THE ROLE OF MICRORNA-378a-5p AND ITS TARGET GENES IN DIFFERENTIATION AND FUSION OF BeWo CELLS

UZMA NADEEM

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ABSTRACT

MicroRNAs are the post-transcriptional modulators of gene expression and have been shown to play vital roles in the placental development. The placenta is a multifunctional organ that plays critical roles throughout the pregnancy and fetal development. Bathing in the maternal blood and formed from the fusion of mononucleated cytotrophoblast (CTBs), syncytiotrophoblasts (STBs) are the primary site of numerous placental functions such as exchange of respiratory gases and waste products, transport of nutrients and hormones production. We have previously shown that miR-378a-5p inhibits STB differentiation by targeting cyclin G2 and targets Nodal that inhibits trophoblast invasion in extravillous trophoblast cells (EVTs). In this study, we have further investigated the role of miR-378a-5p and its target genes in STB differentiation by using a choriocarcinoma cell line, BeWo. Using luciferase reporter assays, we showed that miR-378a-5p targets CREB. Transfection of miR-378a-5p decreased, while antimiR-378a-5p increased CREB protein and mRNA levels. We found that a miR-378a-5p target gene, Nodal, induced STB differentiation by activating CREB. Nodal is downregulated by miR-378a-5p but upregulated during Forskolin (an adenylate cyclase activator) induced STB differentiation. Treatment with recombinant Nodal (rhNodal) resulted in increased cell fusion and expression of several STB markers such as syncytin-1, syncytin-2, galactoside-binding soluble lectin13 (LGALS13) and chorionic gonadotropin beta (CGB). Treatment with rhNodal resulted in increased syncytin-1 and decreased E-cadherin protein expression. On the other hand, knockdown of Nodal inhibited cell fusion, decreased syncytin-1 protein expression and several STB markers but increased E-cadherin

protein expression. Furthermore, silencing of CREB using siRNA attenuated the effect of Nodal, and overexpression of CREB and Nodal reversed the inhibitory effect of miR-378a-5p. We showed that miR-378a-5p target gene, cyclin G2, increased cell fusion and STB marker genes, while siRNA cyclin G2 decreased cell fusion and marker genes expression. Nodal induced cyclin G2 promoter activity, suggesting that downregulation of Nodal and cyclin G2 by miR-378a-5p modulate STB differentiation. In conclusion, our study demonstrates that miR-378a-5p exerts an inhibitory role in STB differentiation, in part by down-regulating CREB, Nodal and cyclin G2 expression.

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LIST OF ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2
AGO	Argonaute
ALPP	Alkaline phosphatase
ALK	Activin-like kinase
ASCT1	Alanine, serine and cysteine transporter subfamily 1
ASCT2	Alanine, serine and cysteine transporter subfamily 2
BM	Basal plasma membrane
BMPs	Bone morphogenic proteins
CaMK	Ca^{+2}/ calmodulin-dependent protein kinase
CCNG2	Cyclin G2
CDK	Cyclin-dependent kinase
CGB	Chorionic gonadotropin beta
CREB	Cyclic AMP response element binding
CRH	Corticotropin releasing hormone
CTD	Carboxyl-terminal domain
Cx43	Connexin 43
C19MC	miRNA gene cluster on chromosome 19
DAPI	4',6-diamidino-2-phenylindole
DGCR8	DiGeorgio Critical Region 8
ECL	Enhanced Chemi-Luminescence
EGF	Epidermal growth factor
EPO-R	Erythropoietin receptor
ER	Estrogen receptors
ERK	Extracellular signal-regulated kinase
EVT	Extravillous trophoblast
FBS	Fetal Bovine Serum
FISH	Fluorescence in situ hybridization
FoxO	Forkhead box class O
FZD5	Frizzled 5
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCM-1	Glial cells missing homolog 1
GDFs	Growth differentiating factors
hCG	Human chorionic gonadotropin
HERV	Human endogenous retrovirus
IGF-1	Insulin like growth factor-1

JNK	Jun N-terminal kinase
KID	Kinase inducible domain
LGALS13	Galactoside-binding soluble lectin 13
MAPK	Mitogen activated protein kinase
MEPM	Murine embryonic palate mesenchymal
MFSD2	Major Facilitator Superfamily Domain Containing 2
MSK	Mitogen/stress-activated kinase
MVM	Microvillous membrane
NTD	Amino-terminal domain
PBS	Phospho buffer saline
PCR	Polymerase Chain Reaction
PPARγ	Proliferator-activated receptor gamma
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PML-NBs	Promyelocytic leukemia nuclear bodies
PP1	Protein phosphatases type 1
PP2A	Protein phosphatases type 2A
PPARGC1β	Proliferator-activated receptor gamma co-activator 1 beta
PVDF	Polyvinylidenedifluoride
qRT-PCR	Quantitative Real time PCR
rhNodal	Recombinant human Nodal protein
RISC	RNA-induced silencing complex
RSK	Ribosomal S6 kinase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Spc1	Furin
Spc4	Pace4
Skp 1/2	S-phase kinase-associated protein 1/2
STB	Syncytiotrophoblast
TAD	Transactivation domain
TGF-β	Transforming growth factor beta
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor

CHAPTER 1
LITERATURE REVIEW

I. THE HUMAN PLACENTA

The placenta is a highly specialized extra-embryonic tissue that plays a multitude of critical roles in the prenatal environment and is essential for a healthy pregnancy and fetal development [1]. Histological classification categorized the human placenta as a hemochorial type and considered it as the most invasive placenta because of the direct contact between the chorionic trophoblast cells and maternal blood [1,2]. Functionally, the placenta is considered as a complex transient organ and is involved in multiple tasks such as exchange of nutrients, respiratory gases and waste elimination between mother and the growing fetus. Moreover, it provides immunity to the growing fetus and serves as an endocrine organ by producing a number of hormones and growth factors that regulate pregnancy, provide support and growth to the developing fetus [3].

1. ANATOMY OF THE HUMAN PLACENTA

The placenta begins to develop at implantation stage and continually grows to keep up with the demand of the growing fetus. In humans, the full term placenta is discoid in shape, averages about 15-20 cm in diameter with a thickness of 2-3 cm and a weight of about 500gm [4]. The placenta is implanted in the uterine wall and connects the fetus via umbilical cord which is approximately 50-60 cm in length [5].

The maternal surface of the placenta has several grooves that subdivides the basal surface of the placenta into lobes called cotyledons and consist of anchoring villi which make contact with the decidua [6,7] [Figure 1A]. The chorionic villi make up the main structure of the maternal-facing side of the placenta. The villi or villous trees connect to the fetal surface (chorionic plate)

and the maternal surface (basal plate). During the gestational period, these villi go through several stages of development and on the histological basis villi are categorized into five types [4].

i) **Mesenchymal villi:** Mesenchymal villi are formed from the tertiary villi via primary and secondary villi. These villi have a cytotrophoblast (mononuclear layer of cells) layer that surrounds the villous core and an outer syncytiotrophoblast (multinucleated layer of cells) that covers the villous surface. They lack proper vascularization and develop a sprout at their tips which are referred as villous sprouts. Mesenchymal villi are very important as they are the place of villous proliferation and a site of endocrine activities [6,8].

ii) Immature intermediate villi: Mesenchymal villi differentiate into immature villi and acquire more bulbous and peripheral position as well as develop more blood vessels. At this stage, cytotrophoblasts (CTBs) stop growing but syncytiotrophoblast (STB) grows throughout the development process. These are the principal site of exchange during the first and second trimesters and are considered as the growth center of the villous trees [9,10]

iii) Mature Intermediate villi: Mature Intermediate villi are derived from Immature intermediate villi and are long and slender in shape. At this stage, Mature Intermediate villi develop a high degree of fetal vascularization and play vital roles in fetal maternal exchange [6,10]

iv) Stem villi: Stem villi are formed from Immature Intermediate villi and are composed of CTB layer and connect to the chorionic plate, and contain large vessels and microvessels. Their function is to support the structure of the villous trees [9].

v) Terminal villi: Terminal villi are the final product of the villous tree. They have a high degree of capillarization. At this stage fetal capillary vessels and STB are separated by a minimal distance

that facilitate the diffusive exchange process between the mother and fetus and are considered as the functional unit of the placenta [10,11].

On the other hand, the fetal surface of the placenta is flat smooth disk, and have an umbilical cord at the center [6,7] [Figure 1.1A]. The fetal side is covered with two layers. The inner layer is called amnion and the outer layer is called chorion. Chorion is the embryo derived portion of the placenta that forms from the trophectoderm layer and develops into the chorionic villi of the placenta. These two layers go around the developing baby and form an amniotic sac that contains a protective liquid called amniotic fluid for the growing fetus [4]. The maternal decidua can be divided into three parts. The decidua capsularis forms the third membrane around the fetus. The decidua basalis forms a contact with the chorionic villi and lastly, decidua parietalis makes the rest of the part of the decidua [6] [Figure 1.1B].

The placenta is a highly unique and vascularized organ and forms two separate circulatory systems for the blood. The maternal-placental or uteroplacental blood circulation consists of decidual spiral arteries that bring oxygenated blood and nutrients to the terminal villi in the intervillous space and waste products are eliminated through the uterine veins [1]. The fetal-placental or feto-placental blood circulation consists of the umbilical arteries that carry the deoxygenated blood and waste products from the fetus to the villous core fetal vessels. The oxygenated blood and nutrients are returned to the fetal circulation by umbilical vein. Placental vascularization increases with the growing fetus demands, and at full term the rate of maternal blood flow to the uterus reaches approximately 600-700ml/minute [12] [Figure 1.1C].

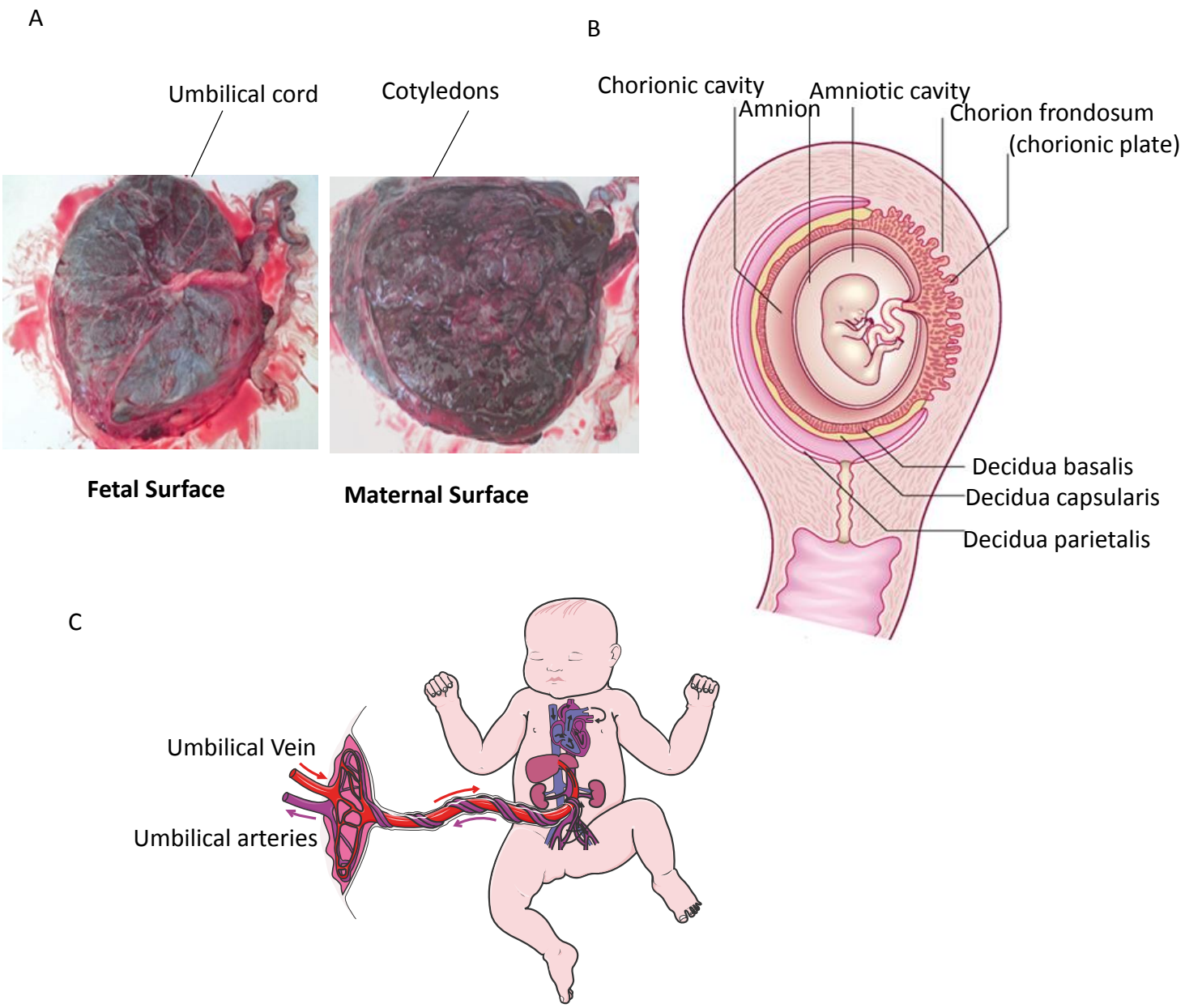


Figure 1.1 A) fetal and maternal side of the placenta. Fetal surface of the placenta is flat and smooth with an umbilical cord at the center. Maternal side of the placenta has several grooves called cotyledons. Figure was modified from an open source article [Ref 19]. **B) Extra embryonic membranes and Placenta formation.** Chorion is the outer most layer and forms from the trophoectoderm layer and develops into the chorionic villi of the placenta. The innermost layer called amnion forms the fluid filled cavity that encapsulates the fetus. Maternal decidua can be divided into three parts. The decidua capsularis forms the third membrane around the fetus. The decidua basalis forms a contact with the chorionic villi. The decidua parietalis, non-interacting part, makes the rest of the part of the decidua. Figure was used with permission from clinicalgate.com. **C) Fetal placental circulation.** It is comprised of umbilical arteries that carry de-oxygenated blood and waste products from fetus to villous vessels. The oxygenated blood and nutrients returned to the fetal circulation by umbilical vein. Figure was modified by using Servier Medical Art template licensed under a CC BY 3.0 license.

2. DEVELOPMENT OF THE HUMAN PLACENTA

Development of the placenta is a highly intricate and continuous process that begins at the time of fertilization. During embryonic development, the fertilized egg undergoes a series of mitotic divisions resulting in a ball of mass called "Morula" [13]. Compaction of the morula is marked as the initiation of differentiation process in which cells in morula align themselves in the outer peripheral region and create a fluid-filled cavity called blastocoele. At this stage, the embryo is called "Blastocyst" and is characterized by the two primary cell types, the outer trophectoderm cells are called trophoblasts, which later develop into the placenta and fetal membranes, and the inner cell mass will form the embryo [13] [Figure 1.2].

After fertilization, the implantation of blastocyst into a receptive endometrium is a critical step of a successful pregnancy [14]. To prepare for successful implantation, endometrium of the receptive uterus undergoes a progesterone-induced decidualization process and does not depend on the presence of blastocyst [15]. During decidualization, progesterone released from the corpus luteum of the ovary transforms the endometrium into a dense cellular matrix of spongy and secretory tissue called decidua [16,17]. The process of implantation begins six to seven days after fertilization [18] and is composed of three stages: apposition, attachment, and invasion [Figure 1.3].

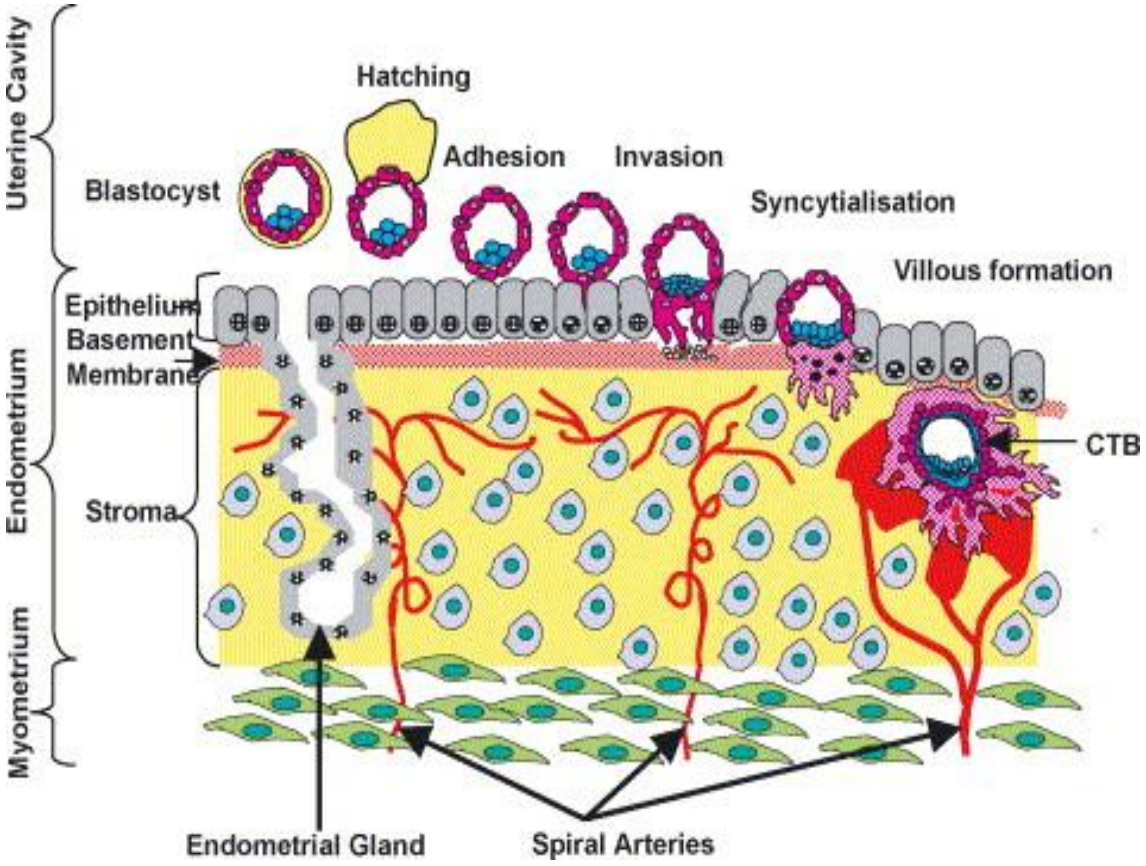


Figure 1.2 Blastocyst implantation steps. Approaching of blastocyst from the series of mitotic divisions result in hatching the blastocyst. Adhesion and invasion result in the digestion of uterine epithelium and implantation of the blastocyst in the uterus. Fusion of mononuclear cytotrophoblasts result in a multinucleated syncytium or syncytiotrophoblast. Used with permission from Bischof P (2005) [13].

i) Apposition:

It is the stage in which blastocyst makes the first contact with the decidua. During this stage, the outer layer of blastocyst interacts through the microvilli that develop at their apical surface with the endometrial epithelial protrusions called "pinopodes" [19]. The appearance of pinopodes can be seen during the window of receptivity in both rodents and humans [20]. The exact function of pinopodes is still unknown, but it is suggested that they arise from the uterine endometrium during the window of receptivity and facilitate the contact between blastocyst and decidua by extracting and decreasing the uterine fluid [20,21]. E-cadherin present in the pinopodes epithelial membrane has been suggested to play an adhesive role in attachment of blastocyst to the decidual membrane [19].

ii) Attachment:

The apposition leads to a next step that is marked by an increase in physical contact between the blastocyst and decidua. Several proteins play vital roles to make this adhesion process stable such as Immunoglobulin superfamily members, integrin and cadherin family [22].

iii) Invasion:

This is the final stage of implantation where a blastocyst invades into the uterine wall by replacing the decidual cells and makes a firm contact with the decidua to initiate placentation. This process is controlled by the component of invading blastocyst and decidual cells through molecular and cellular interaction [23]. This process continues until the cells of the invading blastocyst reach the inner-third of the myometrium and establish a direct contact with the maternal blood and remodel the maternal vasculature [24].

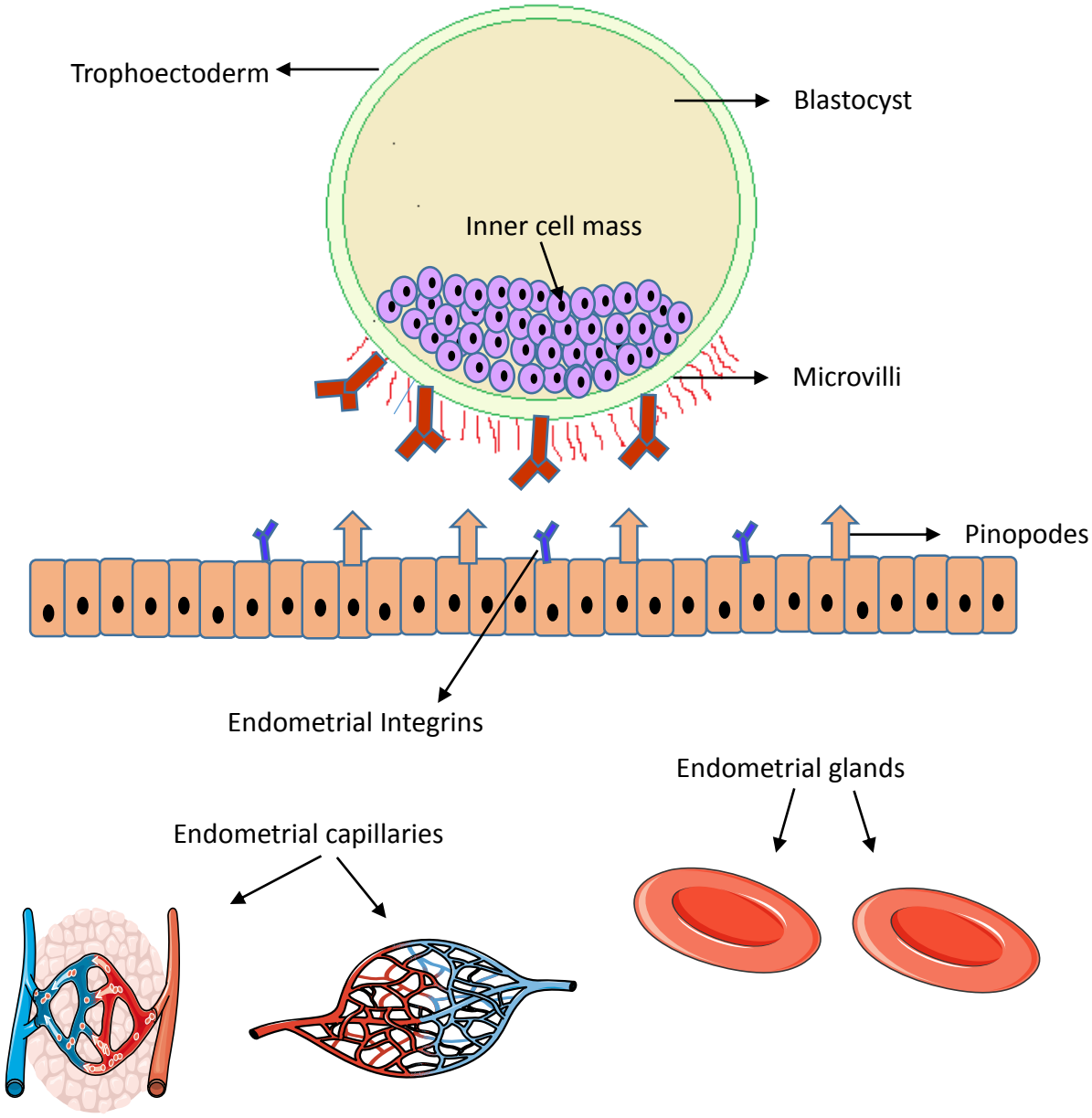


Figure 1.3 A schematic representation of a blastocyst approach in the endometrium. Interaction of blastocyst through the microvilli with the endometrial epithelial protrusions (pinopodes) have been shown. Figure was created with Servier Medical Art template licensed under a CC BY 3.0 license.

3. PATHWAYS OF THE HUMAN PLACENTAL TROPHOBLAST DIFFERENTIATION

Cytotrophoblasts, the precursor cells of the placenta, are the progenitor cells that differentiate into two distinct lineages during embryogenesis: extravillous (EVT) trophoblast and villous trophoblast [3] [Figure 1.4]. EVTs are involved in the remodeling of the maternal vasculature and contain the heterogeneous population of cells that favor the invasive pathway to sustain the growing fetus [25]. On the other hand, in villous pathway mononuclear CTBs fuse to form multinucleated STB cells whose primary functions are secretion and transport [26].

3.1. Extravillous trophoblast: Invasive pathway

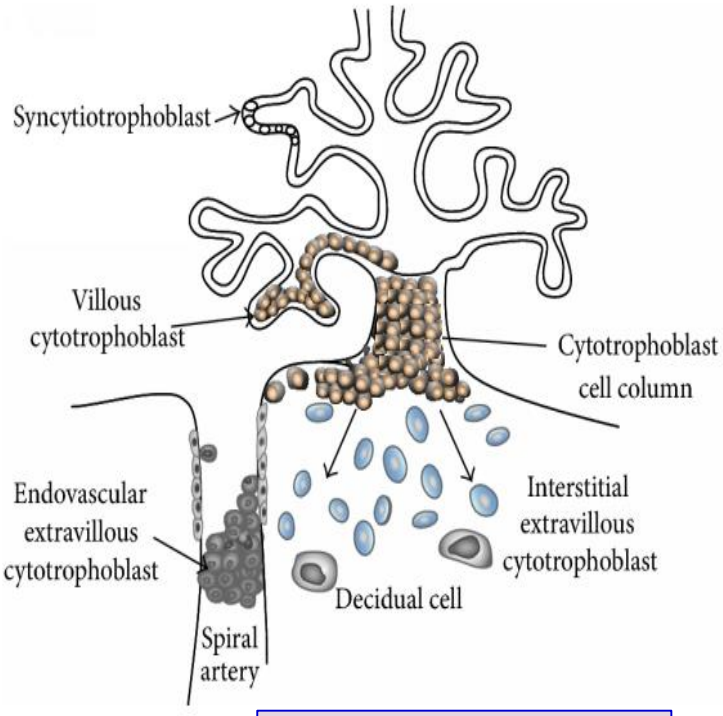
Extravillous trophoblasts consist of a heterogeneous population of CTBs that have highly migratory, proliferative and invasive properties. They arise from the tips of anchoring villi and attach to the decidua. EVTs are primarily mononucleated cells and are found in the chorionic plate, the smooth chorion, cell columns, cell islands, placenta septa and in the lumen and walls of uteroplacental vessels [27].

In the anchoring villi, CTBs proliferate and form multiple layers of CTB cell columns. The cumulation of cell columns result in the proliferative burst of CTBs through the syncytium into the extravillous space and called EVTs. Cells at the proximal end of the portion are highly proliferative and considered as the stem cells of the EVT differentiation pathway [28], while cells at the distal end of the cell column are non-proliferative and form invasive EVTs. Some EVTs

present in the chorionic plate and cell islands exhibit migratory behavior, and this phenotype of EVTs is called migratory EVTs [25].

The invasive EVTs further differentiate into two types: interstitial EVTs that do not invade the blood vessels and endovascular EVTs that invade the uteroplacental vessels and reach the inner-third portion of the myometrium. This precise cellular communication is controlled by factors released and/or expressed by trophoblastic cells and maternal environment such as E-cadherin, cytokines, proteases, integrins, interleukins and several growth factors [29]. These factors facilitate cell migration and invasion by degrading the extracellular matrix proteins such as collagen, laminin, fibronectin and vitronectin. On the other hand, decidua produces a variety of inhibitory proteins and controls trophoblastic invasion. As a consequence of migration and invasion, uterine arteries remodel and facilitate the blood flow to the growing fetus [30]. The endovascular EVTs further differentiate into either intramural EVTs or intra-arterial EVTs. The intramural EVTs invade into the uteroplacental walls and convert decidual vessels into uteroplacental vessels. While intra-arterial EVTs replace the endothelial cells of the maternal vessels and acquire endothelial phenotype through a process called vascular mimicry or pseudovasculogenesis [11,31] [Figure 1.4]. Failure in invasion and remodeling of spiral artery could lead to pathological conditions such as preeclampsia or intrauterine growth restriction [32,33].

A



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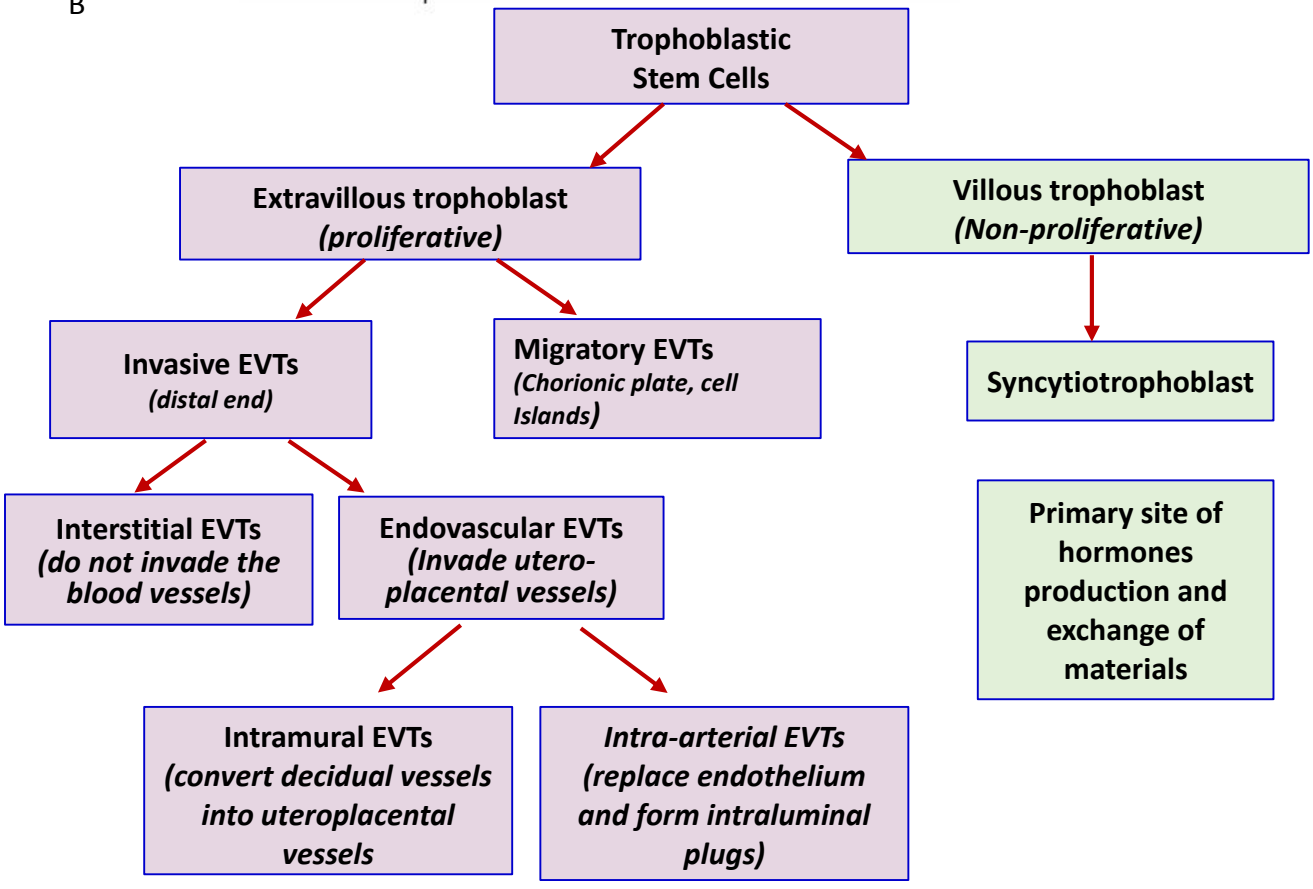

Figure 1.4 Trophoblast Differentiation Pathways. A) Cytotrophoblast stem cells proliferate and form cell columns (CC). At the distal end non-proliferating, extravillous trophoblasts form invasive extravillous trophoblast by detaching from the cell columns and migration into the maternal decidua. Fig 4A was adapted from an open source reference and used with permission from reference [33]. B) Flow chart was drawn and based on reference [23].

3.2. Villous trophoblast: Fusion pathway

In the villous pathway, mononuclear CTBs fuse into multinucleated STB cells and form the syncytial layer that covers the placental villous tree. Fusion of CTBs into STBs results in syncytium formation and this process is referred as syncytialization. STBs are the functional and transport unit of the placenta and are involved in the exchange of gases, nutrients and waste products between mother and fetus. In addition, STBs are considered as the endocrine unit of the placenta and are responsible for the production of various hormones and enzymes that play a vital role in fetal growth and maintenance of pregnancy [3].

3.2.1. Syncytiotrophoblast layer

STBs are the specialized layer of epithelial cells that cover the placental villous tree. These cells are continuously replenished by the fusion of the underlying CTB cell layer. The surface area of STB is measured around $5m^2$ at the 28th week of gestation and reaches to 11-12 m^2 at term [1]. CTBs are characterized by a single nucleus, numerous free cytoplasmic ribosomes and organelles, while the cells are separated by well-formed desmosomes. On the other hand, STBs have multiple nuclei, complex cytoplasmic contents and lack defined cell borders [34].

Unlike STBs, CTBs are proliferative in nature, and therefore the maintenance of syncytial layer is solely dependent on the fusion of CTBs throughout the gestation. The CTB nucleus undergoes morphological changes during fusion process and develops apoptotic features by displaying an annular chromatin aggregation in parts of STB. This annular chromatin aggregation is referred as syncytial knots that later shed from the apical STB into the maternal circulation [35]. This process is essential to keep the biological balance of STB by disposing of the aged cytosolic

contents or apoptotic materials into the maternal circulation and replenishing it with the cellular materials [36–38][Figure 1.5].

Another hallmark of fusion is the loss of plasma membrane during cell fusion. The STBs surface is highly enriched with phosphatidylserine (PS), and externalization of PS from the inner to the outer leaflet of plasma membrane results in the differentiation and fusion of CTBs into STB. Studies show that blocking the PS antigen by a monoclonal antibody blocked the intercellular fusion process in trophoblast models [39]. However, the externalization of PS alone is not sufficient to induce STB fusion and requires more tissue specific recognition mechanisms. The xenobiotic/lipid transporter, ATP-binding cassette sub-family G member 2 (ABCG2) is upregulated during fusion process and showed that it counterbalances the increased PS externalization and regulates PS asymmetry by trafficking PS within plasma membranes [36,40].

3.2.2. Functions of syncytiotrophoblast layer

STBs form a continuous layer that covers the entire surface of chorionic villi of the placenta and have diverse roles throughout the pregnancy.

a) Serve as a placental transport unit

The developing fetus needs energy to grow, and it is therefore essential that nutrients such as glucose, amino acids and fatty acids are available from maternal circulation for the fetus to sustain the appropriate fetal development [41]. The STBs are bathed in the maternal blood of the

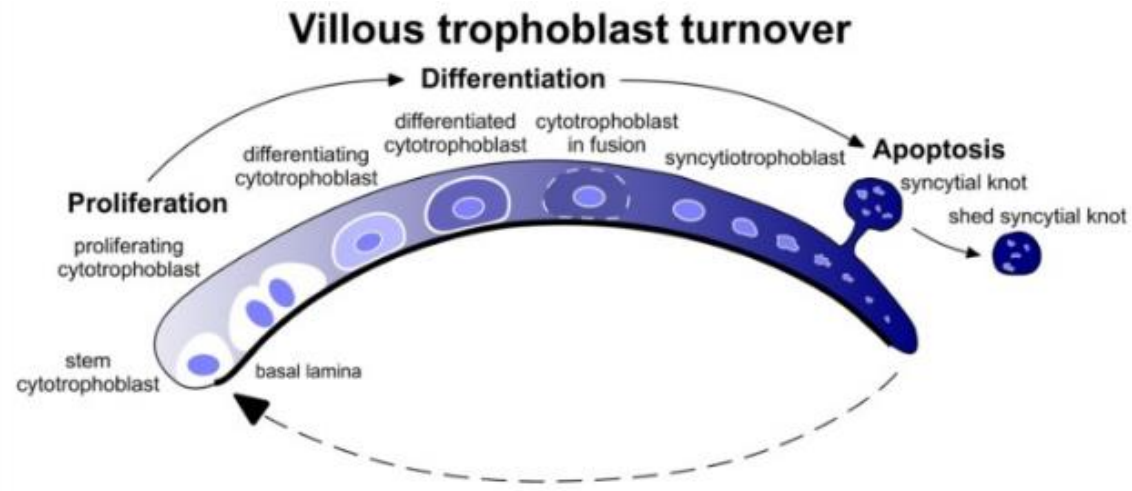

Figure 1.5 Schematic representation of trophoblast turnover. Fusion of CTBs result into multinucleated syncytiotrophoblast. Morphological and functional maintenance of the STB is highly regulated on the fusion of CTBs. The continuous input of syncytial fusion must need to counter balanced by a continuous extrusion of the apoptotic material that accumulates in result of aged nuclei. This apoptotic material is packed into a membrane sealed structure called syncytial knots and released into the maternal circulation as tightly packed corpuscles. Figure was used from an open source reference and used with permission from reference [42].

intervillous space. The anatomical configuration of the placenta revealed that it has transport proteins, electrochemical gradients and diffusion channels for nutrients exchange across the interface. STBs are composed of two polarized membranes: a microvillous membrane (MVM) that faces the maternal circulation and the basal plasma membrane (BM) that faces the fetal circulation [42]. The BM is selectively permeable to molecules such as glucose and amino acids and, based on the size of the solute, it acts as a barrier and rate-limiting step of nutrients transport into the fetal circulation. In addition, passive and active transport, transcellular and facilitated transport, protein transporter, exocytosis and endocytosis also facilitate nutrients transport to the fetal circulation [43,44] [Figure 1.6]. Furthermore, it is also responsible for the exchange of gases and waste products across the materno-fetal interphase by diffusion.

b) Serve as a placental endocrine unit

STBs serve as an endocrine organ throughout the pregnancy. Several lines of evidence show that STB is a site of synthesis of pregnancy-related hormones. For instance: human chorionic gonadotropin (hCG) is synthesized and secreted from STBs and is a key hormone that is involved for the maintenance of pregnancy. It activates several signaling pathways required to sustain the pregnancy and fetal growth [45]. Another hormone, Corticotropin-releasing hormone (CRH) is synthesized and released from the STB and plays a functional role in human parturition [46]. Moreover, leptin is expressed and secreted in the STB and acts as an autocrine/paracrine hormone. Its role in pregnancy and placenta are still unclear, but several studies show that it plays important roles in fetal growth and development [47–49].

c) Serve as a placental immunomodulator unit:

STBs serve as an immune-modulatory role in pregnancy. The hCG secreted from STB plays an immunomodulatory role at the maternal-fetal interface by reducing T-cell activation and cytokine production [50]. STBs synthesize and secrete enzymes such as galactoside-binding soluble lectin 13 (LGALS13, also known as PP13) and alkaline phosphatase (ALPP or PALP). Both enzymes are localized to the syncytiotrophoblast apical membrane and play important roles in maternal-fetal immunity [51,52]. LGALS13 secreted from STBs activates maternal immune cells and facilitates trophoblast invasion and conversion of maternal spiral arterioles [53], on the other hand, ALPP transfers maternal IgG to the fetus [52,54,55].

3.2.3. Regulation of syncytiotrophoblast layer

The differentiation of CTB into STB is governed by a variety of signaling molecules and proteins that play crucial roles in mediating this fusion process.

a) Membrane proteins

Fusion of CTBs into STB cells or syncytium formation is an event of membrane fusion, and the proteins that mediate a key role in STB differentiation belongs to human endogenous retrovirus (HERV) family. HERVs constitutes 8% of the human genome and its members are

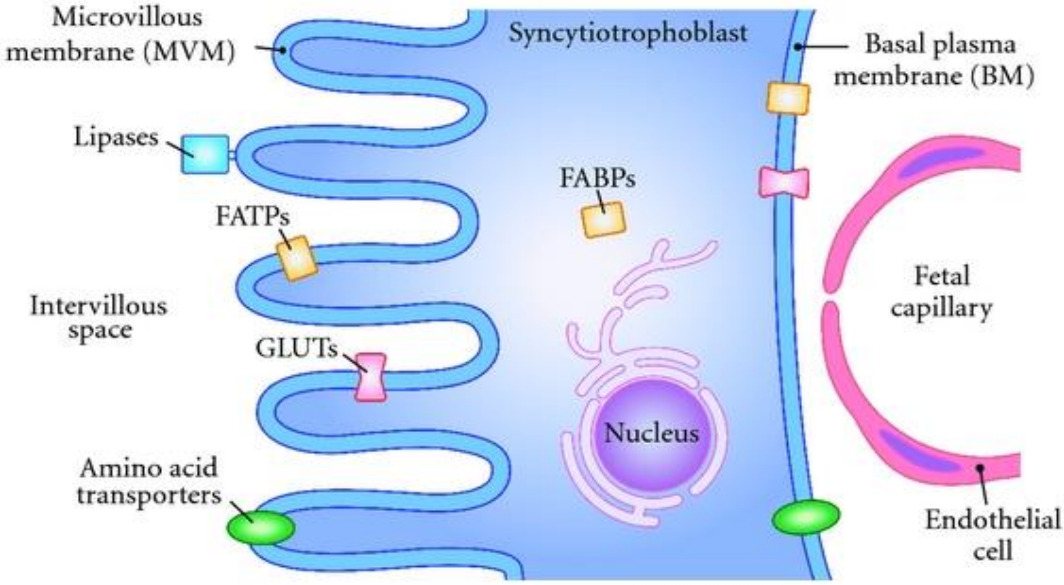

Figure 1.6 Anatomical configuration of syncytiotrophoblast: Syncytiotrophoblasts are composed of two polarized membranes. The one that faces the maternal circulation side is referred as microvillous membrane (MVM) while the membrane facing the fetal circulation is called basal plasma membrane (BM). Several transporters such as amino acids, glucose (GLUTs) and fatty acids (FATPs) are expressed in both MVM and BM of the STB. Lipid contents are available to fetus when extracellular lipases act on maternal lipoprotein from the blood pools in the intervillous space and releases fatty acids that binds to intracellular binding proteins (FABPs) to guide the fatty acids in the cytosol of STB. Figure was obtained from an open source reference and used with permission from reference [45].

glycoprotein in nature [56]. Several members of HERVs are expressed in the placenta, but studies showed that HERV-W envelope glycoprotein (syncytin-1) and HERV-FRD envelope glycoprotein (syncytin-2) played a pivotal role in STB differentiation pathway [57].

The accumulating evidence showed that syncytin-1 is exclusively expressed in the placenta [56]. It is expressed in STB, detected in the placental tissues and is essential for the fusion of CTBs into STBs [36,58]. The name is based on its fusogenic properties to form a syncytial layer of the placenta and is a bonafide gene of fusion [56]. Many factors can regulate syncytin-1 expression. forskolin, an adenylate cyclase activator increased the fusion index in trophoblast cell lines and cultured primary trophoblast [59]. In contrast, silencing of syncytin-1 resulted in a decrease in cell fusion [36]. Syncytin-1 transcripts levels decreased in several pathological conditions such as preeclampsia. Syncytin-1 is a transmembrane protein and induces cell fusion by interacting with mammalian type D receptors, ASCT1 or ASCT2 (alanine, serine and cysteine transporter subfamily 1 and 2) [60].

Evolution of syncytin genes and phylogenetic tree of primates showed that Syncytin-2 is more evolutionarily ancient than syncytin-1, but as this protein was identified after syncytin-1, therefore it is named syncytin-2 [61]. It is an envelope protein of the HERV family FRD and plays critical roles in cell fusion [56]. Syncytin-2 is localized in both CTB and STB and have controversial reports [62]. Some studies reported that syncytin-2 expression increased during forskolin-induced STB differentiation, and its inhibition resulted in a decrease in syncytium

formation [63]. In addition, syncytin-2 plays an important role in the fusion of human trophoblast cells and possesses some immunosuppressive properties [64]. Syncytin-2 interacts with a member of the carbohydrate transporter superfamily, MFSD2 (Major Facilitator Superfamily Domain Containing 2) [65]. In addition to syncytin 1 and 2, another member of HERV family known as ERV-3, has been investigated and shown that its overexpression induced differentiation in a human trophoblast cell model. However, the functional role of Env-3 in not known in the placenta development [66].

Another important membrane protein, CD98, is a multifunctional protein and is expressed in CTBs and STBs [36]. Studies conducted in trophoblast cell model, BeWo, showed that CD98 expression levels increased following forskolin treatment and knockdown using siRNA approach decreased syncytium formation. In addition, CD98 is involved in the cell fusion and regulates amino acid transport during syncytialization [67].

Gap junctional communication between CTBs and STBs has been shown to be essential for syncytialization process [68]. Gap junctions are composed of connexin clusters that act as transmembrane channels. Connexin 43 (Cx43) is detected in both CTBs and STBs and showed that it increased cell fusion, whereas knockdown of Cx43 by antisense Cx43 resulted in decrease in hCG and syncytin levels in primary trophoblast cells suggesting its role in STB pathway [69].

b) Signaling pathways

The coordination of cellular activities and actions in the cellular environment is controlled by signaling pathways. Signaling pathways act as control knobs of the cells that can induce or

block the pathways and play vital roles in the determination of cell fates. Formation of syncytium or fusion of CTBs into STBs is a highly regulated process and several pathways have been shown to regulate STB differentiation. One of the main key regulator of STB differentiation is cyclic AMP (cAMP). Studies have reported that cAMP or its analogs stimulates syncytin gene and induces STB differentiation [70]. Also, an activator of protein kinase C induced STB differentiation in trophoblast cell models, BeWo cells [71]. The CTBs isolated from the term placentae show that ERK1/2 regulates STB differentiation in human placenta [72].

II. TRANSCRIPTION FACTORS

Transcription factors play crucial roles in the fusion of CTBs into STBs. Some of the transcription factors that play a vital role in STB differentiation are peroxisome proliferator-activated receptor gamma (PPARγ)**,** Glial cells missing homolog 1 (GCM1) and cAMP response element binding protein (CREB).

1. PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ)

PPAR-γ, a member of the superfamily of nuclear receptors, is essential for the fusion of CTBs into STB [73]. Studies show that PPARγ deficiency interferes with trophoblast differentiation and vascularization of the placenta [74] and PPARγ signaling pathways play important roles in development and function of the placenta [75]. PPARγ is expressed in STB, and it increases the transcript levels and secretion of hCG [76]. It has been recently reported that knockdown of PPARγ/ RXRα in BeWo cells inhibits the syncytin-1 expression in contrast to controls [36]. Moreover, stimulation of PPARγ/ RXRα signaling by Protein kinase A via forskolin (an adenylate cyclase activator) results in the induction of leptin gene and syncytin-1 gene expression, and hCG secretion [59].

2. GLIAL CELLS MISSING HOMOLOG 1 (GCM1)

GCM1 is considered as a master regulator of the placental cell fusion in mammals. GCM1 is a member of zinc binding transcription factor and regulates differentiation of CTBs into STB. Studies have shown that GCM1 regulates the syncytin-mediated trophoblastic cell differentiation

[77,78] Furthermore, it has demonstrated that forskolin and PKA stimulate GCM1 mediated transcriptional activation and GCM1 controls the expression of syncytin gene that regulates STB formation [79].

3. CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN (CREB)

CREB is one of the best characterized stimulus-induced transcription factor that was originally identified in 1987 as a target of the cAMP signaling pathway regulating somatostatin. CREB belongs to leucine zipper family and activates transcription of target genes in response to a diverse array of extracellular and intracellular signals such as calcium, neurotrophins and growth factors [80] . It is a nuclear transcription factor and ubiquitously expressed in the cells [81].

3.1. Structural features of CREB

In humans, CREB is mapped to chromosome 2, consists of 341 protein residues, and has a molecular mass is 43kDa [82,83]. Three main domains have been characterized in CREB protein structure: an amino (NH_2) terminus activation domain, a dimerization unit and carboxyl (COOH) terminus bZIP DNA-binding domain [84]. The N-terminus of CREB is the activation domain that consists of glutamine rich 1 region (Q1 domain) that is followed by the kinase-inducible domain (KID) and glutamine rich 2 region (Q2 domain). The Q2 domain is also referred to as constitutive activation domain (CAD) that interacts with DNA binding protein factors, and recruits a RNA polymerase complex to initiate the transcription process [85]. Kinase inducible domain is a regulatory region that harbors the phosphorylation sites, in particular at serine 133 and 142, for various kinases and play key roles in CREB activation. The carboxyl terminus of CREB contains a basic DNA-binding domain that binds to the cAMP response elements (CRE) and a leucine

zipper dimerization domain that homo-hetrodimerize with the bZIP transcription factors [84–86] [Figure 1.7].

3.2. CREB activation and signaling

Stimulus-induced CREB activation is mediated by phosphorylation. It is well documented that exposure of forskolin (an activator of adenylyl cyclase) results in CREB phosphorylation at a specific residue, serine 133 (Ser 133) via PKA pathway [82]. Phosphorylation of this site is required for signal induced transcription, and mutation of Ser 133 abolished the transcriptional response to elevated cAMP [87]. CREB can be phosphorylated by various kinases such as protein kinase A (PKA), protein kinase B (Akt/PKB), protein kinase C (PKC), Ca^{+2}/CaM-dependent kinase (CaMK) II and IV, mitogen/stress-activated kinase (MSK), ribosomal S6 kinase (RSK), and MAPKAP (MK2) [70,85]. Studies have shown that CREB activates stimulus-dependent transcription within 30 minutes. The phosphorylated form of CREB, in turn, initiates the transcription of its target genes. GCM1 is the master regulator of STB differentiation, and it has been reported that CREB binds and stimulates the GCM1 promoter activity whereas knockdown of CREB using siRNA mediated approach decreased GCM1 mRNA and activity [88–90]. Furthermore, CREB increases the GCM1 protein stability and facilitates differentiation [90] [Figure 1.8].

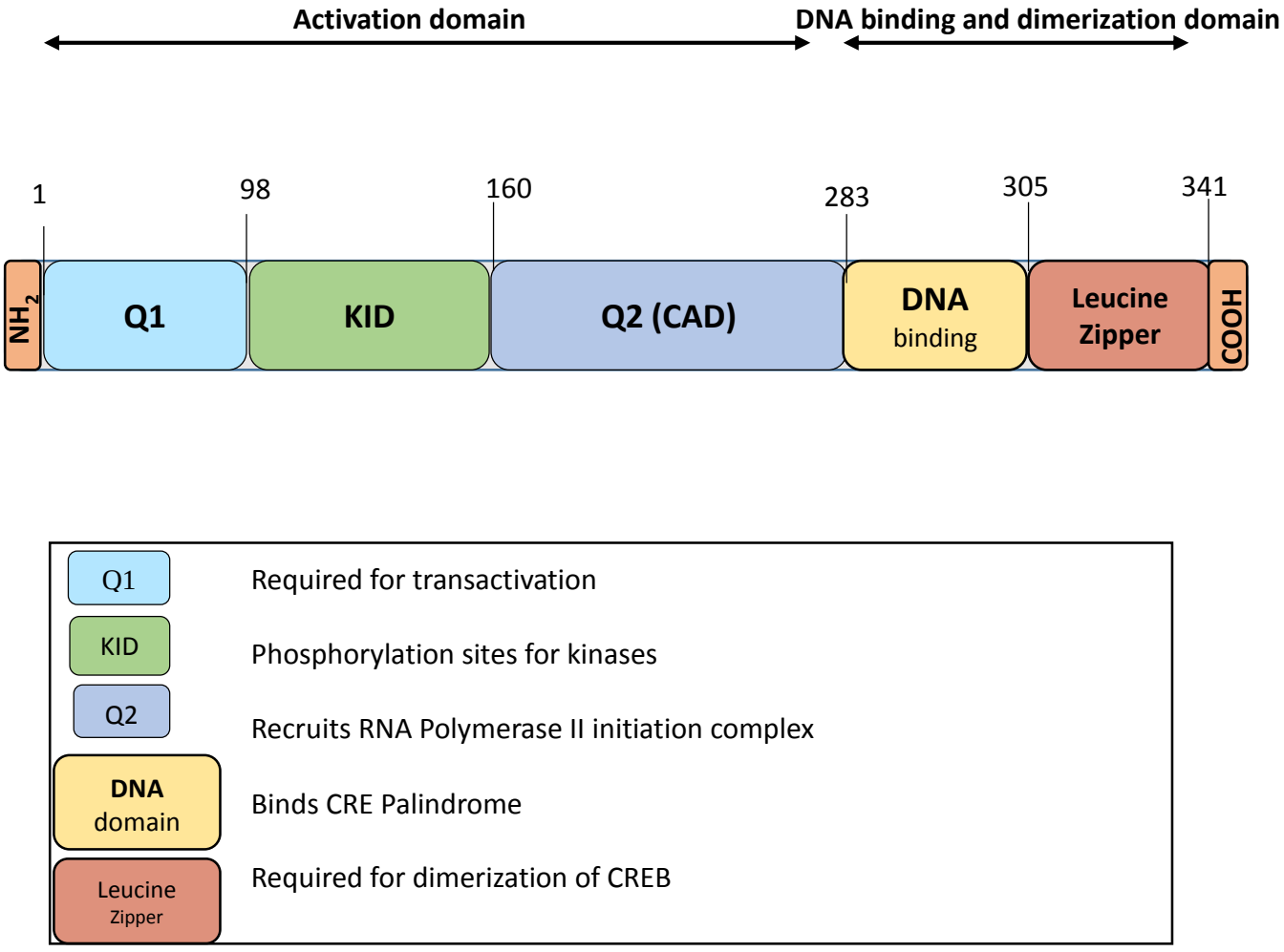

Figure 1.7 Schematic representation of CREB protein: Three main domains of CREB has been characterized. The amino-terminal domain has the activation domain that consists of glutamine rich regions (Q1 and Q2). It has a centrally located kinase inducible domain (KID) that contains phosphorylation sites for the activation of CREB. The carboxyl-terminal domain contains the basic region and the leucine zipper that is important for DNA binding and dimerization, respectively. Figure was drawn based on references [83-85].

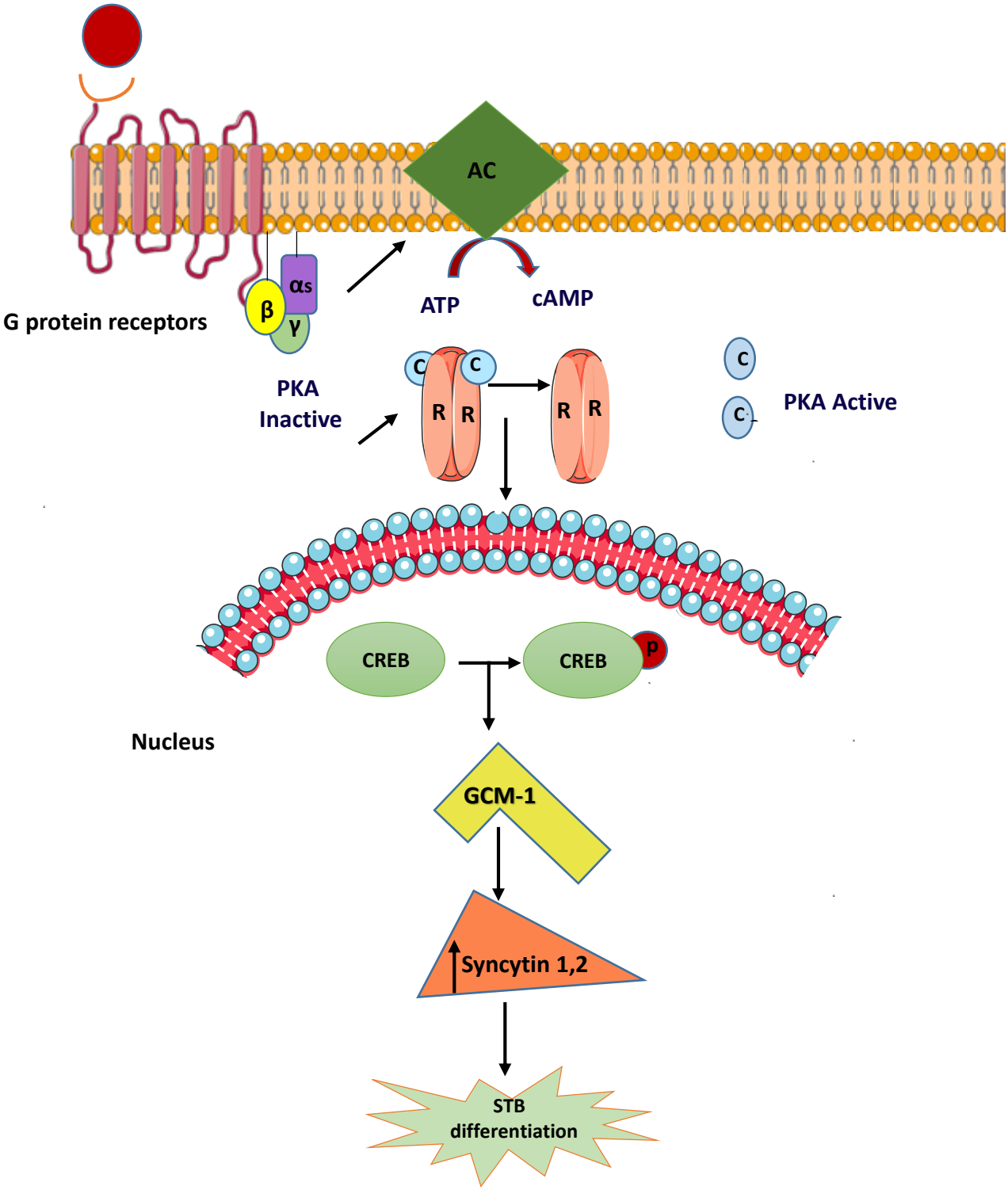

Figure 1.8 Activation of CREB by the cAMP/PKA signaling pathway. Binding of ligand to heterotrimeric G-protein coupled receptors activates the α-stimulatory subunit of G-protein that activates adenylyl cyclase (AC) and catalyzes the production of cAMP. The cAMP-dependent protein kinase (PKA) phosphorylates CREB. The phosphorylated CREB binds to Glial cells missing homolog 1 (GCM1) promoter, one of the most important transcription factor necessary for the differentiation of CTBs into STB, and increases the GCM1 protein stability. GCM1 upregulates the syncytin transcription by binding to its promoter and induces cell fusion. Figure was created with Servier Medical Art template licensed under a CC BY 3.0 license by using references [84-88].

3.3. CREB inactivation and regulation

The inactivation of CREB depends on the transcriptional activation of the target genes and mediated by the Ser/Thr-specific protein phosphatases type 1 (PP1) and 2A (PP2A) [91,92]. It has been reported that phosphodiesterase type IV (PDE4), degrades cAMP and indirectly inactivates CREB [93]. Furthermore, DNA damage can trigger CREB inactivation by phosphorylating multiple Ser residues (Ser 108,111,114 and 117) [93].

It is generally accepted that phosphorylation of CREB at Ser-133 play a key role in regulation of CREB mediated transcription, but several other post-transcriptional modifications are reported that can influence the CREB activity.

a) Acetylation: It has been reported that CREB activation domain contains three Lysine residue (Lys-91, Lys-96 and Lys-136) and CBP can acetylate at these residues that results in increased CREB-dependent transcription [93].

b) Ubiquitination and SUMOylation: It has been shown that hypoxia treatment in T84 cells (human colon carcinoma cell line) resulted in decreased CREB-dependent transcription by time-dependent repression of PP1 levels that results in hyperphosphorylation, ubiquitination and proteasomal degradation of CREB [94]. However, prolonged hypoxia conditions may results in SUMOylation of a fraction of CREB protein and provide stabilization of CREB protein and enhanced the CREB-mediated transcription [95]. Another study conducted on *Aplysia* (an invertebrate species) has reported that stimulation with 5-HT (serotonin) activates PKC that results in increase in ubiquitination and CREB repressor degradation [96].

c) Glycosylation: The Q2 domain of CREB has glycosylation sites and covalent modification of these sites within the amino acid residues (256-261) decreases the CREB activity. Using HeLa

cells, it was demonstrated that glycosylation resulted in inhibition of CREB-mediated transcription [97].

In addition to the post-transcriptional modifications, CREB can be regulated at the transcriptional levels. The non-coding small RNAs called microRNAs (miRNAs) have been shown to bind to the 3'UTR of CREB mRNA and repress CREB expression. Several studies have reported that miRNAs target CREB and regulate its expression at the translation levels. A brain specific miRNA, miR-134, increased in hippocampus due to a deficiency of NAD-dependent deacetylase Sirtuin-1 resulting in impaired synaptic plasticity [98]. CREB is a proto-oncogenic transcription factor and is overexpressed in gliomas. It has been shown that CREB binds to the regulatory sequence of miR-23a and enhances its expression at the translational levels [99]. Similarly, miR-34b targets CREB in acute myeloid leukemia [100].

3.4. Functions of CREB:

CREB regulates diverse cellular responses such as proliferation, survival and differentiation. CREB family members play an essential role in learning and memory [101] as well as neuronal adaptation to drug abuse [102]. CREB is vital for hormonal control of metabolic processes and regulates gluconeogenesis by insulin and glucagon [103]. CREB regulates the rate limiting enzyme (3-hydoxy-3-methylglutaryl coenzyme A) of cholesterol biosynthesis and sterol regulatory element-binding proteins [104]. CREB is known to regulate the adiponectin gene that is responsible for adipocytes differentiation and control metabolic processes including glucose regulation and fatty acid oxidation [105].

Cell survival is one of the critical function of CREB. CREB upregulates an anti-apoptotic gene Bcl-2 that is required for nerve cell growth [106]. CREB is also involved in the survival and differentiation of adipocytes [107]. Furthermore, several studies have reported that CREB plays important role in the maintenance and function of heart and angiogenesis [81].

The signaling cascade cAMP-PKA-CREB has been well known in placental gene activation [70]. It has been reported that CREB binds and stimulates the promoter activity of GCM1 which is the master regulator of placental cell fusion and STB differentiation [90]. In addition, it regulates a fusogenic protein syncytin-2 involved in STB formation. [88]. CREB regulates furin expression and transcription during STB differentiation. Furin is a proprotein convertase enzyme that activates many proteins such as TGF-β, insulin-like growth factor (IGF)-1 and its receptors and vascular endothelial growth factor (VEGF) to regulate trophoblast cell fusion [108]. Furthermore, it has been demonstrated that CREB mediated signals are essential for cell survival during early mouse development [109].

III. MICRORNAs

MicroRNAs (miRNAs) are small (20-22 nucleotides), endogenous single-stranded non-coding RNAs that play essential roles in cell proliferation, differentiation, growth, apoptosis and many physiological processes by regulating gene expression [110]. This sub-set of non-coding RNAs constitute about 1-5% of the total genome, and a single miRNA can target many genes [111]. In addition, bioinformatics analysis of mammalian genomes showed that more than 60% of mRNAs can be targeted by a single miRNA, [111] and any aberrant expression of miRNA could lead to pathological conditions such as cancers, [112], diabetes [113], cardiovascular diseases [114], preeclampsia [115] and neurological disorders [116].

In addition to the abundance of intracellular miRNAs, considerable amounts of miRNAs have been found outside the cells and are referred to as extracellular miRNAs (ECmiRNAs). These ECmiRNAs are expressed in almost all biological fluids such as cerebrospinal, peritoneal, amniotic, synovial, follicular and seminal, as well as in blood, saliva, tear, urine, breast milk and colostrum [111]. These ECmiRNAs are remarkably stable in the RNase-rich extracellular environment, but it is still unclear what makes these miRNAs more vulnerable and susceptible to quick degradation. However, many hypotheses have been proposed to explain the possible mechanism of miRNAs stability in circulation against RNase activity. One of the earliest theories proposed that miRNAs may conjugate with proteins that protect them against enzymatic activity [117] which later on showed that miRNAs are protected by lipoprotein complexes in the plasma [118]. Another hypothesis is that miRNAs are wrapped with membrane vesicles that protect them from the RNase activity in the extracellular environment. This, later on, showed by isolation of miRNAs from membrane vesicles such as exosomes, microparticles and apoptotic bodies

[119,120]. These ECmiRNAs may deliver to the recipient cells by specific pathway and can regulate the transcriptional activity of target genes [121].

1. DISCOVERY OF MICRORNAs:

More than two decades ago **(**1993), Victor Ambros and his colleagues discovered the first miRNA, *lin-4*, in the nematode *Caenorhabditis elegans (C.elegans)* as native RNA fragments that control the timings of post-embryonic development. They demonstrated that *lin-4* gene did not code a gene product but suppressed the *lin-14* expression by binding to the 3'UTR of the *lin-14* gene [122]. Seven years later (2000), the second miRNA, *let-7* was discovered in *C.elegans* and it was demonstrated that one miRNA could target the 3'UTR of several miRNAs and one mRNA 3'UTR can be targeted by many miRNAs [123]. Subsequently, the *let-7* homologs were detected in different species such as human, zebrafish, *Drosophila* (fruitfly), annelids and molluscs. Currently, online databases have annotated about 2500 miRNAs, and among them 1000 of these are validated in humans alone. Furthermore, studies have shown that miRNAs influence gene expression in normal and pathological conditions [111]. The continuous research of miRNA in the different fields suggested that miRNAs constitute an important regulatory network and controls nearly all aspects of cellular processes from development to aging.

2. BIOGENESIS OF MICRORNAs

Based on the genomic location, miRNAs are generally classified into "intergenic" or "intronic". Intergenic miRNAs are transcribed as independent transcription units while intronic miRNAs transcribed in parallel with their host gene transcripts and share the promoter with their host genes. Therefore, intronic or intragenic miRNAs have common expression patterns and regulatory mechanisms with their host genes [124]. However, recent research shows that human

miRNAs can be transcribed as independent transcription unit. Approximately, half of the mammalian miRNAs are processed from introns of protein-coding genes or genes of other non-coding RNAs [125].

Biogenesis of miRNA is a multi-step process that requires enzymes and binding proteins and consists of both nuclear and cytoplasmic phases. MicroRNA genes are transcribed typically by RNA polymerase II into primary transcripts (pri-miRNAs) in the nucleus and generate a primary (pri-mRNA) transcript that consists of several kilobases in length and has a 5' cap and a 3' poly-A tail. The long pri-miRNA transcripts are processed and cleaved into a stem-loop ~70 nt hairpin precursor (pre-miRNA) in the nucleus by a microprocessor complex, composed of RNA-binding protein DiGeorgio Critical Region 8 (DGCR8) and nuclear RNase III endonuclease enzyme Drosha [126,127]. The pre-miRNA is exported out of the nucleus into the cytoplasm by a member of the nuclear transport receptor family, Exportin 5 and a nuclear protein Ran-GTP complex and then subsequently processed by cytoplasmic RNase III endonuclease Dicer. The cleavage by Dicer removes the terminal loop of pre-miRNA, resulting in a 22 nucleotide double-stranded miRNA:miRNA* duplex [128] [Figure 1.9]. Finally, the miRNA duplex unwinds and undergoes the coordinated process of RNA-induced silencing complex (RISC) assembly and loaded onto Argonaute (AGO) proteins. These proteins are the core components of the RISC complex, and in humans there are four main types of AGO (AGO1 to AGO4) proteins have been identified. These proteins act as the target recognition modules and regulate the expression of target genes by adding to a complementary sequence in the 3'UTR of target mRNAs following the Watson and Crick principle of base pairing [129]. Gene silencing largely depends on the perfect complementarity which takes place in the seed region (nucleotide 2-8) of miRNA and target mRNA. Although several mechanisms for miRNA actions have been proposed but a unified

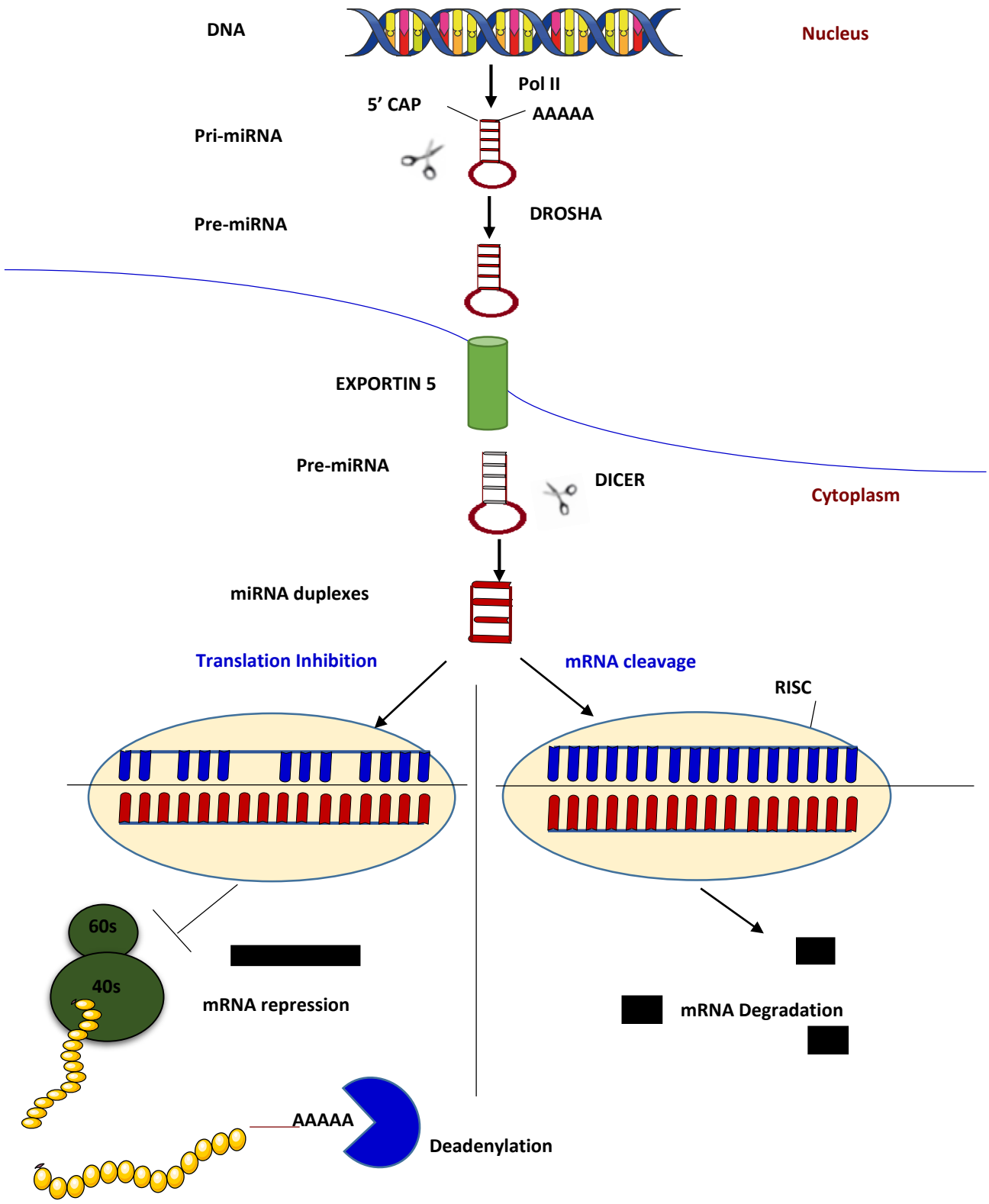

Figure 1.9 Biogenesis of microRNAs: MicroRNA genes are transcribed into primary transcripts (pri-miRNAs) by RNA polymerase II into the nucleus. Primary miRNAs are processed and cleaved into precursor miRNAs (pre-miRNAs) by Drosha and then exported out of the nucleus into the cytoplasm by Exportin 5. Subsequent processing of pre-miRNA by RNase III endonuclease Dicer results in formation of miRNA duplexes. The duplex unwinds and mature miRNA is incorporated into the RISC complex, binds to the 3'UTR of target mRNAs and induces gene silencing through mRNA repression, de-adenylation or degradation. Figure was created from references [99-105].

model is still undefined [130]. In general, perfectly complementary mRNA targets are cleaved while partially complementary targets are silenced through translational repression, mRNA deadenylation and degradation [131]. In addition, it has been reported that some miRNAs can bind to the target sites in the 5'UTR as efficiently as 3'UTR and are able to suppress the target mRNAs [132].

3. CLASSIFICATION OF MICRORNAs

Victor Ambros presented the universal guidelines for the identification and annotation of newly discovered miRNAs, as a consistent naming scheme was quite essential to facilitate the exponential discovery of novel miRNAs and database construction [133]. As sometimes multiple miRNAs arise from the primary transcript and contain multiple hairpins for different miRNA, therefore Ambros suggested that miRNA must follow certain expression and biogenesis criteria. The naming criteria of a miRNA must begin with three letter prefix that designates the species of origin, such as "hsa" for human miRNAs. The precursor hairpins are annotated by "mir, while the mature miRNA used a "miR" prefix and followed a unique identification number (e.g., miR-378). In addition, paralogous miRNAs are annotated by a letter suffix (e.g., miR-378a, miR-378-b) [133].

Finally, the naming of the two strands (miR:miR* duplex) was initially thought on the basis of the thermal stability of miRNA. It was thought one strand of miRNA is biologically active and referred as "miR", while the other strand was considered as non-functional or an inactive strand and represented by miR* (miRNA star or passenger strand). It was believed that miR star will be degraded and act as a minor product in some lineages but further investigation revealed the unique

biological functions and similar expression level to the guide strand. Therefore, mature miRNAs from the same precursor miRNA are designated as -5p (processed from the 5' arm) and -3p (processed from the 3' arm), e.g., miR-378a-5p and miR-378a-3p [134].

4. MICRORNA TARGET PREDICTION AND VALIDATION

The accurate prediction and validation of miRNA targets are paramount to study the function of miRNAs. Several computational methods have been proposed for the identification of target mRNAs for miRNAs [135]. MicroRNA targets are often recognized through the perfect complementarity between miRNA seed region and target mRNA, but not all of these canonical sites are equally effective and create a high signal to noise ratios in predictive algorithms and make target recognition more complex and challenging [136].

There are five canonical "seed" types that have been defined. The most effective canonical site has the highest conservation and efficacy in miRNA suppression are the 8mer targets in which Watson-Crick pairing matches to the 2-8 position of miRNA seed sequence and adenosine across position one. The preference for adenosine (A) nucleotide is independent of miRNA sequence identity and facilitates adenosine-binding pocket in AGO to mediate target recognition. Slightly less effective seed types, 7mer-m8 (match at position 2-8) or 7mer-A1 site (match at position 2-7 with an A opposite position 1) [137]. The other two canonical site types are associated with lower efficacy and weak preferential conservation are 6mer (match at position 2-7) and offset-6mer (match at position 3-8) [138].

Target prediction based on the seed-region complementarity alone is not sufficient to provide the target predictions with high specificity, therefore many algorithms in addition to the

conserved target sites use a combination of different influencing factors. For instance, duplex thermal stability, secondary structure of the miRNA/mRNA duplex, RNA binding proteins and their influence on target sites, sequence matching between miRNA and mRNA, and three-dimensional complex analysis are some of the factors that have been taken into account to increase the predictive power of the functional targets. Some of the computational prediction programs used to identify the potential target genes for miRNA are miRanda, TargetScan, TargetScanS, RNAhybrid, DIANA-microT, PicTar, RNA22 and FindTar [135].

Validation of a predicted target gene of a miRNA can be done by using several experimental approaches such as reporter assays, proteomic analysis, real-time PCR and immunofluorescence. Luciferase reporter gene assay is the most direct method to confirm a miRNA target gene. In this assay, the luciferase coding sequence is fused with the 3'UTR of a potential target gene. The decrease in luciferase activity shows that 3'UTR of a potential target gene has a specific target site of a particular miRNA. In addition, biochemical approaches have been used to determine the miRNA:mRNA complexes by immunoprecipitation (IP) or pull-down of labeled miRNA from RISC components [135]. Moreover, Western blot analysis and quantitative real-time PCR are commonly used to determine the changes in protein expression and mRNA levels in miRNA transfected or knock-down cells [139].

5. ROLE OF MICRORNAS IN SYNCYTIOTROPHOBLAST DIFFERENTIATION

MicroRNAs have been shown to regulate pathways that play vital roles in placental development. Several studies have demonstrated the role of microRNAs in STB differentiation. In the human placenta, highly expressed miRNAs are derived from miRNA gene cluster on chromosome 19 (C19MC) [140]. This is the largest miRNA gene cluster that spans approximately 100 kb at chromosome 19q13.41 and encodes 56 mature miRNAs [141]. Small RNA library

sequencing and miRNA histochemical analysis using human placenta chorionic villi revealed that villous trophoblast expressed several placenta specific miRNAs [141].

Several studies have shown that miRNAs derived from the C19MC cluster are highly expressed in villous trophoblast and involved in STB differentiation. *In situ* hybridization data showed that one of the chromosome 19-derived miRNA (miRNA-517B) is highly expressed and released from STB via exosomes into the maternal circulation and targets maternal tissues [141]. Laser microdissection (LMD) followed by RT-qPCR showed that miR-512-3p, miR-518b, miR-520a, miR-524 and miR-1323 were predominantly expressed in villous trophoblast and in BeWo cells (a choriocarcinoma cell line to study STB differentiation). Among these miRNA, miR-512-3p targets protein phosphatase 3, regulatory subunit B, alpha isoform (PPP3R1) that encodes a calcium-dependent protein, Calcineurin [142]. Calcineurin is involved in the activation of transcriptional regulatory proteins and catalyzes dephosphorylation events in the placenta [142,143]. *In situ* hybridization shows that C19MC derived miRNA (miRNA-371) is expressed in both CTB and STB [144]. MicroRNA microarray analysis of a recent study has demonstrated that several C19MC derived miRNA-515 family members such as miR-519e-5p, miR-515-5p, miR-518f, miR-519c-3p, miR-515-3p, miR-520d-5p, miR-524-5p, miR-520a-5p, miR-516a-5p, and miR-518b were significantly down-regulated in STB differentiation [145]. Further investigation of miR-515-5p by using RT-qPCR and Luciferase assay demonstrated that miR-515-5p targets and inhibits the expression of aromatase (hCYP19A1), Glial cell missing-1 (hGCM1) and frizzled 5 (FZD5) [6] that are essential for STB differentiation. GCM-1 is the most important transcription factor in trophoblast differentiation and promotes trophoblast fusion, in part by enhancing the expression of fusogenic proteins syncytin1 and 2 [77,78] whereas aromatase is highly expressed in STB [46,146] and FZD5 plays a critical role in STB differentiation [147].

Another miRNA gene cluster is a miR-17~92 family that is located on chromosome 13 and encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a) [148]. Microarray and TaqMan qRT-qPCR analysis showed miRNA-17~92 cluster and its paralog, miR-106a~363, are downregulated during STB differentiation and target hCYP19A1 and hGCM1 gene expression and inhibited STB differentiation[149].

Some other miRNAs reported in addition to chromosome 19 and 13 are miR-122A and miR-125B. These miRNAs are located on chromosome 18 and 11 respectively and expressed in villous trophoblast and villous stroma cells. We showed that miR-378a-5p, located on chromosome 5, inhibits STB differentiation by targeting cyclin G2 (CCNG2) in a choriocarcinoma cell line, BeWo cells, and overexpression of CCNG2 induces STB differentiation [150].

Syncytiotrophoblast involves in the feto-maternal exchange of nutrients and gases and acts as an endocrine unit of the human placenta, thus any alteration of the morphological differentiation is concomitant with a decrease in functional differentiation and can affect the placental development.

6. ROLE OF MIR-378a-5p IN DEVELOPMENT AND DISEASE

MicroRNA (miR-378a-5p) was the most abundant miRNA that was cloned from promyelocytic leukemia (HL-60) cells. In humans, it is located on chromosome 5 (chr5: 149,732,829-149,732,850) within the first intron of peroxisome proliferator-activated receptor gamma co-activator 1 beta (PPARGC1β) gene. MicroRNA-378a-5p is co-expressed with PPARGC1β host gene that works as a co-activator of nuclear hormone receptor such as Peroxisome proliferator-activated receptor gamma (PPARγ) and regulates the transcription of

various genes [128]. The transcript of miR-378 generates two mature miRNAs namely, miR-378a-3p and miR-378a-5p.

It has been reported that miR-378a-5p plays important roles in regulating cellular processes. We have previously demonstrated that miR-378a-5p is expressed in the placenta throughout pregnancy and promotes trophoblast cell proliferation, migration and invasion by targeting Nodal in HTR8/SVneo, a cell line derived from EVTs [151]. Recently, we demonstrated that miR-378a-5p inhibits the differentiation and fusion of trophoblast and identifies cyclin G2 (CCNG2) as its target gene [150].

In addition to miR-378a-5p role in placenta, it is expressed in granulosa cells of the porcine ovary and decreases the expression of aromatase enzyme that is required for the biosynthesis of estradiol in female reproduction [152]. It also regulates nephronectin mediated differentiation in the osteoblastic cell line [153] and promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression [154]. It regulates skeletal muscle differentiation [155] and has been identified as a target of the c-Myc oncoprotein and promotes cellular transformation [156]. Moreover, it is expressed in the mammalian heart and controls cardiac hypertrophy by suppressing the mitogen-activated protein kinase (MAPK) signaling pathway [157].

IV. NODAL: DEVELOPMENT ROLES AND REGULATION

Nodal was identified as an essential gene from 413-d mutant mice due to the interference with normal embryo development [158]. Later on this isolated candidate was named Nodal, and showed that it encodes a signaling peptide that is essential for mesoderm formation and axial structures in early mouse development [159]. In humans, the Nodal gene is located on chromosome 10 and contains three exons [160]. Its highly conserved homologs have been identified in many species such as mammals, zebrafish, clawed frogs (Xenopus), small marine animals (Amphioxus) and marine invertebrates (ascidians) [161].

Nodal belongs to the Transforming beta superfamily (TGF-β) superfamily. Like most of the TGF-β family members, Nodal is synthesized as a proprotein and post-transcriptionally regulated by subtilisin-like proprotein convertases (a family of calcium-dependent cleavage enzymes) and glycosylation [160]. Two convertases Spc1 (Furin) and Spc4 (Pace4) cleave off the pro-domain of Nodal precursor and form mature Nodal [162]. Studies show that Nodal precursor is significantly stabilized by its pro-domain versus mature Nodal that is very labile in nature [163]. Nodal signaling starts by binding to heterodimeric serine/threonine receptors. There are two type I (ALK4 and ALK7) and type II (activin-like kinase, ActRIIB) receptors have been identified for Nodal [164]. These serine threonine kinase receptors phosphorylate Smad-2 and possibly Smad-3 proteins and these phosphorylated forms interact with the common smad4 and regulate gene expression [161,162]. Smad2 is considered as an intracellular mediator of a Nodal signaling pathway that interacts with Smad4 (common Smad) before translocating to the nucleus [165]. Our lab has previously cloned human ALK7 and demonstrated that Nodal mRNA and several ALK7 transcripts are expressed in the placenta throughout the entire gestational period [166]. We have

also shown that Nodal activates ALK7 and the Smad pathway to inhibit trophoblast proliferation [166,167] and invasion [168].

In addition to ALK receptors, Cripto has been identified as a co-receptor for Nodal. Cripto is a member of the epidermal growth factor-Cripto-1/FRL-1/Cryptic (EGF-CFC) family, and in mammals it promotes Nodal signaling. Cripto facilitates the Nodal processing and translocation to endosomes by forming a complex with Nodal precursor and convertases at the surface of the responding cells [161]. It has been reported that Nodal signaling through ALK-4 is dependent on Cripto while the interaction between ALK7 and Nodal is independent of Cripto, but its overexpression enhances the Nodal-ALK7 signal transduction [162]. In addition, the best studied inhibitors that can modulate Nodal signaling reported are Lefty and Cerberus. Lefty proteins are divergent members of the TGF-β family, and their interaction with Nodal or Cripto results in prevention of Nodal/Activin receptor complex and that results in inhibition of Nodal signaling. On the other hand, Cerberus are cysteine-rich extracellular proteins that can directly block Nodal signaling by targeting Nodal ligands [161,162] [Figure 1.10].

Nodal signals can act as a morphogen: a signal that acts directly over a distance from their site of production and elicits dose-dependent responses in target cells [161]. Studies done in zebrafish showed that Nodal has both short and long-range effects [169] while another study in mouse showed that Nodal ligands could activate the target genes in distant wild-type cells in lateral plate mesoderm and have direct long range-effects [170]. In addition, Nodal signals can induce time and dose-dependent effects in cells. It has been shown different time and concentrations of

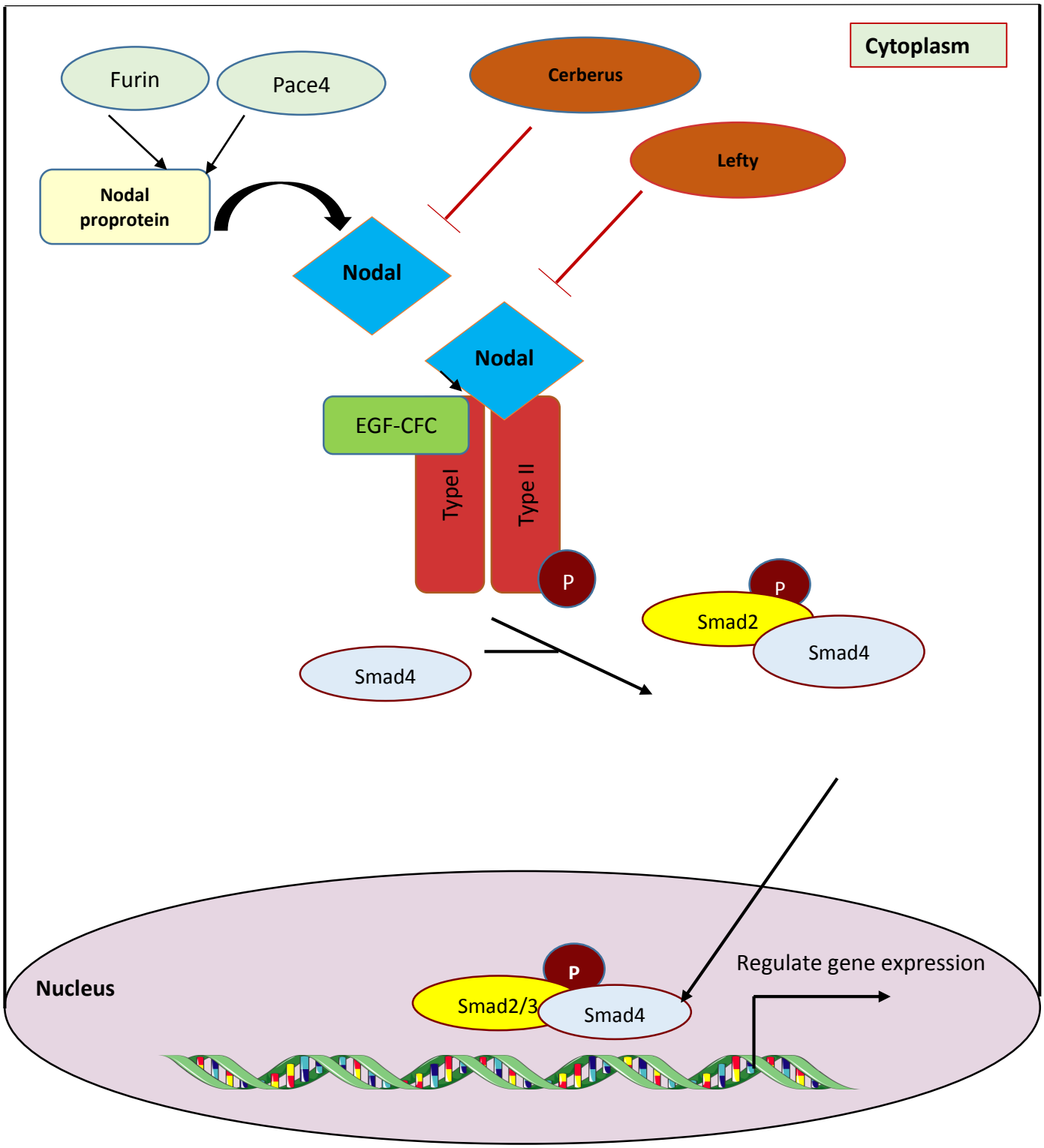

Figure 1.10 Nodal signaling pathway: Nodal is synthesized as a proprotein and post-transcriptionally regulated by Furin and Pace4 that cleave off the pro-domain of Nodal precursor and form mature Nodal. Nodal signaling starts by the interaction of Nodal with activin receptors (Type I/II) and EGF-CFC co-receptors and is inhibited by Lefty and Cerberus. Nodal signaling starts intracellularly by phosphorylation of Smad2 and its association with common Smad4 that translocate it to the nucleus and initiates the transcription of genes regulated by P-Smad2. Figure was drawn on reference 142.

Nodal ligands affect the downstream gene expression *in vivo* models [161]. Nodal plays many important roles such as axes formation, mesendoderm formation, left-right development and neural patterning. It plays critical roles by acting on extraembryonic tissues and controls the axis patterning in embryo development [171].

The convertases (Spc1 and Spc4) are expressed in the extraembryonic ectoderm that stimulates Nodal maturation in the proximal epiblast and induces gene expression of *Lefty 1* and *Cerberus-like* in the distal visceral endoderm, which later becomes the anterior visceral endoderm. Nodal signaling is essential for the positioning of anterior-posterior axis [172]. Nodal is required for the rotation of visceral endoderm and its signaling become restricted by *Lefty 1* and *Cerberus-like* to the posterior region of the epiblast where embryonic ectoderm and primitive endoderm are developing, and where the primitive streaks will form [173]. As development proceeds or in gastrulation, Nodal becomes restricted to the node of the primitive streak and hence name "Nodal" [162].

MicroRNAs are known to regulate Nodal signaling. Nodal ligand Squint and Nodal inhibitors Lefty1 and Lefty2 have been shown to be the targets of miR-430 in zebrafish and reported that miR-430 regulates the endoderm formation by targeting these agonists /antagonist in zebrafish model [174]. Interestingly, microRNA-430 family is evolutionary conserved and abundantly expressed across vertebrate species such as miR-430 (in zebrafish), miR-427 (in Xenopus) and miR-302 (in mammals). These miRNAs are collectively referred as miR-430/427/302 [174]. However, it was shown that target selection of these miRNAs are different in the two species: Xenopus miR-427 targets Nodal ligands (Xnr5 and Xnr6b) and Lefties (Lefty1

and Lefty2) that are required for the proper ectoderm, mesoderm and organization formation, whereas, human miR-302 has been shown to target Lefties and controls the germ layer specification in human embryonic stem cells [175,176]. Also, our lab has demonstrated that miR-378a-5p promotes trophoblast cell survival, migration, and invasion by targeting Nodal [151].

MicroRNAs can modulate Nodal signaling by regulating Nodal receptors. In early *Xenopus* embryos, miR-15 and miR-16 were identified to control the size of the Spemann's organizer by targeting Nodal type II receptor Acvr2a (ActR-IIA) [177]. Moreover, our lab has shown that miR-376c impairs TGF-β/Nodal signaling by targeting ALK5 and ALK7 expression in trophoblast cells [178]. Also, miR-376c suppresses the Nodal induced apoptosis in ovarian cancer cells by targeting ALK7 receptor [179].

V. CYCLIN G2 (CCNG2)

The G type cyclins were first isolated from a rat fibroblast cDNA library in a screen for Src family kinases [180]. Cyclin G was identified as p53 target gene and consists of three members of G type cyclins (cyclin G1, cyclin G2 and cyclin I) that form a distinct sub-group because of high sequence homology and some degree of functional similarity than other cyclins [181,182]. G type cyclins have no active cyclin-dependent kinase (CDK) partner in contrast to promoting cyclins that activate CDKs and form distinct complexes at specific phases of the cell cycle and drive the cells from one stage to another [183]. Cyclin G2 shares high sequence similarity with other G type cyclins but its expression, promoter sequence and regulation differs greatly with the other G-type cyclin (71% homology to cyclin G1 and 41% homology to cyclin I), suggesting that it has distinct and non-compensatory physiological functions [180,184,185]. Cyclin G2 protein is well conserved throughout mammals including human, monkey, dog, rat, cow, mouse and chicken [186].

1. STRUCTURAL FEATURES OF CYCLIN G2

Cyclin G2 was mapped to mouse chromosome 5 at region E3.3-F1.3 by using fluorescence *in situ* hybridization (FISH). This region is homologous to a segment on human chromosome 4 [186]. Cyclin G2 is transcribed from eight exons and spans a total of 8604 base pairs. Cyclin G2 protein consists of 344 amino acids, and a molecular mass is approximately 39 kDa [186]. Cyclin G2 is a nucleocytoplasmic shuttling protein that is primarily localized to detergent-resistant cytoplasmic compartments and associated with the cytoskeletal elements [184].

There are three main domains have been characterized in cyclin G2 protein structure: the amino-terminal domain (NTD), a conserved cyclin box of ~ 110 amino acids and the carboxyl-terminal domain (CTD) [187]. The NTD harbors its cyclin box, stretching from 55-165. The cyclin box domain of cyclin G2 is structurally similar to cyclin A but lacks the conserved α1 and α3 helix sequences present in cyclin A, which is required for interaction with CDK-2, and therefore cyclin G2 complexes lack CDK-like activity [185]. It is possible that cyclin G2 interacts with other regulatory members of the cell cycle outside of the cyclin box. Three cyclin G2 binding partners, cortactin, PP2A and PPARγ have been identified, and these interact with cyclin G2 outside of the cyclin box [188,189]. The CTD of cyclin G2 consists of 46 amino acids and includes a PEST (proline, glutamic acid, serine, and threonine) protein destabilization motif. The PEST domain has been commonly found in rapidly degraded proteins and contains multiple potential phosphorylation sites that may influence its subsequent regulation [190,191]. Moreover, cyclin G2 has two putative Nuclear Receptor (NR) boxes that contain a short signature motif of LXXLL (where L is leucine and X can be any amino acid). These conserved leucine amino acids are LXXLL motifs that play indispensable roles and modulate transcription by binding to NR in a ligand-dependent manner [186,192] [Figure 1.11].

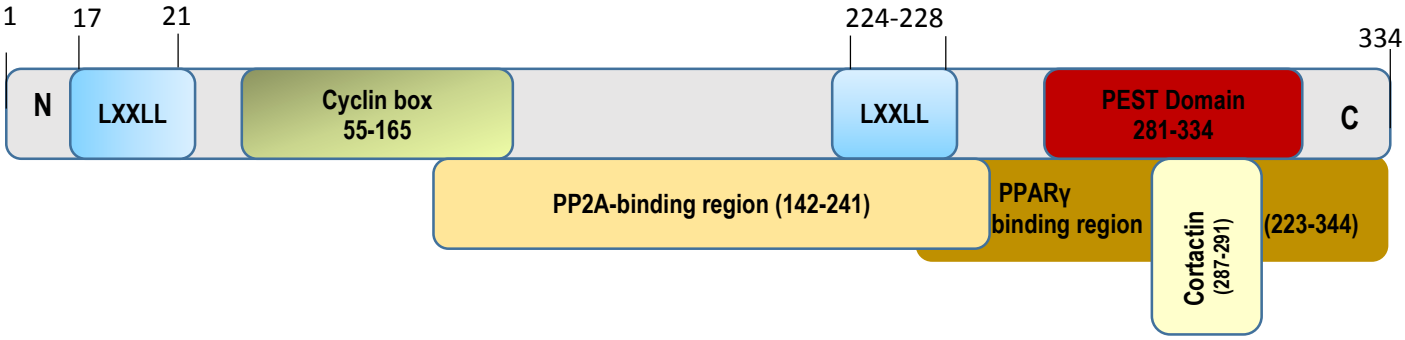

Fig 1.11 Schematic representation of cyclin G2 protein: Three main domains of cyclin G2 have been characterized. The N-terminal domain, centrally located cyclin G2 domain and C-terminal domain. There are two putative nuclear boxes near each terminal. The C-terminal half of cyclin G2 corresponds to the binding region of its various partners such as PP2A, PPAR γ and cortactin. The C-terminal is also linked to cyclin G2 degradation via the PEST domains that destabilizes protein. Figure was drawn on references [187-188].

2. REGULATION OF CYCLIN G2 EXPRESSION

The expression of cyclin G2 at the mRNA level is regulated by a variety of growth inhibitory and mitogenic signals. For instance, TGF-β family members, Nodal and its receptor ALK, robustly upregulate cyclin G2 expression [190]. On the other hand, inactivation of Smads-2 and -3, Nodal-ALK7 downstream effectors, abolished cyclin G2 induction and suggesting that Nodal/ALK7 induces cyclin G2 through the canonical Smad2/3 pathway [190]. Moreover, treatment with hormone receptors or potent growth factors such as estrogen receptors (ER), erythropoietin receptor (EPO-R), insulin growth factor (IGF), human epidermal growth factor receptor (HER)-2 and platelet-derived growth factor (PDGF) decreased cyclin G2 mRNA levels [193–195].

The Phosphoinositide 3-kinase (PI3K) pathway plays an important role in cyclin G2 regulation. Growth factor receptor stimulation results in activation of PI3K which in turn activates downstream effectors such as Akt or protein kinase B [196]. Cyclin G2 promoter has binding sites for FoxO3a, a member of the Forkhead box class O (FoxO) transcription factor family, that positively regulates the expression of cyclin G2 [197]. Activation of PI3K/Akt leads to FoxO3a phosphorylation and subsequent exclusion. On the other hand, inhibition of PI3K/Akt results in dephosphorylation of FoxO3a, which in turn is able to translocate to the nucleus to drive the expression of cyclin G2 [197,198]. Therefore, FoxO action on the cyclin G2 promoter is highly dependent on the PI3K/Akt activity. In addition, FoxO proteins can interact with other transcription factors to increase transcription. Our lab has reported that Nodal increased cyclin G2 transcription by promoting synergistic interaction between FoxO3a and the Smads at the cyclin G2 promoter in ovarian cancer cells [198]. Furthermore, a zinc finger transcription factor, deltaEF1

(enhancer binding factor 1) synergized with FoxO proteins and activate cyclin G2 transcription in both *in vitro* and *in vivo* models [199].

3. REGULATION OF CYCLIN G2 DEGRADATION

Protein degradation is a crucial step in determining the net protein expression and its stability. Cyclin G2 is a highly unstable protein and is degraded quickly by the ubiquitin (Ub) proteasome system (UPS). Our lab has shown that cyclin G2 is physically associated with the ubiquitin and S-phase kinase-associated proteins (Skp1 and Skp2) at the PEST domain. In addition, cyclin G2 protein levels rapidly decreased after transfection and it is degraded through the 26S proteasome pathway; however, cyclin G2 levels increased when cells were treated with 26S proteasome inhibitors [190]. Furthermore, treatment with Nodal inhibited the association of Skp2 with cyclin G2 that results in protection from degradation. Similar to that, we also reported that overexpression of Nodal or its receptors (ALK4/7) increased cyclin G2 transcription in ovarian cancer cells [198]. Taken together, these studies suggest that Nodal overexpression not only regulates cyclin G2 gene activity but it can also protects it from degradation [190,198]. Moreover, we recently reported that cyclin G2 is a target of calpain-mediated proteolysis and Epidermal growth factor (EGF) enhanced cyclin G2 degradation through the calpain-mediated pathway in ovarian cancer cells [200].

4. FUNCTIONS OF CYCLIN G2

Cyclin G2 plays a physiologically important role in the maintenance of cellular growth, arrest or differentiation by either up or down regulation of cyclin G2 or alteration in its downstream binding partners.

4.1. Cell cycle arrest

Cyclin G2 expression oscillates during the cell cycle. Cyclin G2 level is commonly found to be highest at the quiescent or G_o phase of the cell cycle. When cells are stimulated to progress through the G0-G1 transition, a rapid decline in cyclin G2 expression, following re-accumulation of cyclin G2 expression in the late S/G2 and G2/M transition has been reported [187,201] Overexpression of cyclin G2 has been shown to exert a robust cell cycle arrest and inhibited cell proliferation in murine B cells [201], HEK293 (Chinese hamster ovary cells) [184] and several cell lines such as human oral cancer [202], breast cancer [185] and ovarian cancer [190].

4.2. Microtubule Stability

Unlike typical cyclins, cyclin G2 has no known active CDK partners but studies have shown that endogenous and ectopically expressed cyclin G2 forms an active complex with protein phosphatase 2A (PP2A) [203]. PP2A is a heterotrimeric serine/threonine phosphatases complex that is composed of scaffolding, regulatory and catalytic subunits named as A, B and C subunits, respectively [203,204]. PP2A plays a plethora of cellular functions such as signaling events, endosome trafficking, cell cycle and cytoskeletal regulation in cells [205]. The catalytic subunit of PP2A core enzyme has been localized to microtubules, intermediate filaments and centrosomes [206,207]. On the other hand, its regulatory subunit modulates target specificity and directs the entire complex to distinct cellular locations [204]. Cyclin G2 can directly associate with a

regulatory and catalytic subunit of PP2A and co-localize in both the cytoplasm and nucleus. In addition, cyclin G2 may compete with the A subunit of PP2A by acting as a scaffolding protein within the complex and may alter the subset of targeted proteins of PP2A [208]. It has been shown that cyclin G2 and PP2A are co-localized at the centrosome and overexpression of cyclin G2 resulted in the formation of stabilized microtubules that were resistant to re-growth from centrosomes suggesting that cyclin G2 and PP2A play an important role in the cytoskeleton fidelity [189].

4.3. Differentiation

Cell differentiation is a highly coordinated process that is linked to cell cycle arrest. Cyclin G2 levels increased in terminally differentiated cells and it has been associated with enhancing cellular differentiation [201]. Overexpression of cyclin G2 induces terminal differentiation of the luminal and stromal cells following implantation in the mouse uterus [35,209]. It has also been reported that CCNG2 induces adipocyte differentiation by interacting with PPARγ to enhance its transcriptional activity [188,210].

4.4. DNA Damage Repair

DNA damage repair (DDR) is an essential process to maintain the genomic integrity and any failure in this process could lead to genomic instability. Cyclin G2 induction plays a role in the DDR and it has been reported an abnormal response of DNA repair in cells with depleted cyclin G2 expression. Treatment with DNA damaging agents resulted in cell cycle arrest and cyclin G2 was found to be localized at centrosomes [211]. Recently, it has been reported that cyclin G2 is co-localized with PP2A at promyelocytic leukemia nuclear bodies (PML-NBs) following DNA damage by ionization radiation [212]. This study suggested that cyclin G2 is recruited to the sites

of DNA repair and cyclin G2-PP2A complex regulates the dephosphorylation of many factors that are necessary for DNA repair process [212].

5. CYCLIN G2 DYSREGULATION

Dysregulation of cyclin G2 could lead to a variety of pathological conditions. It has been reported that cyclin G2 is significantly downregulated in a variety of malignancies such as ovarian [198], breast [213,214] , gastric [215] , oral [216] , thyroid [217] , esophageal [218], kidney [219], colorectal [220] and prostate cancers [221]. Furthermore, cyclin G2 expression is upregulated in response to anti-tumor agents and it has anti-tumorigenic roles [200,222]. Several studies have reported that overexpression of cyclin G2 inhibits cell proliferation in several cell lines [216,223] and reduced colony formation and cyclin G2 levels are inversely proportional to the tumor and carcinoma stage [222]. Therefore, in addition to its potential as an early mediator of cancer development, cyclin G2 was found to be a marker for prognosis and exoneration and showed that patients who have a higher cyclin G2 levels have a better survival rate and potential for complete exoneration [224].

VI. RATIONALE, HYPOTHESIS, AND OBJECTIVES OF THE STUDY

Over the last decade, miRNAs have been shown the crucial regulator of placental development and modulate gene expression in several processes such as cell proliferation, migration, invasion, apoptosis, angiogenesis and differentiation.

Our lab has demonstrated that microRNA-378a-5p is highly expressed in the placenta throughout different stages of pregnancy, and it increased trophoblast cell proliferation, migration, invasion and survival by repressing the Nodal expression in EVTs [151]. Nodal is expressed in both extravillous and villous trophoblast cell populations in early gestations, and its expression is developmentally regulated [167]. Furthermore, Nodal levels are strongly up-regulated in preeclamptic placenta and its overexpression increases apoptosis, and decreases cell migration and invasion [167], suggesting that Nodal signaling plays important roles in the placenta development. We have demonstrated that miRNA-378a-5p has an inhibitory role in STB differentiation in part by targeting cyclin G2 [150], which is expressed throughout gestation [225]. On the other hand, Nodal increased cyclin G2 expression and protein stability [190], as well as its induces cyclin G2 transcription in ovarian cancer cells [198].

Based on the previous studies findings that 1) Nodal and cyclin G2 are the target genes of miRNA-378a-5p [150,167]; 2) Nodal and cyclin G2 express in human placenta [167,225] and play important roles in trophoblast cell models such as HTR-8/SVneo and BeWo cells [150,167] and 3) Nodal increases cyclin G2 transcription and protein stability in ovarian cancer cells [190,198], I hypothesize that miR-378a-5p and its target genes regulate trophoblast differentiation towards STB pathway.

The overall objectives of my study were to 1) Determine the additional targets of miR-378a-5p to better understand its role in STB differentiation; 2) Investigate the role of Nodal in STB differentiation and 3) Determine the role of cyclin G2 in STB differentiation.

CHAPTER 2

CREB plays a central role in mediating miR-378a-5p and Nodal regulated syncytiotrophoblast differentiation

ABSTRACT

MicroRNAs are considered as the fine tuner of gene regulation that play vital roles in the placenta throughout pregnancy. One of the key events in placenta development is the fusion of mononucleated cytotrophoblasts into multinucleated syncytiotrophoblast (STB). We have previously reported that miR-378a-5p inhibits STB differentiation by targeting cyclin G2 and that miR-378a-5p targets Nodal to inhibit trophoblast invasion. Nodal, a member of the transforming growth factor-β (TGF-β) superfamily, is a critical regulator of embryo development. To further investigate the role of miR-378a-5p in human placental development, we explored the role of its target genes in STB differentiation. Bioinformatics analysis revealed that miR-378a-5p has six potential binding sites at the 3'UTR of CREB. Using a choriocarcinoma cell line, BeWo, we showed that miR-378a-5p decreased the luciferase activity of reporter constructs that contain CREB 3'UTR. In addition, miR-378a-5p decreased, while anti-miR-378a-5p increased, CREB mRNA and protein expression. Furthermore, we found that Nodal is down-regulated by miR-378a-5p but upregulated during forskolin (an adenylate cyclase activator) induced STB differentiation. Treatment of BeWo cells with recombinant Nodal (rhNodal) resulted in an increase in cell fusion, syncytin-1 and a decrease in E-cadherin protein expression. Expression levels of several STB markers, such as syncytin-1, syncytin-2, galactoside binding soluble lectin13(LGALS13) and chorionic gonadotropin beta (CGB) were induced by Nodal. On the other hand, knockdown of Nodal using siRNA inhibited cell fusion, decreased fusogenic marker genes and protein, and increased E-cadherin protein expression. In addition, Nodal activated CREB and silencing of CREB using siRNA attenuated the effect of Nodal. Finally, overexpression of CREB and Nodal reverses the inhibitory effect of miR-378a-5p. Taken together, these findings suggest that Nodal promotes STB differentiation by activating CREB but miR-378a-5p exerts an inhibitory role in STB differentiation, in part by down-regulating CREB and Nodal expression in BeWo cells.

INTRODUCTION

Placenta is a unique, autonomous and transient organ that plays critical roles throughout the entire pregnancy and supports the growth and development of the fetus. Therefore, healthy placentation is a key to a successful pregnancy [3]. During the placenta development, trophoblast progenitor cells differentiate into two lineages [226]. In the invasive or extravillous (EVTs) pathway, cytotrophoblast (CTB) differentiates into either interstitial extravillous trophoblast, which invades into the maternal decidua and myometrium of the uterus, or endovascular extravillous trophoblast, that remodels the maternal vasculature and increases the blood supply to the fetus [227]. On the other hand, in the villous or syncytial pathway, non-proliferative, mononuclear cytotrophoblasts fuse into multinucleated syncytiotrophoblast (STB) and form syncytial layer that covers the placenta villous tree [226]. The STBs are in direct contact with the maternal blood and involved in the exchange of respiratory gases, nutrients and waste products across the materno-fetal interface [3,226,227].

The process of syncytialization is characterized by the loss of E-cadherin [228] and an increase in fusogenic retroviral envelope proteins such as syncytin-1 and syncytin-2 (which are encoded by HERV-W and HERV-FRD) respectively [56,229]. Acting as an endocrine unit of the human placenta, STB synthesizes and secretes various hormones, such as progesterone [230], leptin [47], human placenta lactogen (HPL) [231] and human chorionic gonadotropin (hCG), into the maternal circulation [232]. In addition, STB produces important enzymes such as placental alkaline phosphatase (ALPP or PALP); catalyzes reactions and transfer maternal IgG to fetus [52] and galactoside-binding soluble lectin 13 (LGALS13, also known as PP13); known to have immunoregulatory functions at the materno-fetal interface [51,233].

Previous studies have shown that this fusion process is regulated by a number of transcription factors and one of the major transcription factors that regulates several trophoblast genes is cAMP response element binding protein (CREB) [88–90]**.** CREB is a leucine zipper transcription factor that belongs to CREB/ATF family [234] and regulates diverse cellular responses such as proliferation, survival and differentiation [88,89]. The signaling cascade cAMP-PKA-CREB has been well known in STB pathway [70]. Activation of cAMP is initiated by ligands binding to their specific G-protein coupled receptors (GPCRs) which subsequently activates adenylyl cyclase (AC) that converts ATP to cAMP. Increased intracellular levels of cAMP activate cAMP-dependent protein kinase (PKA) that ultimately phosphorylates various transcription factors, such as CREB. The phosphorylated CREB homodimerizes and binds to cAMP responsive elements (CREs) in the promoter region of its target genes to regulate the transcription process [235]. It has been demonstrated that CREB binds to the promoter of Glial cells missing homolog 1 (GCM1), to increase its transcription [236]. It increases the GCM1 protein stability [90] by acetylation at lysine^{367}, lysine^{406}, and lysine^{409} in the transactivation domain (TAD). The acetylation of these residues protects GCM1 from ubiquitination that results in the increase in transcriptional activity [236]. GCM1 upregulates the syncytin transcription by binding to its promoter and induces the differentiation of CTB into STB [77,78].

Several members of transforming growth factor-β (TGF-β) superfamily are known to regulate trophoblast cell functions, while their dysregulation have implications in the pregnancy-associated diseases [3,237–239]. Nodal, a member of TGF-β family, is known to play critical roles during embryo development [171]. It has been reported that nodal deficient mice showed abnormal placentation with excessive number of giant cells (equivalent to human EVTs), and spongiotrophoblast layers, and nodal suppressed giant cell differentiation in vitro [240], suggesting

that nodal regulates placental development. Two type 1 serine/threonine kinase receptors (ALK4 and ALK7) have been identified for Nodal [164] and these receptors phosphorylate Smad proteins to regulate gene expression [161,162] . Smad2 and Smad3 are intracellular mediators of Nodal signaling pathway [165]. It has been demonstrated that Nodal mRNA and several ALK7 transcripts are expressed in the placenta throughout gestation [241] and Nodal activates ALK7 and the Smad pathway to inhibit trophoblast proliferation [168] and invasion [167].

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules that modulate gene expression through post-transcriptional mechanisms and attenuate protein yield in various signaling networks [242]. Studies have shown that miRNAs modulate regulatory pathways that play vital role in the placental development and regulate proliferation, migration, invasion and differentiation of trophoblast cells [243]. We have previously demonstrated that miR-378a-5p inhibits STB differentiation by targeting cyclin G2 [150] and promotes trophoblast invasion by targeting Nodal [151]. To further elucidate the role of miR-378a-5p in the placenta development, we investigated how miR-378a-5p and its target genes regulate STB differentiation. We demonstrated that miR-378a-5p targets CREB and Nodal in BeWo cells to inhibit cell fusion and STB marker gene expression. We also provide the first evidence that Nodal induces STB differentiation by activating CREB.

MATERIALS AND METHODS

Cell line and cell culture

A human choriocarcinoma cell line, BeWo, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained and cultured in a 1:1 mixture of DMEM (Fisher Scientific) and Ham's F12 (Life Technologies) medium containing 10% heat inactivated Fetal Bovine Serum (Gibco, Life Technologies) in a humidified condition of 5% CO2 at 37^{o}C.

Transfection, Plasmids, and RNA interference

The miR-378a-5p mimic and siRNA duplexes of control, Nodal, and CREB [244] were purchased from Gene Pharma Co (Shanghai, China) and their sequences are listed in Table 1. Transient transfection was carried out by using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's suggested protocols [178]. AntimiR-378a-5p and its corresponding NC were purchased from RiboBio (Guangzhou, China). To confirm gene silencing effects, protein lysates were prepared at 48 hours after transfection and subjected to Western blot analysis.

Generation of CREB reporter and cDNA constructs

Three luciferase constructs containing different region of CREB 3'UTR were generated by Polymerase Chain Reaction (PCR) using the primers listed in Table 1. The cDNA used as a template in PCR was extracted from BeWo cell line and amplified by using Q5 High Fidelity DNA

Polymerase (New England BioLabs). The PCR products were separated by size on 1% agarose gel and isolated with a gel extraction kit (Qiagen). The resulting cDNA fragments were then cloned into the pMIR-REPORT vector (Ambion, Fisher Scientific) downstream of the luciferase coding sequence by double digestion using SpeI and HindIII restriction enzymes. After ligation, plasmid was transformed into DH5α competent cells. Colonies were collected, followed by Taq DNA polymerase PCR to check for the positive clones and selected clones were confirmed by sequencing. Construct-1 contains the second predicted site, construct-2 contains the third and fourth predicted sites, and construct-3 contains the last two predicted sites of CREB.

CREB over-expression plasmid was generated by using pcDNA3.1 vector (GeneScript). In order to construct this vector, PCR was performed by using cDNA from BeWo cells as a template and primers described earlier [244] (Table 1) by using Q5 High Fidelity DNA Polymerase (New England BioLabs). The resulting product was run on 1% agarose gel and DNA was extracted. A double digestion was performed for both DNA fragment and plasmid (pcDNA3.1) using HindIII and EcoRI restriction enzymes. After ligation, plasmid was transformed into DH5α competent cells. Colonies were collected and confirmed with double digestion and sequencing.

Nodal and Forskolin treatment

BeWo cells were treated with 50μM forskolin (Sigma-Aldrich) or DMSO (final concentration 0.04%) as its vehicle control in the presence of 1% Fetal Bovine Serum (FBS) for 24, 48 and 72 h. Recombinant Human Nodal Protein (rhNodal) was purchased from R&D Systems. Cells were seeded in serum free media and then treated with rhNodal (250ng/ml) in 10% FBS at 24, 48 and 72h. At the end of experiments, total RNA extraction, immunofluorescent staining, or protein lysates were prepared to determine the effect of Nodal on cell fusion.

RNA extraction and qPCR

Total RNA was extracted from BeWo cells using Ribozol RNA extraction kit (Amresco) according to the manufacturer's instructions. To determine the mRNA levels of CREB, Nodal and STB markers genes such as syncytin-1, syncytin-2, LGALS13 and CGB, 2µg of total RNA was used to synthesize first strand cDNA by M-MuLV Reverse Transcriptase (New England BioLabs Ltd) according to the manufacturer's protocol. Quantitative Real time PCR (qRT-PCR) was carried out by using gene specific primers and EvaGreen qPCR Master Mix (ABM), following manufacturer directions, on a RotorGene Q thermocycler (Qiagen). All primers used in this study are listed in Table 1 and were validated for specificity with primer-BLAST (NCBI). Amplified products were run on an agarose gel to validate a single band product. The expression levels of mRNA were normalized to GAPDH.

Western blotting

BeWo cells were washed with cold PBS (136 mM NaCl, 2.6 mM KCl, 10mM Na2HPO4.7H2O, 1.8mM KH2PO4 and pH was adjusted to 7.4). The cell lysates were prepared using radio-immunoprecipitation assay buffer (50 mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1% SDS) containing protease and phosphatase inhibitor cocktail (Pierce, Thermo Fischer Scientific). Cell lysates were centrifuged for 15 minutes at 4^{o} C and bicinchoninic acid (BCA) method was used to determine the total protein concentration. Equal amount of protein samples (30µg) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio Rad). The PVDF membranes were blocked with 5% milk in TBST (10mM Tris-Cl pH 8.0, 150 mM NaCl, and

TABLE 1: Sequences of PCR primers and siRNAs

Primer/siRNA	Sequence 5'-------> 3'
CREB 3'UTR-1	**F:**TGGCTTAGAAAGGGCTAGATCC
CREB 3'UTR-1	**R:**AGGAATGCAACAGAACACACA
CREB 3'UTR-2	**F**:GTCAGGCAGCCTATCCCATC
CREB 3'UTR-2	**R**:ACAAGGTTTGAGCCCGTCTT
CREB 3'UTR-3	**F**:CAAGCTTGTGTTGGAAGGCAG
CREB 3'UTR-3	**R**:TGTGTTTTGCAACCTGAGTGT
pcDNA3-CREB1	**F**:TATGACCATGGAATCTGGAG
pcDNA3-CREB1	**R**:TTAATCTGATTTGTGGCAGT
Syncytin-1	**F**: TCATATCTAAGCCCCGCAAC
	R: TGATCTTGCAAGGTGACCAG
Syncytin-2	**F:** TCGGATACCTTCCCTAGTGC
	R: GTATTCCGGAGCTGAGGTTG
LGALS13	**F:** ATTGCCTTCCGTTTCCGAGT
	R: TTTGCCATCCTCAAAGGGCA
CGB	**F:** CCGTCAACACCACCATCTGT
	R: ATTGACAGCTGAGAGCCACG
CCNG2	**F**: GCTGAAAGCTTGCAACTGCCGAC
	R: GGTATCGTTGGCAGCTCAGGAAC
GAPDH	**F:** AAGGTCATCCCTGAGCTGAAC
	R: ACGCCTGCTTCACCACCTTCT
Control siRNA	**Sense**:UUCUCCGAACGUGUCACGUtt
	Anti-sense:ACGUGACACGUUGGAGAAtt
siNodal	**Sense**:AGACAUGAUCGUGGAAGAAtt
	Anti-sense:UUCUUCCACGAUCAUGUCUtt
siCREB-1	**Sense**:AGUAAAGGUCCUUAAGUGCtt
	Anti-sense:GCACUUAAGGACCUUUACUtt
siCREB-2	**Sense:**CAATACAGCTGGCTAACAAtt
	Anti-sense:UUGUUAGCCAGCUGUAUUGtt

TABLE 2: Primary Antibodies for Western Blot

Antibody	Company	Specie	Working Conc.	Dilution	Catalogue No
CREB	Cell Signaling	Mouse	5ug/ml	1:500	9104S
E-cadherin	Santa Cruz	Mouse	200ug/ml	1:500	sc-8426
GAPDH	Santa Cruz	Mouse	200ug/ml	1:1000	sc-365062
Nodal	Abcam	Mouse	1159ug/ml	1:500	ab55676
Phospho CREB	Cell Signaling	Mouse	1600ug/ml	1:500	9196S
Syncytin-1	Santa Cruz	Rabbit	200ug/ml	1:500	sc-50369

0.05% Tween 20) for 1 hour at room temperature with constant agitation and then incubated with specific primary antibodies listed in Table 2 in blocking buffer overnight at 4^{o}C. Next day, membranes were washed three times with TBST for 10 minutes and subsequently probed with HRP conjugated antibody for 1 hr at room temperature. Signals were detected by using Enhance Chemi-Luminescence (ECL) detection reagent (Millipore) according to the manufacturer's instructions. GAPDH was used as the loading control and all the experiments were repeated in triplicate and at least three times. In order to obtain quantitative data from the Western Blots, films were scanned from Canon scanner and signal intensities were analyzed using the Carestream Molecular Imaging software.

Immunofluorescence

BeWo cells were seeded on the coverslips at the density of $4x10^4$ in a 12-well plate. The next day, cells were either treated for rhNodal or transfected with the siRNA targeting Nodal or CREB. Cells were washed with phosphate buffered saline (PBS) and fixed in methanol (-20 ^{o}C) for 10 minutes. Cells were washed three times with PBS and incubated with 3% BSA in PBS for 1 hour. Finally, BSA was removed and cells were incubated with mouse monoclonal anti-E-cadherin antibody (1:100) overnight at 4^{o}C. Next day, coverslips were washed with PBS buffer and incubated with Alexa Fluor 594 conjugated goat anti-mouse secondary antibody (1:300, Life Technologies) at room temperature for 1 hr. DAPI (1:1000, Sigma) was used as a counterstain to stain the cell nuclei. Coverslips were washed, mounted and examined with the inverted fluorescence microscope. Photographs were taken in 10 randomly selected fields per coverslip and cell fusion was analyzed in the cells when 3 or more nuclei were present. Fusion index was calculated as [(N-

S)/T] x 100%, (N = number of nuclei in the syncytia, S = the number of syncytia and T = total number of nuclei in the observed field, as described [245].

ELISA

BeWo Cells were seeded on 12-well plates in serum-free DMEM/F12 media and the next day cells were treated with 250ng/ml rhNodal. Conditioned media were collected at 24, 48 and 72h after treatment and spun down at 12000rpm for 3 minutes. Human chorionic gonadotropin (hCG) was measured using the ELISA βhCG kit (EIA-1911) (DRG) following the manufacturer's direction. Briefly, conditioned media were incubated with enzyme conjugate for 60 minutes at room temperature. Cells were washed and incubated with substrate solution. Enzymatic reaction was stopped by the addition of 0.5M H_2SO_4 and the color reaction was quantified on the BioTek Synergy H4 plate Reader at 450nm. Total βhCG in conditioned media was calculated from the standard curve generated during each assay.

Luciferase Assay

Cells were seeded in 12-well plates at the density of $8x10^4$ /well and co-transfected with 25nM of negative control (NC) or miR-378a-5p mimics, pMIR-Report-CREB 3'UTR plasmids (construct 1, 2 or 3) and pRL-TK internal control (encoding Renilla luciferase) plasmids. Five hours after transfection, cells were recovered in DMEM/F12 for 19 hours. Cells were lysed and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instruction.

Statistical Analysis

All the experiments were done in triplicate in each group and each experiment was performed 3 times. Statistical analysis was performed by using GraphPad Prism 6 ($p<0.05$,95% Cl was considered statistically significant). All of the data was represented as mean ± SEM. Unpaired, two-tailed Student's *t*- test was used to compare the values between two groups. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and two-way ANOVA followed by Sidak's multiple comparisons test were used to determine the differences among several groups.

RESULTS

miR-378a-5p targets CREB

We have previously reported that miR-378a-5p inhibits STB differentiation by targeting CCNG2 [150]. To better understand the role of miR-378a-5p in STB differentiation, bioinformatics tools were used to identify additional targets. Interestingly, FindTar3 (bio.sz.tsinghua.edu.cn) and microRNA.org predicted six potential binding sites of miR-378a-5p at the 3'UTR of CREB (Figure 2.1A). To test this possibility, luciferase reporter constructs were generated. Three CREB 3'UTR fragments containing different predicted targeting sites of miR-378a-5p were cloned into the pMIR-REPORT vector (Figure 2.1B). Cells were transfected with a control reporter without 3'UTR or one of the CREB constructs, (3'UTR-1, 3'UTR-2, 3'UTR-3), along with miR-378a-5p mimics or negative control (NC). Mock transfection was also included as an additional control. Luciferase assays revealed that miR-378a-5p significantly decreased the luciferase activity in cells transfected with CREB 3'UTR-1 or -3 construct (Figure 2.1C).

To further confirm that miR-378a-5p regulates CREB, cells were transfected with miR-378a-5p or NC at 24, 48 and 72 hours, and CREB mRNA and protein levels were determined. CREB protein levels were lower in miR-378a-5p-transfected cells than in the control cells (Figure 2.1D&E). miR-378a-5p also significantly decreased CREB mRNA levels at all time-points measured (Figure 2.1F). Conversely, transfection of anti-miR-378a-5p at 72 hours resulted in an increase in CREB protein (Figure 2.1G&H) and mRNA [Figure 2.1I] levels measured by Western blot and RT-qPCR respectively.

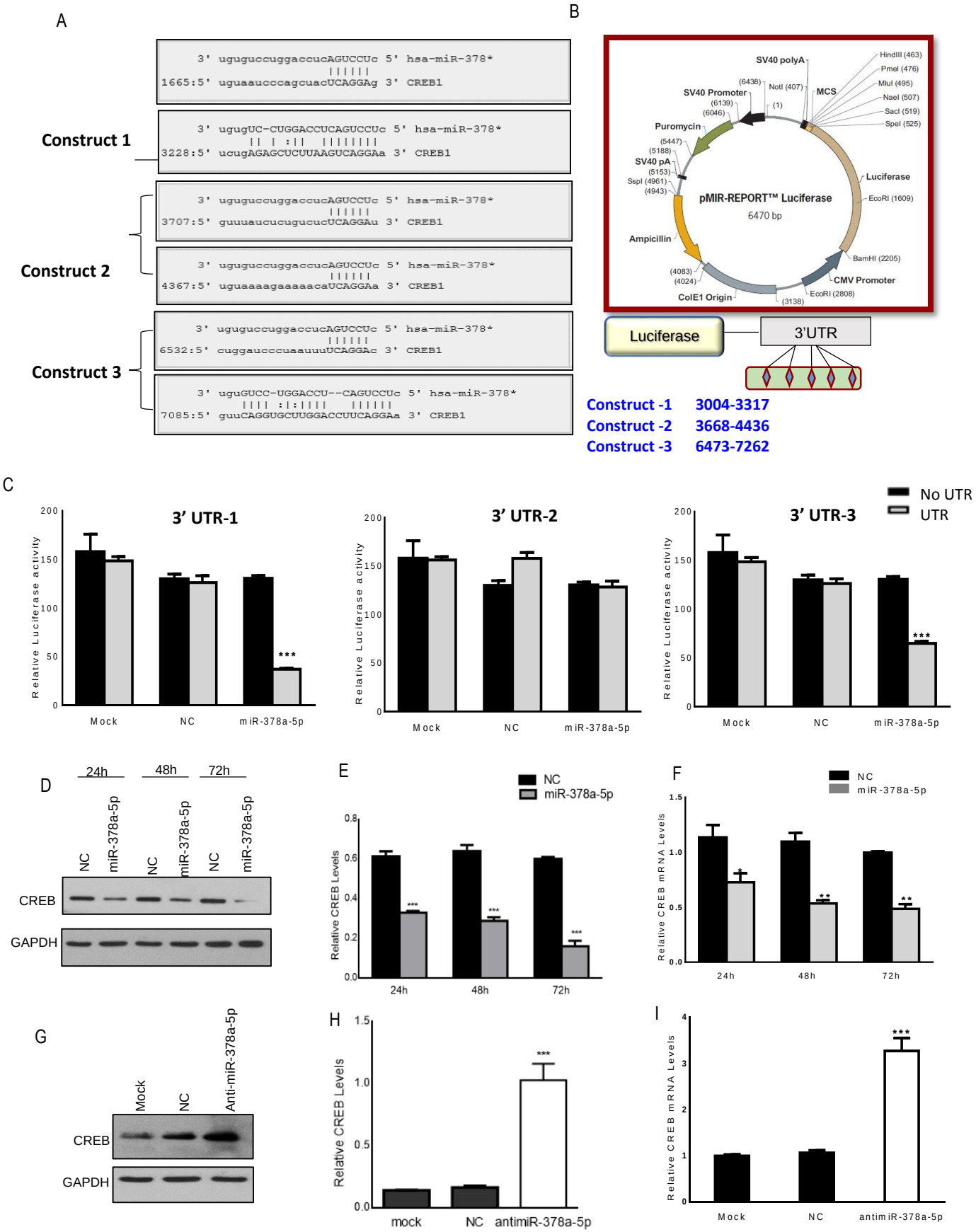

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Figure 2.1 miR-378a-5p targets CREB. A) Predicted binding sites of miR-378a-5p on CREB 3'UTR. B) Three luciferase reporter constructs, each containing a fragment of CREB 3' UTR downstream of the luciferase coding sequence, were generated. 3'UTR-1 contains the second predicted site, 3'UTR-2 contains the third and fourth predicted sites and 3'UTR -3 contains the last two predicted sites. C) Cells were transfected with the control reporter (pMIR-REPORT without 3'UTR) or a CREB 3'UTR construct together with non-targeting control oligo (NC) or miR-378a-5p, or without oligos (mock). Luciferase assay was performed at 24h after transfection. miR-378a-5p significantly decreased the luciferase activity in cells transfected with 3'UTR-1 and 3, when compared to NC and mock groups ***p<0.001 *vs.* Mock and NC. D) BeWo cells were transfected with NC or miR-378a-5p. Cell lysates were collected at 24, 48 and 72h after transfection and Western blotting performed using anti-CREB and GAPDH antibodies. Representative blots from 3 experiments were shown. E) A summary graph depicts the densitometric intensities of CREB levels normalized to its respective GAPDH levels. Data represent mean ± SEM (n=3). ***p<0.01 *vs*. corresponding controls. F) Total RNA was extracted at 24, 48 and 72h after transfection and CREB mRNA levels were determined by RT-qPCR. Data represent mean ± SEM (n=3). *p<0.05, **0.01 *vs.* corresponding controls. G) Cell were transfected with anti-miR-378a-5p or its non-targeting control, or with mock transfection. Cell lysates were collected at 72h after transfection and Western blotting performed using anti-CREB and GAPDH antibodies. Representative pictures from 3 experiments were shown. H) The densitometric intensities of CREB levels normalized to its respective GAPDH levels are shown. Data represent mean ± SEM (n=3). ***p<0.01 *vs*. corresponding controls. I) Cells were transfected without (mock) or with anti-miR-378a-5p or equal amount of NC. Total RNA was extracted at 72h after transfection and CREB mRNA levels were determined by RT-qPCR. Data represent mean ± SEM (n=3). ***p<0.001 *vs.* corresponding controls. Statistical analysis was performed on GraphPad Prism using a two-way ANOVA (p<0.05, 95% Cl) with Sidak's multiple comparison test for (C, E and F). A one-way ANOVA with Tukey's multiple comparison test (p<0.05, 95% Cl) was used for (H and I).

Nodal is down-regulated by miR-378a-5p but up-regulated during forskolin-induced STB differentiation

We have previously found that Nodal is a target of miR-378a-5p [151]. Therefore, we determined if Nodal is also inhibited by miR-378a-5p to regulate STB differentiation. BeWo cells were transiently transfected with miR-378a-5p mimics or its NC at 24, 48 and 72 hours. Figure 2.2A shows that Nodal mRNA levels were significantly lowered in cells transfected with miR-378a-5p at 24, 48 and 72 hours. In addition, transfection of miR-378a-5p mimics resulted in decrease in Nodal protein expression as compared to control cells in a time dependent manner (Figure 2.2B&C).

Forskolin is an activator of adenylate cyclase and known to induce BeWo cell fusion and STB marker gene expression [246]. Therefore, BeWo cells were treated with DMSO or forskolin (50 μM) at 24, 48 and 72 hours. Real-time PCR was used to quantify mRNA levels of syncytin-1 and CREB. As shown in Figure 2.2, treatment with forskolin resulted in significant increase in syncytin-1 and CREB mRNA levels when compared with controls at 24, 48 and 72 hours (Figure 2.2D&E). Treatment with DMSO or forskolin also significantly increased Nodal mRNA (Figure 2.2F) and protein levels (Figure 2.2G&H).

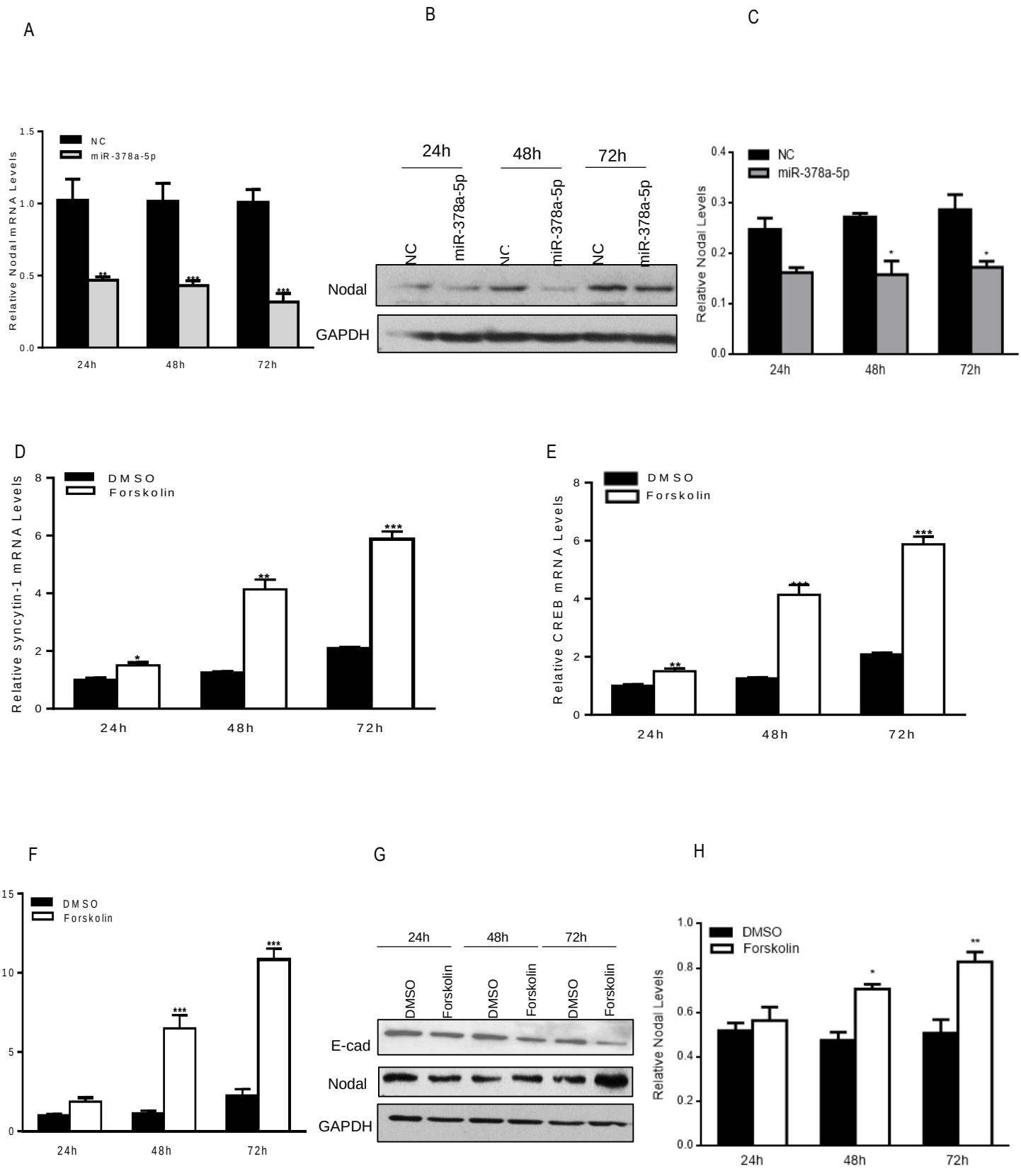

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Figure 2.2 Nodal is down-regulated by miR-378a-5p but up-regulated during forskolin-induced STB differentiation. A) Cells were transfected with non-targeting control oligo (NC) or miR-378a-5p. Total RNA and were collected at 24,48 and 72h after transfection and Nodal mRNA levels were determined by RT-qPCR. Data represent mean ± SEM (n=3). **p<0.01, ***0.001 *vs* corresponding controls. B) Cells were transfected with NC or miR-378a-5p and cell lysates were collected at 24,48 and 72h after transfection and Nodal protein expression was determined by Western blot using anti-Nodal and GAPDH antibodies. Representative blots from 3 experiments were shown. C) A summary graph depicts the densitometric intensities of Nodal levels normalized to its respective GAPDH levels. Data represent mean ± SEM (n=3). *p<0.05 *vs*. corresponding controls. D-E) Cells were treated with DMSO or forskolin for 24, 48 and 72h and total RNA was extracted. mRNA levels of syncytin-1 and CREB and were determined by RT-qPCR and normalized to an internal calibrator glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data represent mean ± SEM (n=3). *p<0.05, **p<0.01, ***0.001 *vs* corresponding controls. F) Cells were treated with DMSO or forskolin, and total RNA were collected at 24,48 and 72h after treatment. Nodal mRNA levels were determined by RT-qPCR. Data represent mean ± SEM (n=3). ***p<0.001 *vs*. corresponding controls. G) Cells were treated with DMSO or forskolin and cell lysates were prepared at 24,48 and 72h after treatment and subjected to Western blot analysis using anti-E-cadherin, anti-Nodal and GAPDH antibodies. Representative blots from 3 experiments were shown. H) A summary graph depicts the densitometric intensities of Nodal levels normalized to its respective GAPDH levels. Data represent mean ± SEM (n=3). *p<0.05, **p<0.01 *vs*. corresponding controls. forskolin induces CREB, syncytin-1 and Nodal mRNA levels. All statistical analysis was done by using a two-way ANOVA with Sidak's multiple comparison test (p<0.05, 95% Cl) by GraphPad Prism.

Nodal induces cell fusion and marker gene expression

To determine if Nodal plays a role in STB differentiation, several sets of experiments were performed. First, BeWo cells were treated with rhNodal and cell fusion was assessed by immunofluorescent staining using anti-E-cadherin antibody at 72 hours. As shown in Figure 2.3A, treatment with rhNodal resulted in a significant increase in cell fusion versus control. Second, the effect of rhNodal on STB marker gene expression was examined. Nodal decreased the E-cadherin and increased syncytin-1 protein expression, as revealed by Western blotting (Figure 2.3B&C). RT-qPCR showed that rhNodal treatment increased the mRNA levels of syncytin-1(Figure 2.3D), syncytin-2 (Figure 2.3E), LGALS13 (Figure 2.3F) and CGB (Figure 2.3G). In addition, treatment with rhNodal significantly increased hCG secretion when compared to the control cells (Figure 2.3H).

Next, we tested the effect of Nodal silencing on STB differentiation. Cells were transfected with a siRNA targeting Nodal and qPCR and western blotting confirmed the knockdown of Nodal (Figure 2.4A). Immunofluorescence using an anti-E-cadherin antibody showed that siNodal significantly decreased the fusion index (Figure 2.4B) and western blots revealed that siNodal decreased syncytin-1 expression while increased E-cadherin levels (Figure 2.4C). Knockdown of Nodal also led to significant decrease in the mRNA level of syncytin-1 and 2 (Figure 2.4D).

Finally, to test whether forskolin-induced STB differentiation is mediated by Nodal, cells were transfected with siRNA targeting Nodal or NC for 24 hours and then treated with DMSO or forskolin (50uM) for 48 hours. RT-qPCR was used to analyze the mRNA levels of syncytin-1, syncytin-2, LGALS13 and Nodal. Figure 2.4E shows that forskolin induces marker genes and Nodal mRNA levels, while siNodal has opposite effect on marker gene expression. On the other hand, cells treated with forskolin in siNodal transfected cells decreased STB marker genes as compared to forskolin alone; suggesting that Nodal play an important role in STB differentiation and forskolin induced STB differentiation is mediated by at least in part by Nodal.

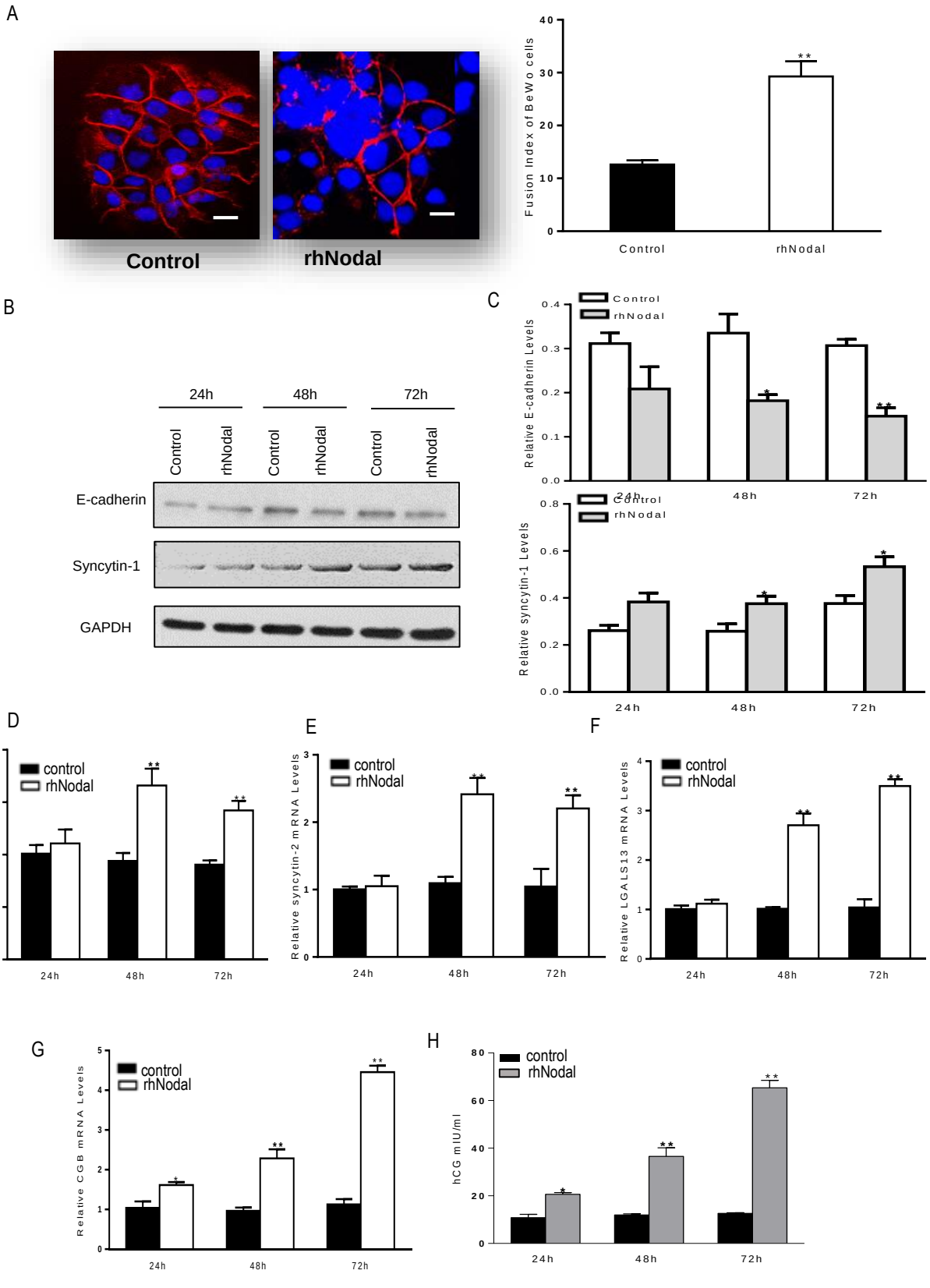

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Figure 2.3 Nodal induces BeWo cell fusion, fusogenic protein and STB marker genes expression. A) Cells were treated with rhNodal for 72h. Immunofluorescent staining using an anti-E-cadherin antibody was performed and fusion index (n=3 wells) was calculated. **, $p<0.01$ *vs.* control. Scale bars=100 μm. B) Treatment of rhNodal in cells at 24, 48 and 72h decreased E-cadherin and increased syncytin-1 protein expression. Representative immunoblot of E-cadherin and syncytin-1. Representative immunoblots from 3 experiments were shown. C) A summary graph depicts the densitometric intensities of E-cadherin and syncytin levels normalized to its respective GAPDH levels. D-G) Total RNA was extracted at 24,48 and 72h after treatment with rhNodal and mRNA levels were determined by RT-qPCR. STB markers, such as syncytin-1, syncytin-2, LGALS13 and CGB were induced by Nodal. Data represent mean ± SEM (n=3). *$p<0.05$ and **$p<0.01$ *vs*. corresponding controls. H) The conditioned media were collected and accessed for hCG by ELISA. Data represent mean ± SEM (n=4 wells) *$p<0.05$ and **$p<0.01$ *vs*. corresponding controls. Statistical analysis was performed on GraphPad Prism using a two-tailed unpaired t-test ($p<0.05$, 95% Cl) for (A) and a two-way ANOVA ($p<0.05$, 95% Cl) with Sidak's multiple comparison test for (C-F).

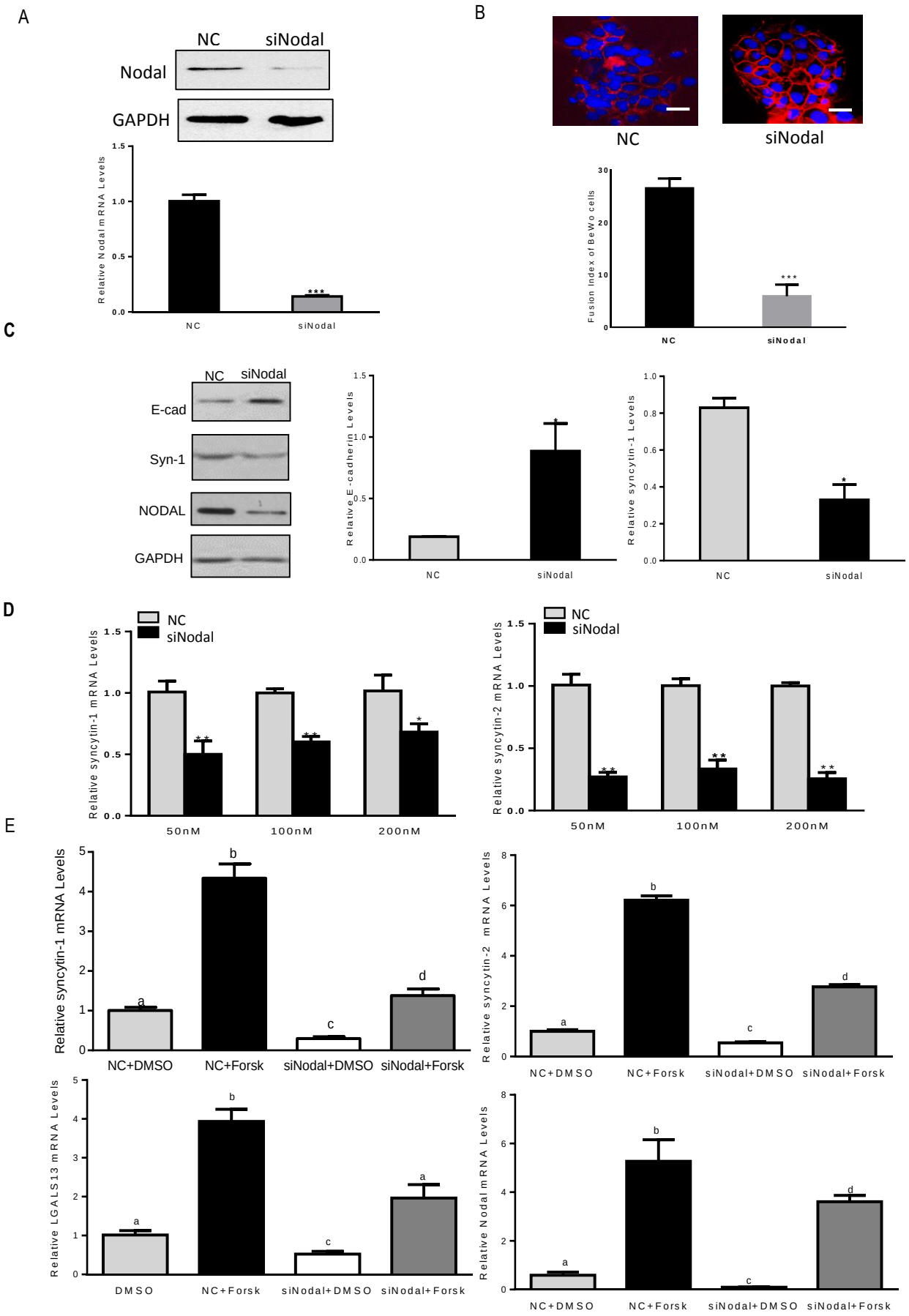

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Figure 2.4 siNodal inhibits cell fusion and decreases STB marker gene expression. A) Cells were transfected with non-targeting control oligo (NC) or siNodal for 48h. Total RNA and cell lysate were collected and Nodal mRNA and protein expression were determined by RT-qPCR and western blotting respectively. B) Cells were transfected with siNodal or NC and immunofluorescent staining using an anti-E-cadherin antibody was performed at 48 h after transfection and fusion index (n=3 wells) was calculated. ***$p<0.001$ *vs.* NC. Cell membrane was stained with anti-E-cadherin (red) and cell- nuclei were DAPI stained (blue). Representative pictures of NC and cells transfected with siNodal showed siNodal decreased cell fusion. Scale bars=100 μm. C**)** Cells were transfected with siNodal (100nM) or NC and E-cadherin, syncytin-1, Nodal and protein expression were determined by Western blotting. A summary graph represents the densitometric intensities of E-cadherin and syncytin levels normalized to its respective GAPDH levels. D) Cells were transfected with different concentrations (50, 100 and 200nM) of siNodal or NC. Total RNA was extracted at 48 h after transfection and mRNA levels of syncytin-1 and syncytin-2 were determined by RT-qPCR. Knockdown of Nodal results in decrease in syncytin-1 and syncytin-2 mRNA levels. Data represent mean ± SEM (n=3). *$p<0.05$ and **$p<0.01$ *vs*. corresponding controls. E) Cells were transfected with siNodal (100nM) or NC for 24 hours and then treated with either DMSO or forskolin (50 μM) for 48h. mRNA levels of syncytin-1, syncytin-2, LGALS13 and Nodal were determined by RT-qPCR. The marker gene mRNA levels were normalized to GAPDH. Knockdown of Nodal using siRNA in forskolin treated cells decreased STB marker genes as compared to forskolin alone. Data represent mean $\pm$ SEM (n=3). Different letters above bars denote statistical significance. Statistical analysis was performed on GraphPad Prism using a two-tailed unpaired t-test ($p<0.05$, 95% Cl) for (A-C). A two-way ANOVA ($p<0.05$, 95% Cl) with Sidak's multiple comparison test for (D) and a one-way ANOVA with Tukey's multiple comparison test ($p<0.05$, 95% Cl) was used for (E).

Nodal activates CREB to induce STB differentiation

The maintenance of the syncytiotrophoblast is a highly regulated process and controlled by different transcription factors. CREB is a stimulus-induced transcription factor and regulates several trophoblast genes [89,90,108]. To investigate the potential mechanism underlying Nodal-induced STB differentiation, cells were treated with rhNodal or its control and activation of Smad2 was examined. Unlike TGF-β, treatment with rhNodal only induced a small increase in phopho-Smad2 levels (Figure S2.1). Therefore, we tested if CREB may be activated by Nodal. Cell lysates were collected at 0, 15, 30, 45 and 60 min after treatment with rhNodal. Western blot analyses showed that rhNodal strongly increased phospho-CREB Levels (Figure 2.5A). To further explore the role of CREB in Nodal-induced STB differentiation, CREB siRNAs were used to silence CREB expression. Cells were transfected with NC or siRNAs targeting CREB (100nM) for 48 hours. Figure 2.5B shows that siCREB-1 and siCREB-2 reduced CREB protein expression, increase E-cadherin and decrease syncytin-1 protein expression. Next, cells were transfected with siCREB-1, siCREB-2, or NC and were treated with rhNodal (250ng/ml) or control and immunofluorescent staining was performed using anti-E-cadherin antibody at 48 hours. Total RNA was also collected and analyzed subsequently. As shown in Figure 2.5C, transfection with siCREBs resulted in decrease in cell fusion versus cells treated with Nodal. There was a significant increase in cell fusion in cells treated with Nodal but siRNA CREB 1 and 2 reduced the effect of Nodal. Similar to the immunofluorescence results, knockdown of CREB using siRNA in cells treated with rhNodal decreased syncytin-1, syncytin-2 and LGALS13 mRNA levels [Figure 2.5D-G].

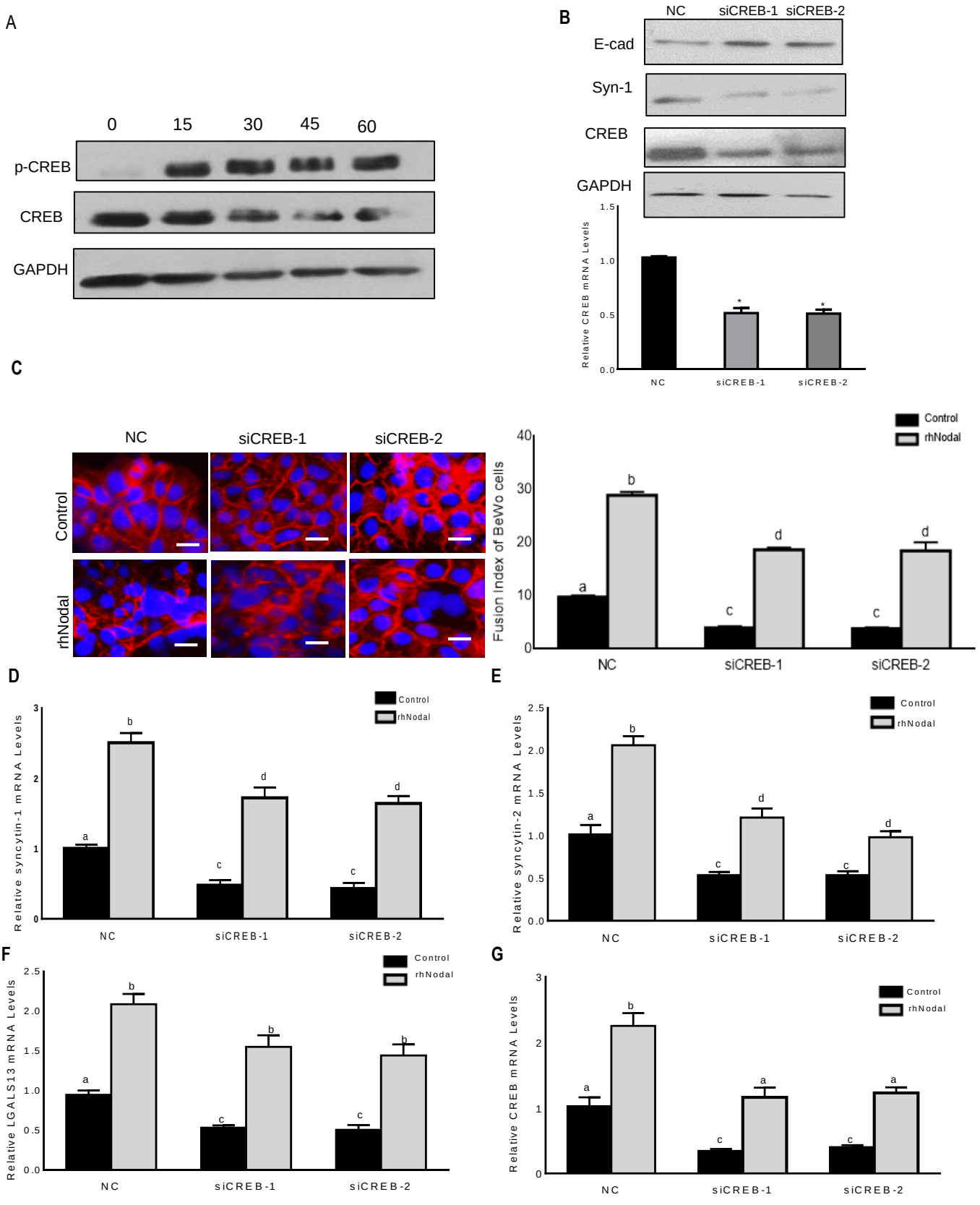

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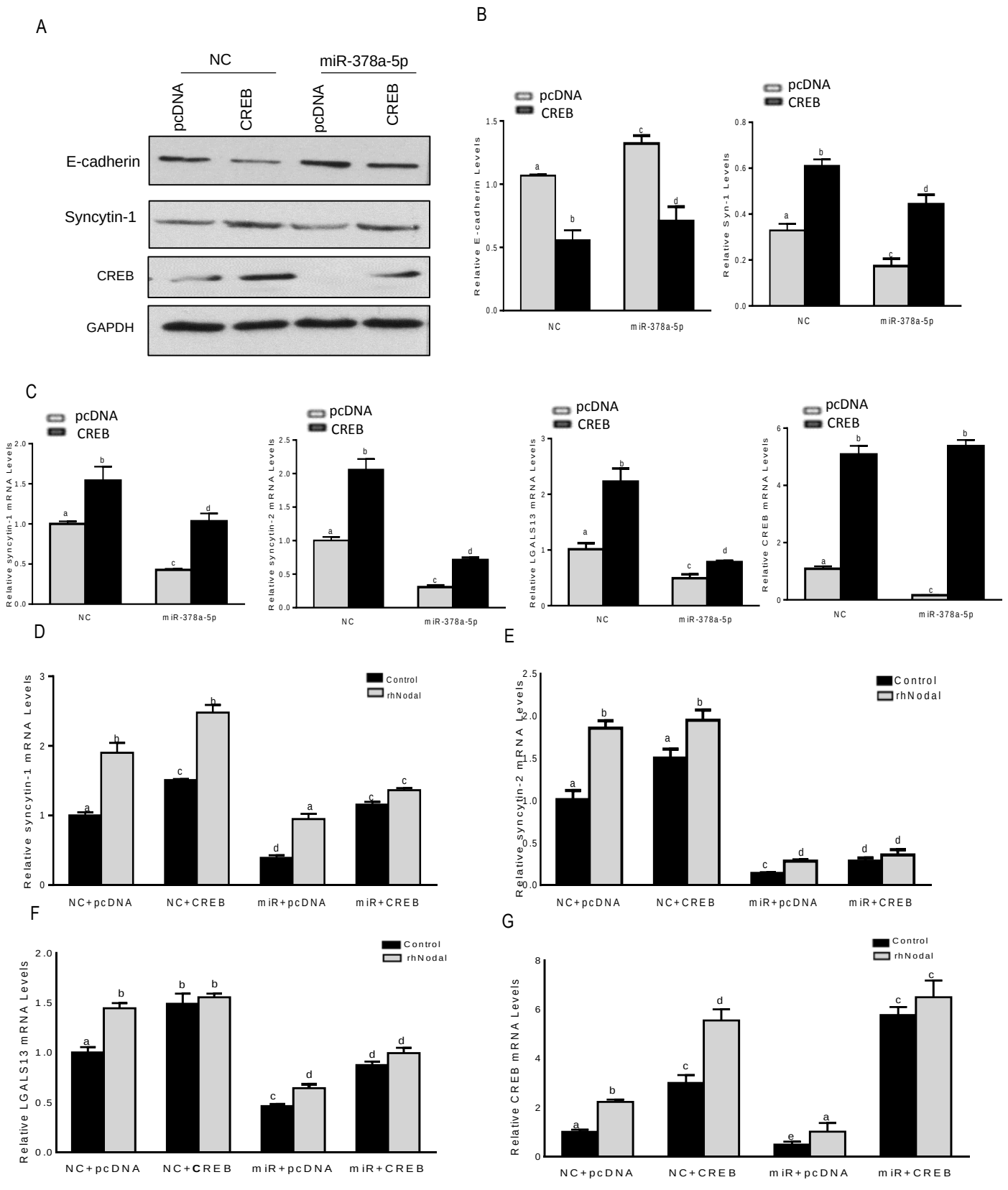
Figure 2.5 Nodal activates CREB to induce STB differentiation. A) Cells were treated with or without rhNodal for 0, 15, 30, 45 and 60 minutes. Cell lysates were collected at respective time points after Nodal treatment and subjected to immunoblotting using anti-phospho-CREB, CREB and GAPDH antibodies. Representative immunoblots are shown. B) Cells were transfected with non-targeting oligo (NC) or siRNAs targeting CREB for 48h. Total RNA and cell lysates were collected and analyzed. Knockdown of CREB was shown by RT-qPCR and Western blotting. E-cadherin and syncytin-1 were determined by Western blot. C) Cells were transfected with siRNA CREB-1, siCREB-2 or NC, and then treated with rhNodal (250ng/ml) or control for 48h. Immunofluorescent staining using an anti-E-cadherin antibody was performed after transfection and fusion index (n=3 wells) was calculated. Different letters above bars denote statistical significance. Representative pictures are shown. Scale bars=100 μm. D-G) Total RNA was collected at 48 h after transfection with siRNA CREB-1, CREB-2 or NC for 48 hours and then treated with rhNodal or control. mRNA levels of syncytin-1, syncytin-2, LGALS13 and CREB were determined by RT-qPCR. Silencing of CREB using siRNA CREB-1 and CREB-2 attenuated the effect of Nodal. Data represent mean ± SEM (n=3). Different letters above bars denote statistical significance. Statistical analysis was performed on GraphPad Prism using a one-way ANOVA with Tukey's multiple comparison test ($p<0.05$, 95% Cl) for (B) and a two-way ANOVA ($p<0.05$, 95% Cl) with Sidak's multiple comparison test for (D-G).

Overexpression of CREB and Nodal reverse the inhibitory effect of miR-378a-5p

Since miR-378a-5p targets CREB and Nodal, rescue experiments were performed to see whether overexpression of CREB and/or treatment with rhNodal can reverse the inhibitory effect of miR-378a-5p on STB differentiation. Cells were transfected with miR-378a-5p mimics or NC, along with EV or CREB plasmid. Cell lysates and RNA were collected at 48 hours after transfection. Western blot analyses were performed using anti-Ecadherin and anti-syncytin-1 antibodies and RT-qPCR was used to analyze the mRNA level of syncytin-1, syncytin-2, and LGALS13. Overexpression of CREB, as confirmed by Western blotting (Figure 2.6A) and qPCR [Figure 2.6BC], resulted in a reduction in E-cadherin and increase in syncytin-1 protein levels and reversed the effect of miR-378a-5p on E-cadherin and syncytin-1 expression [Figure 2.6A&B]. In consistent with the protein data, overexpression of CREB significantly increased syncytin-1, syncytin-2, and LGALS13 mRNA levels and attenuated the inhibitory effect of miR-378a-5p on these genes [Figure 2.6C]. We also tested if CREB and Nodal have additive effects. Cells were transfected with miR-378a-5p mimics or its NC, along with EV or CREB. After five hours of transfection, cells were treated with rhNodal or its vehicle control. Both rhNodal treatment and CREB overexpression increased marker gene expression and reduced the inhibitory effect of miR-378a-5p (Figure 2.6D-G). In a cell fusion assay, CREB overexpression and rhNodal treatment has significantly higher effects in inducing cell fusion and reversing the inhibitory effect of miR-378a-5p when compared to either CREB overexpression or rhNodal treatment alone [Figure 2.6H].



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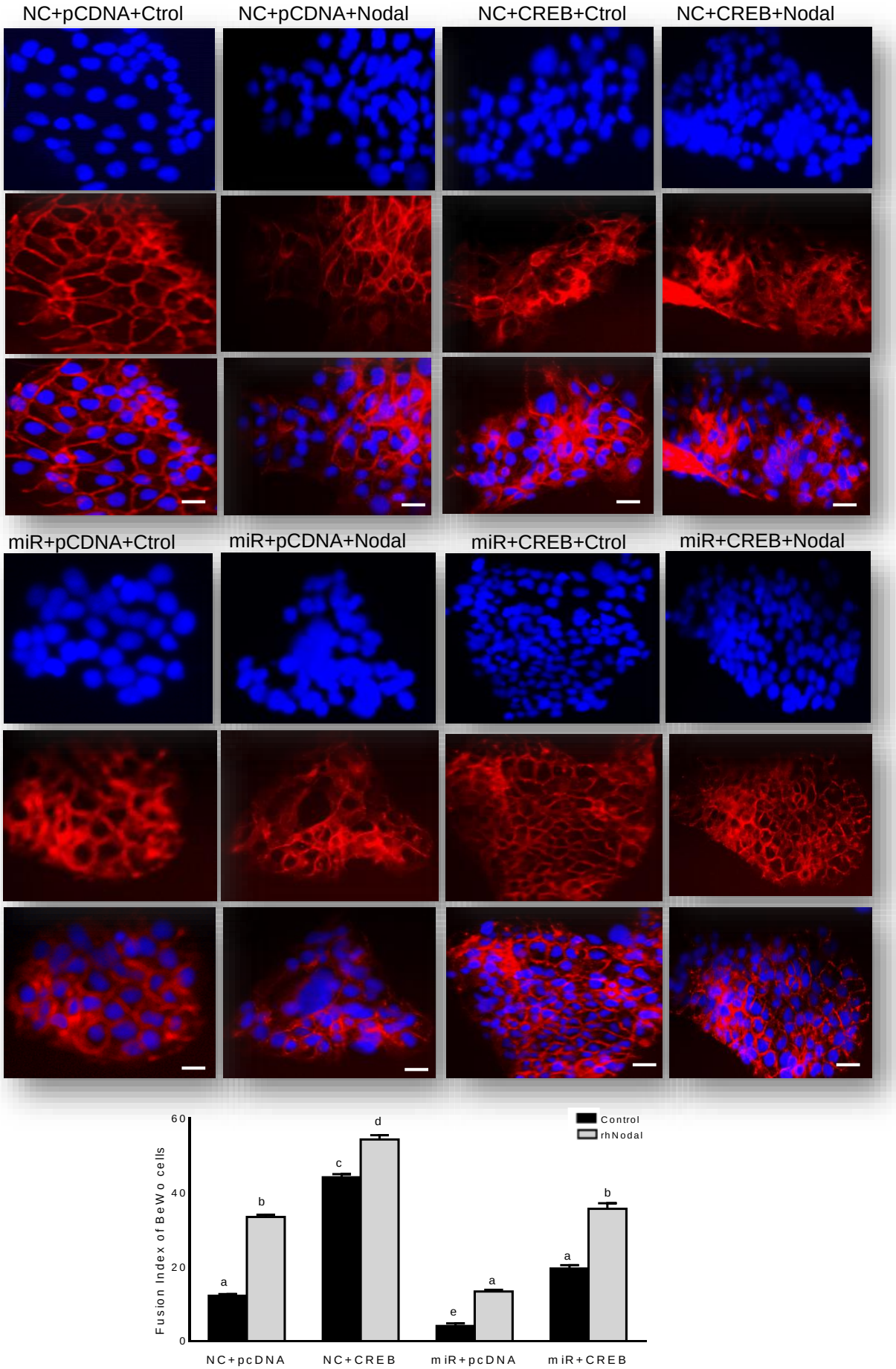

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Figure 2.6 Overexpression of CREB and Nodal reverse the inhibitory effect of miR-378a-5p. A) Cells were transfected with non-targeting control oligo (NC) or miR-378a-5p, along with control vector (pCDNA) or CREB plasmid for 48 hours. Cell lysates were prepared and immunoblotting performed using anti-E-cadherin, syncytin-1 and GAPDH antibodies. Representative immunoblots are shown. B) A summary graph depicts the densitometric intensities of E-cadherin and syncytin levels normalized to its respective GAPDH levels. C) Total RNA was extracted after transfection with NC or miR-378a-5p along with CREB or pCDNA plasmid, and mRNA levels of syncytin-1, syncytin-2, LGALS13 and CREB were determined by RT-qPCR. Data represent mean ± SEM (n=3). D-H) BeWo cells were transfected with miR-378a-5p mimics or its NC, along with pCDNA or CREB and rhNodal or its control. Total RNA was collected and Immunofluorescent staining using an anti-E-cadherin antibody was performed at 48 h after transfection and rhNodal treatment. mRNA levels were determined by RT-qPCR and fusion index (n=3 wells) was calculated respectively. H) Representative pictures of each conditions are shown. Different letters above bars denote statistical significance. Different letters above bars denote statistical significance. Scale bars=100 μm. Statistical analysis was performed on GraphPad Prism using a one-way ANOVA with Tukey's multiple comparison test ($p<0.05$, 95% Cl) for (B-C) and a two-way ANOVA with Sidak's multiple comparison test ($p<0.05$, 95% Cl) was performed for (B-H).

DISCUSSION

We have previously reported that miR-378a-5p targets *CCNG2* and inhibits STB differentiation [150]. In this study, we further investigated the role of miR-378a-5p by using a trophoblast cell model, BeWo cells, in STB differentiation. We identified CREB as a target gene of miR-378a-5p and demonstrated that miR-378a-5p inhibits STB differentiation in part, by down-regulating CREB in the BeWo cell model. In addition, we also demonstrated that miR-378a-5p targets Nodal, which activates CREB and induces cell fusion. These findings demonstrate that CREB plays a pivotal role in mediating miR-378a-5p- and Nodal-regulated STB differentiation.

In the present study, a choriocarcinoma cell line, BeWo cells, was used to examine the role of miR-378a-5p in STB differentiation pathway. BeWo cell line is the most extensively used *in vitro* model to study syncytialization because of the high degree of similarity to the normal placental trophoblast [232]. The STB differentiation can be studied by morphological (aggregation and fusion of cell nuclei) and biochemical differentiation (expression of STB marker genes) *in vitro* studies [247]. The differentiation of CTB into STB is a cascade of events that involves cell aggregation, remodeling of the cortical cytoskeleton and fusion of the plasma membranes and form a well-organized cytoskeleton containing multiple nuclei called syncytium. During STB differentiation, mononuclear CTBs fuse and form a multinucleated STB. This process is marked by the loss of E-cadherin [228] and by the expression of STB specific genes such as syncytin-1, syncytin-2, LGALS13 and CGB [51,52,56,248]. In addition to the morphological and biochemical changes, cell fusion event is accompanied by a series of cell maturation changes such as increased capacity for estrogen and progesterone synthesis, formation of microvilli, smooth membrane vesicle and rough endoplasmic reticulum [26,53,249].

This study identifies CREB as a target gene of miR-378a-5p. Several studies have reported the role of CREB in syncytium formation and regulation of STB specific gene expression [90,234,250]. For example, CREB is highly expressed in STB, it upregulates a transcription factor GCMa that serves as a master regulator in cell fusion [90], and regulates STB marker genes CGB [89,251] and syncytins [88]. Several miRNAs have been reported to target CREB. For instance, miR-27 inhibits adipocyte differentiation by suppressing CREB expression [252], miR-34b targets CREB in acute myeloid leukemia and inhibits tumor growth [100], miR-182 targets CREB and suppresses cell gastric adenocarcinoma cell growth [253], miR-433 targets CREB and inhibits liver cell migration [254] and miR-200b targets CREB and suppresses tumor activity in human malignant glioma [255]. We showed that miR-378a-5p decreased the luciferase activity of reporter constructs that contain 3'UTR, as well as CREB protein and mRNA levels, whereas blocking the endogenous miR-378a-5p levels by antimiR-378a-5p had opposite effects. These results, together with the finding that overexpression of CREB reversed the inhibitory effect of miR-378a-5p on STB differentiation, demonstrate that CREB is an important target of miR-378a-5p.

Consistent with our previous findings that Nodal is a target gene of miR-378a-5p [151], we showed that miR-378a-5p decreased Nodal protein and mRNA levels in BeWo cells. Interestingly, we found Nodal mRNA levels and protein expression increased in cells treated with forskolin, an adenylate cyclase activator, suggesting that Nodal is upregulated during forskolin induced STB differentiation. Several lines of evidence support the positive role of Nodal in STB differentiation. First, immunofluorescence results showed that rhNodal significantly increased, while siNodal significantly decreased, the number of cells that have multiple nuclei, indicating that Nodal induces cell fusion. In addition, rhNodal decreased E-cadherin and increased syncytin-1 protein expression while siNodal has opposite effects on protein expression. Finally, mRNA

levels of STB marker genes such as syncytin-1, syncytin-2, LGALS13 and CGB were upregulated by rhNodal but downregulated by siNodal. Nodal also increased the levels of secretion of βhCG. Interestingly, we observed that treatment with forskolin in siNodal transfected cells results in decrease in STB marker gene expression in BeWo cells as compared to the forskolin treated cells alone, suggesting that Nodal is an important player in STB differentiation and mediates, in part, or at least by cAMP-induced STB differentiation. To the best of our knowledge, the role of Nodal in STB pathway has not been reported. However, a recent study reported that treatment with rhNodal reduces E-cadherin levels in breast cancer cell lines and in BeWo cells [256]. Our results are consistent with the recent study report in terms of E-cadherin that rhNodal reduced E-cadherin in BeWo cells and these cells are well known model to study syncytialization *in vitro* studies. Several studies have reported that loss of E-cadherin is an indicator of syncytial trophoblast in BeWo cells in cell culture, and the intracellular fusion of BeWo cells can be studied by the rearrangement of E-cadherin [70].

The role of Nodal and other members of the TGF-β superfamily, such as bone morphogenic proteins (BMPs), TGFβs, and activin, in trophoblast differentiation has been reported in human embryonic stem cells (hESCs). hESCs originates from the inner cell mass of the blastocysts and have been shown to differentiate after BMPs and fibroblast growth factor (FGF). A recent study has reported that in addition to BMPs and FGF signaling, activin/nodal signaling regulates the terminal differentiation and STB formation [257]. However, there are controversial reports of TGFβ on trophoblast differentiation in different cells and cell lines. A study conducted on TGFβ isoform 1 and 3 (TGFβ1 and TGFβ3) in villous explants and BeWo cells showed a decrease in GCM1 and syncytin-1 protein expression [237]. On the other hand activation of TGF beta isoforms

(TGFβ1, TGFβ2 and TGFβ3) induces differentiation of a mouse placental progenitor cell line, SM10 whereas Nodal and Activin has opposite effects [258].

Nodal signaling starts by binding to heterodimeric serine/threonine receptors (ALK4 and ALK7) [164]. Interaction of Nodal through these receptors phosphorylate cytoplasmic Smad 2 and/or Smad3 proteins that interact with Smad4 (common Smad) and form translation complexes [161,162] to regulate gene expression. Several studies have reported that TGF- β activates non-Smad signaling pathways such as Mitogen activated protein kinase (MAPK), Extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) and regulates various cellular processes including cell differentiation [259,260]. In this study, we tested whether Nodal can induce Smad2 pathway in BeWo cells or not. Our results show that Nodal can activate Smad2 pathway in BeWo cells but this induction was not as strong as compared to the positive control (TGF-β). It could be possible that Nodal may regulate gene expression by non-Smad pathways.

One of the most significant finding from this study is that Nodal activates CREB and induces STB differentiation. We found that Nodal strongly increased phospho-CREB levels. Furthermore, Inhibition of CREB expression by siRNA resulted in a decrease in Nodal-induced cell fusion and STB marker genes, demonstrating that CREB mediates, at least in part, the effect of Nodal on STB differentiation. CREB is considered as one of the best understood phosphorylation-dependent transcription factor that responds to multiple extracellular signals, and its transcriptional activity is positively regulated by the phosphorylation of a Serine (Ser) residue 133 [83]. The phosphorylation of Ser-133 allows the recruitment of the coactivator paralogs CREB binding protein (CBP)/p300 to the cAMP response element (CRE) in the promoter region of target genes and initiates the transcription of CREB responsive genes [83,261]. To date, the role of CREB in Nodal signaling has not been reported. CREB is known to mediate signaling by growth factors,

basic fibroblast growth factor [262], epidermal growth factor [263], TGF-β [264] and hormones such as catecholamines [265], glucocorticoids , androgens [266], estrogens [267]) and adenylate cyclase activator such as forskolin [268]. TGF-β has been reported to induce CREB phosphorylation in murine embryonic palate mesenchymal (MEPM) cells [269], mink lung CC164 cells [269], and hepatic stellate cells [270]. CREB phosphorylation by PKA has been well reported by several studies [79,271,272]. Studies conducted in mink lung cells have shown that TGF- β activates PKA without increasing intracellular cAMP levels and that PKA activation is dependent on TGF- β induced interaction of a Smad3/Smad4 complex with the regulatory subunits of PKA. [264]. However, how Nodal activates CREB remains to be investigated.

To the best of our knowledge this is the first study that has demonstrated CREB as a target gene of miR-378a-5p and showed that it plays a central role in mediating miR-378a-5p regulated STB differentiation in a placenta developmental cell line model. Taken together, this study provides further insight of miR-378a-5p in trophoblast differentiation and advances the miRNA knowledge in the placenta development.

Supplementary Data

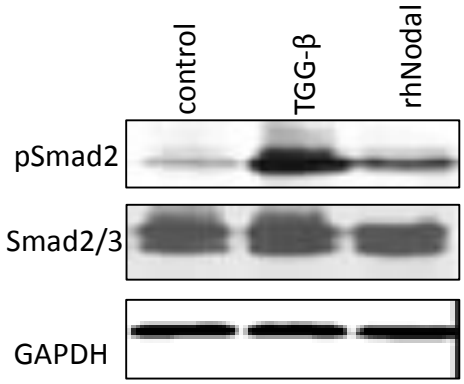

Figure S2.1 Nodal **activates Smad2.** BeWo cells were treated with 10ng/ml of TGF- β and 250ng/ml of rhNodal for 1 hr. Cell lysates were subjected to SDS-PAGE and immunoblot analyses. Western Blot for pSmad2 and total Smad2/3.

CHAPTER 3

Functional role of CCNG2 in differentiation and fusion of BeWo cells

ABSTRACT

Differentiation of mononucleated cytotrophoblasts (CTB) into multinucleated syncytiotrophoblast (STB) is a key event in the placenta development. We have previously demonstrated that miR-378a-5p is a negative regulator of STB differentiation and this inhibitory effect is mediated in part via the down-regulation of cyclin G2 (CCNG2) and identified *CCNG2* as miR-378a-5p target gene. CCNG2 is an unconventional cyclin that inhibits cell cycle progression and its levels increased during cell cycle arrest and cell differentiation. In this study, we further investigated the functional role of CCNG2 in trophoblast differentiation, specifically in the STB pathway. Using a choriocarcinoma cell line, BeWo, we found that overexpression of CCNG2 promoted STB differentiation, as evidenced by an increased expression of STB marker genes such as syncytin 1, syncytin-2 and galactoside binding soluble lectin13 (LGALS13), as well as cell fusion. On the other hand, knockdown of *CCNG2* using siRNA inhibited STB differentiation. In addition, Nodal, a member of transforming growth factor-β superfamily, increased CCNG2 mRNA and transcription. Taken together, these findings further support the role of CCNG2 in promoting STB differentiation.

INTRODUCTION

Placenta is a transient and multifaceted organ that plays crucial roles throughout the pregnancy, and provides nourishment, protection and support of the developing fetus [3]. Trophoblasts are the precursor cells of the placenta that play vital roles during blastocyst implantation and pregnancy hormone production. In addition, trophoblasts are responsible for immune protection of the fetus, increase in maternal vascular blood flow into the placenta and formation of the maternal-fetal interface [226,273]. During placental development, trophoblast progenitor cells differentiate into two distinct pathways. In the extravillous (EVT) or invasive pathway, cytotrophoblast (CTB) in the anchoring villi proliferates to create cell columns that attach to the decidua. Some CTBs detach from the cell column and differentiate into invasive extravillous trophoblasts (EVTs). These EVTs penetrate the maternal decidua and myometrium and are important for the remodelling of maternal vasculature, which leads to an increase in the blood supply to the fetus. In the villous or syncytial pathway, fusion of mononuclear CTBs result in the formation of multinucleated syncytiotrophoblast (STB), forming the syncytial layer that covers the placental villous tree and this process is called syncytialization [274]. These cells are in direct contact with the maternal blood and therefore responsible for the exchange of gases, nutrients and waste products across the materno-fetal interface. In addition, STB synthesizes and secretes various hormones and enzymes [47,230,231].

The two hallmarks in STB formation are loss of E-cadherin and increase in fusogenic retroviral envelope proteins such as syncytin-1 (encoded by HERV-W) and syncytin-2 (encoded by HERV-FRD) [56,229]. Several studies have shown that microRNAs are highly expressed in

the placenta and modulate several cellular processes by regulating gene expression [275,276]. MicroRNAs are endogenous non-coding single stranded RNAs that post transcriptionally regulate a vast number of gene expressions that play vital roles in the placenta development [3,276]. We have reported earlier that miRNA-378a-5p promoted trophoblast cell survival, proliferation, migration and invasion by targeting Nodal in EVTs, expressed throughout the pregnancy [151] and inhibits STB differentiation by targeting cyclin G2 [150].

Human cyclin G2 (CCNG2) is a family of unconventional cyclins that includes G1 and cyclin I and unlike typical cyclins, they inhibits cell cycle progression [201] . CCNG2 levels are increased during cell cycle arrest [201] and cell differentiation [188]. Studies show that CCNG2 induces adipocyte differentiation by interacting with PPARγ [188]. In addition it has been reported that CCNG2 is expressed in the human placenta and its levels increased in mid-gestational stage and then it decreases sharply at term [225]. Immunohistochemical analysis of CCNG2 in the placenta revealed its high expression in the term placentae obtained from compromised pregnancy, such as preeclampsia as compared to the placentae from healthy subjects [225].

We have previously reported that miR-378a-5p inhibits STB differentiation and target CCNG2 [150], suggesting that CCNG2 promotes STB differentiation. In the current study, we further investigated the functional role of CCNG2 in STB differentiation. We have reported that Nodal, a member of transforming factor-β superfamily, increased CCNG2 transcription in ovarian cancer cells [198] and Nodal promotes STB differentiation (Chapter 2), therefore, we also sought to determine if Nodal regulates CCNG2 in trophoblasts.

MATERIALS AND METHODS

Cell line and cell culture

A human choriocarcinoma cell line, BeWo, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained and cultured in a 1:1 mixture of DMEM (Fisher Scientific) and Ham's F12 (Life Technologies) medium containing 10% heat inactivated Fetal Bovine Serum (Gibco, Life Technologies) in a humidified condition of 5% CO2 at 37^{o}C.

Transfection, Plasmids, and RNA interference

The miRNA mimics and siRNA duplexes of control, Nodal and CCNG2 were purchased from Gene Pharma Co (Shanghai, China) and their sequences are listed in Table 1. Transient transfection was carried out by using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's suggested protocols [178]. The FLAG-cyclin G2 plasmid was generated as described previously [190]. To confirm gene silencing effects, protein lysates were prepared at 48 hours after transfection and subjected to Western blot analysis.

RNA extraction and qPCR

Total RNA was extracted from BeWo cells using Ribozol RNA extraction kit (Amresco) according to the manufacturer's instructions. To determine the mRNA levels of CCNG2, and STB markers genes such as syncytin-1, syncytin-2 and LGALS13, 2µg of total RNA was used to

synthesize first strand cDNA by M-MuLV Reverse Transcriptase (New England BioLabs Ltd) according to the manufacturer's protocol. Quantitative Real time PCR (qRT-PCR) was carried out by using gene specific primers and EvaGreen qPCR Master Mix (ABM), following manufacturer directions, on a RotorGene Q thermocycler (Qiagen). All primers used in this study are listed in Table 1 and were validated for specificity with primer-BLAST (NCBI). Amplified products were run on an agarose gel to validate a single band product. The expression levels of mRNA were normalized to GAPDH.

Western blotting

BeWo cells were washed with cold PBS (136 mM NaCl, 2.6 mM KCl, 10mM Na2HPO4.7H2O, 1.8mM KH2PO4 and pH was adjusted to 7.4). The cell lysate was prepared using radio-immunoprecipitation assay buffer (50 mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1% SDS) containing protease and phosphatase inhibitor cocktail (Pierce, Thermo Fischer Scientific). Cell lysates were centrifuged for 15 minutes at 4^{o}C and bicinchoninic acid (BCA) method was used to determine the total protein concentration. Equal amount of protein samples (30µg) were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio Rad). 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 with constant agitation and then incubated with specific primary antibodies listed in Table 2 in blocking buffer overnight at 4^{o}C. Next day, membranes were washed three times with TBST for 10 minutes and subsequently probed with HRP conjugated antibody for 1 hour at room temperature. Signals were detected by using Enhanced Chemi-Luminescence (ECL) detection reagent (Millipore) according to the manufacturer's instructions. GAPDH was used as the loading control and all the experiments were

repeated in triplicate and at least three times. In order to obtain quantitative data from the Western Blots, films were scanned from Canon scanner and signal intensities were analyzed using the Carestream Molecular Imaging software.

Immunofluorescence

Cells were seeded on the coverslips at the density of $4x10^4$ in a 12-well plate. The next day, cells were either transfected with CCNG2 plasmid, miR-378a-5p along with EV or CCNG2 or siRNA CCNG2. Cells were washed with phospho buffer saline (PBS) and fixed in methanol (-20 ^oC) for 10 minutes. Cells were washed three times with PBS and incubated with 3% BSA in PBS for one hour. Finally, BSA was removed and cells were incubated with mouse monoclonal anti-E-cadherin antibody (1:100) overnight at 4^oC. Next day, coverslips were washed with PBS buffer and incubated with Alexa Fluor 594 conjugated goat anti-mouse secondary antibody (1:300, Life Technologies) at room temperature for one hour. DAPI (1:1000, Sigma) was used as a counterstain to stain the cell nuclei. Coverslips were washed, mounted and examined with the inverted fluorescence microscope. Photographs were taken in 10 randomly selected fields per coverslip and cell fusion was analyzed in the cells when 3 or more nuclei were present. Fusion index was calculated as [(N-S)/T] x 100% as described [245].

N = number of nuclei in the syncytia,
S = the number of syncytia
T = total number of nuclei in the observed field

TABLE 1: Sequences of PCR primers and siRNAs

Primer/siRNA	Sequence 5'-------> 3'
Syncytin-1	**F:** TCATATCTAAGCCCCGCAAC **R:** TGATCTTGCAAGGTGACCAG
Syncytin-2	**F:** TCGGATACCTTCCCTAGTGC **R:** GTATTCCGGAGCTGAGGTTG
LGALS13	**F:** ATTGCCTTCCGTTTCCGAGT **R:** TTTGCCATCCTCAAAGGGCA
GAPDH	**F:** AAGGTCATCCCTGAGCTGAAC **R:** ACGCCTGCTTCACCACCTTCT
Control siRNA	**Sense**:UUCUCCGAACGUGUCACGUtt **Anti-sense:**ACGUGACACGUUGGAGAAtt
CCNG2	**Sense:**GCTGAAAGCTTGCAACTGCCGACtt **Anti-sense:** GGTATCGTTGGCAGCTCAGGAACtt

TABLE 2: Primary Antibodies for Western Blot

Antibody	Company	Specie	Working Conc.	Dilution	Catalogue No
Nodal	Abcam	Mouse	1159ug/ml	1:500	ab55676
E-cadherin	Santa Cruz	Mouse	200ug/ml	1:500	sc-8426
GAPDH	Santa Cruz	Mouse	200ug/ml	1:1000	sc-365062
Syncytin-1	Santa Cruz	Rabbit	200ug/ml	1:500	sc-50369

Luciferase Promoter Assay

Cells were seeded in 12-well plates at the density of $8x10^4$/well and co-transfected with CCNG2 promoter construct plasmids, pCS2 or N56 plasmid and pRL-TK internal control (encoding Renilla luciferase) plasmid. The CCNG2 promoter construct was generated as described previously [198]. Cells were recovered in DMEM/F12 for the next 19 hours. Cells were lysed and luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instruction.

Statistical Analysis

All the experiments were done in triplicate in each group and each experiment was performed 3 times. Statistical analysis was performed by using GraphPad Prism 6 ($p<0.05$,95% Cl was considered statistically significant). All of the data was represented as mean ± SEM. Unpaired, two-tailed Student's *t*- test was used to compare the values between two groups and one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test was used to determine the difference among several groups.

RESULTS

CCNG2 induces cell fusion and marker gene expression

We have previously demonstrated that miR-378a-5p inhibits STB differentiation by targeting cyclin G2 (CCNG2) and overexpression of CCNG2 decreased E-cadherin and increased syncytin-1 protein expression [150]. On the other hand, knockdown of *CCNG2* using siRNA resulted in increased E-cadherin and decreased syncytin-1 protein expression [150]. In order to assess if overexpression of CCNG2 could reverse the inhibitory effect of miR-378a-5p on STB differentiation, cells were transfected with negative control or miR-378a-5p along with empty vector (EV) or CCNG2 plasmid for 72 hours and immunofluorescence was performed. As shown in Figure 3.1(A-B), transfection of miR-378a-5p reduced cell fusion but this effect was lost in cells co-transfected with CCNG2, suggesting that CCNG2 promotes STB differentiation. This result has been included in a manuscript, published in Biology of Reproduction [150].

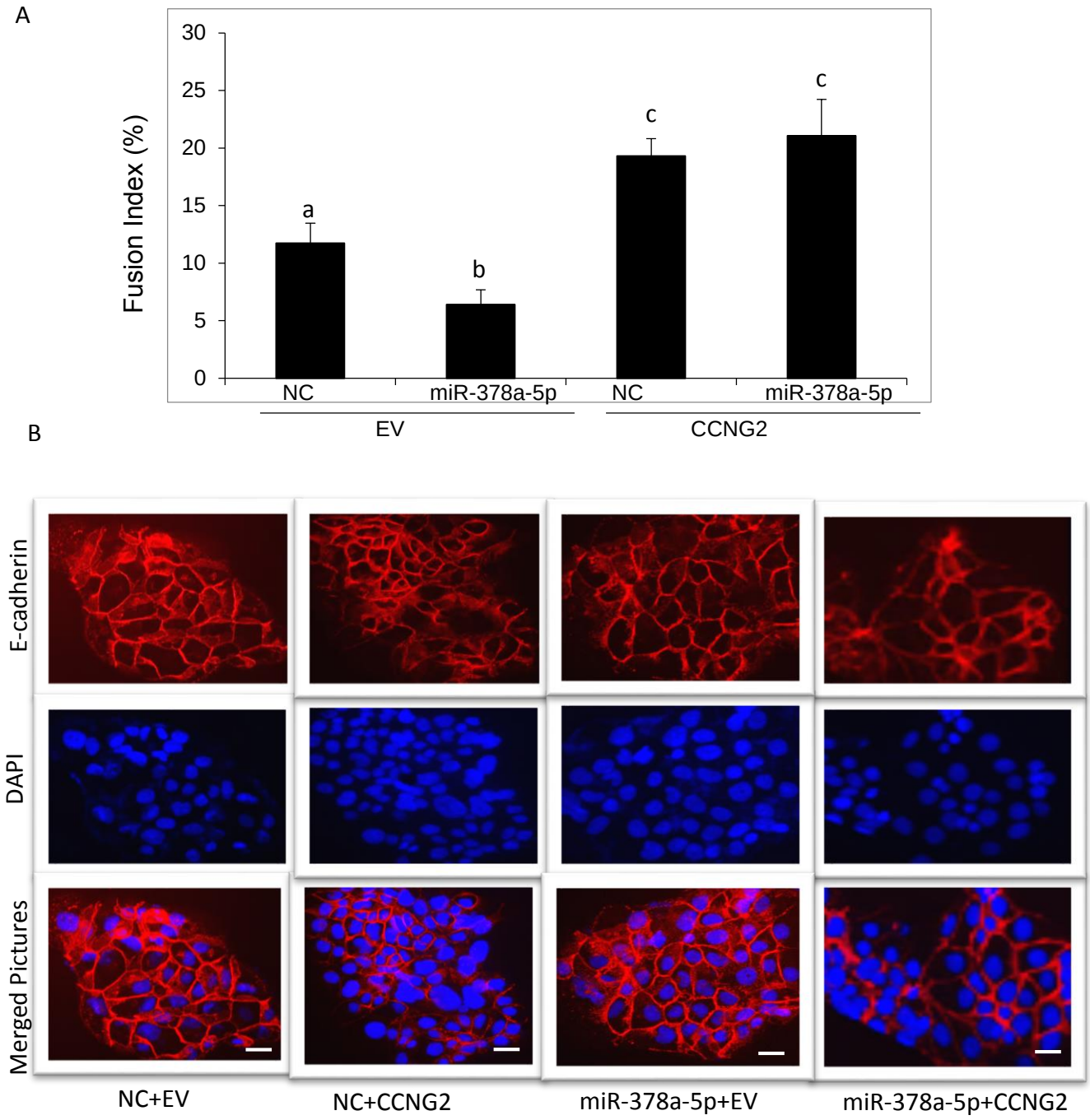

Figure 3.1 Overexpression of CCNG2 reverses the inhibitory effect of miR-378a-5p on cell fusion. A) Cells were transfected with non-targeting control oligo (NC) or miR-378a-5p, along with control vector (EV) or CCNG2 plasmid. Immunofluorescent staining using an anti-E-cadherin antibody was performed at 72 h after transfection and fusion index (n=3) was calculated. Transfection of miR-378a-5p reduced cell fusion but this effect was lost in cells co-transfected with CCNG2. Different letters above bars denote statistical significance. B) Representative pictures of each condition. Statistical analysis was performed on GraphPad Prism using a two-way ANOVA ($p<0.05$, 95% Cl) with Sidak's multiple comparison test.

In order to further examine the role of CCNG2 in differentiation and fusion of BeWo cells, various experiments were performed. Cells were transfected with control plasmid vector (EV) or plasmid expressing CCNG2 and real-time-qPCR was used to quantify mRNA levels of STB marker genes. As shown in Figure 3.2A, transfection with CCNG2 plasmid resulted in significant increase in mRNA levels of syncytin-1, syncytin-2 and LGALS13. In addition, immunofluorescence analysis showed that overexpression of CCNG2 induces cell fusion versus control [Figure 3.2B]. Overexpression of CCNG2 is also showed by using flag antibody. To further evaluate the role of CCNG2 in STB differentiation, siRNA-mediated gene silencing approach was used. BeWo cells were transfected with siRNA targeting CCNG2, NC or mock control. Silencing of *CCNG2* expression was confirmed by qPCR (Figure 3.3A), resulting in decreased in syncytin 1 and 2 and LGALS13 marker gene expression (Figure 3.3B-D). Finally, the effect of CCNG2 silencing on cell fusion was examined. Immunofluorescent staining revealed that transfection with siRNA CCNG2 resulted in a significant decrease in cell fusion versus control (Figure 3.4 A&B).

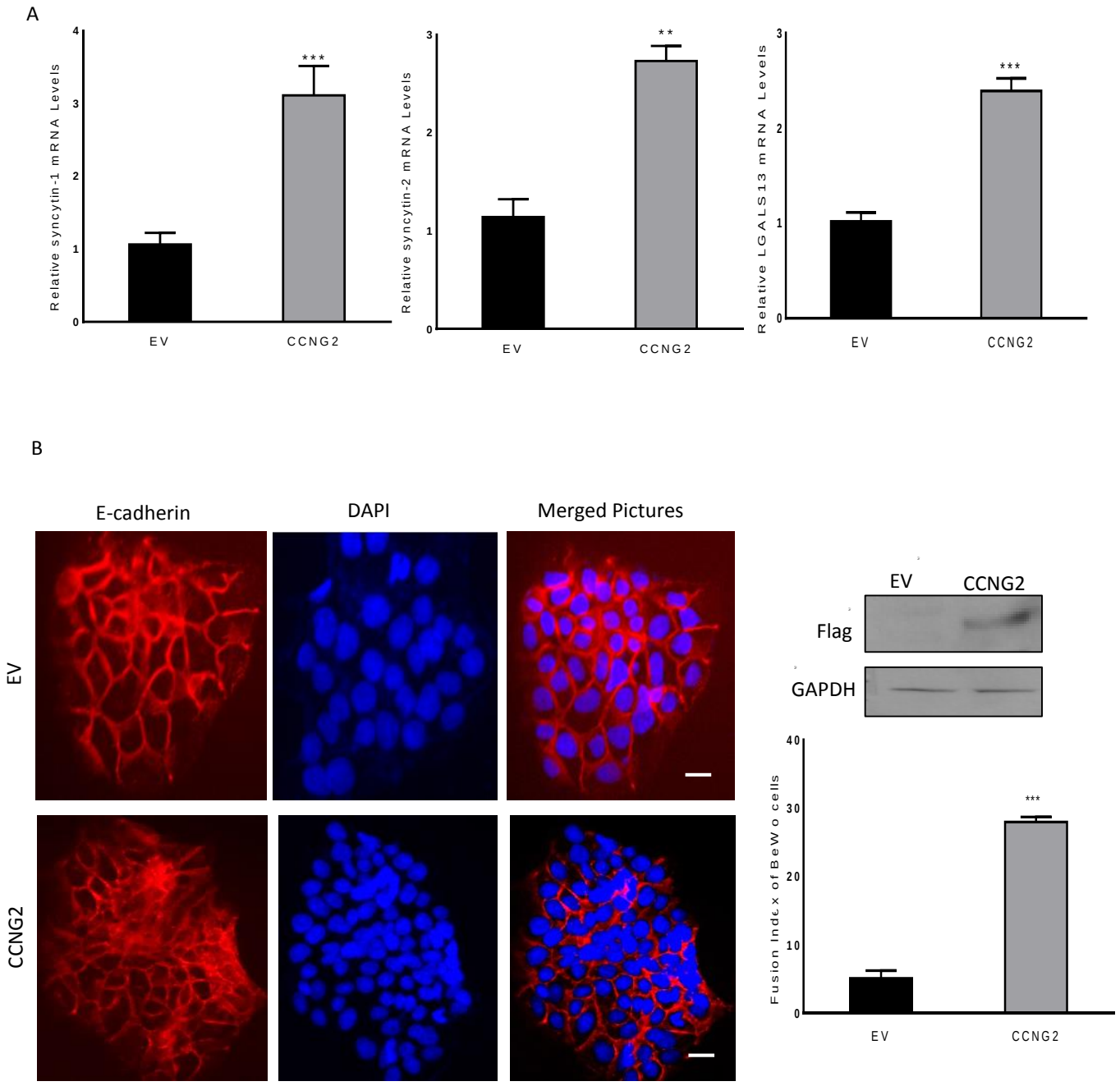

Figure 3.2 Overexpression of CCNG2 induces cell fusion. A) Cells were transfected with control vector (EV) or CCNG2 plasmid. mRNA levels of syncytin 1, syncytin-2 and LGALS13 were determined by RT-qPCR and normalized to an internal calibrator glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Overexpression of CCNG2 resulted in a significant increase in fusogenic syncytin (1 and 2) and LGALS13 mRNA levels. Data represent mean ± SEM (n=3). **p<0.01, ***p<0.001 *vs*. EV. B) Cells were transfected with EV or CCNG2 plasmid. Immunofluorescent staining using an anti-E-cadherin antibody was performed at 72 h after transfection and fusion index (n=3 wells) was calculated. ***, p<0.001 *vs*. EV. Representative pictures of EV and cells transfected with CCNG2. Cell membranes were stained with anti-E-cadherin (red) and nuclei were DAPI stained (blue). Scale bars=100 µm. Cells were transfected with EV or CCNG2 and cell lysate was collected and analyzed by Western blot. Overexpression of CCNG2 was determined by using anti-Flag and GAPDH antibodies. Statistical analysis was performed on GraphPad Prism using a two-tailed unpaired t-test (p<0.05, 95% Cl) for (A and B).

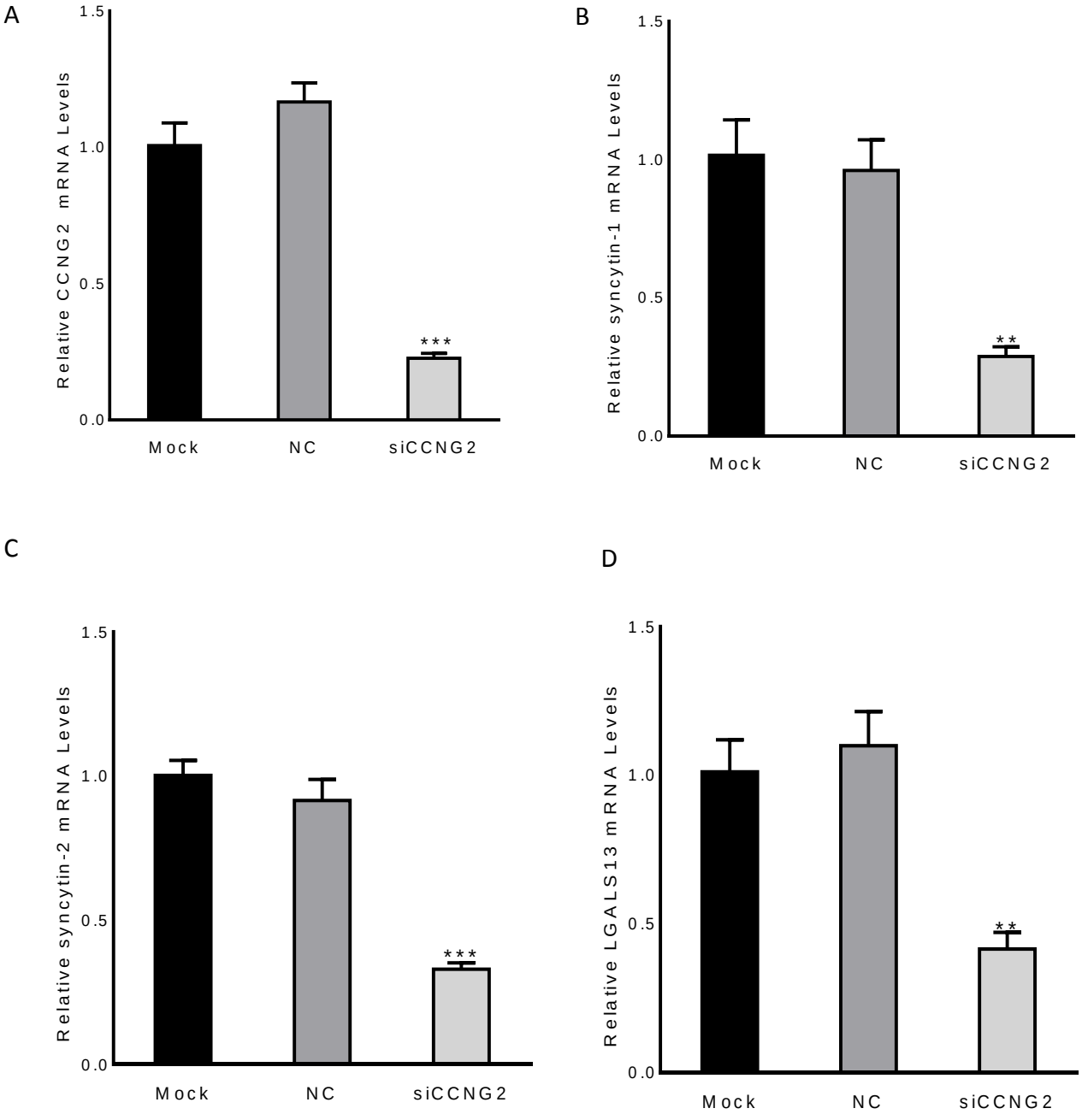

Figure 3.3 Silencing of *CCNG2* expression inhibits STB marker gene expression. Cells were transfected with non-targeting control oligo (NC), siRNA targeting CCNG2 (siCCNG2) or without oligos (mock). Total RNA was extracted at 72 h after transfection and mRNA levels of CCNG2, syncytin-1, syncytin-2 and LGALS13 were determined by RT-qPCR. The marker gene mRNA levels were normalized to GAPDH. Data represent mean ± SEM (n=3). **p<0.01, ***p<0.001 *vs*. corresponding controls. Statistical analysis was performed on GraphPad Prism using a one-way ANOVA with Tukey's multiple comparison test (p<0.05, 95% Cl) for (A-D).

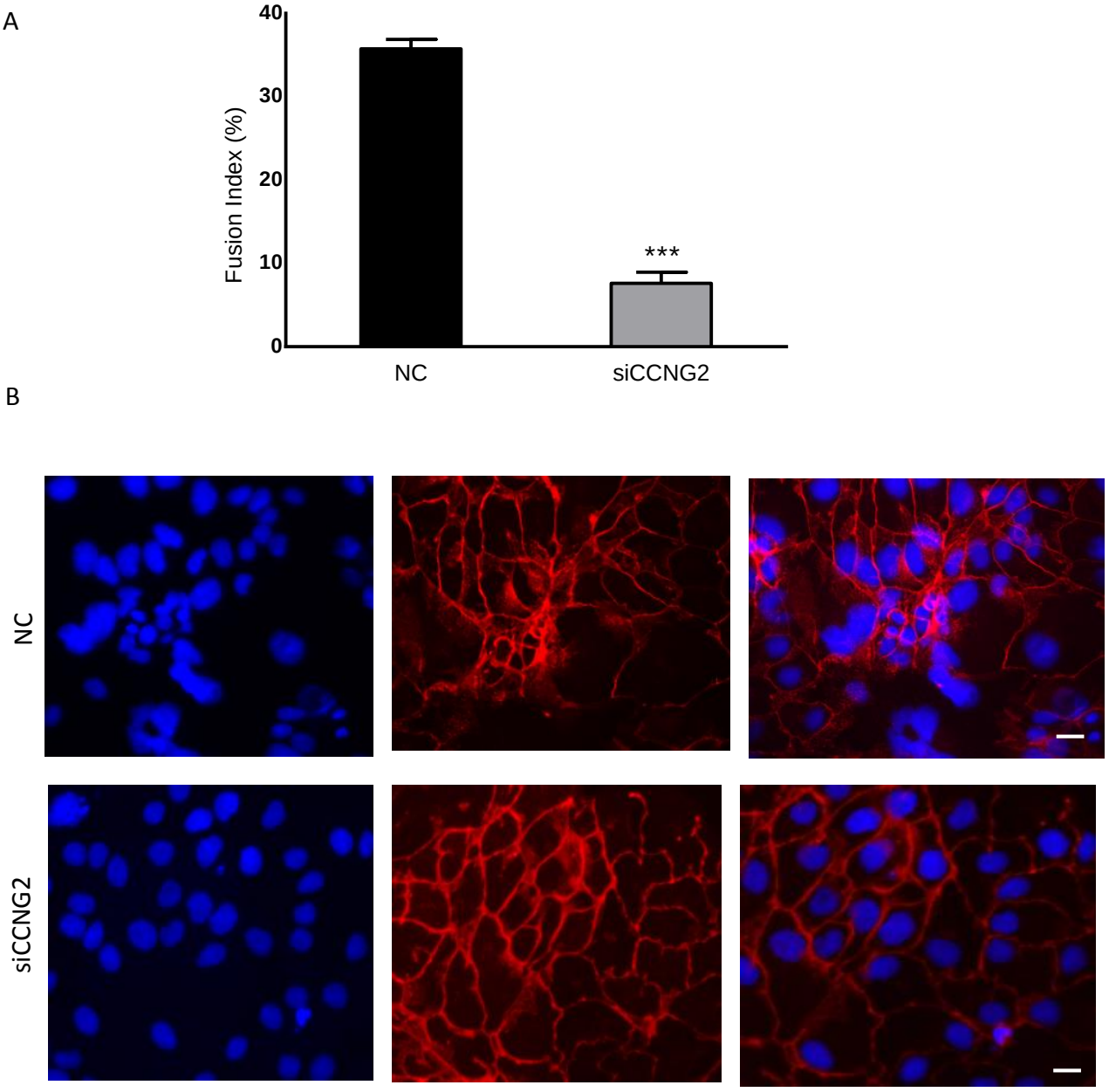

Figure 3.4 Silencing of *CCNG2* expression inhibits cell fusion. Cells were transfected with non-targeting control oligo (NC) or siRNA targeting CCNG2 (siCCNG2). A) Immunofluorescent staining using an anti-E-cadherin antibody was performed at 72 h after transfection and fusion index (n=3 wells) was calculated. ***, $p<0.001$ *vs*. NC. B) Representative pictures of NC and cells transfected with siCCNG2. Cell membrane was stained with anti-E-cadherin (red) and nuclei were DAPI stained (blue). Fused nuclei were shown in the merged pictures. Scale bars=100 µm. Statistical analysis was performed on GraphPad Prism using a two-tailed unpaired t-test ($p<0.05$, 95% Cl).

Nodal induces CCNG2 promoter activity

We have previously reported that Nodal increased CCNG2 mRNA and protein levels [190] and transcription [198] in ovarian cancer cells. Therefore, we tested whether Nodal could induce CCNG2 expression in BeWo cells. Treatment with rhNodal increased CCNG2 mRNA levels (Figure 3.5A). Conversely, silencing of Nodal using siRNA significantly decreased the CCNG2 mRNA levels (Figure 3.5B). Using a full length CCNG2 promoter construct, we found that overexpression of Nodal resulted in a significant increase in CCNG2 promoter activity (Figure 3.5C]. Similarly, treatment with rhNodal also increased CCNG2 promoter activity [Figure 3.5D].

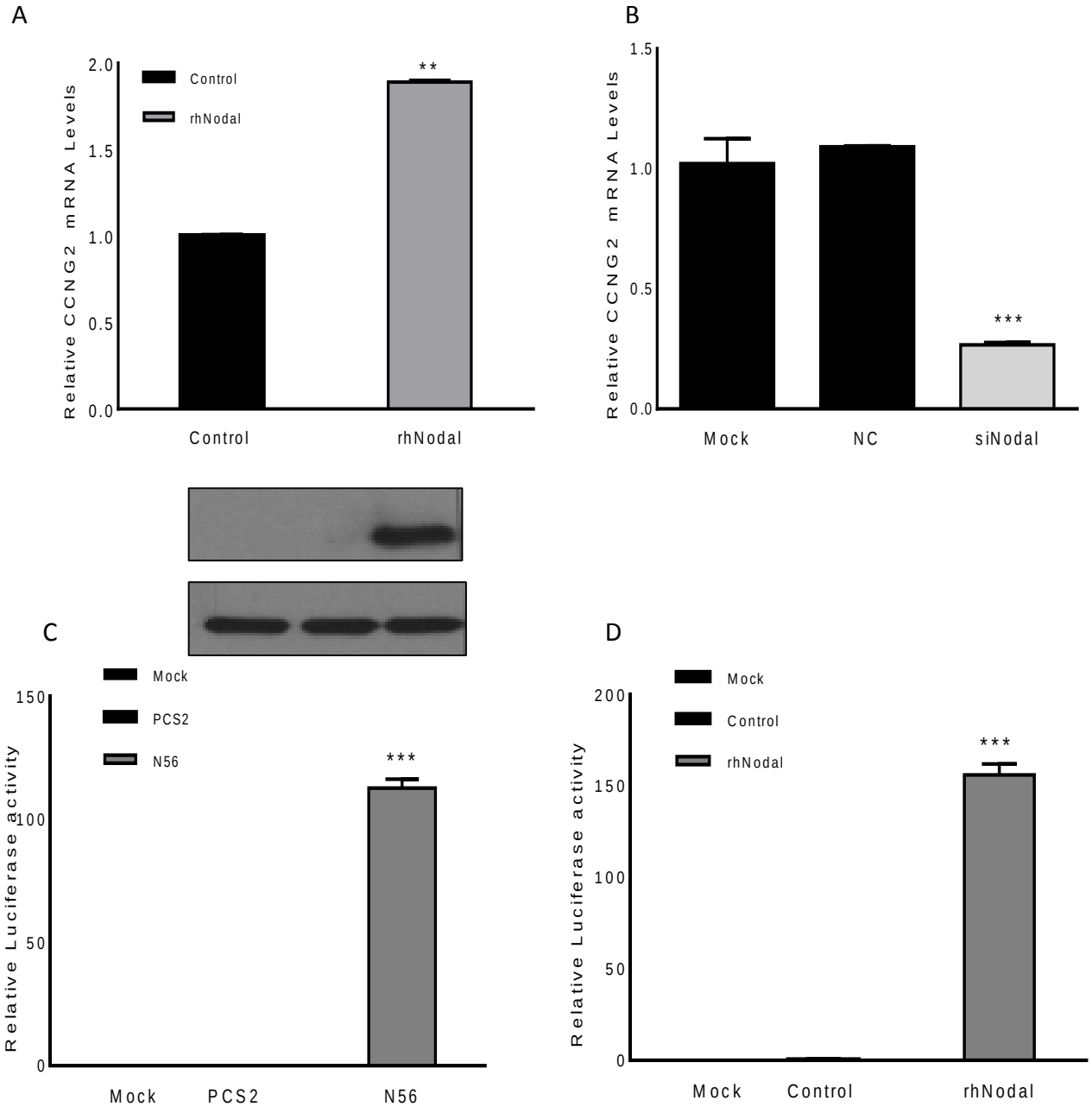

Figure 3.5 Nodal induces CCNG2 Transcription. A) Cells were treated with rhNodal or its control and total RNA was extracted after 48 hrs. treatment. CCNG2 mRNA levels were determined by RT-qPCR. B) Cells were transfected with non-targeting control oligo (NC), siRNA targeting Nodal (siNodal) or without oligos (mock). Total RNA was extracted at 48 h after transfection and CCNG2 mRNA levels were determined by RT-qPCR. The marker gene mRNA levels were normalized to GAPDH. Data represent mean $\pm$ SEM (n=3). **p<0.01, ***p<0.001 *vs* corresponding controls. Nodal increased CCNG2 while siNodal decreased CCNG2 mRNA levels. C-D**)** Cells were transfected with a full-length CCNG2 promoter construct, luciferase construct, together with a control pCS2 (EV), mature Nodal (N56) or with recombinant Nodal (rhNodal). Luciferase assays were performed at 24h after transfection. Data represent mean $\pm$ SEM (n=4). ***p<0.001 vs corresponding controls. Statistical analysis was performed on GraphPad Prism using a two-tailed unpaired t-test (p<0.05, 95% Cl) for (A) and a one-way ANOVA with Tukey's multiple comparison test (p<0.05, 95% Cl) for (B-D).

DISCUSSION

We have previously reported that miR-378a-5p targets *CCNG2* and inhibits STB differentiation [150]. In this study we further investigated the role of CCNG2 in STB pathway and demonstrated that CCNG2 induces STB differentiation.

Several lines of evidence support the positive role of CCNG2 in STB differentiation. First, overexpression of CCNG2 promoted STB differentiation and reversed the inhibitory effect of miR-378a-5p. Next overexpression of CCNG2 increased the expression levels of several STB markers such as syncytin-1, syncytin-2 and LGALS13. Furthermore, immunofluorescence results showed that CCNG2 increased the number of cells that have multiple nuclei, indicating it induces cell fusion. Finally, knockdown of *CCNG2* using siRNA inhibited cell differentiation and decreased fusogenic markers. CCNG2 is an atypical cyclin and its highest levels are observed in quiescent and terminally differentiated cells [188]. Studies have shown that CCNG2 is expressed in the mouse uterus and act as a pro-apoptotic inducer of the terminally differentiated cells at the site of the blastocyst after implantation, suggesting its role in implantation and decidualization [209]. Another study reported that CCNG2 is expressed in placental tissues and its levels increased in mid-gestational stages and then decreases sharply at term. Fusion of CTB into STB is a highly controlled process and several studies have reported that cytokines, hormones, protein kinases, transcription factors and membrane proteins play key roles in regulation and fusion of CTBs into STB. Dysregulation of any factor may results in the aberrant villous trophoblast turnover and could lead to either restricted or exaggerated cytotrophoblast-syncytiotrophoblast fusion that has been suggested to trigger preeclampsia and other pathological conditions [277]. As nuclei in the STB lack mitotic activity therefore, apoptosis plays an important role in STB by continuous disposal of

the aged cytosolic contents into the maternal circulation by forming syncytial knots and thus providing fresh cellular contents to maintain the homeostasis of the STB [277]. Several studies have reported that exaggerated cytotrophoblast-syncytiotrophoblast fusion could lead to increased apoptosis that may result into placental pathologies such as preeclampsia [278–281], early pregnancy loss [282,283], Intrauterine growth restriction (IUGR) [279,284] and gestational trophoblastic diseases [285,286]. On the other hand, restricted fusion may results in depletion of cellular material in the STB and that leads to exhaustion of the syncytial layer which acts as barrier, gaseous exchange, nutrients and waste transport across mother and fetus, and could affect the pregnancy outcomes [277].

Since all of this study has been done by using a cell line model, therefore it is essential to confirm these findings by using placenta explants models. Placenta villous samples from the first and second trimester can be used and floating villi can be incubated with CCNG2 or siCCNG2 with their respective controls at 48 hours. Immunocytochemistry of villous tissues will confirm these findings. In addition, placenta villous samples treated with CCNG2 or siCCNG2 can be analyzed for protein and marker gene expression. These results can be further confirmed by using the primary cytotrophoblast cells from the term human placenta tissues. Key findings from this study can be repeated in primary cells isolated from the whole placental tissues. As to date a very limited knowledge is available of CCNG2 in placenta and its function in placenta is still unknown, therefore further investigation of the role of CCNG2 will advance the potential mechanism and its dysregulation in STB pathway. Cyclin G2 is a target gene of Nodal [190]. Nodal belongs to TGF-β superfamily and plays critical roles during embryo development [171]. We have previously reported that Nodal increased CCNG2 mRNA, protein and transcription levels in ovarian cancer cells [198]. In addition, Nodal [151] and *CCNG2* [150] are both target genes of miR-378a-5p, and Nodal induces STB differentiation (Chapter 2). Therefore, it was investigated whether Nodal could

induce CCNG2 transcription in trophoblast cells. This study showed that overexpression of Nodal increased CCNG2 mRNA levels, and transcription. It is therefore possible that Nodal regulates CCNG2 and increases its stability to induce cell fusion in trophoblast cell models, BeWo cells.

It has been reported that TGF-β increased CCNG2 mRNA by 10-14 folds after 29h of treatment [201]. Our lab has demonstrated that overexpression of Nodal and its receptors ALK-7 resulted in increase in CCNG2 mRNA levels as well as CCNG2 protein stability. [190]. Here, we provided initial evidence that Nodal induces CCNG2 at transcription levels in trophoblasts. However, it remains to be investigated how Nodal induced CCNG2 transcription. In order to understand the role of Nodal signaling in CCNG2-induced STB pathway, various experiments can be performed. Cells can be treated with control or Nodal along with EV or CCNG2 and E-cadherin and syncytin-1 protein levels can be analyzed. STB marker genes can be analyzed to see whether Nodal signaling can be further induced STB differentiation. In order to further confirm these findings, cells can be transfected with or without siRNA Nodal. Briefly, cells can be transfected with siRNA Nodal and its negative control (NC) along with CCNG2 or its control vector. Protein expression level of E-cadherin and syncytin-1 can be determined by using Western blot. Decrease in marker genes or syncytin-1 in cells transfected with siRNA Nodal with CCNG2 *vs.* control group will confirm the role of Nodal signaling in CCNG2 induced STB differentiation. Nodal acts through ALK7 [167] and phosphorylates Smad2 [162] and possibly Smad3 [161] to regulate gene expression. There are several Smad-binding sites within the CCNG2 promoter [198]. Whether or not Nodal acts through Smads in CCNG2 induced STB differentiation can be further investigated in future.

Taken together, this study provides further insight into the role of CCNG2 in STB pathway and advances the role of CCNG2 in the placenta development.

CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

I. SUMMARY

In this dissertation, I have investigated the potential role of miR-378a-5p in trophoblast cell differentiation into the STB pathway. We have previously demonstrated that miR-378a-5p inhibits STB differentiation, in part, by down-regulating CCNG2 expression [150] and promotes trophoblast invasion by targeting Nodal [151]. The overall objectives of this study were to 1) investigate the additional target genes of miR-378a-5p in STB differentiation; 2) determine the role of Nodal in STB differentiation; and 3) investigate the role of cyclin G2 in STB differentiation pathway.

1. miR-378a-5p targets *CREB*

To further understand the role of miR-378a-5p in STB differentiation, bioinformatics tools were used to identify additional targets involved in miR-378a-5p regulated differentiation. Using a choriocarcinoma cell line, BeWo, we showed that miR-378a-5p targets *CREB* (Chapter 2). Transfection with miR-378a-5p decreased CREB protein and mRNA levels and blocking the endogenous levels of miR-378a-5p by antimiR-378a-5p resulted in increased CREB protein and mRNA levels. In addition, overexpression of CREB reverses the inhibitory effect of miR-378a-5p by decreasing E-cadherin and upregulating syncytin protein and other STB marker genes expression.

2. Nodal induces cell fusion and differentiation by activating CREB

To investigate the functional role of a miR-378a-5p target gene, Nodal, in STB differentiation, we first determined the endogenous level of Nodal in cAMP induced STB

differentiation. We showed that that treatment with forskolin (an adenylate cyclase activator) resulted in a significant increase in Nodal mRNA and protein levels in a time-dependent manner. In addition, we found that treatment with rhNodal induced cell fusion, decreased E-cadherin and increased fusogenic protein syncytin-1 expression. In concomitant with the protein expression, Nodal treatment increased mRNA levels of marker genes such as syncytin-1, syncytin-2, LGALS13 and CGB. In addition, treatment with rhNodal significantly increased the hCG levels.

To further evaluate the role of Nodal in STB differentiation, we used the siRNA-mediated gene silencing approach. Transfection with a siRNA targeting Nodal in BeWo cells resulted in increased E-cadherin and decreased syncytin-1 protein levels. Moreover, transfection with siNodal resulted in a significant decrease in syncytin-1 and syncytin-2, mRNA levels as well as cell fusion. Finally, to determine whether forskolin-induced STB differentiation is mediated by Nodal, cells were transfected with Nodal-specific siRNA or control siRNA and then treated with forskolin. We observed that knockdown of Nodal in forskolin treated cells resulted in decrease in STB marker genes suggesting that forskolin induced STB differentiation is mediated by at least in part by Nodal

Nodal and CREB are both target genes of miR-378a-5p. In addition, Nodal and TGF- β belongs to the same family and TGF- β has been shown to induce CREB phosphorylation [280,281]. Therefore, we investigated whether Nodal can induce CREB phosphorylation or not. Interestingly, we found that Nodal activates CREB, as evidenced by a significant increase in phospho-CREB levels in cells treated with Nodal. We further demonstrated that inhibition of CREB activity by using siRNA in Nodal-treated cells resulted in a decrease in cell fusion and marker genes when compared with cells treated with Nodal alone, suggesting that CREB mediates the effect of Nodal on STB marker gene expression. We further examined whether CREB and Nodal can reverse the inhibitory effect of miR-378a-5p on STB differentiation. Several

experiments such as cell fusion assay, protein expression and mRNA levels of STB marker genes show that overexpression of CREB and Nodal can reverse the inhibitory effect of miR-378a-5p, suggesting that Nodal and CREB play important roles in miR-378a-5p regulated STB differentiation.

3. Role of cyclin G2 in differentiation and fusion of BeWo cells

We have reported earlier that miR-378a-5p is a negative regulator of STB differentiation and this inhibitory effect is mediated in part via down-regulation of cyclin G2, which induces cell differentiation [150]. In this study, we showed that overexpression of cyclin G2 increased expression of STB marker genes such as syncytin 1, syncytin 2 and LGALS13, as well as cell fusion. Knockdown of cyclin G2 using a siRNA mediated approach resulted in a decrease in STB marker genes and cell fusion. We have reported that Nodal increased cyclin G2 transcription in ovarian cancer cells [198] and Nodal induces STB differentiation (chapter 2). Nodal and cyclin G2 are both target genes of miR-378a-5p and treatment of Nodal or overexpression of cyclin G2 resulted in STB differentiation. Therefore, we further investigated whether Nodal could induce CCNG2 transcription in BeWo cells. Interestingly, we found that Nodal increased the cyclin G2 transcription in trophoblast cell model, BeWo.

II. FUTURE DIRECTIONS AND PROSPECTS

In chapter 2 of this dissertation, we have reported that miR-378a-5p targets CREB and its target gene, Nodal induces STB differentiation via CREB activation. In this study, we also showed that Nodal could induce the phosphorylation of Smad2. However, there are still a few questions that need to be addressed.

1. Do Smads play a role in miR-378a-5p regulated STB differentiation?

Bioinformatics analysis (microRNA.org) showed that miR-378a-5p has five potential binding sites for Smad2 and three potential binding sites for Smad3 and Smad4. Our preliminary data showed that miR-378a-5p decreased Smad2 and Smad3 mRNA and protein levels (Figure 4.1A&B) and it is possible that it can target these mediators and block the Nodal induced STB differentiation. To confirm whether miR-378a-5p targets Smad2 and Smad3 in BeWo cells, Smad2 and Smad3 constructs will be generated. BeWo cells will be transfected with these constructs, along with miR-378a-5p mimics or NC and a control reporter without 3'UTR and 3'UTR luciferase assay will be performed. Furthermore, rescue experiments can be performed such as cells will be transfected with miR-378a-5p mimics or its NC, along with control or Smad2 and Smad3 overexpressing plasmid, and cell fusion assay, protein expression levels and STB marker genes will be analyzed in future studies.

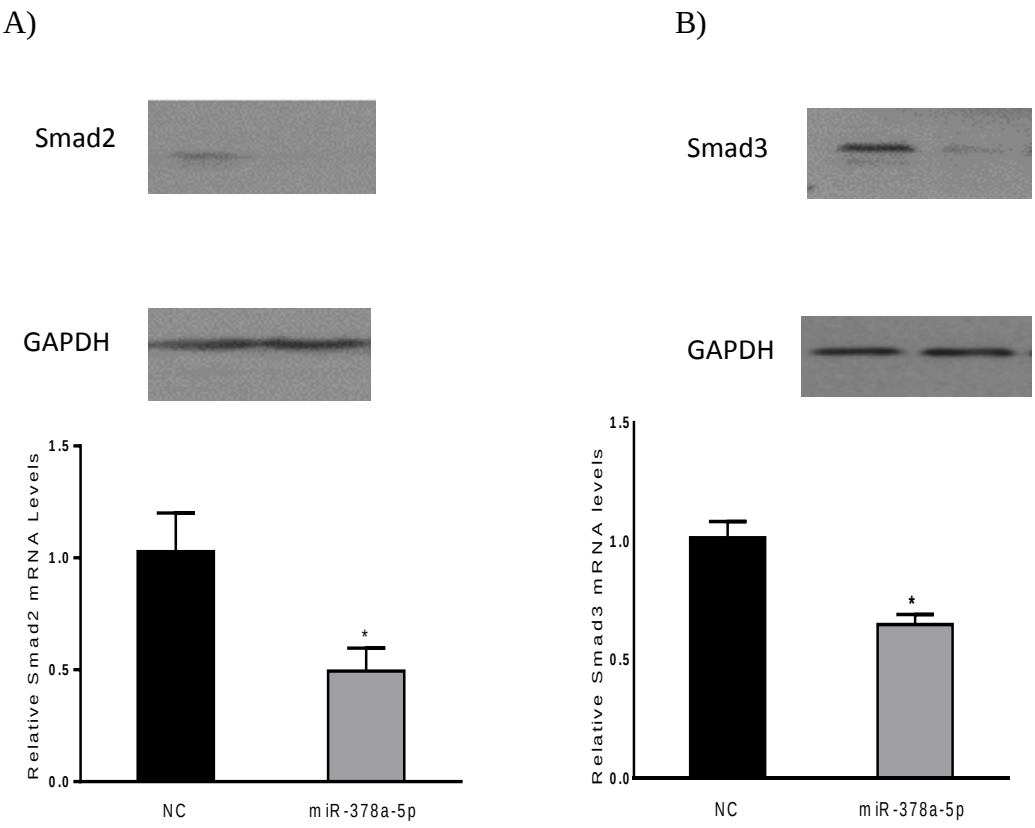

Figure 4.1 MicroRNA-378a-5p decreases Smad2 and 3 expressions. A-B) BeWo cells were transfected with non-targeting control oligo (NC) or miR-378a-5p. Total RNA and cell lysates were collected at 48 h after transfection. mRNA levels were determined by RT-qPCR and Western blotting performed using anti-Smad2 and Smad3 antibody. Representative immunoblots are shown. Data represent mean ± SEM (n=3). **p<0.05 *vs.* NC.

2. What signaling pathways are involved in Nodal-induced STB differentiation?

In the future, we can further investigate the potential signaling pathway involved in CREB activation in Nodal induced STB differentiation. CREB induced the expression of target genes in response to various signals such as hormones, cytokines and growth factors [269]. It has been shown that CREB can be regulated by cAMP [287], AKT [288] cAMPK [79] MAPK and PKA [289] pathways. To analyze the signaling pathway involved in CREB activation during Nodal-induced STB differentiation, cells can be treated with or without prospective signaling inhibitors (PKA, cAMPK, AKT or ERK) inhibitors for an hour and can be treated with or without Nodal for half an hour. CREB phosphorylation can be determined by Western blotting. Furthermore, cells can be treated with or without inhibitors and then can be treated with or without Nodal at different time points. Fusogenic protein and STB marker genes can be analyzed after treating the cells with or without inhibitor and Nodal treatment to determine which signaling pathway could be involved in Nodal induced STB differentiation pathway. Finally, to obtain the quantitative results of CREB phosphorylation, commercially available phospho-CREB (Ser_{133}) and total CREB cellular assay kits can be used.

Nodal signaling through Smad3 has been reported by many studies [39,198,290]. Furthermore, it has been demonstrated that CREB and Smad3 are required for the TGF-β induction [291] and TGF-β induced phosphorylation of Smad3 modulates the interaction with transcriptional coactivator CREB binding protein (CBP) and regulates gene expression [292]. It may be possible that Nodal acts through Smad3 and activates CREB to induce STB differentiation. In future, it can be further tested whether Nodal can activate Smad3 or not. To evaluate the Smad3 activation, cells

will be treated with rhNodal at different time points and phospho-Smad3 levels will be determined by Western blot.

Our lab has shown that Nodal activates ALK7 and inhibits trophoblast proliferation and invasion [167]. Nodal has been suggested to act through both ALK4 and ALK7 receptors and activates Smad2 and Smad3 [290]. Therefore, to investigate whether Smad2/3 or its receptors ALK4/7 mediates the effect of Nodal on cell differentiation, siRNA-mediated gene silencing approach will be used. Cells will be transfected with siRNA Smad2/3 or siRNA ALK4/7 and STB marker genes, fusogenic proteins and E-cadherin expression will be analyzed. Nodal acts through these receptors and by blocking the effect of these receptors will determine what receptors are involved in Nodal induced STB differentiation, as well as confirm the role of Smads in Nodal induced STB differentiation. Furthermore, the interaction of transcriptional coactivator CBP and Smads can be studied in the future.

3. What potential mechanism is involved in cyclin G2 induced STB differentiation?

In Chapter 3 of this dissertation, we have reported the functional role of cyclin G2. In the future, we can further investigate the potential mechanism by which cyclin G2 induced STB differentiation as well as how Nodal increased the cyclin G2 transcription in trophoblast cell model, BeWo cells. Several studies have reported that PPARγ, an important transcription factor, regulates the function and development of the placenta. PPARγ is expressed in STB and increases the CGB mRNA levels and the secretion of hCG, placental lactogen, growth hormone, and leptin [76,293]. Using Bioinformatics analysis, we found that Nodal promoter has two potential binding sites of PPARγ. Using luciferase assay we showed that Nodal induces PPARγ promoter activity,

protein and mRNA levels while knockdown of Nodal resulted in a decrease in PPARγ protein and mRNA levels (Figure 4.2), suggesting that Nodal regulates PPARγ. Cyclin G2 interacts with PPARγ and induces adipocyte differentiation [188]; therefore, it is possible that cyclin G2 regulates STB differentiation through PPARγ co-activation. However, further studies are required to confirm whether cyclin G2 interacts with PPARγ or not. To determine whether cyclin G2 interacts with PPARγ or not, co-immunoprecipitation can be performed.

Interestingly, PPARγ promoter has two consensus SMAD responsive elements. In our study, we showed that Nodal could activate Smad pathway (Chapter 2, S2.1) and it is therefore possible that Nodal may regulate PPARγ by activating Smads. To test this hypothesis whether Nodal may regulate PPARγ by activating Smads, cells will be transfected with siRNA Smad2/3 and then treated with or without rhNodal, and PPARγ levels can be analyzed.

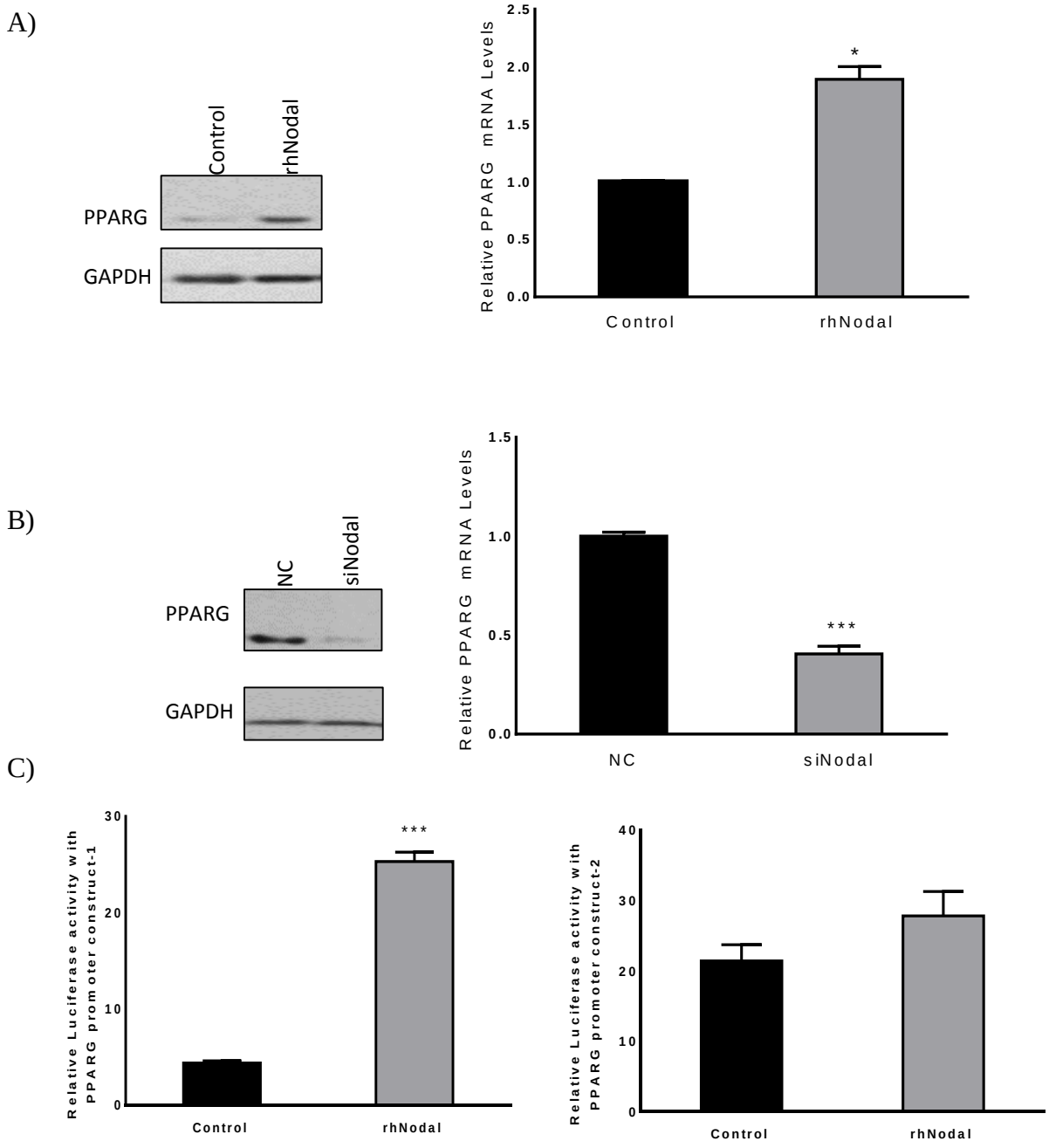

Figure 4.2 Nodal induces PPARγ. A) Cells were treated with rhNodal and control. Cell lysates were prepared at 48h and subjected to immunoblotting using anti-PPARγ. Real-time PCR was used to quantify mRNA levels of PPARγ. B) Cells were transfected with non-targeting control oligo (NC) or siRNA targeting Nodal for 48 hours and PPARγ protein and mRNA levels were analyzed. *p<0.05, ***p<0.001 *vs*. control C) Luciferase promoter assays were performed at 24h after rhNodal treatment. Nodal significantly induces PPARγ promoter activity of construct-1. Data represent mean ± SEM (n=3). ***p<0.001 *vs.* control.

CONCLUSION

To date very limited knowledge is available on how miRNAs regulate STB differentiation. We have previously reported that miR-378a-5p is a negative regulator of STB pathway. In the present study we demonstrated the role of miR-378a-5p target genes. We provide the first evidence that miR-378a-5p targets CREB and that Nodal activates the CREB to induce STB differentiation. CREB is the most important transcription factor of STB differentiation; it initiates the master regulator of trophoblast differentiation, GCM1 [88,90] that regulates the fusogenic proteins (syncytin 1 and 2) and induces STB differentiation [77,78]. Based on our novel findings and the known functions of CREB and other transcription factors in STB differentiation, we propose that miR-378a-5p inhibits STB differentiation by down-regulating a pathway involving Nodal, CREB, and CCNG2. Nodal induces CREB activation and CCNG2 transcription. CREB and Nodal may also induce PPARγ, which could form a complex with cyclin G2 to induce GCM1, which in turn, induces syncytins [Figure 4.3].

Taken together, this study provides further insight into the role of miR-378a-5p target genes in trophoblast cells and advances the miRNA knowledge in STB pathway and placental development.

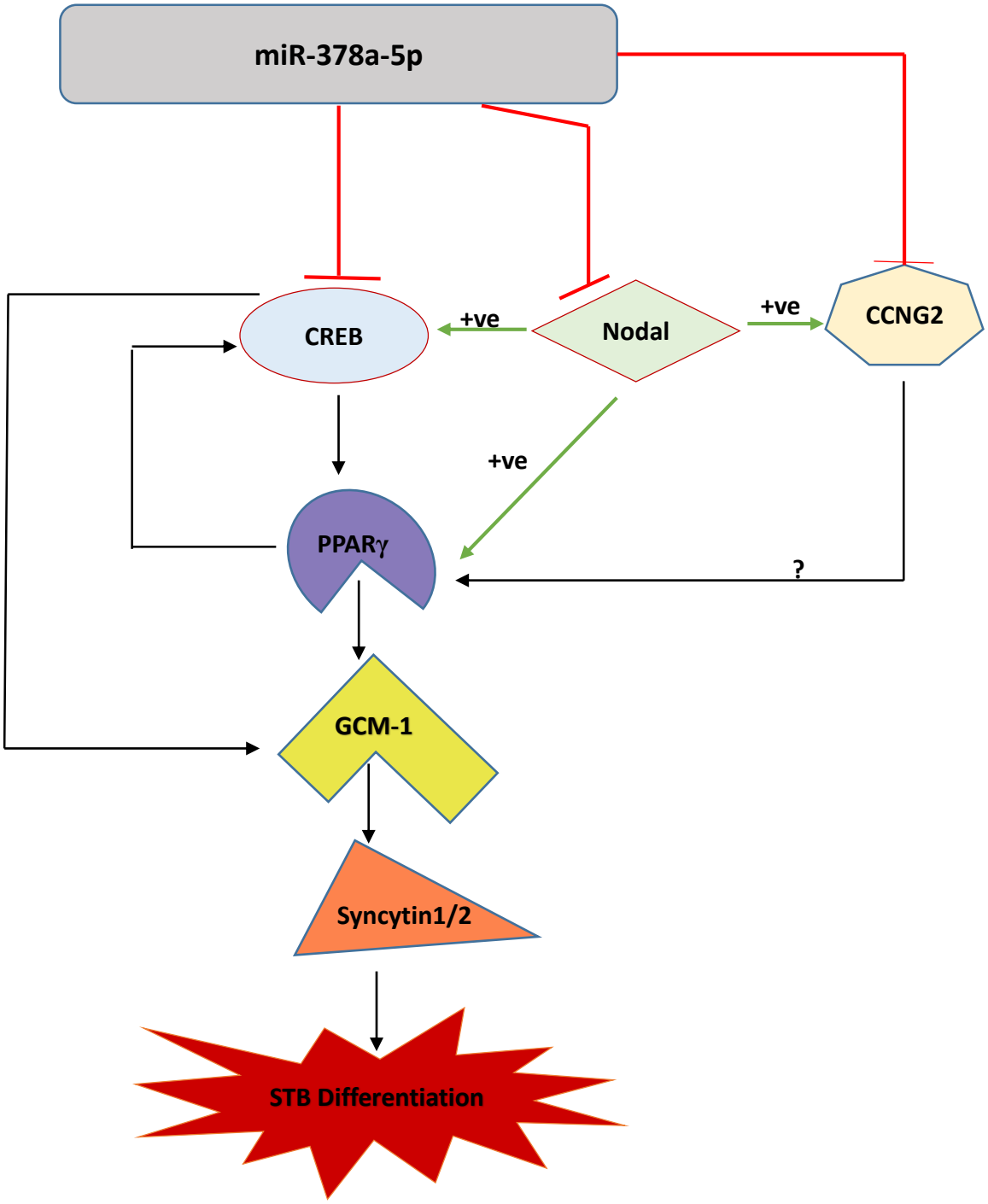

Figure 4.3 Proposed mechanism of miR-378a-5p in STB differentiation. MicroRNA-378a-5p inhibits CREB, Nodal and CCNG2 expression. Nodal activates CREB, regulates PPARγ and cyclin G2. Inhibiting the CREB expression by miR-378a-5p affects Nodal that regulates PPARγ and cyclin G2. PPARγ regulates GCM-1 and syncytin pathway that controls STB differentiation.

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APPENDIX : ADDITIONAL PUBLICATIONS

1. MicroRNA-378a-5p targets cyclin G2 to inhibit fusion and differentiation in BeWo cells

Nadeem U, Ye G, Salem M, and Peng C.

Department of Biology, York University, Toronto, Canada

Biology of Reproduction. 2014 Sep;91(3):1-10

I designed, performed and analyzed all the experiments. I was involved in the preparation and revision of the manuscript. I performed the requested revisions for the manuscript.

2. MicroRNAs: crucial regulators of placental development

Hayder H, O'Brien J, **Nadeem U**, Peng C.

Department of Biology, York University, Toronto, Canada

Reproduction. 2018 Jun;155(6):259-271

I contributed to this review by writing a part on the role of microRNA in differentiation of syncytiotrophoblast. I was closely involved in the revision of the final draft for submission.