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Intact feto-placental growth in microRNA-210 deficient mice

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Abstract

MicroRNA-210 (miR-210) has been implicated in homeostatic adaptation during hypoxia. We hypothesized that miR-210 deficiency impacts feto-placental growth. As expected, mir-210 knockout (ko) mice exhibited markedly reduced placental miR-210 expression, compared to wild-type (wt) mice. Mating of mir-210 heterozygotes resulted in near Mendelian progeny distribution, with insignificant differences between wt and ko animals with regard to embryo or placental weight and gross morphology. Intriguingly, exposure of mice to non-severe hypoxia (O₂=12%) between E11.5-E17.5 reduced placental miR-210 expression, with slight expression changes of some miR-210 target mRNAs. Thus, miR-210 is likely dispensable for feto-placental growth in normoxia or non-severe hypoxia.

Keywords

Placenta; Trophoblast; miR-210; Hypoxia; Knockout

Introduction

Diverse miRNA species are expressed in the human or mouse placenta, yet only a few of them have been mechanistically shown to regulate trophoblast function [1-4]. Ample evidence suggests that miR-210 is a critical regulator of tissue response to hypoxia and is likely regulated by HIF1 α to drive downregulation of target genes [5-8]. miR-210 has also been associated with hypoxia in placental trophoblasts and shown to be upregulated in diseases attributed to hypoxia, such as preeclampsia, [9, 10]. Luo et al. [11] provide evidence that miR-210 downregulates Thrombospondin Type I Domain Containing 7A (THSD7A) in human placental trophoblasts, possibly contributing to the preeclamptic phenotype. Low oxygen tension also leads to placental mitochondria dysfunction, in which

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elevated miR-210 levels downregulate the Iron Sulfur Cluster (ISCU) gene and inhibition of miR-210 protects trophoblast cells from oxidative stress [12]. We therefore surmised that miR-210 was crucial for placental response to hypoxia and thus for fetal growth.

Methods

The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all experimental protocols. We used STOCK mir210^{tm1Mtm/Mmjax} female mice that were first crossed with B6.129S4-*Gt(ROSA)26Sor^{tm1(FLP1)Dym}/RainJ* males to delete the Neo cassette, followed by crossing with B6.C-Tg(CMV-cre)1Cgn/J to delete mir-210. (Mice were obtained from Jackson Laboratory, Bar Harbor, ME.) To produce all genotypes, heterozygous males and females were crossed, and pregnant dams were kept either in normal atmosphere or in a hypoxia chamber (Coy Laboratory Products, Grass Lake, MI) in a 12% O₂ atmosphere between E11.5-E17.5. On E17.5 the mice were sacrificed, and placentas and embryos were weighed. Genotyping was performed by standard PCR (Veriti, Thermo Fisher, Waltham, MA), using specific primer sets (Supplemental Table S1) and genomic DNA isolated from fetal tail by the HotSHOT (Hot Sodium Hydroxide and Tris) method, adapted from Truett et al [13]. Sex determination of the fetuses was performed based on SRY (Sex-determining Region Y) expression using a specific primer set (Integrated DNA Technologies, Table S1) and standard PCR. Amplification products were visualized on agarose gel with 2% bromide ethidium. Paraffin-embedded sections of 4% paraformaldehyde-fixed placentas were stained with hematoxylin and eosin and inspected under a bright light microscope (Nikon 90i microscope, Nikon, Tokyo, Japan). For analysis of miR-210 and target gene expression by qPCR, we took extra care to trim the placental edges to avoid contamination by decidual cells. Collected placental fragments were soaked in RNA later and frozen at -80°C. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) and purified with EconoSpin columns (Epoch Life Science, Missouri City, TX) following the manufacturer's instructions. The quantity and quality of RNA were assessed using a NanoDrop 1000 spectrometer (Thermo Scientific, Wilmington, DE). Reverse transcription and subsequent qPCR for miRNA expression were performed using, respectively, the miScript-II RT Kit and miScript SYBR Green PCR Kit with miScript assay (Qiagen, Valencia, CA), as we have described [14]. For mRNA expression, cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit, followed by real-time PCR using SYBR Select Master Mix (Thermo Fisher) and specific primer sets (Integrated DNA Technologies, Coralville, IA; Supplemental Table S2). Relative transcript expression was calculated using the delta delta Ct method [15] and normalized to RNU6 or to mouse ribosomal protein L32. Statistical differences were calculated using two-way ANOVA, followed by the Bonferroni post-hoc test, with significance set at p<0.05 (Prism 6, GraphPad Software, San Diego, CA). All data are shown as mean ± SD.

Results and Discussion

We initially confirmed that miR-210 expression was markedly reduced in mir-210 ko placentas, compared to placentas from wt mice (Fig. 1A). We also confirmed the reduction of miR-210 in the kidneys from ko fetuses (16-fold) and that the expression of miR-210

increased 3-fold in primary human trophoblasts cultured in hypoxic conditions for 48 h compared to those cultured in standard conditions (false discovery rate = 0.0042, RNAseq, data not shown). We found normal Mendelian distribution of fetuses resulting from breeding mir-210 heterozygous males and females (Fig. 1B). The mean litter size was 8.7 embryos (range 7-11) for animals maintained in standard conditions, which was slightly greater than the mean litter size of 7.2 embryos (range 5-10) for mice exposed to hypoxia (n=11 pregnant females/group, p=0.023, unpaired t-test). Embryo and placental weights were not different between wt and ko animals (Fig. 1C, D). This observation was confirmed using analysis of wt vs. ko animals within each litter (not shown).

We found that hypoxia decreased miR-210 expression level in wt placentas (Fig. 1A). While miR-210 levels were comparable between male and female placentas in standard conditions, in hypoxia the level of miR-210 in the male placentas was approximately 25% lower than the level in female placentas (p<0.05, not shown). As expected, the weight of wt fetuses was reduced when exposed to hypoxia, compared to standard conditions (Fig. 1C, p<0.05). When stratified by fetal sex, the differences were insignificant within each sex (Supplemental Fig. S1A). In contrast, exposure to hypoxia caused a small, yet significant, increase in placental weight in both wt and ko groups (p<0.05; Fig. 1D). This effect was also found in the male placentas, but not in the female placentas (Supplemental Fig. S1B). Placental histomorphology (based on hematoxylin and eosin stain at E18.5) was not different between the groups (not shown). To investigate the possible effect of altered miR-210 expression on known miR-210 targets, we analyzed placental expression of HOXA1, EFNA3, NDUFA4, and ISCU [5, 9, 12]. While we found small differences in mRNA levels between standard and hypoxic conditions, none of the genes were differentially expressed between wt and ko mice (Fig. 2A-D).

We found that ablation of mir-210 in the fetus and placenta did not lead to altered intrauterine growth in standard conditions, suggesting that miR-210 is not necessary for intrauterine development in mice. Moreover, there was no upregulation of placental miR-210 in response to moderate maternal hypoxia. Notably, we used a moderate level of hypoxia (O₂=12%) because, in our previous studies, we found that this degree of hypoxia caused fetal growth restriction by 10-20%, without excessive fetal loss [16]. In addition, we initiated hypoxia at E11.5, as our goal was to assess the effect of hypoxia on the established placenta and avoid exposure to hypoxia earlier in gestation, when the fetoplacental unit develops during physiological hypoxia [17-20]. Indeed, during this period, miR-210 might have a role in attenuating trophoblast invasion [21], as blocking miR-210 in hypoxia increases trophoblast invasion and migration [6]. These processes were not assessed in our study. Thus, while our data cannot be extrapolated to the entire pregnancy or to miR-210 response at any level of hypoxia or other stressors, our results do not support a role for miR-210 in placental adaptation to hypoxia. Interestingly, we recently also found that placenta-specific overexpression of miR-210 had no effect on the mouse fetal and placental weight (Supplemental Fig. S2).

While it has been reproducibly shown by others and by us that miR-210 is upregulated in trophoblasts cultured in hypoxia [6, 22 and Sadovsky et al., unpublished], the evidence for miR-210 function and regulation *in vivo* is less clear [7, 23, 24]. Although miR-210

expression was increased in severe preeclampsia [10], the levels of this miRNA in patients with mild preeclampsia were lower than in normal pregnancies. It is also possible that the inconsistent results represent the variable level of hypoxia used in the different experiments [19, 25]. Together, our data suggest that compensatory or redundant mechanisms [26-28] regulate placental adaptation to hypoxia in mice and in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The weight and morphology of mir-210 knockout placentas and fetuses are indistinguishable from those of wild-type mice.
- Exposure of mice to non-severe hypoxia (O₂=12%) between E11.5-E17.5 reduced placental miR-210 expression.
- miR-210 is likely dispensable for fetoplacental growth in normoxia or non-severe hypoxia.

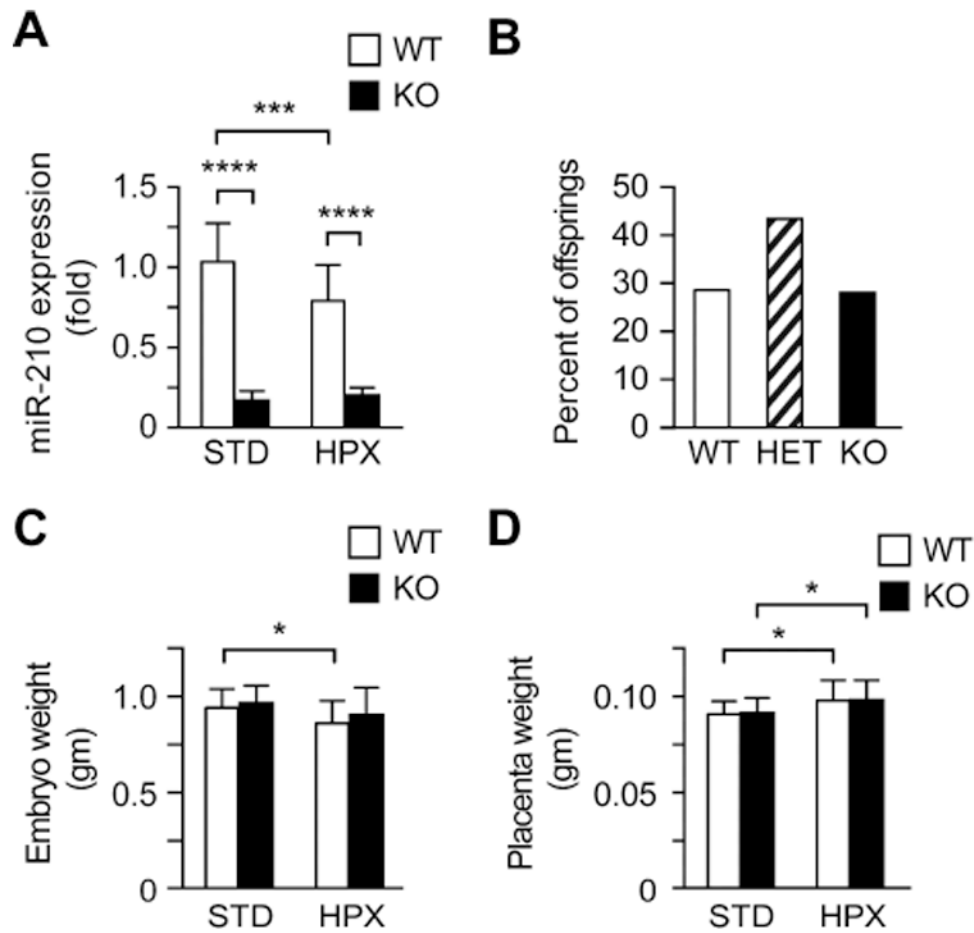


Fig 1. Characterization of the feto-placental phenotype in mir-210 ko mice exposed to standard conditions (STD) or to moderate hypoxia (HPX, O₂=12%)

(A) Relative miR-210 expression in wt vs ko placentas, normalized to expression in wt placentas in STD conditions (n=15-17/group). (B) Genotype distribution of fetuses generated by crossing mir-210 heterozygous males and females (n=11 pregnancies, Chi square test p=0.2193). (C) Mean weight (grams) of wt and ko embryos subjected to either STD or HPX (n=18-22 per condition). (D) Mean weight (grams) of wt and ko placentas subjected to either STD or HPX (n=18-22 per condition). Data presented as mean ± SD. * p<0.05, *** p<0.001, **** p<0.0001.

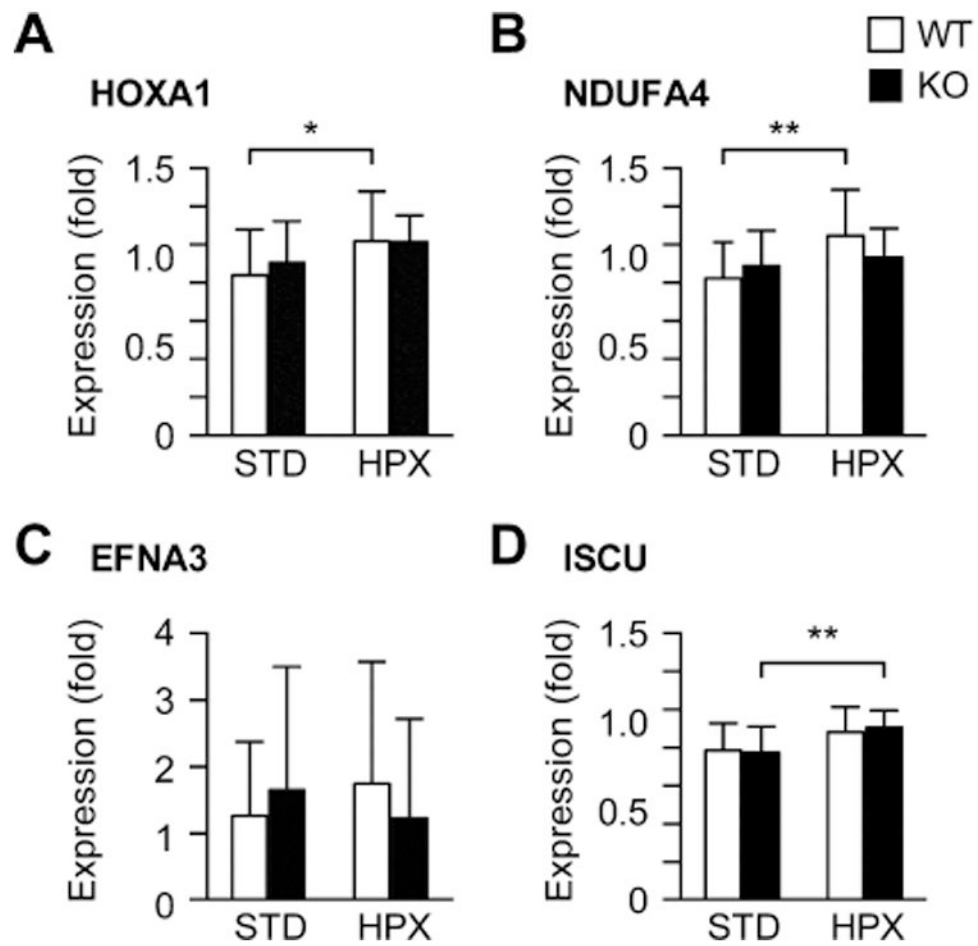


Fig 2. The relative expression of selected miR-210 target mRNAs in wt and ko placentas subjected to STD or HPX conditions (n=15-17 placentas in each condition) (A) HOXA1; (B) NDUFA4; (C) EFNA3; (D) ISCU. All data presented as mean \pm SD, relatively to L32. * p<0.05, ** p<0.01.