

Developmental expression of the homeobox protein Distal-less 3 and its relationship to progesterone production in mouse placenta

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Abstract

Distal-less 3 (Dlx3) is a homeobox factor that functions as a placental-specific transcriptional regulator. Dlx3 null mice (-/-) have compromised placental development and do not survive *in utero* past embryonic day (E) 9.5. The current studies were undertaken to examine the expression of Dlx3 in mouse placenta during gestation, and to determine whether Dlx3 was involved in placental progesterone production. Dlx3 was not detectable at E8.5 but was detected in E9.5 placenta with continuing but diminished expression through E15.5. Dlx3 immunolocalization was restricted to the labyrinth, was nuclear and was found in cytokeratin-positive cells. Previous studies in choriocarcinoma cell lines support the conclusion that Dlx3 is required for expression of 3 β -hydroxysteroid dehydrogenase VI (3 β HSD VI), an obligate enzyme in the production of progesterone by trophoblast giant cells. In a

rat trophoblast stem cell line (Rcho-1), Dlx3 expression was non-detectable in Rcho-1 cells induced to differentiate using mitogen withdrawal. *In vitro* progesterone production in placental cultures and 3 β HSD VI mRNA from Dlx3 (+/+), (+/-) and (-/-) mice were equivalent. *In situ* hybridization for 3 β HSD VI revealed mRNA expression restricted to trophoblast giant cells with no detectable expression in the labyrinth suggesting that Dlx3 and 3 β HSD VI were not colocalized within the placenta. These studies support the conclusion that Dlx3 protein expression is restricted to the labyrinth region of the murine placenta into late gestation and that Dlx3 does not appear to be expressed in trophoblast giant cells. Further, loss of Dlx3 was not correlated with synthesis of progesterone from E9.5 mouse placentas.

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Introduction

Distal-less 3 (Dlx3) is a member of a larger family of homeobox-containing transcriptional regulators known to have important roles in development. Dlx3 expression is restricted to keratinocytes within the epidermis, branchial arches, dental tissues and the placenta (Bendall & Abate-Shen 2000). Dlx3 expression in the bone has also been reported demonstrating a role in osteoblast differentiation (Hassan *et al.* 2004). The importance of Dlx3 as a transcriptional regulator in these tissues is demonstrated based upon characterization of the human tricho-dentosseus syndrome (TDO) (Price *et al.* 1998).

Interestingly, genetic disruption of the Dlx3 locus results in retarded placental development beginning on embryonic day (E) 9.5 leading ultimately to fetal death apparently due to abnormal placental/vascular morphogenesis (Morasso *et al.* 1999). The establishment of the mouse placenta requires a large number of transcriptional

regulators (reviewed by Cross 2000), including Dlx3, that reflects the complexity of establishment of the maternal-fetal interface. Recent studies from our laboratory demonstrate that Dlx3 is an important basal transcriptional regulator in cells of trophoblast origin. Regulation of Dlx3 promoter activity in human choriocarcinoma cells requires a CCAAT box element within the Dlx3 promoter that binds to CCAAT box/enhancer binding protein β (C/EBP β) (Holland *et al.* 2004). The relationship between regulation of the Dlx3/Dlx4 bi-gene cluster and the Dlx3 promoter by C/EBP β in cells of trophoblast origin requires further study. An important gene target of Dlx3 is the human glycoprotein hormone α subunit gene promoter expressed in trophoblast cells (Roberson *et al.* 2001). The α subunit is the common subunit of the heterodimeric glycoprotein hormones including chorionic gonadotropin (CG), a uniquely important luteotropin in early pregnancy in primates and horses (Liu *et al.* 1995, France *et al.* 1996). Dlx3 appears to be a component of an

array of cis regulatory elements that directs expression of the α subunit gene promoter in trophoblasts. In the human placenta, *Dlx3* expression is localized to syncytial- and cytotrophoblasts within the microvilli at a time when CG production is maximal. This observation supports speculation that *Dlx3* is crucial for human placental function during early pregnancy supporting the timed expression of CG (Roberson *et al.* 2001). *Dlx3* is also present in primary cultures of term human placental trophoblasts (Holland *et al.* 2004) suggesting that *Dlx3* expression may be maintained throughout gestation in the primate.

In addition to regulation of the glycoprotein hormone α subunit gene promoter, *Dlx3* has also been associated with regulation of the gene promoter for 3β -hydroxysteroid dehydrogenase type VI (3β HSD VI), an obligate enzyme in the production of progesterone in the murine placenta (Peng & Payne 2002). The 3β HSD VI enzyme catalyzes the conversion of pregnenolone to progesterone and is responsible for progesterone production in the mouse placenta (Peng *et al.* 2002). Transfection studies in human placental cells have identified two novel trophoblast-specific enhancer elements within the 3β HSD VI gene promoter which bind *Dlx3* and activator protein (AP)- 2γ , a combination of regulatory factors shared with the glycoprotein hormone α subunit promoter (Peng & Payne 2002). The conserved nature of these two transcriptional regulators supports the possibility that *Dlx3* and AP- 2γ are a critical placental-specific combinatorial 'code' necessary for expression in trophoblasts. Further, *Dlx3* may be necessary for normal progesterone production in the murine placenta, providing a possible rationale for fetal death with the *Dlx3* ($-/-$) mouse. The aim of the current studies was to characterize localization of expression of *Dlx3* in the mouse placenta during gestation. We then sought to determine whether *Dlx3* plays a role in placental progesterone production in *Dlx3* ($-/-$) mice.

Materials and Methods

Animal preparation

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee prior to implementation of any animal work. Wild-type mice (CF-1 female and males; Charles River, Wilmington, MA, USA) were mated and females were examined for vaginal plugs. For timed pregnancies, 1200 h was designated as 0.5 days *post coitum* (pc). Animals were euthanized by CO₂ asphyxiation. Mouse placentas were collected at days 8.5 through 15.5 of gestation. Individual fetoplacental units were dissected and placed into 4% paraformaldehyde for 24 h, then dehydrated in 70% ethanol and embedded into paraffin. Mice heterozygous for *Dlx3* were mated as described above to generate three genotypes: wild-type

(+/+), heterozygous (+/-) and null (-/-) animals. On day 9.5 pc female mice were euthanized by CO₂, and the uterus was dissected out and placed into ice-cold Dulbecco's phosphate buffered saline. Embryos were removed from individual fetoplacental units and placed in liquid nitrogen for subsequent genotyping. Genotyping was performed as described by Morasso *et al.* (1999) to identify placentas as (+/+), (+/-), or (-/-). Placentas were processed for paraffin embedding as described above.

Antibodies

Rabbit polyclonal *Dlx3* antibody was generated by the Cornell Polyclonal Antibody Service (Cornell University, Ithaca, NY, USA) against a 16-mer synthetic peptide containing amino acids 242–256 of the murine *Dlx3* protein. Anti-*Dlx3* was used at a concentration of 1:900 for immunocytochemistry and at 1:2500 for Western blot. The cytokeratin antibody (WSS; Dako, Carpinteria, CA, USA) was used at a concentration of 1:150. Anti-biotin (Vector Laboratories, Burlingame, CA, USA) was used at a concentration of 1:5000.

Western blot

Placentas from mice at different gestational ages were homogenized on ice in 500 μ l radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors as described (Holland *et al.* 2004) and protein concentration was determined using the Bradford assay. Samples were stored at -80°C until analysis. Ten micrograms protein extract were added to an equal volume of $2 \times$ SDS loading buffer (100 mM Tris, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol and 200 mM dithiothreitol (DTT)). Protein samples were then boiled for 5 min and chilled briefly on ice prior to loading on gels. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting. Membranes were blocked in 5% nonfat dried milk in Tris-buffered saline (10 mM Tris, pH 7.5, 150 mM sodium chloride) containing 0.1% Tween 20 (TBST). The *Dlx3* antibody was used at a concentration of 1:2500 in TBST, 5% nonfat dried milk. Proteins were visualized by chemiluminescence (Perkin Elmer Life Sciences, Boston, MA, USA). In some experiments, JEG3 choriocarcinoma cell nuclear extracts (NE) were used as a positive control. JEG3 cell NE were prepared as previously described (Holland *et al.* 2004).

Immunocytochemistry

On day 1, tissue sections were de-paraffinized through a series of three xylene changes followed by rehydration through a series of decreasing ethanols (100%–70%). Endogenous peroxidase activity was quenched by incubating sections in methanol containing 0.6% H₂O₂. Slides

were then rinsed with water for 30 min. Next, tissue sections were put through an antigen retrieval method using 0.01 M citrate buffer, pH 6.0, and a microwave. Slides were heated to boiling in the microwave in the 0.01 M citrate buffer for 15 min then allowed to cool to room temperature. Slides were then rinsed with 150 mM NaCl, 40 mM K₂HPO₄, 10 mM KH₂PO₄, pH 7.4 (KPBS), for 45 min and incubated in primary antibody at 4 °C overnight. The next morning, on day 2, the tissue sections were rinsed seven times over 45 min with KPBS then incubated for 1 h at room temperature in biotinylated donkey anti-rabbit IgG (Vector Laboratories) at a concentration of 1:1000 in KPBS with 0.4% Triton X-100. Rinsing with KPBS was then repeated, followed by incubation in avidin-biotin complex (ABC) solution (0.3% ABC in KPBS with 0.4% Triton-X; Elite ABC kit, Vector Laboratories) for 1 h at room temperature. The tissue sections were rinsed three times first with KPBS then with 0.01 M Tris, pH 7.2. The antibody-biotin-peroxidase complex was visualized with a solution containing 0.1 mg/ml 3,3 diaminobenzidine-HCl (DAB) and 0.0025% H₂O₂. The staining reaction was monitored intermittently under the microscope. When satisfactory staining was achieved, slides were immersed in 0.01 M Tris, pH 7.2, to stop the reaction. After rinsing with KPBS, tissue was counterstained with Fast Green or eosin to visualize placental morphology, dehydrated in graded alcohols, cleared in xylene and cover-slipped with Histomount (National Diagnostics, Atlanta, GA, USA). Groups of tissue across ages or genotypes were immunostained together in a single run to allow for qualitative comparisons.

Microscope details

Photomicrographs were taken using a SPOT slider RT digital camera mounted on a Nikon E400 using SPOT advance software v. 4.0.5 (Diagnostic Instruments, Sterling Heights, MI, USA). Micron bars are displayed on the figures to define magnification.

Rcho-1 model

The Rcho-1 trophoblast stem cell line was developed from a transplantable rat choriocarcinoma and can be manipulated to proliferate or differentiate along the trophoblastic giant cell pathway (Yamamoto *et al.* 1994). The Rcho-1 trophoblast stem cell line is routinely maintained in subconfluent conditions with NCTC-135 medium (Sigma-Aldrich) supplemented with 20% fetal bovine serum (FBS) to maintain its proliferative state. Differentiation of this cell line was induced by growing to confluence in the FBS-supplemented culture medium and then serum supplementation was replaced with 1% horse serum. Six-well plates were washed with ice-cold PBS and

scraped at 4 °C into Dignam A buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) containing a cocktail of protease inhibitors (Sigma-Aldrich). Swollen cell preparations were then vortexed vigorously to disrupt cells and crude nuclear preparations were obtained by centrifugation at 10 000 × *g* for 10 s. Crude nuclear preparations were resuspended at 4 °C in HEPES-buffered saline (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT) to extract nuclear proteins. Protein content of the NE was determined by the Bradford assay, aliquoted and stored at -80 °C. Western blot was performed as described above for Dlx3.

Northern blot analysis

Northern blot analysis was carried out on implantation site tissue from (+/+), (+/-) and (-/-) (*n*=3 per genotype) fetuses. Total RNA was isolated using Trizol reagent (Invitrogen) as described by the manufacturer. Twenty micrograms total cellular RNA were resolved in denaturing gels and Northern blot hybridization was carried out as described (Roberson *et al.* 1995). The 3βHSD isoform VI cDNA probe contained a 1057 base pair fragment corresponding to exon 4 of the mouse 3βHSD VI enzyme. This plasmid was a generous gift from Dr Anita Payne (Stanford University, Stanford, CA, USA). Hybridization was carried out for 18 h and blots were washed under high stringency. Bands were detected by autoradiography.

Non-radioactive in situ hybridization

Probe preparation To generate the antisense 3βHSD VI riboprobe, the vector described above was linearized with BglII and transcribed with T7 RNA polymerase yielding a 359 nucleotide fragment of the coding region. The *in vitro* transcription reaction contained Biotin-16-UTP (Roche Applied Science, Indianapolis, IN, USA), 1 μg BglII-linearized 3βHSD VI, 4 mM DTT, 20 U RNasin ribonuclease inhibitor (Promega), 40 U T7 RNA polymerase, 0.7 mM UTP, and 1 mM each of CTP, ATP and GTP. Following a 2-h incubation at 37 °C, the reaction was stopped by the addition of 1 μl 0.5 M EDTA, pH 8.0. For the sense probe, the 3βHSD VI plasmid was linearized with SacI and transcribed with T3 RNA polymerase to yield a 380 base pair probe of which 359 base pairs correspond to the 3' UTR.

Hybridization and detection On day 1, tissue sections of mouse placenta spanning E8.5 through E15.5 days were de-paraffinized, re-hydrated and endogenous peroxidase activity was quenched as described above. Sections were then incubated in proteinase K (25 μg/ml) for 5 min at room temperature. Sections were rinsed twice with 0.1 M triethanolamine (TEA, pH 8.0) and then incubated in

0.25% acetic anhydride in 0.1 M TEA, pH 8.0, for 10 min at room temperature. The placental sections were subsequently rinsed in $2 \times$ SSC (0.3 M NaCl, 0.33 M Na citrate) for 10 min at room temperature. Tissue sections underwent a prehybridization step for 2 h at 37 °C during which sections were incubated in hybridization buffer (50% deionized formamide, 10% dextran sulfate, $1 \times$ Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% acetylated BSA), 0.3 M NaCl, 8 mM Tris, pH 8.0, 0.8 mM EDTA, pH 8.0, 15% RNase free H₂O) containing denatured torula yeast RNA (0.1 mg/ml; Ambion, Austin, TX, USA) to aid in decreasing non-specific binding. Following the prehybridization step, sections were rinsed in $2 \times$ SSC for 10 min. The biotinylated 3 β HSD VI riboprobe (600 ng/ml) and torula yeast RNA (0.025 mg/ml) were denatured at 100 °C for 5 min, put on ice for 5 min then added to hybridization buffer. Each slide received 300 μ l of the 3 β HSD VI riboprobe plus torula yeast RNA. The tissue was covered with a plastic coverslip and sections were incubated in humidified chambers at 37 °C overnight.

On day 2, placental sections were rinsed in $4 \times$ SSC for 40 min, followed by incubation in 10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 0.05 M EDTA, pH 8.0, and 20 μ g/ml DNase-free RNase A (Roche Applied Science) at 37 °C for 30 min. Tissue was rinsed and then incubated in 10 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.05 M EDTA, pH 8.0, for 30 min. After 40 min rinsing with $2 \times$ SSC, sections were incubated in $0.1 \times$ SSC for 1 h at 42 °C. Tissue sections were then rinsed for 40 min in KPBS. Tissue sections were incubated in anti-biotin overnight at 4 °C. The next morning, day 3, tissue sections were processed in the manner as described above for day 2 of immunocytochemistry.

Placental short-term culture

Mice heterozygous for *Dlx3* were time mated to generate implantation sites that were wild-type (+/+), heterozygous (+/-) and null (-/-) for *Dlx3*. Placentas were collected at E9.5 and embryos were collected for genotyping. Implantation sites were placed in 24-well plates containing DMEM with hydrocortisone (0.1 μ g/ml) and a cocktail of insulin, transferrin, sodium selenite (ITS; 1 μ g/ml; Sigma-Aldrich) at 37 °C. Media were changed and collected at 0, 1, 3 and 5 h after placement in culture. Data are reported as a cumulative value of progesterone in media at times 0, 1 h, 3 h (=progesterone concentration at 0 h + 1h + 3h) and 5 h (=progesterone concentration at 0 h + 1h + 3h + 5h). Progesterone was measured by radioimmunoassay as previously described (Fortune & Eppig 1979). It is important to note that beginning on E8.5 the sole intrauterine source of progesterone is the placenta; from E4.5 to E7.5 the decidua is the sole source of intrauterine progesterone.

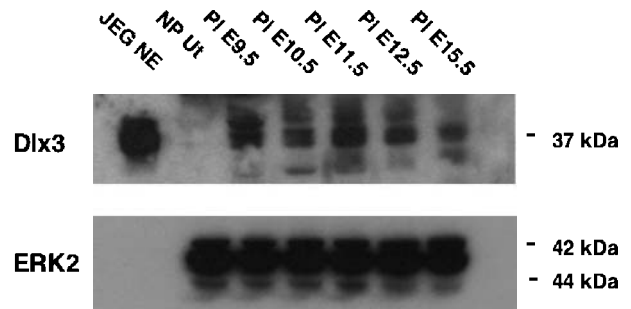


Figure 1 Western blot of *Dlx3* and ERK2 levels in mouse placentas at different times during gestation. *Dlx3* protein was initially detected at E9.5 in mouse placenta (PI) and continued to be present at E15.5. JEG cell nuclear extract (JEG NE) served as a positive control for the *Dlx3* antibody. Non-pregnant mouse uterus (NP Ut) served as a negative control and does not express *Dlx3*. The blot was stripped and re-probed with the antibody against ERK2 to control for lane load. Equal amounts of protein were electrophoresed in each lane of the gel. Molecular size standards are shown on the right of the figure.

Results

Dlx3 expression in mouse placenta

To begin characterizing *Dlx3* expression in mouse placenta, placentas ($n=3$ per time point) were collected throughout gestation and Western blot analyses were performed. *Dlx3* was not detectable in placenta from E8.5 (data not shown) nor was it present in the uterus from non-pregnant mice (Fig. 1). *Dlx3* immunoreactivity appears as one primary band at 37 kDa together with less intense bands that may be associated with phosphorylated forms of *Dlx3* (Park *et al.* 2001). In sections of uterus dissected from between implantation sites, *Dlx3* was also non-detectable (data not shown). *Dlx3* was first detected in placenta from E9.5 and expression continued through E15.5 (Fig. 1). Nuclear extracts from JEG3 choriocarcinoma cells were used in these experiments as a positive control for *Dlx3* immunoreactivity. The blots were stripped and re-probed with an antibody directed towards ERK2 (a mitogen-activated protein kinase) to verify lane load (ERK2 was absent from JEG3 cell nuclear extracts since ERK2 is not appreciably nuclear localized in the absence of cell stimulation by a mitogen).

Immunocytochemistry was performed to determine the spatial and temporal specificity of *Dlx3* expression in mouse placenta. Antibody specificity was initially verified using mouse placenta from midgestation (E10.5). The staining pattern observed in Fig. 2A shows that *Dlx3* expression was restricted to the labyrinthine layer of the placenta and the staining was nuclear (inset panel). When normal rabbit serum (NRS) was used in place of the antibody to *Dlx3*, no staining was observed (Fig. 2B). *Dlx3* localization in mouse placenta during gestation is

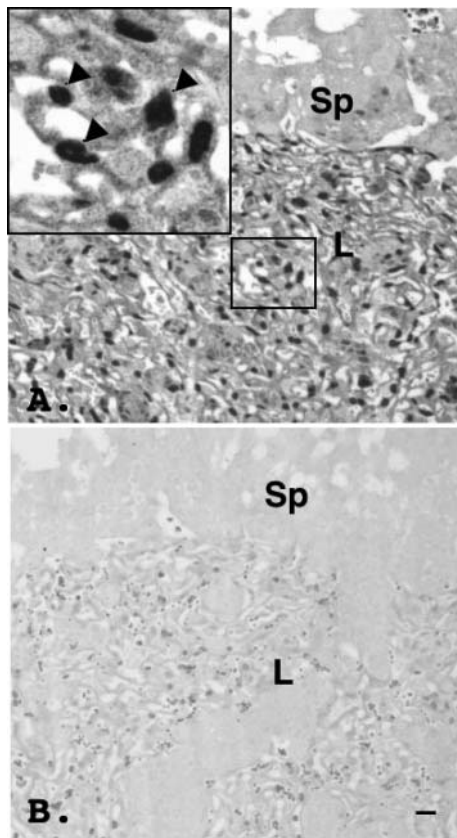


Figure 2 Specificity of the Dlx3 antibody in mouse placenta. (A) Dlx3 staining in the labyrinth (L) of mouse placenta on E10.5; Dlx3 was nuclear localized and abundant in the labyrinth while immunoreactivity was low/absent in the junctional zone of the spongiotrophoblast (Sp) region of the placenta; the boxed inset in panel A corresponds to the region magnified in the upper right portion of the figure. Bar=10 μ m. (B) Normal rabbit serum was used as a negative control on this E10.5 placental section. Bar=20 μ m, A and B are at the same magnification.

illustrated in Fig. 3. As seen with Western blot analysis, Dlx3 is not present at E8.5. Dlx3 was first observed at E9.5 in the labyrinth. As the labyrinth develops and becomes more folded, Dlx3 staining was observed throughout this compartment, notably on E10.5. Moreover, as the labyrinth compacts at later gestational ages (E15.5), Dlx3 staining continued to be confined to the labyrinth.

Based on the observation that Dlx3 was localized in the labyrinth, placentas were stained for cytokeratin to determine whether Dlx3-expressing cells were also of trophoblastic lineage. Cytokeratin immunostaining was observed throughout the labyrinth of the midgestation mouse placenta (Fig. 4A). High power micrographs of adjacent sections confirm that Dlx3 was expressed in the nuclei of cytokeratin-positive trophoblast cells in the labyrinth (Fig. 4B and 4C).

Analysis of the role of Dlx3 in placental progesterone production

To determine if Dlx3 was involved in progesterone production/secretion in rodent placental models, two parallel sets of studies were conducted. The Rcho-1 trophoblast stem cell line is a model of rat trophoblast giant cells, a critical cell type involved in progesterone production by the rodent placenta. The Rcho-1 model can be induced to differentiate along the trophoblastic giant cell pathway following growth to confluence and mitogen withdrawal. In the initial studies, nuclear extracts were obtained from proliferating and differentiated Rcho-1 cells and were subjected to Western blot analysis to determine relative Dlx3 expression levels. The results are shown in Fig. 5. Rcho-1 cells in the proliferative state express low levels of Dlx3 compared with JEG3 cell nuclear extracts which served as a positive control for Dlx3 immunoreactivity in this study. Dlx3 protein expression was beneath detectable limits of this assay in nuclear extracts from Rcho-1 cells induced to differentiate into trophoblast giant cells.

In the second study, short-term murine placental cultures were carried out to more closely investigate the possibility that Dlx3 was involved in placental progesterone production. Mice heterozygous for Dlx3 (+/-) were time mated. Placentas were collected on E9.5 for short-term culture and embryos were collected for genotyping. Placentas of different genotypes (determined *post priori*) were immediately placed in culture for 5 h with media collected at 0, 1, 3 and 5 h. Results of the short-term culture are depicted in Fig. 6. Placentas containing one or two copies of Dlx3 ((+/-), (+/+)) respectively) were combined into one group based upon similar progesterone secretion within the time frame measured. Placental progesterone concentrations were then compared between Dlx3 (-/-) and Dlx3 (+/+ and +/-) implantation sites. No differences in progesterone secretion patterns were observed in any of the three genotypes (Fig. 6). Northern blot analysis showed that relative steady state levels of 3 β HSD VI mRNA in placentas of (+/+), (+/-; data not shown) and (-/-) were similar (Fig. 7). *In situ* hybridization for 3 β HSD VI was performed on placentas with the three Dlx3 genotypes to determine cellular localization of 3 β HSD VI. These studies revealed similar mRNA expression of 3 β HSD VI in trophoblast giant cells of the junctional zone in the spongiotrophoblast region of the placenta (Fig. 7). Consistent with earlier studies (Figs 2 and 3), Dlx3 staining was restricted to the labyrinth while the 3 β HSD VI mRNA was predominantly in the junctional zone and notably absent from the labyrinth.

Discussion

The studies presented here examined Dlx3 expression across gestational ages, identifying the labyrinth as the

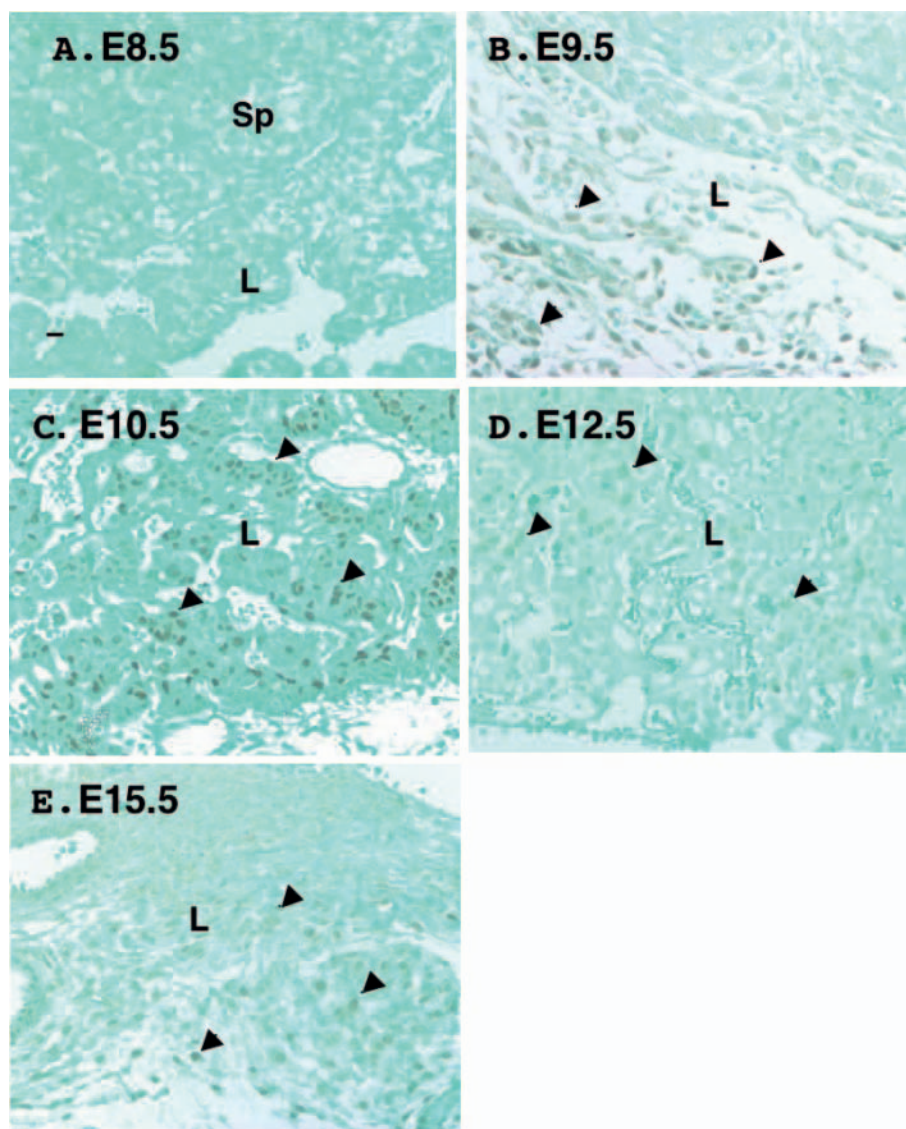


Figure 3 Immunocytochemical localization of Dlx3 in mouse placenta throughout gestation. (A) At E8.5 Dlx3 was not yet expressed in the labyrinth (L) or in the junctional zone of the spongiotrophoblast (Sp). Placentas at (B) E9.5, (C) E10.5, (D) E12.5 and (E) E15.5 expressed Dlx3 protein exclusively in the labyrinth. Bar=20 μ m.

primary region of localized expression beginning at E9.5 through E15.5. Co-localization of Dlx3 and cytokeratin within the labyrinth of the mouse placenta was evident at each stage of gestation examined (Fig. 4 and data not shown). Consistent with current studies, cytokeratin-positive trophoblast cells were present throughout the labyrinth as shown by others (Adamson *et al.* 2002), and in the present studies all cytokeratin-positive cells examined also expressed Dlx3. The pattern of Dlx3 expression was continuous rather than an intermittent pattern or a discrete window of expression. This is consistent with our previous studies in human tissues demonstrating

trophoblast-restricted expression of Dlx3 at 8 weeks gestation coincident with peak CG production, and in purified trophoblasts from normal term human placentas (Roberson *et al.* 2001, Holland *et al.* 2004). The protein expression of Dlx3 at E15.5 appeared to be diminished relative to earlier time points in gestation (Figs 1 and 3). While placental sections were all examined within the same immunocytochemical experimental replicate, we are cautious in our interpretation of relative changes in expression level based upon the caveat that this methodology lacks quantitative rigor. However, diminished expression of Dlx3 (per μ g total protein) in the mouse

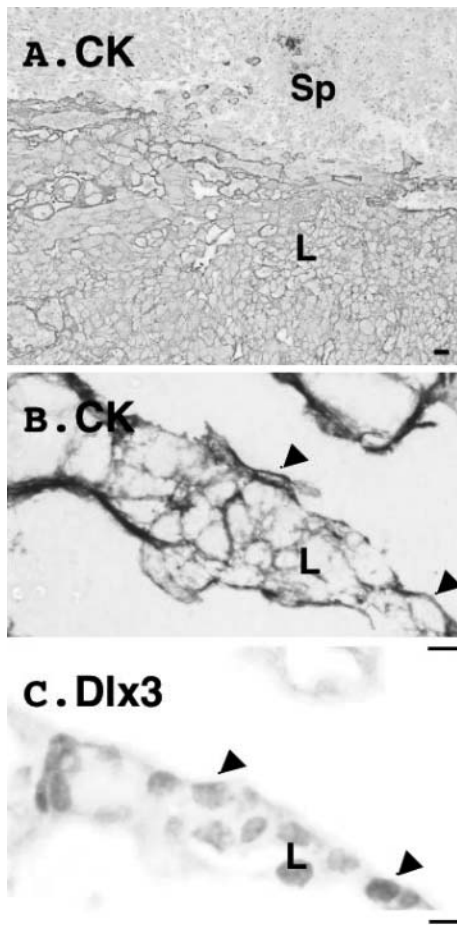


Figure 4 Immunocytochemical colocalization of Dlx3 with cytokeratin. (A) In wild-type mouse placenta, cytokeratin (CK) was present throughout the labyrinth (L). Expression of cytokeratin was reduced or absent in the junctional zone of the spongiotrophoblast (Sp) layer. Bar=20 μ m. (B) CK and (C) Dlx3 localized in the same area of the labyrinth (L) as depicted by the arrows. Bars=5 μ m.

placenta at these time points was evident in Western blot analysis, supporting this conclusion. Whether gestational age-dependent changes in Dlx3 expression is significant with respect to placental function is unknown at this time. The persistence of Dlx3 in the labyrinth during this time frame suggests that its role in placental development and differentiated placental function is ongoing.

Interestingly in Dlx3 ($-/-$) mice, placental expression of another homeodomain gene, Esx1, was down-regulated. The Esx1 gene is located on the X chromosome and has been shown to be expressed in the labyrinth (Li & Behringer 1998, Morasso *et al.* 1999). The direct effects of Dlx3 on Esx1 expression may be a rational prediction based upon the Dlx3 ($-/-$) mutant, since sequence analysis reveals that several consensus Dlx3 binding sites (central core TAAT) are present within the 5' flanking

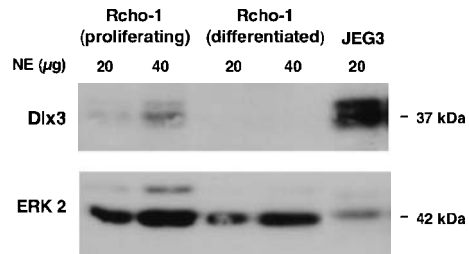


Figure 5 Dlx3 was present at low relative levels in proliferating Rcho-1 cells but was non-detectable in differentiated Rcho-1 cells. Varying amounts of Rcho-1 nuclear extracts (NE; 20 or 40 μ g) were subjected to Western blot analysis for Dlx3. The blot was stripped and reprobbed with an antibody against ERK2 for control for lane load. JEG3 cell NE (JEG3) served as a positive control for Dlx3 immunoreactivity. Molecular size standards are shown on the right of the figure.

region of the Esx1 gene (K A Berghorn and M S Robertson, unpublished observations). The first of these putative Dlx3 binding sites is located 583 nucleotides upstream of the putative transcription initiation site (Li *et al.* 1997). Esx1 mRNA is present in the labyrinth beginning at E9.5 and persists through E17.5 (Li & Behringer 1998) similar to the expression of Dlx3 reported here. Mutation in the Esx1 gene results in vascular defects in the labyrinth potentially acting through trophoblast control of vascular development (Li & Behringer 1998). Abnormal placental morphogenesis in the Esx1 mutant mice was evident by E11.5, a time frame slightly later than the important timing of Dlx3 up-regulation at E9.5. Finally, consistent with the current studies using Dlx3 ($-/-$) mice, Esx1 mutants do not appear to have compromised trophoblast

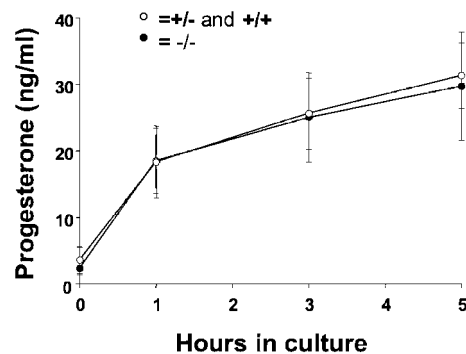


Figure 6 Progesterone production in short term placental cultures was similar among Dlx3 genotypes. Timed pregnancies were set up and tissue collected at E9.5. Embryos were collected for genotyping (*post priori*) and placentas were placed in short-term culture for 5 h. Progesterone production over time did not vary between wild-type (+/+) or heterozygous (+/-) placentas for Dlx3 so data for these 2 groups were combined to compare with Dlx3 ($-/-$) placentas. Progesterone production was then compared between Dlx3 (+/+), (+/-) placentas ($n=4$) and Dlx3 ($-/-$) placentas ($n=6$). Progesterone concentrations over time and total progesterone production were similar between these 2 groups.

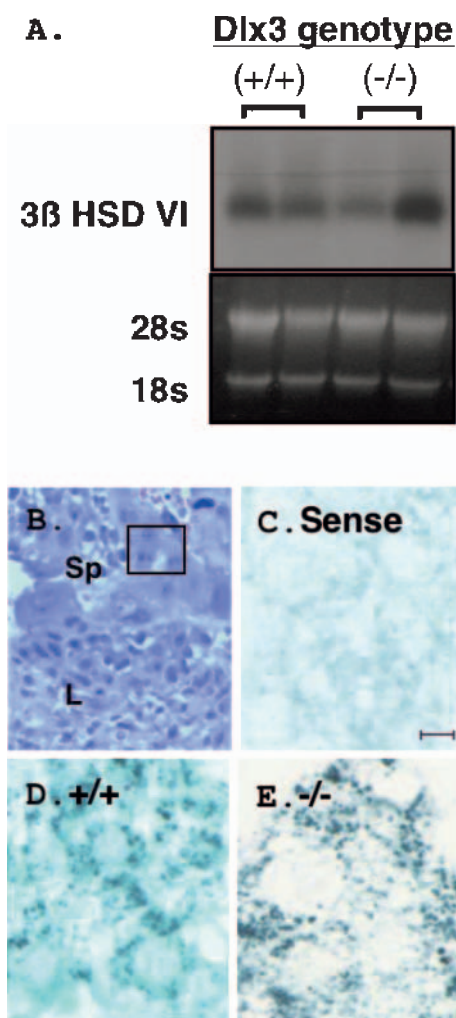


Figure 7 3β HSD VI mRNA expression was similar among *Dlx3* genotypes. Timed pregnancies were set up and tissue collected at E9.5. Embryos were collected for genotyping (*post priori*) and placentas were either frozen at -80°C for isolation of RNA for Northern blot analysis or placed in 4% paraformaldehyde for *in situ* hybridization (see Materials and Methods for details). (A) Northern blot analysis reveals similar 3β HSD VI mRNA expression in (+/+), (-/-) and (+/-; data not shown) placentas. (B) Histological section stained with hematoxylin and eosin showing placental morphology of mouse placenta. The box represents an area of the junctional zone seen at high power in panels C-E and is morphologically representative of the 3β HSD VI signal in (+/+), (+/-) and (-/-) placentas. Bar=20 μm . (C) Sense negative control. (D and E) 3β HSD VI mRNA expression was restricted to the trophoblast giant cells in the junctional zone in *Dlx3* (+/+), (-/-), (+/-; data not shown), and placentas. Bar=5 μm . L, labyrinth; Sp, spongiotrophoblast.

giant cell function (Li & Behringer 1998). It is reasonable to speculate that *Dlx3* and *Esx1* are key elements in the elegant regulation of placental morphogenesis and are under precise genetic control within the same pathway.

Additional studies are required to determine if *Dlx3* is a direct regulator of *Esx1* promoter activity in placental trophoblasts.

Our data do not support the hypothesis that *Dlx3* contributes to murine placental progesterone production, given that *Dlx3* was found localized only in the labyrinth of the mouse placenta, and not in the trophoblast giant cells. This is consistent with reduced or absent expression of *Dlx3* in the differentiated Rcho-1 rat trophoblast stem cell line (Fig. 5). *In vitro* studies in choriocarcinoma cells demonstrated that *Dlx3* binds to the 3β HSD VI promoter and was required for trophoblast-specific expression of 3β HSD VI (Peng & Payne 2002). Moreover, the glycoprotein hormone α subunit and the 3β HSD VI promoters share the transcriptional regulators *Dlx3* and AP-2 γ , which supports speculation that these two transcriptional regulators are an important combinatorial 'code' for trophoblast-specific expression. Given the similarities between the two promoters, our prediction was that *Dlx3* may be necessary for normal progesterone production by the mouse placenta. Our results do not support this conclusion based upon localization studies and the ability of *Dlx3* (-/-) implantation sites to produce progesterone at levels comparable to *Dlx3* (+/+) implantation sites when placed in short term culture. Progesterone production by placentas of the different genotypes increased equivalently over a five-hour culture period, suggesting that 3β HSD VI was functional. 3β HSD VI mRNA expression was essentially equivalent among the different *Dlx3* genotypes, supporting this conclusion. Others have reported that decidual expression of 3β HSD VI (thus maternal progesterone production) does not occur after E7.5 in the murine placenta (Peng *et al.* 2002). In the present studies, all of the progesterone measured in culture from implantation sites collected on E9.5 was presumably of trophoblast giant cell origin. The observation that there were similar expression patterns of 3β HSD VI mRNA in the junctional zone of E9.5 *Dlx3* (+/+), (+/-) and (-/-) mouse placentas is in agreement with another report where 3β HSD VI was shown to be expressed in trophoblast giant cells by E9.5 (Peng *et al.* 2002). Not consistent with our finding, however, was the identification of *Dlx3* in DNA binding complexes with a cis element from the 3β HSD VI gene promoter, using electrophoretic mobility shift assays (Peng & Payne 2002). In those studies, a *Dlx3* antibody super-shifted a putative *Dlx3*-containing complex formed in the context of nuclear extracts from mouse trophoblast giant cells, suggesting that *Dlx3* was capable of binding this cis element within the 3β HSD VI promoter. The possibility exists that contaminating labyrinth within the giant cell preparations may have been the source of *Dlx3* in the subsequent analysis of nuclear extracts in these DNA binding studies. Moreover, similar studies performed with the human placental-specific 3β HSD (3β HSD type I) promoter found that *Dlx3* is not required for placental-specific expression of

human 3 β HSD (Peng *et al.* 2004). While these observations suggest that Dlx3 may not be of key importance within these binding complexes, it is plausible that another giant cell-specific homeobox transcription factor (other than Dlx3) binds to and participates in the transactivation of the 3 β HSD VI promoter.

The present studies provide an important framework with which to move forward in delineating a functional role for Dlx3 in the mouse placenta. These results support the conclusion that Dlx3 is restricted to the labyrinth, consistent with abnormal placental morphogenesis demonstrated in the Dlx3 (–/–) mouse (Morasso *et al.* 1999). Our data demonstrate that Dlx3 is likely not involved in placental progesterone production in the mouse. The labyrinth is a region of maternal–fetal interface, where maternal vasculature interdigitates with the fetoplacental unit to allow for the exchange of nutrients and gases (Adamson *et al.* 2002). Dlx3, along with transcriptional regulators such as Esx1, may be requisite factors within a common pathway critical for vascular morphogenesis in placental development.

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