

Variant Hepatocyte Nuclear Factor 1 is required for visceral endoderm specification

Elena Barbacci^{1,*}, Michaël Reber^{1,*}, Marie-Odile Ott², Christelle Breillat¹, François Huetz³ and Silvia Cereghini^{1,‡}

¹U423 INSERM, Hôpital Necker-Enfants Malades, 75743 Paris Cedex 15, France ²U257 INSERM, ICGM, 75014 Paris, France

³Unité d'Immunogénétique, Institut Pasteur, 75724 Paris Cedex 15, France

*These authors contributed equally to this work

‡Corresponding author (e-mail: cereghin@necker.fr)

Accepted 30 July; published on WWW 6 October 1999

SUMMARY

Genetic and molecular evidence indicates that visceral endoderm, an extraembryonic cell lineage, is required for gastrulation, early anterior neural patterning, cell death and specification of posterior mesodermal cell fates. We show that variant Hepatocyte Nuclear Factor 1 (vHNF1), a homeodomain-containing transcription factor first expressed in the primitive endoderm, is required for the specification of visceral endoderm. *vHnf1*-deficient mouse embryos develop normally to the blastocyst stage, start implantation, but die soon afterwards, with abnormal or absent extraembryonic region, poorly organised ectoderm and no discernible visceral or parietal endoderm. However, immunostaining analysis of E5.5 nullizygous mutant embryos revealed the presence of parietal endoderm-like cells lying on an abnormal basal membrane. Homozygous mutant blastocyst outgrowths or differentiated embryonic

stem cells do not express early or late visceral endoderm markers. In addition, in *vHnf1* null embryoid bodies there is no activation of the transcription factors HNF-4 α 1, HNF1 α and HNF-3 γ . Aggregation of *vHnf1*-deficient embryonic stem cells with wild-type tetraploid embryos, which contribute exclusively to extraembryonic tissues, rescues periimplantation lethality and allows development to progress to early organogenesis. Our results place vHNF1 in a preeminent position in the regulatory network that specifies the visceral endoderm and highlight the importance of this cell lineage for proper growth and differentiation of primitive ectoderm in pregastrulating embryos.

Key words: vHNF1, Transcription factor, Gene targeting, Visceral endoderm, Embryoid bodies, HNF-4

INTRODUCTION

During mammalian development, the blastocyst prepares for implantation within the receptive uterine epithelium by the generation of the first two cell lineages, the primitive endoderm and the trophoblast, which will contribute to the formation of the yolk sac and placenta, respectively (Rossant, 1995). The primitive endoderm appears between 4 and 4.5 days of gestation in the mouse, as a layer of cells on the blastocoelic surface of the inner cell mass (ICM), and contributes to the endoderm of the extraembryonic, but not embryonic tissues of the conceptus (Gardner and Beddington, 1988). At implantation, it rapidly gives rise to two distinct cell lineages; the visceral and then the parietal endoderm. Parietal endoderm cells migrate along the inner surface of the trophoblast layer and secrete a basement membrane (Reichert's membrane) between themselves and the trophoblast. While parietal endoderm cells remain loosely associated, visceral endoderm (VE) cells form a layer of closely associated polarised cells at the periphery of the egg cylinder. Recent studies indicate that VE, although exclusively an extraembryonic tissue, also participates in other embryonic developmental processes

preceding and during gastrulation, such as ectoderm cavitation (Coucouvanis and Martin, 1999), early anterior neural patterning (Beddington and Robertson, 1998) and specification of posterior mesodermal cell fates (Belaousoff et al., 1998). Organised as an absorptive/secretory epithelium, the VE is the first structure to provide nutritional and hematopoietic function, before the hemochorial placenta is formed, and at early postimplantation stage, it secretes a wide spectrum of serum proteins (Rossant, 1995). While some of these VE functions persist during gestation in the yolk sac, most of them are subsequently replaced by primitive gut derivatives, in particular the foetal liver and intestine. Although the VE and liver have different embryonic origins, the similarity of their function has led to the suggestion that both tissues share similar gene regulation pathways (Rossant, 1995).

Variant Hepatocyte Nuclear Factor 1 (vHNF1) is a homeodomain-containing transcription factor structurally related to HNF1 α . Both proteins share extensive identity in their DNA-binding domains, bind DNA as a bimolecular molecule and heterodimerise with each other (Cereghini, 1996). Initially identified by its interaction with a sequence essential for liver-specific transcription of several genes

(Cereghini et al., 1988), *vHNF1* is a member of a complex regulatory network composed of a heterogeneous class of transcription factors including HNF-4, HNF-3 α , β and γ (Cereghini, 1996). The temporal and spatial expression pattern of *vHnf1* is consistent with the hypothesis that it plays a role in early development. In early postimplantation mouse embryos, *vHnf1* is detected from 5.5 days (E5.5) of gestation in the VE, preceding and/or coinciding with the induction of several putative target genes in this cell lineage (Cereghini et al., 1992). *vHnf1* is also highly induced upon in vitro differentiation of the embryonal carcinoma cell line F9 into either visceral or parietal endoderm (Cereghini et al., 1992), a system that mimics the features of extraembryonic endoderm differentiation upon implantation of the mouse embryo (Hogan et al., 1981). During early organogenesis, *vHnf1* is expressed in the polarised epithelia of the gut and in the developing kidney, pancreas, liver and lung, as well as transiently in the neural tube (Cereghini, 1996; Ott et al., 1991). These observations have led to the suggestion that *vHNF1* participates in early developmental decisions in different cell lineages.

To examine the specific requirement for *vHNF1* in embryonic development, we inactivated the murine gene by targeted mutation. In this study, we provide new insights into the mechanism of VE formation. We show that *vHnf1*-deficient (*vHnf1*^{-/-}) embryos die soon after implantation, displaying no extraembryonic region and severely disorganised ectoderm. In vitro analysis shows that *vHnf1*^{-/-} ICM outgrowths and differentiated embryonic stem (ES) cells lack both the surrounding layer of VE and the expression of its early and late markers. In chimeric embryos, VE derived from wild-type (wt) tetraploid embryos rescues the periimplantation lethality and allows development to proceed to early organogenesis. These results indicate that the periimplantation defect of *vHnf1*^{-/-} embryos is non-cell autonomous and suggest an essential role for the VE in the normal development of both primitive ectoderm and extraembryonic tissues in pregastrulation embryos.

MATERIALS AND METHODS

Construction of a *vHNF1* targeting vector and transfection of ES cells

A lambda Dash II mouse 129/SVJ genomic library was screened with the full-length mouse *vHnf1* cDNA; one out of 14 clones isolated contained exon 1, 5 kb of 5' flanking sequences, exon 2 and part of the intron 2 (Power and Cereghini, 1996). The original pGN targeting vector was modified by the introduction of the SV40 Nuclear Localisation Signal just upstream from the first methionine of β -gal. A 1153 bp (-981 to +172) and a 4600 bp genomic fragment encompassing the entire first intron were used as the 5' and 3' arms respectively, and cloned in the same orientation as the *NLSlacZ* and *neomycin* reporter genes. The entire coding sequence of the first exon was therefore deleted (Fig. 1A). 20 μ g of linearised targeting vector were electroporated into 2×10^7 HM1 ES cells in 800 μ l of DMEM medium without serum at 180 V, 1050 μ F. Transfected cells were grown for 7-8 days in medium containing 350 μ g/ml of G418. PCR screening for homologous recombination involved a sense primer located upstream of the 5' targeting sequence (-1164 to -1145 from the transcriptional initiation site of *vHnf1*) and an antisense primer located in the *lacZ* sequences (positions 133 to 153 relative to the ATG); amplification of a 1300 bp fragment indicated a homologous

recombination event. Single integration and correctly targeted events were verified by Southern blot hybridisation with probes internal or external to the targeting construct (Fig. 1A). Five correctly targeted clones were identified from 319 examined, one of which contained an additional insertion. ES clones were microinjected into the blastocoel cavity of 3.5 day C57BL/6J blastocysts and implanted into 2.5 day pseudopregnant females.

Genotyping and PCR assays

DNA from ES cells and 2-week-old mouse tails, was isolated by overnight digestion at 37°C in tail buffer (50 mM Tris-HCl pH 7.6, 250 mM NaCl, 25 mM EDTA, 0.25% SDS, and 1 mg/ml proteinase K). One volume of DNA Now (Biogentek) was added and the DNA was isolated according to the manufacturer's protocol. Blastocysts, yolk sacs from E8.5-10.5 embryos and embryos aged E5.5-8.0, were incubated overnight at 55°C in 20-50 μ l of water containing 1 mg/ml of proteinase K. The proteinase K was inactivated by incubation at 75°C for 10 minutes. Genotyping was performed using the following PCR conditions: one cycle of 3 minutes at 94°C, followed of 30 cycles of 30 seconds at 94°C, 60 seconds at 58°C, and 60 seconds at 72°C. Primers P1 (5'-TGCATCTTGCCGAAAGCTGAG-3') and P2 (5'-AGGAGTGTTCATAGTCGTCGC-3') and P1 and P3 (5'-CTCTTCGCTATTACGCCAGCTG-3') generated PCR products of 518 bp and 410 bp for the wt and mutant *vHnf1* alleles, respectively (Fig. 1D).

β -galactosidase staining and histological analysis

Embryos up to E10.5 and embryoid bodies were fixed for 5-30 minutes at 0°C in PBS containing 0.2% glutaraldehyde, 2% formaldehyde, 2 mM MgCl₂ and 5 mM EGTA, washed in PBS and stained for 15-48 hours as described by Hogan et al. (1994). Stained specimens were embedded in 7.5% gelatine, 15% sucrose in PBS and cryostat sectioned (6-10 μ m). Embryos older than E11.5 were fixed 30-90 minutes in 4% paraformaldehyde in PBS, and cryostat sections (7-14 μ m) were stained for β -gal activity for 24-48 hours. Uteri from females plugged during a 2 hour mating period were fixed for 2 hours in Bouin's fixative at room temperature, extensively washed in 70% alcohol, and paraffin embedded and sectioned. EB thin sections were prepared by fixation in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, embedded in EPON, sectioned and stained with toluidine blue.

Blastocyst culture and immunohistochemical staining

3.5-day blastocysts were collected as described by Hogan et al. (1994) and cultured individually in gelatine-coated 24-well plates in 1 ml of ES medium without LIF for 7 days. Blastocyst outgrowths and E5.5 embryos were fixed for 5 minutes at 4°C in 3% formaldehyde in PBS, permeabilised 15 minutes in methanol at 4°C and washed in PBS. Blocking consisted of 30 minutes incubation in 10% heat-inactivated foetal calf serum, 1% bovine serum albumin in PBS at room temperature. Primary antibody was incubated overnight in 1% bovine serum albumin, 1% heat-inactivated foetal calf serum in PBS. After three washings in PBS, specimens were incubated for 1 hour at room temperature with secondary FITC- or Texas red-labelled antibody, washed and photographed under fluorescent microscopy. The primary antibody dilutions were as follows: 1/400 rabbit anti-AFP (ICN), 1/400 goat anti-GATA4 (SantaCruz), 1/100 mouse anti-laminin- γ 1 (Hybridoma Bank), 1/100 mouse anti- β -galactosidase (Sigma), 1/5 rat-Troma-3 (kindly provided by R. Kemler), 0.5 mg/ml FITC-SJA (Sigma). The secondary antibodies were used at a 1/100 dilution.

RNA and protein analysis

Total RNA extraction from EBs and first strand cDNA synthesis were performed as described by Cereghini et al. (1992). 3 μ l aliquots were amplified in a 30 μ l PCR reaction for 18 cycles. PCR products were electrophoresed on 1.8% agarose gel, transferred to nylon membrane (NX, Amersham) and hybridised with the corresponding internal

probes. Sequences of the specific primers used for RT-PCR analysis shown in Fig. 6 are available upon request. Nuclear extracts from EBs and western blots were performed as reported by Cereghini et al. (1992).

Generation of chimeric embryos by ES cell aggregation

Two 4-cell stage CD1 tetraploid embryos were aggregated with a single loose clump of 15–20 ES cells, and tetraploid chimeric embryos were generated as reported by Nagy and Rossant (1993). To generate diploid aggregation chimeras, loose clumps of ES cells were aggregated with wt CD1 morulae and the resulting blastocysts were transferred into pseudopregnant females.

RESULTS

Targeted disruption of the murine *vHnf1* gene

The murine *vHnf1* gene was disrupted by homologous recombination in ES cells. The strategy, outlined in Fig. 1, consisted of replacing the first exon encoding the N-terminal dimerisation domain and part of the distantly related to the POU-A subdomain or B-domain of vHNF1 (Cereghini, 1996), with the *lacZ* and *neomycin* genes (Fig. 1A). This deletion

impairs DNA binding and prevents a potential dominant negative effect through heterodimerisation with HNF1 α . In the disrupted allele, *vHnf1* ^{Δ Exon1-SC}, the *lacZ* gene is under the control of the *vHnf1* regulatory sequences and functions as a marker for cell lineage analysis. The linearised targeting vector was electroporated into the HM1 ES cells (Magin et al., 1992) which were then subjected to G418 selection. ES clones that had undergone homologous recombination were identified by genomic Southern blot analysis (Fig. 1B). Following injection of targeted ES clones into C57BL/6J blastocysts, one clone transmitted the mutant allele through the germ line (Fig. 1C).

To determine whether the homozygous deletion of *vHnf1* ^{Δ Exon1-SC} causes loss of gene function, we generated *vHnf1*^{-/-} ES cell lines by culturing independent heterozygous ES clones in high concentrations of G418 (Mortensen et al., 1992) (Fig. 1B). Two homozygous, a heterozygous and the wt HM1 ES cell lines were chosen for subsequent analysis. After spontaneous in vitro differentiation, these clones were examined for the presence of vHNF1 by western blot assay. Neither normal nor aberrantly sized vHNF1 was detected in differentiated *vHnf1*^{-/-} ES cells (Fig. 1E). vHNF1 was not detected in non-differentiated ES cells, as previously reported

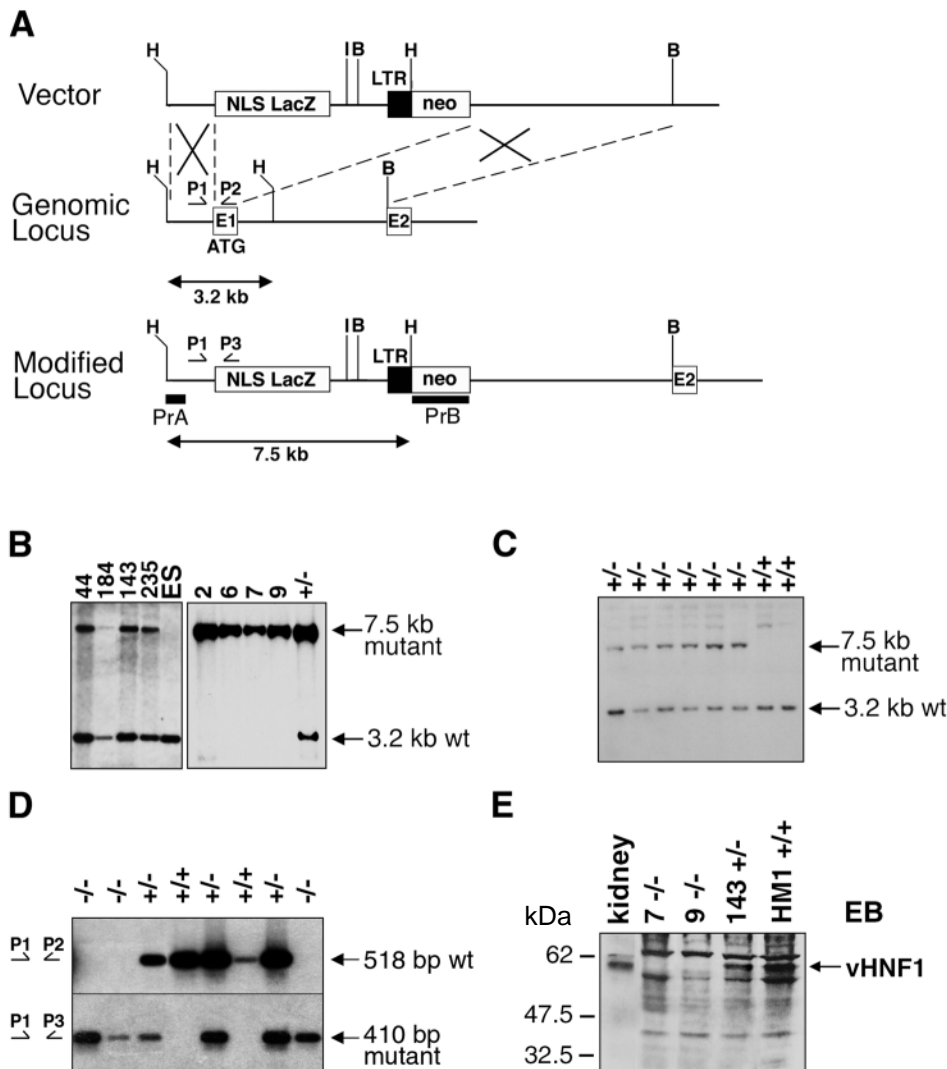


Fig. 1. Targeted disruption of *vHnf1*. (A) Targeting strategy. Targeting vector (top), wild-type gene locus (middle), and *vHnf1* modified locus (bottom). Homologous recombination replaces the first exon with the *NLSlacZ* and the *neomycin* resistance (*neo*) genes. B, *Bgl*II; H, *Hind*III; I, *I-Sce*I. (B) Correct 5' and 3' recombination events were verified by Southern blot analysis using a 5' probe (PrA) and 3' probe (PrB, not shown). The 7.5 kb and 3.2 kb *Hind*III-digested fragments correspond to the mutant and wt alleles, respectively. (C) Southern blot analysis of DNA from F1 intercross litters. (D) PCR analysis of blastocyst progeny from a heterozygous intercross. Primers P1 and P2 were used for detection of the wt allele and P1 and P3 were used for the mutated allele. (E) Western blot analysis of nuclear extracts from wt (HM1), heterozygous (clone 143) and *vHnf1*^{-/-} (clones 7 and 9) 14 day EBs, using a polyclonal α -vHNF1 antibody raised against amino acids 293–375 of the murine vHNF1. A specific band of approximately 60 kDa was only detected in kidney (control) and heterozygous or wt EBs.

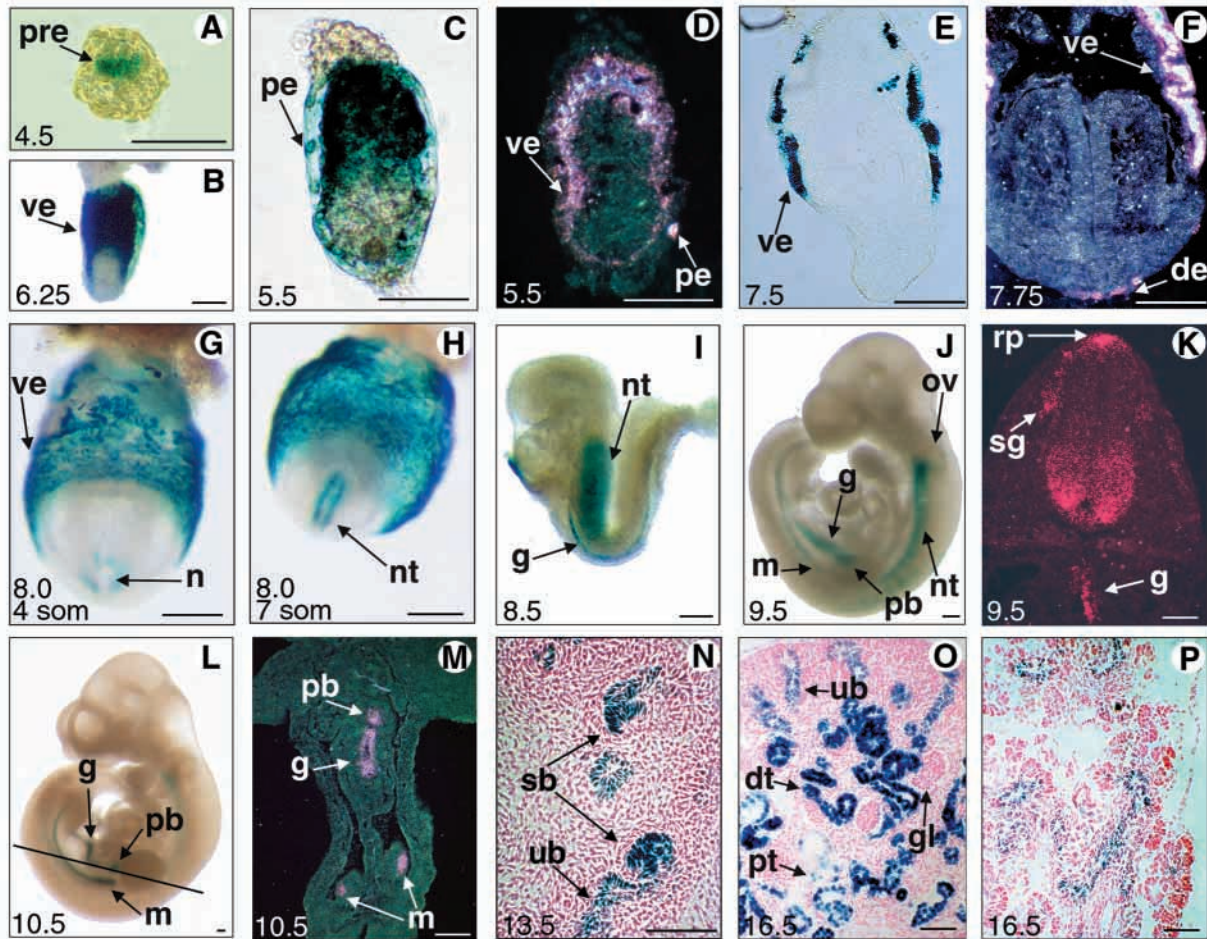


Fig. 2. Analysis of the β -galactosidase expression pattern in *vHnf1* heterozygous embryos. (A) β -gal expression in the primitive endoderm of E4.5 blastocyst. (B) E6.25: expression in the VE. (C-D) β -gal expression in parietal and visceral endoderm surrounding the primitive ectoderm, (C) in toto and (D) in sagittal section. On these embryos, as well as on the embryo shown in B, the Reichert's membrane was left intact to detect the β -gal activity in the parietal endoderm cells. (E) E7.5: labelled VE in sagittal section. (F) E7.75: expression in definitive endoderm seen in frontal section. (G) Early E8.0: a few positive cells flank the node. (H) Stained neural tube at the 7 somite stage. (I) E8.5: expression in the gut epithelium. (J) E9.5: expression in mesonephros and pancreatic primordium. (K) E9.5 neural tube cross section. (L) E10.5 β -gal staining in toto. (M) Cross section at the level indicated by the line. (N) E13.5: developing kidney in sagittal section. (O-P) E16.5: sagittal section of kidney and pancreas, respectively. (D,F,K,M) β -gal staining appears pink under dark-field optics. de, definitive endoderm; dt, distal tubule; g, gut; gl, glomerulus; m, mesonephric duct; n, node; nt, neural tube; ov, otic vesicle; pb, pancreatic bud; pe, parietal endoderm; pt, proximal tubule; rp, roof plate; sb, s-shape body; sg, spinal ganglia; ub, ureteric bud; ve, visceral endoderm. Scale bars in A-F, 100 μ m; in G-P, 200 μ m.

(Cereghini et al., 1992; see below). Thus, the homozygous state of *vHnf1* Δ Exon1-SC resulted in a null mutation.

***vHnf1-lacZ* expression pattern during early mouse development**

A preliminary screening of heterozygous embryos at different stages indicated that the pattern of *lacZ* expression was identical to that previously described for *vHnf1* (Cereghini, 1996; Ott et al., 1991). Hence, the use of *lacZ* allowed a more precise analysis of *vHnf1* expression from pre-implantation to post-gastrulation stages (Fig. 2).

lacZ expression was first detected at E4.5 in the primitive endoderm (Fig. 2A). At E5.5, β -gal staining was observed in the parietal endoderm and, with increasing intensity, in both the proximal (embryonic) and distal (extraembryonic) VE cells (Fig. 2C,D). Subsequently, this expression became

restricted to the columnar VE surrounding the proximal part of the egg cylinder (Fig. 2E). Up to the early stages of gastrulation, expression was confined to the extraembryonic lineage. From early headfold stage at E7.75, few strongly positive cells flanking the node were detected, which appeared to correspond to emerging definitive endoderm (Fig. 2F,G) (Lawson et al., 1991). The node itself and the primitive streak were negative. A high level of expression was subsequently observed in the forming neural tube and the entire gut (Fig. 2H-I). Expression in the neural tube was initially strong (E8.0-9.0) but gradually decreased, until, after E11.5-12.5, it was undetectable. At E8.5, staining of the neural tube was homogenous, but subsequently became predominant in the ventral part and in the roof plate (Fig. 2K). At E9.5, the domain of *vHnf1* expression in the neural tube extended from the posterior border of the otic vesicle to

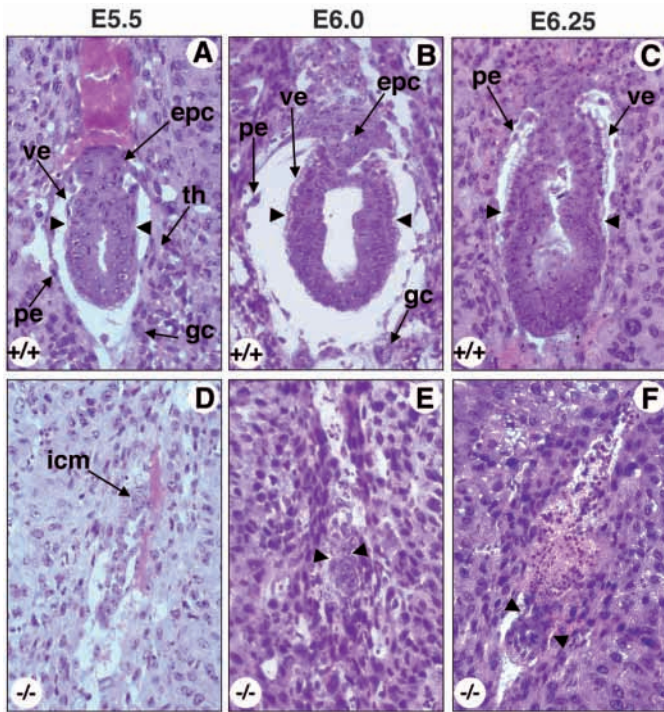


Fig. 3. Histological analysis of normal and *vHnf1* mutant embryos. wt (A-C) and presumptive *vHnf1* nullizygous (D-F) embryos at E5.5 (A,D), E6.0 (B,E) and E6.25 (C,F). 7 μ m sections were stained with hematoxylin and eosin. Arrowhead marks demarcation line between embryonic and extraembryonic regions. epc, ectoplacental cone; gc, giant trophoblastic cells; icm, inner cell mass; th, trophoblast. Magnification, 20 \times .

below). During kidney development, from E9.0, the mesonephric tubules and the Wolffian duct were labelled as well as, from E10.5, the ureteric bud emerging from the most caudal portion of the Wolffian duct. At E13.5, β -gal expression was strongly evident in the comma-shaped, and then in the S-shaped bodies (Fig. 2N). At E16.5, the expression pattern was similar to the adult (Cereghini, 1996) with both the proximal and distal tubules staining positive while the glomeruli were negative (Fig. 2O).

Thus, *vHNF1* is one of the earliest transcription factors expressed in the mouse embryo in the primitive endoderm and its derivatives. After gastrulation *vHnf1* expression is restricted to the forming neural tube and the primitive gut, and later to the developing meso- and metanephros.

Homozygosity for the *vHNF1* mutation leads to embryonic lethality before gastrulation

Mice heterozygous for *vHnf1* ^{Δ Exon1-SC} were phenotypically normal (size, health, fertility and longevity) and were indistinguishable from their wt littermates in either the outbred (129/Sv \times C57BL/6j or 129/Sv \times B6D2F1) or inbred (129/Sv) genetic background. However, no homozygous mutant was born among 304 live-born offspring from heterozygous

forelimb bud level (Fig. 2J); neural crest cells and early spinal ganglia were also positive.

By E9.5, expression was detected in the epithelial cells of the hind- and mid-gut as well as in the dorsal foregut, and in the hepatic and pancreatic primordia (Fig. 2J,L-M and see

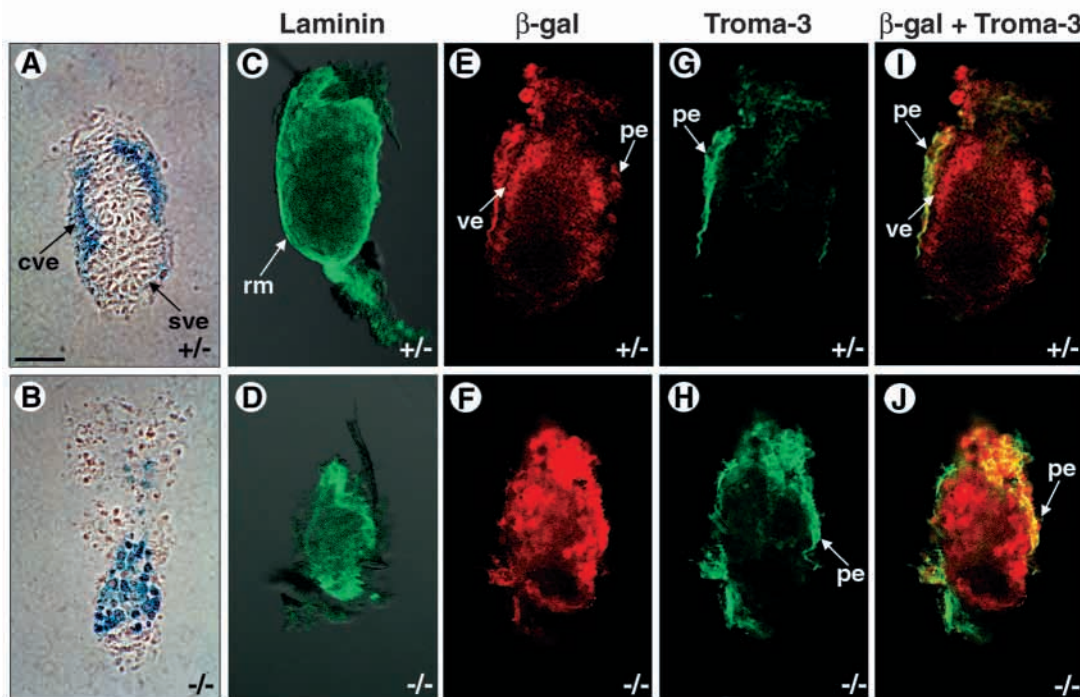


Fig. 4. Characterisation of E5.5 mutant phenotype. (A,B) Sagittal sections of β -gal stained heterozygous (A) and *vHnf1*^{-/-} (B) embryos, showing impaired development. (C-J) Immunohistochemical staining of heterozygous (C,E,G,I) and homozygous mutant (D,F,H,J) whole embryos. (C,D) Laminin- γ 1 antibody reveals the Reichert's membrane in the heterozygous embryo (C) and a heterogeneous staining in a nullizygous embryo (D). (E,F) β -gal immunohistochemistry using Texas red labelled secondary antibody. (G,H) Troma-3-FITC labelling reveals the presence of parietal endoderm in both heterozygous (G) and homozygous mutant (H) *vHNF1* embryos.

(I,J) Double staining with anti- β -gal (Texas red) and Troma-3 (FITC) antibodies showing that not all β -gal-positive cells in *vHnf1*^{-/-} embryos (J) are parietal endoderm-like cells. Abbreviations: cve, columnar visceral endoderm; rm, Reichert's membrane; sve, squamous visceral endoderm. Scale bar, 50 μ m.

Table 1. Morphologies and genotypes of embryos obtained from *vHnf1* heterozygous intercrosses

Litters	Stage	Genotype				Total
		+/+ (Normal phenotype)	+/- (Normal phenotype)	-/- (Abnormal phenotype)	Resorbed (Abnormal phenotype)	
49	3 weeks	104 (34%)	200 (66%)	– (0%)		304
7	E8.5	14 (40%)	21 (60%)	– (0%)	22	35
2	E8.0	9 (53%)	8 (47%)	– (0%)	4	17
6	E7.5	18 (39%)	27 (59%)	1* (2%)	11	46
4	E7.25	6 (24%)	19 (76%)	– (0%)	9	25
10	E6.5	21 (33%)	43 (67%)	– (0%)	18	64
6	E6.25	7 (20%)	19 (56%)	8‡ (24%)	2	34
19	E3.5	35 (28%)	59 (46%)	33 (26%)		127

*The embryo was resorbed.
‡2 of 8 embryos were resorbed.
In parentheses appear the percentages corresponding to the different genotypic classes.
Resorbed embryos that could not be typed were not included in the total count.

matings (Table 1), irrespective of the genetic background. The results presented below were obtained from heterozygous crosses in a mixed genetic background.

To define the stage of lethality of *vHnf1*^{-/-} embryos, litters from heterozygous matings were isolated at sequential stages. From E6.5 to E8.5, harvested embryos were either wt or heterozygous for *vHnf1*^{ΔExon1-SC}, showing no significant deviation from a 2:1 heterozygote/wt ratio. Analysis of 127 E3.5 blastocysts established that *vHnf1*^{-/-} embryos were present (Fig. 1D) at the expected Mendelian proportion (26%), demonstrating that they were viable prior to implantation (Table 1). Thus, *vHnf1*^{-/-} concepti were viable until the blastocyst stage but died upon implantation and before gastrulation.

To investigate the fate of the *vHnf1*^{-/-} embryos, we performed histological analysis of serially sectioned E5.5–6.5 embryos. At E5.5, presumptive homozygous mutant embryos already showed significantly impaired growth and differentiation as compared to normal littermates (Fig. 3A,D). E5.5 mutant embryos (27%: 7/26) had induced a uterine reaction, but still resembled implanting blastocysts which had started to collapse (Fig. 3D). At E6.0 presumptive mutant embryos (27%: 8/29) lacked distinct extraembryonic tissue or a visible VE and parietal endoderm cell layer, and ectoderm cells were severely disordered (Fig. 3E). Proamniotic and ectoplacental cavities were absent and resorption of mutant embryos was complete by E6.5 (29%: 5/17).

Further characterisation of the mutant phenotype at E5.5 showed that genotyped *vHnf1*^{-/-} embryos were invariably reduced in size and displayed the characteristic rounded shape observed in the histological sections (Figs 3E and 4B). A striking feature of the *vHnf1*^{-/-} embryos is that the β-gal-positive cells appeared rounded and loosely associated (Fig. 4B), instead of forming a layer in close contact with neighbouring ectodermal cells. Hence, we examined the expression of the parietal endoderm markers laminin-γ1, a Reichert’s membrane protein secreted by the parietal endoderm (Hogan et al., 1980), and Troma-3 (Kemler et al., 1981). Immunostaining with an antibody against laminin-γ1 resulted in a heterogeneous sparse staining in *vHnf1*^{-/-} embryos (Fig.

4C,D) indicating the presence of an extracellular matrix, but consistent with an abnormal basal membrane structure. The staining pattern obtained using the monoclonal antibody Troma-3 confirmed the presence of parietal endoderm-like cells in *vHnf1*^{-/-} embryos (Fig. 4E,F). Co-immunostaining using Troma-3 and an antibody against β-galactosidase showed that only a fraction of β-gal-positive cells did indeed correspond to parietal endoderm-like cells (Fig. 4J).

These results show that *vHnf1* activity is required for appropriate differentiation of extraembryonic endoderm cell lineages and postimplantation morphogenesis of the epiblast. Lethality occurs approximately 24 hours after *vHnf1* induction in the primitive endoderm, concomitant with a sharp increase in its expression in the VE. The presence of parietal endoderm-like cells in *vHnf1*^{-/-} embryos suggests that the primary defect lies in the inability of the primitive endoderm to differentiate into VE.

Impaired differentiation of visceral endoderm in *vHnf1* null blastocyst outgrowths

vHnf1^{-/-} embryos die at a time which coincides with the dramatic increase in epiblast cell proliferation between E5.5 and E6.5 in normal mouse embryos (Snow, 1973). It was therefore possible that the defects observed in mutant ectoderm cells, where *vHnf1* is not expressed, could be due to a generalised growth failure or an inadequate nutrition caused by a lack of or impaired VE differentiation.

To determine the extent to which mutant preimplantation embryos can proliferate, E3.5 blastocysts derived from heterozygous intercrosses were isolated and individually cultured in vitro. *vHnf1*^{-/-} blastocysts appeared morphologically identical to both the wt and heterozygous blastocysts. After 7 days in culture, *vHnf1*^{-/-} blastocysts showed a clear outgrowth of both trophoblast and ICM. Although *vHnf1*^{-/-} ICM outgrowths were sometimes smaller than that of wt or heterozygotes (Fig. 5C), they did not appear to exhibit impaired growth (Fig. 5G,K). In agreement with these results, *vHnf1*^{-/-} ES cells showed a growth rate similar to that of wt ES cells (data not shown). Thus, *vHnf1* deficiency does not critically affect the proliferation and/or survival of the ICM.

After 4 days of blastocyst in vitro culture, the ICM differentiates into a core of ectoderm cells surrounded by a discernible VE layer (Gonda and Hsu, 1980). In our experiments, none of the mutant ICM outgrowths ever showed distinct overlying endodermal layer as was typically observed in heterozygotes and wt blastocysts. This suggests that VE differentiation is defective in *vHnf1*^{-/-} blastocyst outgrowths. To further investigate this possibility, we analysed the expression of several known markers of this cell lineage using immunohistochemistry. As shown in Fig. 5, neither the VE early marker *GATA4* (Soudais et al., 1995) nor the late marker *AFP* were expressed in 10/10 *vHnf1*^{-/-} blastocyst outgrowths. The absence of VE in mutant ICM outgrowths was confirmed by the lack of staining with FITC-labelled *Sophora japonica* agglutinin (SJA) (6/6 *vHnf1*^{-/-} blastocysts) which specifically reacts with the mature VE (Sato and Muramatsu, 1985).

Thus, while *vHnf1*^{-/-} blastocyst are capable of forming the ICM and the trophoblast cell layer outgrowths, further differentiation of the ICM is blocked and VE differentiation

fails. These results are consistent with the *in vivo* observations of *vHnf1*^{-/-} embryos.

vHnf1-deficient embryoid bodies exhibit a specific visceral endoderm differentiation defect

The role of vHNF1 in VE differentiation was characterised through the morphological and biochemical analysis of mutant and wt *in vitro* differentiated ES cells. When ES cells are cultured in suspension they develop into multicellular structures called embryoid bodies (EB) composed of an outer endoderm layer similar to the VE, surrounding an inner ectodermal layer, resembling primitive ectoderm at the egg cylinder stage (Doetschman et al., 1985; Robertson, 1987). Thus, EBs provide an useful model system to investigate VE differentiation.

Heterozygous *vHnf1* EBs exhibited a clearly discernible outer layer of endoderm cells after 5-7 days of differentiation, and later most of the EBs formed large cystic structures surrounded by visceral yolk sac endoderm (Fig. 6A). In contrast, *vHnf1*^{-/-} EBs failed to form an external VE cell layer, remained smaller and formed abortive cavitated bodies instead of cysts (Fig. 6B,F). Sections of 7-day β -gal-stained EBs showed that the *vHnf1* expression was restricted to the columnar VE layer in heterozygous EBs (Fig. 6C). Importantly, in *vHnf1*^{-/-} EBs most of the β -gal positive cells formed a flat disorganised layer at the surface (Fig. 6D), lacking features of fully differentiated VE, such as apical phagocytic vacuoles and abundant microvilli (Fig. 6G-H). These abnormal features on the surface of *vHnf1*^{-/-} EBs persisted at later stages suggesting a specific block in VE differentiation. However, the presence of rhythmically contracting areas indicated that other differentiation pathways, i.e. cardiac myocytes, were not affected.

RNA analysis of EBs was performed by semiquantitative RT-PCR (Fig. 6I) to place vHNF1 in the proposed transcriptional regulatory cascade involved in VE differentiation (Duncan et al., 1997; Morrissey et al., 1998). In contrast to mutant blastocyst outgrowths (Fig. 5D), *GATA4* expression was not affected in mutant EBs (Fig. 6I). It is likely that this expression comes from primitive endoderm-like cells and/or from early cardiac myocytes. The expression of other genes known to be required for proper differentiation of either VE, such as *GATA6* (Koutsourakis et al., 1999; Morrissey et al., 1998) and *Smad-4* (Sirard et al., 1998) or both VE and parietal endoderm, like *evx-1* (Spyropoulos and Capecchi, 1994), was not affected in mutant EBs at any stage of differentiation. Since *Smad-4* and *evx-1* are both expressed at high levels in ES cells before and after differentiation (Fig. 6I), it was difficult to assess whether a specific activation of these two genes was linked to VE differentiation.

Hnf-4 has been shown to be required for complete VE differentiation *in vivo* and *in vitro* (Duncan et al., 1997). In addition, an alternative splice variant of *Hnf-4*, *Hnf-4 α 7*, has been recently identified as being expressed in ES cells (Nakhei et al., 1998). Remarkably, even though *Hnf-4* gene targeting disrupts both *Hnf-4 α 1* and *Hnf-4 α 7* isoforms, EBs derived from *vHnf1*^{-/-} ES cells lacked the expression of *Hnf-4 α 1*, but not that of *Hnf-4 α 7*. As expected, null mutant EBs lacked the expression of the genes encoding several serum proteins reported to be controlled either by vHNF1 (*AFP*, *α At-1* and *albumin*, Fig. 6I), or by HNF-4 (*ApoB*, *α At-1* and *transferrin*,

Fig. 6I and data not shown) (Duncan et al., 1997). Interestingly, the induction of both *Hnf1 α* and *Hnf-3 γ* expression upon ES differentiation was not observed in *vHnf1*^{-/-} EBs (Fig. 6I), suggesting an additional role for vHNF1 in the transcriptional activation of these genes.

Taken together, our results indicate that, in the absence of vHNF1, primitive endoderm cells do not differentiate into the VE. Moreover, vHNF1 is required, directly or indirectly, for the activation of the *Hnf-4 α 1*, *Hnf1 α* and *Hnf-3 γ* transcription factors and their target genes.

Wild-type extraembryonic endoderm rescues the periimplantation defect of vHnf1 mutant embryos

To demonstrate that periimplantation lethality in *vHnf1*^{-/-} embryos is due to the absence of the VE, we generated tetraploid aggregation chimeras using *vHnf1*^{-/-} ES cells. In this procedure tetraploid embryonic cells, which contribute only to extraembryonic tissues, were aggregated with ES cells (Nagy and Rossant, 1993); the resulting foetuses are derived entirely from the ES cells. Since the defects due to *vHnf1* inactivation were observed at the periimplantation stage, we focused our analysis on the chimeric embryos from gastrulation to early organogenesis stages.

Rescued chimeric embryos were obtained by combining two independently derived *vHnf1*^{-/-} ES cell clones (7 and 9) with wt tetraploid embryos. The chimeric embryos obtained displayed a stronger *lacZ* expression than heterozygous embryos (compare Figs 2J and 7B), indicating that vHNF1 is not involved in an autoregulatory process. These embryos did not show any gross external morphological defects up to E9.5 although they showed signs of necrosis at the level of the anterior and posterior neural pore closure (Fig. 7B). Tetraploid rescued embryos also had kinks in the neural tube in the trunk region and histological analysis revealed that the ventral neuroepithelial cells were disorganised (Fig. 7C-D). A similar neural tube defect was observed in chimeric embryos generated by morula aggregation with heterozygous ES cells (see below Fig. 7E-H), suggesting that this disorganisation was not due to the absence of *vHnf1*.

We also generated chimeric embryos by aggregation of heterozygous or homozygous mutant ES cells and wt diploid morulae (Nagy and Rossant, 1993). Using this approach, the ES cells are excluded from the extraembryonic lineages, while the resulting embryos are formed from a mix of mutant and wt cells. Therefore, this provides a means of investigating the ability of the *vHnf1*^{-/-} cells, when they are in competition with wt cells, to contribute to the tissues that normally express *vHnf1*. The extent of chimerism in these tissues was estimated by the presence of β -gal-positive cells. Chimeric embryos presenting more than 95% mutant cells in the tissues expressing *vHnf1* were morphologically very similar to the rescued embryos generated by tetraploid aggregation (Fig. 7B,J). The E9.5 chimeras generated with either null or heterozygous ES cell clones presented similar neural tube disorganisation (Fig. 7C-D,G-H,K), strongly suggesting that this defect was inherent to the parental ES cell line. Finally, β -gal-stained cells were clearly detected in the neural tube, in the pancreatic and liver primordia, and in the dorsal region of the foregut (Fig. 7K-M), demonstrating that the mutant cells were not specifically excluded from any of these cell lineages.

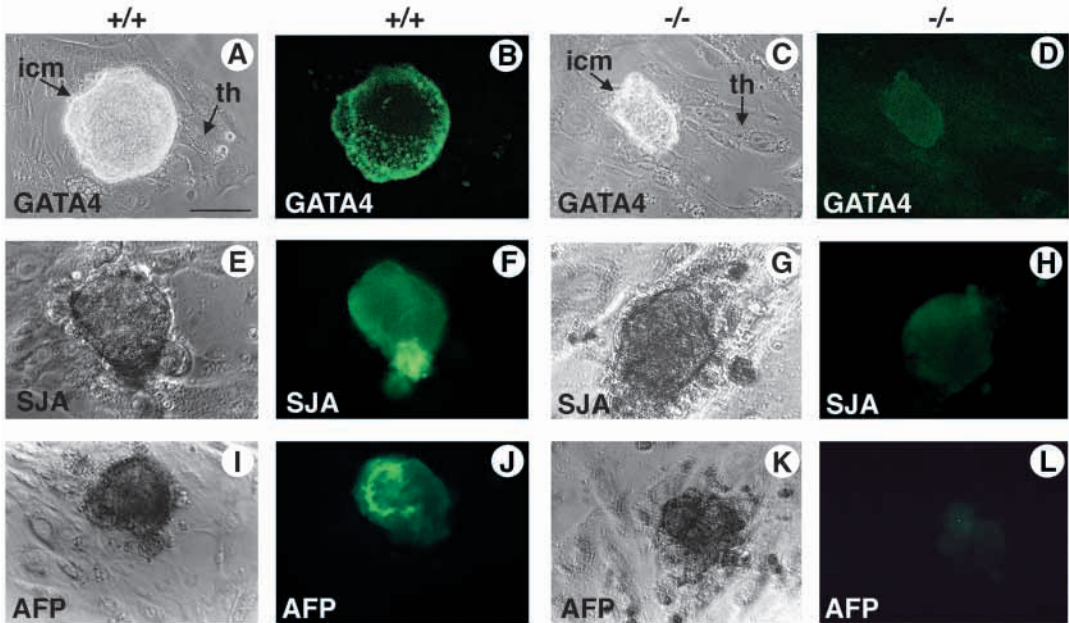


Fig. 5. Expression of visceral endoderm-specific markers in in vitro blastocyst culture. Control (A,B,E,F,I,J) and *vHnf1*^{-/-} (C,D,G,H,K,L) 7-day cultured blastocysts. Immunohistochemistry analysis (B,D,F,H,J,L) of blastocysts and the corresponding bright-field images (A,C,E,G,I,K). Visceral endoderm markers, GATA4 (B), SJA (F), and AFP (J) are detected in control blastocyst cultures. In *vHnf1*^{-/-} blastocyst outgrowths there is no expression of GATA4 (D), SJA (H), and AFP (L). Scale bar 100 μm.

In conclusion, these results definitely confirm that the defect observed at E5.5 in the primitive ectoderm of mutant embryos is exclusively due to impaired function of extraembryonic tissues, and further demonstrate the importance of the VE for growth and organisation of both primitive ectoderm and extraembryonic tissues in pregastrulating embryos.

DISCUSSION

We have examined the role of vHNF1 during early mouse development by generating genetically deficient mice and homozygous mutant ES cells. Using three different experimental approaches, our study demonstrates that vHNF1 is required for early embryogenesis and plays an essential role

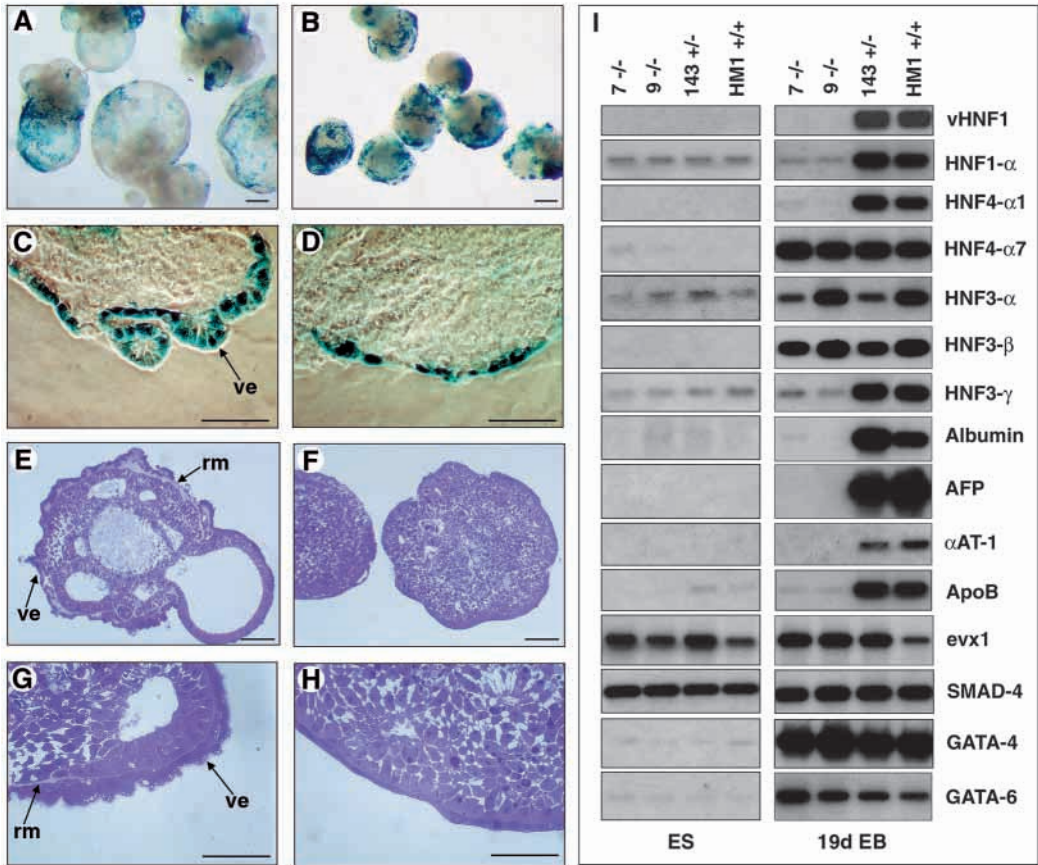


Fig. 6. Morphology and structure of wild-type and *vHnf1* mutant embryoid bodies. (A-D) β-gal staining of heterozygous (A,C) and mutant (B,D) EBs. (A) 14-day heterozygous EBs formed a cystic cavity. (B) *vHnf1*^{-/-} EBs give no sign of cysts and are smaller in size. (C) 7-day heterozygous EBs have a defined β-gal stained VE layer. (D) In *vHnf1*^{-/-} EBs the β-gal-positive cell layer is disorganised. (E-H) Toluidine blue-stained 1 μm sections of heterozygous (E,G) and *vHnf1*^{-/-} (F,H) EBs after 8 days differentiation. (I) RT-PCR analysis of the indicated markers in wt (HM1), heterozygous (143 clone) and homozygous mutant (7 and 9 clones) in ES cell clones and in 19 day EBs. Scale bars in A,B and E,F, 200 μm; in C,D and G,H, 100 μm.

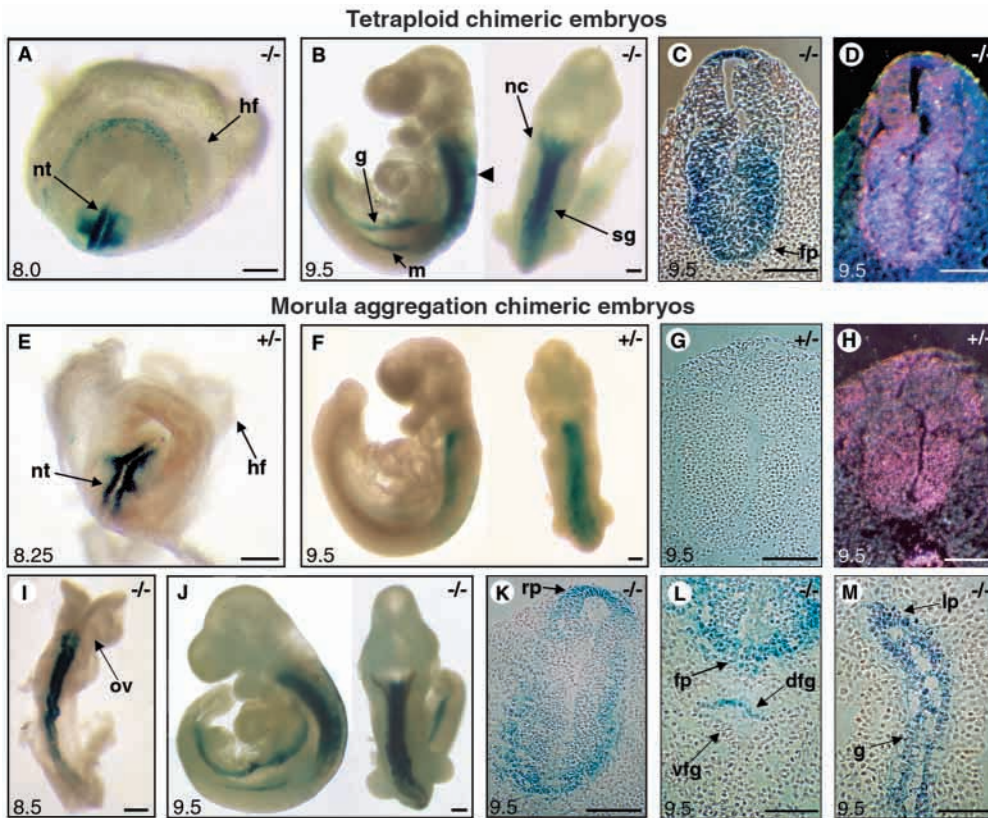


Fig. 7. Wild-type tetraploid extraembryonic tissues rescue the lethal periimplantation phenotype of the *vHnf1* mutation. β -gal expression and histological analysis of tetraploid rescued embryos (A-D) and morula aggregation chimeras (E-M). (A) Ventral views of an E8.0 embryo derived from *vHnf1*^{-/-} ES clone 7, showing abnormal proliferation of β -gal-positive cells on both sides of neural tube. (B) Side and back views of β -gal-stained embryos derived from *vHnf1*^{-/-} ES clone 9 at E9.5. (C,D) Neural tube cross section at the level indicated in B (arrowhead) under bright (C) and dark (D) field optics. (E-H) Chimeric embryos derived from ES heterozygous clone 143 at E8.25 (E) and E9.5 (F). (G-H) Cross section of the chimeric embryo in F at the same level as indicated in B under bright (G) and dark (H) field optics. (I,J) Chimeras derived from homozygous ES clone 9 at E8.5 (I) and at E9.5 (J). (K,M) Histological sections of the neural tube (K), the foregut (L) and the liver primordium (M) of the embryo in J (cross sections). dfg, dorsal foregut; fp, floor plate; hf, headfold; lp, liver primordium; nc, neural crest; vfg, ventral foregut. Embryos in A,B and I,J were β -gal stained for 15 hours, while embryos in E,F were stained for 48 hours. Scale bars in A,B, E,F, I,J, 200 μ m; in C,D, G,H, K,M, 50 μ m.

headfold; lp, liver primordium; nc, neural crest; vfg, ventral foregut. Embryos in A,B and I,J were β -gal stained for 15 hours, while embryos in E,F were stained for 48 hours. Scale bars in A,B, E,F, I,J, 200 μ m; in C,D, G,H, K,M, 50 μ m.

in VE specification. Nullizygous *vHnf1* blastocysts appear normal, invade the uterine epithelium and start implantation. Shortly after implantation, at E5.5, mutant embryos are severely growth retarded, exhibiting an abnormal or absent extraembryonic region, poorly organised ectoderm and no discernible VE. By E6.5 the *vHnf1*^{-/-} concepti are resorbed.

In normal embryos, the earliest expression of *vHnf1* is seen in the primitive endoderm (Fig. 2A), the precursor of both visceral and parietal endoderm. The onset of phenotypic defects coincides with the increased levels of *vHnf1* expression in the VE. In the absence of vHNF1, the primitive endoderm lacks the ability to generate a VE epithelium, while the ability to differentiate into parietal endoderm is retained. However our results do not exclude the possibility that parietal endoderm differentiation may also be defective as a consequence of abnormal cell-cell interactions (Gardner, 1982; Hogan et al., 1981). Our observations using tetraploid chimeric embryos further demonstrates that the early lethal phenotype of the *vHnf1* null mutation is essentially due to defective extraembryonic tissue formation. Consistent with in vivo observations, both *vHnf1*^{-/-} blastocyst outgrowths and mutant EBs do not form the characteristic external VE layer. Similar results were obtained at early and late stages of ES cell differentiation indicating that there was not a lag in the differentiation process. More importantly, *vHnf1*^{-/-} EBs do not express early or late markers of the VE cell lineage, including the transcription factors HNF-4 α 1, HNF1 α and HNF-3 γ and downstream genes. Thus, this mutant phenotype indicates that

vHNF1 is a critical regulator in the differentiation of the VE from the primitive endoderm.

The complex pleiotropic pattern of defects displayed by *vHnf1*^{-/-} postimplantation embryos are consistent with the lack of a functional VE. The absence of the VE and a poorly organised ectoderm are features distinctive to *vHnf1*^{-/-} embryos. Mutation of the transcription factor *Hnf-4*, expressed in the primitive endoderm and VE, leads to impaired gastrulation (Chen et al., 1994). Death is caused by disruption of VE function. This is brought about through reduced production of several serum proteins resulting in increased cell death within the embryonic ectoderm and a dramatic reduction in epiblast size (Duncan et al., 1997). Recently, the targeted mutation of the *GATA6* transcription factor has been described (Koutsourakis et al., 1999; Morrissey et al., 1998). *GATA6* homozygous mutant embryos correctly specify the VE but display a similar phenotype to *Hnf-4*-deficient embryos, consistent with the observation of the absence of *Hnf-4* expression (Morrissey et al., 1998). Unlike *Hnf-4* and *GATA6*, *vHnf1* is required for the differentiation of primitive endoderm into an organised VE cell layer. Thus, the *vHnf1* null phenotype is not entirely explained by the inability of the VE to provide histotrophic nutrition to the embryonic ectoderm. Since the VE is not formed in *vHnf1*^{-/-} embryos, not only is its specialised nutritional role impaired, but also its other early postimplantation embryonic functions. Thus, signals such as those involved in programmed cell death in the embryonic ectoderm and subsequent proamniotic cavity formation

(Coucounanis and Martin, 1999), and in early anterior neural patterning (Beddington and Robertson, 1998) are also abrogated in *vHnf1*^{-/-} embryos.

The severity and timing of the defects observed in *vHnf1*^{-/-} embryos are strikingly similar to those described in *evx-1* mutants (Spyropoulos and Capecchi, 1994), a transcription factor expressed at E5.0 in the VE. Both *vHnf1*- and *evx-1*-deficient embryos fail to differentiate into distinct embryonic and extraembryonic tissues soon after implantation, raising the possibility that these two transcription factors control similar differentiation processes. The expression profile of *evx-1* in *vHnf1*^{-/-} EBs was found to be unaffected (Fig. 6I). Furthermore, *evx-1* is expressed at high levels in non differentiated ES cells (Fig. 6I), where *vHnf1* is not expressed. Thus, *evx-1* and *vHnf1* could be linked in a regulatory cascade, with *evx-1* acting upstream of vHNF1, or alternatively these two factors may cooperate in the differentiation of extraembryonic endoderm cell lineages. Taken together, these results further demonstrate that VE interactions are required for both normal embryonic and extraembryonic development.

The specification and differentiation of the VE has been proposed to be a multistep process (Gardner, 1982) involving a transcriptional regulatory cascade (Duncan et al., 1997; Morrissey et al., 1998). In this context, previous studies suggested that *vHnf1* could act upstream of *Hnf-4*. The proximal promoter of *Hnf-4* contains a HNF1 binding site, that interacts with both vHNF1 and HNF1 α (Taraviras et al., 1994; Zhong et al., 1994; and unpublished data). Moreover, *vHnf1* and *Hnf-4* genes display a strikingly similar spatial and temporal expression pattern, more notably in the primitive and visceral endoderm, kidney and gut derivatives (Duncan et al., 1997; and this study). We have demonstrated that only HNF-4 α 1 and not HNF-4 α 7, which is very likely generated by an alternative upstream promoter (Nakhei et al., 1998), is affected by lack of vHNF1. These results strongly suggest that vHNF1 acts as a direct transactivator of the *Hnf-4* proximal promoter. Remarkably, lack of vHNF1 results in a strong decrease in the induction of *Hnf1* α and *Hnf-3* γ transcripts, and in the absence of HNF1 α protein (Fig. 6I and data not shown). *Hnf1* α has been proposed as a target gene of *Hnf-4* (Kuo et al., 1992). However, *Hnf-4* inactivation has essentially no effect on *Hnf1* α expression (Duncan et al., 1997), indicating that the lack of HNF1 α in *vHnf1*^{-/-} EBs is not a secondary effect due to the absence of HNF-4. The possible involvement of vHNF1 in *Hnf-3* γ regulation has already been proposed (Hiemisch et al., 1997). Thus, at least in the VE cell lineage, *vHnf1* acts upstream of *Hnf-4*, *Hnf1* α and *Hnf-3* γ in a transcriptional hierarchy.

In summary, the present study has defined a crucial role for the transcription factor vHNF1 in the differentiation of the primitive endoderm into the VE. Death of *vHnf1*^{-/-} embryos prior to gastrulation is due to impaired VE formation. Although the mechanisms of VE specification remain largely unknown, our results, together with the observations of other mutations affecting VE function (Duncan et al., 1997; Koutsourakis et al., 1999; Morrissey et al., 1998; Spyropoulos and Capecchi, 1994), strongly suggest that the specification of the VE cell lineage involves a complex regulatory network between the different signals and regulators rather than a linear transcriptional cascade. Placement of vHNF1 in a preminent position in this regulatory network is consistent with its early expression in the primitive endoderm.

Our experiments with chimeric embryos produced by aggregation of mutant cells with either tetraploid or diploid embryos, further show that *vHnf1* null ES cells are also capable of contributing to the neural tube, the primitive gut and its derivatives, indicating that absence of vHNF1 does not affect their specification. Future studies, involving more detailed chimera analysis and conditional tissue-specific gene inactivation, will determine whether cells that normally express *vHnf1* in these tissues carry out their normal functions upon further differentiation and morphogenesis.

We are grateful to S. Power, M. Zakin, J. P. Concordet and V. Kalatzis for critical reading of the manuscript. We thank M. Sich, D. Rocancourt, H. Le Mouellic, L. Zakin, M. Souyri, J. F. Nicolas, C. Deschatrette, F. Ringeisen, A. Begue for advice and materials, Y. Deris for artwork, and Y. Goureau for confocal microscopy. This work was supported by the Biotechnology program of the EEC under contract BIO2-CT93-0319, ARC (number 1704 and number 9215) and INSERM (to S. C.). E. B. is a fellow from the EEC Biotech program (ERB4001GT9). M. R. is a fellow from ARC.

REFERENCES

- Beddington, R. S. and Robertson, E. J. (1998). Anterior patterning in mouse. *Trends Genet* **14**, 277-284.
- Belaoussoff, M., Farrington, S. M. and Baron, M. H. (1998). Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. *Development* **125**, 5009-5018.
- Cereghini, S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. *Faseb J.* **10**, 267-282.
- Cereghini, S., Blumenfeld, M. and Yaniv, M. (1988). A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. *Genes Dev.* **2**, 957-974.
- Cereghini, S., Ott, M. O., Power, S. and Maury, M. (1992). Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. *Development* **116**, 783-797.
- Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bachvarova, R. F. and Darnell, J. E., Jr. (1994). Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* **8**, 2466-2477.
- Coucounanis, E. and Martin, G. R. (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. *Development* **126**, 535-546.
- Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87**, 27-45.
- Duncan, S. A., Nagy, A. and Chan, W. (1997). Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of *Hnf-4*(-/-) embryos. *Development* **124**, 279-287.
- Gardner, R. L. (1982). Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. *J. Embryol. Exp. Morphol.* **68**, 175-198.
- Gardner, R. L. and Beddington, R. S. (1988). Multi-lineage 'stem' cells in the mammalian embryo. *J. Cell Sci. Supplement* **10**, 11-27.
- Gonda, M. A. and Hsu, Y. C. (1980). Correlative scanning electron, transmission electron, and light microscopic studies of the in vitro development of mouse embryos on a plastic substrate at the implantation stage. *J. Embryol. Exp. Morphol.* **56**, 23-39.
- Hiemisch, H., Schutz, G. and Kaestner, K. H. (1997). Transcriptional regulation in endoderm development: characterization of an enhancer controlling *Hnf3g* expression by transgenesis and targeted mutagenesis. *EMBO J.* **16**, 3995-4006.
- Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo*. New York: Cold Spring Harbor Laboratory Press.
- Hogan, B. L., Cooper, A. R. and Kurkinen, M. (1980). Incorporation into

- Reichert's membrane of laminin-like extracellular proteins synthesized by parietal endoderm cells of the mouse embryo. *Dev. Biol.* **80**, 289-300.
- Hogan, B. L., Taylor, A. and Adamson, E.** (1981). Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* **291**, 235-237.
- Kemler, R., Brulet, P., Schnebelen, M. T., Gaillard, J. and Jacob, F.** (1981). Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J. Embryol. Exp. Morphol.* **64**, 45-60.
- Koutsourakis, M., Langeveld, A., Patient, R., Beddington, R. and Grosveld, F.** (1999). The transcription factor GATA6 is essential for early extraembryonic development. *Development* **126**, 723-732.
- Kuo, C. J., Conley, P. B., Chen, L., Sladek, F. M., Darnell, J. E., Jr. and Crabtree, G. R.** (1992). A transcriptional hierarchy involved in mammalian cell-type specification. *Nature* **355**, 457-461.
- Lawson, K. A., Meneses, J. J. and Pedersen, R. A.** (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113**, 891-911.
- Magin, T. M., McWhir, J. and Melton, D. W.** (1992). A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. *Nucl. Acids Res.* **20**, 3795-3796.
- Morrissey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S. and Parmacek, M. S.** (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12**, 3579-3590.
- Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. and Seidman, J. G.** (1992). Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell Biol.* **12**, 2391-2395.
- Nagy, A. and Rossant, J.** (1993). Production of completely ES cell-derived fetuses. In *Gene Targeting: A Practical Approach* (ed. A. L. Joyner), pp. 147-179. Oxford UK: IRL Press.
- Nakhei, H., Lingott, A., Lemm, I. and Ryffel, G. U.** (1998). An alternative splice variant of the tissue specific transcription factor HNF4alpha predominates in undifferentiated murine cell types. *Nucl. Acids Res.* **26**, 497-504.
- Ott, M. O., Rey-Campos, J., Cereghini, S. and Yaniv, M.** (1991). vHNF1 is expressed in epithelial cells of distinct embryonic origin during development and precedes HNF1 expression. *Mech. Dev.* **36**, 47-58.
- Power, S. C. and Cereghini, S.** (1996). Positive regulation of the vHNF1 promoter by the orphan receptors COUP- TF1/Ear3 and COUP-TFII/Arp1. *Mol. Cell Biol.* **16**, 778-791.
- Robertson, E. J.** (1987). Embryo-derived stem cell lines. In *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach* (ed. E. J. Robertson), pp. 71-112. Oxford: IRL Press.
- Rossant, J.** (1995). Development of the extraembryonic lineages. In *Seminars in Developmental Biology*, vol. **6**, 237-247.
- Sato, M. and Muramatsu, T.** (1985). Reactivity of five N-acetylgalactosamine-recognizing lectins with preimplantation embryos, early postimplantation embryos, and teratocarcinoma cells of the mouse. *Differentiation* **29**, 29-38.
- Sirard, C., de la Pompa, J. L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S. E. et al.** (1998). The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev.* **12**, 107-119.
- Snow, M. H.** (1973). Abnormal development of pre-implantation mouse embryos grown in vitro with (3 H) thymidine. *J. Embryol. Exp. Morphol.* **29**, 601-615.
- Soudais, C., Bielinska, M., Heikinheimo, M., MacArthur, C. A., Narita, N., Saffitz, J. E., Simon, M. C., Leiden, J. M. and Wilson, D. B.** (1995). Targeted mutagenesis of the transcription factor GATA-4 gene in mouse embryonic stem cells disrupts visceral endoderm differentiation in vitro. *Development* **121**, 3877-3888.
- Spyropoulos, D. D. and Capecchi, M. R.** (1994). Targeted disruption of the even-skipped gene, *evx1*, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8**, 1949-1961.
- Taraviras, S., Monaghan, A. P., Schutz, G. and Kelsey, G.** (1994). Characterization of the mouse HNF-4 gene and its expression during mouse embryogenesis. *Mech. Dev.* **48**, 67-79.
- Zhong, W., Mirkovitch, J. and Darnell, J. E., Jr.** (1994). Tissue-specific regulation of mouse hepatocyte nuclear factor 4 expression. *Mol. Cell Biol.* **14**, 7276-7284.