INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurodegenerative disorder (see recent reviews by Jones et al., 1997; Wellington and Hayden, 1997), which is caused by an expansion of a variable stretch of glutamine (CAG) codons (>38) in the first exon of the HD gene encoding a widely expressed 348 kDa cytoplasmic protein (huntingtin) of unknown function. This apparently gain-of-function mutation has been correlated with the appearance of neuronal intranuclear inclusions containing aggregates of an amino-terminal fragment of huntingtin in association with ubiquitin in the cortex and striatum of HD patients (DiFiglia et al., 1997). Analogous inclusions were first detected in mice carrying an HD exon 1 transgene (~1 kb) with 115-156 CAG triplets, which developed a progressive neurological phenotype simulating features of HD (Mangiarini et al., 1996; Davies et al., 1997). In contrast, targeted null mutations of the murine homologue of the HD gene (Hdh) result in developmental retardation, dysmorphogenesis and early death of nullizygous embryos (Nasir et al., 1995; Duyao et al., 1995; Zeitlin et al., 1995).

To investigate further the cause of lethality in Hdh null mutants and at the same time examine the function of huntingtin in early embryonic development, we used chimeric analysis taking advantage of the developmental bias of embryonic stem (ES) cells to preferentially colonize the embryonic ectoderm (epiblast), when injected into host blastocysts (see Beddington and Robertson, 1989). Thus, we asked whether embryos formed after injection of Hdh null ES cells into wild-type host blastocysts are rescued from lethality. Conversely, when wild-type ES cells are injected into Hdh null blastocysts, the chimeric embryos are morphologically indistinguishable from Hdh null mutants derived from natural matings, and die shortly after gastrulation. Therefore, the primary defect in the absence of huntingtin lies in extraembryonic tissues, whereas the epiblast and its derivatives are affected secondarily. It is likely that the mutation results in impairment of the nutritive functions of the visceral endoderm, which otherwise appears to differentiate normally, as evidenced by the expression of several specific marker genes. Consistent with preliminary histochemical analysis indicating that at least the transport of ferric ions is defective in Hdh mutants and in conjunction with the known localization of huntingtin in the membranes of vesicles associated with microtubules, we hypothesize that this protein is involved in the intracellular trafficking of nutrients in early embryos.

SUMMARY

Mouse embryos nullizygous for a targeted disruption of the Huntington’s disease gene homologue (Hdh), which encodes a protein (huntingtin) of unknown biochemical function, become developmentally retarded and disorganized, and die early in development. Using chimeric analysis, we demonstrate that extensively chimeric embryos derived by injection of Hdh null ES cells into wild-type host blastocysts are rescued from lethality. In contrast, when wild-type ES cells are injected into Hdh null blastocysts, the chimeric embryos are morphologically indistinguishable from Hdh null mutants derived from natural matings, and die shortly after gastrulation. Therefore, the primary defect in the absence of huntingtin lies in extraembryonic tissues, whereas the epiblast and its derivatives are affected secondarily. It is likely that the mutation results in impairment of the nutritive functions of the visceral endoderm, which otherwise appears to differentiate normally, as evidenced by the expression of several specific marker genes. Consistent with preliminary histochemical analysis indicating that at least the transport of ferric ions is defective in Hdh mutants and in conjunction with the known localization of huntingtin in the membranes of vesicles associated with microtubules, we hypothesize that this protein is involved in the intracellular trafficking of nutrients in early embryos.

Key words: Hdh gene; huntingtin; chimera; visceral endoderm

MATERIALS AND METHODS

Generation and characterization of ES cell lines

Hdh(+/−) heterozygous mice (129/SvEv × C57BL/6J hybrids) were crossed with 129/SvEvTac partners for four generations. Of the progeny, female heterozygotes were crossed with males homozygous for the ROSA26 (R) transgene (Friedrich and Soriano, 1991). Hdh(+/−)/R offspring were then intercrossed to obtain E3.5 blastocysts. Embryos were allowed to attach to feeders of STO fibroblasts (see Robertson, 1987) and cultured for 4-5 days in the presence of 1,000 units/ml of mLIF (Gibco-BRL). The inner cell mass obtained from the embryo outgrowths was then trypsinized in the presence of chicken serum and cultured as described by Robertson (1987); Abbondanzo et al. (1993). After 4 days in culture, surviving cells with the appropriate morphology were expanded and stored frozen.

Southern analyses were performed using a probe that distinguishes
between the targeted and wild-type \textit{Hdh} alleles (Zeitlin et al., 1995). Western analyses were performed using antibodies recognizing either the amino- or the carboxyl-terminal region of huntingtin (Ab-1 and HF-1, respectively; DiFiglia et al., 1995; Persichetti et al., 1995).

Briefly, ES cells were grown to 80% confluency, and, after harvesting, suspended in hypotonic lysis buffer (10 mM Hepes pH 8.3, 1.5 mM MgCl$_2$, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin) for 10 minutes on ice, pelleted, resuspended in one-fifth volume of the same buffer and homogenized by douncing. Following centrifugation, the supernatant was stored at -80°C. Protein concentration was determined using a dye binding assay (BioRad), and 25-50 μg of extract was fractionated on a 5% polyacrylamide gel with a 3% stacking gel containing 4.5% of DA TD cross-linker. The concentration was determined using a dye binding assay (BioRad), and an additional high-stringency wash step (0.2 M SSC) was used after hybridization; and color development using BMB-purple reagent (Boehringer Mannheim) was allowed to proceed for 5 days at room temperature.

**Generation of chimeras**

\textit{Hdh}(-/-)/R ES cells (12-16) were injected into wild-type C57BL/6j host blastocysts using standard procedures and transferred into the uterine horns of pseudopregnant females as described by Bradley (1987). At the time of transfer, the age of embryos was considered as E2.5 (embryonic day 2.5). Foster mothers were killed at different times of gestation, to obtain embryonic material for analysis.

\textit{Hdh} (+/+) /R ES cells (12-16) were injected into host blastocysts obtained from intercrosses between \textit{Hdh} (+/-) heterozygotes. Foster mothers were killed at E8.5 and E9.5 and embryos were processed for X-gal staining. In this set of experiments, the genotype of host blastocysts was determined retrospectively by PCR analysis of DNA extracted from parietal endoderm and trophoblast cells, as described by Zeitlin et al. (1995), except that the pair of primers for detection of the targeted allele were: forward 5'-aaccgcagecagcccc-3' and reverse 5'-agacagaggaaggtgcctggttg-3'.

**X-gal staining**

Staining of whole mounts to visualize lacZ expression in chimeras was performed as described by Hogan et al. (1994). For staining of sections (10-15 μm) as described by Hogan et al. (1994), chimeric embryos were first fixed overnight at 4°C in 0.1 M PIPES pH 6.9, 2 mM MgCl$_2$, 5 mM EGTA containing 0.2% paraformaldehyde, and then cryopreserved. Frozen sections were postfixed for 5 minutes at room temperature. Slides were washed three times (5 minutes each) at 4°C in PBS, and then incubated in a 1:200 dilution of a rabbit anti-mouse α-fetoprotein polyclonal antibody (ICN Biochemicals) of established specificity (see Tyner et al., 1990) in blocking solution containing 1% Mannheim), 10% heat-inactivated goat serum (HINGS), 0.1% Triton X-100 for 60 minutes at room temperature. The sections were then rinsed twice with PBS and incubated for 10 minutes at room temperature. After three washes with PBS, the slides were developed in a Co$^{2+}$-enhanced diaminobenzidine/H$_2$O$_2$ solution (Sigma).

**In situ hybridization analysis**

In situ hybridization using frozen sections was performed as described by Wilkinson (1992) with a mixture of three digoxigenin-labeled antisense RNA probes corresponding to \textit{Hdh} cDNA positions 281-1425, 3836-5000 and 8025-9266. To provide controls, sections of \textit{Hdh}(-/-) embryos were also hybridized with the same probes. To increase the sensitivity and specificity of these analyses, probes were used at a combined concentration of 1.5 μg/ml in the hybridization mixture; an additional high-stringency wash step (0.2x SSC) was used after hybridization; and color development using BMB-purple reagent (Boehringer Mannheim) was allowed to proceed for 5 days at room temperature.

**Immunohistochemical staining for α-fetoprotein**

Decidua were fixed for 1 hour at 4°C in 4% paraformaldehyde/PBS, washed three times (30 minutes each) at 4°C in PBS, and then cryopreserved. Frozen sections were postfixed for 5 minutes at room temperature. Slides were washed three times (5 minutes each) in PBS, and then incubated overnight at 4°C in a 1:6,000 dilution of a rabbit anti-mouse α-fetoprotein polyclonal antibody (ICN Biochemicals) of established specificity (see Tyner et al., 1990) in blocking solution containing 1% HINGS. Slides were washed three times (5 minutes each) by adding blocking solution containing 1% HINGS (0.5 ml/slide), and then incubated in a 1:200 dilution of a sheep anti-rabbit IgG secondary antibody conjugated to peroxidase (Boehringer Mannheim) for 30 minutes at room temperature. After three washes with PBS, the slides were developed in a Co$^{2+}$-enhanced diaminobenzidine/H$_2$O$_2$ solution (Sigma).

**Histochemical staining for iron**

The histochemical distributions of Fe$^{3+}$ and Fe$^{2+}$ were determined as described by Morris et al. (1992), using the following modifications. For Fe$^{3+}$ staining, decidual cryosections were postfixed for 5 minutes in 4% paraformaldehyde/PBS, rinsed three times (5 minutes each) in PBS and incubated for 30 minutes at room temperature in 1% potassium ferrocyanide, 0.12N HCl. After washing with PBS, the endogenous peroxidase activity was quenched by incubation in 0.3% H$_2$O$_2$ in methanol for 20 minutes at room temperature. Sections were then rinsed twice with PBS and incubated for 10 minutes at room temperature in diaminobenzidine/H$_2$O$_2$ (Sigma FAST-DAB tablets). Positive Fe$^{3+}$ staining was visible as a reddish brown to dark brown color. In the absence of the H$_2$O$_2$ bleaching step, faint staining from endogenous peroxidase activity was observed in the extraembryonic mesoderm of normal embryos. Control sections were incubated in
0.12N HCl without potassium ferrocyanide. The same procedure was followed for Fe²⁺ staining, except that potassium ferrocyanide was replaced with 10% potassium ferricyanide.

RESULTS

Generation of marked ES cells

To follow the fate of injected ES cells in chimeras, we used as a marker the ROSA26 gene-trap retroviral integration, which includes a lacZ reporter sequence expressed ubiquitously throughout embryogenesis, as can be monitored by X-gal (blue) staining of tissues (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). Using embryos carrying the ROSA26 transgene (hereafter referred to as R), we verified, by staining of sections and whole mounts, that the marker is expressed in both the embryonic and extraembryonic regions of the mouse conceptus (see below). We then intercrossed mice that were heterozygous for the Hdh mutation and carried the transgenic reporter [Hdh(+/-)/R], and derived Hdh(+/-)/R, Hdh(+/-)/R and Hdh(-/-)/R ES cells from isolated blastocysts (see Materials and Methods). The genotypes of these cells were verified by Southern and western analyses (Fig. 1).

Rescue of Hdh null embryos

Previously, we reported that Hdh(-/-) nullizygous embryos examined at E6.5 do not differ significantly in morphology from normal (wild-type or heterozygous) siblings, but they become retarded and disorganized in comparison with the controls at E7.5, although they do gastrulate (Zeitlin et al., 1995). In the extraembryonic region of the conceptus, the visceral and parietal endoderm are abnormal, whereas in the distal portion of the epiblast there is a high incidence of apoptotic cell death. Subsequently, there is little further development and the mutant embryos die between E8.5 and E10.5.

Because the entire conceptus was affected, we could not discriminate whether the absence of huntingtin function is detrimental for the cell lineages of both the primitive endoderm and embryonic ectoderm or if a defect in only one of these tissues affects the other indirectly. Thus, the abnormality of the epiblast might be the indirect consequence of impairment of endodermal nutritive function. Alternatively, inability of the dying embryonic ectoderm to elaborate mesoderm properly might result incidentally in abnormalities of the visceral yolk sac.

To investigate these possibilities, we generated chimeras by injecting Hdh(-/-)/R ES cells into wild-type host blastocysts, which were then transferred into pseudopregnant females. Using this approach, we expected that the mutant cells would preferentially colonize the epiblast, but would contribute infrequently to the primitive endoderm lineage. Therefore, if huntingtin is not directly involved in the development of the epiblast and its derivatives, the Hdh mutation should be inconsequential for this lineage in the chimeras possessing a mostly wild-type visceral endoderm, and the embryonic lethality should be bypassed. If, on the other hand, huntingtin has an indispensable function in the cell lineage of the embryonic ectoderm, the embryonic region of the chimeric conceptus should be abnormal and morphologically similar to that of nullizygous embryos obtained from matings between Hdh(+/-) heterozygous mice.

If the colonization of injected mutant ES cells is poor, rescue from lethality will occur, and this result will not be informative. Therefore, we classified the embryos that were obtained according to the degree of their chimerism: weak, moderate and strong chimeras (see Table 1), and selected for further analysis only specimens from the latter two classes.

Chimeras were first examined in sagittal and transverse histological sections of decidua dissected from the uteri of pregnant females at E6.5. X-gal staining showed that the Hdh(-/-)/R ES cells had extensively colonized the embryonic ectoderm, whereas the visceral endoderm, the parietal endoderm and the trophoblast giant cells were of host origin (Fig. 2B,D). As expected, the extensively chimeric embryos were identical morphologically to wild-type embryos of the same age obtained from natural matings (Fig. 2B,D; compare with A and C).

At E7.5, extensively chimeric embryos had gastrulated and X-gal staining was detected in the embryonic ectoderm and mesoderm (Fig. 2F). In contrast to Hdh(-/-) embryos of the same age, which are smaller than their control siblings, the chimeric embryos were comparable in size to wild-type littermates not colonized by ES cells.

Importantly, chimeras dissected at E8.5 were also indistinguishable from wild-type controls at the gross morphological level, and dramatically different from Hdh nullizygotes, in which the mutant egg cylinder was
significantly underdeveloped and the extraembryonic portion of the embryo exhibited many blebs and invaginations. X-gal stained sections of E8.5 chimeras demonstrated that the embryos exhibited all the features of normal controls at this stage of development, including headfolds and somites (Fig. 2G). Consistent with previous observations, the Hdh(-/-)/R cells did not colonize the visceral endoderm layer of the yolk sac, whereas ES cell descendants (stained blue) were detected in all derivatives of the epiblast (Fig. 2G).

To determine whether the chimeras had a potential for further development, we examined X-gal stained whole-mounts at E9.5 and E12.5. Again, the extensively chimeric embryos were identical morphologically to wild-type controls (Fig. 2H-J). Sections through E12.5 stained embryos (not shown) revealed that the null ES cells had colonized a high percentage of all cell types in the embryo, including those in the developing brain.

As summarized in Table 1, the majority of the generated chimeras were analyzed before E12.5. However, when a number of them were examined at more advanced developmental ages, they were again indistinguishable in gross morphology from normal embryos. We note that, when some pregnancies were allowed to come to term, extensively chimeric progeny were not observed, although several partial chimeras were obtained and studied (to be presented elsewhere).

**Indispensable role of huntingtin in extraembryonic tissues**

Although the results described above do not rule out the possibility that a small fraction of wild-type host cells persisting in the ectoderm lineage were able to sustain the development of Hdh null embryos (see Discussion), a more likely interpretation is that the mutant epiblast and its derivatives were rescued by the normal extraembryonic tissues of the chimeras.

To test directly whether huntingtin plays a crucial role in the extraembryonic tissues of the mouse conceptus, we also performed a converse chimeric analysis, reasoning that, if our hypothesis were correct, embryos derived from wild-type ES cells should be compromised in an Hdh null host environment.
Thus, we injected Hdh(+/+)yR ES cells into blastocysts obtained from intercrosses between Hdh(+/-) heterozygous mice. In this experimental design, not only Hdh(-/-) blastocysts, but also blastocysts with Hdh(+/-) and Hdh(+/+) genotypes were injected, to provide controls. The genotypes of all host blastocysts were determined from isolated parietal yolk sacs, after dissection of chimeric embryos that were obtained in an approximately mendelian ratio (Table 2).

We observed that the chimeric embryos generated by injection of Hdh(+/-) and Hdh(+/+) blastocysts were phenotypically normal at E8.5, as expected. In contrast, the embryonic portion of the chimeras with an Hdh(-/-) extraembryonic component resembled that of nullizygous mutants from natural matings, despite derivation of the embryos from wild-type ES cells. These embryos were underdeveloped and disorganized, although gastrulation had occurred and an allantois was present (Fig. 3A,C-E). The extraembryonic portion was also abnormal and exhibited numerous blebs and invaginations of the yolk sac, as expected. Overall, extensive X-gal staining was observed in the embryonic ectoderm and mesoderm. Some regions of the definitive endoderm were stained, and extensive patches of blue cells, located in the extraembryonic mesoderm of the yolk sac, were also evident. In contrast, sections of stained whole-mounts revealed that the regions of visceral and parietal endoderm were devoid of marked cells (Fig. 3F,G). Analogous observations were made with E9.5 chimeras derived from injections of Hdh(-/-) blastocysts, which exhibited features similar to those of nullizygous embryos of the same age. Thus, the extraembryonic membranes had somewhat increased in size, but the embryonic portion of the chimeric conceptus was almost completely resorbed (Fig. 3B).

**Ubiquitous expression of the Hdh gene in early mouse embryos**

Our results with chimeras derived from marked wild-type ES cells and Hdh null blastocysts indicated that embryonic lethality is the indirect consequence of elimination of huntingtin function in extraembryonic tissues, (see Discussion). However, we were previously unable to detect Hdh gene expression in the visceral or parietal endoderm of wild-type embryos by whole-mount in situ hybridization analysis (Zeitlin et al., 1995). Thus, to correlate the observed phenotypes of chimeras with the expression pattern of Hdh, we now performed in situ hybridization on fresh frozen sections of wild-type and nullizygous embryos at E7.0-E8.5 using a protocol of higher sensitivity (see Materials and Methods). The results of these analyses showed that Hdh is expressed relatively uniformly in all embryonic and extraembryonic tissues (Fig. 4B,D-F). As expected, no hybridization with an Hdh probe was detected in sections of control nullizygous embryos (Fig. 4A,C).

**Expression of marker genes in extraembryonic tissues of Hdh nullizygous embryos**

To investigate further the defect in the extraembryonic tissues of Hdh null mutants, we assayed for the expression of marker genes using RT-PCR (Fig. 5). For these analyses, we selected a panel of genes expressed in the visceral endoderm and the trophoblast. The RNA used in the assays was prepared from E8.5 embryos or extraembryonic tissues. To ascertain the quality of the RNA substrates, amplification with β-actin primers was performed in parallel as a positive control. In addition, we assayed for Hdh expression in the RNA preparations from the mutants to exclude contamination with maternal tissue. Finally, to exclude the presence of

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**Fig. 3.** X-gal staining of sections and whole mounts of chimeras generated by injecting Hdh(+/+)yR ES cells (clone M) into host blastocysts obtained from intercross between Hdh(+/-) heterozygotes. (A) E8.5 chimeric littermates. The embryos on the left and right were derived from Hdh(-/-) and Hdh(+/+) host blastocysts, respectively. (B) E9.5 littermates derived from Hdh(-/-), Hdh(+/-), and Hdh(+/-) blastocysts, from left to right respectively. The chimera indicated by an arrowhead is also shown in higher magnification (inset). In this specimen, retaining its extraembryonic membranes, the embryonic portion of the conceptus was almost completely resorbed. (C-E) Examples of E8.5 chimeras derived from Hdh(-/-) host blastocysts. The allantois (al) and parietal endoderm (pe) are indicated. (F,G) Sagittal and transverse sections of the embryos in C and D, respectively photographed by interference contrast (Nomarski) microscopy. The embryonic ectoderm (ee), embryonic mesoderm (me), definitive endoderm (de), and extraembryonic mesoderm (exm) stained blue are indicated. The parietal endoderm (pe) and visceral endoderm (ve), which are derived from the host, remain unstained. The bars correspond to 50 μm.
contaminating genomic DNA in the RNA preparations, amplifications with each pair of primers were performed in the absence of reverse transcriptase.

To determine whether Hdh expression is required for early visceral endoderm differentiation, we first assayed for the presence of transcripts of the genes Gata-4 (Soudais et al., 1995; Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997) and Hnf-4 (Duncan et al., 1994; Taraviras et al., 1994; Chen et al., 1994). The Gata-4 and Hnf-4 genes play essential roles in the differentiation of endodermal cells, as they encode transcription factors regulating the local expression of other

**Table 2. Generation of chimeras by injection of Hdh(++)/R ES cells into blastocysts**

<table>
<thead>
<tr>
<th>Genotype of host blastocyst</th>
<th>Embryo phenotype (E8.5 and E9.5)</th>
<th>No. of embryos*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hdh(+/+)</td>
<td>normal</td>
<td>7</td>
</tr>
<tr>
<td>Hdh(+/−)</td>
<td>normal</td>
<td>12</td>
</tr>
<tr>
<td>Hdh(−/−)</td>
<td>mutant</td>
<td>5</td>
</tr>
</tbody>
</table>

*The observed relative frequency of phenotypes approximates to the expected mendelian ratio of 1:2:1. The % colonization by the ES cells was >75% in all chimeras.
Ihh

Product sizes are as predicted, except for parallel. A subset of these markers (m) is included in each panel. Therefore, within the limitations of these assays, we conclude (Acampora et al., 1995), thought to be involved in patterning. 

predicted 225 bp). The anomalous migration of this product may be due to high GC content (74%).

fragments (MspHdhembryos, with the exception of Hdhnullizygous embryos, we observed that amplification products were obtained for both the Gata-4 and Hnf-4 differentiation markers (Fig. 5A). Consistent with the expression of the HNF-4 regulator, positive results were also obtained for Hnf-4 and Tfn (the gene encoding transferrin), both of which are late markers of fully differentiated visceral endoderm. Expression of another late marker, Ihh (Indian hedgehog; Becker et al., 1997), was also detected. Moreover, positive results were obtained for two homeobox-containing genes, Hesx-1 (Thomas and Beddington, 1996) and Otx-2 (Acampora et al., 1995), thought to be involved in patterning. Therefore, within the limitations of these assays, we conclude that the differentiation of the visceral endoderm per se does not appear to be perturbed in the absence of huntingtin. Interestingly, the results of additional RT-PCR assays showed that transcripts encoding the huntingtin-interacting proteins HAP-1 and HIP-1 (see Discussion) are present in early embryos, although differences between Hdh nullizygotes and wild-type controls were not observed.

The marker genes used to examine expression in the trophoblast cell lineage are shown in Fig. 5B. Of these, Err2, encoding a nuclear receptor (estrogen receptor related), is expressed in trophoblast progenitor cells between E6.5 and E7.5 (Pettersson et al., 1996). Mash2, encoding a transcription factor, is strongly expressed in cells of the trophodermal lineage after E7.5 (Guillen et al., 1994). Pem, encoding a homeodomain protein, is expressed in the extraembryonic portion of the visceral endoderm, in extraembryonic ectoderm and in trophoblast cells (Lin et al., 1994). Lgals1, encoding a galactose-binding lectin is expressed at high levels in giant trophoblast cells and in the ectoplacental cone (Poirier et al., 1992). Finally, Gjb3, encoding a gap junction protein (connexin 31), is expressed exclusively in the extraembryonic ectoderm and in the ectoplacental cone (Reuss et al., 1997). The results of RT-PCR analyses demonstrated that all of these markers are expressed in Hdh mutants.

Histochemical analysis of visceral endoderm transporting functions

Our results are consistent with the hypothesis that the early embryonic lethality of Hdh nullizygous mutants is due to the inability of the visceral endoderm to provide histotrophic nutrition (also see Discussion). Before the placenta becomes functional at around E10-E11 (see Kaufman 1992; Cross et al., 1994), to provide hemotrophic nutrition, development of the conceptus is sustained by locally available histotroph (extravasated maternal blood, endometrial breakdown remnants and secretions of uterine glands). Macromolecules in this nutritive mixture, for example proteins, which are filtered through the Reichert’s (basement) membrane secreted by the parietal endoderm into the visceral cavity, are endocytosed by the visceral endoderm, and, after their degradation in lysosomes, amino acids are transferred to the embryo (see Schlüter, 1980; Beck, 1981; Lloyd, 1990; Beckman et al., 1997). Whereas uptake of nutrients during E6.0 and E7.0 is served by both types of visceral endodermal cells, i.e. those covering the epiblast (squamous) and those present in the extraembryonic region (columnar), only the latter type perform this function at later times (Schlüter, 1980). This is not unexpected, as the primitive endoderm is progressively replaced by definitive endoderm derived from the epiblast, beginning at the distal region of the egg cylinder in embryos of the early primitive streak stage (see Tam and Beddington, 1992). Endodermal cells in this region of cultured embryos are unable to take up horseradish peroxidase (HRP) added to the medium at E7.5, in contrast to the rest of the endoderm (see Kadokawa et al., 1987). Whether this related to the incidence of programmed cell death occurring preferentially in the distal epiblast (Poellmann, 1980), is unknown. It is appealing, however, to speculate that the significant increase of apoptosis occurring in this region of Hdh null embryos (Zeitlin et al., 1995) is related to increased vulnerability of distal epiblastic cells which are located in the vicinity of a region with temporarily poor nutritive function, especially when nutrition is curtailed overall. Considering that serum proteins are expressed in Hdh mutants, at least as exemplified by the positive results for Tfn transcription (see above), we performed a preliminary analysis to investigate nutritive function by examining whether the visceral endoderm was defective in the transport of nutrients or ions.
First, we assayed for the expression of α-fetoprotein (AFP), a classic marker of endodermal cells. Although the exact biological roles of AFP are not fully established, it is thought that, among other potential functions, this protein serves as a carrier for transport of various substances, including steroids, fatty acids and heavy metals (for a review, see Chen et al., 1997). Transcription of the gene encoding AFP in visceral endoderm, as shown by in situ hybridization (Dziadek and Andrews, 1983; see also Downs et al., 1989), has indicated that the cognate protein, detected immunohistochemically (Dziadek and Adamson, 1978; Dziadek and Andrews, 1983), is synthesized locally. It was shown that at E6.5-E7.0, but not earlier, AFP was confined to the squamous visceral endodermal cells covering the epiblast. This localization changed at E7.5 and only endodermal cells at the midgirth of the embryo exhibited immunoreactivity. Columnar visceral endodermal cells in the extraembryonic region of the conceptus (yolk sac) became positive for AFP expression only after E8.5. It was also reported that from E7.5 onward, labeling was detected in the luminal surface of the epiblast, as well as in mesoderm, amnion and allantois. Therefore, we sought to examine by immunostaining, whether AFP, considered as a putative transporting protein, was reaching the epiblast in Hdh null embryos. We found, however, that the premise of this assay was not valid, as the published data (Dziadek and Adamson, 1978) were only partly confirmed. In both normal and mutant embryos, AFP immunoreactivity, confined exclusively to visceral endoderm, was observed at E7.5 and E8.5, whereas labeling of the epiblast, mesoderm, amnion and allantois was undetectable even in the controls (Fig. 4G-J). Nevertheless, these results strengthened our previous conclusion that the visceral endoderm of Hdh mutants is capable of synthesizing serum proteins.

Our AFP data and similar observations of other carrier proteins, like transferrin (Adamson, 1982) and apolipoprotein AI (Shi and Heath, 1984), which are synthesized in visceral endoderm, but are not detected in the epiblast, suggested that it would be difficult, if not impossible, to assess the integrity of transport processes in Hdh mutants by examining the status of transporters by immunohistochemistry. We sought, therefore, to address the same question by assaying for transported substances, and examined mutant and control embryos for the presence of iron, as an example. The ferric ion, which is a co-factor of many metalloenzymes and an essential component of erythrocytes in the developing blood islands of the yolk sac, is supplied to the embryo bound to maternal transferrin (see Young et al., 1997). This carrier protein is endocyted by visceral endodermal cells by a mechanism not mediated by the transferrin receptor, which is not expressed in this tissue (Drake and Head, 1990). After release of ferric ions, transferrin is apparently degraded in lysosomes instead of being recycled (Young et al., 1997). Transport of iron is presumably served by transferrin synthesized endogenously in visceral endoderm (Adamson, 1982), but the details of this process remain obscure. If this carrier reaches the embryo (the epiblast and also the embryonic and extraembryonic mesoderm possess transferrin receptors; Drake and Head, 1990), it may be subject to rapid turnover precluding detection. The results of our histochemical analysis showed that ferric ions were present in the extraembryonic visceral endoderm of both normal and Hdh mutant embryos at E7.5 (Fig. 4K,L). Therefore, at least qualitatively, the uptake of iron was not affected by the mutation, consistent with the unperturbed endocytosis of HRP that we reported previously (Zeitlin et al., 1995). However, the transport of iron (by whatever mechanism) appeared to be compromised in E8.5 mutants. In normal embryos of this age, ferric ions diffusely located in the cytoplasm were present in both the endodermal and mesodermal layers of the visceral yolk sac, including the blood islands, and also in scattered cells of the head mesenchyme (Fig. 4N,N'). In contrast, the iron present in yolk sac endoderm in the mutants was concentrated in small vesicles or granules, whereas staining in the mesodermal layer was below detection limits (Fig. 4O,O'). We note that the results of staining for ferrous ion were negative for both mutants and controls (data not shown).

**DISCUSSION**

We have shown that the function of huntingtin is indispensable for the physiological role of extraembryonic tissues during early mouse embryogenesis. Although we cannot exclude a role for huntingtin in the proper functioning of the trophoblast cell lineage, including the extraembryonic ectoderm, we think that the primary defect in Hdh null embryos, which becomes secondarily detrimental for the development of the epiblast, lies in the visceral endoderm. This we infer from the observation that, in contrast to the abnormalities of the visceral and parietal endoderm revealed by histological analysis of Hdh nullizygotes, the ectoplacental cone and the trophoblast giant cells are morphologically normal (Zeitlin et al., 1995). In this regard, it is interesting to compare the Hdh null phenotype with that of mutants lacking the Nf2 tumor suppressor gene. A defect in the trophoblast cell lineage of Nf2 nullizygotes results in failure to initiate gastrulation that leads to embryonic death between E7.5 and E8.5, although some of these mutants die shortly after implantation (McClatchey et al., 1997). In embryos lacking Nf2, the extraembryonic region is poorly developed and the extraembryonic ectoderm is disorganized (McClatchey et al., 1997). Like Hdh, the Nf2 gene is expressed ubiquitously throughout the conceptus, and the nullizygous Nf2 mutant phenotype can be rescued in chimeras derived from null ES cells injected into wild-type host blastocysts. We note that other mutants in which the trophectodermal cell lineage is affected exhibit a phenotype more severe than that manifested in the absence of Nf2 or Hdh expression and die before implantation. Such preimplantation lethality results, for example, from mutations of the vav proto-oncogene (Zmuidzinas et al., 1995), of Cdx2, which is one of three mouse homologues of the Drosophila homeotic gene caudal (Beck et al., 1995; Chawengsaksophak et al., 1997), and of the genes encoding the cell adhesion molecule E-cadherin (Larue et al., 1994; Riethmacher et al., 1995) and the cadherin-associated protein α-E-catenin (Torres et al., 1997).

Whether additional morphological abnormalities detected in the parietal endoderm of Hdh null embryos (Zeitlin et al., 1995) contribute to the lethal phenotype is unclear and resolution of this issue requires further analysis.

Assuming that the interpretation that we propose is correct, the Hdh null phenotype can be attributed to a failure of the visceral endoderm to fulfill its nutritive functions, although this tissue also appears to be involved in patterning through
reciprocal inductive interactions with the underlying epiblast (Thomas and Beddington, 1996) and potentially provides signals for formation of the proamniotic cavity (Coucouvanis and Martin, 1995). Because early embryos lack circulation and the epiblastic cells are anatomically isolated and unable to receive nutrients directly from the visceral cavity, nourishment is supplied by the visceral endoderm (see Lloyd, 1990). Apparently, this specialized physiological role of endodermal cells is strictly dependent on the function of huntingtin, which cannot be compensated in this tissue of the mutants. In contrast, the Hdh null epiblastic cells, which only receive and process nutrients, are not intrinsically handicapped. This view can also resolve the apparent paradox that, while huntingtin is expressed ubiquitously (see Results) and presumably has the same biochemical function in all tissues of the conceptus, the detected primary defect is tissue-specific, rather than universal, against expectation. The alternative view that both the endodermal and epiblastic cells are compromised in the absence of huntingtin is unlikely. For this model to hold, it is necessary to postulate that, when Hdh null ES cells are injected into normal blastocysts, a small number of wild-type ICM cells participating in the formation of the epiblast as a minor component are able to rescue the chimeric embryos from lethality. This possibility cannot be excluded formally from our data, but it is highly unlikely, as huntingtin is not a secreted protein that could be involved in rescue by paracrine action.

Observations with Hnf-4 null embryos (Chen et al., 1994) that bear a striking phenotypic similarity to Hdh nullizygotes are consistent with the view that the nutritive functions of the visceral endoderm are impaired in both cases, although the pathogenetic mechanisms differ. In early embryos, the transcription factor encoded by Hnf-4 is expressed exclusively in the visceral endoderm (Duncan et al., 1994; Chen et al., 1994), where it becomes involved in the expression of several transcription factor encoded by Hnf-4 pathway. In early embryos, the protein that could be involved in rescue by paracrine action. For example, lysosomal lesions, are not excluded. The alternative view that both the endodermal and epiblastic cells are compromised in the absence of huntingtin is unlikely. For this model to hold, it is necessary to postulate that, when Hdh null ES cells are injected into normal blastocysts, a small number of wild-type ICM cells participating in the formation of the epiblast as a minor component are able to rescue the chimeric embryos from lethality. This possibility cannot be excluded formally from our data, but it is highly unlikely, as huntingtin is not a secreted protein that could be involved in rescue by paracrine action.

In Hdh nullizygous mutants, the genes encoding Hnf-4, α-fetoprotein and transferrin are expressed (see Results). Therefore, transporters are available, but nutrients or co-factors are not supplied, as can be inferred from the phenotypic similarity with Hnf-4 nullizygotes and as demonstrated experimentally at least for the transport of iron (see Results). On this basis, we propose that in the absence of huntingtin one or more transporting processes are defective in visceral endodermal cells, although alternate pathophysiological causes, for example lysosomal lesions, are not excluded. Nevertheless, our model is consistent with the known subcellular localization of huntingtin in neural tissues and fibroblasts and presumably in all other cell types. When the available evidence from subcellular fractionations and electron microscopic immunohistochemical analyses is considered altogether (DiFiglia et al., 1995; Gutekunst et al., 1995; Sharp et al., 1995; DeRootj et al., 1996; Wood et al., 1996; Sapp et al., 1997), the emerging picture is that huntingtin is predominantly a cytoplasmic protein, which is membrane-bound and also present in the cytosol. It is possible, therefore, that this protein is in equilibrium between these two cellular compartments. In fractions enriched in vesicles, huntingtin immunoreactivity was found to overlap with the distribution of proteins present in vesicular membranes (synaptophysin, SV2 and transferrin receptor; DiFiglia et al., 1995). Interestingly, localization of huntingtin on vesicles associated with microtubules was detected (DiFiglia et al., 1995; Gutekunst et al., 1995). Huntingtin is not a transmembrane protein, however, as it can be dislodged from membranes by high-salt washes (Sharp et al., 1995). This suggests that huntingtin is bound directly or indirectly to the periphery of vesicular membranes by electrostatic forces.

Although the exact biochemical function of huntingtin continues to remain elusive, the available clues, including our data, are consistent with the proposal that this protein plays a role in microtubule-mediated trafficking of transport vesicles (DiFiglia et al., 1995). This view is strengthened further by considering that huntingtin has a HEAT domain also found in other vesicular proteins (Andrade and Bork, 1995). The relationships of the huntingtin-associated polypeptides HIP1 and HAP1 with other proteins are also informative. HIP1 (Kalchman et al., 1997; Wanker et al., 1997), which interacts with huntingtin and is also a protein loosely associated with membranes, has sequence homology (I/LWEQ module; McCann and Craig, 1997) with mammalian (talin) and yeast (Spa2p) actin-binding cytoskeletal polypeptides. Although the second huntingtin-interacting protein HAP1 (Li et al., 1995) has no known homologies, it is interesting that it interacts with Duo, a polypeptide containing domains involved in cytoskeletal protein-protein interactions (Colomer et al., 1997). Even more interesting in the context of this discussion is the information that HAP2 binds to p150Glued (Engelender et al., 1997), the largest subunit of the dynactin complex, which in turn binds and activates the motor protein dynein for vesicle transport along microtubules. Because both HIP1 and HAP1 are transcribed in early embryos as we have shown (see Results), it is appealing to speculate that huntingtin may be an important component of large protein complexes involved in intracellular transport pathways.

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