic lineage and further as a potential therapeutic target of asthma and allergic diseases [250].

*GATA-3 and Nervous System*

The expression of the gene in the central nervous system and the study of the genomic sequences conferring this expression pattern [251, 252] led to a pharmacological rescue of the embryonic lethality in the *GATA-3* mutant mice. Early embryonic development was rescued by providing the embryos in vivo with catechol intermediates to stabilize noradrenaline production [253]. This rescue

established an important role of the protein in the sympathetic nervous system and furthermore implies that absence of GATA-3 in this system is actually the primary cause of the previously described embryonic lethality [239]. In the rescued embryos, subsequent necessary functions of the protein were revealed in a number of other tissues and organs. Derivatives of the cephalic neural crests, like lower jaw and part of the ear were affected and organs like thymus and kidneys were hypoplastic [253]. As explained above, thymus development was not surprisingly affected and GATA-3 was already implicated in human kidney development [254] as well as in the differentiation of the hair cells in the cochlea of the mouse ear [255]. More interestingly, *GATA-3* haplo-insufficiency has been recently associated with the hypoparathyroidism, sensorineural deafness, renal anomaly (HDR) syndrome in humans [256]. This also holds true in mice, at least with regard to deafness (van der Wees, unpublished).

Since the gene is expressed in multiple sites in the central and peripheral nervous system [226], a more detailed analysis of the pharmacologically rescued mutants may reveal more distinct functions of GATA-3 protein within these systems. However, there are some sites in the nervous system in which GATA-3 function has already been explored. As mentioned earlier, induction of *GATA-3* expression by the Hoxb1 induced GATA-2 in rhombomere 4 is critical for the correct projection and migration of neurons originating from this rhombomere [234]. Furthermore, the study of chimeric embryos generated with *GATA-3* mutant ES cells implicated the protein in the development of serotonergic neurons in the caudal raphe nuclei and in their function in locomotion [257].

### *GATA-4*

*Expression and mutant phenotype*

GATA-4 is the best studied member of the heart and endoderm related subfamily of GATAs. During embryogenesis, it is expressed in the visceral yolk sac endoderm, in the cardiogenic mesoderm and subsequently in the developing heart, in proximal and distal gut, testis, ovary and liver [258, 259]. Expression persists in the adult heart, ovary, testis, liver and small intestines [259]. *GATA-4* inactivation in the mouse results in embryonic lethality around day 8.5 due to defects in heart morphogenesis and ventral closure of the foregut [20, 21]. The cardia bifida displayed by the mutant embryos is the result of a failure in ventral fusion of the lateral aspects of the embryo and the cardiac tissue that is present bilaterally is fully differentiated. Although *in vitro* studies in P19 cells suggested that GATA-4 is required for terminal cardiac differentiation [260], *GATA-4 -/-* ES cells are able to contribute to all three heart layers in chimeric embryos and the mutant cardiomyocytes in such embryos have a fully differentiated phenotype [261]. Furthermore, in embryos mutant for the cardiac basic helix-loop-helix protein dHAND, there is no *GATA-4* expression in the cardiac mesoderm and yet cardiac differentiation and morphogenesis proceed to latter stages when embryos

die due to underdeveloped right ventricle and abnormal outflow tract [262]. These data imply that the GATA-4 protein is not essential for terminal differentiation of cardiomyocytes and suggests that other GATAs, such as GATA-5 or GATA-6, may compensate for the lack of GATA-4.

*Primary defect in mutants embryos*

The phenotype of the *GATA-4* null embryos points to a more general defect in the proper ventral closure of the embryo resulting from an abnormal morphogenesis of the definitive endoderm. However, this is not due to an intrinsic defect in the definitive endoderm lineage but rather due to a defective signaling from the primitive yolk sac endoderm. When *GATA-4 -/-* ES cells were differentiated in vitro, the mutant embryoid bodies were deprived from fully differentiated visceral yolk sac endoderm [22]. *In vivo*, chimeric embryos exclusively derived from *GATA-4* mutant ES cell were normal due to the wild type primitive yolk sac endoderm that is provided by the wild type host blastocyst [76]. In the quail system, transplantation of normal anterior primitive endoderm is able to rescue defects in heart tube morphogenesis caused by absence of *GATA-4* expression due to vitamin A deficiency [263]. These observations are in support for a role of GATA-4 in the extraembryonic primitive endoderm and although in the *GATA-4* mutant embryos the yolk sac was formed, its function and signaling ability has not been assessed. Additionally and more interestingly, it was observed that one third of the *GATA-4* null embryos were arrested before gastrulation when the gene is normally expressed in the visceral yolk sac endoderm [20]. The null embryos that were characterized at embryonic day 8.5 are likely to represent the ones that survived due to a compensatory effect of the GATA-6 protein, which has been shown to also be expressed in the primitive endoderm and it is upregulated in the *GATA-4* mutant embryos [20, 21, 264].

*Role in cardiogenesis*

Although the *in vivo* data from the *GATA-4* null embryos and ES cells suggest redundancy among the GATA factors during heart development there are numerous *in vitro* studies demonstrating the transcriptional regulation of cardiac specific genes by GATA-4. Very few of these transcriptional regulations are specific for GATA-4 but there are some protein interactions appearing to be specific for GATA-4 protein. Furthermore clinically associated conditions have been specifically connected to GATA-4 function. The protein was shown to regulate a number of structural cardiac genes such as - and *-myosin heavy chain* ( -, *-MHC*) [265], *cardiac troponin-C* (*cTNC*) [266], *atrial natriuretic factor* (*ANF*) [267], *brain natriuretic peptide* (*BNP*) [268], *cardiac troponin-I* [269, 270], *sodium/calcium exchanger* (*NCX1*) [271], *cardiac-restricted ankyrin repeat protein* (*CARP*) [272], *A1 adenosine receptor* [273], *m2 muscarinic receptor* [274] and the *myosin light chain 1/3* [275]. Among all these cardiac genes only - and - *myosin heavy chain* gene are preferentially regulated by GATA-4 due to higher affinity of

the protein for their promoters [276]. For most of the other genes there is cooperative interactions among the GATAs, and especially GATA-6, which is coexpressed with GATA-4 in both myocardium and endocardium [258, 259, 277]. The two proteins are able to form an heterotypic complex that binds a single GATA site and synergistically activates transcription [276]. The complexity of the heart with the dynamic, but non fully overlapping, expression pattern of all three GATAs during heart morphogenesis suggests that only heart specific gene depletion of each one of the members will reveal a possible unique function during cardiogenesis. In humans, a chomosomal deletion resulting in *GATA-4* haploinsufficiency has been associated with congenital heart diseases [278] and although other genes may also be involved, they are certainly not *GATA-5* or *–6*.

*Protein interaction with Nkx 2.5*

An interesting regulation and interaction is with the homeobox gene *Nkx 2.5* (the homologue of the Drosophila *tinman*), which is required for proper looping of the heart [279]. In promoter studies of *Nkx 2.5*, GATA sites were identified which are functional at different stages during cardiogenesis [280, 281]. From GATA-4 dominant negative experiments in Xenopus it has even been suggested that GATA factors may normally antagonize transcription of *Nkx2.5* thereby limiting the boundaries of its expression domain during specification of the cardiogenic field [282]. The two proteins are co-expressed very early during the specification of the cardiac mesoderm and in the chick embryo they were shown to be direct mediators of the cardiac inductive signal of the bone morphogenetic proteins (BMP) [283-285]. However, neither of them alone is able to induce the full cardiogenic program, both proteins need to be present [286, 287].

This observation led to the hypothesis that the two genes may function in concert to induce cardiac development. Indeed, the study of the *ANF* and *cardiac -actin* promoter proved that there is a synergistic transcriptional activation of both genes by Nkx 2.5 and GATA-4 proteins. Additionally, a direct physical interaction of the two proteins was demonstrated, which involves the carboxy-terminus part of GATA-4 (including the zinc finger) and the carboxy-terminus part (with the homeodomain) of Nkx2.5 [288, 289]. The result of such an interaction depends on the promoter and the binding sites present. If Nkx2.5 binds to the DNA its autorepressive domain is blocked by interacting with GATA-4 allowing Nkx2.5 mediated transcriptional activation [290]. When only a GATA site is present, interaction with Nkx2.5 reduces the ability of GATA-4 to activate transcription [291]. Interestingly, this interaction with Nkx2.5 appears to be the only specific one for GATA-4, for example GATA-6 cannot substitute for GATA-4 [290].

*Other important interactions*

Other proteins such as the MADS box proteins, serum response factor (SRF) and myocyte enhancer factor-2 (MEF-2), also interact with the carboxy-

terminus of GATA-4 to activate cardiac specific gene expression [292, 293]. Similar to GATA-1, interaction with the general factor CBP was shown to stimulate transcription dependent on GATA-4 [217]. The amino-terminal zinc finger of GATA-4 mediates an interaction with the transcriptional modifying protein friend of GATA–2 (FOG-2) [294-296]. It is uncertain if FOG-2 acts as a transcriptional activator or repressor of GATA-4, but its interaction with the repressor protein CtBP2, implies that FOG-2 may be a transcriptional repressor of GATA-4, -5 and –6 [297]. This interaction is likely to play an important role in regulating GATA factor-dependent gene expression in the heart since *FOG-2* null mice die during embryogenesis with significant cardiac abnormalities [298, 299].

Last but not least, a very interesting interaction with significance for heart failure is that with the Nuclear Factor of Activated T-cells 3 (NF-AT3). GATA-4 was implicated in hypertrophic heart development that results in heart failure by the upregulation of its expression, necessary for the induction of genes required for the growth of the cardiac tissue [300-302]. The interaction of GATA-4 with NF-AT3 (GATA-5 and –6 can also interact) upon translocation of the latter to the nucleus is one of the ways that this induction is mediated. Since NF-AT3 translocates to the nucleus subsequent to calcium dependent phosporylation, it suggests a link between the increased calcium levels detected in heart failure and the resulting cardiac gene transcription [303, 304].

*Gonadal differentiation*

Among all the other tissues expressing *GATA-4*, the dynamic expression pattern of the gene in the gonads implicated GATA-4 in early gonadal differentiation and sexual dimorphism. By embryonic day 11.5, when there is no sex-specific differentiation, *GATA-4* is expressed in the primitive gonads of both male and female embryos. Shortly after the histological differentiation of the ovaries, expression is markedly reduced while in the testis expression persists throughout development and adulthood [305]. Specifically, *GATA-4* expression is associated with the somatic cell lineages, the Sertoli and Leydig cells in the testis and the granulosa cells in the ovary, rather than the primordial germ cells [306, 307]. However, in humans expression of *GATA-4* has been reported in fetal germ cells and prepubertal spermatogonia and it is downregulated after puberty [308].

Consistent with the role of GATA-4 in sexual dimorphism of the gonads, the protein was shown to regulate expression of the Mullerian inhibiting substance, which is necessary for male sex differentiation [305]. This regulation is mediated by protein interaction with another sex determination protein, the steroidogenic factor-1 (SF-1) [309, 310]. Furthermore, *GATA-4* and the co-expressed *GATA-1* were shown to transactivate *inhibin/activin beta-B*-subunit gene transcrption in testicular cells [311].

Although the gene is downregulated in the embryonic ovaries, there is strong expression after birth in the granulosa cells of the follicles. This expression

declines as the cells differentiate or undergo apoptosis, suggesting a function of GATA-4 as a cell survival factor in granulosa cells, analogous to GATA-1 in erythroid cells [312]. Another role in reproduction has been speculated for GATA-4, though not directly relevant to gonadal development. Gene expression was shown in migrating gonadotropin-releasing neurons and the protein was shown to regulate the promoter of the *gonadotropin-releasing hormone* (*GnRH*) gene [313, 314].

*GATA-4 and definitive endoderm*

Within the definitive endoderm lineage, not much is know about specific functions of GATA-4. GATA-factor-binding activity has been described in gut endodermal cells at their pluripotent stage. Such activity, at least half contributed to GATA-4, potentiates gene expression and endoderm specification to organs such as the liver, as it will be discussed later [80, 81]. This was studied in the liver specific albumin gene and gave insight not only in a liver specific GATA-regulation but in a general mechanism of gut endoderm specification [80]. Another gene that is expressed in the liver and is activated by GATA-4 is the homeobox gene *Hex*. GATA-4 together with Hepatocyte Nuclear Factor-3b (HNF-3b) transactivate the gene specifically in this organ [315]. In the small intestines, expression of all three *GATAs* has been described in different vertebrates. *GATA-4* and *–5* seem to have overlapping expression pattern and they have been both associated with terminal differentiation of epithelial cell at the tips of the villi. *GATA-6* has a distinct, complementary expression pattern [316].

## *GATA-5*

*Expression and mutant phenotype*

During embryogenesis, *GATA-5* is expressed in the allantois, heart, outflow tract, lung mesenchyme, urogenital ridge, bladder and gut epithelium. Expression persists in the small intestine, stomach, bladder and lungs in adult life [317]. Surprisingly, for a GATA protein, targeted disruption of *GATA-5* in the mouse did not result in embryonic lethality. Male mutant mice are indistinguishable from wild type mice and only in the mutant females there are some abnormalities in their genitourinary tract [318]. Specifically, these females show malpositioning of the urogenital sinus, vagina and urethra, a phenotype mimicking a condition of proximal hypospadias in human females. This result clearly demonstrates that GATA-5 has a unique function during female genitourinary tract development and that in other organs expressing the protein other GATAs may compensate for its lack.

*Role in cardiogenesis*

During cardiogenesis, *GATA-5* has a more restricted expression pattern that overlaps with both *GATA-4* and *–6*. At the early primitive steak stage it is expressed in the precardiac mesoderm and later, during and after heart looping, *GATA-5* transcripts are detected in both atrial and ventricular chambers [317]. Expression markedly declines at midgestion and becomes restricted to the endocardium and finally it ceases during late fetal and postnatal development. Given this restricted expression pattern and the already mentioned fact that most cardiac genes can be equally efficient regulated by all GATA proteins, the normally formed hearts in the *GATA-5* null embryos did not come as a surprise. However, GATA-5 specific cardiac regulation has been suggested in rat cardiomyocytes due to higher affinity of the protein for some promoters [319]. Furthermore, specific protein interaction with the ubiquitously expressed transcriptional activator p300 was shown to regulate transcription of the cardiac restricted *atrial natriuretic factor* gene (*ANF*) [320]. Also transcription of the -*myosin heavy chain* gene in myocardial cells undergoing leukemia inhibitory factor-induced hypertrophy was attributed specifically to GATA-5 [321]. Interestingly, in zebrafish, mutation of the *GATA-5* affects the production of normal numbers of myocardial procursors, the expression of cardiac specific genes and the migration of the cardiac primordia to the embryonic midline [322]. In fact the phenotype is quite reminiscent of the mouse embryos mutant for *GATA-4*, suggesting a potential reversal in the roles of these two GATA proteins between mammals and fish.

*GATA-5 and definitive endoderm*

As can be predicted from the mouse *GATA-4* null phenotype, endoderm morphogenesis is also affected in the zebrafish *GATA-5* mutants [322]. However, Xenopus is an organism in which GATA-5 plays a key role in the endodermal lineage. As in other vertebrates, during gastrulation endoderm is one of the three germ layers that subsequently gives rise to most of the thoracic and abdominal organs. During Xenopus development, *GATA-5* is specifically expressed in the cell population that will become the endoderm. But more importantly, when it is ectopically expressed in another germ layer, like mesoderm, it is able to respecify the cells towards an endodermal fate [64]. In higher vertebrates GATA-5 was shown to regulate expression of the *pepsinogen* gene in the developing chicken stomach and its own expression is affected by epithelial-mesenchymal interaction [323]. Additionally, there is the study of intestine epithelial cell differentiation, which is already mentioned in the function of GATA-4. The two genes are coexpressed at the villus tip where there is extensive cell differentiation and they have been both implicated in activation of terminal differentiation genes like *intestinal fatty acid binding protein* (*IFABP*) [316].

*GATA-6*

*Expression pattern*

GATA-6 is presently the last member of the GATA family of transcription factors and it is expressed in a number of different tissues. When the expression domains of all the other members are also considered, there is hardly any tissue of mesodermal or endodermal origin that does not express one of the GATAs at some stage during development. *GATA-6* is first expressed at the blastocyst stage in part of the inner cell mass and in the trophectoderm (chapter2). During and after gastrulation, expression can be seen clearly in the derivatives of the primitive endoderm, visceral yolk sac and parietal endoderm. Subsequent to mesoderm formation, the gene is expressed in the lateral plate mesoderm and it marks the cardiogenic plate [23, 258] (chapter2). Throughout heart tube development transcripts are present in both myocardium and endocardium. However, subsequent to the formation of the four chambered heart, high expression levels are found in the left ventricle and atrium as well as in the endocardial cushions of the outflow tract, the atrioventricular canal and the interventricular septum [258, 324]. Parallel to the dynamic expression during heart tube formation and morphogenesis, strong expression is detected in the developing primitive gut and throughout foregut, midgut and hindgut development. Later during embryonic and fetal development, *GATA-6* transcripts are detected in the bronchial epithelium of the developing lungs, in the liver, dorsal aorta, urogenital ridge, granulosa cells of the ovary, Sertoli cells of the testis, arterial smooth muscle cells and in the epithelial layer of the stomach, small and large intestines [258, 307, 312, 325]. Postnatally, *GATA-6* expression persists in the heart, aorta, lung, stomach, small intestines, gonads, adrenal cortex and bladder [258, 326].

*Mutant phenotype*

Although GATA-6 is the last member of the family it is actually the one that is required first during development since mice depleted of its function die around embryonic day 6 [23] (chapter2). The lethality is caused by a defect in the primitive endoderm lineage, most likely in the visceral yolk sac endoderm. Evidence in support of this is the expression pattern of the gene in this tissue and the dowregulation of visceral endoderm specific genes, like *Hepatocyte Nuclear Factor- 4* (*HNF-4*) and *GATA-4*, in the null embryos. Additionally, the mutant embryoid bodies developed *in vitro* show a defective visceral endoderm with a similar effect on gene expression [23].

Consistent with this explanation is the study using chimeric embryos (chapter2). The analysis is based on a previous observation that in chimeric embryos the visceral endoderm as a derivative of the extraembryonic primitive endoderm, receives very low, if any at all, contribution from the injected ES cells [327]. Therefore, the generation of chimeric embryos by using different combinations between wild type or *GATA-6* mutant blastocysts and ES cells can

attribute the defect to the embryo or to an extraembryonic tissue. When *GATA-6-/-* ES cells were used to generate highly chimeric embryos, which were almost exclusively *GATA-6 -/-*, the early lethality was overcome. This was due to the presence of wild type extraembryonic tissues that were provided by the host blastocyst. In the complementary experiment, *GATA-6 -/-* blastocysts that were injected with wild type ES cells did not survive later than embryonic day 5.5. This implies that the lack of GATA –6 in the extraembryonic tissues provided by the mutant blastocyst is sufficient to cause the lethality.

Morphology of both in vitro and in vivo developed mutant embryos suggests that visceral endoderm is the extraembryonic tissue that is defective in the absence of GATA-6 and indirectly influences the growth and survival of the underling embryo (chapter 2). Further study and comparison of wild type and mutant embryoid bodies revealed that GATA-6 directly regulates the expression of the mitogen-responsive phoshoprotein *Dab2* in the visceral endoderm. This regulation is specific to GATA-6 since Dab2 is not detected in the visceral endoderm of *GATA-6* null embryos and GATA-4 not only is unable to confer any regulation in vitro but also its absence in vivo does not affect expression of the Dab2 protein [328].

*Role in cardiogenesis*

The early lethality of *GATA-6* null embryos does not allow direct assessment of its potential role during cardiogenesis. As mentioned earlier, regulation of different cardiac specific genes has been attributed to both GATA-4 and GATA-6 proteins [84, 193] and in the case of *ANF* and *BNF* the two proteins form an heterotypic complex to cooperatively activate their transcription [276]. Furthermore, Nkx2.5 regulates an element in both mouse and chicken *GATA-6* genes, which is required for gene expression in the cardiogenic mesoderm [329, 330]. Considering the fact that enhancers controlling *Nkx2.5* expression in the heart contain essential GATA binding sites [281, 331], it seems that the two factors cross regulate one another's expression in the cardiac crescent and early heart tube. However, it is not known which factor starts the feedback loop of this cross regulation but it is likely that this loop serves to reinforce an expression induced by other molecules and/or endodermal inductive signals [330].

When *GATA-6 -/-* ES cells were differentiated in vitro, beating cardiomyocytes were formed indicating that the cardiogenic program was completed although expression of individual genes including *Nkx2.5* has not been assessed (chapter2). Additionally, in chimeric embryos generated with *GATA-6* mutant ES cells, contribution is evident in the early heart tube suggesting that there is no cell autonomous defect in cardiomyocytes depleted for GATA-6 [23] (chapter2). These data point to a redundant function of the protein during cardiogenesis and only the generation of heart specific mutants will reveal any possible unique functions of protein. Interestingly, in Xenopus cardiomyocyte *GATA-6* expression has been associated with maintenance of the precursor stage of

the cells and differentiation is the result of *GATA-6* downregulation. When expression of the gene was kept constant normal differentiation was blocked [332].

*GATA-6 and differentiation*

Association between *GATA-6* expresssion and differentiation has been studied in human and rat vascular smooth muscle cells (VSMCs). The VSMCs of the normal adult vessels are quiescent cells and display characteristics of a differentiated phenotype [325]. Upon stimulation to proliferate by mitogens or following an arterial injury *GATA-6* expression normally declines. When GATA-6 levels are sustained, sebsequent stimulation with mitogens has no effect since GATA-6 induces cell cycle arrest by activating the general cyclin-dependent kinase inhibitor p21 [333]. In agreement, in balloon injured rat carotid arteries, *GATA-6* expression normally declines but when it is artificially sustained, the intimal hyperplasia associated with the injury is inhibited [334]. At the molecular level, GATA-6 was shown to physically interact with the general transcriptional activator p300 in order to induce expression of the *smooth muscle-myosin heavy chain* gene (*Sm-MHC*), a specific marker for the differentiated phenotype of VSMCs [335]. This specific function of GATA-6 in the control of the VSMCs phenotype suggests a potential role of the protein in the pathogenesis of atherosclerotic and restenotic lesions in humans. Recently, a similar function has been described in the glomerular mesangial cells (GMCs) of the kidneys. In these cells *GATA-6* expression is also associated with quiescent phenotype and in a similar fashion when the gene was overexpressed, proliferation arrest was induced by upregulation of p21 [336, 337].

*GATA-6 and definitive endoderm; function in the lungs*

In derivatives of the definitive endoderm *GATA-6* expression has been described in the gastric endocrine cells of the rat [338] and in the stomach epithelium the protein activates expression of the *Trefoil Factor Family 1* and *2* genes (*TFF1* and *2*) [339]. In the intestinal epithelium the gene is expressed in a pattern complementary to that of *GATA-4* and *–5*. While the two other *GATAs* are expressed in the differentiating epithelium of the villus tip, *GATA-6* is expressed in the region of the proliferating progenitor cells at the proximal sites of the villus tip [316]. During embryonic development, GATA-factor binding activity has been associated with endoderm specification to liver promordia, as mentioned already for GATA-4 [80, 81]. Additionally, the GATA-6 protein was shown to regulate in vitro lung endoderm specific genes like *Thyroid Transcription Factor-1* (*TTF-1*) and *surfactant protein-A* [340, 341].

The only *in vivo* data implying an important role of the protein in a definitive endodermal lineage was the observation that *GATA-6 -/-* ES cells do not contribute to the broncial epithelium of the developing lungs in chimeric embryos [23]. This observation led to a widely accepted conclusion that GATA-6 protein is required for early pulmonary endoderm specification [165]. When a more

extensive study of the contribution of the *GATA-6* mutant ES cells into the lung endoderm was carried out, a different and more specific role of the protein was revealed (chapter3). The generation of chimeric embryos by using genetically marked blastocysts (ROSA26), the progeny of which could be traced in the developing embryos, and higher number of injected mutant ES cells demonstrated that *GATA-6 -/-* cells are able to form pulmonary endoderm. Nevertheless, this mutant endoderm does not branch properly and its further differentiation is affected (chapter3).

In support of an important function of the protein in branching morphogenesis and epithelial differentiation are the data from *in vivo* overexpression of *GATA-6* in the lung epithelium (chapter4). When a pulmonary epithelium specific promoter (from the *human surfactant protein-C* gene) was employed to direct *GATA-6* expression in the lungs of transgenic mice, both branching and differentiation were affected. Specifically, the poorly branched epithelium was able to differentiate only along the proximal pathway. Distal epithelium which normally expresses *GATA-6* showed a lack of terminal differentiation to TypeI or TypeII pneumocytes, necessary for gas exchange and surfactant protein production, respectively (chapter4).

*Function of GATA-6*

GATA-6 has several functions. First, it is required in the primitive endoderm for the proper formation of the visceral yolk sac endoderm, which further supports the growth and the survival of the embryo (chapter 2). Subsequently, during organogenesis GATA-6 function is crucial for the proper branching and differentiation of the pulmonary endoderm to form a functional respiratory tree (chapter3). Within the distal epithelium, expression levels of the protein are critical for differentiation and when *GATA-6* expression is elevated, terminal differentiation is blocked (chapter4). Thus, GATA-6 is an important factor during endoderm development in the mouse embryo. Such a unique role for a GATA factor in the mouse endoderm does not come as a surprise. As already mentioned, *xGATA-5* was shown to be necessary and sufficient for endoderm specification in the xenopus embryo [64] and in the Caenorhabditis elegans the GATA factor END1 is crucial for the formation of the E cells, the progenitors of the endoderm [187, 342]. In flies, the GATA factor serpent determines the endodermal midgut [188, 189].

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Chapter 2

# The transcription factor GATA-6 is essential for early extraembryonic development

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# 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 trophectoderm. 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-$X_2$-Cys-$X_{17}$-Cys-$X_2$-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 splanchic 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; Molkentin 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 (Molkentin 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 02 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′-TAACGCCAGGGTTTTCCCAG-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% $CO_2$ 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 ClaI* site and the plasmid *ClaI* 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 μl of anti-GATA4 antibody and resulted in a supersbift (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

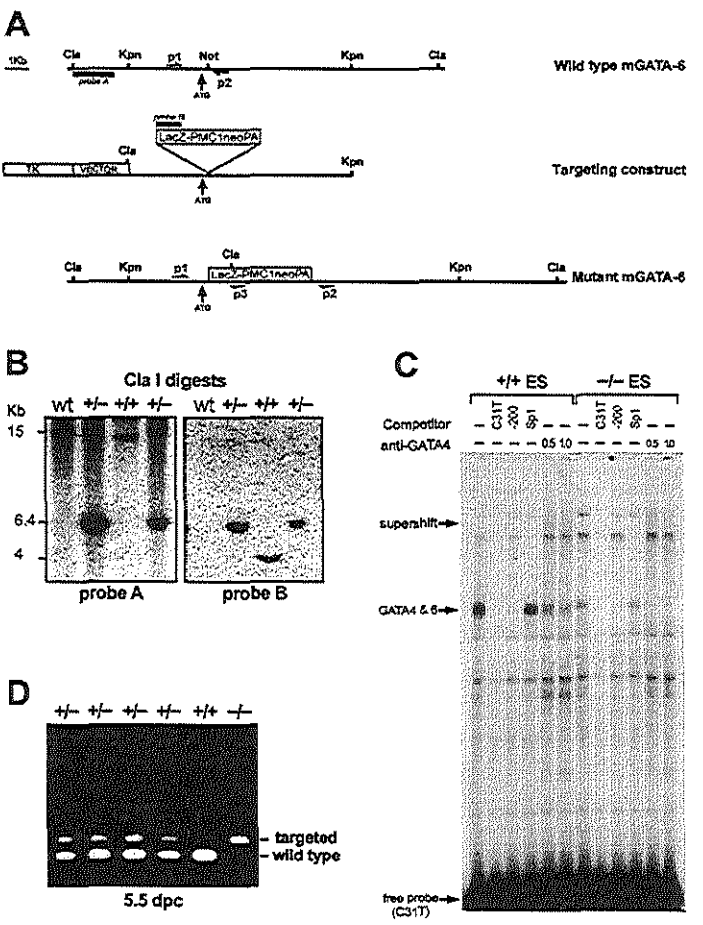


**Fig. 1.** Targeted disruption of the *GATA6* gene. (A) Schematic representation of the targeting strategy. Top line: Partial restriction map of the murine *GATA6* locus in which the ATG is indicated with the arrowhead. Middle line: Targeting vector containing the polyoma enhancer-herpes simplex virus 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 *ClaI*-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.

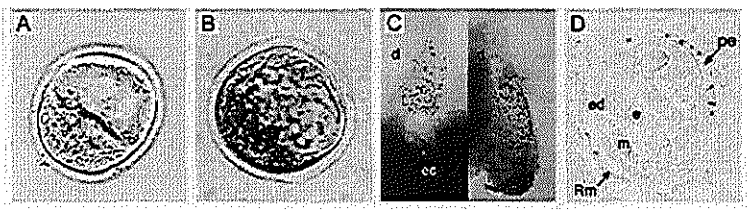


Fig. 2. Expression of *GATA6* gene, as detected by β-galactosidase activity, in early mouse embryos heterozygous for the *lacZ* insertion. (A,B) 3.5 dpc embryos, blastocysts stage, from heterozygote intercrosses with clear difference in the intensity of the staining, presumably corresponding to the presence 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 μm 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

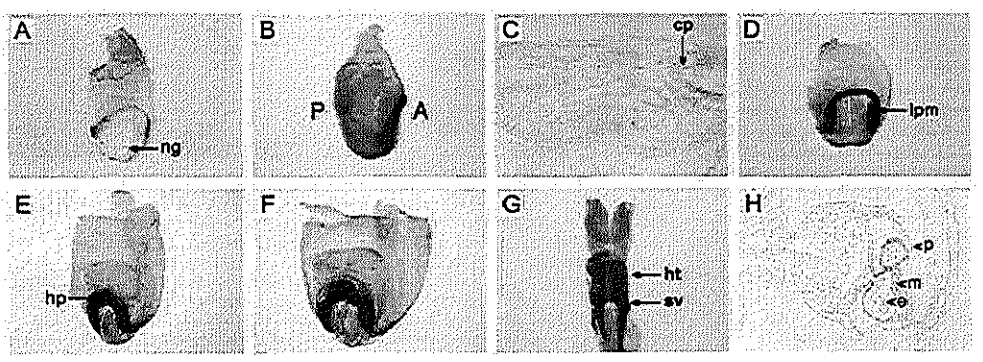


Fig. 3. Embryonic expression of the mouse *GATA6* gene, as demonstrated by β-galactosidase activity in mouse embryos heterozygous for the *lacZ* insertion in the *GATA6* locus. (A) An advanced primitive streak embryo at 7.5 dpc (ng, neural groove) shows mesodermal expression, which is better visualized 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).

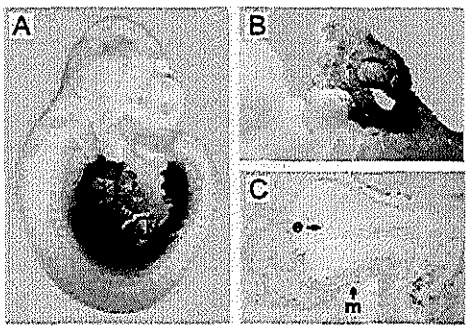


Fig. 4. *lacZ* expression in a *GATA6*$^{+/-}$ embryo at embryonic day 9.5. (A) Whole-mount β-gal staining. Expression persists in the heart tube, mainly in the inflow and outflow tracts, and very strong expression can be seen in the foregut and hindgut as well as in the midgut. (B) Higher magnification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

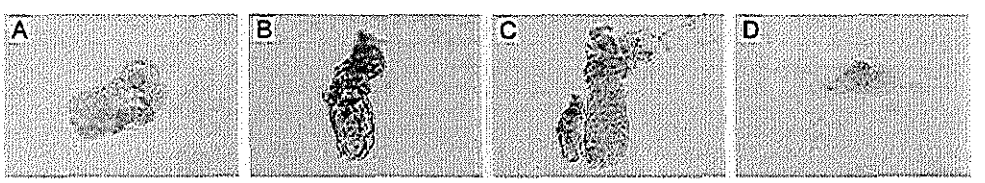


Fig. 5. GATA6 expression during the phase of peri-implantation lethality. (A) A 4.5 dpc heterozygous embryo in which the β-gal staining is restricted to a few cells on one side of the blastocoelic surface of the inner cell mass adjacent to the trophoectoderm. (B) An implanted 5.5 dpc embryo from heterozygous 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.

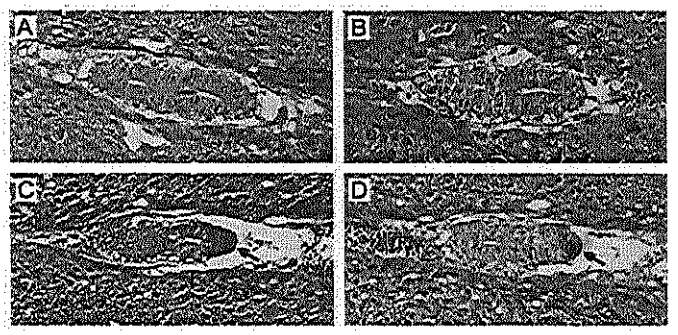


Fig. 6. Haematoxylin and eosin-stained sagittal sections of 5.5 dpc embryos from GATA6 heterozygous intercrosses. (A,B) Normally developed embryos; (C,D) embryos showing abnormal visceral endoderm surrounding the embryonic ectoderm (arrow).

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 trophectoderm 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

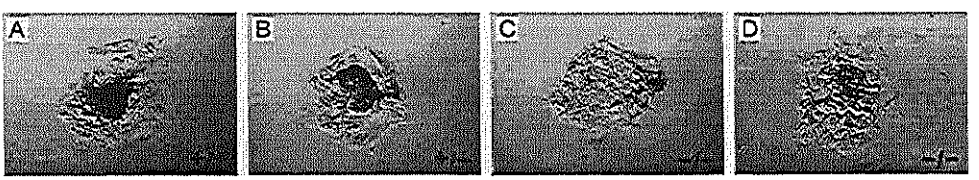


Fig. 7. In vitro blastocyst outgrowths after 5 days in culture. (A,B) Wild-type and heterozygous blastocysts, respectively, with the expected outgrowth of trophoblast cells and the formation of an inner cell mass surrounded by the visceral endoderm. (C,D) Homozygous mutants, which exhibit normal outgrowth of the trophectoderm 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.

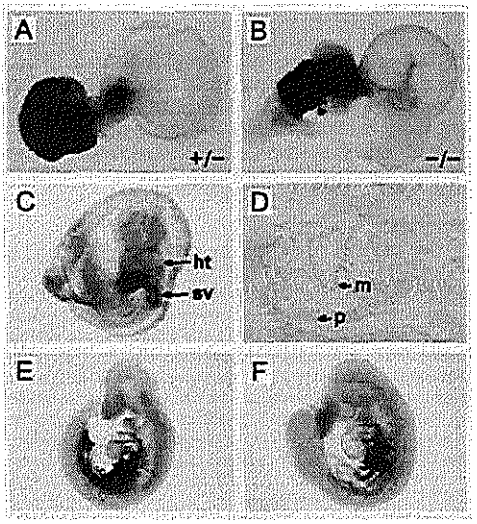


Fig. 8. Developmental potential of the *GATA6*$^{-/-}$ ES cells. (A,B) β-gal-stained embryoid bodies derived from ES cells differentiated in vitro for 23 days in suspension culture. There is no morphological difference between heterozygote and homozygote mutants and they both display contraction activity. 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*)

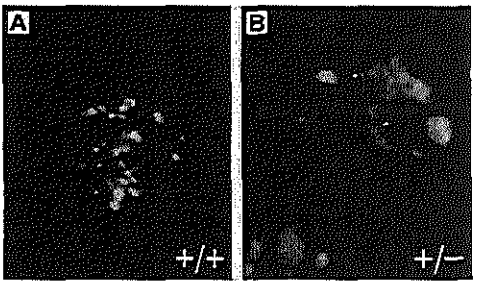


Fig. 9. FISH genotyping of Reichert's membrane nuclei derived from 7.5 dpc chimeric embryos generated by injecting wild-type ES cells into blastocysts from *GATA6*$^{+/-}$ intercrosses. A cocktail probe was used with a cosmid for the HIRA locus as a wild-type control (red) and a plasmid with the *lacZ*-neo 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 linaeges. 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 splanchic 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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Chapter 3

# The transcription factor GATA-6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development

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# The transcription factor GATA6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development

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## SUMMARY

**Recent loss-of-function studies in mice show that the transcription factor GATA6 is important for visceral endoderm differentiation. It is also expressed in early bronchial epithelium and the observation that this tissue does not receive any contribution from *Gata6* double mutant embryonic stem (ES) cells in chimeric mice suggests that GATA6 may play a crucial role in lung development. The aim of this study was to determine the role of GATA6 in fetal pulmonary development. We show that *Gata6* mRNA is expressed predominantly in the developing pulmonary endoderm and epithelium, but at E15.5 also in the pulmonary mesenchyme. Blocking or depleting GATA6 function results in diminished branching morphogenesis both in vitro and in vivo. TTF1 expression is unaltered in chimeric lungs whereas SPC and CC10 expression are attenuated in abnormally branched areas of chimeric lungs. Chimeras generated in a ROSA26 background show that endodermal cells in these abnormally branched areas are derived from *Gata6* mutant ES cells, implicating that the defect is intrinsic to the endoderm. Taken together, these data demonstrate that GATA6 is not essential for endoderm specification, but is required for normal branching morphogenesis and late epithelial cell differentiation.**

Key words: GATA6, Branching morphogenesis, Lung, Endoderm differentiation, Mouse

## INTRODUCTION

The formation of a lung requires a well organized program to coordinate the balanced interplay between activation and repression of gene transcription as it develops from a cluster of cells in the floor of the primitive foregut to the highly vascularized gas exchange organ (Hogan, 1999; Warburton et al., 2000). Several evolutionarily conserved signaling pathways have been implicated in the different stages of fetal pulmonary development. In particular members of the fibroblast growth factor, bone morphogenetic protein, hedgehog/Gli, wingless and epidermal growth factor families have been demonstrated to be key regulatory factors for lung morphogenesis and epithelial differentiation, but lung endoderm specification is less well understood (for reviews see Hogan, 1999; Warburton et al., 2000). Recently, a member of the GATA family of transcription factors, GATA6, has been implicated in endoderm specification (Morrisey et al., 1998). In vertebrates, the GATA family of transcription factors contains a conserved zinc-finger motif that binds to the consensus sequence A/TGATA/G resulting in transcriptional regulation of genes in different cell lineages (for reviews see Charron and Nemer, 1999; Evans, 1997; Jordan and Van Zant, 1998; Orkin, 2000). The GATA transcription factors can be subdivided into two main groups. First, GATA1, GATA2 and GATA3, which all have unique functions in the hematopoietic system (Leonard et al., 1993; Pandolfi et al., 1995; Pevny et al., 1991; Simon et al., 1992; Ting et al., 1996; Tsai et al., 1994; Weiss et al., 1994; Whyatt et al., 2000) and, in case of GATA2 and GATA3, in many other tissues, including the nervous system and the kidneys. Second, GATA4, GATA5 and GATA6, which are mainly expressed and involved in the formation of the extra-embryonic and embryonic endoderm, as well as in the cardiogenic mesoderm (Arceci et al., 1993; Koutsourakis et al., 1999; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997a; Morrisey et al., 1997b; Morrisey et al., 1998).

Radioactive in situ hybridization experiments have demonstrated that GATA6 mRNA is expressed in the developing embryonic bronchial epithelium and in chimeric experiments this endodermally derived tissue did not receive any contribution from *Gata6*$^{-/-}$ ES cells (Morrisey et al., 1996; Morrisey et al., 1998). The conclusion of these studies was that

GATA6 is required for specification of lung endoderm. In addition, the promoters of several lung specific genes, such as the genes for human thyroid transcription factor 1, human surfactant protein C, mouse surfactant protein A and mouse Clara cell marker 10, contain GATA motifs (Ikeda et al., 1995; Korfhagen et al., 1992; Ray et al., 1993; Wert et al., 1993) and GATA6 has also been shown to activate both surfactant protein A and thyroid transcription factor 1 reporter gene transcription in vitro (Bruno et al., 2000; Shaw-White et al., 1999). Taken together, these data strongly suggest that GATA6 plays an important role during lung development.

We therefore searched to identify the specific role(s) of GATA6 in fetal pulmonary development. In order to obtain a more detailed insight into the spatial-temporal distribution of GATA6 mRNA, non-radioactive in situ hybridization during fetal lung development in vivo was performed. The observed expression patterns in early embryonic lungs suggest a role for GATA6 in branching morphogenesis, which we investigated using organotypic explant cultures with antisense oligonucleotides. To confirm and further investigate the role of GATA6 in lung development in vivo, we also generated highly chimeric embryos by injecting wild-type blastocysts with *Gata6*$^{-/-}$ embryonic stem (ES) cells. The latter experiments enabled us to overcome the early embryonic lethality in *Gata6*$^{-/-}$ mice, which is due to extra-embryonic defects (Koutsourakis et al., 1999; Morrisey et al., 1998), because in such chimeras, the extra-embryonic tissues are provided by the wild-type host blastocyst. Highly chimeric lungs were histologically analyzed and the expression of molecular markers for endoderm specification and epithelial cell differentiation (thyroid transcription factor 1, surfactant protein C and Clara cell marker 10) was investigated using immunohistochemistry and in situ hybridization.

Branching morphogenesis was dramatically affected in vitro when antisense oligonucleotides for *Gata6* were added to lung cultures. In chimeric embryos, pulmonary endoderm was formed but it branched abnormally and failed to undergo late epithelial cell differentiation, based on diminished surfactant protein C (SPC; Sftpc – Mouse Genome Informatics) and Clara cell marker 10 (CC10; Utg – Mouse Genome Informatics) expression. Using ROSA26 recipient blastocysts, we demonstrate that this abnormal epithelium is derived from *Gata6*$^{-/-}$ ES cells. We conclude that GATA6 is essential for the later stages of branching morphogenesis and late epithelial cell differentiation, but not for lung endoderm specification.

## MATERIALS AND METHODS

### Whole-mount in situ hybridization

Wild type embryonic day (E) 10.5 and E12.5 embryos were isolated from pregnant FVB female mice according to standard methods (Hogan et al., 1994) and the lungs were dissected from these embryos using microsurgical techniques. The lungs were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 to 6 hours, dehydrated in a graded series of ethanol and kept in 100% methanol at –20°C until used. The whole-mount in situ hybridization protocol was adapted from the protocol described by Wilkinson (Wilkinson, 1993). In short, following hydration, the lungs were bleached for 1 hour in 6% (v/v) hydrogen peroxide in PBS containing 0.1% (v/v) Tween-20 (PBT) and permeabilized with proteinase K (10 $\mu g.ml^{-1}$ in PBT) for 15 minutes at room temperature. Subsequently, the lungs were post-fixed in 4% (w/v) paraformaldehyde in PBS and prehybridized in hybridization mixture, containing 50% formamide, 5× SSC (NaCl 0.75 mol/l, sodium citrate 0.075 mol/l, pH 4.5), 1% sodium dodecyl sulphate, 5 $\mu g.ml^{-1}$ yeast tRNA, 50 $\mu g.ml^{-1}$ heparin in water for 1 hour at 70°C. Hybridization was carried out for 16 to 18 hours at 70°C using the same hybridization mixture, but now containing a digoxigenin (DIG)-labeled sense or antisense GATA6 RNA probe. A 1.5 kb fragment of the 5′ end of the mouse *Gata6* gene that was described previously (Brewer et al., 1999) was used to generate a probe for in situ hybridization on sections. A *PstI-NotI* subclone from the 5′ part of this 1.5 kb fragment was used to generate a probe for whole-mount in situ hybridization. The cDNA fragments were DIG-labeled according to a protocol provided by the manufacturer (Roche Diagnostics, Almere, The Netherlands). The next day, the lungs were stringently washed and treated with RNAse to avoid nonspecific background staining. Following a blocking step using 10% (v/v) sheep serum in 0.14 mol/l NaCl, 2.7 mmol/l KCl, 25 mmol/l Tris/HCl pH7.5 and 0.1% (v/v) Tween-20 in water (TBST) including 2 mmol/l levamisole, the lungs were incubated with an alkaline phosphatase-coupled antibody (1:2000), against DIG for 16 to 18 hours at 4°C. For at least 3 days the lungs were then washed in TBST and 2 mmol/l levamisole. The lungs were changed to 0.1 mol/l NaCl, 0.1 mol/l Tris/HCl, pH 9.5, 0.05 mol/l $MgCl_2$ and 0.1% (v/v) Tween-20 (NTMT) and the hybridized probe was visualized using 337.5 $\mu g.ml^{-1}$ NBT and 175 $\mu g.ml^{-1}$ BCIP mixture as a substrate. Nonspecific labeling was removed in 95% ethanol, and the lungs were kept at 4°C in PBT containing 1 mmol/l EDTA.

### Branching morphogenesis in vitro with antisense oligonucleotides

E11.5 and E12.5 lungs were harvested from wild-type embryos under a dissection microscope. The lungs were transferred to porous membranes (8 μm pore size) from Nucleopore (Whatman, NY), and incubated in 12-well culture plates from Costar (Corning, NY). The membranes were pre-soaked in MEM (Gibco) for 1 hour before the explants were placed on them. The explants were incubated as floating cultures in 500 μl Dulbecco's modified Eagle's medium, nutrient mixture F-12 (Gibco) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 mg/ml ascorbic acid. The explants were cultured at 37°C in 95% air and 5% $CO_2$. Ten cultured lungs were treated with 40 μM phosphorothioated oligonucleotides, targeted against the translation initiation site of the murine *Gata6* mRNA in the antisense direction with the following sequence: GTCAGTCAAGGCCAT. Ten cultured lungs were treated with the same concentration sense-orientated oligonucleotides with the following sequence: ATGGCCTTGACTGAC. Ten untreated cultured lungs served as controls. The lungs were cultured for up to 72 hours and branching morphogenesis was monitored daily and images captured using a dissecting microscope (Leica MZ12) and the Leica Digital Imaging Systems.

### Generation of chimeric embryos

In contrast to standard procedures, we injected more than 20 *Gata6*$^{-/-}$, or *Gata6*$^{+/-}$ for the control experiment, ES cells into each blastocyst in order to obtain highly chimeric embryos. These injections were performed either in C57BL/6 or ROSA26 (Friedrich and Soriano, 1991) blastocysts and the ES cell lines used have been previously described (Koutsourakis et al., 1999). Chimeric lungs were dissected at E12.5, E13.5, E15.5 or E18.5 and either processed for explant cultures (E13.5), as described above, or fixed and used for histological analysis and in situ hybridization, as described above (E12.5, E15.5 and E18.5). Head or tail tissue of the embryos was used to determine the percentage of chimerism using Glucose Phosphate Isomerase (GPI) electrophoresis (Hogan et al., 1994).

### In situ hybridization and immunohistochemistry

Non-radioactive in situ hybridization on 6 μm sections with SPC and

CC10 RNA probes was carried out essentially as described before by Motoyama et al. (Motoyama et al., 1998). In short, tissue sections were rehydrated and washed in PBS. Pretreatment included postfixation in 4% paraformaldehyde for 15 minutes, followed by proteinase K digestion (20 mg/ml) for 15 minutes at room temperature and acetylation (0.1 mol/l triethanolamine and 0.25% acetic anhydride) for 10 minutes at room temperature. Sections were then dehydrated and air-dried before addition of the hybridization solution. Digoxigenin-labeled probes were added to freshly prepared hybridization solution (50% deionized formamide, 10% dextran sulfate, 1.5× Denhardt's reagent, 0.5 mg/ml of yeast tRNA, 0.3 mol/l NaCl, 5 mmol/l EDTA and 25 mmol/l Tris, pH 7.5) at a concentration of 1 ng/μl. Following denaturation at 80°C, the probe was added to the tissue section and incubated for 16 to 18 hours at 55°C. After brief washes with 5× SSC and 50% formamide at 55°C, the tissue was treated with RNase A (10 μg/ml) for 30 minutes at 37°C. The digoxigenin nucleic acid detection kit (Roche Diagnostics, Almere, The Netherlands) was used for immunological detection of the hybridized probe. Tissue was then counterstained with Methyl Green and prepared for viewing.

Mouse specific SPC and CC10 cDNA fragments (330 and 315 bases, respectively) were DIG labeled according to a protocol provided by the manufacturer (Roche Diagnostics, Almere, The Netherlands). Immunohistochemistry with a monoclonal antibody against thyroid transcription factor 1 (TTF1) (Neomarkers, CA, USA) in a concentration of 1 in 100 was carried out as described before (Keijzer et al., 2000).

## RESULTS

### Spatial-temporal expression of *Gata6* mRNA during fetal lung development

Starting from E10.5, when five primary lung buds have just evaginated from the foregut, *Gata6* mRNA was detected in developing lung endoderm (Fig. 1A). At this stage, the *Gata6* mRNA was predominantly expressed at the tips of the growing lung buds. Two days later at E12.5, when branching morphogenesis of the lung buds is proceeding rapidly in order to establish the future bronchial tree, *Gata6* mRNA was still observed in the branching endoderm. However, at this stage the signal was observed in the entire endoderm lining the developing lung buds, instead of being limited only to the tips of the growing buds (Fig. 1B). On sections of wild-type E15.5 lungs, in which branching morphogenesis is almost completed, and differentiation and vascularization are beginning, *Gata6* mRNA was expressed in the endoderm lining the growing airways, as well as in parts of the mesenchyme surrounding these airways, albeit at a lower level (Fig. 1C). At E18.5, when the bronchial tree is complete, and the lung tissues are differentiating into the different cell types that will constitute the lung after birth, *Gata6* mRNA was observed mainly in epithelial cells lining the bronchioli, but also to a lesser extent in the epithelial cells lining the sacculi (Fig. 1D). At this stage, the signal in the mesenchyme was difficult to distinguish, possibly because of the thinning of this tissue layer. Hybridization with sense RNA GATA6 probes generated no specific signal in either whole-mount or section in situ hybridization experiments at all stages (results not shown).

### Antisense oligonucleotides against *Gata6* reduce branching morphogenesis in vitro

Based on the mRNA expression pattern of *Gata6*, in particular expression in the endoderm lining the early lung buds, an important role for GATA6 in branching morphogenesis was anticipated. Antisense oligonucleotides targeted against the translation initiation site of *Gata6* were used in explant cultures of isolated fetal lungs to investigate this role. When E11.5 lungs were cultured for 72 hours in the presence of antisense GATA6 oligonucleotides (Fig. 2B), branching morphogenesis of these lungs was clearly reduced when compared with the patterns of branching morphogenesis of lungs exposed to sense GATA6 oligonucleotides (Fig. 2A) or to lungs cultured in medium alone without oligonucleotides (not shown). A similar reduction in branching morphogenesis was observed when E12.5 lungs were cultured for 72 hours with antisense (Fig. 2D) oligonucleotides. Again, lungs exposed to sense oligonucleotides (Fig. 2C) and control lungs (not shown), which were cultured without oligonucleotides, demonstrated similar patterns of normal branching morphogenesis.

### *Gata6*$^{-/-}$ chimeric lungs display diminished branching morphogenesis in vitro

In all experiments highly chimeric (more than 50%) embryos and lungs were selected based on GPI analysis (results not shown). Isolated E12.5 *Gata6*$^{-/-}$ chimeric lungs were smaller in size and had less lung buds (Fig. 3B) when compared with non-chimeric littermates (Fig. 3A). From a different litter, E13.5 *Gata6*$^{-/-}$ chimeric and non-chimeric lungs were cultured for 4 days as organotypic explants. At the time of isolation, both lungs were comparable in size, but the *Gata6*$^{-/-}$ chimeric lungs had fewer branches (Fig. 3D) than the non-chimeric lungs (Fig. 3C). After 4 days of culture, the *Gata6*$^{-/-}$ chimeric lungs demonstrated diminished branching morphogenesis in certain areas of the lung (Fig. 3F, arrow), whereas in other areas, branching morphogenesis patterns were the same as wild type (Fig. 3E).

### *Gata6*$^{-/-}$ chimeric lungs display diminished branching morphogenesis in vivo resulting in respiratory insufficiency

When *Gata6*$^{-/-}$ chimeric lungs were isolated at E15.5 they appeared similar to lungs isolated from non-chimeric littermates. The size was comparable, the lungs had four lobes on the right, one lobe on the left and no defect in branching morphogenesis was observed macroscopically (Fig. 4A,B). However, when these lungs were analyzed microscopically, a clear defect in branching morphogenesis indicated by the large airspaces was observed in the *Gata6*$^{-/-}$ chimeric lungs (Fig. 4D, arrow), when compared with non-chimeric lungs (Fig. 4C). When chimeras were generated with the parental heterozygous ES cells, which were used to generate the double mutant lines (Koutsourakis et al., 1999), no abnormalities were observed in highly chimeric E15.5 lungs (results not shown). Chimeric E18.5 lungs, generated with *Gata6*$^{-/-}$ ES cells (Fig. 4F) were again the same size as the non-chimeric lungs (Fig. 4E) from their littermates. In addition, chimeric lungs had four lobes on the right and one lobe on the left. However, at this stage a clear defect in branching morphogenesis was observed macroscopically in some *Gata6*$^{-/-}$ chimeric lungs (Fig. 4F, arrows). This defect in branching morphogenesis was confirmed at the microscopic level (Fig. 4H).

Eight pups were delivered by Caesarian section at E18.5 following blastocyst injection. Without knowing their

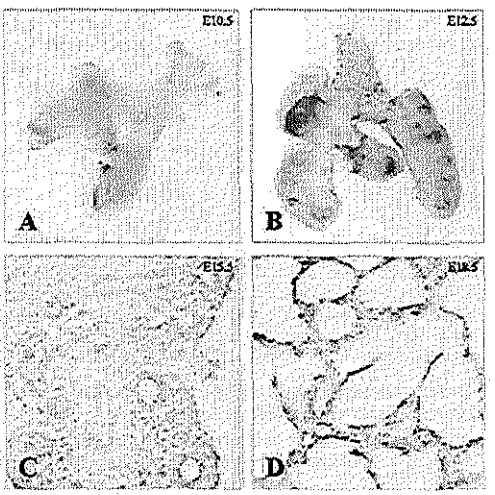


**Fig. 1.** Spatial-temporal distribution of *Gata6* mRNA during embryonic and fetal pulmonary development. (A) At E10.5, *Gata6* mRNA is predominantly expressed at the tips of the primary lung buds. (B) Two days later at E12.5, *Gata6* mRNA expression has expanded and is now expressed in the entire endoderm lining the actively branching lung buds. (C) When branching morphogenesis is almost completed at E15.5, *Gata6* mRNA is observed in the endoderm lining the growing airways, and in particular in the distal endoderm. In the mesenchyme, *Gata6* mRNA is also observed, but at a lower level. (D) Finally, during maturation and differentiation at E18.5, *Gata6* mRNA is expressed in epithelial cells lining the sacculi.

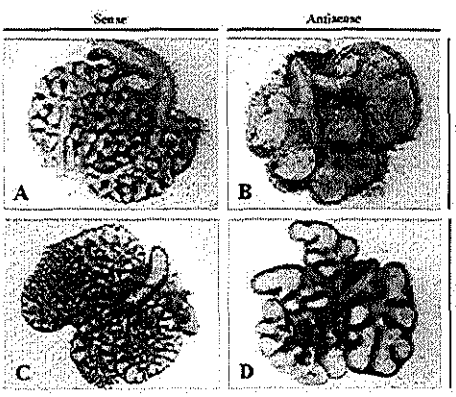


**Fig. 2.** GATA6 antisense oligonucleotides inhibit branching morphogenesis in vitro. Representative pictures of experiments with E11.5 (A,B) or E12.5 (C,D) wild-type lungs, cultured as organotypic explants in the presence of sense (A,C) or antisense (B,D) oligonucleotides targeted against the translation initiation site of *Gata6*. All pictures were taken at the same magnification.

chimerism, the pups were stimulated to breathe. Out of the eight pups, two were mummified and three were born dead. Out of the three pups that were born alive, two appeared purplish-blue and one was pink. All three breathed normally. GPI electrophoresis demonstrated that only the pink pup was non-chimeric, and all other seven pups were highly chimeric (results not shown).

### Expression of SPC, CC10 and TTF1 in *Gata6*$^{-/-}$ chimeric lungs

In order to investigate if cell differentiation was altered in *Gata6*$^{-/-}$ chimeric lungs, non-radioactive in situ hybridization with probes for SPC, a marker for type II cells that indicates distal epithelial cell differentiation, and CC10, a marker for Clara cells that indicates proximal epithelial cell

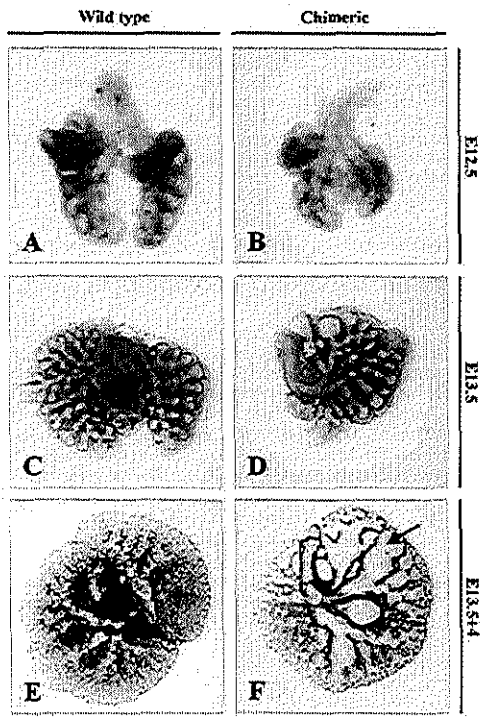


**Fig. 3.** *Gata6*$^{-/-}$ chimeric lungs display diminished branching morphogenesis in vitro. When isolated at E12.5, *Gata6*$^{-/-}$ chimeric lungs (B) are smaller and have fewer branches than wild-type lungs isolated from a littermate (A). E13.5 wild-type (C) and *Gata6*$^{-/-}$ chimeric (D) lungs at day 0 of culture as organotypic explants. After 4 days of culture (E,F), the *Gata6*$^{-/-}$ chimeric lungs had areas of normal and areas of diminished branching morphogenesis (F), whereas in the wild type lung branching morphogenesis had occurred as normal (E). All pictures are representative of a series of experiments, and are at the same magnification. (Arrow indicates area with diminished branching morphogenesis.)

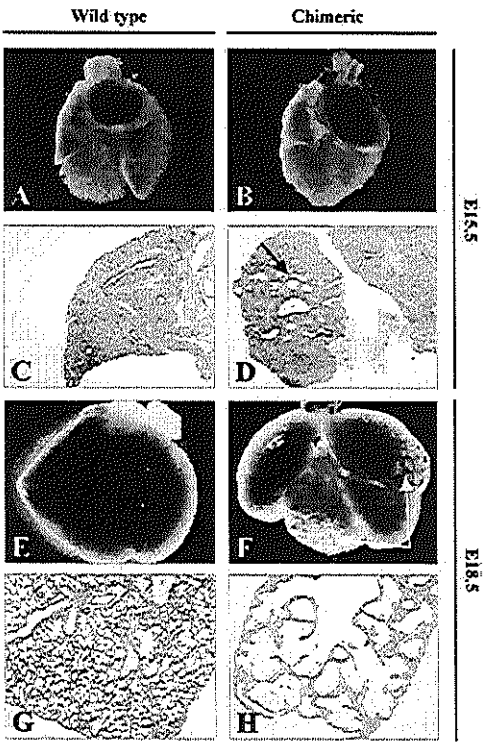


**Fig. 4.** Branching morphogenesis in vivo is diminished in *Gata6*$^{-/-}$ chimeric lungs when compared with wild-type lungs. At E15.5 (A-D), lungs appeared macroscopically indistinguishable when isolated from wild-type (A) or *Gata6*$^{-/-}$ chimeric embryos (B). However, when microscopic sections were analyzed, *Gata6*$^{-/-}$ chimeric lungs had bigger airspaces (D) that the lungs of wild-type embryos (C). At E18.5, *Gata6*$^{-/-}$ chimeric lungs (F,H) displayed diminished branching morphogenesis both macroscopically (F) and microscopically (H) when compared with lungs of wild-type littermates (E,G). All pictures are representative for a series of experiments and are at the same magnification. (Arrows indicate areas with diminished branching morphogenesis.)

differentiation, was performed. The promoters of the genes for both these proteins have previously been demonstrated to contain GATA motifs, indicating that the transcription of these genes may be regulated by GATA transcription factors (Ray et al., 1993; Wert et al., 1993). Another epithelial cell marker which has been shown to be regulated by GATA6 in vitro is TTF1. Localization of TTF1 protein was investigated in the *Gata6*$^{-/-}$ chimeric lungs.

At E15.5 (Fig. 5A-C) and E18.5 (Fig. 5D-F), normal SPC expression was observed in *Gata6*$^{-/-}$ chimeric lungs in the areas that appeared to have branched normally (Fig. 5B,C,E). In contrast, no SPC expression was observed in areas with abnormal big sacculi (Fig. 5B,C,F). Normal SPC expression was observed in the wild-type lungs in distal epithelium at E15.5 (Fig. 5A) and in type II cells at E18.5 (Fig. 5D).

At E15.5, no CC10 expression was observed in either wild-type or *Gata6*$^{-/-}$ chimeric lungs, which was expected, because the gene for CC10 is not expressed at that stage (results not shown). At E18.5, CC10 was expressed in the proximal airways of wild-type lungs, as well as in the normally branched parts of the *Gata6*$^{-/-}$ chimeric lungs (Fig. 5G,H). In the abnormally branched parts of the *Gata6*$^{-/-}$ chimeric lungs, no CC10 expression was observed (Fig. 5I).

At E15.5 and E18.5 TTF1 protein was localized normally in epithelial cells of both wild-type and *Gata6*$^{-/-}$ chimeric lungs (Fig. 6A-F). No differences were observed between areas with normal or abnormal branching morphogenesis in the *Gata6*$^{-/-}$ chimeric lungs.

### Contribution of *Gata6*$^{-/-}$ ES cells in chimeric lungs

In order to identify the origin of the endoderm of the abnormally branched areas, *Gata6*$^{-/-}$ ES cells (white) were injected into ROSA26 blastocysts (blue). In the targeted ES cell clones we used for generation of the chimeras, *Gata6* was inactivated by insertion of a *lacZ* marker gene (Koutsourakis et al., 1999). However, when we investigated β-galactosidase activity in early embryonic lungs, we could not detect activity in developing airways lined with pulmonary endoderm (results not shown). In contrast, β-galactosidase activity was detected in developing major vessels later during lung development (results not shown). Therefore, we did not expect any interference between β-galactosidase activity in ROSA26- and ES cell-derived endoderm.

Using this approach, a similar phenotype of E12.5 lungs was observed upon isolation as in the previous experiments. The *Gata6*$^{-/-}$↔ROSA26 chimeric lungs were smaller and had developed fewer lung buds (Fig. 7A). More interestingly, after detection of β-galactosidase activity, completely white endodermal buds as well as mixtures of white and blue endodermal cells and completely blue endodermal buds were observed in highly chimeric lungs (Fig. 7B). In E15.5 *Gata6*$^{-/-}$↔ROSA26 chimeric lungs, the same observations were made (Fig. 8A,B). Areas with reduced branching morphogenesis had almost completely white endoderm derived from *Gata6*$^{-/-}$ ES cells (Fig. 8A,B, arrows), whereas areas with normal branching morphogenesis had completely blue endoderm, derived from the wild-type ROSA26 host blastocyst cells (Fig. 8A,B).

## DISCUSSION

Based on experiments using chimeric embryos and extrapolation of results obtained in other organs and model systems, GATA6 was presumed to be essential for the specification of foregut endoderm into pulmonary endoderm (Kalb et al., 1998; Morrisey et al., 1998). Using experiments with antisense oligonucleotides in organotypic explant cultures, and (in particular) chimeric experiments with *Gata6*$^{-/-}$ ES cells, we provide evidence for a different and more extensive role for GATA6 in fetal pulmonary development than the previously proposed role of endoderm specification. In lung explants with already specified lung

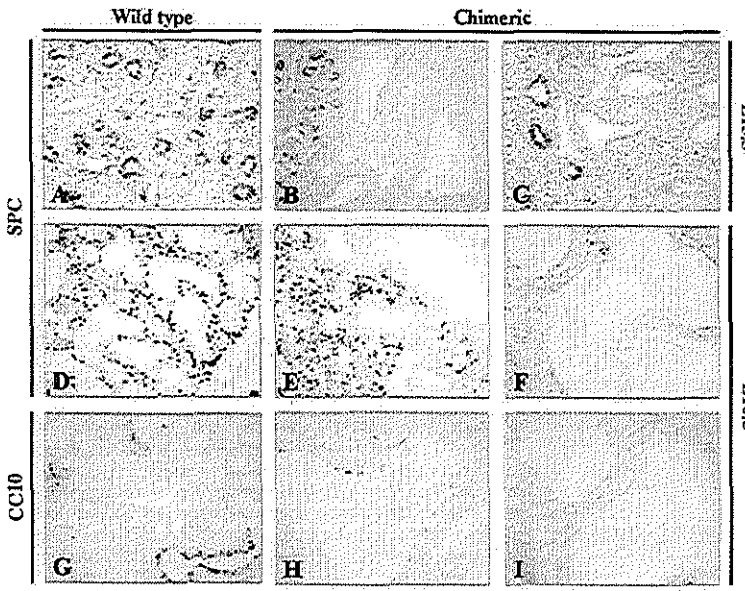


Fig. 5. mRNA distribution of molecular markers for distal (SPC) (A-F) and proximal (CC10) (G-I) epithelial cell differentiation. In both E15.5 (A-C) and E18.5 (D-F) embryos, SPC mRNA was expressed in normally branched areas of wild-type (A,D) and *Gata6*$^{-/-}$ chimeric (B,E) lungs. No SPC mRNA expression was observed in abnormally branched areas of *Gata6*$^{-/-}$ chimeric lungs (B,C,F). At E18.5, CC10 mRNA was expressed in normally branched areas of both wild-type (G) and *Gata6*$^{-/-}$ chimeric lungs (H), whereas in abnormally branched areas of *Gata6*$^{-/-}$ chimeric lungs, no CC10 mRNA expression could be observed. All pictures are at the same magnification, except B, which is a low-power overview of C.

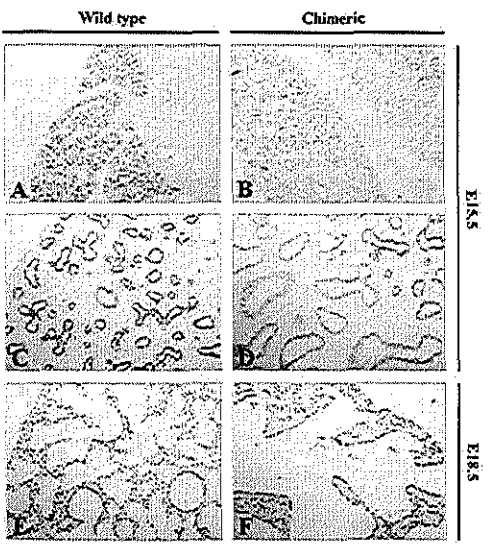


Fig. 6. Thyroid transcription factor 1 (TTF1) protein distribution as a marker for pulmonary endoderm specification. In wild-type (A,C,E) and *Gata6*$^{-/-}$ chimeric (B,D,F) lungs, similar patterns of TTF1 protein distribution were observed at E15.5 (A-D) and E18.5 (E,F). (A,B) Low-power overview of E15.5. (C-F) Same magnification.

endoderm (wild type) we could abrogate branching morphogenesis in vitro by culturing the explants in the presence of antisense oligonucleotides against *Gata6*. In agreement with this, highly chimeric lungs displayed diminished branching morphogenesis both in vitro and in vivo at different gestational ages. Proximal and distal epithelial cell differentiation was attenuated in chimeric lungs, as indicated by diminished expression of markers for distal and proximal epithelial cell differentiation, SPC and CC10 mRNA, respectively. TTF1, an early pulmonary epithelial cell marker that has also been shown to be regulated by GATA6 in vitro, was expressed as normal in chimeric lungs both at E15.5 and E18.5, indicating that endoderm specification occurred normally. In accordance with this, we found that *Gata6*$^{-/-}$ ES cells contribute to pulmonary endoderm in highly chimeric lungs, and, consequently, the observed phenotype of reduced branching morphogenesis and attenuated epithelial cell differentiation is the result of loss of function of GATA6 in pulmonary endoderm.

### GATA6 is essential for branching morphogenesis

Using both in vitro and in vivo experiments, we demonstrate here that GATA6 plays an essential role during certain stages of branching morphogenesis in the lung. Specification of foregut endoderm into pulmonary endoderm occurred normally in the chimeric lungs, because we obtained highly chimeric embryos with lungs that had undergone morphogenesis. Outgrowth of two endodermally derived primary lung buds initiates the process of lung formation. Thus, highly chimeric lungs successfully completed the earliest phase of lung development, i.e. the specification of pulmonary

endoderm. The next event in lung development, formation of four primary lung buds on the right and one primary lung bud on the left in mice, also occurred normally in highly chimeric lungs, since all isolated lungs at E12.5, E13.5, E15.5 and E18.5 had four lobes on the right and one lobe on the left side. This indicates that primary branching morphogenesis does not require GATA6. Consequently, the observed phenotype of reduced branching morphogenesis in lungs of highly chimeric embryos is the result of an essential role for GATA6 in secondary branching morphogenesis, the next stage of early pulmonary development. Either by blocking wild-type GATA6 translation with antisense oligonucleotides, or by depleting GATA6 function in chimeric lung endoderm, we demonstrate that absence of GATA6 gives rise to reduced branching morphogenesis. This resulted in lungs with very big airspaces. However, these lungs appeared no smaller than wild-type lungs, and when a marker for proliferation (Ki-67) was investigated in chimeric and wild-type lungs, no differences were observed (results not shown). Thus, the observed defect in branching morphogenesis is not the result of growth or proliferation inhibition by GATA6. For future studies it will be very interesting to investigate expression patterns of other molecules that have been demonstrated to play an important role during branching morphogenesis. Endodermally expressed markers like sonic hedgehog (SHH) (Litingtung et al., 1998), bone morphogenetic protein 4 (BMP4) (Bellusci et al., 1996) and hepatocyte nuclear factor/forkhead homolog 4 (HFH4; FOXJ1 – Mouse Genome Informatics) (Chen et al., 1998), as well as mesenchymally expressed markers like fibroblast growth factor 10 (FGF10) (Sekine et al., 1999) and GATA5 (Morrisey et al., 1997a) are likely candidates.

### GATA6 is essential for late epithelial cell differentiation, but not endoderm specification

In highly chimeric lungs, we could not detect expression of SPC and CC10 mRNA in abnormally branched areas, indicating that both proximal and distal epithelial cell differentiation is disturbed in these parts of the lungs. In areas that had undergone normal branching morphogenesis SPC and CC10 were normally expressed. These data provide evidence for a functional role of the GATA motifs in the promoters of the genes for human SPC and murine CC10 in vivo (Ray et al., 1993; Wert et al., 1993). Another gene that is thought to be regulated by GATA6 on the basis of in vitro studies is TTF1 (Shaw-White et al., 1999). In addition, TTF1 alone has been demonstrated to regulate transcription of SPC and CC10 (Kelly et al., 1996; Zhang et al., 1997). Nevertheless, we could not detect any differences in protein expression of TTF1 between chimeric and wild-type lungs using immunohistochemistry with an antibody against TTF1. This indicates that downregulation of the expression of the late epithelial markers SPC and CC10 does not function through down-regulation of TTF1, which suggests that if GATA6 and TTF1 regulate SPC and CC10 gene transcription, they do it through different pathways. The fact that we observed normal expression of TTF1 protein, a marker for lung endoderm specification, indicates that specification of foregut endoderm into pulmonary endoderm occurred normally in the chimeric lungs. Hence, we conclude that GATA6 is not involved in pulmonary endoderm specification, as has been suggested before (Morrisey et al., 1998). This conclusion corroborates with recent data obtained in experiments with *Xenopus* in which both GATA4 and GATA5 were demonstrated to induce early endodermal marker genes, whereas GATA6 was demonstrated not to induce these endodermal markers (Weber et al., 2000).

### *Gata6*$^{-/-}$ ES cells do contribute to pulmonary endoderm

Using ROSA26 blastocysts (blue) as host blastocysts for *Gata6*$^{-/-}$ ES cells (white), we were able to demonstrate contribution of *Gata6*$^{-/-}$ ES cells to pulmonary endoderm. Abnormally branched areas in E15.5 chimeric lungs had consistently white endoderm (*Gata6*$^{-/-}$) and normally branched areas blue endoderm (wild type) and therefore we

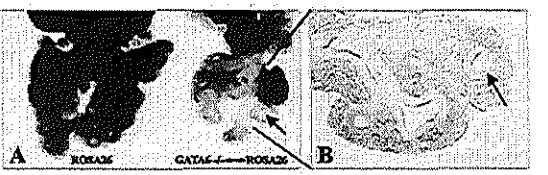


**Fig. 7.** Distribution of *Gata6*$^{-/-}$ ES cell contribution into ROSA26 host lungs. (A) *Gata6*$^{-/-}$ ES cells (white) contribute to both mesenchyme and endoderm of E12.5 chimeric lungs (right), which are smaller and have fewer branches than lungs from wild-type littermates (left). In a section of the chimeric lung (B), blue (wild-type ROSA26), mixed white and blue, and white (*Gata6*$^{-/-}$) endoderm are observed, clearly indicating that *Gata6*$^{-/-}$ ES cells do contribute to pulmonary endoderm (please note, as opposed to the usual experiment, that the white areas are abnormal and the blue areas normal). Arrows indicate white endoderm derived from *Gata6*$^{-/-}$ ES cells.

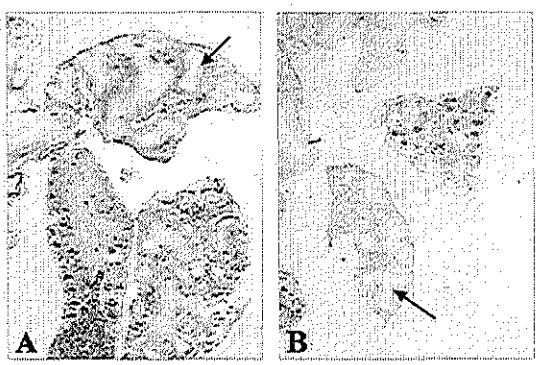


**Fig. 8.** Distribution of *Gata6*$^{-/-}$ ES cell contribution in E15.5 lungs. Both sections of different *Gata6*$^{-/-}$ chimeric lungs contain areas with abnormal branching morphogenesis that have white endoderm (*Gata6*$^{-/-}$) and areas with normal branching morphogenesis which have blue endoderm coming from the wild-type ROSA 26 blastocyst. (Arrows indicate white endoderm derived from *Gata6*$^{-/-}$ ES cells).

conclude that reduced branching morphogenesis in *Gata6*$^{-/-}$ chimeric lungs is the result of defective GATA6 mutant endoderm. Since we observed different areas with either blue, white or mixed white and blue endodermal cells and not lungs or lobes that were completely white or blue, it is likely that the lungs originate from a substantial number of cells from the foregut. Our observations are in contrast with data presented by another group, demonstrating that *Gata6*$^{-/-}$ ES cells do not contribute to the bronchial epithelium in chimeric lungs, and concluded that GATA6 is required for establishment of the endodermally derived bronchial epithelium (Morrisey et al., 1998). The contradiction in observations can be explained by the different approach that we used in order to obtain animals that were highly chimeric. Whereas regular protocols suggest the injection of nine to 12 ES cells into blastocysts to generate chimeric embryos (Hogan et al., 1994), we used double the number of ES cells to obtain highly chimeric embryos. GPI electrophoresis revealed that most chimeric embryos were derived from at least 50% *Gata6*$^{-/-}$ ES cells and only chimeric embryos that were derived from more than 50% *Gata6*$^{-/-}$ ES cells were used in our experiments. If we assume that selection by competition between wild-type and mutant cells occurs during the earliest stages of lung development in favor of wild-type cells, then the regular amount of *Gata6*$^{-/-}$ ES cells (as used by Morrisey et al.) may not have progeny in pulmonary endoderm. However, by using more *Gata6*$^{-/-}$ ES cells, mutant cells were able to survive competition and to contribute to pulmonary endoderm. To overcome any interference that resulted from the percentage of wild-type cells present during early pulmonary specification of these chimeric lungs, it will be of interest to generate embryos exclusively derived from *Gata6*$^{-/-}$ ES cells. However, in all our experiments we never found embryos that were close to 100% chimeric (based on GPI electrophoresis), although a number of implantation sites with resorbed embryos were present. Based on the fact that GATA6 is not only expressed in extra-embryonic tissues, but also in embryonic tissues (Koutsourakis et al., 1999; Morrisey et al., 1996; Morrisey et al., 1998) it is likely that embryos exclusively derived from *Gata6*$^{-/-}$ ES cells do not survive to later stages of gestation. Hence, it is unlikely that the use of methods such as the generation of tetraploid embryo chimeras would answer this question. It will probably require the generation of conditional knockout mice.

### Role for GATA6 in epithelial-mesenchymal interactions

Since branching morphogenesis is highly dependent on epithelial-mesenchymal interactions (Hogan and Yingling, 1998), it is tempting to speculate which tissue layer causes the defect that reduces branching morphogenesis. Since the spatial-temporal distribution of *Gata6* mRNA was predominantly observed in developing lung endoderm, it is most logical to postulate defective endoderm as the site where the primary defect is localized. Defective mutant *Gata6*$^{-/-}$ endoderm appears unable to undergo normal branching morphogenesis and late epithelial cell differentiation resulting in the observed phenotype in the chimeric lungs. This would lead to the conclusion that the observed phenotype is based on a cell-autonomous defect in *Gata6*$^{-/-}$ endodermal cells. However, there is an alternative explanation. Specification of pulmonary endoderm and primary branching morphogenesis occurs normally in a *Gata6*$^{-/-}$ environment, but at the initiation of subsequent branching morphogenesis, as yet unidentified signals produced by the endoderm are not processed normally by the mesenchyme, which in turn sends abnormal or no signals to the (up till that moment normal) endoderm. From then on, the endoderm would be instructed incorrectly by the mesenchyme, and would not undergo normal branching morphogenesis, resulting in big airspaces and attenuated epithelial cell differentiation. As a consequence, the observed phenotype is based on a cell-nonautonomous defect. Whichever scenario proves right, further investigation is warranted, and studies using transgenic and conditional knockout mice would be invaluable. Another important question not addressed in this study is what the early target genes for GATA6 in pulmonary development are. It is of interest whether known 'master' genes of branching morphogenesis such as *Bmp4* (Bellusci et al., 1996), *Shh* (Litingtung et al., 1998) and members of the fibroblast growth factor family (Post et al., 1996; Sekine et al., 1999) are also transcriptionally regulated by GATA6. Future studies using immunoprecipitation, yeast two-hybrid screens and DNA microarray techniques, as well as detailed promoter analysis, should bring more insight.

### GATA6 function during pulmonary development: a hierarchical model

Based on the results of our experiments, a reconsideration of the position of GATA6 in the hierarchy of factors involved in pulmonary development is warranted. To date *Gata6* was considered to be one of the master genes in lung development (Whitsett and Tichelaar, 1999). Together with HNF3β (Ang and Rossant, 1994) and TTF1 (Kimura et al., 1996), GATA6 was positioned high up in the hierarchy because all three factors were thought to be essential for the specification of foregut endoderm into pulmonary endoderm. However, in this study, we provide evidence that GATA6 is not essential for pulmonary endoderm specification, but plays an important role in branching morphogenesis and late epithelial cell differentiation. Therefore, we postulate that GATA6 functions (in concert with TTF1) in the process of branching morphogenesis and late epithelial cell differentiation.

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Chapter 4

# Branching and differentiation defects in pulmonary epithelium with elevated GATA-6 expression

*submitted for publication*

# Branching and differentiation defects in pulmonary epithelium with elevated GATA6 expression

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Running title:GATA-6 overexpression and lung development

## ABSTRACT

The transcription factor GATA-6 is expressed in the fetal pulmonary epithelium of the developing mouse lung and loss of function studies strongly suggested that it is required for proper branching morphogenesis and epithelial differentiation. We have further investigated the role of GATA-6 in this process by utilizing a pulmonary epithelium specific promoter to maintain high levels of GATA-6 protein during fetal lung development. Transgenic mice expressing a myc-tagged *GATA-6* cDNA under the control of the human *Surfactant Protein-C* (*SP-C*) promoter were generated and their lungs were analyzed during fetal stages. Transgenic lungs exhibit branching defects as early as embryonic day (E) 14.5 and molecular analysis just before birth (E18.5) shows a lack of distal epithelium differentiation whereas proximal epithelium is unaffected. Electron microscopic analysis and glycogen staining confirm the lack of differentiation to mature Type II cells. Thus, elevated levels of GATA-6 protein affect early lung development and in analogy to other GATA factors in other tissues, GATA-6 also plays a crucial role in the terminal differentiation in this case of the distal pulmonary epithelium.

Key words: GATA-6, overexpression, differentiation, branching, distal pulmonary epithelium.

## INTRODUCTION

The complexity of the lung tissue with respect to morphogenesis and cell type constitution reflects the complexity in the control of its development. Early specification (around embryonic day 9.5 in the mouse) is far from understood and molecules like HNF-3β that is thought to be involved, has a more general role in foregut and midgut formation (Ang and Rossant, 1994; Dufort *et al.*, 1998). For the initial lung bud outgrowth mesenchymal-epithelial interactions are crucial since genetic studies have shown that molecules like FGF-10, Gli-2 and Gli-3, all expressed in the lung mesenchyme, are key factors (Min *et al.*, 1998; Motoyama *et al.*, 1998). Mesenchymal-epithelial interactions are also important throughout branching morphogenesis and in the current concept of lung budding, localized high expression of *FGF-10* induces the endoderm to bud (Hogan, 1999; Weaver *et al.*, 2000). This pre-programmed process is reiterated and leads to the establishment of an extensive respiratory tree which, by the end of the pseudoglandular stage of lung development (E16.5), consists of proximal and distal airways. At this stage, epithelial cells undergo extensive differentiation to give rise to a number of distinct cell types (ciliated, nonciliated secretory, goblet, basal, alveolar Type I and Type II cells), which will establish a functional lung. Although some studies describe these different cell types morphologically and in terms of surfactant protein expression (Ten Have-Opbroek, 1991; Ten Have-Opbroek *et al.*, 1988; Ten Have-Opbroek *et al.*, 1990) their differentiation program remains obscure at the molecular level.

*BMP-4* expression in the endoderm is thought to contribute to the acquisition of cell fate with distal epithelium resulting from exposure to high growth factor concentration (Weaver *et al.*, 1999). A number of other molecules including FGFs, glucocorticoids and some transcription factors like TTF-1, HFH-4 and GATA-6 have been implicated in epithelial cell differentiation and surfactant protein production (Perl and Whitsett, 1999; Warburton *et al.*, 2000; Whitsett and Tichelaar, 1999). TTF-1 was shown to positively influence BMP-4 levels and its absence results in a lack of distal structures and the lack of surfactant proteins expression (Minoo *et al.*, 1999). The HNF3/forkhead homologue-4 (HFH-4) has been associated with induction of proximal fate and specifically the appearance of ciliated cells (Tichelaar *et al.*, 1999). GATA-6, a member of the GATA DNA binding family of zinc finger transcription factors, is expressed in the early branching endoderm and it was thought to be essential for bronchial endoderm specification (Morrisey *et al.*, 1998). However, recent studies have shown that the protein is not required for early specification but for normal branching morphogenesis and epithelial differentiation. Lung endoderm depleted of GATA-6 fails to branch normally and to acquire either distal or proximal fate (Keijzer *et al.*, 2001). Furthermore, GATA binding sites have been identified in the promoters of

Nkx2.1, Clara cell marker-10 and Surfactant Protein-A and –C (Bruno *et al.*, 2000; Shaw-White *et al.*, 1999; Wert *et al.*, 1993).

GATA proteins have been shown to play key roles in the differentiation of a number of distinct cell lineages and their levels can influence cell choices between differentiation and proliferation (Molkentin, 2000). GATA-1 functions in erythrocytes and alteration of its levels results in a block of differentiation (Pevny *et al.*, 1991; Whyatt *et al.*, 1997). Overexpression of GATA-2 blocks differentiation of early haematopoietic progenitors (Briegel *et al.*, 1993) and overexpression of GATA-3 is involved in the differentiation of T-cells (Hendriks *et al.*, 1999; Zheng and Flavell, 1997). In vivo loss of function studies in mice have shown distinct roles for GATA-4, -5 and –6 during development and both GATA-4 and –6 primarily function in the extraembryonic visceral endoderm (Koutsourakis *et al.*, 1999; Kuo *et al.*, 1997; Molkentin *et al.*, 1997; Morrisey *et al.*, 1998). *In vitro* studies have implicated GATA-6 in the differentiation program of vascular smooth muscle cells, VSMCs (Mano *et al.*, 1999), and more recently in glomerular mesangial cells, GMCs (Nagata *et al.*, 2000). In Xenopus embryos, it has been demonstrated that decreasing *GATA-6* expression is associated with differentiation of cardiac precursors. When GATA-6 levels were sustained high, the cardiac differentiation program was blocked (Gove *et al.*, 1997).

In this study we utilized a previously characterised promoter/enchancer from the human *Surfactant Protein-C* (*SP-C*) gene (Wert *et al.*, 1993) to keep *GATA-6* expression levels elevated in the pulmonary epithelium. Transgenic embryos were generated and their lungs were analyzed morphologically at various stages. Just before birth molecular as well as Electron Microscopic analysis were performed to evaluate the status of differentiation of the lung epithelium. Our results parallel data from other GATA factors and show that elevated *GATA-6* expression in the lung epithelium, *in vivo*, not only affects its normal branching pattern but also impairs the differentiation program in the distal airways.

## MATERIALS AND METHODS

### *Construction of the GATA-6 transgene*

The full *GATA-6* cDNA was constructed by joining the two *Eco*RI fragments which were isolated from a mouse E11.5 library (CLONTECH) (Brewer *et al.*, 1999; Koutsourakis *et al.*, 1999). 3' UTR was eliminated by PCR from the unique *Pst*I site to the stop codon (144bp) and a unique site was introduced in the 3' PCR primer in order to clone the last non-coding exon, intron and polyA (PA) (2.8 kb) from the human *-globin* gene (Drabek *et al.*, 1997). PCR between the first ATG and the unique *Not*I (520bp) site was used to eliminate 5' sequences. In the 5' PCR primer the myc epitope (EQKLISEEDL) was introduced immediately after the ATG of *GATA-6* (Elefanty *et al.*, 1996). The sequence of all PCR generated clones was confirmed before further cloning. The complete (1.7 kb) myc-tagged *GATA-6* cDNA was transfected in COS-1 cells and the overexpressed protein was immunoreactive in situ with both the anti-myc (9E10) and the anti-GATA-6 (SantaCruz) antibodies (data not shown). The human SP-C promoter (Wert *et al.*, 1993) was cloned as a 3.7 kb blunted *Nde*I-*Sal*I fragment in blunted *Sal*I of pBS and the *myc-GATA-6-globinPA* as an *Xho*I fragment. The transgene was released with *Cla*I between the pBS polylinker and the *Cla*I that was present at the end of the *PA*. The 8.2 kb fragment was gel purified with CONCERT™ Matrix (GIBCO BRL) and passed through ELUTIP-D column (Schleicher & Schuell).

### *Generation of transgenic embryos*

Transgenic mouse embryos were generated by pronuclear injection of FVB/N oocytes with transgene concentration 2-3 ng/μl according to standard protocols (Hogan *et al.*, 1994). Day of injection and transfer to pseudopregnant females was considered as day 0.5 for the subsequent staging of the dissected embryos and lungs. Part of the tail of the dissected embryos was used for southern blot genotyping using the human *-globin PA* as a probe.

### *In situ hybridization and immunohistochemistry*

Fetal or newborn lungs were dissected out and fixed in 4% PFA overnight at 4°C before processing for paraffin embedding according to routine protocols. 5-7μm thick sections were used for H&E staining, RNA in situ hybridization (Motoyama *et al.*, 1998) and immunohistochemistry (Keijzer *et al.*, 2000). Digoxigenin labeled RNA probes were made from cDNA fragments of 1.5kb for *GATA-6*, 0.33 kb for *SP-C* and 0.315 kb for *CC-10* according to the protocol supplied by Roche Diagnostics. For TTF-1, a monoclonal antibody (Neomarkers, CA, USA) was used in 1:100 dilution and the sections were microwave boiled in citrate buffer for

15min. The same dilution was used for the monoclonal anti-myc antibody (9E10) but for antigen retrieval, trypsin treatment (0.6mg/ml for 5min at RT) was used.

### *Electron Microscopy*

The caudal lobes of E18.5 fetal lungs were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer pH 7.3 at 4°C for a few days until the genotypes were known. One trangenic and one wild type littermate lobe were washed in 0.1 M cacodylate buffer and postfixed in 1% $OsO_4$ in 0.1 M cacodylate buffer and finally embedded in epon as has been previously described (De Bruijn and Den Beejen, 1975). Ultrathin sections were contrasted with Uranyl-acetate and Lead-citrate prior to Electron Microscopic analysis (Hanaichi *et al.*, 1986).

### *PAS staining*

From the epon-embedded E18.5 lungs, semithin (1μm) sections were used for periodic acid Shiff's staining as previously described (Nevalainen *et al.*, 1972).

## RESULTS

### *GATA-6 expression and over-expression in the pulmonary epithelium*

Expression of *GATA-6* in the developing embryonic bronchial epithelium has been described previously (Morrisey *et al.*, 1996). In a recent study (Keijzer *et al.*, 2001), a more extensive analysis of *GATA-6* expression revealed that initially it is expressed at the tips of the growing buds (E10.5) and by E12.5 it is expressed in the entire endoderm lining the developing buds. By E15.5, *GATA-6* mRNA is predominantly expressed in the endoderm lining the growing airways and to a lower extent in parts of the surrounding mesenchyme (Fig. 1A). This expression pattern, although not at a comparable level, is quite reminiscent to that of *SP-C* at the same developmental stage (Fig. 1B). This observation led us to employ the 3.7kb promoter/enhancer of the human *SP-C* gene (Wert *et al.*, 1993) in order to sustain high *GATA-6* expression levels in the developing epithelium *in vivo*. The complete mouse GATA-6 cDNA was cloned downstream of the *SP-C* promoter and was followed by the human *-globin* last exon, intron and polyA for mRNA stability. Sequences coding for the myc epitope were introduced in frame with the first ATG of the gene (Fig. 1C). The *SPC-mycGATA6* transgene was injected into fertilized eggs and lungs were isolated from embryos at different developmental stages, E14.5, E16.5 and E18.5. Embryos were genotyped by Southern blotting (data not shown) and transgenic lungs were identified. All the analysis was performed in $F_0$ transgenic founder embryos. Transgenic lungs were not significantly different in size from their wild type littermates (data not shown).

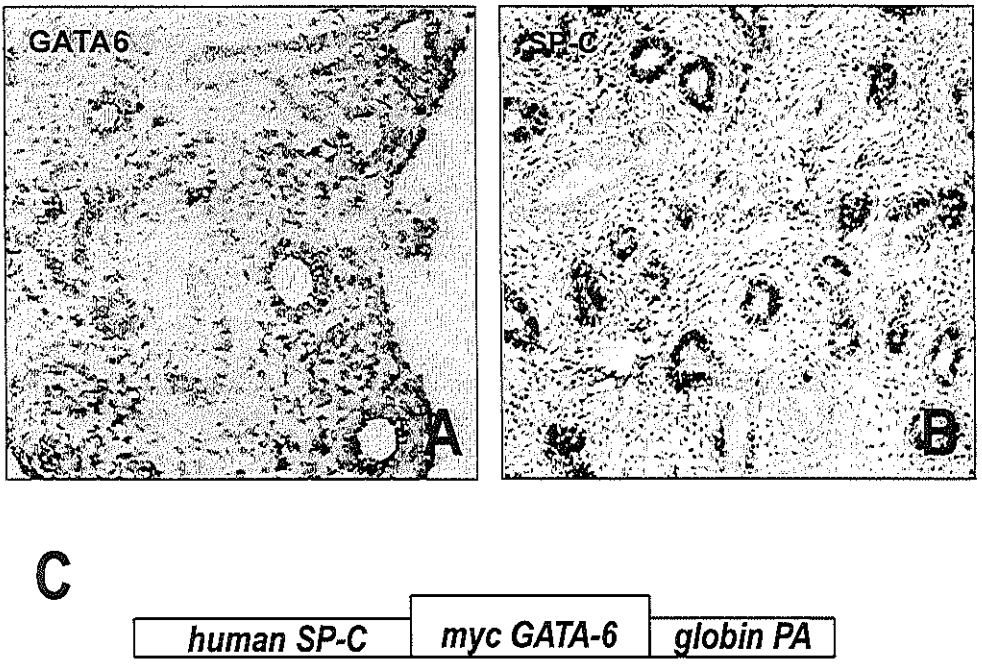



**Figure 1**
Distribution of *GATA-6* (A) and *SP-C* (B) mRNA in E15.5 lungs. Both genes, although at a different level, are expressed in cells lining the distal epithelium (blue is the detected mRNA and the tissue is counterstained with methyl-green). In C the transgene used to overexpress *GATA-6* in the pulmonary epithelium is schematically depicted. A myc-tagged version of the complete *mGATA-6* cDNA is under the control of the 3.7 kb human *SP-C* promoter and it is followed by the human *-globin* last intron and polyadenylation signal.

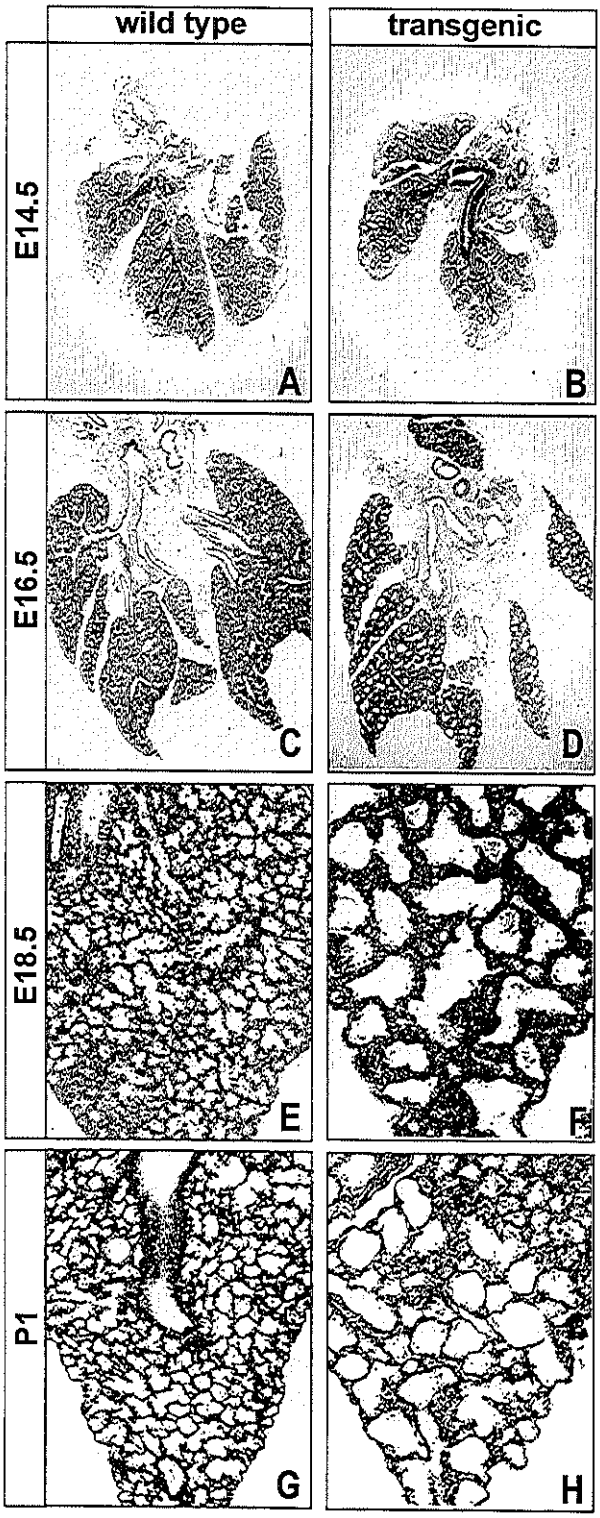
Only after careful examination of the whole transgenic lungs, a difference in the texture was noticed, especially in the affected lungs at E18.5 (data not shown). At this stage, out of 5 transgenic founder, 3 were expressing the transgene and they all had a comparable phenotype (Fig. 2F and 4H). Sections from the same transgenic E18.5 lung were used for both morphological (Fig. 2F) and transgene expression analysis (Fig. 3B&D). Expression analysis of molecular markers (Fig. 4) and EM (Fig.5) were performed on sections from a different transgenic E18.5 lung.

***Branching morphogenesis in the SP-C/GATA-6 transgenic fetal lungs***

Starting at embryonic day 14.5, the transgenic lungs looked similar in size with the wild type but they had undergone less extensive branching judged by the fewer terminal buds (Fig. 2B). Two days later, at E16.5, the defect in branching morphogenesis was more pronounced because of the presence of grossly dilated terminal buds separated by excess of mesenchyme (Fig. 2D). Just before birth, at E18.5, the phenotype was comparable to that seen at E16.5 and the overall growth of the tissue was not severely affected (Fig. 2F). The mesenchyme appears to be thickened although it is at present not clear whether this is due to an actual increase in the number of mesenchymal cells or the lack of being intersected by branching endoderm. Postnatally, although the remains of a transgenic pup were found subsequent to its perinatal death, few transgenic pups were born alive and they were breathing normally. These transgenic pups were left to breathe for few hours before they were sacrificed for genotyping and histologic analysis. In figure 2H a section of an abnormal lung among the transgenic pups is shown with a phenotype similar to the clinical centro-acinar aeration pattern. The severity of the phenotype was less than that seen in E18.5 lungs (Fig. 2F) most likely due to the lower expression levels of the transgene. Expression of the transgene in the affected E18.5 lungs was confirmed by immunostaining for the myc epitope (Fig. 3B) and *GATA-6* mRNA, transgenic and endogenous, was detected by in situ hybridization (Fig. 3 C and D).

***Molecular analysis of differentiation in the GATA-6 over-expressing epithelium***

Since extensive differentiation and the initiation of surfactant production start at the saccular stage of lung development (E17.5), which extends even after birth (to P5), we decided to investigate epithelium differentiation in E18.5 transgenic lungs. Thyroid Transcription Factor-1 (TTF-1 or Nkx2.1) protein, a marker for specified pulmonary endoderm (Minoo *et al.*, 1999), was abundantly present in transgenic lungs (Fig. 4B and C). However, the staining in all cells lining the dilated terminal buds is more intense than the characteristic staining for this stage of development as seen in wild type E18.5 lungs (Fig. 4A). *Clara Cell marker-10* mRNA (Ray *et al.*, 1996) was expressed in transgenic lungs at a normal level indicating the presence of non-ciliated sectretory cells in the proximal epithelium (Fig. 4D-F). In


wild type
transgenic
E14.5
E16.5
E18.5
P1
A
B
C
D
E
F
G
H

the distal epithelium, topologically represented by the dilated buds, *Surfactant Protein-C* (Kalina *et al.*, 1992) expressing cells could be detected among the cells lining the epithelium (Fig. 4H and I) although the number of cells and their distribution were very different from that observed in wild type lungs (Fig. 4G). *SP-C* is expressed in the Type II cells which function mainly in surfactant production and they can further differentiate into Type I pneumocytes, the functional cells for gas exchange.

### *Morphological analysis of differentiation in the GATA-6 over-expressing epithelium*

From morphological studies (Ten Have-Opbroek, 1991; Ten Have-Opbroek *et al.*, 1988) it was shown that before maturation, Type II cells have an excess of glycogen present in their cytoplasm and their nucleus is less round. We therefore stained the epithelium of the transgenic lungs (E18.5) for glycogen (Fig. 4J-L) and used Electron Microscopy to examine the ultrastructure of the cells lining the dilated buds (Fig. 5). Periodic acid Shiff's staining (PAS) for glycogen revealed that a very high percentage of cells lining the distal epithelium of the transgenic lungs is positive for glycogen in their cytoplasm (Fig. 4K and L). Normally at this stage, due to advanced differentiation, glycogen positive cells are hardly detectable in the lining of the distal epithelium (Fig. 4J). The caudal lobe of a transgenic (Fig. 4) and a wild type littermate lung were processed for Electron Microscopy. In the epithelium of the wild type lung, normal Type II cells could be easily identified by the cuboidal shape, the almost round nuclei and the presence of several multilamellar bodies (Fig. 5C). Squamous Type I cells were also observed surrounding capillaries (Fig. 5E, arrows indicate capillaries). Both Type II and I cells were represented in every alveoli that was examined. In contrast, examining the epithelial lining in the dilated alveoli in the transgenic lung we were not able to find any typical Type II or I cells. Instead, a number of cells with more irregular nuclei and numerous glycogen fields in their cytoplasm were found (Fig. 5D, glycogen fields are indicated by arrowheads). Although formation and localization

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**Figure 2**
Histological analysis of *SP-C/GATA-6* transgenic lungs during fetal lung development. At E14.5, transgenic lungs (B) are smaller than wild type (A) and they have fewer terminal buds. Two days later, at E16.5 the branching defect is more prominent with big dilated distal buds present in the transgenic lungs (D) while fine branching is already apparent in wild type littermates (C). This phenotype is more dramatic just before birth, at E18.5, with the presence of abnormally shaped and sized distal alveoli (F) in contrast to normal alveolization that can be seen in wild type lungs (E). A few transgenic pups were born alive and the most severely affected one (H) had a milder branching phenotype when compared to most of the E18.5 transgenic lungs (F). This phenotype is similar to the clinical central-acinar aeration pattern. All pups were sacrificed few hours after birth.

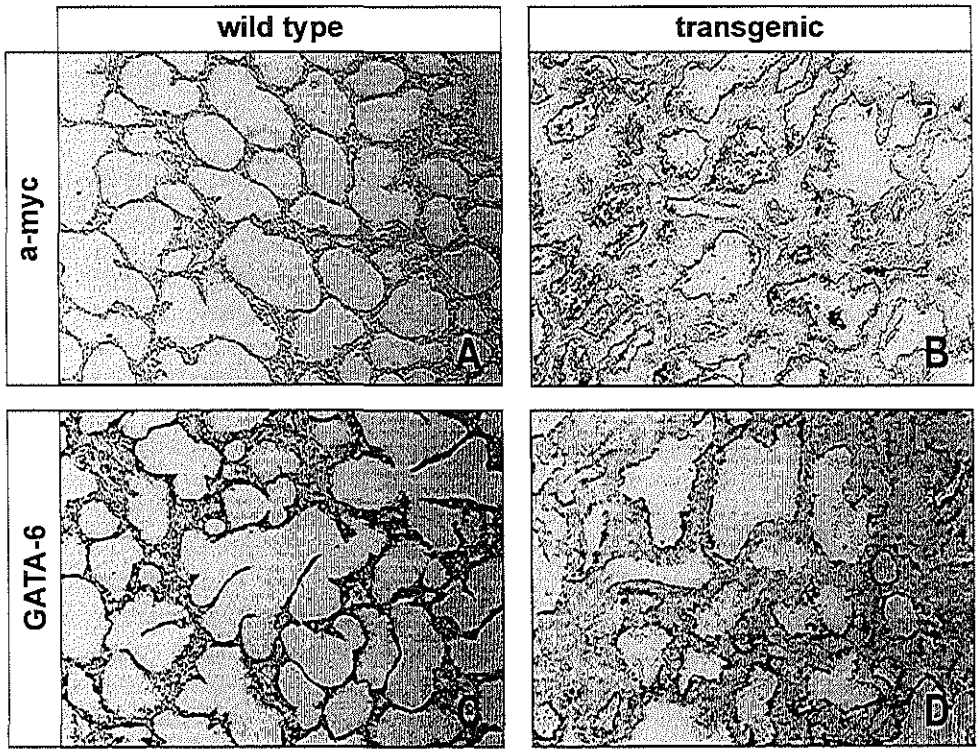


**Figure 3**
Expression of the *SP-C/GATA-6* transgene in E18.5 lungs. The anti-myc antibody was used to detect chimeric protein in transgenic lungs (B). Intense staining can be seen in the epithelium lining the abnormally dilated alveoli (brown). Wild type lungs show no staining (A). *GATA-6* mRNA was detected in both wild type and transgenic lungs by in situ hybridization. In C, normal *GATA-6* expression in parts of the epithelium and in the surrounding mesenchyme can be seen in wild type E18.5 lungs (blue). In transgenic lungs (D) the signal for RNA expression (blue) is very intense in the lining of the epithelium as seen with the anti-myc antibody staining (B). For this analysis, sections from the lung that is shown on Figure 2F were used.

of capillaries appeared normal, the squamous cells present around them had different morphology than that of a typical Type I cell and some even had glycogen in their cytoplasm (Fig. 5F, arrow indicates capillary and arrowhead glycogen). Proximal epithelium appeared normal and ciliated cells were observed as in the wild type epithelium (data not shown).

## DISCUSSION

Depletion of *GATA-6* expression in the pulmonary endoderm, both *in vitro* and *in vivo*, resulted in lack of branching morphogenesis and failure of the epithelium to differentiate (Keijzer *et al.*, 2001). To better understand the role of this transcription factor in lung development we used the *SP-C* promoter to artificially maintain high levels of *GATA-6* expression in the fetal pulmonary epithelium *in vivo*. This alteration also resulted in defective branching morphogenesis and epithelial differentiation. However, the branching phenotype was manifested later during development and in a less severe form probably due to the late onset of the transgene expression (Wert *et al.*, 1993). The promoter is active after pulmonary endoderm specification, initial budding and lobe determination but before the differentiation program starts at the pseudoglandular stage (E14.5) of lung development (Perl and Whitsett, 1999; Warburton *et al.*, 2000). Hence, *GATA-6* expression in the *SP-C/GATA-6* lungs is already elevated in the primordial epithelium before any proximal or distal fate had been acquired. Nevertheless, this overexpression affected only distal epithelium where the gene is normally expressed. Thus, this study shows that GATA-6 has a specific regulatory role in the differentiation program of the distal epithelium and also confirms that GATA-6 protein levels are crucial during both lung morphogenesis and cell type specification (Keijzer *et al.*, 2001)

### *Branching morphogenesis in the SP-C/GATA-6 transgenic lungs*

The interaction between epithelium and mesenchyme is a determining factor in lung development starting from the initial budding and continuing during branching morphogenesis involving well-studied molecules like FGF-10, BMP-4 and SHH (Hogan, 1999; Weaver *et al.*, 2000). The localized high expression of *FGF-10* in the mesenchyme promotes the endoderm to bud and to express high levels of *BMP-4*. Subsequently, BMP-4 together with SHH downregulates *FGF-10* to prevent new budding at the same position while allowing the newly formed bud to grow. When a certain distance has been reached, the high expression domains of *FGF-10* present on either side or both sides of the growing bud induce new budding resulting in lateral or dichotomous branching, respectively. *GATA-6* expression in the endoderm coincides with branching morphogenesis but unlike

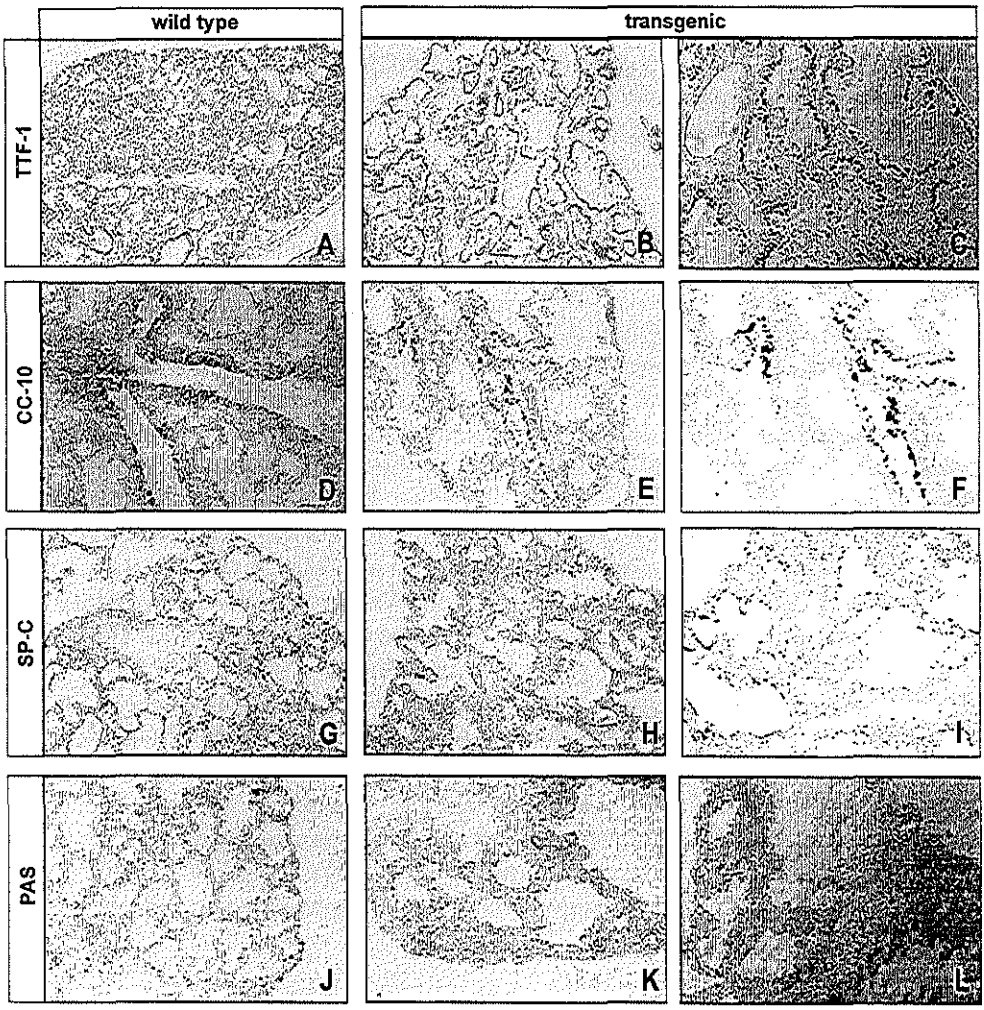


**Figure 4**
Molecular analysis of differentiation in the transgenic epithelium at E18.5. TTF-1 protein localization in wild type (A) and transgenic (B and C) epithelium shows the abnormally, for this stage, TTF-1 positive epithelium which lines the complete transgenic alveoli (brown). In D and E, F the normal expression of *CC-10* mRNA, a marker for proximal non-ciliated cells, is shown in both wild type and transgenic lungs, respectively (blue). In the distal epithelium, expression of endogenous *SP-C*, a marker for Type II cells, can be seen in a wild type lung (G) and the decreased levels with the different distribution in the transgenic lung (H and I) (blue). Periodic Acid Shiff's staining for glycogen (purple) revealing that in contrast to the wild type lung (J), the transgenic lung has an alveolar epithelium which is lined almost exclusively by glycogen positive cells (K and L).

*BMP-4* expression (Weaver *et al.*, 2000), there are no detailed *GATA-6* expression data during the induction and growth of a bud. In E10.5 lungs *GATA-6* mRNA has been localized to the growing tips of the initial buds (Keijzer *et al.*, 2001). At that stage, the *SP-C* promoter becomes active resulting in high expression of *GATA-6* in all SP-C expressing cells throughout the transgenic endoderm. This alteration of *GATA-6* expression results in the branching defects in the transgenic lungs which could be explained in two ways. Either by distorting a molecular pathway intrinsic to the branching endoderm or by interrupting a pathway involved in processing an inductive signal from the mesenchyme.

In cardiac development, FGFs and BMPs have been placed upstream of GATAs (Schultheiss *et al.*, 1997; Monzen *et al.*, 1999; Reifers *et al., 2000*). Cardiac induction involves GATA-4 and its co-factor Nkx 2.5 and they are both necessary to establish cardiac phenotype (Durocher *et al.*, 1997). An analogous regulatory pathway appears to operate in the lung endoderm. TTF-1 (or Nkx 2.1), a gene from the same Nkx homeobox family and GATA-6 are expressed in the lung endoderm from early pulmonary specification and both are required for branching morphogenesis (Lazzaro *et al.*, 1991; Keijzer *et al.*, 2001). It has been suggested that TTF-1 expression may be downstream and depends on GATA-6 expression (Shaw-White *et al.*, 1999), however, TTF-1 expression is normal in *GATA-6 -/-* endoderm showing that TTF-1 expression is independent of GATA-6 (Keijzer et al., 2001). Unfortunately it is not known whether GATA-6 is still expressed in *TTF-1 -/-* embryos (Minoo *et al.*, 1999)

***GATA-6 levels and pulmonary epithelium differentiation***

Expression of *GATA-6* starts in the primordial epithelium and it is restricted to the distal epithelium by E15.5. The levels of its expression are difficult to quantitate by in situ hybridization. By expressing *GATA-6* under the control of *SP-C*, a constant high level of the protein was present from the onset of differentiation resulting in a distal epithelium attenuated of any terminal differentiation. Unlike overexpression of *TGF- 1* (Zhou *et al.*, 1996) and *HNF-3* (Zhou *et al.*, 1997) that resulted in endodermal arrest at the primordial or pseudoglandular stages, *GATA-6* overexpression did not affect initiation of the differentiation program. The pathway of proximal epithelium differentiation was unaffected since both ciliated cells, seen in the EM analysis, and non-ciliated secretory cells, visualized by *CC-10* expression, were detected as normal. Along the distal epithelium no mature Type II cells were present and all the cells were still expressing *TTF-1*. Normally the presence of numerous glycogen fields in the cells lining the distal airways is indicative of their differentiating status (Sorokin, 1965). This has been described in detail in an extensive morphological study of the features of Type II alveolar epithelial cells by Ten Have-Opbroek (1988). Later during lung development, before birth, a multi-step formation of multilamellar bodies (MLB) is thought to

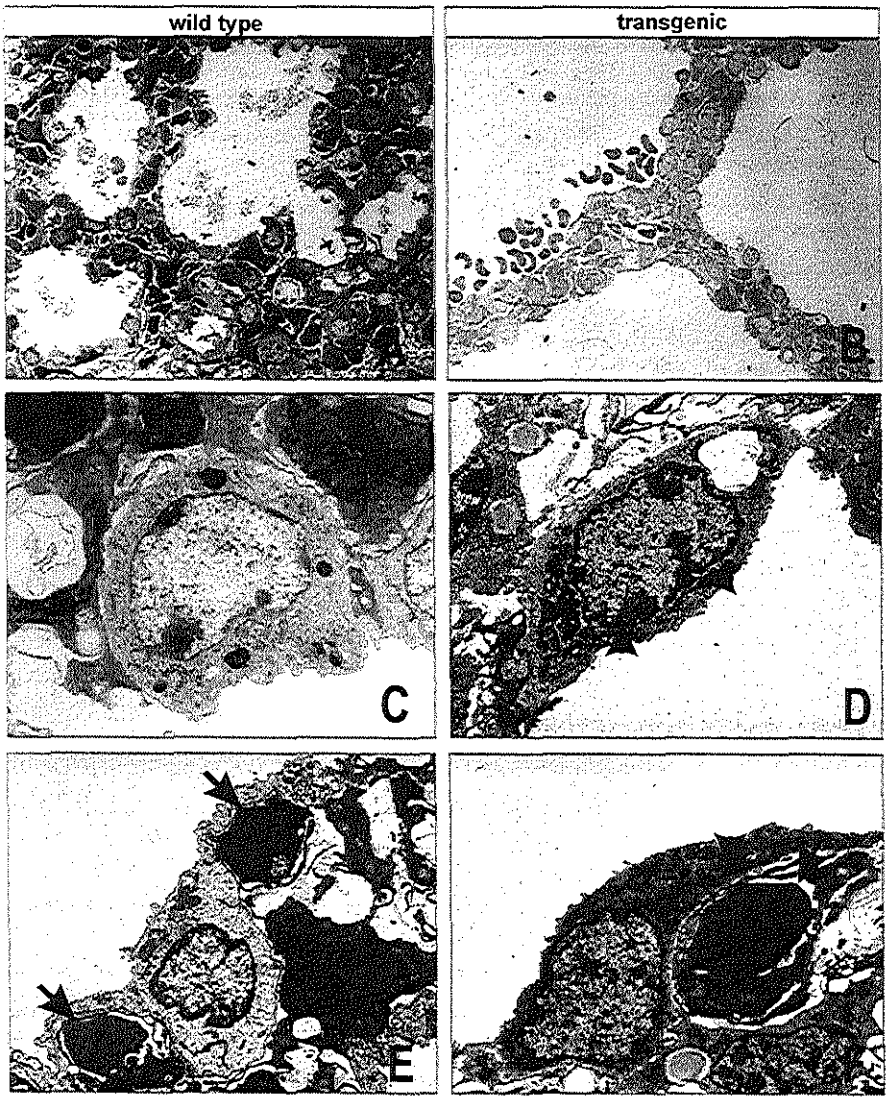


**Figure 5**
Electron Microscopic analysis of the distal epithelium at E18.5. A low power overview of a wild type and a transgenic lung is shown in A and B, respectively. In the distal epithelium of a wild type lung both typical Type II cells, with multilamellar bodies in their cytoplasm (C) and flat Type I pneumonocytes, around capillaries, can be detected (E). In the transgenic lung atypical cells lining the alveoli are detected and the presence of glycogen in their cytoplasm is a common feature (D). Flat cells are present around the capillaries (F) although they look distinct from wild type Type I cells and some of them still have glycogen in their cytoplasm (arrowheads point to glycogen fields and arrows indicate capillaries, magnifications: A and B X620, C-F X5800).

compartmentalize glycogen (Ten Have-Opbroek *et al.*, 1990). Our data show a continued presence of glycogen in the distal epithelial cells suggesting that GATA-6 is involved in a particular stage of this maturation process. When the levels are elevated, the cells do not initiate the final differentiation step which is characterized by multilamellar bodies formation. Instead, most of the cells lining the alveolar epithelium have glycogen in their cytoplasm, as visualized by periodic acid Shiff's staining (Fig. 4K and L), indicating a block in the Type II differentiation pathway. The flat Type I cells which function in gas exchange, are thought to originate from further differentiation of Type II cells although it is not clear whether they are derived from a mature Type II cell or from one of the intermediate stages during their maturation. In the *GATA-6* overexpressing lungs, flat epithelial cells surrounding the capillaries were observed and their shape and localization suggest that they are Type I cells. However, some of them still had glycogen fields, they were not as flat as normal Type I cells and their nucleus had an irregular shape. At this final step, differentiation is thought to be driven by growth and particularly by the intercalation of the forming capillaries with the epithelium (Ten Have-Opbroek *et al.*, 1988). Thus, the apparent Type I-like cells we see, could be the same precursors of Type II cells as discussed above, except that they are in close proximity with the capillaries and have less glycogen fields. This would suggest that elevated levels of GATA-6 would prevent the formation of fully differentiated Type I cells even when in contact with capillaries. In conclusion our data demonstrate that GATA-6 plays an important role in lung organogenesis and especially during the multi-step process of maturation of Type II cells. Elevated GATA-6 protein levels result in a block of terminal differentiation to mature Type II and Type I pneumocytes.

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# Chapter 5

# Concluding remarks

## Concluding remarks

Among the members of the GATA family of transcription factors GATA-4, -5 and –6 constitute a subfamily, based on high sequence homology and overlapping expression patterns during embryogenesis. Initially, it was anticipated that the proteins are important for cardiogenesis based on their dynamic expression pattern during heart development. As a result, they have been described as the heart related subfamily of GATAs [1]. However, in view of the recent data there is a number of reasons to question this name. First, targeted inactivation of *GATA-4* revealed that the gene is required for normal ventral-to-lateral folding of the embryo rather than for heart-specific morphogenesis and differentiation [2, 3]. Further analysis of chimeric embryos with *GATA-4-/-* ES cells demonstrated that the primary requirement for the protein is actually in the extraembryonic visceral endoderm [4]. Second, *GATA-6* inactivation in the mouse pointed to a primary function of the protein in the visceral endoderm [5] (chapter 2). In addition, chimeric loss of function and transgenic gain of function of *GATA-6* in the lung endoderm showed that the levels of GATA-6 protein are crucial for normal pulmonary morphogenesis and differentiation (chapter 3 and 4). Third, experimental evidence from Xenopus as well as zebrafish suggest a role for GATA-5 in early endoderm formation in these species [6, 7]. However, endoderm formation in the mouse is not affected by GATA-5 [8]. Thus, endoderm is emerging as the common theme for this subfamily of transcription factors. This also holds true during evolution since analogies can be found not only in frogs but also in flies and worms [9-11]. Therefore it may be more appropriate to refer to GATA-4, -5 and -6 as the endoderm related subfamily of GATAs.

*Primary function of GATA-6*

GATA-6 appears to be required earlier than any other GATA factor during mouse embryonic development. Mutant embryos cannot survive past the implantation stage and they die around embryonic day 5.5 [5] (chapter 2). Prior to implantation, the mouse embryo is a very simple structure and yet only part of it, the epiblast, gives rise to embryonic tissues. The rest of the cells are organized in the surrounding membranes, which play a crucial role in the support and the further growth of the underlying epiblast. However, the parietal and the visceral endoderm do not just provide a filtrative or nutritive function by their attachment to the uterus or the embryo. In the last few years a more specific function of the visceral endoderm has been revealed. It has been shown to pattern the underlying totipotent cells of the epiblast and this inductive function contributes significantly to our understanding of gastrulation and the events preceding it [12]. Based on molecular expression patterns and by using ES cell technology to either inactivate a gene or generate chimeric embryos with different genotypes in embryonic and extraembryonic tissues, the recent data assigned anterior-posterior patterning activity to the visceral endoderm [13]. This function is mediated by part of the

visceral endoderm that is referred to as anterior visceral endoderm (AVE), simply because it marks the anterior site of an epiblast that still consists of totipotent cells and has no sign of primitive streak formation.

The precise expression pattern of *GATA-6* in the visceral endoderm is still not very clear. Radioactive in situ hybridization on sections of E5.5 embryos gives a signal around the embryo and makes localization to parietal endoderm of the Reichert's membrane or to visceral yolk sac endoderm very difficult. Moreover, any localized expression within the visceral endoderm cannot be easily recognized [5]. The *-galactosidase* reporter gene that we inserted into the *GATA-6* locus gave a more or less ubiquitous expression pattern at the blastocyst stage, which is subsequently restricted to very few cells at E4.5. Based on their localization, these cells could be primitive endoderm, however, they can only be a small portion of it and it is unlikely that they represent cells already differentiating to visceral endoderm [14, 15]. By E5.5, expression completely disappears and appears again later at E7.5, in the parietal cells of the Reichert's membrane (chapter 2). This expression pattern is very intriguing but subsequent findings that the *LacZ* gene does not faithfully recapitulate *GATA-6* expression in all lineages at later developmental stages (chapter 3), mandate a more cautious interpretation of β-galactosidase reporter activity. The validity of the *LacZ* expression pattern could only be confirmed by comparison to the expression pattern of the endogenous *GATA-6* gene. However, attempts to detect *GATA-6* mRNA on whole mount preparations of early pre- and post-implantation embryos were not successful. Although the precise expression pattern of GATA-6 during visceral endoderm differentiation is not clear, the implication of the protein in the differentiation of this cell lineage is well supported. It is based on the morphology of the E5.5 null embryos and the *in vitro* outgrowth of mutant blastocysts (chapter 2). Additionally, expression of *HNF-4*, which is considered as an important factor for visceral endoderm differentiation [16, 17], was markedly reduced in the mutant embryos [5]. It was even suggested, based on *in vitro* experiments, that the gene is a downstream target of GATA-6 [5]. Differential screening of wild type and *GATA-6* mutant embryoid bodies revealed additional GATA-6 target genes in this lineage. Of particular interest is the gene encoding the mitogen-responsive phosphoprotein *Dab2*, which is not expressed in *GATA-6* null visceral endoderm and in *in vitro* studies it is directly and specifically regulated by GATA-6 [18]. Thus, the possibility remains that loss of *HNF-4* expression is simply due to a poor differentiation of visceral endoderm in the *GATA-6* mutants.

While the regulatory targets of GATA-6 have not been extensively analyzed, data from our study (chapter 2) and from others [5] indicate that GATA-6 function is essential for the differentiation of the visceral yolk sac endoderm. Although it is unlikely that the protein is involved in any subsequent patterning activity of this tissue, these findings add a new crucial gene function for visceral endoderm differentiation to a very small list. In addition to TGF-β signaling [19, 20], only HNF-4 [17] and vHFN-1 [21, 22] have been implicated in the

differentiation of this lineage. The way that GATA-6 regulates differentiation is not yet clear and it will be very interesting to identify more target genes of the protein. Furthermore, the early ubiquitous expression pattern of *GATA-6* in the blastocyst raises the question why the protein is initially present in almost all cell types and during the subsequent 24 hours it is restricted to one cell population affecting its differentiation. If perhaps maintenance of *GATA-6* expression was a requirement for visceral endoderm specification, then the regulation of the gene at the blastocyst stage would be an interesting issue. Unfortunately, the main obstacle in addressing such interesting questions further is the small size of the embryos at these stages and the limited amounts of available material.

*GATA-6 in lung development*

In addition to the described expression pattern of *GATA-6* in the lung endoderm [23], an observation was published showing that *GATA-6* mutant ES cells do not contribute to the pulmonary epithelium [5]. Consequently, GATA-6 protein was considered as one of the first factors crucial for pulmonary endoderm specification [24-26]. We decided to further study the role of GATA-6 in the specification of the anterior foregut to lung endoderm by generating highly chimeric embryos with *GATA-6* mutant ES cells. The prediction was that embryos almost exclusively derived from mutant ES cells would have no specified lung endoderm. To our surprise, highly chimeric embryos had lungs, which had undergone initial branching and lobe formation (chapter 3). The use of genetically marked blastocysts (ROSA26) clearly indicated that chimeric lungs at E12.5 had some endodermal buds in their lobes that were almost exclusively *GATA-6 -/-*. The implication of these findings is that foregut can be specified to lung endoderm in the absence of GATA-6. Although the signals for lung endoderm specification are not known yet [27], it seems that GATA-6 deficient cells are able to process them normally and even undergo initial lung morphogenesis and differentiation. But these findings are also in sharp contrast to the previous observation about exclusion of *GATA-6* mutant cells from pulmonary epithelium [5]. In both studies the ES cells used were of the same genotype, namely *GATA-6-/-*, and therefore any resulting defect in gene regulation was common. Thus, the difference in the data can only be explained by the difference in procedure, in particular the number of the injected ES cells. In our experiments we used double or more, the number of ES cells that is normally injected since we were aiming for highly chimeric embryos. Although the number of injected ES cells was not mentioned in the other study [5], it is likely that normal numbers of ES cells were used, perhaps even less than normal. It is common practice that when a gene has an essential function and it is required early in embryogenesis, chimeras are initially generated with low numbers of ES cells to avoid any interference with normal development which may be seen in highly chimeric embryos. Assuming this difference in procedure, we envisage that if there are only a few *GATA-6* mutant cells in the population of foregut cells that is destined to form lung any advantage of the wild type cells in

growth would result in the subsequent elimination of mutant cells from the lungs. However, a high percentage of mutant cells in the foregut would not allow such a stringent selection resulting in GATA-6 mutant endodermal buds.

Another implication of our data is that there must be a substantial number of cells in the foregut that are specified as lung endoderm. If there were only few cells, the picture would have been black and white in the chimeric experiments and give either an exclusively mutant or an exclusively wild type lung. Instead, a variety of patterns were observed in the generated chimeric lungs. Some buds were exclusively blue (*ROSA26*-wild type blastocysts), others were completely white (*GATA-6-/-* ES cells) and others were a mixture of blue and white cells (chapter 3).

The ability of *GATA-6* mutant endoderm cells to undergo morphogenesis and differentiation is limited to the early stages of lung development. Subsequent to initial budding and lobe formation, endoderm consisting of *GATA-6* mutant cells does not branch properly to generate a normal respiratory tree. The molecular mechanism underling this defect is not yet clear. In the current molecular model for branching morphogenesis, epithelial-mesenchymal interactions play a critical role. FGF signaling initiating in the mesenchyme and BMP-4, Shh and TTF-1 (or Nkx2.1) activities in the epithelium seem to mediate these interactions (Figure 4) [28-30]. How does GATA-6 fit in this model? In cardiac induction, GATAs are downstream of BMPs [31, 32] and they act in concert with Nkx2.5 to promote cardiogenesis [33]. In zebrafish it was even suggested that both *GATA* and *Nkx* are induced by FGFs [6]. Although *in vitro* studies suggested that GATA-6 regulates *TTF-1* expression [34], the normal expression of the gene in the *GATA-6* mutant endoderm supports the idea of a co-function as opposed to cross regulation (chapter 3). Unfortunately, *BMP-4* mutant lungs are not available in order to investigate expression of *GATA-6*, but an option would be to use transgenic lungs for the BMP-4 antagonist *Noggin* [35]. If *GATA-6* were induced in the epithelium directly by the FGF-10 from the mesenchyme the implication would be that GATA-6 protein has a role similar to *BMP-4* during branching morphogenesis. Such a requirement for GATA-6 in the epithelium in order to respond to the proliferative and chemoattractant action of FGF-10 can be easily tested. Highly chimeric endoderm can be generated with *GATA-6* mutant ES cells into *ROSA26* blastocysts. This endoderm can be used in the *in vitro* FGF-10 bead-based branching model [28] and the genotype of the endoderm that has branched successfully and surrounded the bead can be determined. If the FGF-10 inductive activity is mediated by GATA-6, any endoderm around the bead has to be blue (wild type). On the other hand if mutant endoderm responds normally to FGF-10, then GATA-6 function in the epithelium is more likely to be independent of FGF signaling. The implication would be that GATA-6 is part of a completely different pathway that regulates branching morphogenesis.

If the latter possibility holds true, two directions will be important to establish a GATA-6 based model for branching morphogenesis. First, the identification of target genes for GATA-6 protein in the lung endoderm is required.

This can be achieved by differential expression screening of blue and white endoderm from chimeric lungs. Second, a yeast two-hybrid screen with parts of the GATA-6 protein could be explored to identify other proteins from lung endoderm that may interact with GATA-6. One protein, FOG-2 has been found to interact with GATA-4 and –6 [36-38]. It appears to be expressed in the embryonic lung endoderm (M van Dooren, unpublished), suggesting a potential co-function with GATA-6 during lung development. Target genes and interacting proteins will also help to elucidate the mechanism by which GATA-6 controls distal differentiation. The connection with BMP-4 function in epithelial cell fate acquisition is another interesting aspect for investigation. According to the distal signaling center model, high *BMP-4* expression induces distal fate while in its absence cells acquire proximal fate [35]. Since overexpression of *GATA-6* results in a block in terminal distal differentiation (chapter 4), it appears that the two molecules actually antagonize each other. This in turn suggests that they function in concert to induce and fine tune epithelial differentiation.

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## Summary

Members of the GATA family of transcription factors control differentiation of distinct cell lineages by binding to GATA sites in the promoter of lineage specific genes. GATA-6 is the last member of this family and it is expressed during early heart and gut development. We studied the role of GATA-6 protein during embryogenesis by targeted gene inactivation in mouse Embryonic Stem (ES) cells.

*GATA-6* heterozygote mutant mice were generated and when intercrossed no homozygote mutant mice were born alive. The lack of the protein results in an early embryonic lethality just after implantation at embryonic day 5.5 (E5.5). Histological analysis of *in vivo* and *in vitro* developed null embryos suggested that the defect is in the visceral endoderm, which is a derivative of the primitive endoderm and give rise to the visceral endoderm of the yolk sac. Confirmation of this observation was obtained by generating chimeric embryos. In such embryos the genotype of the embryonic tissues and of the extraembryonic visceral endoderm was different, corresponding to the genotype of the injected ES cells and the host blastocyst, respectively. When *GATA-6*-/- ES cells were injected into wild type blastocysts, the resulting chimeric embryos had embryonic tissues mutant for *GATA-6* and they survived to E9.5 appearing normal. In the converse experiment, when *GATA-6* mutant blastocysts were injected with wild type ES cells, the *GATA-6* mutation was confined to the extaembryonic tissues, including the visceral endoderm. Such embryos did not survive later than E5.5, proving that the primary cause of lethality in the *GATA-6* homozygote mutant embryos is the lack of the GATA-6 protein in an extraembryonic tissue.

In chimeric embryos generated by *GATA-6* mutant ES cells the rescued early lethality by the wild type extraembryonic tissues allowed the study of GATA-6 function in embryonic tissues expressing the gene. Pulmonary endoderm is a tissue in which *GATA-6* is the only member of the GATA family to be expressed. Analysis of lungs from highly chimeric embryos revealed that *GATA-6* mutant cells form lung endoderm, which subsequently shows branching defects and lack of epithelial differentiation. These defects lead to respiratory failure at birth. The importance of the GATA-6 protein levels for normal lung development is further demonstrated by the *in vivo* overexpression of *GATA-6* under the control of the human Surfactant Protein-C promoter. Transgenic embryos have abnormally branched endoderm and although differentiation to proximal epithelium appears normal, terminal differentiation to distal epithelium is blocked. Both mature Type I (gas exchanging pneumocytes) and Type II (surfactant producing cells) cells are absent in the GATA-6 overexpressing lung epithelium.

Thus, GATA-6 protein is first required early in embryogenesis (E5.5) for normal differentiation of the visceral endoderm, which gives rise to the yolk sac endoderm . Subsequently, the protein plays an important role during pulmonary branching morphogenesis and differentiation to distal epithelium.

## Samenvatting

De GATA familie van transcriptie factoren regelen de differentiatie van verschillende celsoorten door middel van binding aan GATA sequenties in de promoter van weefsel specifieke genen. Het meest recent ontdekte lid van deze familie van genen, GATA-6, komt tot expressie in een vroeg stadium van de ontwikkeling van het hart en de darm. Wij hebben de rol van GATA-6 bestudeerd tijdens de embryogenese door een inaktivatie van het GATA-6 gen in Embryonale Stam (ES) cellen.

Homozygote GATA6 muis mutanten worden niet geboren na kruising van heterozygote muizen. Het onbreken van het GATA-6 eiwit resulteert in een vroeg embryonale dood kort na implantatie op dag 5.5 (E5.5) van de ontwikkeling. De histologische analyse van *in vivo* en *in vitro* gegroeide GATA-6 negative embryo's suggereert dat er een defect is in het viscerale endoderm, wat zelf ontwikkelt uit het primitieve endoderm. Een bevestiging van deze waarneming werd verkregen uit het maken van chimere embryo's. In zulke embryo's is het genotype van de embryonale weefsels en dat van het extraembryonale viscerale endoderm verschillend en komen respectievelijk overeen met het genotype van de geinjecteerde ES cellen en dat van de ontvangende blastocyst. Wanneer GATA-6-/- ES cellen geïnjecteerd worden in wild type blastocysten, is het resulterende embryonale weefsel mutant voor GATA-6, maar de embryo's overleven zeker tot E9.5 en zien er normaal uit. In de tegenovergestelde proef , wanneer GATA-6 -/- blastocysten worden geïnjecteerd met wild type ES cellen is de GATA-6 mutatie beperkt tot de extra-embryonale weefsels, inclusief het viscerale endoderm. Zulke embryo's overleven niet later dan E5.5 en laten zien dat de primaire doodsoorzaak door de afwezigheid van GATA-6 in het vroege embryo gelegen moet zijn in een extra-embryonaal weefsel

Het feit dat de GATA6-/- embryo's gered worden van een vroeg embryonale dood het wild type extraembryonale weefsel, maakte het mogelijk om de functie van GATA-6 te bestuderen in de weefsels van deze chimere embryo's'. Long endoderm is een weefsel waarin GATA-6 als enige van de familie van GATA factoren tot expressie komt. Analyse van de long van zeer chimère embryo's liet zien dat de GATA-6 -/- cellen long endoderm vormen, maar dat het daarna een longvertakkingsdefect heeft en dat de differentiatie van het epitheel afwezig is. Deze defecten leiden tot de dood na de geboorte door ademhalingsgebrek. Het belang van de juiste concentratie van GATA-6 voor een normale ontwikkeling van de longen werd ook nog aangetoond door GATA-6 tot overexpressie te brengen *in vivo* door het GATA-6 gen te plaatsen onder de controle van de promoter van het Surfactant Protein-C gen. Zulke transgene embryo's hebben een abnormaal vertakt endoderm. De differentiatie naar proximaal epitheel lijkt normaal, maar de differentiatie naar distaal epitheel is geblokkeerd. Zowel volwassen Type I (gas uitwisselende pneumocyten) als Type II cellen (surfactant producerende cellen) zijn afwezig in het epitheel dat GATA-6 tot overexpressie brengt.

GATA-6 is dus eerst noodzakelijk in de vroege embryogenese (E5.5) voor de normale differentiatie van het viscerale endoderm waaruit het dooierzak endoderm gevormd wordt. Later speelt het eiwit een belangrijke rol in het vertakkingproces van de longen en de differentiatie van het distale long epitheel.

## Curriculum Vitae

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| **1969** | Started the 1st of October in Metohia Frantzeskiana, Rethymnon, Crete |
| **1988** | General examination of Greece, subjects: Biology, Chemistry, Physics and Greek |
| **1988-1992** | Degree in Medical Laboratory Science in the School of Health and Welfare Professions of the Technological Educational Institution (TEI) of Athens |
| **1992** | Six months practise at the Leicester Royal Infirmary, Leicester UK |
| **1993-1994** | Master of Science in Molecular Genetics at the University of Leicester, UK |
| **1994-2001** | Department of Cell Biology & Genetics, Erasmus University Rotterdam |
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