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Distal-less 3 haploinsufficiency results in elevated placental oxidative stress and altered fetal growth kinetics in the mouse

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Abstract

Distal-less 3 (*Dlx3*)^{-/-} mice die at E9.5 presumably due to an abnormal placental phenotype including reduced placental vasculature and secretion of placental growth factor. To examine the role of *Dlx3* specifically within the epiblast, *Dlx3* conditional knockout mice were generated using an epiblast-specific *Meox2*^{CreSor} allele. *Dlx3*^{-/fl}, *Meox2*^{CreSor} animals were born at expected frequencies and survived to weaning providing indirect evidence that loss of *Dlx3* within the trophoectoderm plays a critical role in fetal survival in the *Dlx3*^{-/-} mouse. We next examined the hypothesis that loss of a single *Dlx3* allele would have a negative impact on placental and fetal fitness. *Dlx3*^{+/-} mice displayed reduced fetal growth beginning at E12.5 compared with *Dlx3*^{+/+} controls. Altered fetal growth trajectory occurred coincident with elevated oxidative stress and apoptosis within *Dlx3*^{+/-} placentas. Oral supplementation with the superoxide dismutase mimetic, Tempol, rescued the fetal growth and placental cell death phenotypes in *Dlx3*^{+/-} mice. To determine the potential mechanisms associated with elevated oxidative stress on the *Dlx3*^{+/-} placentas, we next examined vascular characteristics within the feto-placental unit. Studies revealed reduced maternal spiral artery luminal area in the *Dlx3*^{+/-} mice receiving water; *Dlx3*^{+/-} mice receiving Tempol displayed maternal spiral artery luminal area similar to control *Dlx3*^{+/+} mice. We conclude that reduced *Dlx3* gene dose results in diminished fetal fitness associated with elevated placental cell oxidative stress and apoptosis coincident with altered vascular remodeling. Administration of antioxidant therapy ameliorated this feto-placental phenotype, suggesting that *Dlx3* may be required for adaptation to oxidative stresses within the intrauterine environment.

Keywords

Placenta; fetal growth; oxidative stress; labyrinth; vascular remodeling; mouse

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Introduction

Distal-less 3 (Dlx3) is a homeodomain-containing transcription factor that is involved in key developmental events in several tissues including placental morphogenesis in the mouse as well as expression of key endocrine products of the human and mouse placentas (1-5). In general, Dlx3 plays a role in the differentiation of epithelial compartments such as skin. For example, ectopic expression of Dlx3 in the basal layer of mouse epidermis results in cessation of proliferation of basal cells and enhanced differentiation of keratinocytes (6). Complete genetic loss of Dlx3 in mice results in mid-gestation lethality presumably due to inadequate placental development, suggesting an important role for this transcription factor at the maternal fetal interface (2); however, clearly delineated roles for Dlx3 within the trophoectoderm and epiblast in the Dlx3^{-/-} mouse model have not been established. In trophoblast cell culture models, Dlx3 is a required transcriptional regulator of the α subunit of chorionic gonadotropin (CG) (7). Immunohistochemical studies of human placental sections obtained at 8 weeks gestation suggest that Dlx3 is expressed in villous cyto- and syncytial trophoblasts at a time when CG biosynthesis is high (4,7). Recently, Murthi and colleagues reported that Dlx3 transcript levels increased coincident with *in vitro* differentiation of human villous cytotrophoblasts where differentiation was measured by increased expression of the β subunit of CG as well as 3 β -hydroxysteroid dehydrogenase (8). These studies support the conclusion that Dlx3 may be playing a role in the regulation of expression of α and β subunits of CG as well as steroid hormone biosynthesis in the human placenta.

Pathological conditions such as intrauterine growth restriction (IUGR) and pre-eclampsia (PE) have been linked to failure in placental morphogenesis correlated with aberrant gene expression, abnormalities in proangiogenic growth factor production and secretion, endothelial cell dysfunction and improper placental vascularization; all of which can lead to reduced placental perfusion and elevated levels of reactive oxygen species (ROS) within the placenta (9-12). Interestingly, the Dlx3 null mice display dysregulated vascularization within the placental labyrinth (2,4) coincident with reduced expression and secretion of the proangiogenic placental growth factor (PGF), a direct target of Dlx3 action (13). Notably in women, reduced PGF levels in maternal serum serve as a key biomarker of PE (10,14); in addition, the BPH/5 mouse model of PE also displays altered labyrinth morphogenesis, reduced PGF expression and maternal vascular remodeling (15-16). Taken together, these studies suggest that reduced expression of Dlx3 may be associated with placental insufficiency due to inappropriate expansion of the labyrinth within an environment of a reduced angiogenic potential; however, since the Dlx3 null animal is embryonic lethal, little is known regarding the role of Dlx3 governing the feto-placental unit past E9.5.

The present studies provide evidence that conditional loss of Dlx3 specifically within the epiblast (rather than within the trophoectoderm and epiblast in the Dlx3^{-/-} model) results in viable albeit phenotypically abnormal mice. These data provide indirect support for the conclusion that loss of Dlx3 within the placenta in the Dlx3^{-/-} model is likely central to embryonic lethality. Loss of a single Dlx3 allele has marked effects on intrauterine fetal growth patterns coincident with elevated oxidative stress and vascular dysfunction in murine placentas. These Dlx3-dependent placental defects are rescued with oral administration of an antioxidant given prior to conception and throughout gestation providing evidence that Dlx3 may be playing a role in the adaptation of the murine placenta to oxidative stresses during gestation.

Materials and Methods

Animals

All mice used in these studies were maintained and used in compliance with protocols approved by the Cornell University Institutional Animal Care and Use Committee. $Dlx3^{+/-}$ and $Dlx3^{floxed}$ (designated $Dlx3^{fl}$; 17) mice were a generous gift from Dr. Maria Morasso (NIAMS, NIH). $Meox2^{CreSor}$ mice (18) were purchased from the Jackson Labs (Bar Harbor, ME). Genotyping for the $Dlx3^{fl/fl}$ alleles was carried out by PCR using previously described methods (2,17). The $Meox2^{CreSor}$ allele was determined using a PCR strategy described by the Jackson Labs. All animals examined were litters derived from mating $Dlx3^{+/-}$, $Meox2^{CreSor}$ males to $Dlx3^{fl/fl}$ females. The $Dlx3^{fl/fl}$, $Meox2^{CreSor}$ animals provide the unique opportunity to examine loss of $Dlx3$ specifically within the epiblast of the developing animal while the trophectoderm is spared this genetic excision due to the fact that $Meox2^{CreSor}$ Cre recombinase expression is epiblast-specific. $Dlx3^{fl/fl}$, $Meox2^{CreSor}$ animals were subjected to a pathological assessment including gross and histological analyses of skin, teeth, reproductive organs, submandibular salivary glands, adrenals and bone. Initial studies examined fetal growth characteristics in $Dlx3^{+/+}$ and $Dlx3^{+/-}$ animals. Fetuses were weighed and photographed at E9.5, 10.5, 12.5, 15.5 and 18.5. Crown rump measurements of each animal were obtained from photographs using the Image J Analysis Software (NIH). In a subsequent study, the superoxide dismutase mimetic, Tempol (Sigma, St. Louis MO), was administered in the drinking water (2 mM) of the $Dlx3^{+/+}$ and $Dlx3^{+/-}$ female mice as previously described (19). Sterile drinking water was used as the control. All animals in this study were killed at E12.5.

Oxidative stress and Western Blot analyses of protein lysates

Placental disk samples from $Dlx3^{+/+}$ and $Dlx3^{+/-}$ animals were collected at E10.5, 12.5, and 15.5. Protein complexes were resolved on SDS-polyacrylamide (10%) gel electrophoresis and transferred to polyvinylidene difluoride membranes. Some blots were assayed using the Oxidation Detection Kit, (Oxyblot™; Millipore, Billerica, MA) as per the manufacturer's instructions. Actin levels (Santa Cruz Biotechnology, Santa Cruz CA) were determined as a lane loading control. Band intensity within the OxyBlots was determined using ImageJ Analysis Software (NIH) and standardized for the actin levels within each gestational age and plotted as relative fold change. Other blots were probed with a $Dlx3$ and actin antibodies as described previously (4). Relative density of each band was determined and standardized to actin levels.

Assessment of Placental Apoptosis

Placental tissues were collected from $Dlx3^{+/+}$ and $Dlx3^{+/-}$ in the Tempol and normal water treated groups at E12.5. Placental tissues were fixed in 10% formalin and paraffin embedded. Sections were cut at 10μm and subjected to analyses using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Apoptosis Detection kit (Millipore, Billerica, MA) according to manufacturer's instructions. Stained sections were scanned and digitized using an Aperio Scanscope (Vista CA). Data are reported as number of TUNEL positive cells/10x field within the labyrinth and maternal decidua.

Assessment of Placental Labyrinth, Artery and Vein Area

Some formalin-fixed sections were incubated with mouse anti-human smooth muscle actin (SMA) monoclonal antibody (1:50; DakoCytomation, Glostrup, Denmark) to examine maternal arteries and veins (n = 6-8/animal/ treatment group). Some sections were incubated with biotinylated GSL I-isolectin B4 (1:100, Vector Laboratories, Burlingame, CA) to delineate vasculature within the labyrinth. AEC (Invitrogen, Carlsbad, CA) was used as

chromogen to visualize antigen localization, and the sections were lightly counterstained with hematoxylin and eosin (H&E). Stained sections were imaged on an Aperio Scanscope (Vista, CA). Sections representing the widest portion of the placental disk were evaluated. Full placental area and area of the labyrinth and total area and luminal area of maternal vessels were quantitated using Aperio software. Data are presented as area of the labyrinth as a proportion of the total placental area and luminal area of a vessel as a proportion of the total vessel area.

Statistical Analysis

Data was analyzed using analysis of variance. Comparisons were made by Student's *t*-test. All data are expressed as means $\pm$ standard error of the mean. A *p* value of <0.05 was considered statistically significant.

Results

Mice with conditional epiblast-restricted deletion of the *Dlx3* gene survive to weaning

Our initial studies examined the hypothesis that conditional deletion of *Dlx3* within the epiblast and embryonic loss of *Dlx3* would result in embryonic lethality consistent with the *Dlx3*^{-/-} mouse model. To examine this hypothesis, we bred *Dlx3*^{-/fl} animals expressing Cre recombinase in the epiblast using a *Meox2*^{CreSor} allele (18). Surprisingly, *Dlx3*^{-/fl}, *Meox2*^{CreSor} animals survived to weaning and beyond at expected genetic ratios albeit with substantial phenotypic defects (Figure 1 and Table 1). *Dlx3*^{-/fl}, *Meox2*^{CreSor} mice were generally smaller than gender-matched littermate controls and grossly displayed abnormal hair growth patterns (Figure 1A and data not shown).

Histological examination of several tissues revealed marked differences between the *Dlx3*^{-/fl}, *Meox2*^{CreSor} animals and their littermate controls consistent with the role of *Dlx3* during development (3,6,20-22). For example, the epidermis was characterized by 1-2 cells thick and hair follicles contain well-formed, pigmented hairs in wild type mice. In *Dlx3*^{-/fl}, *Meox2*^{CreSor} mice, the epidermis is up to 4 cells thick and follicles contain amorphous keratin (Figure 1B). In teeth, the dentin and enamel of the wildtype mouse was homogenous and lined by a single layer of columnar odontoblasts and ameloblasts. In *Dlx3*^{-/fl}, *Meox2*^{CreSor} mice, the dentin was irregular and lined by an attenuated layer of odontoblasts. The enamel was lost during processing (Figure 1B). Bony trabeculae in the bone of wild type mouse were composed of a small amount of remnant cartilage covered by a thick layer of osteoid, which often enveloped osteocytes. Plump osteoblasts lined the trabeculae. In *Dlx3*^{-/fl}, *Meox2*^{CreSor} mice, bony trabeculae were composed of thick cartilaginous cores covered by a thin layer of osteoid (Figure 1B). Altogether these results indicate the impact of loss of *Dlx3* in the developing embryo was substantial in many tissues that have been shown to express *Dlx3* as part of a developmental program (3,17,23-24). Moreover, this model provides support for the conclusion that sparing loss of *Dlx3* within the trophoectoderm/placenta results in *Dlx3*-deficient embryos reaching birth and beyond, providing indirect evidence that the placental lesions within the *Dlx3*^{-/-} fetoplacental unit likely are an important contributor to embryonic lethality.

Dlx3 gene dosage affects fetal growth trajectory

Dlx3^{-/-} animals die at E9.5, which restricts assessment of the role of *Dlx3* later in gestation (2,4-5). To determine a role for *Dlx3* later in gestation, we examined the impact of haploinsufficiency of *Dlx3* on fetal growth and placental viability during gestation. We initially determined that *Dlx3*^{+/-} did indeed express reduced levels of *Dlx3* in the placenta at E12.5 (Figure 2). Once established, embryos from *Dlx3*^{+/-} matings were weighed at embryonic days (E) 9.5, 10.5, 12.5, 15.5 and 18.5 and assigned to genotype. Fetal weights

were similar at E9.5 and E10.5 in both genotypes. However, at E12.5 and 15.5, weight gain was delayed in $Dlx3^{+/-}$ fetuses compared to controls (Figure 3A). By E18.5, the fetal weights were indistinguishable between genotypes (data not shown). Since $Dlx3$ is expressed in both the embryo as well as the placental labyrinth, we next focused on the impact of loss of $Dlx3$ within the placental disk.

Loss of one $Dlx3$ allele results in elevated placental oxidative stress

Placental disks were collected from $Dlx3^{+/+}$ and $Dlx3^{+/-}$ animals at 3 different gestational time points (E10.5, 12.5 and 15.5). Samples were assayed for changes in carbonylation of cellular protein, which serves as a marker of oxidative stress (25). Figure 3B reveals an increased abundance of carbonyl modifications of protein in the $Dlx3^{+/-}$ mouse placentas; particularly at E12.5 and 15.5 (3 to 8 fold; Figure 3C), coincident with the fetal growth phenotype described in Figure 3A.

Antioxidant therapy rescues the $Dlx3^{+/-}$ fetal and placental phenotype

To determine if there were causal links between elevated placental oxidative stress and fetal growth, we examined the effects of antioxidant therapy on fetal and placental outcomes in the $Dlx3^{+/-}$ mouse. The superoxide dismutase mimetic Tempol has been used as an antioxidant in several experimental settings including the BPH/5 mouse (19, 26-27). Beginning 5 days prior to mating and throughout gestation, $Dlx3^{+/+}$ and $Dlx3^{+/-}$ females received Tempol in their drinking water or received normal drinking water as a vehicle control. We have shown previously that water intake in mice is not affected by the presence of Tempol in the drinking water (19). Animals were euthanized at E12.5; fetoplacental units were removed for analyses. Consistent with our initial studies, $Dlx3^{+/-}$ fetuses had reduced fetal weight and crown rump measurements compared to $Dlx3^{+/+}$ littermates at E12.5 (Figure 4A, B and C). The addition of Tempol in the drinking water effectively rescued this fetal growth phenotype. Surprisingly, oral administration of Tempol revealed a very small subset of $Dlx3^{-/-}$ animals (nine litters of $Dlx3^{+/-}$ mothers receiving Tempol resulted in 101 fetuses; we obtained 4 confirmed $Dlx3^{-/-}$ embryos or 3.96%) at E12.5 (Figure 4A), fully 3 days beyond the normal timing of fetal demise of these animals (2). It should be noted that Tempol had no effect on fetal growth in $Dlx3^{+/+}$ mice.

Increased TUNEL positive cells were detected throughout the $Dlx3^{+/-}$ labyrinth compared to $Dlx3^{+/+}$ animals receiving water (Figure 5A and B). Interestingly, the effects of loss of $Dlx3$ within the labyrinth (the site of $Dlx3$ expression) were paralleled with elevated cell death within the maternal decidua in $Dlx3^{+/-}$ animals receiving water (Figure 5C and D) suggesting possible paracrine communication between labyrinth trophoblasts and maternal decidua. In both compartments, administration of Tempol reduced the apoptotic index in the $Dlx3^{+/-}$ animals compared to water controls.

One potential cause of elevated oxidative stress within the developing placenta is altered remodeling of placental vascularization leading to reduced placental perfusion. Morasso and colleagues (2) described a reduced and compacted labyrinth area with reduced fetal vasculature in the $Dlx3^{-/-}$ placenta and increased density within the decidua. Based upon these observations, we assessed placental labyrinth area and cross-sectional area of maternal vasculature in the $Dlx3^{+/-}$ using histological techniques. For these studies, the labyrinth and vascular compartments were identified using H&E, isolectin staining and immunohistochemistry for SMA (Figure 6). Initially, we quantitated total placental and labyrinth area at the widest portion of the histological sections in the $Dlx3^{+/+}$ and $Dlx3^{+/-}$ mice receiving water or Tempol. Unlike the $Dlx3^{-/-}$ placenta, total labyrinth area did not appear reduced or compacted in the $Dlx3^{+/-}$ and was unaffected by Tempol (Figure 6A). In contrast, maternal spiral artery luminal area was reduced in the $Dlx3^{+/-}$ mice compared to $Dlx3^{+/+}$

mice receiving normal water (Figure 6B and C), and this was normalized in $Dlx3^{+/-}$ mice receiving Tempol. Luminal area of maternal veins within these placentas were unaffected by genotype or antioxidant treatment (data not shown).

Discussion

Complete loss of $Dlx3$ in the developing placenta has a clear impact on placental morphogenesis as well as fetal fitness (2). Our studies indicate conditional loss of $Dlx3$ within the epiblast of the developing embryo does not affect the ability of $Dlx3^{-/fl}$, $Meox2^{CreSor}$ animals to survive the neonatal period and reach weaning (Figure 1A and Table 1); however, defects in skin, teeth and bone were observed in $Dlx3^{-/fl}$, $Meox2^{CreSor}$ animals consistent with a role for $Dlx3$ in the development of these tissues (3,17,23-24). These results support two important conclusions: our genetic approach for epiblast-specific $Dlx3$ gene excision was effective in altering developmental outcomes in tissues known to be regulated by $Dlx3$ in the fetus and adult animal; and sparing $Dlx3$ gene excision from the trophoctoderm cell lineage in this model resulted in a normal distribution of $Dlx3$ -deficient animals reaching weaning suggesting that $Dlx3$ -dependent effects within the trophoctoderm play a fundamental role in fetal survival of the $Dlx3^{-/-}$ animal. Despite these findings, there are important limitations to this study that need to be considered carefully. Unlike the $Dlx3^{-/-}$ animal, $Meox2^{CreSor}$ animals do not display epiblast-specific Cre expression until ~E6.0 (18). Thus, key $Dlx3$ -dependent developmental events may have occurred prior to gene excision in the $Dlx3^{-/fl}$, $Meox2^{CreSor}$ embryo. Additionally, Cre is expressed widely within the $Meox2^{CreSor}$ embryos; however, Cre expression is considered slightly mosaic (18). Despite these caveats, the $Dlx3^{-/fl}$, $Meox2^{CreSor}$ animals display predictable phenotypes in skin, teeth and bone as have been previously described in patients with $Dlx3$ deficiencies and during mouse development in tissues that are known to express $Dlx3$ (2-3,28). These studies indirectly support the conclusion that mid-gestational embryonic lethality associated with $Dlx3^{-/-}$ mice is linked to placental lesions caused by loss of $Dlx3$ within placental trophoblasts.

Loss of a single $Dlx3$ allele resulted in increased oxidative stress and apoptotic index within the placental labyrinth coincident with reduced or delayed fetal growth (Figures 2 and 4) in midgestation. By E18.5, fetal weight in the $Dlx3^{+/-}$ animals appears to catch up leaving open the possibility that other mechanisms governing fetal growth provide compensation for the loss of a $Dlx3$ allele; conversely, the role of $Dlx3$ in later gestation may shift in importance relative to late gestation fetal growth. Future studies will examine this issue. Oxidative stress induced by loss of $Dlx3$ is clearly causal relative to the cell death and retarded fetal growth phenotypes since oral administration of a potent antioxidant (Tempol) prior to conception and throughout gestation protected against these phenotypes (Figures 4 and 5). The use of Tempol in these studies was patterned after studies of oxidative stress in the BPH/5 model since key water intake and pharmacodynamic studies carefully defined the parameters of Tempol administration and uptake in mice (19). In those studies, oral Tempol administration did not affect water intake in mice; and electron spin resonance spectrophotometry demonstrated significant *in vivo* plasma levels of Tempol radicals suggesting this route of administration was appropriate and effective. What remains to be determined is if $Dlx3$ is playing a direct or indirect role (or both) in processes leading to oxidative stress in trophoblasts expressing $Dlx3$ and in extra-labyrinth compartments within the feto-placental unit (such as within maternal decidua) that do not express $Dlx3$. Markers of oxidative stress in maternal blood and urine indicate that under normal circumstances, pregnancy is characterized by high oxidative stress largely due to the high metabolic demand by the placenta (29-31) coupled with reduced placental ROS scavenging potential (32). Placental oxidative stress is further exacerbated in pregnancies complicated by PE, IUGR, and diabetes (reviewed in 33-34). Although a precise mechanism(s) associated with increased

placental ROS generation in *at risk* pregnancies has not been fully elucidated, one compelling hypothesis arises from observations of placental vasculature associated with PE. Pregnancies complicated by PE exhibit reduced remodeling of maternal spiral arteries (35) resulting in impaired placental perfusion leading to potential harmful effects on fetal growth kinetics (36). A similar relationship has also been reported in the BPH/5 mouse where reduced angiogenic potential is associated with incomplete vascular remodeling (16). Our own studies have demonstrated that *Dlx3* has a direct effect on transcriptional regulation of the mouse PGF gene promoter and loss of *Dlx3* results in reduced secretion of PGF in placental explants cultured *in vitro* (13). VEGF and PGF are thought to be important biomarkers of PE (37,38). This putative loss of angiogenic potential may have a direct effect of vascular remodeling, effectively reducing or delaying the normal dilation of maternal spiral arteries and maintaining relatively high vascular resistance and impaired placental perfusion. This notion would be consistent with the role of PGF as a proangiogenic factor in modulating vascular remodeling in some tumor models (39). Hedlund and colleagues showed that tumor-derived PGF appeared to contribute to increased microvessel organization, branching morphogenesis and diameter, including the ability of PGF to contribute to vascular remodeling in spontaneously arising human choriocarcinomas. These studies provide support the conclusion that loss of *Dlx3*-dependent PGF may be one causative factor in altered or delayed maternal vascular remodeling in the *Dlx3*^{+/-} model system through paracrine actions of PGF on maternal vasculature.

Therapeutic antioxidant treatments represent a potentially powerful defense from the ROS-mediated placental abnormalities during pregnancy. Supplementation of vitamin C and E during the second trimester of pregnancy in women decreased biochemical incidence of ROS (40). Further, gestational supplementation of vitamins C and E indicate a significant reduction in syncytiotrophoblast apoptosis associated with placental hypoxia (41). However, not all of the studies examining the utility of antioxidant supplementation during *at risk* pregnancies have resulted in positive effects (reviewed by 42). Critical timing of antioxidant therapy is essential to effectively reduce oxidative stress within the placenta. Inadequate supplementation during pregnancy could address some of the controversy in the therapeutic value of antioxidant therapy. Myatt and colleagues have indicated antioxidant therapy appears to be most effective when administered prior to conception and during early gestation (34). The timing of Tempol administration in the present study is consistent with this recommendation. The present studies suggest that loss of full expression of *Dlx3* and or *Dlx3* target genes within placental trophoblasts is linked to accommodation or adaptation to oxidative stresses leading to loss of fetal well-being coincident with altered placental vasculature. This may be reinforced more directly by the anecdotal observation that Tempol therapy rescued a small number of *Dlx3* null fetuses beyond their normal timeframe of demise supporting speculation that the loss of *Dlx3* within the placenta and fetus is linked to mechanisms supporting the adaptation to oxidative stress. Antioxidant therapy prior to and during gestation ameliorates abnormal placental morphogenesis and placental angiogenesis induced by loss of *Dlx3*; the outcome of which clearly leads to improved fetal fitness.

In sum, the present studies support the conclusions that loss of a single *Dlx3* allele results in abnormal levels of placental oxidative stress linked to elevated placental apoptosis and reduced or delayed fetal growth and maternal spiral artery remodeling. The effects of *Dlx3* on trophoblast populations may include reduced expression of proangiogenic growth factors such as PGF that likely help explain effects of loss of *Dlx3* in placental compartments distal to the labyrinth (the site of *Dlx3* expression) such as within the maternal spiral arteries and decidua. Hence the impact of *Dlx3* and the *Dlx3* gene network on adaptation to oxidative stress within the feto-placental unit may be more far-reaching than previously appreciated.

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Figure 1. *Dlx3* epiblast-conditional knockout survives to parturition with growth abnormalities
A. Female $Dlx3^{-/-}, Meox2^{CreSor}$ animals are considerably smaller in stature at 6 weeks of age and grossly display abnormal hair growth patterns in contrast to wild type gender matched control littermates. Male $Dlx3^{-/-}, Meox2^{CreSor}$ mice display a similar growth and hair pattern phenotype compared to female $Dlx3^{-/-}, Meox2^{CreSor}$ animals (data not shown).
B. Hematoxylin and Eosin (H&E) stained sections of skin, teeth and bone from $Dlx3^{-/-}, Meox2^{CreSor}$ animals indicate structural and developmental irregularities compared to wild type littermate controls. F = follicle; E = epidermis; En = enamel; D = dentin; Od = odontoblasts; A = ameloblasts; Os = osteoid; C = cartilage.

Figure 2. Dlx3 protein levels are reduced in Dlx3^{+/-} placentas

A. Dlx3 heterozygous (Dlx3^{+/-}) animals were mated and sacrificed at E 12.5 (n=6/group). Fetal genotypes were determined using PCR. Placental disks were dissected free and homogenized. Equal placental protein was loaded in each lane (20 μ g) and proteins were resolved using SDS-PAGE and transferred to PVDF membranes. Dlx3 antibody was used to detect Dlx3 levels in placentas from Dlx3^{+/+} and ^{+/-} placentas. The Dlx3 and Actin bands are noted. The band labeled “ns” is a non-specific band. **B.** Relative levels of Dlx3 protein are depicted for the placental samples measured. Relative density of the Dlx3 band was standardized for individual actin levels. Dlx3^{+/+} samples were then set to 1.0 for comparison to Dlx3^{+/-} placentas. * differs in pairwise comparison indicated, p<0.05.

Figure 3. $Dlx3^{+/-}$ mice exhibit reduced intrauterine growth and elevated relative abundance of protein carbonyl groups within the placenta

A. $Dlx3$ heterozygous ($Dlx3^{+/-}$) animals were mated and sacrificed at four different gestational ages, E9.5, 10.5, 12.5, 15.5 ($n=6-12/\text{group}$). $Dlx3^{+/-}$ and $Dlx3^{+/+}$ embryos display similar growth patterns at E9.5 and 10.5. By E12.5 and then again at E15.5, $Dlx3^{+/-}$ embryos weighed less ($p < 0.05$) than $Dlx3^{+/+}$ littermates. * $p < 0.05$ vs $Dlx3^{+/+}$. **B.** $Dlx3^{+/+}$ and $Dlx3^{+/-}$ placentas were harvested at 3 different gestational time points, E10.5, 12.5, and 15.5 ($n=3-4/\text{group}$). Equal amounts of placental tissue lysates were subjected to an Oxyblot assay to determine the abundance of protein carbonyl side chains which are indicative of oxidative stress. Actin was used to standardize carbonyl staining. **C.** Using ImageJ software, intensity

of specific protein carbonyl bands (<a>, and <c> in panel B) was determined and standardized to the actin bands. The data are expressed as relative fold change where the $Dlx3^{+/+}$ observation within embryonic day is standardized to 1.0. * differs in pairwise comparison indicated, $p < 0.05$.

Figure 4. Antioxidant therapy rescues the fetal growth phenotype in $Dlx3^{+/-}$ mice
 $Dlx3^{+/+}$ and $Dlx3^{+/-}$ females were pretreated with Tempol (2 mM) in their drinking water five days prior to mating with $Dlx3^{+/+}$ and $Dlx3^{+/-}$ males, respectively, and were compared to water-treated controls; treatments continued throughout gestation. All animals were sacrificed at E12.5. **A.** Images of representative fetuses from individual genotypes are depicted. Fetal weights (mg) (**B.**) and crown rump measurements (mm) (**C.**) were reduced in $Dlx3^{+/-}$ compared with $Dlx3^{+/+}$ fetuses in animals receiving water. Tempol administration rescued the fetal weight and crown rump measurement in the $Dlx3^{+/-}$ animals at E12.5. * differs in pairwise comparison indicated, $p < 0.05$.

Figure 5. Chronic Tempol administration ameliorates apoptosis in Dlx3^{+/-} placentas
 Dlx3^{+/+} and Dlx3^{+/-} females were pretreated with Tempol (2mM) in the drinking water or normal water as control five days prior to mating with Dlx3^{+/+} and Dlx3^{+/-} males, respectively; treatments continued throughout gestation. All animals were sacrificed at E12.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on placental sections was used to determine apoptotic index in the placentas of Dlx3^{+/+} and Dlx3^{+/-} fetuses at E12.5 (n = 6/genotype/treatment) receiving either Tempol or water as control. **A.** Data reflect number of TUNEL-positive cells per 10x visual field within the labyrinth. **B.** Representative images of apoptotic nuclei (black arrows) within the labyrinth are shown for Dlx3^{+/+} and ^{+/-} placentas from mothers receiving water. **C.** Data reflect number of TUNEL-positive cells per 10x visual field within the maternal decidua. **D.** Representative images of apoptotic nuclei (black arrows) within the decidua are shown for Dlx3^{+/+} and ^{+/-} placentas from mothers receiving water. The black bars in B and D reflect the magnification of the image. * differs in pairwise comparison indicated, p<0.05.

Figure 6. Loss of a single *Dlx3* allele results in altered maternal spiral artery remodeling
Standard histological analysis using H&E, iso-lectin staining and immunohistochemistry using a smooth muscle actin antibody was used to examine placental labyrinth and maternal vasculature within the placental disk (n = 6-8/animal/treatment group). See Methods for details. **A.** Placental labyrinth area (identified by H&E and iso-lectin staining and the presence of nucleated fetal red blood cells; shown as a percentage of total placental area) is depicted for Dlx3^{+/+} and Dlx3^{+/-} receiving Tempol or water as control. **B.** Maternal spiral artery cross sectional area (defined by cross-section area of a vascular lumen surrounded by smooth muscle actin staining and the presence of non-nucleated red blood cells; shown as a percentage of total placental area) is depicted for Dlx3^{+/+} and Dlx3^{+/-} receiving Tempol or water as control. * differs in pairwise comparison indicated, p<0.05. **C.** Representative images of smooth muscle actin staining within the decidua are shown for Dlx3^{+/+} and +/- placentas from mothers receiving water or Tempol. Red staining indicates smooth muscle actin. The black bar reflects the magnification of the image.

Table 1

Litter size and distribution of genotypes in $[Dlx3^{+/-}, Meox2^{CreSor}] \times [Dlx3^{fl/fl}]$ matings.

<u># of litters</u>	<u>Total pups</u>	<u>% normal (expected)</u>	<u>% mutant (expected)</u>
33	135	74.8 (75)	25.2 (25)