Roles of miR-1246, miR-146, and miR-155 and their Targets in Human Trophoblast Differentiation

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INTRODUCTION

Normal human placentation is crucial for embryonic development and successful pregnancy outcome. Defective placental implantation is believed to be the cause of a number of pregnancy-related diseases, including intrauterine growth retardation and preeclampsia (PE), a hypertensive disorder of pregnancy that is a leading cause of maternal and neonatal morbidity and mortality. The molecular mechanisms underlying normal and abnormal placental development remain incompletely defined.

The capacity of the human placenta to synthesize estrogens is increased greatly after the 9th week of gestation, in association with cytotrophoblast invasion and enlargement of the uterine spiral arteries, increased blood flow and O_2 availability. Placental estrogens are synthesized by aromatization of C_{19}-steroids produced by the fetal adrenals via the action of aromatase P450 (product of *hCYP19A1* gene). These estrogens, acting through estrogen receptor α (ERα), are believed to play an autocrine role in human trophoblast differentiation and hCYP19A1 expression. Trophoblast stem cells and cytotrophoblasts do not express *hCYP19A1*. However, when mononuclear cytotrophoblasts isolated from mid-gestation human placenta are cultured in a 20% O_2 environment, they spontaneously aggregate and fuse to form multinucleated syncytiotrophoblast and *hCYP19A1* is markedly induced **(Fig. 1**).

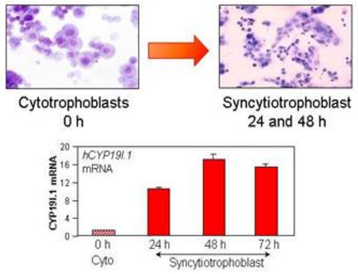


Figure 1. Expression of Placenta-Specific *hCYP19A1* (*hCYP19l.1*) is Induced During Human Trophoblast Differentiation

To further define the cellular and molecular mechanisms that underlie human trophoblast differentiation and induction of *hCYP19A1* gene expression, we have investigated the potential roles of microRNAs (miRNA, miR), small noncoding RNAs of 20-25 nucleotides in length. MicroRNAs repress gene expression by binding via their seed sequences to complementary sequences within the 3' untranslated regions (3'-UTR) of target mRNAs. This results in degradation of the mRNA target and/or inhibition of translation. Individual miRNAs can bind to and regulate networks of mRNAs with related biological functions and can act as rheostats or as on-off switches of gene expression, while multiple miRNAs can target a single mRNA.

To identify miRNAs involved in human trophoblast differentiation and the associated induction of aromatase/*hCYP19A1*, we performed miRNA microarray analysis of total RNA from freshly isolated human cytotrophoblasts, before and after differentiation to syncytiotrophoblast in primary culture. We observed that miR-146a/b-5p, miR-155 and miR-1246 were highly upregulated during trophoblast differentiation **(Fig. 3)**. In the present study, we confirmed the induced expression of these miRNAs, which are proven and/or predicted to target important inhibitors of trophoblast differentiation.

METHODS

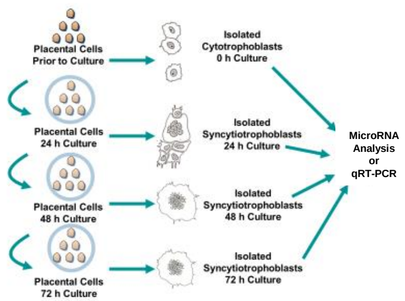
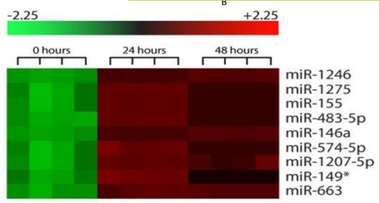


Figure 2. Isolation and Culture of Mid-gestation Human Placental Cells

Cytotrophoblasts were isolated from mid-gestation human placental tissues by trypsin digestion and Percoll density gradient centrifugation. Some of the cells were immediately harvested as starting cytotrophoblasts. The remaining cells were placed in monolayer culture for 24, 48 and 72 h and RNA was isolated at each time point using a miRNeasy® kit (Qiagen). The RNA was either subjected to miRNA microarray or analyzed for expression of miR-1246, miR-146, miR-155 by RT-qPCR using sequence-specific primers and Taqman probes (Applied Biosystems), and for their potential target, Jarid2, using SyBR Green RT-qPCR. Expression of placenta-specific exon I.1 of *hCYP19A1* was analyzed in parallel, as an index of trophoblast differentiation. Delta-Ct values were calculated for miRNAs relative to U6 RNA, as internal control; mRNA expression was analyzed using RPLP0 as internal standard.

Figure 3. miR-1246, miR-155 and miR-146a/b-5p are Dramatically Upregulated in Human Trophoblasts During Differentiation in Culture

A



A. RNA from freshly isolated cytotrophoblasts and from syncytiotrophoblast after 24 h and 48 h of culture was analyzed using miRNA microarray (LC sciences, Houston, TX). Statistical analysis of the microarray data revealed that miR-146a/b-5p, miR-155 and miR-1246 were markedly and significantly upregulated during trophoblast differentiation at 24 h and 48 h compared to 0 h. .

B

miRNA	Putative Target Genes of Interest (TargetScan, DIANA-mt, miRanda)
hsa-miR-1246	JARID2, AXIN2, GSK3B
hsa-miR-155	ZNRF3, JARID2, HIF1A
hsa-miR-146 a/b	ZNRF3, JMJD3

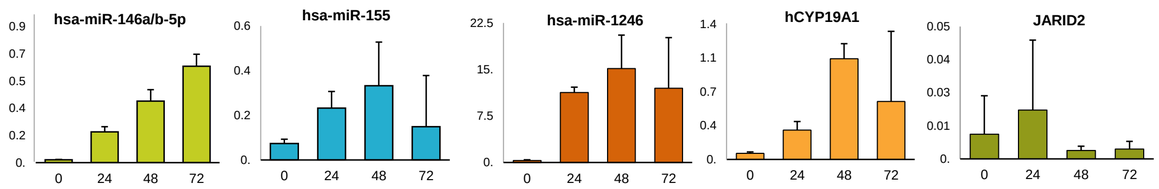
B. By comparative analysis of three target prediction programs (TargetScan 6.2, DIANA-mt, miRanda), a number of predicted target genes of these miRNAs were identified. The listed miRNA targets are known to be of importance in trophoblast differentiation.

Relevance of Predicted Targets

All three miRNAs are predicted to target components of Wnt/β-catenin signaling, which plays an important role in trophoblast differentiation. miR-146a/b-5p and miR-155 target ZNRF3, a ubiquitin E3 ligase that promotes degradation of the Wnt receptor complex, FZD/LRP5/6, while miR-1246 is predicted to target AXIN2 and GSK-3β, components of the cytoplasmic β-catenin 'destruction complex'. Collectively, these differentially regulated miRNAs, which target negative regulators of the Wnt signaling pathway, may serve as potent inducers of human trophoblast differentiation. Moreover, their aberrant expression can underlie placental pathogenesis leading to PE or intrauterine growth retardation.

miR-155 and miR-1246 also are known and predicted, respectively, to target JARID2, which recruits the polycomb repressive complex (PRC) 2 to developmentally-regulated genes. PRC2 catalyzes methylation of histone H3 on lysine 27. This chromatin mark promotes recruitment of the PRC1 complex, resulting in repressed chromatin. This causes transcriptional repression of developmentally-regulated genes. Since these miRNAs increase dramatically during human trophoblast differentiation, we postulate that their induction will inhibit JARID2 expression, which in turn, will result in increased expression of genes involved in trophoblast differentiation. In this study, we also analyzed changes in JARID2 expression during differentiation of cultured human trophoblasts.

Figure 4. miR-155 and miR-1246 are Induced with hCYP19A1 during Trophoblast Differentiation in Culture, while Target JARID2 Declines



To confirm the findings of our miRNA microarray, RNA isolated from mid-gestation human trophoblasts before and after culture for 24, 48 and 72 h was analyzed for miR-1246, -155 and -146a/b-5p using Taqman-based RT-qPCR. RNA from the same samples was analyzed for hCYP19l.1 and JARID2 using SYBR Green RT-qPCR. Data are the mean of 3 independent experiments analyzed in triplicate.

SUMMARY AND CONCLUSIONS

- Prior to differentiation, miR-146a/b-5p, miR-155 and miR-1246 are at expressed at low levels within cytotrophoblasts.
- As cytotrophoblasts fuse and differentiate to syncytiotrophoblasts, levels of all three miRNAs were confirmed to increase dramatically.
- miR-1246 and miR-155 peak at 48 h while miR-146 continues to increase with time in culture.
- Expression of JARID2, the putative and confirmed target of miR-1246 and miR-155, respectively, falls with syncytiotrophoblast differentiation.

FUTURE DIRECTIONS

- Analyze effects of overexpression of anti-miRs (antagonists) of miR-146a/b-5p, miR-155 and miR-1246 on differentiation of human trophoblasts in culture
- Validate JARID2 as a miR-1246 and miR-155 target
- Analyze expression of miR-1246, miR-155 and JARID2 in placentas from preeclamptic and gestationally-matched normotensive women at term.

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