Developmental Biology 373 (2013) 72–82

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Generalized disruption of inherited genomic imprints leads to wide-ranging placental defects and dysregulated fetal growth

K.P. Himes [a], E. Koppes [b], J. Richard Chaillet *[,b]

[a] Magee-Womens Research Institute, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine, 204 Craft Avenue Pittsburgh, PA 15213, USA

[b] Magee-Womens Research Institute, Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, 204 Craft Avenue Pittsburgh, PA 15213, USA

ARTICLE INFO

Article history:
Received 5 June 2012
Received in revised form
2 October 2012
Accepted 8 October 2012
Available online 17 October 2012

Keywords:
Imprinting
Placenta
Fetal growth
DNMT1
Methylation

ABSTRACT

Monoallelic expression of imprinted genes, including ones solely expressed in the placenta, is essential for normal placental development and fetal growth. To better understand the role of placental imprinting in placental development and fetal growth, we examined conceptuses developing in the absence of maternally derived DNA (cytosine-5-)-methyltransferase 1o (DNMT1o). Absence of DNMT1o results in the partial loss of methylation at imprinted differentially methylated domain (DMD) sequences in the embryo and the placenta. Mid-gestation E9.5 DNMT1o-deficient placentas exhibited structural abnormalities of all tissue layers. At E17.5, all examined placentas had aberrant placental morphology, most notably in the spongiotrophoblast and labyrinth layers. Abnormalities included an expanded volume fraction of spongiotrophoblast tissue with extension of the spongiotrophoblast layer into the labyrinth. Many mutant placentas also demonstrated migration abnormalities of glycogen cells. Additionally, the volume fraction of the labyrinth was reduced, as was the surface area for maternal fetal gas exchange. Despite these placental morphologic abnormalities, approximately one-half of DNMT1o-deficient fetuses survived to late gestation (E17.5). Furthermore, DNMT1o-deficient placentas supported a broad range of fetal growth. The ability of some DNMT1o-deficient and morphologically abnormal placentas to support fetal growth in excess of wild type demonstrates the importance of differential methylation of DMDs and proper imprinting of discrete gene clusters to placental morphogenesis and fetal growth.

Introduction

Genomic imprinting is a highly conserved epigenetic process that distinguishes parental alleles of a small number of genes such that only one parental allele is transcriptionally active. For the majority of imprinted genes, this difference is absolute; one allele is expressed and the opposite allele is silent (Bartolomei and Tilghman, 1997). With few exceptions, the approximately 100 known mouse imprinted genes are organized into 16 clusters (http://www.mousebook.org/catalog.php?catalog=imprinting.) harboring a variable number of imprinted genes. Importantly, the imprinted, monoallelic expression of genes in the same cluster is determined by a small contiguous set of sequences called the imprint control region (ICR) (Reinhart et al., 2002). In general, deletion of ICR sequences leads to complete (biallelic) transcriptional silencing or to biallelic expression, depending on the cluster and the particular gene in the cluster. These findings are consistent with the notion that imprinted gene expression is a complex regulatory phenomenon involving many *cis*- and *trans*-acting factors, but mediated primarily through ICRs (Reinhart and Chaillet, 2005).

The function of ICR sequences in regulating the imprinted expression of genes appears to be largely determined by differentially methylated domains (DMDs) within the ICR sequence. DMDs originate during gametogenesis through the action of DNA (cytosine-5-)-methyltransferase 3 alpha (Bourc'his et al., 2001; Hata et al., 2002; Kaneda et al., 2004). After fertilization, DMD methylation is maintained in all cells of the conceptus by different isoforms of the DNMT1 cytosine-5 methyltransferase (Cirio et al., 2008a; Hirasawa et al., 2008).

Proper inheritance of methylation imprints is essential for normal development. Insight into the mechanism of this epigenetic inheritance during preimplantation has come from examining the expression of isoforms of DNMT1. The enzyme DNMT1o exhibits a unique, stage-specific role in the inheritance of DMD methylation during preimplantation. DNMT1o is synthesized in the maternal oocyte and maintains DMD methylation in the 8-cell embryo (Cirio et al., 2008b; Doherty et al., 2002; Howell et al., 2001). In contrast the longer somatic DNMT1s form maintains methylation at other preimplantation cleavage stages (Cirio et al., 2008a; Hirasawa et al., 2008). Post-implantation embryos derived from DNMT1o-deficient oocytes have lost methylation on ~50% of the normally

* Corresponding author. Fax: +1 412 641 3899.
E-mail address: chailletjr@mwri.magee.edu (J.R. Chaillet).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.10.010

methylated alleles of their DMDs. Loss of DNMT1o results in generation of epigenetic mosaic embryos (Cirio et al., 2008b; Howell et al., 2001; Toppings et al., 2008). These are produced by the combined effects of a loss of DNMT1o maintenance methyltransferase activity followed by the normal process of random chromosome segregation. The loss of methylation leads to altered expression of imprinted genes, manifest as biallelic expression of some genes or loss of expression of most genes. Methylation of non-imprinted DNA is unchanged.

Imprinted genes are highly and in some cases uniquely expressed in the placenta. Of the 16 imprinted gene clusters, seven (*Peg*10, *H*19/*Igf*2, *Mest*, *Kcnq*1, *Grb*10, *Dlk*1/*Meg*3, and *Igf*2*r*) are of particular interest. Not only do these clusters contain genes that are highly expressed in the placenta, but mutations in these genes have been associated with placental maldevelopment and/or dysfunction (Frost and Moore, 2010). For example, ablation of the maternally expressed allele of *Ascl*2 results in fetal death on embryonic day 10.5 (E10.5), with poorly developed labyrinthine vasculature, reduced population of spongiotrophoblasts, and an excess of trophoblast giant cells (Guillemot et al., 1994). Deletion of *Peg*10 results in a similar placental phenotype (Ono et al., 2006).

While much has been learned about the role of imprinted genes in placental development from targeted inactivating or over-expressing individual imprinted genes, a comprehensive study of the role of imprinting in placental function should include removal of the inherited DNA methylation at DMD sequences. This can be conferred by DNMT1o deficiency. The loss of DNMT1o in embryos from homozygous $Dnmt1^{\Delta 1o/\Delta 1o}$ female mice provides an opportunity to analyze the functions of multiple imprinted genes by stochastic elimination of DMD methylation at many sites. Here we tested the hypothesis that DNMT1o deficiency adversely affects placental development and function.

Materials and methods

Animals

The mutant $Dnmt1^{\Delta 1o}$ allele was maintained in the wild type (wt) 129/SvTac strain background. Embryos derived from wild-type and homozygous $Dnmt1^{\Delta 1o/\Delta 1o}$ dams mated with 129/SvTac males were compared. For studies of parent-specific methylation of differentially methylated domains (DMDs), $Dnmt1^{\Delta 1o/\Delta 1o}$ or wt 129/Sv/Tac female mice were crossed to inbred CAST/Ei male mice. $Dnmt1^{\Delta 1o}$ mice were genotyped using a PCR assay as previously described (Howell et al., 2001). Primers are included in Supplemental Table 2. All experiments were performed in compliance with guidelines established by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Placenta dissection and extraction

Copulation was determined by the presence of a vaginal plug and embryonic day zero (E0) was assumed to be midnight. Conceptuses were collected from the uteri of female mice at E9.5, E12.5, E15.5 or E17.5. Placentas for DNA and RNA extraction were dissected from embryos, decidua and yolk sac tissue and kept individually in RNALater (Sigma). Placentas for histologic and morphometric analyses were dissected from embryo and yolk sac but decidua was preserved.

Determinations of DMD methylation

Genomic DNA was extracted from E9.5 and 12.5 placentas using AllPrep DNA/RNA Micro Kit and from one half of sagittal sections of E15.5 and E17.5 using AllPrep DNA/RNA Mini Kit (Qiagen). Genomic DNA methylation patterns of DMDs in F1 hybrid mice obtained from crosses between 129/Sv/Tac female mice and CAST/Ei male mice were determined using the method of bisulfite genomic sequencing (Lucifero et al., 2002). Five DNMT1o-deficient E9.5 placentas and six DNMT1o-deficient E17.5 placentas were analyzed. Maternal and paternal alleles were distinguished by single nucleotide polymorphisms in the different DMDs. Primers are included in Supplemental Table 2. For more widespread quantitative methylation analysis the EpiTYPER application (Sequenom) was used as previously described (Ehrich et al., 2005). DNA methylation standards (0, 50, and 100%) were used to correct for bias. Primers for the EpiTYPER experiments are included in Supplemental Table 2.

Standard histology and stereology

Placentas were collected, bisected in the midline and half of placenta fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in PBS. Specimens were then dehydrated and embedded in paraffin. Sections (5 μm) were stained with either hematoxylin and eosin or periodic acid-Schiff (PAS) stain. Volume fraction of spongiotrophoblast and labyrinth was determined by counting points that fell in the relevant placental layer compared to total placental reference space. Data is presented as percent spongiotrophoblast or labyrinth of total. Four wild type (2 litters) and twelve DNMT1o-deficient placentas from 5 litters were evaluated.

RNA in situ hybridization (ISH)

E9.5 and E17.5 placentas were dissected in PBS and fixed in fresh 4% PFA. PFA-fixed samples were immersed in 10%, then 20% sucrose in PBS, followed by OCT embedding. We used digoxigenin-labeled cRNA probes, synthesized using digoxigenin RNA labeling kit (Roche, Basel, Switzerland). Cryosections (10 μm) of the OCT-embedded placentas were used for ISH as previously described (Barak et al., 1999).

Immunohistochemistry

Sections (5 μm) were obtained after fixing placentas in 4% PFA overnight, dehydration and paraffin embedding. Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector laboratories) and DAB substrate per manufacturer recommendations (Vector Labs). To identify fetal endothelium, a 1:200 dilution of rabbit polyclonal anti-CD31 antibody (Abcam-ab28364) was used. Hematoxylin and acid alcohol were used for counter stain. The ratio of vasculature surface area to labyrinthine volume was determined using a cycloid arc grid as previously described (Baddeley et al., 1986; Coan et al., 2004). A cycloid arc grid was overlaid on high-magnification images of CD31 stained sagittal placental sections from wt or DNMT1o-deficient placentas. Cycloid line intersections with vascular walls were counted to determine vasculature surface area and points within the reference space counted to determine volume. Measurements were performed on systematic random samples of 24 images per placenta using every fifth section from each placenta. Five wt and 10 DNMT1o-deficient placentas were analyzed. The data are presented as maternal and fetal surface area, and expressed as vasculature surface area per volume of labyrinth (cm^2/cm^3).

Quantitative measurements of imprinted gene transcripts

RNA was extracted using either the AllPrep DNA/RNA Micro or Mini Kit (Qiagen). Contaminating DNA was removed by DNAse treatment according to manufacturer's instruction. Complementary DNA was prepared from 1 μg RNA using the high capacity