

# Expression and Function of the LIM Homeobox Containing Genes *Lhx3* and *Lhx4* in the Mouse Placenta

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The LIM homeobox containing genes of the LIM-3 group, *Lhx3* and *Lhx4*, are critical for normal development. Both genes are involved in the formation of the pituitary and the motoneuron system and loss of either gene causes perinatal lethality. Previous studies had shown that *Lhx3* is overexpressed in hyperplastic placentas of mouse interspecies hybrids. To determine the role of LHX3 in the mouse placenta, we performed expression and function analyses. Our results show that *Lhx3* exhibits specific spatial and temporal expression in the mouse placenta. However, deletion of *Lhx3* does not produce a placental phenotype. To test whether this is due to functional substitution by *Lhx4*, we performed a phenotype analysis of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> double-mutant placentas. A subset of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas exhibited abnormal structure of the labyrinth. However, absence of both LIM-3 genes did not interfere with placental transport nor consistently with expression of target genes such as *Gnrhr*. Thus, LHX3 and LHX4 appear to be dispensable for placental development and function. *Developmental Dynamics* 237:1517–1525, 2008.

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## INTRODUCTION

A functional placenta is an absolute prerequisite for development to term in all eutherian mammals, including man, and defects that severely compromise placental function will result in fetal lethality (Rossant and Cross, 2001; Cross et al., 2003). Significantly impaired placental transport function that does not jeopardize survival of

the developing fetus will still cause fetal undernutrition and intrauterine growth restriction (IUGR), which is again associated with a high incidence of perinatal lethality (Cross, 2006). However, even newborns that survive this critical perinatal period will suffer from late to very late effects of IUGR on brain development (Lane et al., 2001), energy metabolism (Sim-

mons et al., 2001; Tsirka et al., 2001), cardiac function (Gagnon, 2003; Leipala et al., 2003; Vonnahme et al., 2003; Xu et al., 2006), and other organ systems (Vonnahme et al., 2003). These findings stress the pivotal role of the placenta in normal fetal development. In recent years, numerous genes have been identified in the mouse that, when mutated, cause ab-

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normal placental phenotypes. This clearly demonstrates that these genes are important in normal placentation (Rossant and Cross, 2001; Hemberger and Cross, 2001; Cross et al., 2003). However, the functional roles of many other genes expressed in the placenta have not been revealed to date.

We had previously reported global mRNA expression profiles of murine hyperplastic placentas (Singh et al., 2004) generated by interspecific hybridization between different mouse species (interspecies hybrid placental dysplasia; IHPD) (Zechner et al., 1996, 2002), reproductive cloning (Tanaka et al., 2001), and mutation of the X-linked gene *Esx1* (Li and Behringer, 1998). In that study, we had identified a considerable number of genes that are differentially expressed between normal and hyperplastic placentas, thus forming a pool of potential placentation genes. Altered expression of some of these genes could reflect secondary changes due to disturbed gene expression cascades or shifts in tissue proportions. Indeed, deletion of some genes identified in our previous study (Singh et al., 2004) did not cause any placental phenotypes (Singh et al., 2005, 2006a). On the other hand, deletion of other such genes was associated with placental phenotypes, thus providing evidence for their functional roles in placental development (Singh et al., 2006b, 2007). Thus, analysis of more genes from this set of data is likely to increase our knowledge about gene function in mouse placentation.

A gene that was shown to be upregulated in hyperplastic IHPD placentas, *Lhx3*, encodes a LIM homeodomain transcription factor (Singh et al., 2004). Together with *Lhx4*, *Lhx3* forms the LIM-3 group within the LIM homeobox gene family, which is characterized by a conserved homeodomain that it is distinctive from that of other homeodomain-containing families (Hobert and Westphal, 2000). As shown by gene targeting in the mouse, the LIM-3 group genes are important in pituitary and motor neuron development (Sheng et al., 1996, 1997; Sharma et al., 1998; Mullen et al., 2007; Raetzman et al., 2002; Ellsworth et al., 2008). Interestingly, *Lhx3* and *Lhx4* have both redundant and complementary functions in these developmental processes (Sheng

et al., 1997; Sharma et al., 1998). Thus, formation of the definite Rathke's pouch is controlled in a redundant manner by both *Lhx3* and *Lhx4* as shown by the analysis of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> mouse embryos. However, the presence of one wild-type allele, such as in *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>-/-</sup> or *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>+/-</sup> is sufficient for formation of a definitive pouch (Sheng et al., 1997). For the next developmental step, formation of a proper pituitary structure and specification of pituitary lineages, *Lhx3* expression is an absolute requirement (Sheng et al., 1997). Thus, the presence of one *Lhx3* wild-type allele is sufficient for pituitary development, but *Lhx4* wild-type alleles are not able to substitute for *Lhx3* (Sheng et al., 1997). In a subsequent step, both *Lhx3* and *Lhx4* together control proliferation and differentiation of pituitary-specific cell lineages (Sheng et al., 1997). In contrast to this, during the differentiation of motor neurons *Lhx3* and *Lhx4* act in a plain redundant manner, that is, either *Lhx3* or *Lhx4* alone are competent to specify motor neuron identity (Sharma et al., 1998).

Up-regulation of *Lhx3* in abnormal placentation raised the possibility that this transcription factor could be important in placental development. To test this possibility, we performed *Lhx3* in situ hybridization to characterize the spatio-temporal expression pattern of this gene. We furthermore analyzed *Lhx3* placentas and, when these failed to exhibit any obvious phenotypes, *Lhx3*; *Lhx4* double mutant placentas. Unexpectedly, even though *Lhx3* and *Lhx4* are both expressed in the spongiotrophoblasts, double-homozygous mutant placentas exhibited a specific but not fully penetrant phenotype characterized by defective labyrinth structure. This finding suggests that other, to date unidentified, genes can substitute for both LIM-3 transcription factors in mouse placental development and function.

## RESULTS

### Spatio-Temporal Expression of *Lhx3* and *Lhx4* mRNA in the Placenta

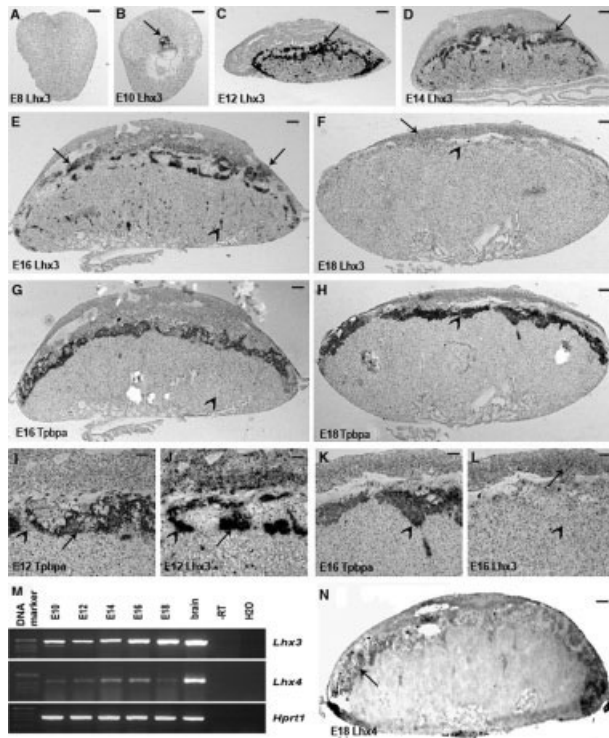
*Lhx3* expression was assayed on E8, E10, E12, E14, and E18 wild type placentas. No expression of *Lhx3* was de-

tected at E8 (Fig. 1A). At E10, the expression was in the ectoplacental cone and the developing spongiotrophoblast (Fig. 1B, arrow). From E12 onwards, the expression was predominantly in parts of the spongiotrophoblast (Fig. 1C, arrow) and in many small foci in the labyrinth (see Supplemental Fig. 1, which can be viewed at [www.interscience.wiley.com/jpages/1058-8388/suppmat](http://www.interscience.wiley.com/jpages/1058-8388/suppmat)). Use of several markers for placental cell types, such as *Tpbpa*, *Prl3d1*, *Prl3b1*, *Eomes*, and *Tfeb*, did not allow identification of the cell types present in these foci. Similar expression was seen at E14 and E16 (Fig. 1D, E, arrows). Between E12 and E16, only a limited overlap in the spatial expression patterns of *Tpbpa* and *Lhx3* could be observed, showing that not all spongiotrophoblasts expressed *Lhx3* (Fig. 1E-L). At E14 and E16, weak expression was seen in the glycogen cells situated in the decidua (arrows in Fig. 1E,F). *Lhx3* expression declined after E16 and extremely weak expression was detected in the spongiotrophoblast of E18 placentas (Fig. 1F). Decidua glycogen cells, however, expressed *Lhx3* at comparable levels in E16 and E18 placentas (arrows in Fig. 1F and E). Expression was not observed in giant cells and in the glycogen cells present in spongiotrophoblast or labyrinth.

*Lhx4* in situ hybridization was carried out only on E18 wild type placentas. *Lhx4* expression was detected specifically in the spongiotrophoblast region (Fig. 1N). Temporal expression pattern of both *Lhx3* and *Lhx4* was determined by RT-PCR. In contrast to the *Lhx3* in situ analysis, RT-PCR showed no major changes in expression levels for both genes between E10 and E10 of gestation. Expression of other LIM-homeobox genes, *Lhx1*, *Lhx2*, *Lhx4*, *Lhx5*, *Lhx6*, *Lhx8*, and *Lhx9*, was also assessed by RT-PCR only. By this method, transcripts from all genes were detected in the E18 mouse placenta (not shown).

### *Lhx3* Mutation Causes No Changes in Placental Weight and Morphology

From the *Lhx3* het × het matings, four litters with a total of 42 E18 conceptuses were obtained. These con-



**Fig. 1.** In situ expression analysis of *Lhx3* and *Lhx4*. **A:** E8 conceptus shows no expression of *Lhx3*. **B:** At E10, the expression is seen in the ectoplacental cone and the invasive trophoblasts (arrow), whereas the embryo is devoid of any expression. **C:** In E12 placentas, predominant expression is detected in many parts of spongiotrophoblast (arrow) and multiple foci in the labyrinth. Expression in the rare patches of the decidua is not clearly seen at this magnification. **D:** A similar expression pattern is seen in E14 placentas, but expression is reduced in spongiotrophoblast and increased in the decidua (arrow). **E:** At E16, decidual expression is even stronger (arrows), but further reduced in spongiotrophoblast and in the labyrinth (arrowhead). **F:** Finally at E18, weak expression is seen only in the decidua (arrows) with no expression in spongiotrophoblast (arrowhead), which earlier was a major site for *Lhx3* expression. Overviews of *Tpbpa* expression on sections adjacent to those shown in E and F show that *Lhx3* is expressed only in a subset of spongiotrophoblasts at E16 and confirms that it is not expressed in this tissue at E18. **I, J:** Higher magnification views of *Lhx3* and *Tpbpa* hybridizations on adjacent sections of an E12 placenta show that in some parts of the spongiotrophoblast expression is spatially separated, whereas in other nearby parts they overlap. Decidual expression of *Lhx3* in E12 placenta can be seen (J). **K, L:** At E18, *Lhx3* and *Tpbpa* are not co-expressed anymore. **M:** RT-PCR analysis of *Lhx3* and *Lhx4* expression during placental development. **N:** *Lhx4* expression in E18 placentas, arrows show positive signal (in spongiotrophoblast). Scale bars = 200  $\mu\text{m}$  (A, B), 300  $\mu\text{m}$  (C), 400  $\mu\text{m}$  (D), 500  $\mu\text{m}$  (E–H), 125  $\mu\text{m}$  (I, J), 100  $\mu\text{m}$  (K, L), 250  $\mu\text{m}$  (N).

sisted of 25 heterozygous (14 females and 11 males), 10 homozygous mutant (5 females and 5 males), and 7 wild type (5 females and 2 males) conceptuses. Weights of *Lhx3*<sup>+/+</sup>, *Lhx3*<sup>+/-</sup>, and *Lhx3*<sup>-/-</sup> placentas were  $88.7 \pm 10.0$ ,  $87.4 \pm 11.5$ , and  $89.2 \pm 13.6$  mg, respectively. Thus there was no consistent effect of the *Lhx3* mutation on the wet placental weight and this effect was independent of the fetal sex (not shown).

Isolectin B4-stained sections were studied for changes in placental morphology. Light microscopy did not reveal any significant differences. Furthermore, 2 isolectin B4-stained

sections from 2 placentas of each genotype and sex combination, altogether 24 sections, were analyzed by semi-quantitative morphometry for subtle differences in the section-areas occupied by decidua, spongiotrophoblast and labyrinth. As with placental weight, no significant difference was found between different genotypes (not shown). Furthermore, in situ hybridization revealed that the spatial expression patterns of the placental cell type-specific markers *Tpbpa*, *Prl3d1*, *Prl3b1*, *Eomes*, and *Tfeb* were not altered in *Lhx3* mutant placentas (not shown). Together these results indicated that loss of LHX3 does not

cause any obvious placental phenotypes.

### Deletion of Both *Lhx3* and *Lhx4* Causes an Inconsistent Labyrinthine Phenotype

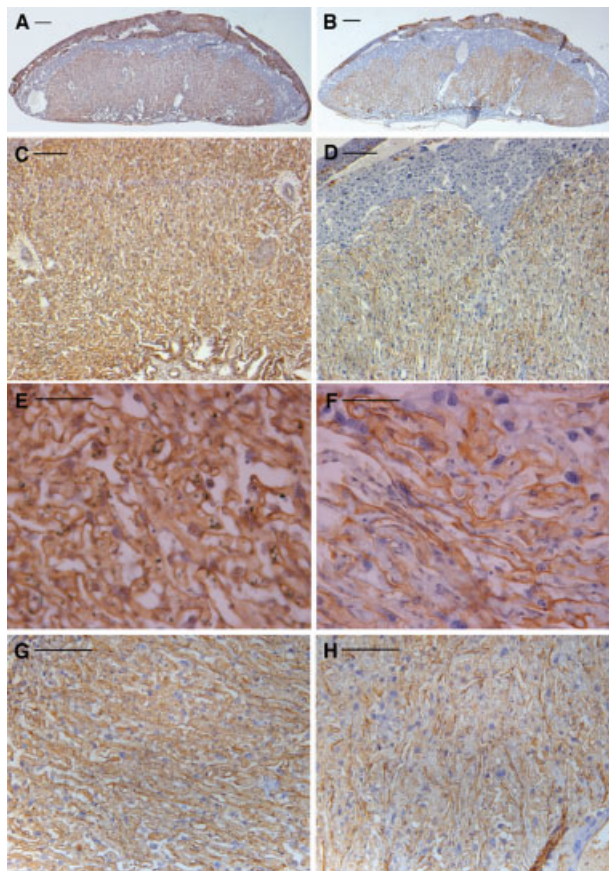
As it is known that in pituitary and motoneuron development, LHX3 and LHX4 may substitute for each other, the possibility existed that expression of *Lhx4* could compensate for loss of LHX3. This possibility was supported by the similar expression of *Lhx3* and *Lhx4* in the mouse placenta. However, as assessed by RT-PCR, *Lhx4* transcript levels did not vary between *Lhx3*<sup>+/+</sup> and *Lhx3*<sup>-/-</sup> placentas (not shown). Thus, *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup> double het  $\times$  het matings were performed. A total of 17 E18 litters with 176 conceptuses were obtained and among these 7 *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> conceptuses were identified (4%). No significant differences in placental weight were observed between the different genotypes (Table 1). Furthermore, histological analysis of placentas showed no discernible difference between any of the genotypes, with the exception of some *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas (see below). This suggests that the presence of a single LIM-3 allele, as in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>+/-</sup> or *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>-/-</sup>, is fully compatible with normal placental development. However, in three E18 litters (litter 1, 3, and 5; Suppl. Table 2), double mutant fetuses were smaller as compared to other fetuses in the same litter (Table 1). Comparative histological analysis of isolectin B4 stained sections from 5 *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas from the same litters showed that 3 double-mutant placentas exhibited a specific abnormal phenotype, which did not correlate with reduced fetal size (Suppl. Table 2). This phenotype was characterized by loose organization of labyrinthine architecture, with reduced density of isolectin B4-positive trophoblast basement membranes (Fig. 2A–F). This finding was supported by ASMA staining, which revealed a similar disorganization of pericytes in the 3 abnormal *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas (Fig. 2G,H). Peri-



**TABLE 1. Placental and Fetal Weights of *Lhx3*<sup>-/-</sup>, *Lhx4*<sup>-/-</sup> Double Mutants and Other Genotypes<sup>a</sup>**

Litter number and litter size (N)	Average placental weight (mg)	<i>Lhx3</i> <sup>-/-</sup> ; <i>Lhx4</i> <sup>-/-</sup> placental weight (mg)	Average fetal weight (mg)	<i>Lhx3</i> <sup>-/-</sup> ; <i>Lhx4</i> <sup>-/-</sup> fetal weight (mg)
1 (N = 9)	83.9 ± 9.0	89.5	1251.0 ± 67.9	1006.4
2 (N = 10)	93.7 ± 10.1	88.1	924.6 ± 52.2	886.4
3 (N = 10)	82.6 ± 9.3	76.4	930.7 ± 36.7	754.7
4 (N = 6)	124.8 ± 11.7	129.5	882.4 ± 105.5	903.8
5 (N = 10)	100.7 ± 15.3	88.1	947.8 ± 70.8	842.3
6 (N = 11)	87.4 ± 6.8	90.0	876.7 ± 61.7	813.3
7 (N = 10)	111.1 ± 17.6	90.0	743.3 ± 49.2	770.0

<sup>a</sup>Values are given as means ± SD. Average placental and fetal weights do not include the *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> weights.



**Fig. 2.** Morphological analysis of a *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas. **A,C,E,G:** Control placentas. **B,D,F,H:** *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas. **A,B:** Overview of isolectin B4 staining; **C-F:** isolectin B4 staining in labyrinth; **G,H:** ASMA staining. Arrows show positive staining. Scale bar = 1 mm.

cytes function in capillary looping in the labyrinth (Ohlsson et al., 1999). However, in the remaining 2 double-mutant placentas, no difference could be detected compared to control placentas. Semi-quantitative morphometrical analysis, which was performed on three double mutant and six control placental sections stained with isolectin B4, showed no signifi-

cant alterations in placental tissue distribution.

#### Normalization of *Lhx3* and *Lhx4* Expression Levels Does Not Rescue the Placental Phenotypes of AT24 Mice

Like the hyperplastic IHPD placentas, AT24 placentas over-express *Lhx3*

(not shown). In order to establish whether over-expression of LIM-3 group genes is a cause or a consequence of placental over growth in IHPD, we tested the effect of reduction of LHX3 and LHX4 levels in AT24 placentas. From 2 E18 AT24/AT24 × *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup> pregnancies, 18 conceptuses were isolated. Four conceptuses were AT24/+; *Lhx3*<sup>+/+</sup>; *Lhx4*<sup>+/+</sup> and 3 were AT24/+; *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup>. The remaining conceptuses were either *Lhx3*<sup>+/-</sup> or *Lhx4*<sup>+/-</sup>. Mean placental weights were 165.8 ± 10.1 mg for AT24/+; *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup> and 162.5 ± 21.0 mg for AT24/+; *Lhx3*<sup>+/+</sup>; *Lhx4*<sup>+/+</sup>. Thus, reduction of LIM-3 gene expression by half has no influence on the AT24 placental phenotype.

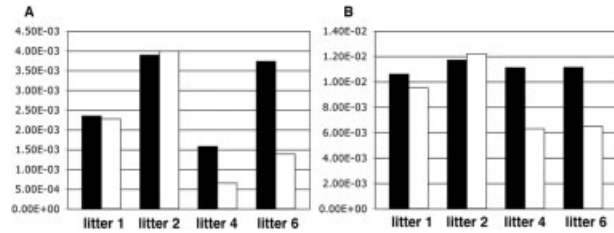
#### Placental Transport Function Analysis in *Lhx3* and *Lhx4* Double Mutant Mice

The abnormal morphology of the labyrinth in a sub-set of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas raised the possibility that placental transport functions could be compromised in these *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas. Therefore, one parameter of placental transport was assessed by determining the accumulation of the essential fatty acids (EFAs) linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6), and the non-essential fatty acids (NEFAs) palmytic acid (16:0) and stearic acid (18:0). EFAs are obtained through the mother's diet and are, therefore, transported to the fetal organism via the placenta. NEFAs are synthesized in the fetus. With this ap-

proach, compromised placental transport function is indicated by distorted ratios of EFAs and NEFAs in favor of the latter (Wu et al., 2003). Our lipid measurements provided no evidence for defective lipid transport function of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas. Thus, in control E18 fetuses the EFAs made up  $22.0 \pm 9.4\%$  (N = 6) of all FAs. In *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> fetuses this value was  $21.2 \pm 4.4$  (N = 2;  $P > 0.05$ ). In the placentas from the same conceptuses, these values were  $23.4 \pm 4.9$  and  $20.6 \pm 11.3\%$ , for control and *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas, respectively ( $P > 0.05$ ). However, this finding does not exclude the possibility that other transport systems are affected in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas.

### Expression of Lhx3/Lhx4 Target Genes in Lhx3 and Lhx4 Double Mutant Mice

One of the known target genes of *Lhx3* that is expressed in the placenta is gonadotropin-releasing hormone receptor (*Gnrhr*) (McGillivray et al., 2005; Granger et al., 2006). To determine if absence of the LIM-3 transcription factors alters expression of genes involved in the gonadotrope pathway, levels of *Gnrhr* and gonadotropin-releasing hormone (*Gnrh*) were determined in 4 *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas and 10 control litter-mate placentas (Suppl. Table 2) by qRT-PCR. Furthermore, expression of *Dlx3* was assessed, as the product of this gene is involved in the regulation of the subunit gene of chorionic gonadotropin (Roberson et al., 2001) and its deletion causes placental failure, which is however associated with down-regulation of *Esx1* (Morasso et al., 1999), a gene shown to be relevant for labyrinthine morphogenesis (Li and Behringer, 1998). These experiments provided no indication for differential expression of *Dlx3* and *Gnrh* (not shown) in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas. However, for *Gnrhr* up-regulation was seen in the phenotypically normal *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas of 2 litters, whereas in 2 litters where the *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas were abnormal, *Gnrhr* expression was not modified by genotype (Fig. 3B; Suppl. Table 2). This finding provided some indication that *Gnrhr* could possibly



**Fig. 3.** *Gnrhr* expression level in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas. **A:** Relative ratio of *Gnrhr/Tubb3* in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas. **B:** Relative ratio of *Gnrhr/Gapdh* in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> and control placentas. Black column: gene expression level in *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas. White column: gene expression level in control placentas. For detailed information on mutant and control placentas, see Supplemental Table 2.

function in placental development. To test this possibility, isolectin B4-stained sections of E18 and E19 *Gnrhr*<sup>+/+</sup> and *Gnrhr*<sup>-/-</sup> littermate placentas were analyzed. At both developmental stages, 2 mutant and 2 wild-type placentas were compared. With this light-microscopy approach, no phenotypic differences could be observed between *Gnrhr*<sup>+/+</sup> and *Gnrhr*<sup>-/-</sup> placentas.

### DISCUSSION

We had previously identified a large number of genes, which exhibited changed expression in the following models: placental over growth (Singh et al., 2004), interspecific hybridization (Zechner et al., 1996), cloning by somatic cell nuclear transfer (Tanaka et al., 2001), and mutation of *Esx1* (Li and Behringer, 1998). For a considerable number of these genes, there were no previous reports that demonstrated or suggested that they had functions in placental development. Therefore, in the present study we initially focused on one gene, *Lhx3*, whose expression was increased in IHPD placentas, as assessed by microarray, Northern, and in situ hybridization analyses (Singh et al., 2004). Our study shows that *Lhx3* is expressed in the mouse placenta in a cell type-specific manner, in that only three cell types, spongiotrophoblasts, decidual glycogen cells, and some cells in the labyrinth zone, express the gene. In the spongiotrophoblast proper, which excludes the glycogen cells also present in the junctional zone, not all cells expressed *Lhx3*. Similarly, *Lhx3* expression in glycogen cells was strictly dependent on their location. The significance of the

differential *Lhx3* expression in these two cell types is presently not clear.

The function of LHX3 as transcription factor and the perinatal lethality of the *Lhx3* deletion suggested that absence of *Lhx3* might also result in abnormal placental development and the finding that deletion of *Lhx3* alone was not sufficient to cause any alterations in placental histology and morphology was unexpected. However, it had previously been shown that in some developmental processes, such as motoneuron formation (Sharma et al., 1998), *Lhx4* is fully competent to substitute for *Lhx3*, which suggested that the same could apply to placental development. This possibility was also supported by the finding that on E18 *Lhx4* is expressed in the spongiotrophoblast, that is, in the tissue layer where high *Lhx3* transcripts levels were detected between E12 and E16. While deletion of both LIM-3 factors resulted in a mild placental phenotype, this was not fully penetrant and in addition affected the labyrinth, whereas both *Lhx3* and *Lhx4* are expressed mainly in the junctional zone. The ectopic phenotype may possibly be explained by the fact that LHX3 and LHX4 are involved in transcriptional regulation of several hormones, such as glycoprotein  $\alpha$ -subunit, prolactin, and follicle stimulating hormone- $\beta$ , which do not act in an autocrine manner (McGillivray et al., 2005). It is difficult to explain the incomplete penetrance, as it seems to be established that no other LIM homeodomain containing transcription factors can substitute for LHX3 and LHX4 (Hobert and Westphal, 2000), which excludes functional redundancy. However, it is important to note that several other LIM-homeobox

genes are expressed in the placenta, including *Lhx5*, which belongs to the related LIN-11 group (Hobert and Westphal, 2000). A simple explanation could be that neither of the two strains has been bred to full homozygosity for a B6 background, so there is the possibility that genetic heterogeneity underlies the incomplete penetrance. Alternatively, it has been shown that co-expression of *Lhx3*, *Nr5a1* (steroidogenic factor 1), and the LIM-homeodomain containing transcription factor *Isl1* enhances expression of *Gnrhr* expression in mouse gonadotrope  $\beta$ T3-1 and L $\beta$ T2 cells (Granger et al., 2006). In non-gonadotrope COS cells, which normally do not express *Gnrhr*, co-expression of these expression factors is also competent to induce *Gnrhr* and interestingly *Nr5a1* and *Isl1* alone induce expression, albeit at lower levels than when *Lhx3* was cotransfected. Co-expression of either *Lhx5* or *Lhx9* did not increase *Gnrhr* activation above that produced by *Nr5a1* and *Isl1* (Granger et al., 2006). Performance of *Lhx4* was not tested in that study. Thus the possibility remains that in the mouse placenta deletion of *Lhx3* and *Lhx4* can be rescued to some degree by expression of *Nr5a1* and *Isl1*, or *Isl2*, which is also competent to induce *Gnrhr* (Granger et al., 2006). However, to the best of our knowledge it is not known if these genes are expressed in the mouse placenta. In addition, it is not clear if the transcription factors encoded by these genes are at all involved in transcriptional regulation of *Gnrhr* in the placenta (Cheng et al., 2000). While we have analyzed only some genes involved in the gonadotrope pathway, as these are known to be expressed in the mouse placenta (Khodr and Siler-Khodr, 1980; Tan and Rousseau, 1982; Miyake et al., 1982; Cheng et al., 2000), it is possible that the inconsistent placental phenotype of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> double-mutant conceptuses is in fact caused by compromised regulation of other target genes such as *Foxl2* (Ellsworth et al., 2006). However, the finding that up-regulation of *Gnrhr* coincides with phenotypical normality of *Lhx3*<sup>-/-</sup>; *Lhx4*<sup>-/-</sup> placentas may suggest that the placental phenotypes were indeed caused by altered GNRHR levels. Over-expression of *Lhx3/Lhx4* in Jcg3

trophoblast tumor cells or trophoblast stem cells in vitro or in transgenic mice in vivo could probably clarify the functions of *Lhx3* and *Lhx4* in placental development, as over-expression does not allow rescue through redundancy (Heintz, 2001).

This study was initiated because of our previous finding that *Lhx3* is up-regulated in enlarged IHPD placentas (Singh et al., 2004). The fact that reduced *Lhx3/Lhx4* expression has no discernible effect on the phenotype of AT24 placentas demonstrates that over-expression of *Lhx3* does not underlie placental overgrowth. However, as demonstrated previously (Singh et al., 2004) *Lhx3* expression in IHPD placentas is ectopic and does not reflect the expression in normal placentas, which shows that *Lhx3* over-expression was not just a consequence of increased spongiotrophoblast. Again, over-expression of *Lhx3* in trophoblasts in vitro or in vivo could possibly provide information of the LIM-3 transcription factors in normal and abnormal placental development.

## EXPERIMENTAL PROCEDURES

### Mice and Tissues

All experiments with mice were conducted according to the guidelines issued by Uppsala University. For isolation of wild-type placentas, C57BL/6 (B6)  $\times$  B6 matings were performed. Pregnant females were killed by cervical dislocation, with the day of vaginal plug being counted as day 1. *Lhx3* and *Lhx4* mutant mice (Sheng et al., 1997, 1996) were kindly given to us by Dr. Sally A. Camper, University of Michigan. Both strains were propagated in the original B6 strain background by mating heterozygous males with wild-type females. However, neither strain was systematically backcrossed to produce inbred B6 genetic background. Fetuses and placentas were weighed. Placentas were halved, and one half was frozen on dry ice for RNA extraction, while the other half was fixed in Serra's fixative overnight at 4–8°C and later processed for paraffin histology. Fetal tissue was frozen prior to DNA extraction for genotyping. To generate *Lhx3* and *Lhx4* dou-

ble mutant mice, het  $\times$  het matings were performed between female *Lhx3*<sup>+/-</sup> and male *Lhx4*<sup>+/-</sup> to produce double heterozygous mutant mice. Subsequently, females and males *Lhx3*<sup>+/-</sup> *Lhx4*<sup>+/-</sup> double heterozygous mice were mated to obtain *Lhx3/Lhx4* double mutants. Females of the AT24 congenic strain (Hemberger et al., 1999; Elliott et al., 2001), kindly provided by Dr. J. Forejt, Prague, were mated with *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup> double heterozygous males to generate heterozygous AT24/+; *Lhx3*<sup>+/-</sup>; *Lhx4*<sup>+/-</sup> conceptuses. AT24 contains a *M. spretus*-derived proximal X chromosome. AT24 mice exhibit a mild but consistent placental hyperplasia that mimics IHPD not only phenotypically (Hemberger et al., 1999), but also in terms of gene expression (Singh et al., 2005). To determine the role of *Gnrhr*<sup>-/-</sup> in placental development, *Gnrhr*<sup>+/-</sup> heterozygous mice were mated to generate *Gnrhr*<sup>+/+</sup> and *Gnrhr*<sup>-/-</sup> conceptuses for comparison (Pask et al., 2005). Placentas were dissected from the uteri of pregnant females on E18 and E19. The extraembryonic membranes were removed and fetal and placental weights were measured prior to fixation of the placentas in 4% paraformaldehyde.

Placental sections were phenotypically assessed independently by at least three investigators who were not aware of the genotypes of the conceptuses.

### Histology and Immunohistochemistry

Isolectin B4 (Vector Labs) and anti-human alpha smooth muscle actin (aSMA; Dako) staining and histology were performed as described before (Singh et al., 2007). Semiquantitative morphometry was performed on isolectin B4 stained sections as described by Salas et al. (2004) using Adobe PhotoshopCS. Values from two randomly selected sections each from two placentas of each genotype were averaged. Sections near the placental midline were used to exclude shifting of placental compartments in lateral sections. The area occupied by the chorionic plate was excluded.



## mRNA In Situ Hybridization

Radioactive and non-radioactive in situ hybridizations were performed as described previously (Meunier et al., 2003; Anson-Cartwright et al., 2000). As placental cell type-specific markers, *Tpbpa* (Lescisin et al., 1988), *Prl3d1* (Guillemot et al., 1994), *Prl3b1* (Monkley et al., 1996), *Eomes* (Ciruna and Rossant, 1999; Russ et al., 2000), and *Tfeb* (Steingrimsson et al., 1998) were applied. A linearized *Lhx3* clone (accession number: AI893926), derived from the cDNA library described in a previous study (Singh et al. 2004), was used as in vitro transcription template. The *Lhx4* probe was generated by RT-PCR. The purified PCR product was cloned into pGEMT Easy vector (Promega). Digoxigenin (Roche) labeled sense and antisense riboprobes were synthesized by in vitro transcription using SP6 or T7 RNA polymerases. Primers used to generate the *Lhx4* in situ probe were: forward, 5'-GTCACCCTCCTTGCTCCTCTG-3'; reverse, 5'-GGTTCTGGTGAGAGG-GATGA-3'.

## DNA Extraction and Genotyping PCRs

Genomic DNA was extracted from fetal tissues by using the Promega Wizard genomic DNA extraction kit. To genotype *Lhx3* mutant mice, two different primer pairs were used in separate reactions. The wild-type allele was amplified using the primers 5'-GTG ACT GCC ATG CTG CTA GA-3' and 5'-GGA TCT CTG GAG TCC TGC TG-3', whereas the neomycin cassette inserted in the knockout allele was amplified using the primer pair 5'-CGG CAT CAG AGC AGA TTG TA-3' and 5'-GCT TCC TCT TGC AAA ACC AC-3'. Reaction conditions were 94°C for 3 min (94°C for 35 sec, 56°C for 30 sec, 72°C for 45 sec) × 35, 72°C for 7 min, for the wild-type allele and 94°C for 3 min (94°C for 30 sec, 59°C for 35 sec, 72°C for 45 sec) × 35, 72°C for 7 min for the mutant allele. To genotype *Lhx4* mutant mice, multiplex primers 5'-CTC GGA AGC TCA GCT CAC TGT CAC TAA CCC-3', 5'-ATC AGG ATG ATC TGG ACG AAG AGC ATC AGG-3' and 5'-CAG AAA ATC CGA CTG TGA CCA CAG CTT GAA-3' were used. Reaction condi-

tions were 94°C for 3 min (94°C for 35 sec, 61°C for 30 sec, 72°C for 45 sec) × 35 cycles, 72°C for 5 min. Fetal sex was determined by a Y chromosome-specific PCR using the primer pair 5'-CAT TTA TGG TGT GGT CCC GTG-3' and 5'-GTG TGC AGC TCT ACT CCA G-3'. Conditions of the reaction were 94°C for 3 min (94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec) × 35, 72°C for 7 min. All genotyping PCRs were performed in PTC-100 PCR machines (MJ Research, Watertown, MA). PCR products were resolved on 1.5% agarose gels. The genotype of *Gnrhr* mice was assessed as previously described (Pask et al., 2005). Exon 1 of the *Gnrhr* gene was amplified by PCR using the following primers: forward, 5'-CTC CAC TCT TGA AGC CTG TCC-3'; reverse, 5'-TCA CCA TGT TCA CAC AAA TTC-3'. Subsequently, the PCR product was digested with *DdeI* and resolved on 3% Nusieve agarose gel (Cambrex Bioproducts, Baltimore, MD).

## RNA Extraction, RT-PCR, and qRT-PCR

RNA was extracted using Trizol (Invitrogen). RQ1 DNase (Promega) was used to treat RNA, which was then reverse transcribed using M-MLV reverse transcriptase and random primers (Promega) as described earlier (Singh et al., 2004). QuantiTect SYBR green PCR mix (Qiagen) and RotorGene RG3000 (Corbett Research) was used for qRT-PCRs and each sample was analysed in duplicate. Melt curve was analysed to ensure specificity of the PCR. Delta-delta Ct method was used for analysis and the ratio of gene expression for each sample was calculated by normalizing the comparative quantitation values to those of *Gapdh* and *Tubb3*. Primer sequences used in qRT-PCRs are listed in Supplementary Table 1.

## Lipid Extraction and Analysis

Total lipid was extracted and purified from 8 fetuses and their placentas (for detailed information see Suppl. Table 2), using methanol/chloroform reagent according to a standardized protocol (Bligh and Dyer, 1959; Wuhrer et al., 2000). Lipids were treated with 500

ml of 1 M HCl and 10 M H<sub>2</sub>O in methanol for 16 hr at 100°C according to Gaver and Sweeley (1965). Fatty acids, released as their methyl esters, were recovered by a 3-fold phase partition using n-hexane. Fatty acid methyl esters were analyzed by gas chromatography/mass spectrometry using a VF 5ms capillary column (60 m, 0.25-mm inner diameter, 0.1-mm film thickness; Varian, Darmstadt Germany) and 2.5 ml/min helium as carrier gas. Temperature was maintained at 50°C for 2 min, raised to 130°C with 40°C/min, and to 300°C with 6°C/min, and was finally maintained at 300°C for 5 min. Fatty acid compounds were registered by mass spectrometry in the positive ion mode after chemical ionization with methanol using an PolarisQ instrument (ThermoQuest Analytical Systems).

## Statistical Analysis

Statistical analysis was carried out by t-test using GraphPad Prism 3.0 (San Diego, CA). A value of  $P < 0.05$  was considered significant. Data are presented as mean ± SD.

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