MicroRNA-378a-5p inhibits the differentiation of cytotrophoblast into syncytiotrophoblast

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO
AUGUST 2013

ABSTRACT

MicroRNAs are expressed abundantly in the placenta throughout pregnancy. We have previously reported that miR-378a-5p promotes trophoblast cell proliferation and invasion. To further understand the role of miR-378a-5p during placental development, we determined if it may also regulate the differentiation of cytotrophoblast (CTB) into the syncytiotrophoblast (STB). Using BeWo cells, we found that miR-378a-5p was down-regulated during Forskolin-induced STB differentiation. Transfection of miR-378a-5p mimic into BeWo cells decreased the formation of multinucleated STB, mRNA levels of differentiation markers and E-cadherin levels. However, inhibition of endogenous miR-378a-5p by anti-miR-378a-5p increased cell fusion, induced STB marker gene expression and decreased E-cadherin levels. Luciferase reporter assay and real-time PCR analysis showed that CCNG2 is a target of miR-378a-5p. Taken together, these findings demonstrate that miR-378a-5p is a negative regulator of STB differentiation. This study also provides initial evidence that CCNG2 promotes trophoblast differentiation and may be involved in miR-378a-5p-regulated STB formation.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor, Dr. Chun Peng, for giving me this precious opportunity to join her lab. I had the privilege of doing this research under the most inspiring, dedicating and passionate supervisor. I greatly appreciate her guidance, support, motivation and encouragement throughout my program. Her insightful advice and knowledge are invaluable to me and allowed me to learn and think scientifically.

I would like to express my heartful gratitude to my supervisory committee member, Dr. Yi Sheng, for her valuable suggestions and encouragement. I would also like to thank my thesis committee members; Dr. Vivan Saridakis and Dr.Amy Wu.

I want to pay gratitude to all the lab members for their encourgament, advice, time and friendship. A special thanks to Dr. Gang Ye, for teaching me all the experimental techniques, offering his advice, answering all my questions and for Luciferase construct. To Dr. Guodong Fu, for teaching me qPCR and its trouble shoot. Thank you Mohammad for your helping hands, Luciferase construct and your constructive criticism to improve my skills throughout this journey. To Heyam Hayder, thank you for your friendship, valuable suggestions and advice for this program. I want to acknowledge Jelena Brkic and Stefanie Bernaudo for their valuable advice. To Yara Zayed, Shahin Khazai and Jasmina Uzunovic thank you so much for a happy sharing time! Thank you to all the previous and current

fellows for maintaining the lab and stock. I also want to show my gratitude to previous lab member, Lubna Nadeem. Thank you for your friendship, time, advice and suggestions in all the hard time.

I would like to express my gratitude to Adrienne Dome, our Graduate assistance, for her guidance, concerns, suggestions and time. In addition, I would also like to thank the funding support from Canadian Institute of Health Research and entrance Graduate Scholarship from York University.

Last but not least, I want to express my appreciation to my wonderful family for their love, care, encouragement and support. My parents and mother-in-law for their love and prayers. My sisters and brother for boosting my moral. My two lovely kids, Ahmed and Rafia, for their smiles, enduring patience, sacrifice, and giving me enough time to adjust in new environment and learn the techniques. Most of all, I want to truly thank my husband, Nadeem, for his never ending love, suggestions, cooperation, invaluable advice and lending an ear to all my problems. Without his consistent support, understanding and encouragement this task would have been impossible for me.

Thank you all of you!

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Acronym	Definition
DGCR8	DiGeorge Critical Region 8
EDTA	Ethylenediaminetetraacetic Acid
EVT	Extravillous
FBS	Fetal Bovine Serum
hCG	Human Chorionic Gonadotropin
HCl	Hydrochloric Acid
hGCM1	Human Glial Cells Missing -1
hPL	Human Placental Lactogen
HRP	Horse Radish Peroxidase
IGF-I	Insulin-Like Growth Factor-I
LGALS13	Lectin, Galactoside Binding, Soluble 13
LIF	Leukemia Inhibitory Factor
mRNA	Messenger RNA
PAGE	Polyacrylamide Gel Electrophoresis
PALP	Placental Alkaline Phosphatase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Pre-eclampsia
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
PKA	Protein kinase A
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Real Time Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RT-qPCR	Real Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
STB	Syncytiotrophoblast
TBST	Tris Buffered Saline/Tween 20
TNF-α	Tumor Necrosis Factor-Alpha
UTR	Untranslated Region

Chapter I. Introduction

I. MicroRNAs

MicroRNAs (miRNAs) are small (20-22 nucleotides), single stranded, non-coding regulatory RNAs that post-transcriptionally modulate the expression of their target genes (1). Generally, miRNAs negatively regulate gene expression by partial complementary binding to the 3' untranslated region (UTR) of target mRNAs, resulting in mRNA cleavage or translational repression (2). The mechanism of silencing or repressing mRNA is dependent on the degree of complementarity between the miRNA and the target sequence. Studies have demonstrated that miRNAs play important roles in cell proliferation (3-4), differentiation (5-6), apoptosis (4-6) and many physiological processes (7-9).

I.1. Discovery of microRNAs

Two decades ago (1993), Victor Ambros and his colleagues discovered the first miRNA, *lin-4*, in *Caenorhabditis elegans (C.elegans)* that controls the timings of post-embryonic development. Interestingly, they found that *lin-4* (22 nucleotide non-coding RNA) does not encode a protein product but represses the protein expression by binding to the 3'UTR of the *lin-14* gene (10). Seven years later, the second miRNA, let-7 (21 nucleotide non-coding RNA) was discovered in *C. elegans.* Subsequently, the let-7 homologs were detected in human, zebrafish, Drosophila, annelids and molluscs (11). A total of over one hundred additional miRNAs were identified in Drosophila, human and worms in less than one year after the discovery of let-7. Furthermore, most of the cloned

miRNAs are highly conserved in closely related species such as human and mouse (12-13) or *C. elegans* and *C. briggsae (14)* while others are broadly conserved among the animal lineages (15-16). For instance, more than one third of the *C. elegans* miRNAs are homologous with human miRNAs (13). To date, more than a thousand of miRNAs have been identified in humans and this number is still increasing. It has been reported that one miRNA targets many mRNAs (17) and many miRNAs can target one mRNA (17-18).

I.2. Biogenesis of miRNA

MicroRNA genes are transcribed into primary transcripts (pri-miRNAs) in the nucleus by RNA polymerase II (pol II). These primary miRNAs are either derived from the introns of protein coding genes, exons of noncoding genes, or from intergenomic regions within the genome (35). Next, pri-miRNAs which are greater than 1000 bases are polyadenylated and capped with 7-methylguanosine are folded into hairpin structures (1, 19). Pri-miRNAs are processed and cleaved into stem-loop precursor miRNAs (pre-miRNAs, 60-100 nucleotides) by the nuclear RNase III enzyme Drosha and its coactivator DGCR8 (20). The pre-miRNAs are transported from the nucleus into the cytoplasm through the interaction with Ran-GTP and receptor Exportin-5. The pre-miRNA is further cleaved by a cytoplasmic RNase III enzyme Dicer. Dicer is a member of the RNase III superfamily of bidentate nucleases and its function is to remove the base pairs and the loop structure of pre-miRNA and form a 22 nucleotide double stranded duplex miRNA:miRNA* (21). Finally, the RNA duplex unwinds and the single stranded mature miRNA is incorporated into the RNA-induced silencing complex (RISC). Argonaute

proteins are the core component of the RISC complex, acting as target recognition modules of the RISC (22-25). The incorporation of miRNA into the RISC regulates the expression of target genes by binding to complementary sequences in the 3'UTR of target mRNAs following the Watson and Crick principle of base pairing (23). Therefore, gene silencing is based on the degree of miRNA-mRNA complementarity which takes place in the seed region of miRNA and target mRNA. MicroRNA which has a perfect or near perfect complementarity with the target mRNA results in mRNA degradation while imperfect or partial complementarity between miRNA and target mRNA results in inhibition of protein synthesis (26). However, it has been reported that some miRNAs can bind to the target sites in the 5'UTR as efficiently as 3'UTR and still successfully repress the target mRNAs (27) [Fig. 1].

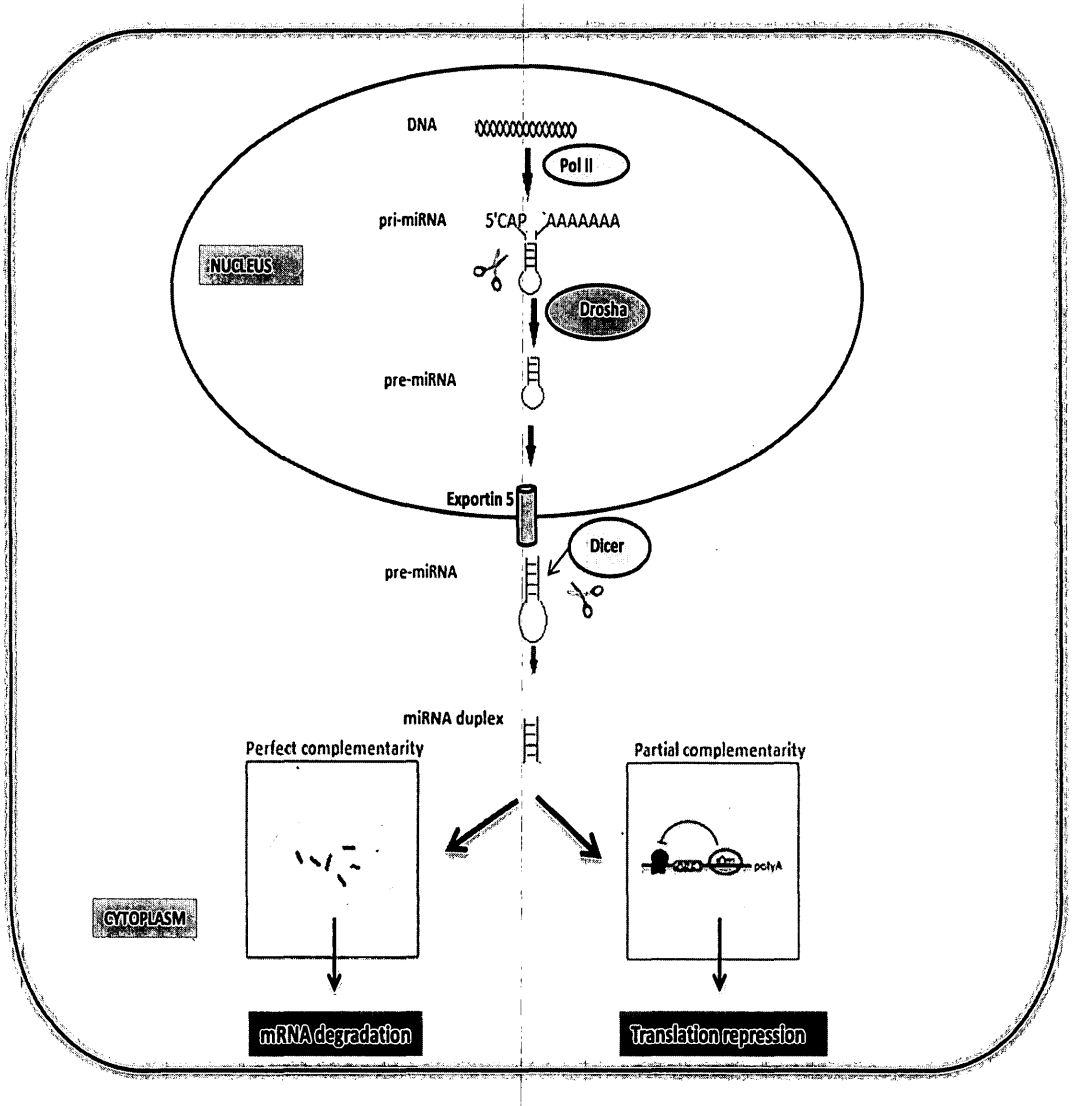


Fig 1. Overview of microRNA biogenesis and regulation. MicroRNA genes are transcribed into primary transcripts (pri-miRNAs) by RNA polymerase II (Pol II). Pri-miRNAs are cleaved by Drosha into precursor miRNAs (pre-miRNAs) that have a stem-loop structure. Pre-miRNAs are exported to the cytoplasm where they are further cleaved by Dicer and form miRNA duplexes. The duplex is unwinded and mature miRNA is incorporated into the RISC complex, binds to the 3'UTR of target mRNAs and induces gene silencing either by causing mRNA degradation or translation inhibition.

I.3. Classification of miRNAs

Discovery of miRNA opened a new chapter in the field of molecular biology. Victor Ambros presented universal guidelines for the identification and annotation of newly discovered microRNAs. Moreover, he suggested that miRNA must follow specific expression and biogenesis criteria (89). The naming criteria of miRNA follows the rule of three –latter prefix, capitalization, hyphenation and italics. The pre-miRNA is annotated by "mir". Mature miRNAs used a "miR" prefix and followed a unique identification number (e.g., miR-378) which are assigned sequentially and not depends on the organism. Therefore, the identical miRNAs have the same number. Letter preceding the "miR" prefix represents the organism in which miRNA is found. For example, hsa-miR-378 refers to *Homo sapiens*. A letter following the identification number represents its relation to another miRNA. These two miRNA are slightly different in sequence from each other by 1 or 2 nucleotide (i.e. miR378a is related to miR378b) (89).

Based on thermal stability it was thought that one strand of miRNA is biologically active and referred as miR, and the other was considered as an inactive strand and that strand was called miR* (miRNA star or passenger strand). It was believed that miR* will be degraded and act as a minor product in some cell lineages. However, more recent work have shown that in many cases, both strands of miRNA are functional and therefore, mature miRNAs from the same pre-miRNA are named as miR-5p and miR-3p (90).

I.4. MicroRNA target prediction and validation

Identification of miRNA target genes is very challenging because of the partial complementarity sequence between the target mRNAs and miRNAs. Therefore, both computational and experimental approaches are needed to identify miRNA target genes (77). Bioinformatic prediction analysis of target genes is mainly based on sequence matching between miRNA and mRNA, duplex thermal stability, secondary structure of the miRNA/mRNA duplex, three-dimensional complex analysis and conservation of the target site (78). Several computational prediction programs, such as miRanda, TargetScan, TargetScanS, RNAhybrid, DIANA-microT, PicTar, RNA22 and FindTar, are available to identify potential target genes.

The validation of a predicted target gene of a miRNA can be done by experimental approaches, including reporter assays, western blot analysis, and real-time PCR. The most direct method to confirm a miRNA target gene is the Luciferase reporter gene assay. In this assay the luciferase coding sequence is fused with the 3'UTR of a potential target gene. If the 3'UTR has a specific target site of a particular miRNA, expression of the miRNA would result in a decrease in the luciferase activity (79). In addition to luciferase assay, quantitative real-time PCR and Western blot methods are commonly used to detect the mRNA and protein levels respectively in miRNA transfected or knock-down cells. These approaches are accurate to quantify the changes in mRNA and protein level of a miRNA target genes (78-79).

I.5. miR-378a-5p

The miR-378a-5p was originally cloned from promyelocytic leukemia and named miR-422b (80). Landgraf *et al.* later showed that the miR-422b and miR-422a loci are unrelated and thus miR-422b was renamed as miR-378 (81). This miRNA was later called miR-378* but more recently, several miRNAs related to miR-378 were characterized and thus miR-378* was renamed to miR-378a-5p.

It has been reported that miR-378a-5p plays important roles in regulating cellular processes. Recently, our lab has demonstrated that miR-378a-5p is predominantly expressed in first and second trimester human placenta and it promotes trophoblast cell survival, migration and invasion by targeting Nodal (82). In porcine ovary, miR-378a-5p is expressed in granulosa cells and decreases the aromatase expression. Aromatase (estrogen synthetase) is a key enzyme in the biosynthesis of estradiol and play an important role in ovarian follicular development and is required for female reproduction (83). It is also a significant regulator in skeletal muscle differentiation (84). In addition, miR-378a-5p regulates nephronectin (extracellular matrix protein) mediated differentiation in the osteoblastic cell line (85) and promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression (86). Furthermore, miR-378a-5p has been identified as a novel target of the c-Myc oncoprotein and promotes cellular transformation (87). Finally, it is strongly expressed in the mammalian heart and controls cardiac hypertrophy by suppressing the MAP kinase signaling pathways (88).

II. Human Placenta

Placenta plays critical roles throughout the entire pregnancy and supports the growth and development of the fetus. Serving as the interface between the maternal and fetal environment, the placenta is involved in the exchange of respiratory gases, nutrients and waste products between the mother and the growing fetus (36). Moreover, it is an endocrine organ which produces a number of pregnancy- associated hormones and growth factors that regulate maternal physiology and fetal development. Furthermore, placenta helps to prevent the rejection of fetal allograft (37). Therefore, placenta plays a vital role in the survival and health of the embryo.

II.1. Implantation

Development of placenta is an extremely regulated and continuous process that begins at the time of fertilization. During embryonic development, the fertilized egg undergoes a series of mitotic divisions and forms a ball of cells called "Morula". Compaction of morula is the first event of morphogenic and cellular differentiation. Cells in morula align themselves in the outer peripheral region and create a fluid filled cavity called "Blastocyst". This blastocyst consists of two primary cell types, the outer trophoectoderm cells called trophoblast, which later form the placenta and fetal membranes, and the inner cell mass forms the embryo (38).

Once the blastocyte reaches the uterine cavity it orients its pole (inner cell mass) towards the uterine epithelium and attaches to the uterine wall. The process of implantation is accomplished by the highly adhesive and invasive trophectodermal cells of the blastocyst (trophoblast) which appose, attach and finally invade the uterine epithelium. Invasion results in the digestion of the epithelial membrane and as a result, the blastocyst reaches the endometrial stroma (38, 39) [Fig. 2].

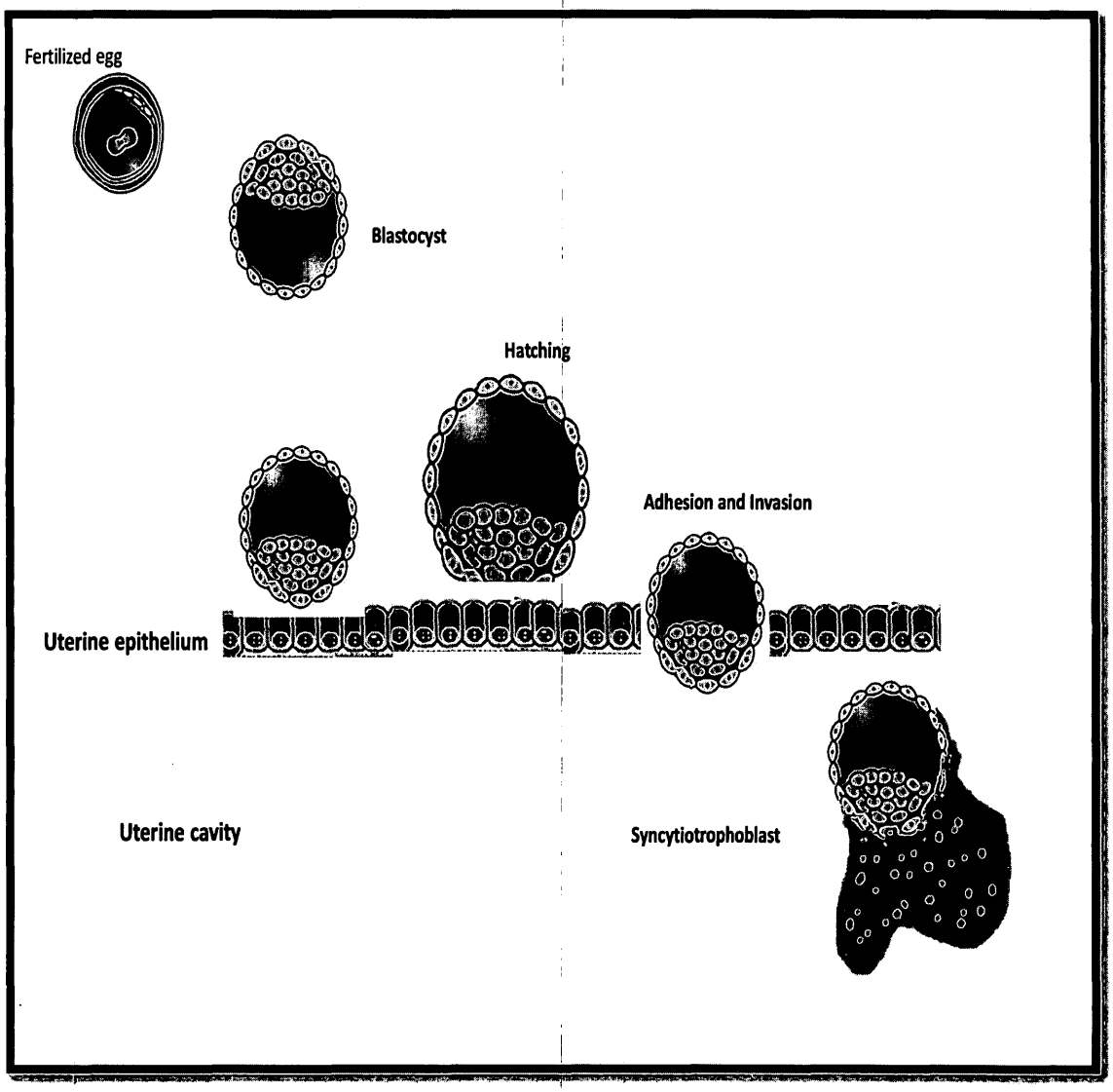


Fig 2. Blastocyst implantation steps. A series of mitotic divisions convert the fertilized egg to morula. The cells in morula align themselves and form blastocyst. Once the blastocyte reaches the uterine cavity, it starts hatching. Later on, adhesion and invasion result in digestion of uterine epithlium and it finally implants itself in the uterus. Mononuclear cytotrophoblasts fuse to form a multinucleated syncytium or syncytiotrophoblast. The image was drawn based on (Bischof P 2005).

II.2. Trophoblast Differentiation

Trophoblasts, the precursor cells of placenta, play a vital role in the embryo implantation and placental development. Trophoblast progenitor cells differentiate into two distinct pathways (extravillous and villous). In the extravillous (EVT) or invasive pathway, cytotrophoblasts (CTB, inner layer of trophoblast) proliferate and differentiate into an invasive phenotype and penetrate into the maternal decidua and myometrium. In the villous or syncytial pathway, fusion of mononuclear CTB results in the formation of a multinucleated structure called syncytium or syncytiotrophoblast (STB) and this process is referred as syncytialization (40-41) [Fig 3].

The multinucleated STB is formed by the fusion of underlying mononucleated CTBs and it covers the floating villi surrounded by the maternal blood. As the STB is non-proliferative and lacks the ability of nuclear division, donation of CTBs is essential for syncytial health and function (37). The STB is responsible for nutrient and gas exchange (42). Moreover, it is the site of synthesis of various growth factors and hormones, such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL) (42-43). STB is the main source of progesterone during pregnancy and its level of synthesis gradually increases over the entire pregnancy period (44).

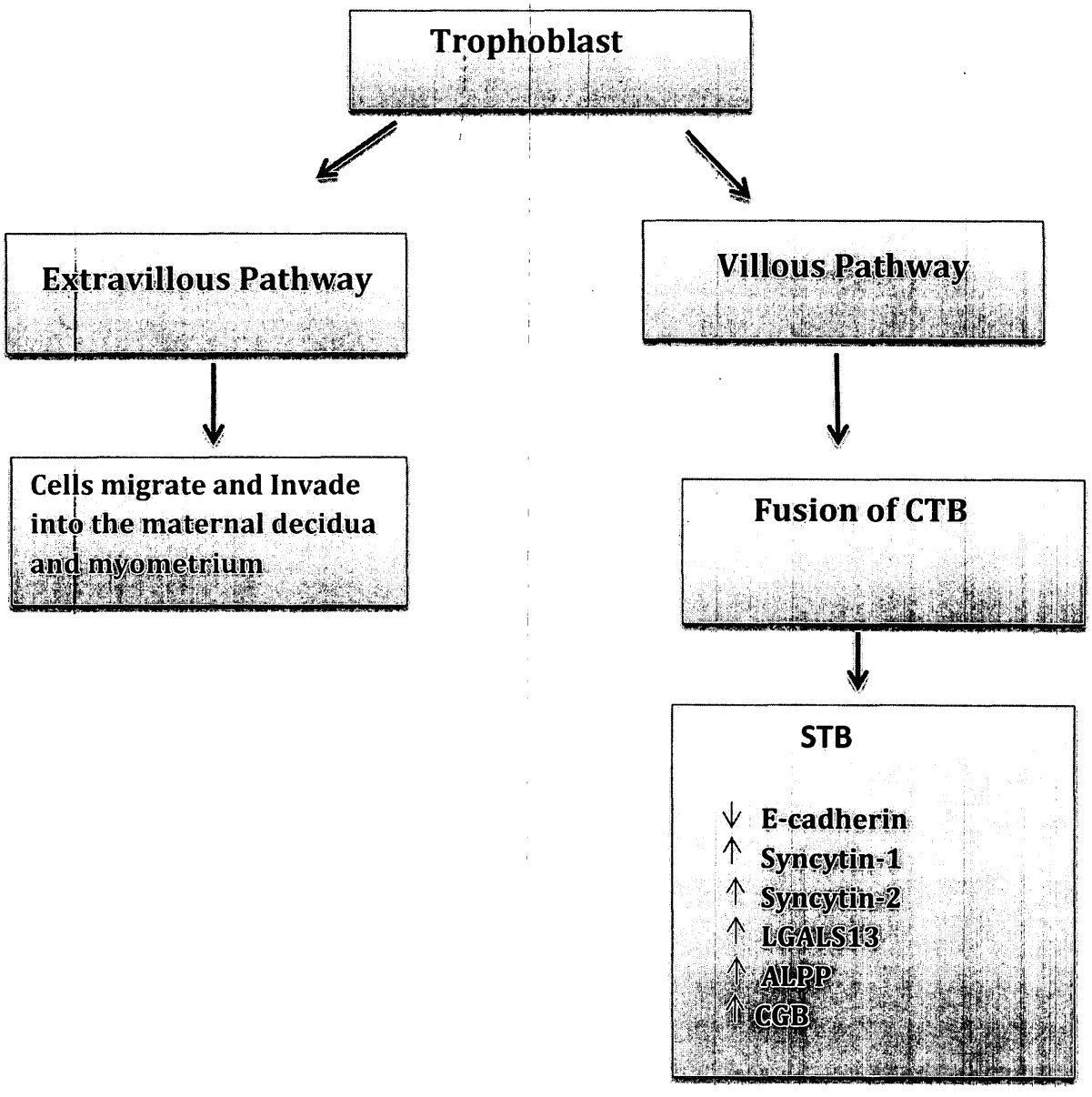


Fig 3. Trophoblast differentiation pathways. Trophoblast differentiates into two distinct pathways during placental development. In the extravillous or invasive pathway cells proliferate, migrate and finally invade into the uterus. In the villous or fusion pathway mononuclear cytotrophoblast (CTB) fuse to form a multinucleated syncytium called syncytiotrophoblast (STB). Formation of STB is associated with changes in expression of marker genes.

Therefore, syncytiotrophoblast formation plays vital roles in human placentation and any disturbance in the trophoblast differentiation could result in pregnancy complications. For example, defective or retarded syncytium formation produces severe pathological conditions such as Down's syndrome or Trisomy of chromosome 21 which occurs because of the hyperactivity of a cytoplasmic enzyme (coded by a gene which is located on chromosome 21) and impairs the trophoblast differentiation towards fusion (46).

Differentiation of CTB into STB can be studied by morphological (aggregation and fusion of cells) and biochemical changes (expression of genes) (17). Morphological differentiation can be studied by microscopic observation of cell fusions through immunofluorescent staining using antibodies against cell surface molecules, such as E-cadherin (17, 65). Loss of E-cadherin is a marker for STB differentiation as E-cadherin is reduced when CTBs are fused to form STB (65-66). During STB differentiation, cells begin to express genes that synthesize and secrete a vast array of endocrine products such as hCG and hPL (57). In addition to these hormones, a number of genes are commonly used as markers of STB differentiation, such as syncytin-1 and 2, galactoside-binding soluble lectin 13 (LGALS13) and placental alkaline phosphatase (ALPP).

II.2.1. Human Chorionic Gonadotropin (hCG) and Placental Lactogen (hPL)

Human Chorionic gonadotropin (hCG) belongs to the glycoprotein family and is composed of α and ß subunits. The hCG has paracrine effects on several processes such as implantation and angiogenesis (58). Moreover, it rescues the corpus luteum from involution by maintaining progesterone secretion (59). Since hCG is produced from the STB, it is used as a marker of syncytialization. Human placental lactogen (hPL) is a polypeptide hormone and is secreted from the STB throughout the pregnancy. It alters the maternal carbohydrate and lipid metabolism for fetal nutrient requirement (59,125).

II.2.2. Syncytin-1 and Syncytin-2

Human endogenous retroviruses (HERVs) constitute about 8% of the human genome and several of HERVs are expressed in the placenta (60). Studies have demonstrated that the trophoblast expresses the two fusogenic retroviral envelope proteins, Syncytin-1 (encoded by HERV-W) and Syncytin 2 (encoded by HERV-FRD) (61). It has been reported that Syncytin-1 plays an essential role in the formation of STB and a decrease in the expression of Syncytin-1 has been associated with pregnancy-associated anomalies, such as preeclampsia (60). Syncytin-2 is also placental specific and plays a vital role in trophoblast cell fusion (62).

II.2.3. LGALS13

The placental-specific Lectin, Galactoside Binding, Soluble 13, LGALS13, (also known as PP13) belongs to the galectin family and is predominantly localized to the STB apical membrane (63). It has been suggested that LGALS13 can be used as a potential serum marker for the diagnosis of pregnancy complications as its levels are reduced in patients with pre-eclampsia (68).

II.2.4. Placental Alkaline Phosphatase

Placental Alkaline Phosphatase (ALPP or PALP) is an enzyme produced by STB during pregnancy, and therefore used as a marker of STB differentiation (64). It is a membrane bound glycosylated enzyme responsible for dephosphorylation of many proteins. ALPP is synthesized by placenta throughout the entire gestation period. ALPP is localized at the apical and basal plasma membrane in term villous trophoblast (67) and is used as an apical marker enzyme for the isolation and purification of placental brush border membranes (67).

II.3.Regulation of syncytiotrophoblast differentiation

Primary cultures of CTB cells or cell lines, such as BeWo, can be induced to differentiate into STB by pharmacological agents that elevate intracellular cAMP levels

(48-49). Many factors, including transcription factors, hormones and growth factors, and miRNAs have been shown to regulate STB differentiation (40).

II.3.1.Transcription factors

Syncytial fusion is stimulated by transcriptional factors such as Glial cells missing - 1 (GCM-1). GCM-1 is localized within the nuclei of a subset of CTBs cells (51) and involved in the transcription of syncytin and placenta specific genes. Transcription factors of the activating protein-2 (AP-2) family members are regulated by cAMP and play important roles in the syncytial fusion. The AP-2 transcription factors family members are required for the expression of the syncytium specific genes such as CGB, hPL or CYP19 (91). Moreover, PPARγ regulates the STB differentiation in a ligand-dependent manner. PPARγ interacts with the promoter region of the CGβ-5 gene and affects CGβ transcription, thus controls the syncytium formation (92-93).

II.3.2. Hormones and growth factors

Trophoblast differentiation into the STB pathway is stimulated by hCG, glucocorticoids and estradiol. Human chorionic gonadotropins act as an autocrine, paracrine, intracrine and endocrine regulator and promote syncytial fusion, thus able to self-regulate the mRNA levels of its own subunits. Estradiol is produced by the fetoplacental unit and is very important hormone because numerous studies have shown that it can modulate placental hormone production (94).

Growth factors, such as colony stimulating factor-1(CSF-1), and insulin-like growth factor-I (IGF-I), promotes the trophoblast differentiation into STB (95). On the other hand, tumor necrosis factor-alpha (TNF-α) impairs the expression of CGß subunit (54), while transforming growth factor-ß1 (TGF ß1) inhibits hCG and hPL secretion (56), suggesting that these growth factors inhibit STB differentiation. Interestingly, leukemia inhibitory factor (LIF) enhances trophoblast fusion but decreases CGß mRNA levels (55). This study, together with a previous report (66), suggests that distinct signalling pathways are involved in cell fusion and CGß expression.

II.3.3.MicroRNA

Recent genome-wide analysis of miRNA (miRNAome) by miRNA array has identified 600 miRNAs in healthy term placenta (17). While the function of most of these miRNAs is unknown, recent studies have shown that miRNAs regulate cell proliferation, migration and invasion. For example, miR-210, miR-37a, and miR-155 inhibit cell proliferation, invasion and migration while miR-378a-5p, miR-376c and miR-195 had the opposite effects. (17-18). In addition, miRNAs have also been implicated in the regulation of STB differentiation by targeting various genes (45). It has been recently reported that miR-19b and miR-106a directly target aromatase expression (estrogen synthesis enzyme) and impair STB differentiation. MicroRNA-19b also targets human GCM1 (hGCM1) to inhibit trophoblast differentiation into STB (47). Similarly, miR-20b also inhibits syncytialization (96).

IV. Cyclin G2

Cyclins play a key role in the regulation of cell cycle by activating the cyclin – dependent kinases (CDKs) enzymes. Mammalian cyclins are classified on the basis of structural similarity and functional, and regulation expression pattern into twelve different types (cyclins A to I) (69). Human cyclin G2 belongs to the cyclin G family of proteins also comprised of cyclin G1 and cyclin I. These cyclins are known as unconventional cyclins because unlike other cyclins, they do not form complexes with any cyclin- dependent protein kinases and they inhibit cell cycle progression (70).

Cyclin G2 (CCNG2) is up-regulated during growth inhibition or apoptosis. (71). CCNG2 is quickly degraded through the ubiquitin–proteasome pathway (72). The short half-life of CCNG2 may provide a way for the quiescent cells to re-enter the cell cycle. Therefore, increase in cyclin G2 expression inhibits the cell cycle progression and maintain the cells in a differentiated state (73).

The up-regulation of cyclin G2 expression at protein and mRNA level has been reported in the process of human adipocyte differentiation process (70). The mechanism by which cyclin G2 induces adipocyte differentiation is by direct binding to the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) and increasing the transcriptional activity of PPARγ (74). The PPARγ is a ligand-activated transcription factor and controls the expression of genes which are involved in different physiological processes, including adipogenesis and glucose homeostasis (75)

Recently, CCNG2 was reported to be expressed in human placenta with an increased level in mid-gestational stage and then decreases sharply at term (76). Immunohistochemical analysis of CCNG2 in placenta revelaed that it is highly expressed in term placentae obtained from compromised pregnancy, such as pre-eclampsia (PE) and gestational diabetes mellitus (GDM) as compared to normal gestation placentae (76). Although the function of CCNG2 in placenta is unknown, its high expression level suggests that it may play a role in placental development (76).

V. Objectives of the study

MicroRNAs have been detected in human placenta and recent studies have suggested that they play important roles in regulating placental development and that their dysregulation is associated with various gestational disorders (29). Our lab has demonstrated miR-378a-5p promotes trophoblast cell proliferation migration and invasion (82). The objective of this study is to further understand the role of miR-378a-5p during placental development. Specifically, I examined if miR-378a-5p regulates trophoblast cell differentiation along the STB pathway and explored the potential mechanisms by which miR-378a-5p controls STB differentiation.

Chapter II. Material and Methods

II.1 Materials:

Dulbecco's modified Eagle's medium (DMEM) was purchased from (Hyclone), Ham's F12 and Fetal Bovine Serum (Gibco), penicillin and streptomycin (Life Technologies), Trypsin (0.2% Gibco), Forskolin (Sigma-Aldrich). Bovine Serum Albumin (BSA) (Sigma), ECL (Millipore), TRIzol Reagent (Life Technologies), M-MuLV Reverse Transcriptase (New England), oligo dT primer (Sigma), SsoFast EvaGreen Supermix (Bio-Rad), Dual Luciferase Reporter Assay System (Promega).

II.2. BeWo cell culture

BeWo cells, human choriocarcinoma cell line, were purchased from American Type Culture Collection (ATCC). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in a 1:1 mixture of DMEM and Ham's F12, and supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin and 100μg/ml streptomycin. Cells between passage 10 and 15 were used in all the experiments.

II.3. Induction of BeWo cell differentiation with Forskolin

Syncytialization of BeWo cells was induced with Forskolin. Forskolin was prepared in 100% DMSO and added to the medium with the final concentration of 50 μM. Controls were treated with the same concentration of DMSO that serves as the vehicle for Forskolin. BeWo cells were treated with or without Forskolin in 1:1 DMEM/F12 medium, supplemented with 1%FBS.

II.4. Transient transfection

Transient transfection of plasmids, miRNA mimics, inhibitors or siRNA were carried out using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's suggested procedures (73). MicroRNA mimics, inhibitors and siRNA were purchased from GenePharma Co. (Shanghai, China). Generation of CCNG2 expressing plasmid (pcDNA4-CG2-V5) was reported previously (72). Luciferase reporter constructs containing different regions of the CCNG2 3'UTR were generated by inserting a 3'URT fragment into the MCS (multiple cloning site) region of pMir Report luciferase vector downstream of the Luciferase coding gene. Construct 1 contains the first predicted site, and construct-2 contains the other three predicted site of miR-378a-5p.

II.5. Protein extraction and Immunoblotting (Western blot)

Cells were washed with cold PBS and cell lysate was prepared using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1% SDS) containing protease inhibitor cocktail. Cell lysates were centrifuged at 12,000g for 20 minutes at 4^{O}C. Protein concentrations of the cell lysate was measured by Bicinchoninic acid (BCA) method using bovine serum albumin as a standard. Equal amount of protein samples (30ug) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF, Bio Rad) membrane. The PVDF membranes were blocked with 5% milk in TBST (10mM Tris-Cl pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 hour at room

temperature and then incubated with primary antibodies in the blocking buffer overnight at 4 °C. Primary antibodies used were: anti-E-cadherin (1:500, Santa Cruz), anti-Syncytin-1 (1:500, Santa Cruz) and anti-GAPDH (1:5000, Santa Cruz). The membranes were washed with TBST and subsequently probed with HRP-conjugated secondary antibody at room temperature for 1 hr. Signals were detected using ECL according to manufacturer's protocol.

II.6. Immunofluorescence

BeWo cells were seeded on the coverslips in a 12-well plate at the density of 4 x 10^5 /well. The next day, cells were treated with 50μM Forskolin or transiently transfected with miR-378a-5p/anti-miR-378a-5p at different time points. Cells were fixed in methanol (-20°C) for 10 minutes and washed with PBS (x 3). Non-specific bindings were blocked with 3% BSA in PBS for 1 hour and then incubated with primary mouse monoclonal anti-E-cadherin antibody (1:50 dilution in 3% BSA in PBS) overnight at 4 °C. The coverslips were washed with PBS buffer (x3) and incubated with goat anti-mouse secondary antibody, Alexa Fluor 594, (1:300 dilution in 3% BSA in PBS, Life Technologies) at room temperature for 1 hr. Cell nuclei were counterstained with DAPI (1:1000, Sigma) for 5 minutes. Finally, coverslips were washed and mounted. Coverslips were examined with the inverted fluorescence microscope and photographed using a Nikon camera and Simple PCI software. Cell fusion was analyzed in randomly 20 selected field of each condition in triplicates and scored when ≥ 3 nuclei shared the

same cytoplasm. The quantification of cell-cell fusion was expressed in percentage and calculated as the ratio of number of nuclei in the syncytia to the total number of nuclei counted in the field.

II.7. RNA extraction and RT-qPCR

Total RNA was extracted from the cultured cells and isolated with Trizol Reagent according to manufacturer's instructions. Two microgram of total RNA was used to synthesize first stand cDNA by M-MuLV Reverse Transcriptase. RT-qPCR was carried out using gene specific primers (Table 1) and SsoFast EvaGreen Supermix. The expression levels of mRNA were normalized to GAPDH. The qPCR reactions were carried out using primer sets indicated in Table 1, with initial denaturation step at 95°C for 1 minute and followed by 40 cycles of 20 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Ct values were used to calculate the expression levels of gene expression by the standard ΔΔCt method.

II.8. miR-378a-5p detection by TaqMan assay

Total RNA was isolated with Trizol reagent from the cultured cells with a slight modification in the protocol as previously reported. Specifically, the precipitation of RNA was carried out at −20°C overnight to enrich the small RNA population (123). Both reverse transcription and qPCR were carried out according to the manufacturer's instructions. Reverse Transcription was carried out by TaqMan

Table 1 Primers used for qPCR: Syncytin-1, Syncytin-2, ALPP, LGALS13 and CGB with their forward and reverse primers.

Primer sets	Sequence
Syncytin-1	
Sense	TCATATCTAAGCCCCGCAAC
Antisense	TGATCTTGCAAGGTGACCAG
Syncytin-2	
Sense	TCG GAT ACC TTC CCT AGT GC
Antisense	GTATTCCGGAGCTGAGGTTG
ALPP	
Sense	ACGGGAAGAATCTGGTGCAG
Antisense	GTGGAGTCTCGGTGGATCT
LGALS13	
Sense	ATTGCCTTCCGTTTCCGAGT
Antisense	TTTGCCATCCTCAAAGGGCA
CGB	
Sense	CCGTCAACACCACCATCTGT
Antisense	ATTGACAGCTGAGAGCCACG

microRNA Reverse Transcription Kit and subsequently qPCR was performed for the detection of microRNAs. U6 was used as a housekeeping gene and amplified for each sample. The relative expression of miR-378a-5p was determined using standard ΔΔCt method.

II.9. Luciferase assay

BeWo cells were seeded in 12-well plates at the density of $8x10^5$ cells/well and co-transfected with 25 nM of negative control or miR-378a-5p mimics, 0.175 μg pMIR-Report-CCNG2 3'UTR plasmids, and 0.1 μg pRL-TK internal control (encoding Renilla luciferase) plasmids. Five hours after transfection, cells were recovered in DMEM/F12 for another 19 hours. Twenty-four hour of post transfection, luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

II.10. Statistical analysis

All the experiments were done at least three times with triplicate in each group. The results are expressed as mean ± SEM. Statistical analysis was performed using Sigma Stat software. The experiments with multiple groups were analyzed by one-way analysis of variance, with Student-Newman-Keuls post hoc test to determine the statistical significant differences among the individual groups. Student's *t*-test was used for comparison between two groups. $P<0.05$ was considered statistically significant.

Chapter III. Results

III.1. Forskolin induces syncytiotrophoblast differentiation

The choriocarcinoma BeWo cell line is the most extensively used *in vitro* model to study syncytialization because of the high degree of similarity to normal placental trophoblast (66). It is well documented that activation of the cAMP pathway, such as addition of cAMP analogue 8-bromo-cAMP or the activator of adenylate cyclase Forskolin, induces BeWo cell fusion and STB marker gene expression (98). In order to establish a positive control for our differentiation assays, BeWo cells were treated with control and different concentrations of Forskolin (25 μM, 50 μM and 100 μM) for 48 hours. Immunofluorescent staining with an E-cadherin revealed that Forskolin strongly induced cell fusion (Fig. 4). Quantification of the number of cells with 3 or more nuclei indicated that 50 μM of Forskolin is the most effective dose in inducing cell fusion. Therefore, this dose of Forskolin was selected and used for further experiments as a positive control.

To determine the time course effect of Forskolin BeWo cells were treated with DMSO (control) or 50 μM dose of Forskolin for 24, 48 and 72 hours. Western blot using an anti-E-cadherin antibody revealed that E-cadherin levels were decreased significantly by Forskolin at 48 and 72 hours after treatment (Fig. 5A). Forskolin also induced the mRNA level of Syncytin-1, a marker gene of STB, at the 48 and 72 hour time points (Fig. 5B).

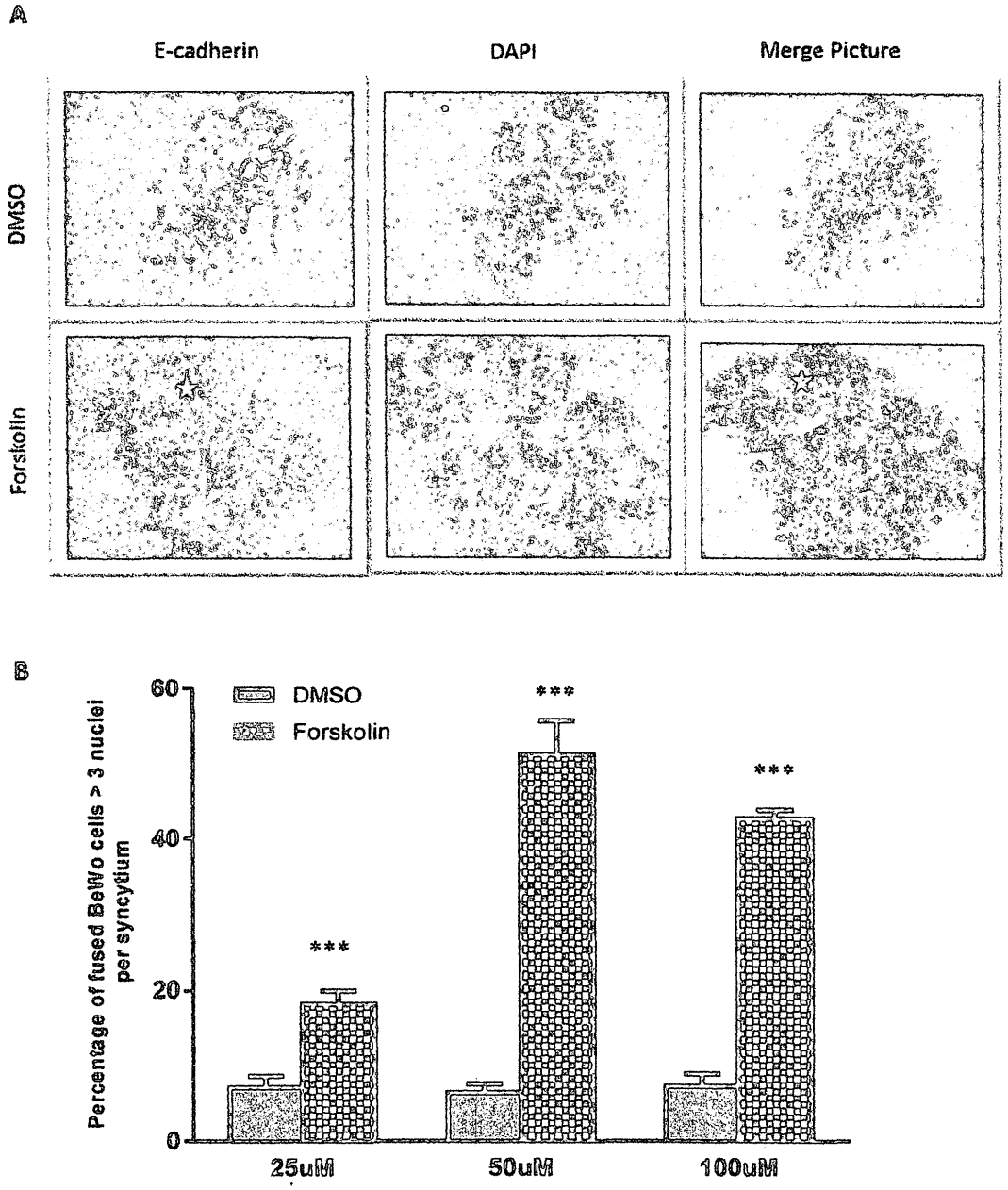


Fig. 4. Forskolin induces trophoblast cell fusion. A) Representative pictures show BeWo cells treated with vehicle only (DMSO) and Forskolin (50 μM) for 48 hours and immunofluorescence was performed. Cell membranes were stained with anti-E-cadherin (red) and cell nuclei were stained with DAPI (blue). Merged pictures were shown in the right panel *, fused nuclei in the merged picture were marked with stars. B). The Immunofluorescence was performed on cells treated with DMSO or different concentrations of Forskolin (25, 50 and 100μM) for 48 hours and fused nuclei were counted. The graph shows that 50 μM is the most effective dose in inducing cell fusion. ,***P<0.001 vs. DMSO control. Data represent mean ± SEM of 3 experiments.

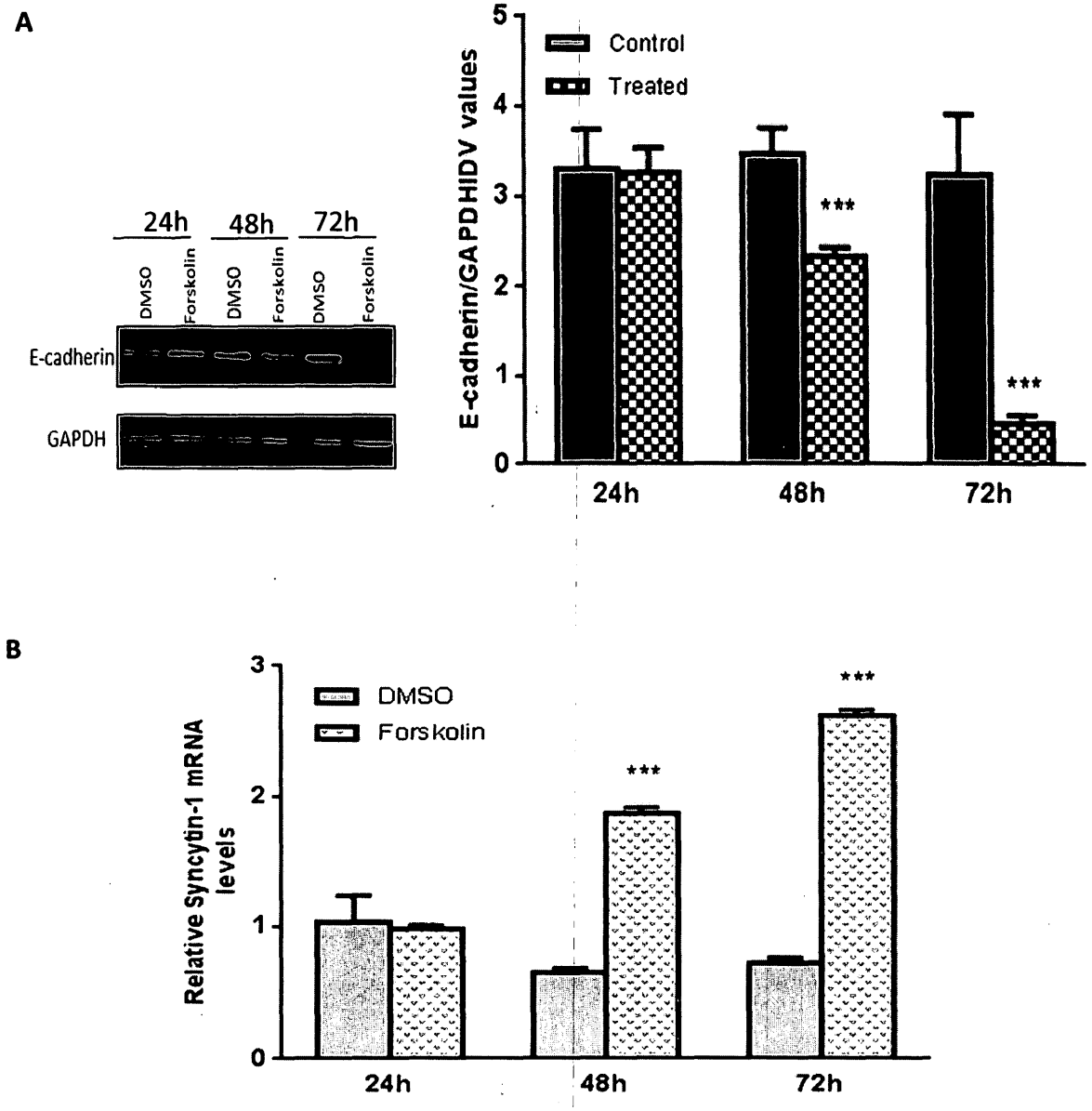


Fig. 5. Forskolin decreases E-cadherin protein expression and increased Syncytin-1 mRNA levels. Cells were treated with DMSO as the control or Forskolin for 24, 48 and 72h. A). A representative blot of E-cadherin and GAPDH and a summary graph from 3 experiments showing that Forskolin inhibited E-cadherin expression at 48 and 72h after treatment. ,***, $p \leq 0.001$ vs. control. B). RT-qPCR of syncytin-1 from BeWo cells treated with DMSO or Forskolin for 24 to 72 hours. Forskolin significantly increased syncytin-1 mRNA levels at 48 and 72h after treatment. ***, $p \leq 0.001$ vs. DMSO control. Data represent mean ± SEM of 3 experiments.

III.2. miR-378a-5p is suppressed during Forskolin-induced syncytialization

To investigate the potential involvement of miRNAs in STB differentiation, I determined the level of several miRNAs in BeWo cells treated with Forskolin and DMSO controls for 24, 48 and 72 hours. Real-time PCR was used to quantify miRNA levels. There was a significant decrease in the endogenous level of miR-378a-5p in the Forskolin-treated group at 48 and 72 hours, as compared to its respective control (Fig. 6).

III.3. miR-378a-5p inhibits syncytiotrophoblast differentiation

The down-regulation of miR-378a-5p during Forskolin-induced STB differentiation suggests that it may inhibit syncytialization. To test this hypothesis, several sets of experiments were performed to determine the effect of miR-378a-5p overexpression on morphological and biochemical differentiation of STB. BeWo cells were transfected with different concentrations (50nM, 100nM and 200nM) of miR-378a-5p, a non-targeting negative control (NC) or with a mock transfection for 72 hours and cell fusion was assessed by immunofluorescence. As shown in Fig. 7A, transfection with miR-378a-5p resulted in a decrease in cell fusion. When the percentage of fused BeWo cells was calculated by counting the number of cells with 3 or more nuclei (Fig. 7B) or 2 or more nuclei (Fig. 7C), a significant decrease in cell fusion was observed in cells transfected with 100 or 200 nM miR-378a-5p, as compared to their corresponding NC and Mock controls.

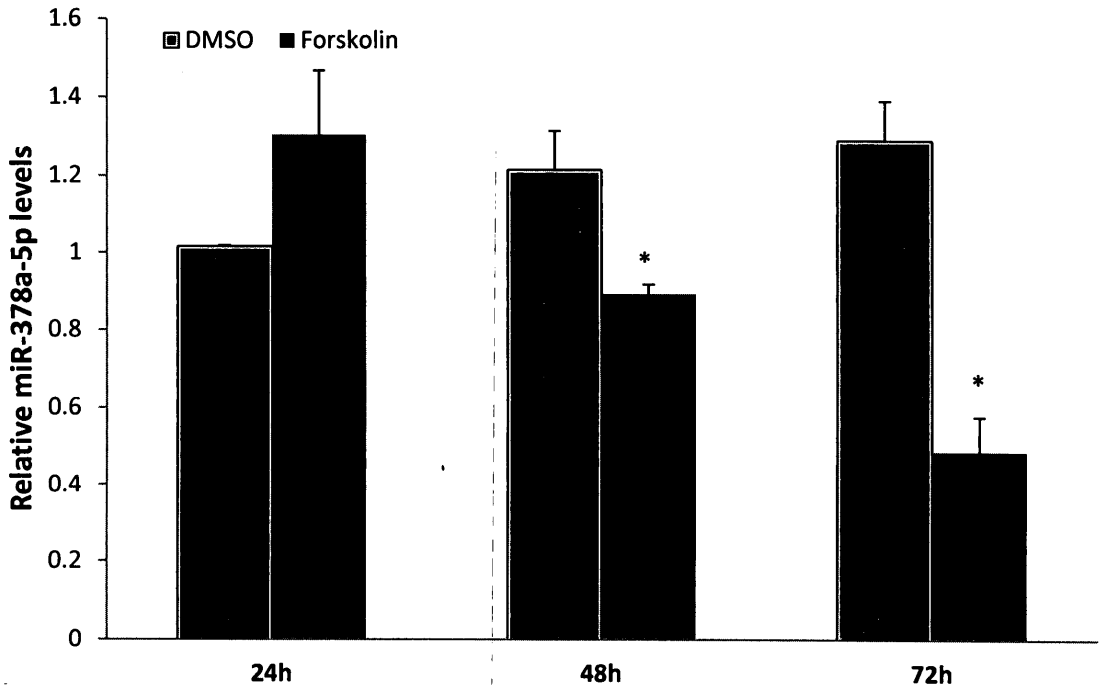
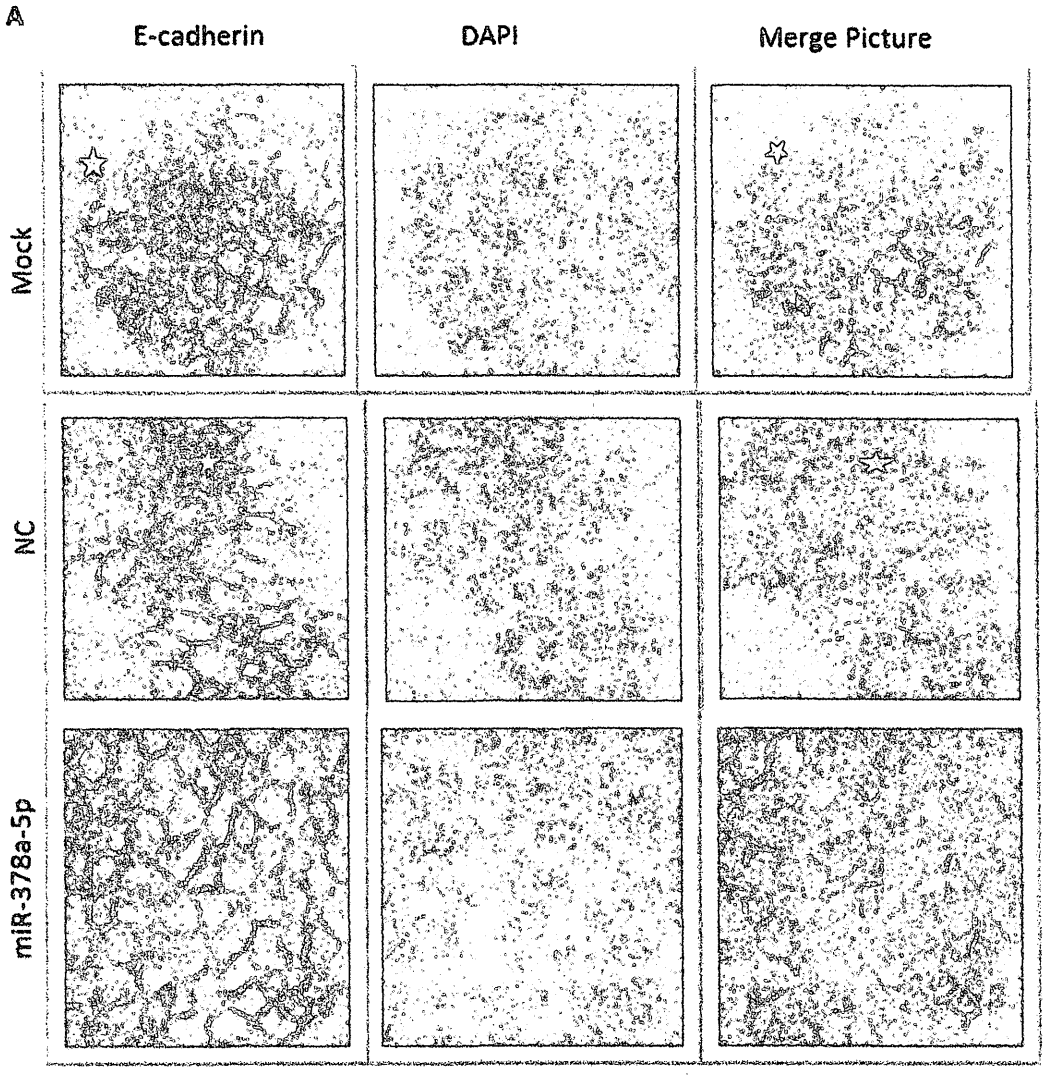


Fig. 6. Forskolin inhibits miR-378a-5p expression. Cells were treated with Forskolin (50μM) or DMSO control for 24 to 72h. Endogenous levels of miR-378a-5p was measured by real-time qPCR and normalized to an internal calibrator U6. A significant decrease in miR-378a-5p levels during Forskolin-induced syncytialization was observed at 48 and 72h time points. *, $p<0.05$ vs. DMSO control . Data represent mean $\pm$ SEM (n=3 experiments).


A
E-cadherin
DAPI
Merge Picture
Mock
NC
miR-378a-5p

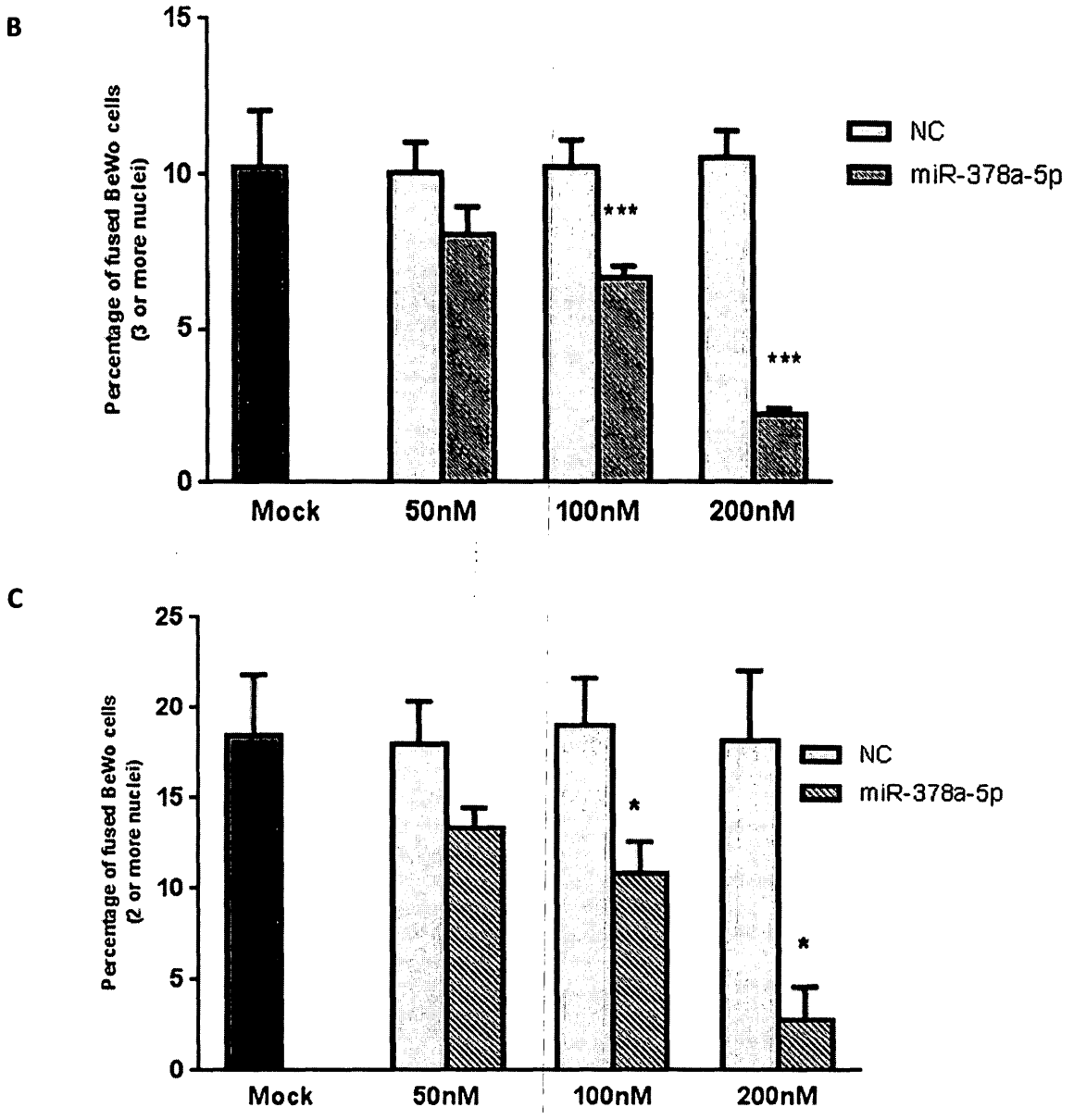


Fig. 7. miR-378a-5p inhibits cell fusion. A) Representative pictures of immunofluorescence staining of E-cadherin in BeWo cells transfected without (mock) or with negative control (NC) or miR-378a-5p (200 nM). Membrane was stained with anti E-cadherin antibody (red) and nuclei were stained with DAPI (blue). Merged pictures (fused nuclei marked with stars) are shown in the right panel. B) A summary graph showing the percentage of cells with 3 or more nuclei in cells transfected with different concentration of miR-378a-5p or NS for 72h. C) A summary graph showing the percentage of cells with 2 or more nuclei.
Data represent mean ± SEM (n=3 experiments).

To further evaluate the role of miR-378a-5p in STB differentiation, RT-qPCR was used to analyze the mRNA level of several STB marker genes, including Syncytin-1, Syncytin-2, ALPP and LGALS13. BeWo cells transfected with 100 and 200nM of miR-378a-5p showed a statistically significant decrease in mRNA level of Syncytin-1 (Fig. 8B and 8C). Transfection of 200 nM miR-378a-5p also decreased Syncytin-2 mRNA levels (Fig. 9C). However, there was no significant difference between the control groups and those transfected with a lower dose of miR-378a-5p (Fig. 9A, B). Similarly, miR-378a-5p also inhibited the expression of ALPP (Fig. 10C) and LGALS13 (Fig. 11C) at the 200nM concentration. Overall, these results showed that miR-378a-5p downregulated STB marker gene expression in a concentration- dependent manner.

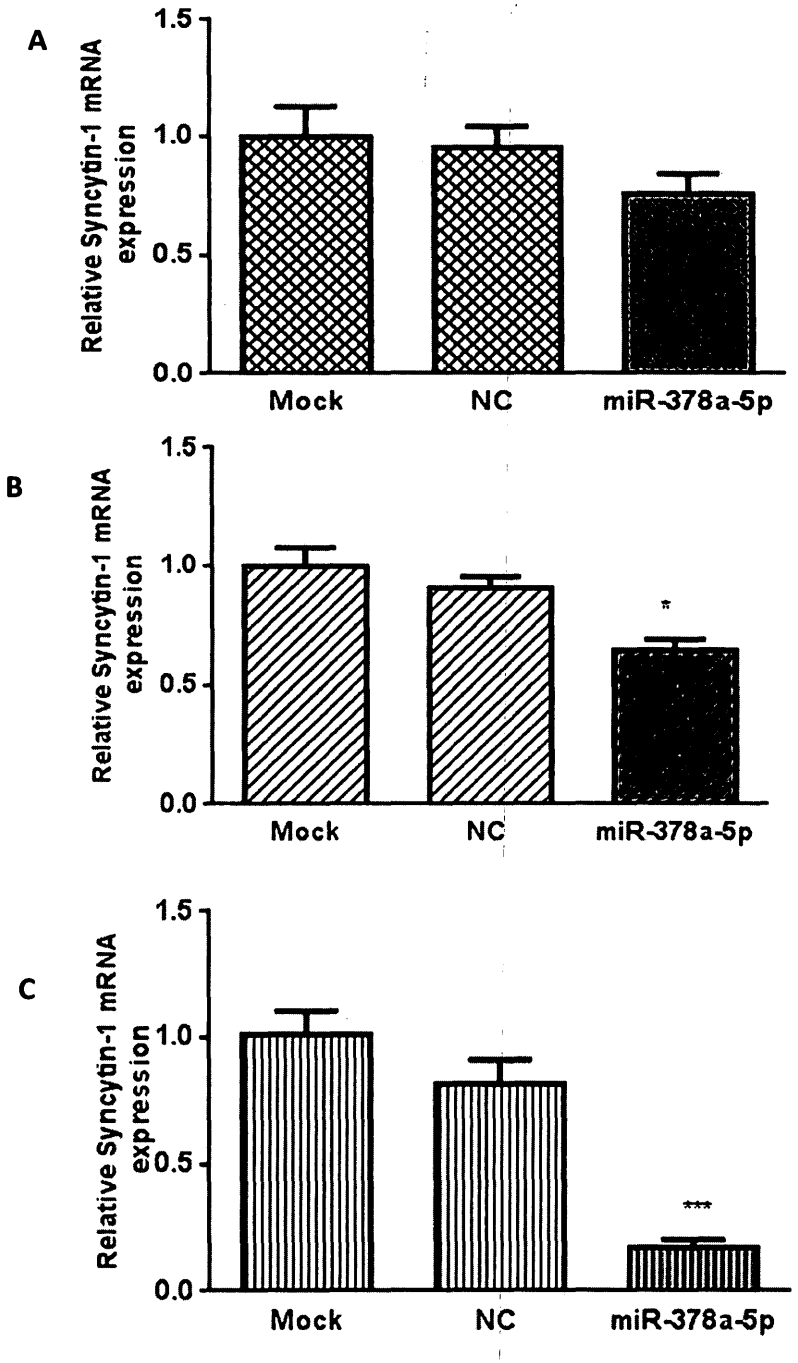


Fig. 8. miR-378a-5p down-regulates the mRNA levels of Syncytin 1. BeWo cells were transfected without (mock) or with 50 nM (A), 100 nM (B), or 200 nM (C) of miR-378a-5p or negative control (NC). Total RNA was extracted at 72h after transfection and syncytin 1 mRNA levels were determined by RT-qPCR. Data represent mean ± SEM of 3 experiments. *, $p<0.05$, ***, $p<0.001$ vs corresponding mock and NC groups.

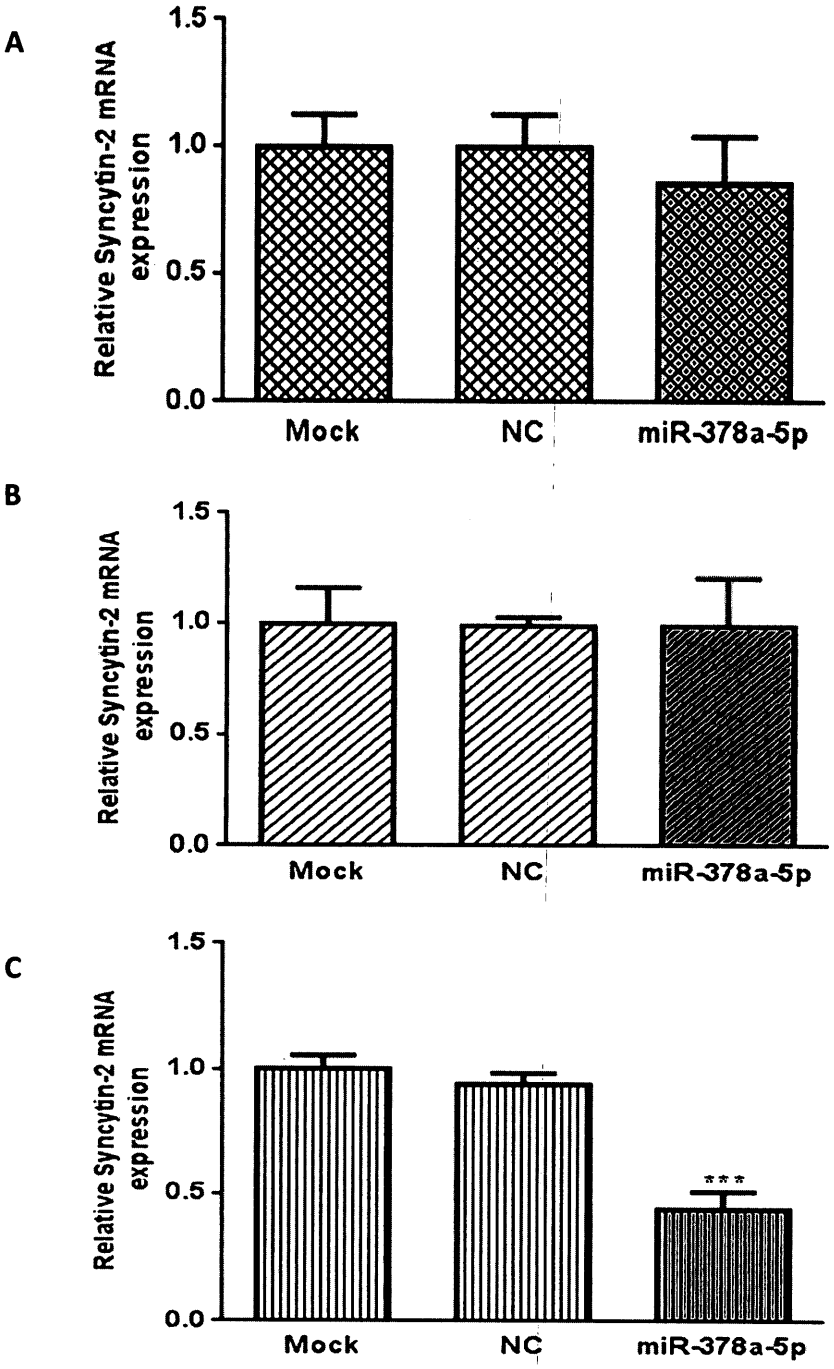


Fig. 9. miR-378a-5p down-regulates the mRNA levels of Syncytin 2. BeWo cells were transfected without (mock) or with 50 nM (A), 100 nM (B), or 200 nM (C) of miR-378a-5p or negative control (NC). Total RNA was extracted at 72h after transfection and syncytin 2 mRNA levels were determined by RT-qPCR. Data represent mean ± SEM of 3 experiments. **, $p<0.01$ vs corresponding mock and NC groups.

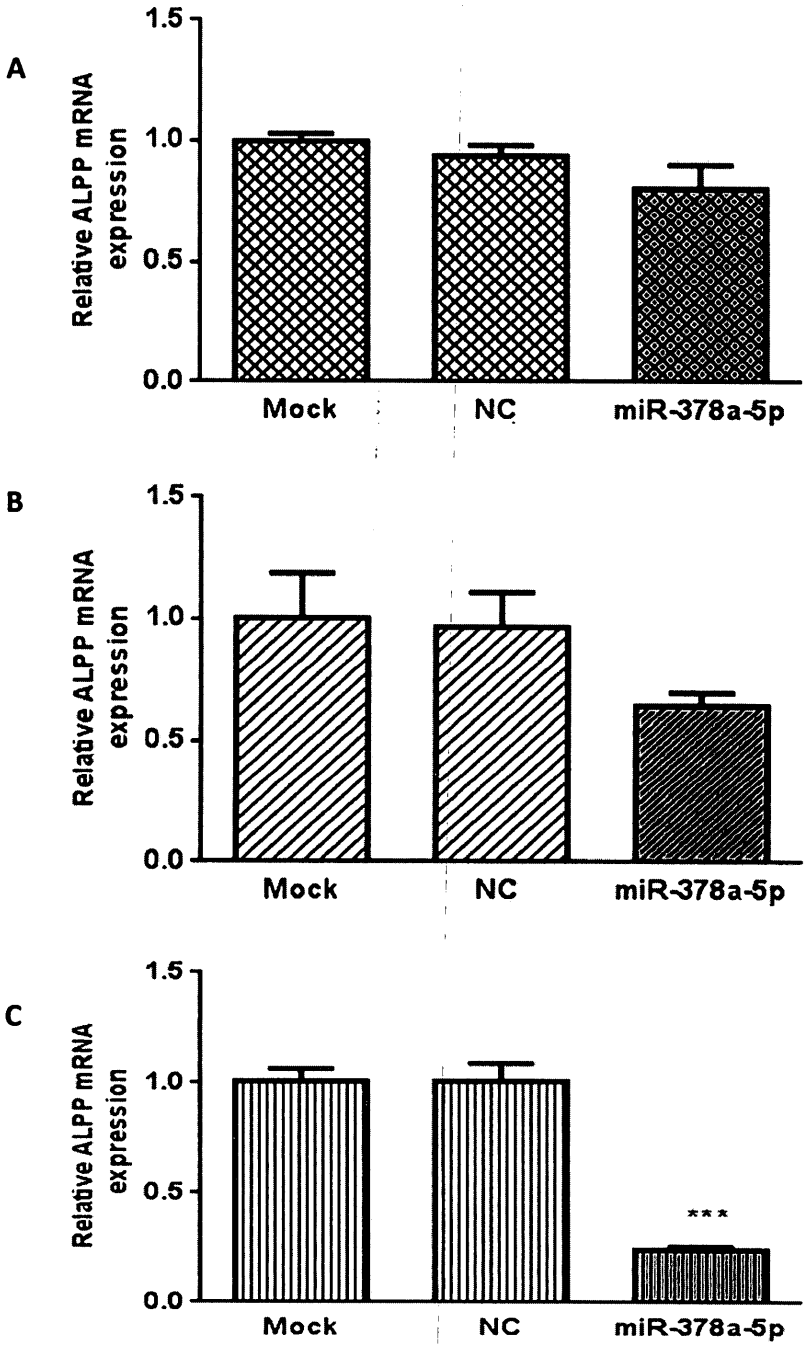


Fig. 10. miR-378a-5p down-regulates the mRNA levels of ALPP. BeWo cells were transfected without (mock) or with 50 nM (A), 100 nM (B), or 200 nM (C) of miR-378a-5p or negative control (NC). Total RNA was extracted at 72h after transfection and ALPP mRNA levels were determined by RT-qPCR. Data represent mean ± SEM of 3 experiments. ***, $p<0.001$ vs corresponding mock and NC groups.

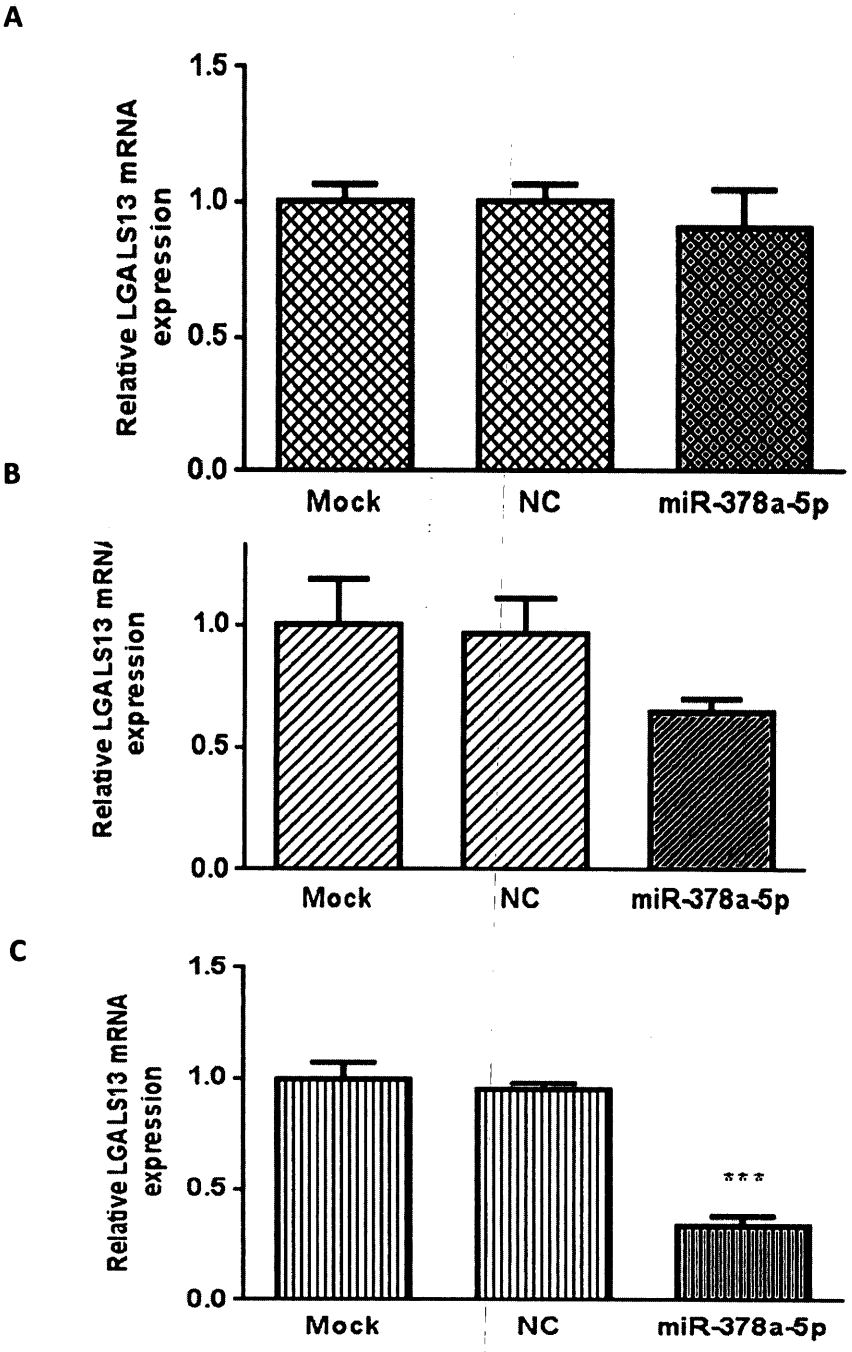


Fig. 11. miR-378a-5p down-regulates the mRNA levels of LGALS13. BeWo cells were transfected without (mock) or with 50 nM (A), 100 nM (B), or 200 nM (C) of miR-378a-5p or negative control (NC). Total RNA was extracted at 72h after transfection and LGALS13 mRNA levels were determined by RT-qPCR. Data represent mean ± SEM of 3 experiments. ***, $p<0.001$ vs corresponding mock and NC groups.

III.4. miR-378-5p inhibits Forskolin-induced syncytiotrophoblast differentiation

To understand the role of miR-378a-5p in STB differentiation, BeWo cells were transfected with miR-378a-5p (200nM) or a negative control (NC) for 24 hours and then cells were treated with DMSO (control) or Forskolin (50uM) for 48 hours. Immunofluorescent studies showed that miR-378a-5p reduced cell fusion not only under basal condition, but also in Forskolin-treated cells (Fig. 12).

E-cadherin is lost during cell fusion. Therefore, a decrease in E-cadherin protein expression is often used as an indicator of STB differentiation (101). To further confirm the inhibitory effect of miR-378a-5p in STB differentiation, Western blot analysis was conducted on cell lysate prepared from cells transfected with miR-378a-5p (200nM) and negative control for 24 hours and treated with DMSO and Forskolin for 48 hr. Western blotting using an anti-E-cadherin and a GAPDH antibody revealed that miR-378a-5p increased E-cadherin protein levels and reversed the inhibitory effect of Forskolin on E-cadherin expression (Fig. 13).

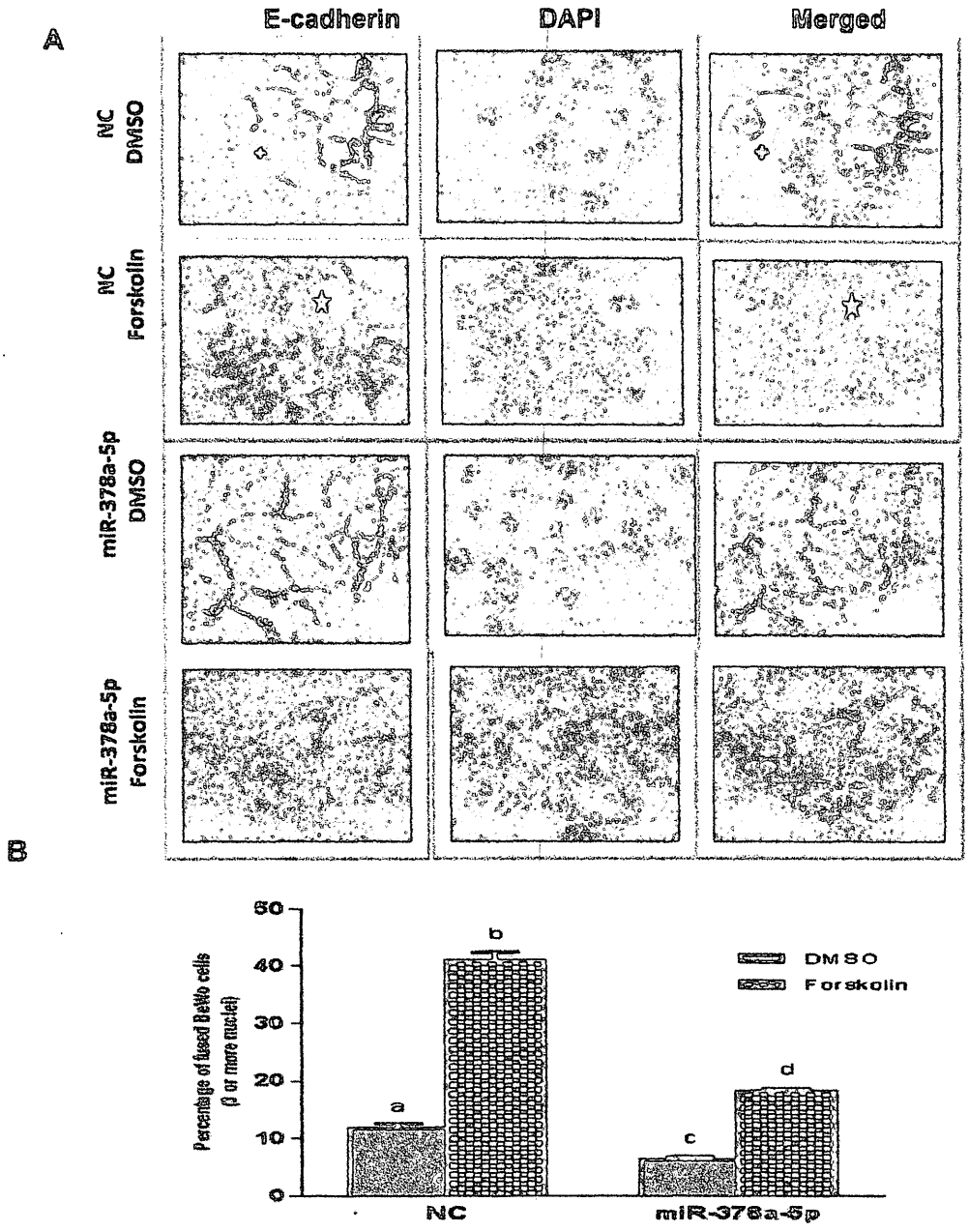


Fig. 12. miR-378a-5p inhibits Forskolin-induced cell fusion. BeWo cells were transfected with miR-378a-5p (200nM) or a negative control (NC) for 24 hours and then treated with either DMSO or Forskolin (50 μM) for 48h. A) Representative pictures of immunofluorescence staining using anti-E-cadherin antibody (red for membrane) and DAPI (blue for nuclei). Fused nuclei were marked with stars. B) Quantified results show that miR-378a-5p decreased basal and Forskolin-induced cell fusion. Different letters above bars denote statistical significance. Data represent mean ± SEM of 3 experiments.

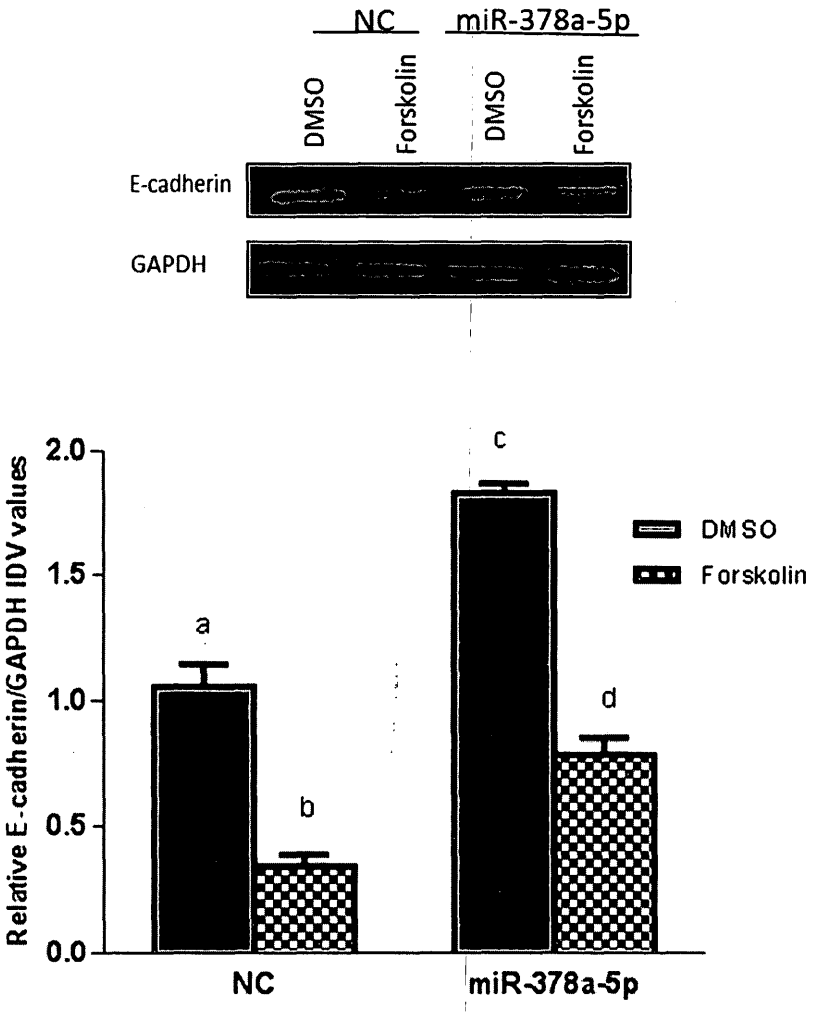


Fig. 13. miR-378a-5p reduces the inhibitory effect of Forskolin on E-cadherin expression. BeWo cells were transfected with miR-378a-5p (200nM) or a negative control (NC) for 24 hours and then treated with either DMSO or Forskolin (50 μM) for 48h. Cell lysates were prepared and Western blot using anti-E-cadherin and GAPDH (as an internal control) antibodies was performed. A representative picture and a summary graph is shown. Different letters above bars denote statistical significance. Data represent mean ± SEM of 3 experiments.

III.5. Anti-miR-378a-5p induces syncytiotrophoblast differentiation

Anti-microRNAs can reduce the endogenous level of a miRNA and result in an increase in target gene expression (35). To confirm the physiological significance of miR-378a-5p in regulating STB differentiation, I investigated the effect of anti-miR-378a-5p on STB differentiation. First, BeWo cells were transiently transfected with anti-miR-378a-5p (200nM) or negative control (NC) or without any oligonucleotide (mock). At 72 hours after transfection, morphological differentiation was analyzed by immunofluorescence. Compared to mock transfection or NC, anti-miR-378a-5p significantly increased cell fusion as indicated by the number of cells with 3 or more nuclei (Fig. 14). Second, RT-qPCR analysis showed that anti-miR-378a-5p increased the mRNA level of STB marker genes, Syncytin-1, -2, ALPP and LGALS13 (Fig. 15) Third, in immunoblot analysis, anti-miR-378a-5p decreased E-cadherin but increased Syncytin-1 protein levels and these effects are opposite to miR-378a-5p overexpression (Fig. 16).

Fusion of CTB into STB is also associated with an increase in the expression of CGB gene (100). I, therefore, determined the effect of miR-378a-5p and anti-miR-378a-5p on CGB mRNA expression. BeWo cells were transiently transfected with 200nM concentration of miR-378a-5p or anti-miR-378a-5p, together with mock and corresponding controls. Total RNA was extracted and mRNA level of CGB was determined by RT-qPCR. Figure 17 shows that CGB levels were decreased significantly in cells transfected with miR-378a-5p. On the other hand, cells transfected with antimiR-378a-5p had an increase in CGB mRNA levels.

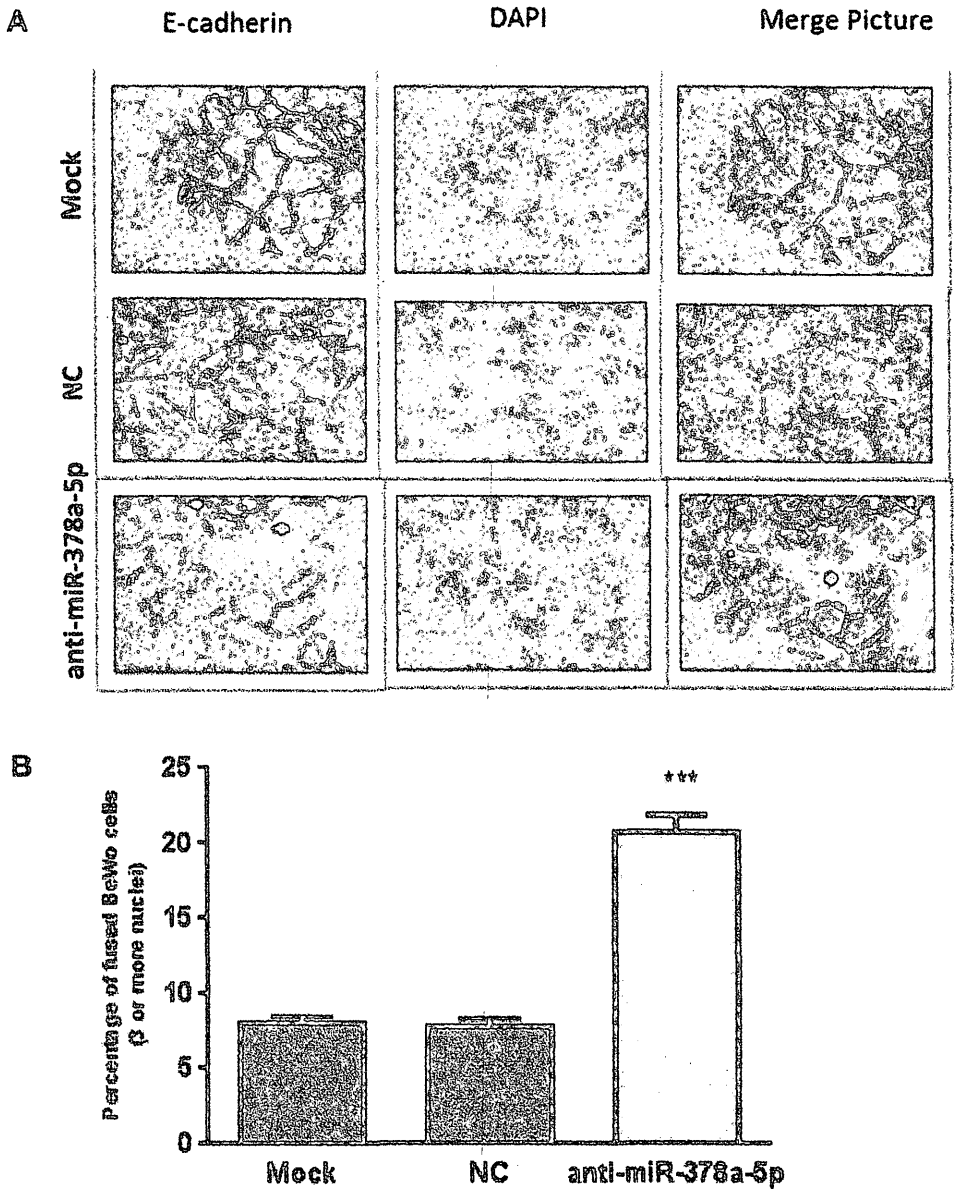


Fig. 14. Anti-miR-378a-5p induces cell fusion. BeWo cells were transfected without (mock) or with anti-miR-378a-5p (200nM) or a negative control (NC). A). Representative pictures of immunofluorescence staining with anti-E-cadherin antibody (red) and DAPI (blue for nuclear staining). Nuclei within an intact membrane are shown with stars. B). Quantified results show that anti-miR-378a-5p induces cell fusion. ***,$p<0.001$ vs. corresponding mock and NC. Data are mean ± SEM of 3 experiments.

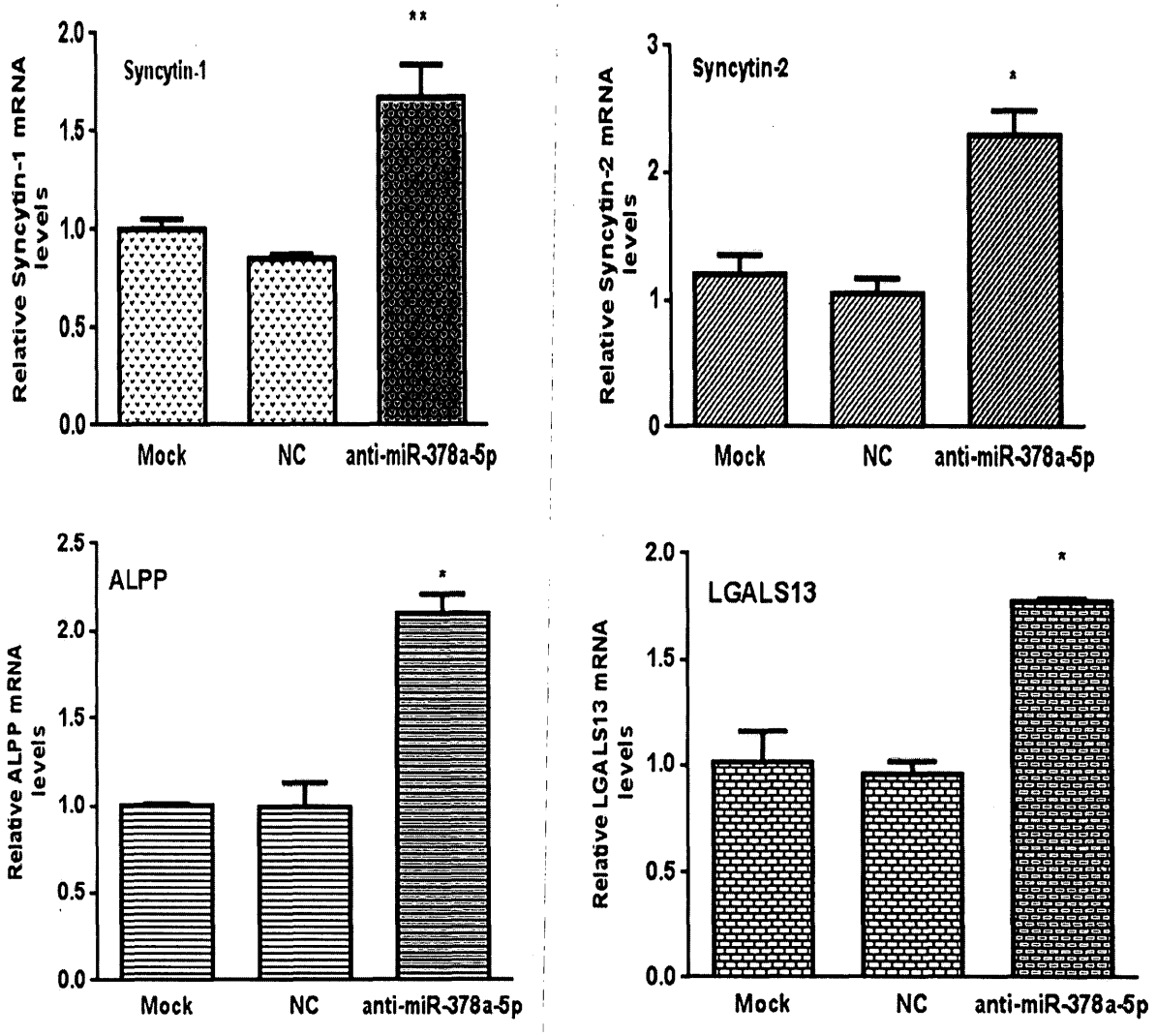


Fig. 15. Anti-miR-378a-5p induces mRNA expression of STB. BeWo cells were transfected without (mock) or with anti-miR-378a-5p (200nM) or a negative control (NC). Total RNA was extracted at 72h after transfection and mRNA levels were determined by RT-qPCR. Data represent mean ± SEM of 3 experiments. *, $p<0.05$, **, $p<0.01$ vs. corresponding mock and NC groups.

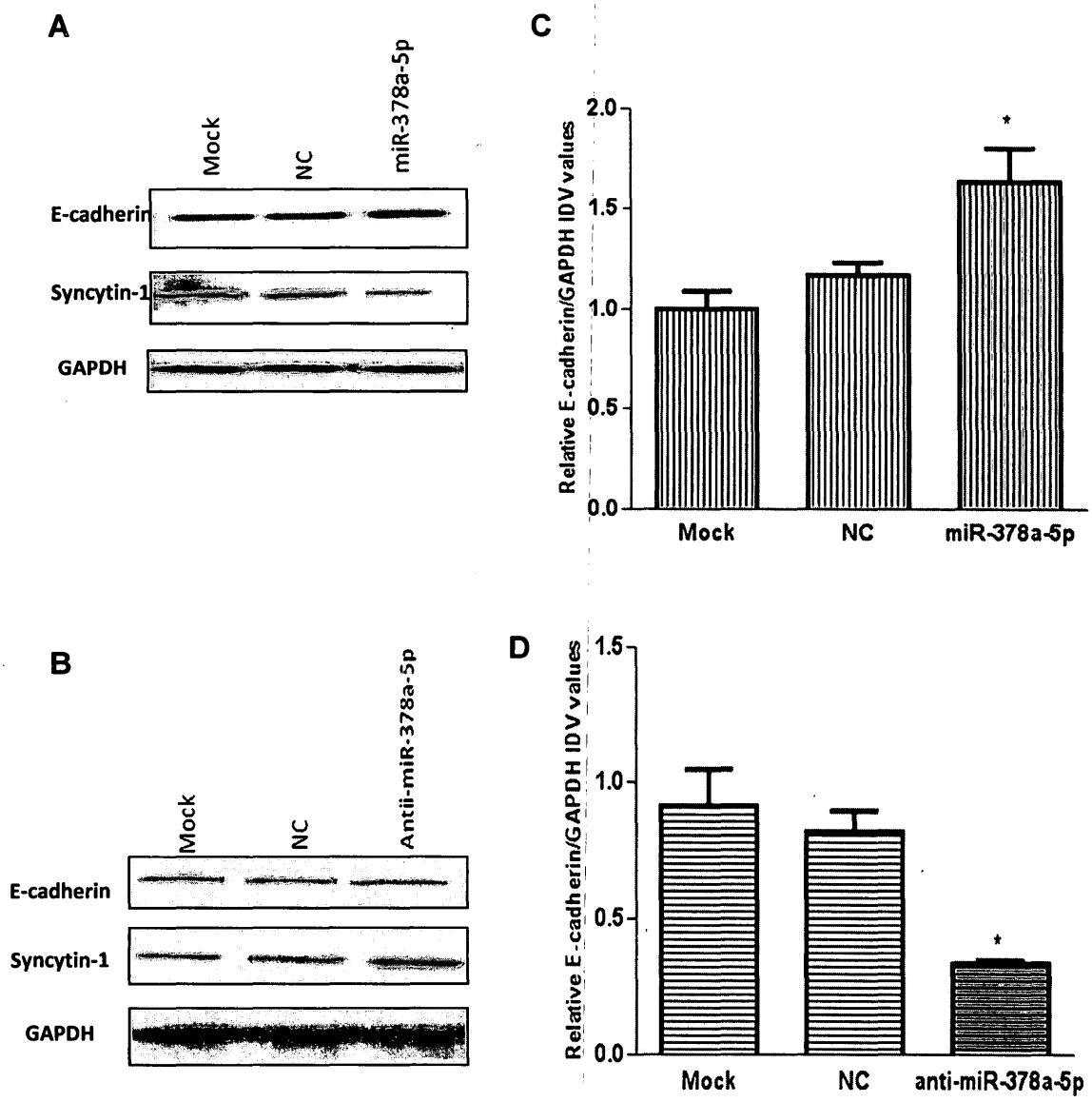


Fig. 16. miR-378a-5p and anti-miR-378a-5p exert opposite effects on E-cadherin and syncytin-1 protein expression. BeWo cells were transiently transfected without (mock), miR-378a-5p, anti-miR-378a-5p, or their corresponding negative controls (NC). Cell lysates were collected after 72 hrs and subjected to immunoblot analyses using E-cadherin and syncytin-1 antibodies. A) Representative blots showing miR-387a-5p increased E-cadherin but decreased syncytin-1 protein levels. B) Representative blots showing anti-miR-387a-5p decreased E-cadherin but increased syncytin-1 protein levels. C) A summary graph depicting miR-378a-5p increasing E-cadherin levels. D). A summary graph showing anit-miR-378a-5p decreases E-cadherin levels. *, $p<0.05$ vs. corresponding mock and NC. Bar graph data are mean ± SEM of 3 experiments.

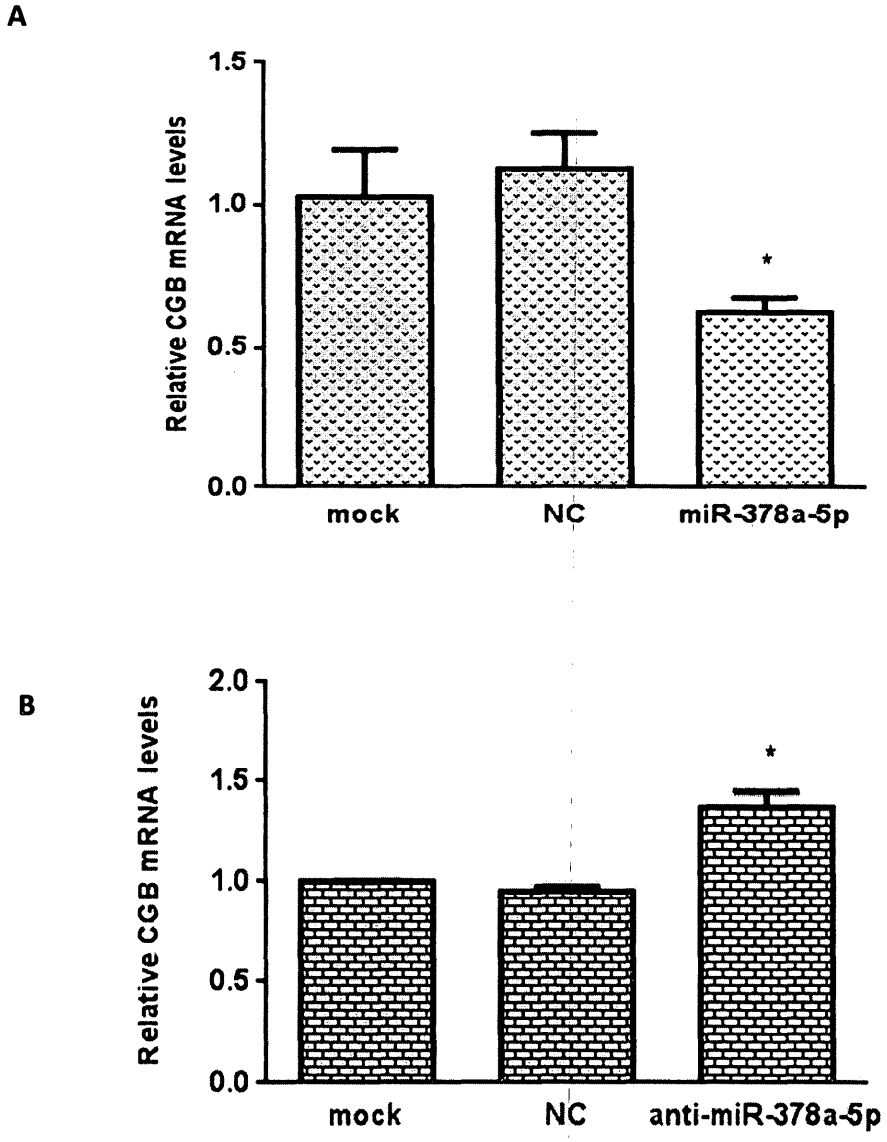


Fig. 17. miR-378a-5p and anti-miR-378a-5p exert opposite effects on the expression of chorionic gonadotropin β-subunit (CGB). BeWo cells were transiently transfected without (mock) or with miR-378a-5p, anti-miR-378a-5p, or their corresponding negative controls (NC). Total RNA was extracted at 72h after transfection and subjected to real-time PCR. A) CGB mRNA levels were decreased in cells transfected with miR-378a-5p. B). CGB mRNA levels were increased in cells transfected with anti-miR-378a-5p. *, $p<0.05$ vs. corresponding mock and NC. Data are mean ± SEM of 3 experiments.

III.6. miR-378a-5p targets cyclin G2

To explore the mechanisms by which miR-378a-5p inhibits the differentiation of STB, bioinformatic tools (FindTar3) was used to examine the predicted target genes of miR-378a-5p. It was found that miR-378a-5p has five potential complementary binding sites at the 3'UTR of CCNG2 (Fig. 18). Since CCNG2 is known to induce adipocyte differentiation (70) and its expression in the placenta has been recently reported (76), we hypothesize that CCNG2 may promote STB differentiation and may be down-regulated by miR-378a-5p. To test this possibility, luciferase reporter assay was first performed. CCNG2 3' UTR is almost 4 kb long and has five predicted target sites of miR-378a-5p, spanning almost the entire 3'UTR (Fig. 18). Two CCNG2 3' UTR fragments (CCNG2 3' UTR-1 and CCNG2 3' UTR-2) were cloned into the pMIR-REPORT vector, downstream of the luciferase coding sequence (Fig. 19A), by a research associate and a PhD student in the lab, respectively. BeWo cells were co-transfected with a control reporter (without 3'UTR) or one of the two constructs of CCNG2 3' UTR construct and miR-378a-5p or its NC or mock controls. Luciferase assays were performed at 24h after transfection. MicroRNA-378a-5p significantly decreased the luciferase activity of CCNG2 3'UTR-1 construct, which contains the first predicted site of miR-378a-5p (Fig. 19B). A decrease in luciferase activity by miR-378a-5p was also observed in the cells transfected with the CCNG2 3'UTR-2 construct; however, the difference is not statistically significant (Fig. 19B).

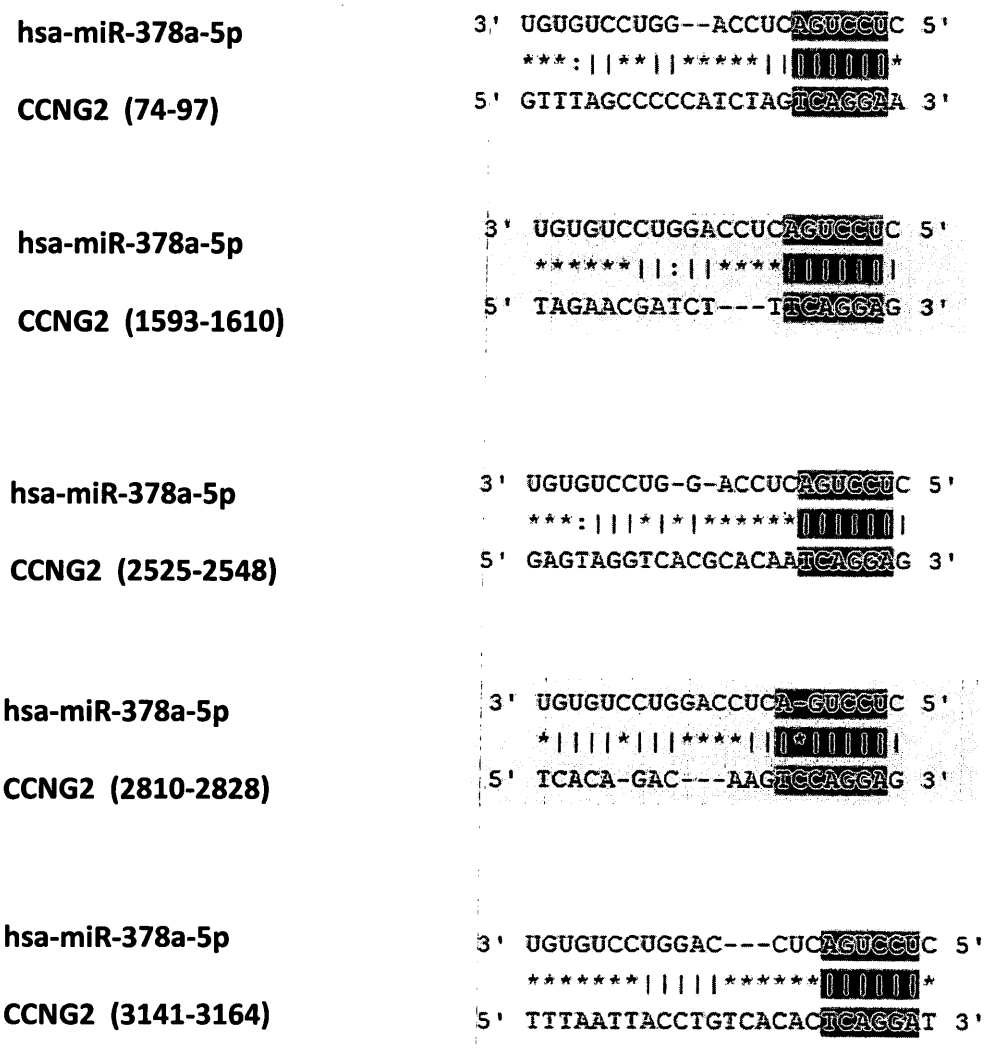


Fig 18. Predicted miR-378a-5p targeting sites on CCNG2 3' UTR. miR-378a-5p has five potential target sites on CCNG2 3'UTR, as predicted by Findtar3.

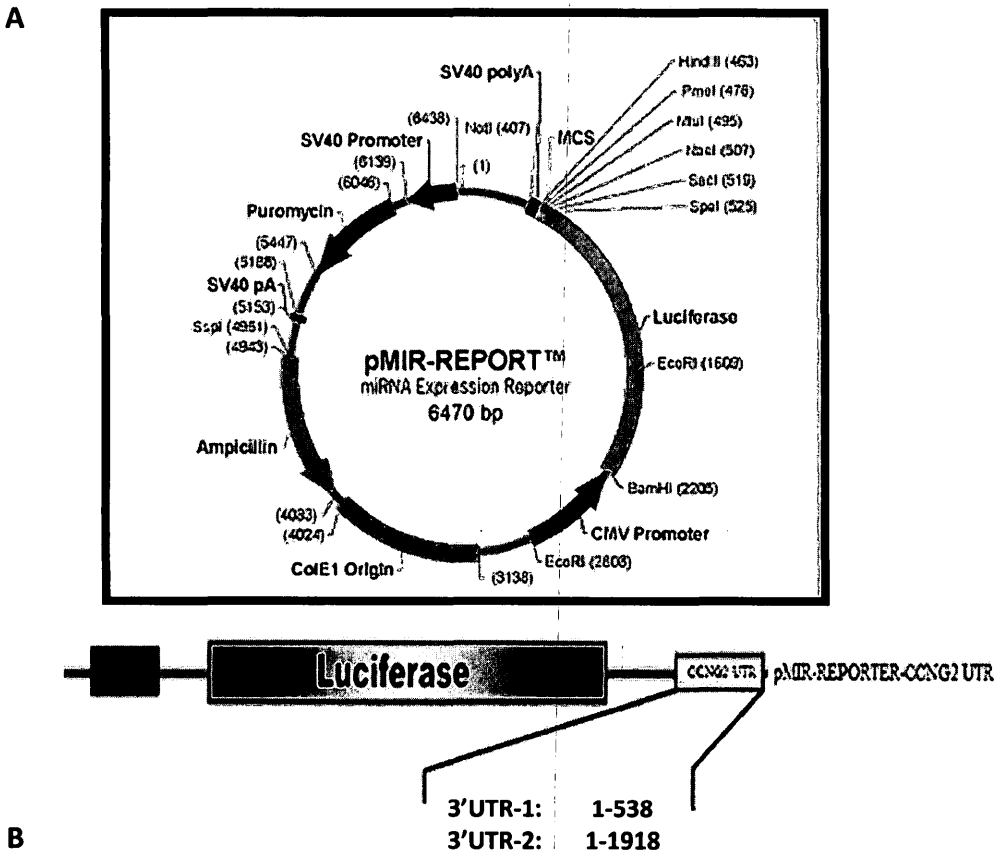
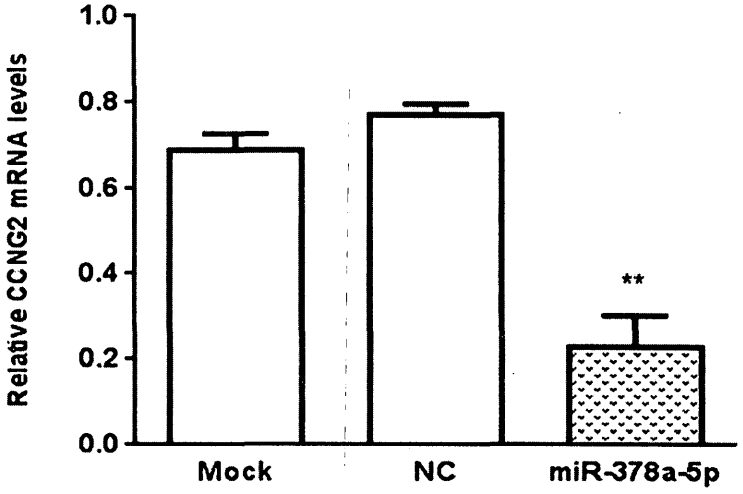


Fig. 19. miR-378a-5p targets CCNG2. A) Two luciferase reporter constructs, each containing a fragment of CCNG2 3' UTR downstream of the luciferase coding sequence, were generated. B) Luciferase assays. BeWo cells were transfected with the control reporter (pMIR-REPORT without 3'UTR) or the CCNG2 3'UTR constructs, together with and without negative control (NC) or miR-378a-5p. In cells transfected with 3'UTR-1, miR-378a-5p significantly decreased the luciferase activity when compared to mock and NC groups. ***$p<0.001$ vs. corresponding mock and NC. Data are mean ± SEM of 3 experiments.

To further confirm that miR-378a-5p regulates CCNG2, BeWo cells were transiently transfected with 200nM concentration of miR-378a-5p or anti-miR-378a-5p, or their corresponding controls, total RNA was extracted at 72h after transfection and mRNA level of CCNG2 was determined by RT-qPCR. Figure 20A shows that CCNG2 mRNA levels were significantly lower in cells transfected with miR-378a-5p than in control cells. Conversely, cells transfected with anti-miR-378a-5p have significantly higher CCNG2 mRNA levels than control cells (Fig. 20B).

Finally, I determined if CCNG2 regulates STB differentiation. Cells were transfected with control plasmid vector (EV) or plasmid expressing CCNG2 (CCNG2). Cell lysates were prepared at 24 hours after CCNG2 expression and Western blot analyses were performed using an anti-E-cadherin and anti-syncytin-1 antibodies. Overexpression of CCNG2 resulted in a significant inhibition of E-cadherin but a strong induction of Syncytin-1 protein levels (Fig. 21A). On the other hand, knockdown of CCNG2 expression using a siRNA-mediated gene silencing approach increased E-cadherin and decreased Syncytin-1 protein levels (Fig. 21B).

A



B

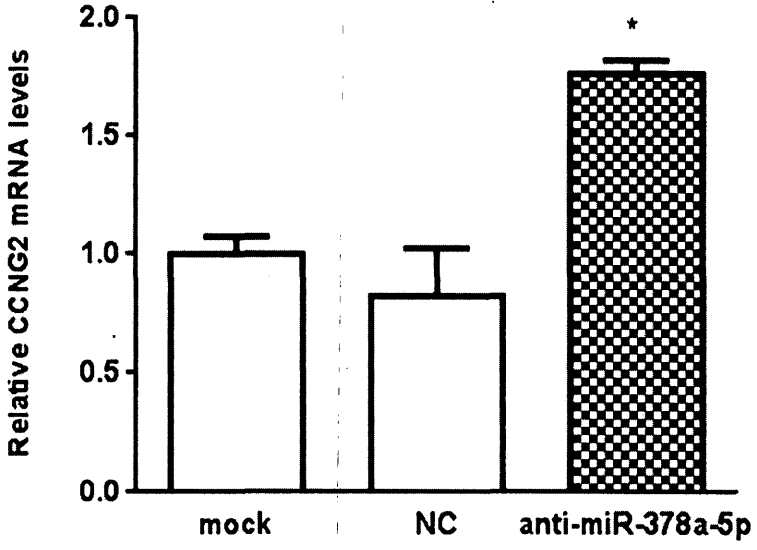


Fig. 20. miR-378a-5p inhibits, while anti-miR-378a-5p increases, CCNG2 mRNA expression. BeWo cells were transiently transfected without (mock) or with miR-378a-5p, anti-miR-378a-5p, or their corresponding negative controls (NC). Total RNA was extracted at 72h after transfection and subjected to real-time PCR. A) CCNG2 mRNA levels were decreased in cells transfected with miR-378a-5p. B). CCNG2 mRNA levels were increased in cells transfected with anti-miR-378a-5p *$p<0.05$, **$p<0.01$ vs. corresponding mock and NC. Data are mean ± SEM of 3 experiments.

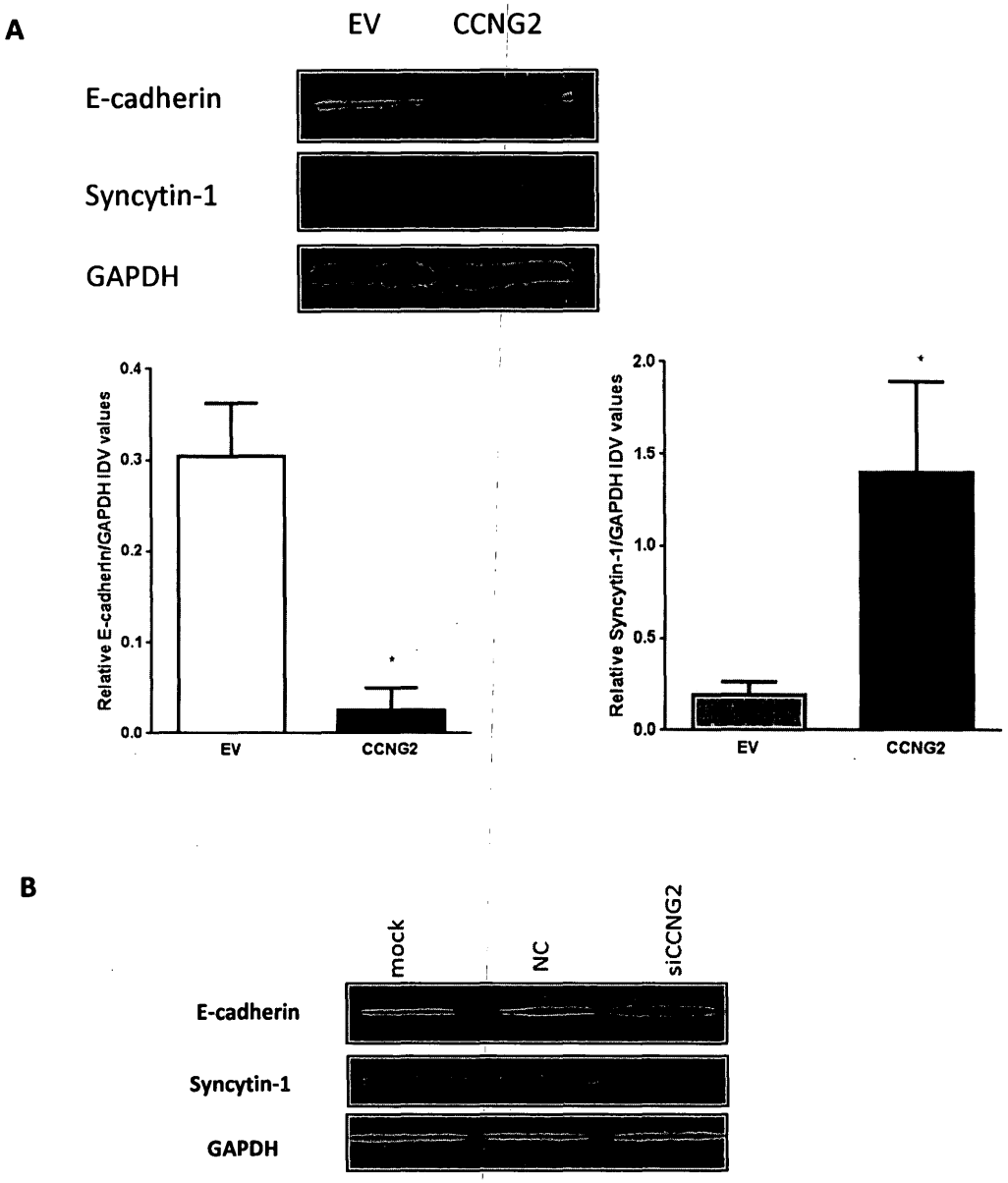


Fig. 21. CCNG2 induces syncytiotrophoblast differentiation. A). BeWo cells were transfected with control plasmid vector (EV) or plasmid expressing CCNG2 (CCNG2). Cell lysates were prepared at 24 h after transfection and subjected to immunoblotting using anti-E-cadherin and anti-syncytin-1 antibody. Representative immunoblots and graphs depicting the densitometric intensities of E-cadherin and syncytin-1 levels are shown. Transfection of CCNG2 resulted in a decrease in E-cadherin and an increase in syncytin-1 protein levels. *$p<0.05$ vs. corresponding empty vector. Data are mean ± SEM of 3 replicate wells in one experiment. B) Silencing of CCNG2 expression increased E-cadherin levels. Cells were transfected without (mock) or with a siRNA targeting CCNG2 (siCCNG2) or its negative control (NC). Cell lysates were prepared at 48h after transfection.

Chapter IV. Discussion

Expression of miRNAs in placenta and their dysregulation in gestational disorders suggest that they are important regulators of placental development and function (29). Recently, we have demonstrated that miR-378a-5p promotes trophoblast cell survival, proliferation, migration and invasion by targeting Nodal (82). Moreover, in human placenta, miR-378a-5p is expressed throughout gestation and its levels were lower in preeclamptic patients than the healthy controls (82), suggesting that it has important regulatory functions in placenta and its abnormal expression may contribute to the development of pregnancy associated disorders, such as preeclampsia. This study was carried out to further investigate the role of miR-378a-5p during placental development, specifically, to examine the potential role of miR-378a-5p in trophoblast cell differentiation into the STB pathway. I demonstrated that miR-378a-5p inhibits the STB differentiation and identified cyclin G2 (CCNG2) as a target gene of miR-378a-5p. These findings further support the notion that miR-378a-5p is an important regulator of human placental development. In addition, this study provides the first evidence that CCNG2 regulates trophoblast cell differentiation.

IV.1. miR-378a-5p is an inhibitor of syncytiotrophoblast differentiation

BeWo cells have been used extensively to study trophoblast cell differentiation along the STB pathway (65,121). In the present study, I used this cell line to examine the role of miR-378a-5p in regulating the differentiation of CTB into STB. During STB differentiation, CTB cells fuse to form multinucleated STB cells and this is accompanied by expression of STB specific genes, such as Syncytin-1, Syncytin-2, ALPP, hCG and LGALS13 (57,61,63,64). Therefore, STB differentiation can be studied by morphological and biochemical approaches. Before proceeding with differentiation assays, I first tested the dose-dependent and time-course effect of Forskolin, which is well documented to induce STB differentiation, (66, 42, 98) to establish a positive control in our assays.

To explore the potential involvement of miRNAs in regulating STB differentiation, I first examined several miRNA levels during Forskolin-induced STB differentiation. I found that miR-378a-5p levels were strongly inhibited by Forskolin treatment. The specific factor(s) which downregulates the miR-378a-5p level remains to be identified. It is well known that activation of adenylate cyclase by Forskolin results in the accumulation of cAMP (98) which activates protein kinase A (PKA) (104). PKA could activate transcription factors that are involved in the transcription of miR-378a-5p gene. A potential transcription factor that may regulate miR-378a-5p is peroxisome proliferator-activated receptor γ (PPARγ) which is involved in cell differentiation, development and metabolism, including adipogenesis and glucose homeostasis (75). A recent study in mouse adipocytes has identified an inhibitory effect of PPARγ on the expression of miR-378, which is a homologue of human miR-378a-5p (Fig. 22A). In human trophoblast cells,

PPARγ promotes the differentiation of STB (117, 119). Therefore, it is possible that PPARγ is involved in the Forskolin-inhibited miR-378a-5p expression. This can be investigated in future studies.

Downregulation of miR-378a-5p during Forskolin-induced STB differentiation suggests that miR-378a-5p may have an inhibitory effect on the differentiation of CTB to STB. To test this possibility, I performed several functional assays and examined the effect of miR-378a-5p overexpression on morphological and biochemical differentiation of STB. First, immunofluorescence results showed that transfection of a miR-378a-5p mimic significantly reduced the number of cells that have multiple nuclei, indicating that miR-378a-5p inhibits cell fusion. Then, RT-qPCR analysis of Syncytin-1, Syncytin-2, CGB, ALPP and LGALS13 showed a significant downregulation in STB marker gene expression in a concentration dependent manner. Finally, miR-378a-5p increased E-cadherin and decreased Syncytin-1 protein expression levels. It is well documented that E-cadherin levels are decreased during STB formation (106, 107). Moreover, Syncytin-1, Syncytin-2, ALPP and LGALS13 have been shown to be expressed in STB. Therefore, a decrease in the expression of these genes provides additional evidences that miR-378a-5p has an inhibitory role in STB differentiation.

I also evaluated if miR-378a-5p could block Forskolin-induced STB differentiation. E-cadherin immunostaining results showed that miR-378a-5p reduced cell fusion not only under basal condition but also in Forskolin-treated cells. In concordance with these results, Western blot analysis revealed that miR-378a-5p increased E-cadherin protein in both control cells and cells treated with Forskolin. The miR-378a-5p increased E-cadherin protein levels and reversed the inhibitory effect of Forskolin on E-cadherin expression.

These results confirm that miR-378a-5p plays an inhibitory role in the STB differentiation pathway.

The physiological role of miR-378a-5p in regulating STB differentiation is further supported by loss-of-function studies. I used antisense oligonucleotide (anti-miR-378a-5p) to inhibit the endogenous miR-378a-5p. First, I found that anti-miR-378a-5p significantly increased the number of cells with multiple nuclei. Then, RT-qPCR analysis showed that anti-miR-378a-5p significantly increased the mRNA levels of STB marker genes, Syncytin-1, Syncyin-2, CGB, ALPP and LGALS13. Finally, E-cadherin immunostaining results showed anti-miR-378a-5p decreased E-cadherin but increased Syncytin-1 protein levels. Anti-miRNAs are modified antisense oligonucleotides that have partially or fully complementary sequence of mature miRNA. They inhibit endogenous miRNAs by inducing their degradation and release target gene repression by the miRNAs (23). Thus, these results suggest that trophoblast cells can be induced to differentiate into the STB pathway by inhibiting endogenous miR-378a-5p.

Collectively, data obtained from this study strongly suggest that miR-378a-5p inhibits STB differentiation. We have previously demonstrated that miR-378a-5p enhanced trophoblast cell migration and invasion. It is therefore possible that miR-378a-5p promotes trophoblast cell differentiation into the invasive EVT pathway but prevents their differentiation into the STB pathway. More studies are required, especially using trophoblast cells prepared from healthy human placenta, to test this hypothesis.

IV.2. Target genes of miR-378a-5p

This study identifies CCNG2 as a target gene of miR-378a-5p. CCNG2 transcript has a 4-kb 3' UTR that contains predicted binding sites for many miRNAs. Interestingly, 5 potential binding sites have been predicted for miR-378a-5p. Several lines of evidence suggest that CCNG2 is a true target of miR-378a-5p. First, miR-378a-5p decreased the luciferase activity of CCNG2 3'UTR-1 construct. Second, mRNA levels of CCNG2 were lower in miR-378a-5p overexpressed cells but higher in the presence of anti-miR-378a-5p. Finally, knockdown of CCNG2 using siRNA mimicked the effect of miR-378a-5p in trophoblast cell differentiation. Additional studies, especially luciferase assays using constructs that contain mutated binding sequence of miR-378a-5p and Western blotting to measure CCNG2 protein levels, will further confirm that CCNG2 is a target gene of miR-378a-5p.

It is well established that a miRNA can target many genes (35). Several of the reported target genes of miR-378a-5p, such as Nodal (82), aromatase (83), and IGF1R (126), may be involved in STB differentiation.

Nodal is a member of transforming growth factor beta (TGF- β) superfamily and plays important roles during embryonic development (110), regulates placental development in mouse and trophoblast differentiation (111), inhibits trophoblast proliferation, migration, and invasion, and induce apoptosis (112,113) and promotes trophoblast cell survival, migration and invasion. We have already demonstrated that Nodal is a target gene of miR-378a-5p and regulates trophoblast cell survival, invasion and

migration (82). Moreover, it has recently reported that Nodal inhibits E-cadherin expression in BeWo cells (114). Future studies will investigate if Nodal promotes STB differentiation and if miR-378a-5p inhibits the STB differentiation by targeting Nodal.

Aromatase has been identified as a target gene of miR-378 in porcine follicular cells (83). Aromatase P450 (P450 arom) is a product of CYP19 gene which catalyzes the conversion of C19 steroids to estrogens and syncytiotrophoblast differentiation is associated with marked induction of CYP19 gene expression and aromatase activity (120, 124,128) (116). In addition, miR-378a-5p targets insulin like growth factor-1 receptor (IGF1R) expression (123). IGFs are the stimulator of tissue growth and regulate the differentiation, mitogenesis and survival of target cells. IGFs exert their effects by binding to IGF1R. The IGF1Rs enhance trophoblast proliferation and differentiation and rescue trophoblast from apoptosis (127). IGF1R expression is increased in BeWo cells during their differentiation into STB, suggesting a role of IGF1R in STB differentiation (103).

Other than these known targets, database mining also revealed that miR-378a-5p has a potential targeting site on hGCM1 3'UTR (Fig. 22B). GCM1 is a placenta-specific transcriptional factor and is required for placental development (47, 103). It is activated by the PKA pathway and promotes STB formation by activating the syncytin protein gene expression (104). It has been reported that overexpression of GCM1 enhanced the syncytin gene expression and cell fusion in BeWo cells (103). In addition, hGCM1 regulates the aromatase (hCYP19I) and CGβ (47). Finally, GCM1 deficient mouse embryos failed to form STB (122). It is recently reported that GCM1 is a target gene of miR-19b and

overexpression of miR-19b mimics result in decrease syncytium formation (47). The possibility that miR-378a-5p suppresses GCM1 to inhibit trophoblast cell differentiation into STB will be investigated in future.

A.

Mature sequence
Mmu-miR-378a-5p

Mature sequence
hsa-miR-378a-5p

B.

hsa-miR-378a-5p

GCM-1 (345-373)

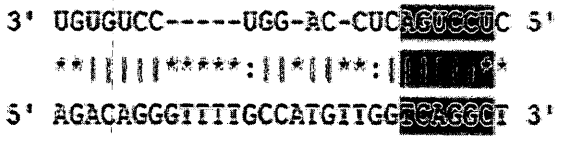


Figure 22. Comparison of human miR-378a-5p with mouse mature sequence of miR-378a-5p and GCM-1 3'UTR. A.) Mouse mature sequence of mir-378a-5p is a homologue of human miR-378a-5p. B). miR-378a-5p has a potential target site on GCM-1 3' UTR, as predicted by Findtar3.

IV.3. Cyclin G2 induces syncytiotrophoblast cell differention

CCNG2 is an unconventional cyclin that inhibits cell cycle progression (69-71). It is expressed in trophoblast cells, including STB, however, its role in human placenta has not been reported. In this study, I provided initial evidence that CCNG2 induces trophoblast differentiation along the STB pathway. BeWo cells transiently transfected with a plasmid containing CCNG2 coding sequence showed a decrease in E-cadherin and increase in syncytin-1 protein levels. On the other hand, knockdown of CCNG2 expression using a siRNA-mediated gene silencing approach resulted in an increase in E-cadherin and a decrease in syncytin-1 protein levels. The results suggest that CCNG2 promotes STB differentiation. However, more experiments have to be done in future to further understand the role of CCNG2 in STB differentiation. Immunofluorescence and quantification of syncytialization markers such as syncytin-1, syncytin-2, ALPP, hCG and LGALS13 in CCNG2 overexpressed and siRNA CCNG2 transfected cells can be further determined in future studies. Although, results from this study suggest that CCNG2 plays a role in STB differentiation, the mechanism by which CCNG2 regulates STB differentiation is yet to be explored.

A recent study demonstrated that CCNG2 interacts with PPARγ to induce adipocyte differentiation (74). It is reported that PPARγ deficiency interferes with trophoblast differentiation and vascularization of placenta (117). PPARγ signaling pathways play important roles in development and function of placenta (118). Interestingly, PPARγ was detected in STB and it increases the β-hCG transcripts levels and secretion of hCG, with the further induction of syncytiotrophoblast hormones such as human placental

lactogen, human placental GH, and leptin (119). The possibility of CCNG2, acting as a co-activator of PPARγ to promote STB differentiation will be investigated in future.

Taken together this study provides the first evidence that CCNG2 regulates trophoblast differentiation and miR-378a-5p is an important regulator of human placental development.

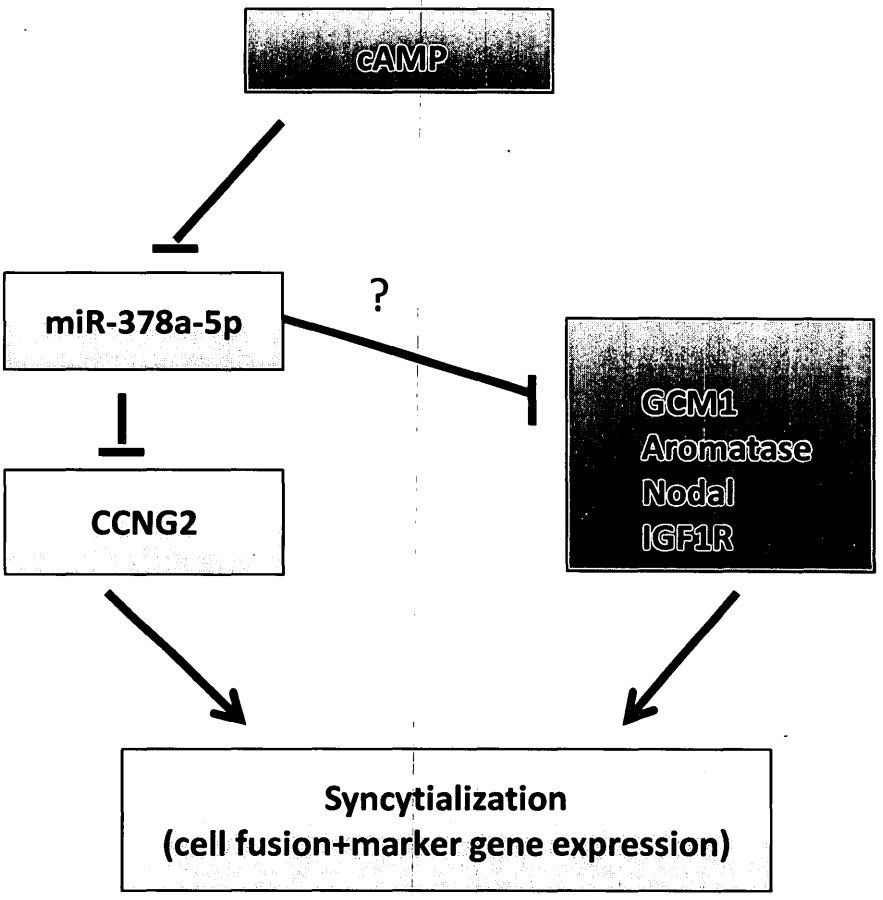


Figure 23. Working model of miR-378a-5p in syncytiotrophoblast differentiation.. miR-378a-5p is down-regulated during cAMP-induced STB differentiation. miR-378a-5p inhibits cell fusion and STB marker gene expression. Cyclin G2, a gene targeted by miR-378a-5p, promotes STB differentiation. Several other potential or confirmed target genes of miR-378a-5p, such as GCM1, aromatase, Nodal, and IGF1R, may also promote STB differentiation. It is possible that miR-378a-5p inhibits STB differentiation by down-regulating the expression of these genes.

V. Summary and Future Directions

The major findings of this study are:

1. miR-378a-5p is a negative regulator of syncytiotrophoblast differentiation

In this study, I examined the role of miR-378a-5p in the regulation of trophoblast cell differentiation along the STB pathway. I identified that miR-378a-5p was strongly inhibited during Forskolin-induced STB differentiation. Subsequently, I performed several functional assays to determine the effect of miR-378a-5p overexpression and inhibition in STB differentiation. First, immunofluorescence results showed that transfection of a miR-378a-5p mimic reduced the number of cells that have multiple nuclei, indicating that miR-378a-5p inhibits cell fusion. Then, RT-qPCR analysis showed that miR-378a-5p downregulated STB marker gene expression. Furthermore, Western blotting revealed that miR-378a-5p increased E-cadherin and decreased syncytin-1 protein levels. On the other hand, inhibiting endogenous miR-378a-5p using an antisense oligonucleotide resulted in an increase in STB differentiation. It was found that anti-miR-378a-5p increased cell fusion, induced STB marker gene expression and decreased E-cadherin levels. Taken together, these findings demonstrate that miR-378a-5p is a negative regulator of trophoblast differentiation into the STB pathway.

We have previously demonstrated that miR-378a-5p promotes cell survival, migration and invasion. The present study provides novel evidence that miR-378a-5p inhibits the STB differentiation. Therefore, it is possible that a physiological role of miR-

378a-5p in placental development is to direct the trophoblast cells into the EVT pathway while limiting their ability to differentiate into the STB pathway. This can be further tested in future studies by using trophoblast cells from healthy placenta.

2. Cyclin G2 is a target gene of miR-378a-5p

Using Bioinformatic tools, luciferase reporter assay, and RT-qPCR, I identified CCNG2 as a novel target of miR-378a-5p. Bioinformatic analysis revealed that miR-378a-5p has five potential complementary binding sites at the 3'UTR of CCNG2. First, miR-378a-5p decreased the luciferase activity of CCNG2 3'UTR-1 construct (contains the first potential binding site). Then, overexpression of miR-378a-5p showed decrease in mRNA levels of CCNG2. Conversely, increase in mRNA levels of CCNG2 was found in cells transfected with anti-miR-378a-5p. Finally, knockdown of CCNG2 using siRNA mimicked the effect of miR-378a-5p, while overexpression of CCNG2 had the opposite effects as miR-378a-5p, on E-cadherin and Syncytin-1 expression. These results strongly suggest that CCNG2 is a target gene of miR-378a-5p. However, additional studies are required to confirm which binding site(s) interacts with miR-378a-5p. Luciferase assays can be conducted using constructs that contain mutated binding sites of miR-378a-5p. In addition, endogenous levels of CCNG2 following miR-378a-5p transfection will be determined by Western blotting.

To determine if miR-378a-5p inhibits STB differentiation by down-regulating, at least in part, CCNG2 expression, several "rescue experiment" can be performed. First,

miR-378a-5p can be co-transfected with a CCNG2-expressing plasmid to test if overexpression of CCNG2 will reverse the inhibitory effect of miR-378a-5p. Second, cells can be co-transfected with anti-miR-378a-5p and CCNG2 siRNA to examine if silencing of CCNG2 expression can prevent anti-miR-378a-5p to induce STB differentiation.

It is well established that a miRNA can target many genes (35) and it is highly possible that all of the observed trends are not due to CCNG2 alone. Several confirmed or predicted targets of miR-378a-5p, such as Nodal, aromatase, hGCM1, and IGFIR, have been shown or suggested to play a role in STB differentiation. Therefore, their involvement in miR-378a-5p-regulated STB differentiation will be explored in future studies.

3. Cyclin G2 induces syncytiotrophoblast trophoblast differentiation

Although CCNG2 has been detected in human placenta, its role during placental development is unknown. In this study, I provided initial evidence that CCNG2 induces trophoblast differentiation along the STB pathway. BeWo cells transfected with CCNG2 showed a decrease in E-cadherin and increase in syncytin-1 protein levels. On the other hand, knockdown of CCNG2 expression showed an increase in E-cadherin and a decrease in syncytin-1 protein levels suggesting that CCNG2 plays a role in STB differentiation. More studies are required to further confirm the role of CCNG2 in trophoblast differentiation. Specifically, the effect of CCNG2 on cell fusion and STB marker gene expression should be determined.

The mechanism by which CCNG2 regulates STB differentiation is yet to be determined. A recent study demonstrates that CCNG2 interacts with PPARγ to induce adipocyte differentiation (74). Since PPARγ has been shown to induce STB differentiation (119), it is possible that CCNG2 promotes the STB differentiation by interacting PPARγ. Co-immunoprecipitation can be used to determine if CCNG2 and PPARγ interact. The effect of CCNG2 on STB differentiation can be tested in the presence of PPARγ agonists and antagonists.

In summary, this study demonstrates that miR-378a-5p inhibits trophoblast differentiation into the STB pathway and suggests that these actions may be partially due to its inhibition of CCNG2 expression. These findings, together with the planned future studies, will provide novel insights into how miRNAs and their regulatory networks control trophoblast differentiation and placental development.

REFERENCES

1. Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell.116: 281-297.

2. Friedman RC, Farh KK, Burge CB, Bartel DP. 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19: 92–105.

3. Bertero T, Gastaldi C, Bourget-Ponzio I, Imbert V, Loubat A, Selva E, Busca R, Mari B, Hofman P, Barbry P, Meneguzzi G, Ponzio G, Rezzonico R. 2011. miR-483-3p controls proliferation in wounded epithelial cells. Faseb J. 25(9):3092-3105.

4. Feng S, Cong S, Zhang X, Bao X, Wang W, Li H, Wang Z, Wang G, Xu J, Du B, Qu D, Xiong W, Yin M, Ren X, Wang F, He J, Zhang B.2011. MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells. Nucleic Acids Res. 39 (15):6669-6678.

5. Crippa S, Cassano M, Messina G, Galli D, Galvez BG, Curk T, Altomare C, Ronzoni F, Toelen J, Gijsbers R, Debyser Z, Janssens S, Zupan B, Zaza A, Cossu G, Sampaolesi. 2011. MJ Cell Biol. miR669a and miR669q prevent skeletal muscle differentiation in postnatal cardiac progenitors 193(7):1197-212.

6. Chen Y, Stallings RL.2007. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. Cancer Res. 1: 67(3): 976-83.

7. Johnston RJ, Hobert O. 2003. A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature. 18; 426(6968): 845-849.

8. Wienholds E, Plasterk RH. 2005. MicroRNA function in animal development. FEBS Lett.;579(26) : 5911-22.

9. Nicholas S. Sokol and Victor Ambros. 2005. Mesodermally expressed *Drosophila microRNA-1* is regulated by Twist and is required in muscles during larval growth. Genes Dev. 1; 19(19): 2343–2354.

10. Victor Ambros. 2008. The evolution of our thinking about microRNAs. Nature Medicine. 14(10).

11. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G. 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2;408 (6808): 86-89.

12. Quintana ML, Rauhut R, Meyer J, Borkhardt A, Tuschl T. 2003. New microRNAs from mouse and human.RNA. 9 : 175–179.

13. Lim LP, Glasner ME, Yekta S, Burge CB, Bartel DP.2003. Vertebrate microRNA genes. Science. 299:(5612): 1540.

14. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP.2003.The microRNAs of *Caenorhabditis elegans.* Genes Dev. 17. 991–1008.

15. Ambros V, Lee RC, Lavanway A, Williams PT, Jewell D.2003.MicroRNAs and other tiny endogenous RNAs in *C. elegans*. Curr. Biol.13 : 807–818.

16. Aravin AA, Quintana ML, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T.2003.The small RNA profile during Drosophila melanogaster development.Dev. Cell. 5 :337–350.

17. Daniel W, Thomson C, Bracken P, and Gregory J. Goodall. 2011. Experimental strategies for microRNA target identification. Nucleic Acids Res. 39(16): 6845–6853.

18. Jingmin *et al.* 2012.Dose-dependent differential mRNA target selection and regulation by let-7a-7f and miR-17-92 cluster microRNAs. RNA Biology. 9 (10): 1275-1287.

19. Cai X, Hagedorn CH, Cullen BR. 2004.Human microRNAs are processed from capped,polyadenylated transcripts that can also function as mRNAs. RNA. 10: 1957–1966.

20. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim *et al.* 2003. The nuclear RNase III Drosha initiates microRNA processing. Nature, 425: 415–419.

21. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. 2004. Nuclear export of microRNA precursors. Science. 303: 95–98.

22. Kleinrouweler CE, van Uitert M, Moerland PD, Ris-Stalpers C, van der Post JA, Afink GB. 2013. Differentially expressed genes in the pre-eclamptic placenta: a systematic review and meta-analysis. PLoS One. 2013 Jul 12;8(7)

23. Bianca C. Bernardoa, Fadi J. Charcharb, Ruby C.Y. Linc and Julie R. McMullena. 2012. A MicroRNA Guide for Clinicians and Basic Scientists: Background and Experimental Techniques.Heart, Lung and Circulation. 21:131–142.

24. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. 2004. Processing of primary microRNAs by the Microprocessor complex. Nature. 432: 231–235.

25. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. 2004.The microprocessor complex mediates the genesis of microRNAs. Nature.432: 235–240.
26. Vasudevan S, Tong Y, Steitz JA. 2007.Switching from repression to activation: MicroRNAs can up-regulate translation. Science. 318: 1931–1934.

27. Lytle JR, Yario TA, Steitz JA. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc Natl Acad Sci. 104:9667–9672.

28. Guo L, Lu Z. 2010. The Fate of miRNA* Strand through Evolutionary Analysis: Implication for Degradation As Merely Carrier Strand or Potential Regulatory Molecule? PLoS ONE 5(6): e11387.

29. Guodong Fu, Jelena Brkić, Heyam Hayder and Chun Peng. 2013. MicroRNAs in Human Placental Development and Pregnancy Complications. Int. J. Mol. Sci. 14: 5519-5544.

30. Dong-bao Chen and Wen Wang. 2013. Human Placental MicroRNAs and Preeclampsia Biol Reprod.113.107805.

31.Line M, Cavaille B, Weber M and Cavaille J. 2009. C19MC microRNAs are processed from introns of large Pol-II, non-protein-coding transcripts. Nucleic Acids Res.37(10): 3464–3473.

32. Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC, Cavaillé J.2004. A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. Genome Res. 14(9):1741-1748.

33. Morales PDM, Chaiwangyen W, Ospina PS, Schneider U, Herrmann J, Gruhn B, Markert UR. 2012. MicroRNA expression profiles of trophoblastic cells. Placenta. 33: 725–734.

34. Donker RB, Mouillet JF, Chu T, Hubel CA, Stolz DB, Morelli AE, Sadovsky Y.2012 The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod. 18(8):417-424.

35. Rooij EV. 2011. The Art of MicroRNA Research.Circulation Research. 108: 219-234.

36.Rossant J and Cross J. 2001. Placental development: Lessons from mouse mutants. Nature Reviews Genetics. 2: 538-548

37.Danniele EB, Malassine A. 2003. Human placenta as an endocrine organ. Growth Horm IGF Res. Suppl A: S34-S37

38. Bischof P, Irminger FI. 2005. The human cytotrophoblastic cell, a mononuclear chameleon. Int J Biochem Cell Biol : 37(1):1-16.

39. Elsebeth SR and Eliezer S. 2005. Human trophoblast function during the implantation process. Reproductive Biology and Endocrinology. 3(56) : 1-12.

40. Gauster M, Moser G, Orendi K, Huppertz B. 2008. Factors involved in regulating trophoblast fusion: potential role in the development of pre-eclampsia. Placenta. 11:1-68.

41.Gavin PC, Elizabeth AL, Christopher WG. 2010. Downregulation of caveolin-1 enhances fusion of human BeWo choriocarcinoma cells. PloS One 5(5):1-7.

42. Benaitreau D, Santos ED, Leneveu MC. 2010. Adiponectin promotes syncytialisation of BeWo cell line and primary trophoblast cells. Reprod Biol Endocrinol. 8:128.

43. Yoshie M, Kaneyama K, Kusama K, Higuma C,Nishi H, Isaka K, Tamura K. 2010. Possible role of the exchange protein directly activated by cyclic AMP (Epac) in the cyclic AMP-dependent functional differentiation and syncytialization of human placental BeWo cells. Human Rep. 25(9): 2229-2238.

44. Tuckey RC. 2005. Progesterone synthesis by the human placenta. Placenta. 26(4):273-281.

45. Ji L, Brkic´ J, Liu M, Fu G, Peng C, Wang YL. 2013. Placental trophoblast cell differentiation: Physiological regulation and pathological relevance to preeclampsia. Molecular Aspects of Medicines.

46. Frendo JL, Thérond P, Bird T, Massin N, Muller F, Guibourdenche J, Luton D, Vidaud M, Anderson WB, Evain BD. 2001. Overexpression of copper zinc superoxide dismutase impairs human trophoblast cell fusion and differentiation. Endocrinology. 142(8):3638-48

47. Kumar P, Luo Y, Tudela C, Alexander JM, Mendelson CR.2013. The c-Myc-regulated microRNA-17~92 (miR-17~92) and miR-106a~363 clusters target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. Mol Cell Biol. 33(9):1782-96.

48. Morrish DW, Dakour J, Li H, Xiao J, Miller R, Sherburne R, Berdan RC, Guilbert LJ. Placenta. 1997. In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium.18(7):577-585.

49. Nulsen JC, Woolkalis MJ, Kopf GS, Strauss JF. 1988. Adenylate cyclase in human cytotrophoblasts: characterization and its role in modulating human chorionic gonadotropin secretion. J Clin Endocrinol Metab. 66(2): 258-65.

50. Daoud G, Amyot , Rassart E, Masse A, Simoneau L and Lafond J. 2005. ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta. J Physiol. 15 (566): 409–423.

51. Baczyk D, Satkunaratnam A, Nait-Oumesmar B, Huppertz B, Cross JC, Kingdom JC. 2004. Complex patterns of GCM1 mRNA and protein in villous and extravillous trophoblast cells of the human placenta. Placenta. 25(6):553-9.

52. Yamada K, Ogawa H, Honda S, Harada N, Okazaki T. 1999. A GCM motif protein is involved in placenta-specific expression of human aromatase gene. J Biol Chem. 274(45):32279-32286.

53. Yu C, Shen K, Lin M, Chen P, Lin C.2002. GCMa regulates the syncytin-mediated trophoblastic fusion. J Biol Chem. 277(51):50062-50068.

54. Leisser C, Saleh L, Haider S, Husslein H, Sonderegger S, Knöfler M. 2006. Tumour necrosis factor-alpha impairs chorionic gonadotrophin beta-subunit expression and cell fusion of human villous cytotrophoblast. Mol Hum Reprod. 12(10):601-609.

55. Nachtigall MJ, Kliman HJ, Feinberg RF, Olive DL, Engin O, Arici A. 1996. The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. J Clin Endocrinol Metab. 81(2):801-806.

56. Morrish DW, Bhardwaj D, Paras MT. 1991. Transforming growth factor beta 1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. Endocrinology. 1991. 129(1):22- 26.

57. Stuart Handwerger. 2009. New insights into the regulation of human cytotrophoblast cell differentiation. Mol Cell Endocrinol. 323(1): 94–104.

58. Głodek A, Kubiczak M, Urbaniak P, Walkowiak G, Nowak-Markwitz E, Jankowska A. 2012. Human chorionic gonadotropin--a well-known hormone with unknown functions. 83(10):766-71.

59. Walker WH, Fitzpatrick SL, Barrera-Saldaña HA, Resendez-Perez D, Saunders GF. 1991. The human placental lactogen genes: structure, function, evolution and transcriptional regulation. Endocr Rev. 12(4):316-328.

60. Muir A, Lever A, Moffett A. 2004. Expression and Functions of Human Endogenous Retroviruses in the Placenta: An Update. Placenta. 25 (18): S16-S25.

61. Chen CP *et al.* 2008. Functional Characterization of the Human Placental Fusogenic Membrane Protein Syncytin 2. Biology of reproduction.79 :815-823.

62. Vargas A, Moreau J, Landry *et al.* 2009. Syncytin-2 plays an important role in the fusion of human trophoblast cells. J Mol Biol. 392(2): 301-18.

63. Than NG, Pick E, Bellyei S, Szigeti A, Burger O, Berente Z, Janaky T, Boronkai A, Kliman H, Meiri *et al.* 2004. Functional analyses of placental protein 13/galectin-13. European Journal of Biochemistry. 271: 1065–1078.

64. Mangal *et al.* 2005. Histochemical Analysis of Placental Alkaline Phosphatase in Hypertensive Disorders complicating Pregnancy. J.Anat.Soc. 54 (2): 1-9

65. Wegmann TG, Guilbert LJ. 1992. Immune signalling at the maternal-fetal interface and trophoblast differentiation. Dev Comp Immunol. 1992 Nov-Dec;16(6):425-30.

66. Orendi *et al.* 2010. The choriocarcinoma cell line BeWo: syncytial fusion and expression of syncytium-specific proteins Reproduction. 140: 759–766.

67. Mangal A, Shrivastava P, Gaur U, Jain A, Goyal U, Rath G. 2005. Histochemical Analysis of Placental Alkaline Phosphatase in Hypertensive Disorders complicating Pregnancy. J.Anat.Soc. 54 (2): 1-9.

68. Spencer K, Cowans NJ, Chefetz I, Tal J, Meiri H. 2007. First-trimester maternal serum PP-13, PAPP-A and second-trimester uterine artery Doppler pulsatility index as markers of pre-eclampsia. Ultrasound Obstet Gynecol. 29: 128–134

69. Martínez GL, Marqués M, García Z, Campanero MR, Carrera AC. 2004. Control of Cyclin G2 mRNA Expression by Forkhead Transcription Factors: Novel Mechanism for Cell Cycle Control by Phosphoinositide 3-Kinase and Forkhead. Mol Cell Biol. 24(5): 2181–2189.

70. Aguilar V, Annicotte JS, Escote X, Vendrell J, Langin D, Fajas L. 2010.Cyclin G2 Regulates Adipogenesis through PPAR Coactivation. Endocrinology. 151(11):5247-5254.

71. Horne MC, Donaldson KL, Goolsby GL, Tran D, Mulheisen M, Hell JW, Wahl AF. 1997. Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J Biol Chem. 272:12650–12661.

72. Xu G, Bernaudo S, Fu G, Lee DY, Yang BB, Peng C. 2008. Cyclin G2 is degraded through the ubiquitin-proteasome pathway and mediates the antiproliferative effect of activin receptor-like kinase 7. Mol Cell. 19 (11):4968-4979.

73. Fu G and Peng C. 2011. Nodal enhances the activity of FoxO3a and its synergistic interaction with Smads to regulate cyclin G2 transcription in ovarian cancer cells. Oncogene. 30: 3953–3966

74. Tontonoz P, Spiegelman BM. 2008. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 77:289-312.

75. Fajas L, Debril MB and Auwerx J. 2001. Peroxisome proliferator-activated receptor from adipogenesis to carcinogenesis. Journal of Molecular Endocrinology. 27: 1–9

76. Uusküla L, Männik J, Rull K, Minajeva A, Kõks S, *et al.* 2012. Mid-Gestational Gene Expression Profile in Placenta and Link to Pregnancy Complications. PLoS ONE 7(11): e49248.

77. Thomas M, Lieberman J, Lal A. 2010. Desperately seeking microRNA targets. Nat Struct Mol Biol. 17(10):1169-1174.

78. Linde et al. 2009. Network Properties for Ranking Predicted miRNA Targets in Breast Cancer. Advances in Bioinformatics. Article ID 182689, 7 pages.

79. Xia W, Cao G, Shao N. 2009. Progress in miRNA target prediction and identification.

Sci China C Life Sci. 52(12):1123-1130.

80. Kasashima K, Nakamura Y, Kozu T. 2004. Altered expression profiles of microRNAs during TPA-induced differentiation of HL-60 cells. Biochem Biophys Res Commun. 322:403-410.

81. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q. 2007. A mammalian microRNA expression atlas based on small RNA library sequencing. Chien M Cell. 129:1401-1414.

82. Luo L, Ye G, Nadeem L, Fu G, Yang BB, Honarparvar E, Dunk C, Lye S, Peng C. 2012. MicroRNA-378a-5p promotes trophoblast cell survival, migration and invasion by targeting Nodal. J Cell Sci. 125: 3124-3132.

83. Xu S, Linher-Melville K, Yang BB, Wu D, Li J. 2011. Micro-RNA378 (miR-378) regulates ovarian estradiol production by targeting aromatse. Endocrinology. 152 (10):3941-3951.

84. Gagan *et al.*, 2011.MicroRNA-378 targets the myogenic repressor MyoR during myoblast differentiation. The Journal of biological chemistry. 286(22): 19431-19438.

85. Kahai S, Lee S-C, Lee DY, Yang J, Li *et al.* 2009. MicroRNA miR-378 Regulates Nephronectin Expression Modulating Osteoblast Differentiation by Targeting GalNT-7. PLoS ONE 4(10): e7535.

86. Lee DY, Deng Z, Wang CH, Yang BB. 2007. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. Proc Natl Acad Sci. 104(51):20350-20355.

87. Feng M, Li Z, Aau M, Wong CH, Yang X and Yu Q. 2011. Myc/miR-378/TOB2/cyclin D1 functional module regulates oncogenic transformation. Oncogene 30: 2242-2251.

88. Ganesan *et al.*2013. MiR-378 Controls Cardiac Hypertrophy by Combined Repression of MAP Kinase Pathway Factors. Circulation. 127: 2075-2077.

89. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T. 2003. A uniform system for microRNA annotation RNA. 9: 277-279

90. Yang JS, Phillips MD, Betel D, Mu P, Ventura A, Siepel AC, Chen KC, Lai EC. 2011. Widespread regulatory activity of vertebrate microRNA* species. RNA. 17:312–326.

91. Loregger T, Pollheimer J and Kno¨ flera M.Placenta. 2003. Regulatory Transcription Factors Controlling Function and Differentiation of Human Trophoblast- A Review. Placenta. 24 Suppl A: S104-110.

92. Schaiff WT, Carlson MG, Smith SD, Levy R, Nelson DM & Sadovsky Y. 2000.Peroxisome proliferator -activated receptor-gamma modulates differentiation of human trophoblast in a ligand-specific manner. J Clin Endocrinol Metab. 85: 3874–3881.

93.Tarrade A, Schoonjans K, Guibourdenche J, Bidart JM, Vidaud M, Auwerx J, Rochette-Egly C & Evain-Brion D. 2001. PPAR gamma/ RXR alpha heterodimers are involved in human CG beta synthesis andhuman trophoblast differentiation. Endocrinology, 142: 4504–4514.

94. Malassiné A, Cronier L. 2002. Hormones and Human Trophoblast Differentiation: a review. Endocrine. 19(1): 3–11.

95. Handwerger S, Aronow B. 2003.Dynamic Changes in Gene Expression During Human Trophoblast Differentiation. Recent Prog Horm Res.58: 263-281.

96.Choi SY, Yun J, Lee OJ, Han HS, Yeo MK, Lee MA, Suh KS.2013. MicroRNA expression profiles in placenta with severe preeclampsia using a PNA-based microarray. Placenta. S0143-4004 (13)00259.

97. Wang W, Feng L, Zhang H, Hachy S, Satohisa S, Laurent LC, Parast M, Zheng J, Chen DB. 2012.Preeclampsia up-regulates angiogenesis-associated microRNA (i.e., miR-17, 20a,

and 20b) that target ephrin-B2 and EPHB4 in human placenta. J Clin Endocrinol Metab. 97: E1051-1059

98. Wice B, Menton D, Geuze H & Schwartz AL.1990. Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. Experimental Cell Research. 186: 306–316.

99. Zachariades *et al.* 2011 Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BeWo): Effects of syncytialization. Int J Mol Med. 27(6): 767-74

100. Knöfler M, Saleh L, Bauer S, Galos B, Rotheneder H, Husslein P, Helmer H. 2004. Transcriptional regulation of the human chorionic gonadotropin beta gene during villous trophoblast differentiation. Endocrinology. 145(4):1685-1694.

101. Coutifaris C, Kao LC, Sehdev HM, Chin U, Babalola GO, Blaschuk OW and Strauss JF III. 1991. E-cadherin expression during the differentiation of human trophoblasts. Development. 113: 767–777.

102. Pötgens AJ, Drewlo S, Kokozidou M, Kaufmann P. 2004. Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action. Hum Reprod Update. 10(6):487-496.

103. Cohran V, Fang J, Milio L, Smith CH, Fant M.1996. Type I insulin-like growth factor receptors in the BeWo choriocarcinoma cell (b30 clone) during cell different. Placenta. 17(5-6):313-20.

104. Knerr *et al.* 2005. Stimulation of GCMa and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions. FEBS Letters. 579 (18):3991–3998.

105. Sha *et al.*2000. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature. 403: 785–789.

106. Fisher SJ, Cui TY, Zhang L, Hartman L, Grahl K, Zhang GY, Tarpey J, Damsky CH. 1989. Adhesive and degradative properties of human placental cytotrophoblast cells *in vitro.* J. Cell. Biol. 109: 891-902.

107. Wheelock MJ, Buck CA, Bechtol KB, Damsky CH. H. 1987.The soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. Cell Biochem. 34:187-202.

108. Capparuccia L, Marzioni D, Giordano A, Fazioli F, De Nictolis M, Busso N, Todros T, Castellucci M. 2002.PPARγ expression in normal human placenta, hydatidiform mole and choriocarcinoma. Mol. Hum. Reprod. 8 (6): 574-579.

109. Schaiff WT, Carlson MG, Smith SD, Levy R, Nelson DM, Sadovsky Y. 2000. Peroxisome Proliferator-Activated Receptor-g Modulates Differentiation of Human Trophoblast in a Ligand-Specific Manner. J Clin Endocrinol Metab. 85(10):3874-3381.

110. Weng W and Stemple DL. 2003. Nodal signaling and vertebrate germ layer formation. Birth Defects Res. C Embryo Today. 69(4) 325–332.

111. Ma GT, Soloveva V, Tzeng SJ, Lowe LA, Pfendler KC, Iannaccone PM, Kuehn MR, Linzer DI. 2001. Nodal regulates trophoblast differentiation and placental development. Dev. Biol. 236(1): 124-135.

112. Munir S, Xu G, Wu Y, Yang B, Lala PK, Peng C. 2004. Nodal and ALK7 inhibit proliferation and induce apoptosis in human trophoblast cells. J. Biol. Chem. 279 (30): 31277–31286.

113. Nadeem L, Munir S, Fu G, Dunk C, Baczyk D, Caniggia I, Lye S, Peng C. 2011.. Nodal signals through activin receptor-like kinase 7 to inhibit trophoblast migration and invasion: implication in the pathogenesis of preeclampsia. Am. J. Pathol. 178(3): 1177–1189.

114. Quail DF, Zhang G, Findlay SD, Hess DA, Postovit LM. 2013. Nodal promotes invasive phenotypes via a mitogen-activated protein kinase-dependent pathway. Oncogene. 1-13.

115. John E, Wienecke-Baldacchino A,Liivrand M, Heinäniemi, Carlberg C and Sinkkonen L. 2012. Dataset integration identifies transcriptional regulation of microRNA genes by PPARγ in differentiating mouse 3T3-L1 adipocytes. Nucleic Acids Res. 40(10): 4446–4460

116. Kamat A, Alcorn JL, Kunczt C, and Mendelson CR. 1998. Characterization of the regulatory regions of the human aromatase (P450arom) gene involved in placenta-specific expression. Mol. Endocrinol. 12: 1764–1777.

117. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM.1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell. 4(4):585-95.

118. Giaginis C, Spanopoulou E, Theocharis S. 2008. PPAR-gamma signaling pathway in placental development and function: a potential therapeutic target in the treatment of gestational diseases. Expert Opin Ther Targets. 12 (8): 1049-1063.

119. Tarrade A, Schoonjans K, Guibourdenche J, Bidart JM, Vidaud M, Auwerx J, Rochette-Egly C, Evain-Brion D.2001. PPAR gamma/RXR alpha heterodimers are involved in human CG beta synthesis and human trophoblast differentiation. Endocrinology. 142(10):4504-4514.

120. Kamat A, Hinshelwood MM, Murry BA and Mendelson CR. 2002.Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. Trends Endocrinol. Metab. 13: 122–128.

121. Neelima PS, Rao AJ. 2008. Gene expression profiling during Forskolin induced differentiation of BeWo cells by differential display RT-PCR. Mol Cell Endocrinol. 281(1-2):37-46.

122. Anson-Cartwright L, Dawson K, Holmyard D, Fisher SJ, Lazzarini RA, Cross JC.2000. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. Naure Genetics 25 (3). 311-314.

123. Ye G, Fu G, Cui S, Zhao S, Bernaudo S, Bai Y, Ding Y, Zhang Y, Yang BB and Peng C. 2011. miR-376c enhances ovarian cancer cell survival by targeting activin receptor-like kinase 7: implication in chemoresistance. J Cell Sci, 124:359-368.

124. Zhou H, Fu G, Yu H, Peng C.2009. Transforming growth factor-beta inhibits aromatase gene transcription in human trophoblast cells via the Smad2 signaling pathway. Reprod Biol Endocrinol. 9(7) :146

125. Handschuh K, Guibourdenche J, Tsatsaris V, Guesnon M, Laurendeau I, Evain-Brion D, Fournier T. 2007. Human chorionic gonadotropin expression in human trophoblasts from early placenta: comparative study between villous and extravillous trophoblastic cells. Placenta. 2007. 28(2-3): 175-84.

126. Knezevic I, Patel A, Sundaresan NR, Gupta MP, Solaro RJ, Nagalingam RS, GuptaM. 2012. A novel cardiomyocyte-enriched microRNA, miR-378, targets insulin-like growth factor 1 receptor: implications in postnatal cardiac remodeling and cell survival.J Biol Chem. 287(16):12913-12926.

127. Forbes K, Westwood M, Baker PN, Aplin JD. 2008. Insulin-like growth factor I and II regulate the life cycle of trophoblast in the developing human placenta. Am J Physiol Cell Physiol. 294(6):1313-13122.

128. Bing J, Amrita K and Mendelson CR.2000.Hypoxia Prevents Induction of Aromatase Expression in Human Trophoblast Cells in Culture: Potential Inhibitory Role of the Hypoxia Inducible Transcription Factor Mash-2 (Mammalian Achaete-Scute Homologous Protein-2). Molecular Endocrinology. 14 (10): 1661-1673.