REGULATION OF HUMAN TROPHOBLAST SYNCYTIALIZATION BY HISTONE DEMETHYLASE LSD1

By

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Abstract:

A successful pregnancy is critically dependent upon proper placental development and function, beginning with differentiation of human trophoblast cells. At the final stage of trophoblast differentiation, the human placenta contains a cell population called syncytiotrophoblasts which provide the exchange surface between the mother and the fetus. However, molecular regulation of syncytiotrophoblast development is poorly understood. In this study, we explored the regulatory pathway of lysine-specific demethylase 1 (LSD1), in combination with transcription factor *GATA2* and cellular signaling, in human trophoblast syncytialization. Using cell culture models, we show that LSD1 is important for human trophoblast syncytialization. Cellular signaling, such as the activation of protein kinase A, together with LSD1 contribute to the recruitment of *GATA2* at the Syncytin-1 and Syncytin-2 loci. Loss of LSD1 in trophoblast cells impairs *GATA2* function, histone modifications and RNA polymerase II recruitment at Syncytin-1 and -2 loci leading to their transcriptional repression and impaired syncytialization. In summary, our data reveals a regulatory pathway demonstrating that LSD1 is essential for human trophoblast syncytialization.

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List of Abbreviations

8Br-cAMP	8-Bromoadenosine 3',5'-cyclic monophosphate
cAMP	cyclic Adenosine monophosphate
CTB	Cytotrophoblast
E(7.5/8.5)	Embryonic day
ERV	Endogenous retrovirus
EVT	Extravillous cytotrophoblast
FAD	Flavin adenine dinucleotide
GCM1	Glial cells missing 1
H3K4(me/me2)	Histone H3 Lysine 4 (monomethyl/dimethyl)
H3K9(me/me2)	Histone H3 Lysine 9 (monomethyl/dimethyl)
hCGα	human Chorionic Gonadotropin alpha
hCGβ	human Chorionic Gonadotropin beta
HELLP	Hemolysis Elevated Liver enzymes and Low Platelets
ICM	Inner cell mass
IUGR	Intrauterine growth retardation
KD	knockdown
LSD1	Lysine-specific demethylase 1
PE	Preeclampsia
PKA	Protein Kinase A
STB	Syncytiotrophoblast
TB	Trophoblast
TCP	Tranylcypromine
TE	Trophectoderm
TSC	Trophoblast stem cell

Chapter I: Introduction

The hemochorial placenta

Proper development and maintenance of the human placenta is essential for a healthy, successful pregnancy. In addition to being the interface for the exchange of blood, nutrients, gases and waste, the placenta protects the fetus from the maternal immune response and secretes hormones and growth factors necessary for development [1]. Human chorionic gonadotropin alpha and beta (hCGα, hCGβ) are hormones secreted during pregnancy to improve the stability of implantation and to establish the proper microenvironment for both the mother and the fetus [1, 2].

Humans possess the invasive hemochorial placenta in which the fetal tissues that branch to form villi are in direct contact with maternal blood, thus establishing the maternal-fetal exchange surface [3]. While this type of placenta is the most invasive, it provides the smallest surface area for exchange [3]. There are three subtypes of hemochorial placenta: hemomonochorial with one trophoblast layer, hemodichorial with two layers and hemotrichorial with three [4]. The human placenta is classified as villous hemomonochorial [5].

In the less invasive endotheliochorial placenta, the endothelial wall of the maternal blood vessels separates the fetus from maternal blood after the destruction of the uterine epithelium and connective tissue post-implantation [3, 4]. These vessels either remain in a coiled structure or they become more parallel and linear as is seen in the hemochorial placenta [6]. The non-invasive epitheliochorial placenta separates the fetus from maternal blood with three layers of tissue because no maternal tissues are destroyed or removed post-implantation [3, 4].

Further variation in placenta ultrastructure includes the surface area for exchange. Villi, which develop from branching fetal tissue, are either surrounded by maternal blood or maternal tissue in the villous placenta [3]. The much larger surface area of the labyrinthine placenta is

formed by a trophoblast network derived from fetal capillaries and maternal blood [3]. Structurally, the trabecular placenta falls in between the villous placenta and the labyrinthine placenta [3]. It is thought that the variation in placental ultrastructure is correlated with life history and environmental factors [3].

Since growth, nourishment and survival of the embryo is dependent upon the placenta, we need to understand how and where developmental mistakes occur in this organ. As a result of elucidating these signaling pathways, we would be able to design therapies for at-risk patients to ensure successful pregnancy.

Trophoblast differentiation

The most important cellular component of the placenta is the trophoblast lineage, which is necessary at every stage of fetal and placental development [1]. Soon after ovulation, fertilization takes place and cell division begins, leading to a mass of cells called the morula [7]. The morula will then make its way from the fallopian tube to the uterine cavity [7]. Implantation can occur once the morula has transitioned to a blastocyst [7], where two specific cell types are defined. The cluster of cells inside the blastocyst is called the inner cell mass (ICM), which will eventually become the fetus [8]. The layer of cells surrounding the blastocyst is the trophectoderm (TE), which then gives rise to trophoblast (TB) cells [1, 8, 9]. TB cells produce the necessary hormones and cytokines for creating an environment conducive for embryonic and placental development, as well as for maternal health [10]. These cells are also responsible for expressing receptors that are required for endometrium invasion [10].

Shortly following implantation to the uterine wall, TB cells begin to differentiate to form new TB populations [1]. As differentiation of these cells progresses, a very crucial structure unfolds. Villous cytotrophoblast (CTB) cells differentiate into a few different TB populations: TB cells that group together form a TB cell column which then makes contact with the maternal decidua [1, 9] and extravillous trophoblasts (EVTs) invade the decidua, remodeling maternal vascular structure and creating an anchor together with the TB column cells and, consequently, the chorionic villi [1, 11].

These chorionic villi are tissues made up of mesenchymal cells and are outlined by two layers of CTBs [9]. Villous CTBs form the first layer, and when these cells become cell cycle-terminated, they fuse to form multinucleated syncytiotrophoblasts (STB), completing the outermost layer [9]. During the first trimester of pregnancy, CTBs form a distinct layer beneath

the STB layer, but further along the developmental timeline, the CTBs become discontinuous leaving just the monolayer of STBs [5]. Upon the development of the STB layer, the connection between the mother and the fetus is established, providing easy exchange of blood, gases and nutrients [1, 8, 11].

Human pathologies, such as intrauterine growth retardation (IUGR) and preeclampsia (PE), have been linked to syncytial malformation [12]. It has been reported that in PE there is a reduction in EVT invasion and placental blood flow, as well as unstable oxygen concentration [11]. The role of trophoblast differentiation in placenta development is essential and its regulation must be understood in order to investigate methods to improve pregnancy success.

Role of endogenous retroviral proteins in the trophoblast

Endogenous retroviruses (ERVs) originated as infectious retroviruses which have been integrated into human DNA [13, 14]. ERVs can be inherited if they infect the germline and colonize, which leads to provirus amplification [14]. These retroviruses are no longer able to produce infectious virions due to the vast amount of mutations accumulated over time. They have proven to be beneficial to human life, particularly in the context of the placenta, including immunosuppressive characteristics that prevent fetal rejection by the mother [13, 14].

Transcription of ERVs seems to be controlled by DNA methylation patterns which are also inheritable [14]. This finding has led to the idea that, through evolution, the need for genetic control over ERVs may have been the reason that DNA methylation began [14, 15]. ERV envelope proteins, such as placenta-specific Syncytin-1 and Syncytin-2 whose transcription is activated upon demethylation [15], contribute their cell-fusion properties to the formation of syncytiotrophoblasts [12, 14, 16]. Syncytins are generally silenced until their function is required for syncytialization and maintenance of the STB layer [11].

Many studies have been devoted to understanding the regulation of these *Syncytin* genes since they play such a crucial role in trophoblast syncytialization. It has been shown that cyclic adenosine monophosphate (cAMP)-induced differentiation of human CTBs and BeWo cells results in the significant upregulation of *Syncytin* genes, and that transcription factor *GATA2* shows similar expression patterns [17, 18]. In addition, transcription factor *GCM1* activates *Syncytin* gene expression, which appears to enhance trophoblast syncytialization. This is supported by the observed increase in Syncytin expression following induced expression of GCM1 [18].

Errors in the regulation of Syncytins, and thereby syncytin-mediated cell fusion, have been implicated in PE, IUGR and Hemolysis Elevated Liver Enzymes and Low Platelets (HELLP) syndrome [11]. Langbein et.al showed that the loss of Syncytin expression leads to impaired STB formation or no STB formation in these pathological placentas. Also, levels of GCM1 and hCGβ expression are diminished in PE/IUGR placentas, and even further downregulation of hCGβ is seen in HELLP/IUGR placentas [11]. It has been suggested that an accumulation of CTBs occurs where STB development is hindered due to the loss of regulation of *Syncytin* genes in these pathological placentas [11]. Therefore, retroviral proteins Syncytin-1 and Syncytin-2 are absolutely necessary for successful placentation.

Epigenetic regulation of trophoblast syncytialization

Epigenetics is the change in gene expression patterns that is unrelated to genomic changes [19]. Epigenetic changes can be inherited but they can also happen due to an environmental factor [19]. The most common examples are DNA methylation and histone modification, the latter achieved by methylation, acetylation, phosphorylation, ubiquitination and sumoylation [19]. DNA methyltransferases typically methylate DNA at sites where there is a high quantity of CpG dinucleotides [19]. Various enzymes are involved in histone modification, including methyltransferases and demethylases, depending on the type of modification necessary [19, 20]. There are specific sites on histone tails where these modifications can take place, namely arginine or lysine side chains, and modifications at these sites can be associated with either transcriptional repression or activation [20-22]. Epigenetic modifications have been discovered to be important mechanisms in several instances, such as disease progression and embryonic development [23].

In order to address pregnancy failure caused by errors in placental development and maintenance, we first needed to understand how these processes are regulated. TB development and differentiation is controlled by transcriptional regulation. However, TB regulatory mechanisms in humans are currently unknown. Chromatin modification is known to be a key factor in trophoblast differentiation [9]. We hypothesized that epigenetic modifier LSD1, along with transcription factor *GATA2*, plays an imperative role in regulating trophoblast syncytialization.

LSD1 has been implicated in mouse trophoblast stem cell (mTSC) differentiation. Previously, Zhu et.al, showed that mouse embryos were dying before embryonic day 7.5 (E7.5) upon depletion of LSD1. In trophoblast-specific LSD1 knockout embryos, there were major

morphological defects, but the embryos displayed delayed lethality. This phenotype reveals LSD1 as an important factor in trophoblast development and maintenance for the survival of an embryo [24].

LSD1 is a histone H3 lysine 4 and lysine 9 (H3K4, H3K9) demethylase, and is unique in its mechanism of demethylation [20-22]. This particular histone demethylase is a monoamine oxidase which operates in the presence of the cofactor Flavin adenine dinucleotide (FAD) by means of an oxidation-reduction of FAD [20, 21, 25]. Since H3K4 is an activation mark, demethylation at this site results in transcriptional repression. Conversely, H3K9 is a repressive mark and demethylation leads to transcriptional activation [20-22, 25]. These post-translational modifications determine which genes are to be expressed or repressed.

Based on our findings, transcription factor *GATA2* appears to interact with LSD1, in that its expression is downregulated upon knockdown of LSD1, and LSD1 is reduced with GATA inhibition. GATA2 is involved in early trophoblast differentiation [17] and is maintained throughout placenta maturation. In the mouse, *Gata2* has been shown to regulate the differentiation of mTS cells to TB giant cells [26, 27, 28]. Thus, we hypothesized that *GATA2* may also play a role in regulating human TB syncytialization. The fate of the embryo is dependent upon the proper regulation of these factors, as well as many others.

In this study, we have been able to elucidate a signaling pathway by which epigenetic factor LSD1, in combination with *GATA2*, controls the expression of Syncytin-1 in order to promote human trophoblast syncytialization.

Chapter II: Results and Methods

Role of LSD1 in trophoblast stem cell differentiation and migration.

It has been established that LSD1 is essential for proper trophoblast differentiation and migration in the mouse [24]. Zhu et.al, demonstrated that a complete knockout of LSD1 lead to early embryonic death but the proper cell lineages were present. An epiblast-specific knockout resulted in delayed lethality with relatively normal morphological features until eventual death before E8.5, which lead to the idea that LSD1 must play an essential role in extraembryonic tissues [24]. Following that result, they developed a trophoblast-specific knockout mouse and observed severe morphological defects before embryonic death at E8.5 [24].

Lack of LSD1 in mTSCs caused the cells to exhibit abnormal characteristics. Cell size increased quickly and there appeared to be a loss of control over trophoblast migration and invasion, which is indicative of early differentiation onset [24]. Because of the large amount of endometrium invasion, proliferation of TSCs is greatly reduced leading to a loss of placental tissues [24]. Interestingly, re-expression of LSD1 rescued TSC migration, bringing it back to an appropriate level [24]. The representative gene expression patterns of differentiation were also altered in LSD1-deficient cells, but did not affect stemness-associated gene expression profile [24]. Moreover, LSD1 appears to be responsible for the fate of TSCs, which suggests that LSD1 is not only required for proper timing of differentiation but also for differentiation into the correct cell type [24].

Based on these results, it would seem that LSD1 plays a role in controlling differentiation-associated gene expression in TSCs. This finding provides evidence of the importance of epigenetic control throughout development, beginning with initiation of differentiation and continuing with maintenance of cell behavior and cell fate [24].

LSD1 is important for trophoblast syncytialization.

LSD1 is present in the cytotrophoblast and syncytiotrophoblast layers of placental tissues during all three trimesters, indicating relevance of this protein in placental development and maintenance (Figure 1A-C). In BeWo cells, LSD1 localizes in the nucleus which is consistent with its function in chromatin modification (Figure 1D). Typically, BeWo cells form multilayered colonies as they grow *in vitro*. Treatment of BeWo cells with 8-Br-cAMP for 48 hours in culture causes a monolayer, differentiation-like morphological change (Figure 2A) which, in this cell type, indicates syncytialization [29].

Syncytialization occurs when cell cycle-terminated cytotrophoblasts fuse to form multinucleated syncytiotrophoblasts [9]. Prior to this cell-cell fusion, retroviral proteins Syncytin-1 and Syncytin-2 are expressed, signaling the cells to undergo syncytialization [16]. This process leads to large amounts of hCGα and hCGβ production which further reveals the beginning of placenta formation [1, 2]. Upregulation of *Syncytin-1*, *Syncytin-2*, *hCGα* and *hCGβ* was seen in the cells which were treated with 8Br-cAMP when their expression was measured by q-PCR (Figure 2B). Notably, LSD1 protein expression level is not altered due to the 8Br-cAMP treatment (Figure 2C).

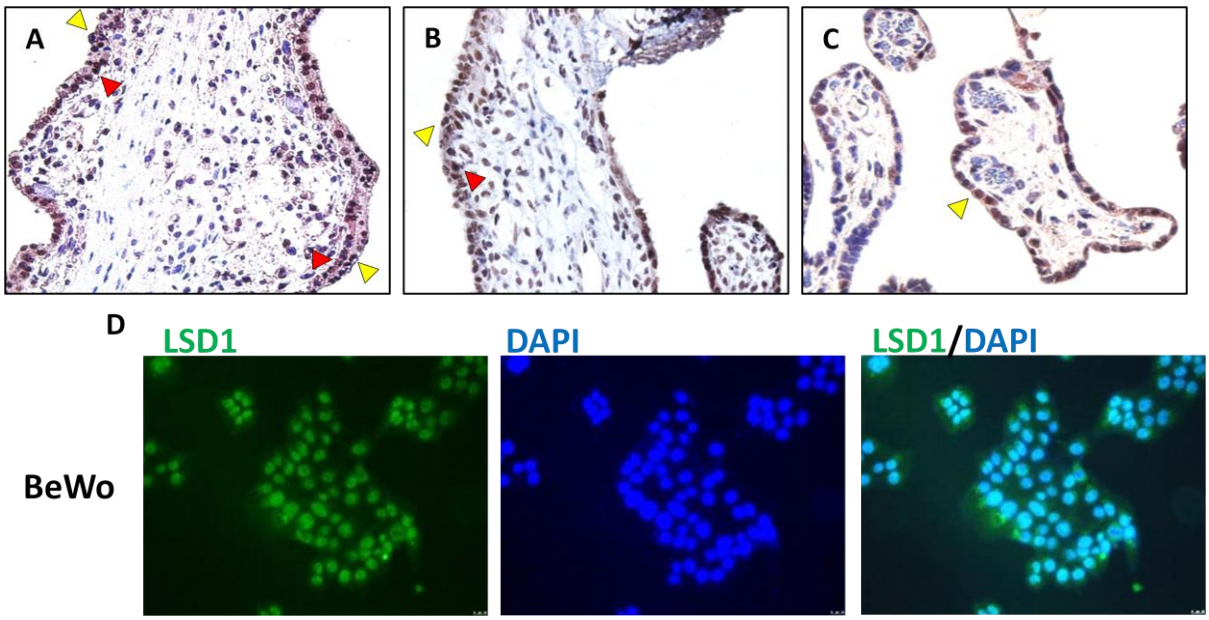

Figure 1. LSD1 is maintained in cytotrophoblast and syncytiotrophoblast populations throughout placental maturation.

Immunohistochemistry staining showing LSD1 in first trimester (A), second trimester (B) and term (C) human placenta tissues. Red arrowhead, CTB; yellow arrowhead, SynTB. (D) Immunofluorescence staining showing LSD1 localized to the nucleus in BeWo cells.

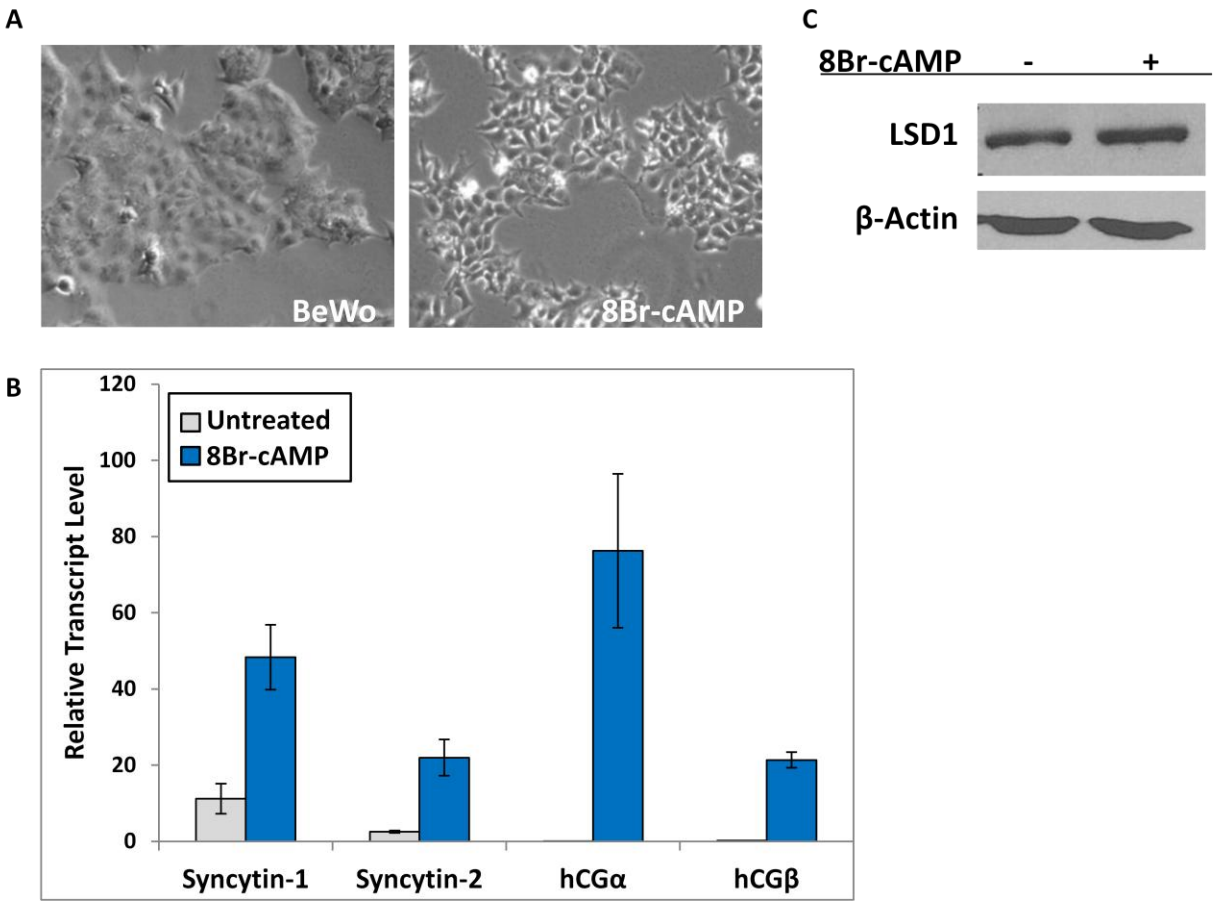

Figure 2. 8Br-cAMP induces syncytialization in BeWo cells.

(A) Morphological change in BeWo cells when treated with 8Br-cAMP. (B) mRNA expression of syncytialization-associated genes and with or without 8Br-cAMP. (C) Protein expression of LSD1 with or without 8Br-cAMP. Loading control, β-Actin.

Depletion of LSD1 represses syncytialization-associated genes.

To expand upon the involvement of LSD1 in syncytialization, LSD1 was depleted in BeWo cells via lentiviral transduction. The shRNA-mediated knockdown of LSD1 showed a nearly 10-fold decrease in RNA expression (Figure 3A), and appeared just as efficient when protein expression was visualized by western blotting (Figure 3B). LSD1 expression was not rescued by treating the LSD1-deficient cells with 8Br-cAMP, just as the treatment in normal BeWo cells did not alter the level of LSD1 (Figure 3B).

In order to assess the loss of LSD1 in relation to syncytialization, the *syncytin* and *hCG* gene expression was measured by qPCR. Reduction in the expression of *syncytin* genes hinders syncytiotrophoblast formation [16]. In the absence of *LSD1*, *Syncytin-1* and *Syncytin-2* were greatly reduced (Figure 3C). Expression levels of *hCGα* and *hCGβ* were also decreased when *LSD1* was knocked down (Figure 3D). This reduction in expression of Syncytin-1, Syncytin-2, hCGα and hCGβ is evidence that the occurrence of syncytialization is impaired upon depletion of LSD1.

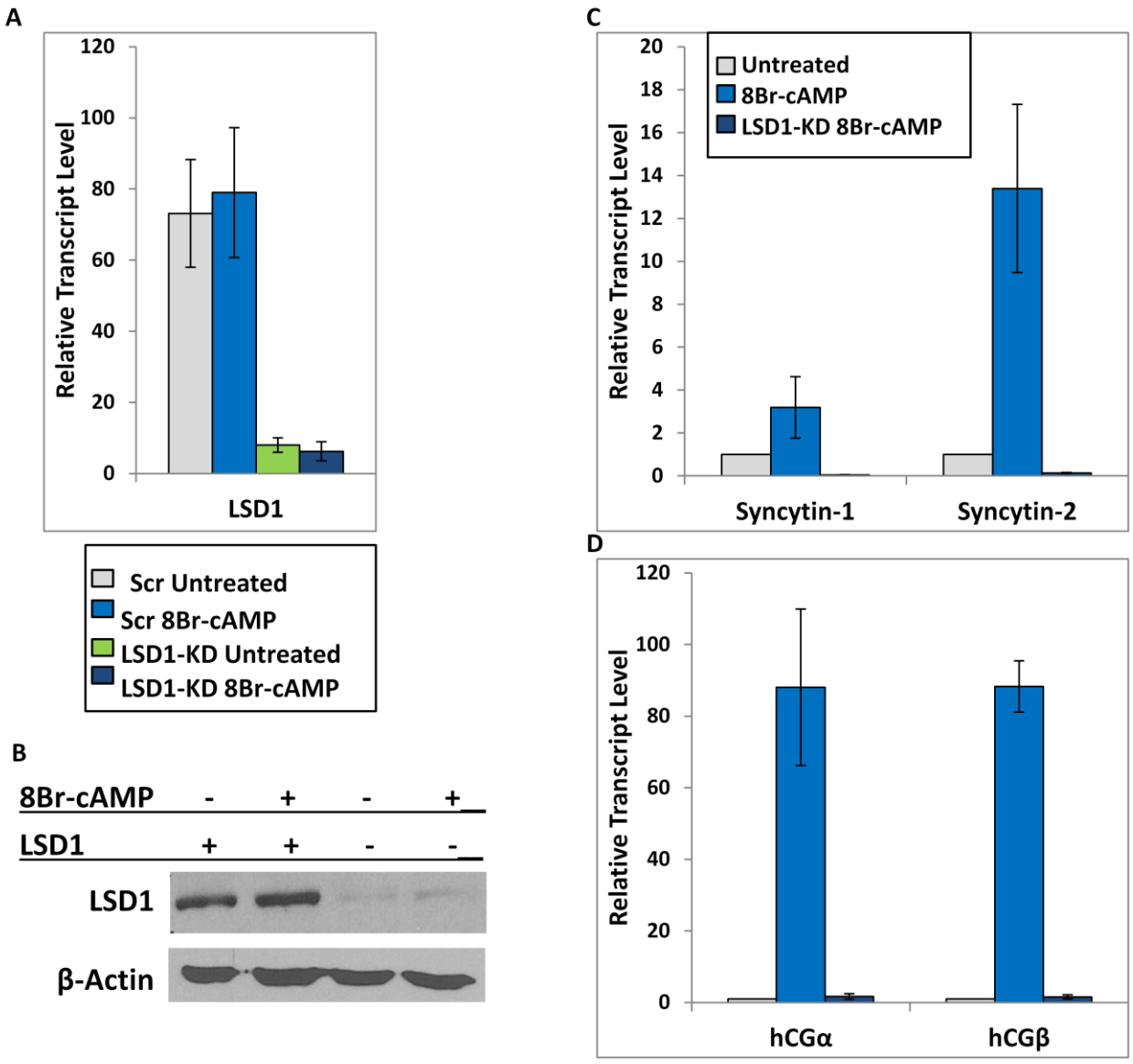

Figure 3. LSD1 is required for trophoblast syncytialization.

mRNA expression (A) and protein expression (B) of LSD1 with or without 8Br-cAMP treatment; LSD1 knockdown by lentiviral transduction. (C) mRNA expression of Syncytin-1 and -2, and (D) hCGα and hCGβ, in BeWo cells treated with 8Br-cAMP versus LSD1-knockdown BeWo cells treated with 8Br-cAMP, normalized to untreated control.

LSD1 alters chromatin state and gene expression profile.

To investigate the histone demethylase properties of LSD1 in the context of syncytialization, we utilized chromatin immunoprecipitation for the methylated histone marks in the BeWo/LSD1-KD model. Where LSD1 is present at normal levels, RNA Polymerase II is recruited at Syncytin-1 and Syncytin-2 loci upon 8Br-cAMP treatment, while there is no recruitment of RNA Polymerase II where LSD1 has been knocked down (Figure 4A-B). In this context, RNA Polymerase II is recruited to *Syncytin* genes to catalyze transcription, but only when syncytialization is induced and LSD1 is expressed at normal levels.

Upon induction of syncytialization by treatment with 8Br-cAMP, there were lower levels of di- and monomethylated H3K9 as compared to the untreated condition at Syncytin-1, Syncytin-2, hCGα and hCGβ (Figures 4C and 4E). Interestingly, treatment with 8Br-cAMP in LSD1-KD cells showed an increase in di- and monomethylation at the genes listed when compared to the 8Br-cAMP treated control BeWo cells (Figure 4D and 4F). More specifically, these genes were repressed under normal conditions but induction of syncytialization derepressed these genes. Loss of LSD1 caused the opposite effect, in that these genes remained repressed even though PKA was activated by the 8Br-cAMP treatment. Therefore, histone modification by LSD1 plays a key role in syncytialization-associated gene expression.

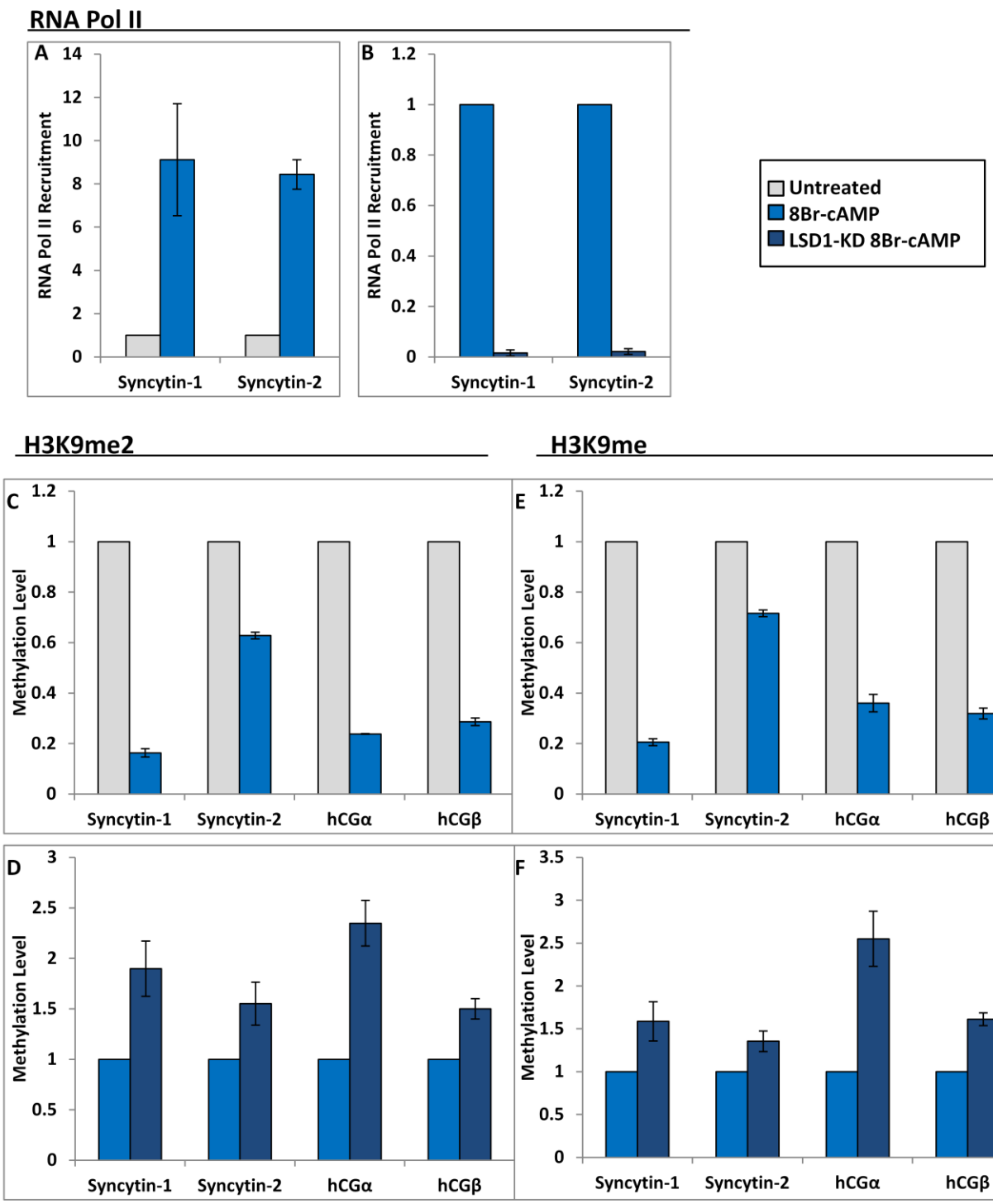
RNA Pol II
A
RNA Pol II Recruitment
14
12
10
8
6
4
2
0
Syncytin-1
Syncytin-2
B
RNA Pol II Recruitment
1.2
1
0.8
0.6
0.4
0.2
0
Syncytin-1
Syncytin-2
Untreated
8Br-cAMP
LSD1-KD 8Br-cAMP
H3K9me2
H3K9me
C
Methylation Level
1.2
1
0.8
0.6
0.4
0.2
0
Syncytin-1
Syncytin-2
hCGα
hCGβ
E
Methylation Level
1.2
1
0.8
0.6
0.4
0.2
0
Syncytin-1
Syncytin-2
hCGα
hCGβ
D
Methylation Level
3
2.5
2
1.5
1
0.5
0
Syncytin-1
Syncytin-2
hCGα
hCGβ
F
Methylation Level
3.5
3
2.5
2
1.5
1
0.5
0
Syncytin-1
Syncytin-2
hCGα
hCGβ

Figure 4. LSD1 is required for H3K9 demethylation and subsequent expression of syncytialization-associated genes.

RNA Polymerase II recruitment at Syncytin-1 and Syncytin-2 in 8Br-cAMP treated BeWo cells, normalized to untreated control (A). Loss of RNA Polymerase II recruitment in 8Br-cAMP treated LSD1-KD BeWo cells (B). H3K9me2 (C) and H3K9me (E) methylation levels in 8Br-cAMP treated BeWo cells. H3K9me2 (D) and H3K9me (F) methylation levels in 8Br-cAMP treated LSD1-KD BeWo cells, normalized to 8Br-cAMP treated BeWo cells.

Inhibition of LSD1 in term cytotrophoblasts impairs syncytialization.

Cytotrophoblasts isolated from a human term placenta were cultured for expression analysis. In culture, these cells fuse when in close proximity to each other, producing hCGα and hCGβ. *LSD1* appeared to be present in a reasonably high quantity when its expression was measured by qPCR (Figure 5A). When the term cytotrophoblasts were treated with Tranylcypromine (TCP), an LSD1 inhibitor, they did not exhibit characteristics of cell fusion in that there was a decrease in *Syncytin-1, hCGα* and *hCGβ* expression after LSD1 inhibition (Figure 5A), revealing the necessity of LSD1 function for the formation of syncytiotrophoblasts. Additionally, the amount of di- and monomethylated H3K9 was much higher at hCGα and hCGβ loci when LSD1 was inhibited (Figures 5B-C). This further supports the idea that syncytialization is repressed where LSD1 activity is lacking.

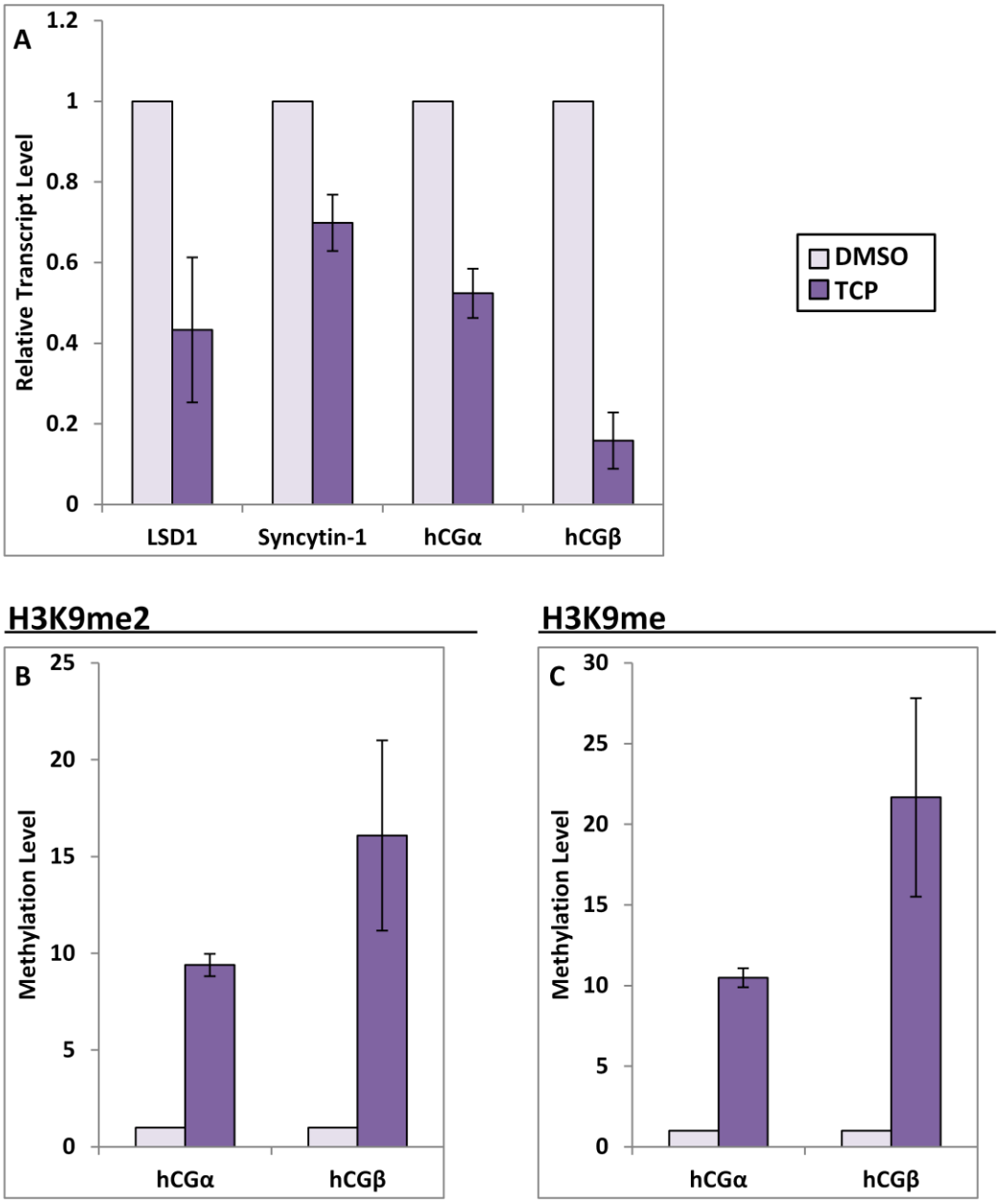

Figure 5. Inhibition of LSD1 prevents syncytialization in human term cytotrophoblasts.

mRNA expression of LSD1, Syncytin-1, hCGα and hCGβ in term CTBs treated with LSD1 inhibitor TCP, normalized to vehicle control (A). H3K9me2 (B) and H3K9me (C) methylation levels at hCGα and hCGβ in term CTBs treated with LSD1 inhibitor TCP, normalized to vehicle control.

GATA2 plays a role in LSD1 regulation of syncytialization.

Transcription factor *GATA2* is important for early trophoblast differentiation [17, 26-28, 30]. Though the amount of GATA2 expression declines over time during placenta formation, it remains present in the syncytiotrophoblast layer during all three trimesters of pregnancy (Figure 6A). Relevance of GATA2 during development is well studied, however its role is yet to be established in regards to human trophoblast syncytialization.

To further understand the involvement of GATA2 in regulation of syncytialization, we first explored the relationship between GATA2 and LSD1. Where LSD1 was knocked down, GATA2 expression was also reduced (Figures 6B and 6D), even with 8Br-cAMP treatment (Figure 6C-D). Chromatin immunoprecipitation showed recruitment of *GATA2* at Syncytin-1 and Syncytin-2 loci when syncytialization was induced by 8Br-cAMP treatment (Figure 7A), while there was a major loss of *GATA2* recruitment in the absence of LSD1 in the treated cells (Figure 7B). Using a GATA-specific inhibitor, K-7174, BeWo cells were treated with either DMSO, 8Br-cAMP or both 8Br-cAMP and K-7174. The combined treatment resulted in a downregulation of *LSD1* and *Syncytin-1* (Figure 7C). Further, the amount of H3K9 di- and monomethylation at the GATA2 locus decreases upon 8Br-cAMP treatment as compared to the level of methylation in the untreated BeWo sample (Figures 7D and 7F). In the absence of LSD1, methylation at GATA2 increases with the 8Br-cAMP treatment, relative to the 8Br-cAMP treated BeWo cells where LSD1 function is maintained (Figures 7E and 7G). Together, our data defines a clear relationship between LSD1 and GATA2 that has an impact on the function of GATA2 in human trophoblast cells.

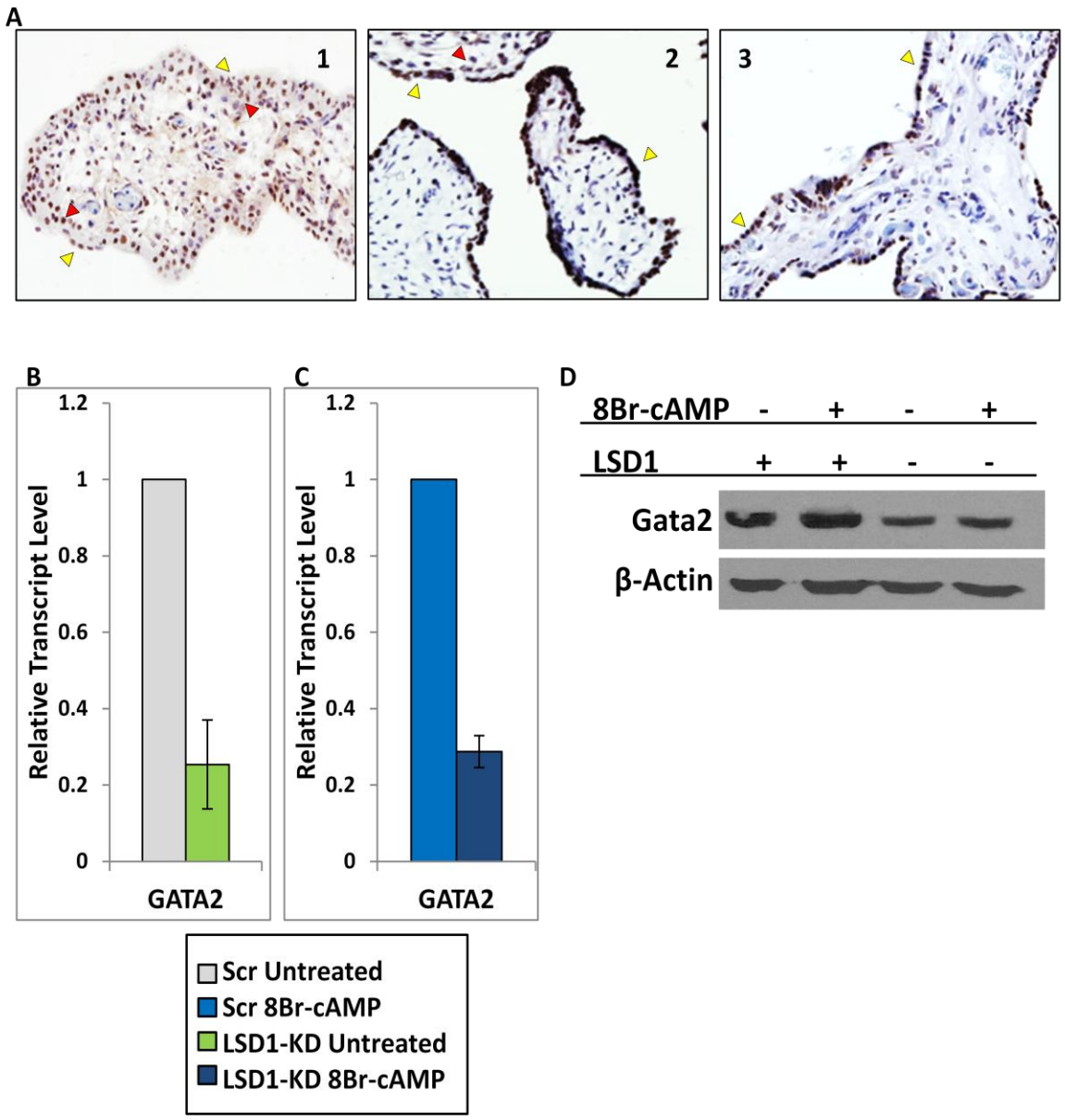

Figure 6. The interrelationship of GATA2 and LSD1.

GATA2 staining in first trimester (A1), second trimester (A2) and term (A3) human placenta. mRNA expression of GATA2 in LSD1-KD cells, normalized to Scramble control (B), and in 8Br-cAMP treated LSD1-KD BeWo cells, normalized to 8Br-cAMP treated Scramble control (C). Protein expression of GATA2 in BeWo cells with or without 8Br-cAMP treatment vs. LSD1-KD cells with or without treatment (D).

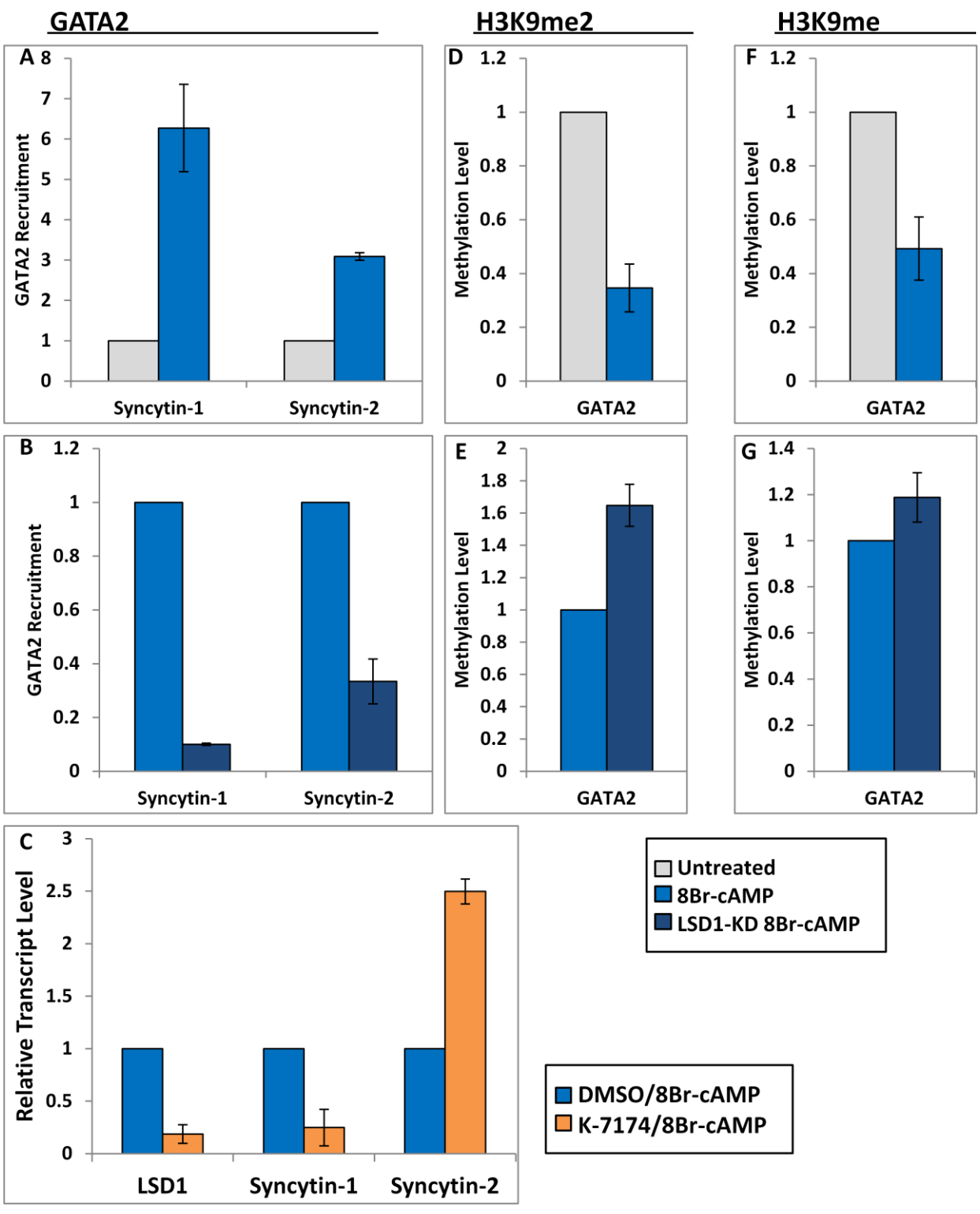
GATA2
H3K9me2
H3K9me
A
GATA2 Recruitment
Syncytin-1
Syncytin-2
D
Methylation Level
GATA2
F
Methylation Level
GATA2
B
GATA2 Recruitment
Syncytin-1
Syncytin-2
E
Methylation Level
GATA2
G
Methylation Level
GATA2
C
Relative Transcript Level
LSD1
Syncytin-1
Syncytin-2
Untreated
8Br-cAMP
LSD1-KD 8Br-cAMP
DMSO/8Br-cAMP
K-7174/8Br-cAMP

Figure 7. Trophoblast syncytialization and associated gene expression is GATA2-dependent.

GATA2 recruitment at Syncytin-1 and Syncytin-2 in 8Br-cAMP treated BeWo cells, normalized to untreated control (A), and in 8Br-cAMP treated LSD1-KD cells, normalized to 8Br-cAMP treated BeWo cells (B). mRNA expression of LSD1, Syncytin-1 and Syncytin-2 in BeWo cells treated with GATA inhibitor K-7174, normalized to vehicle control (C). H3K9me2 (D) and H3K9me (F) methylation levels at GATA2 in 8Br-cAMP treated BeWo cells, normalized to untreated control. H3K9me2 (E) and H3K9me (G) methylation levels at GATA2 in 8Br-cAMP treated LSD1-KD cells, normalized to 8Br-cAMP treated BeWo cells.

MATERIALS & METHODS

Cell culture and reagents. BeWo human choriocarcinoma cells were cultured in DMEM F12 medium with L-Glutamine, 15 mM HEPES, 10% fetal bovine serum and primocin (2 uL/mL). For inducing syncytialization, BeWo cells were cultured for 24 hours and then treated with 250 uM 8-Bromoadenosine 3',5'-cyclic monophosphate (Sigma) for 48 hours.

Human term CTBs were cultured in IMDM medium with 10% fetal bovine serum, 1% L-Glutamine and 1% Pen-strep. Cells were collected at 24 hours for non-syncytialized CTB population. Cells were cultured for 4-5 days for syncytiotrophoblasts. For inhibition of LSD1, CTBs were immediately treated with 500 uM Tranylcypromine, HCl (Millipore) and cultured for aforementioned amounts of time.

Isolation of CTBs from human term placenta. Human placenta is obtained following a cesarean section birth. Process is modified from a published protocol. Tissue was washed using 1X saline and dried on cheesecloth. Tissues were scraped from the chorionic membrane and placed in 1X saline for further washing. After 50 g of tissue was dried and digested in Q-water with 100 mg trypsin, 10 mL dispase, 40 mg DNase and 25 mL 10X Hanks buffered with HEPES for a total volume of 250 mL. Digestion should take place in a shaker incubator at 37℃ at ~200 rpm for 1 hour, but add additional 20 mg trypsin and 20 mg DNase after the first 30 minutes. The contents were then filtered into 50 mL fetal bovine serum and then subjected to a series of centrifugation. Pellets were resuspended in DMEM with 10% FBS and added to discontinuous percoll gradient, followed by centrifugation. Trophoblast cells can be identified by density and extracted for use in experiments.

qPCR. RNA was extracted from cells with the TRIzol reagent (Sigma) and used to synthesize cDNA. Analysis achieved by quantitative Real-time PCR (Applied Biosystems).

Primer	Exons	Forward Sequence Reverse Sequence
LSD1	Ex2/3	GAA GAG AGA AAT GCC AAA GCA CGT CTC CAT ACC CTC CAG AA
Syncytin-1		CTA CCC CAA CTG CGG TTA AA GGT TCC TTT GGC AGT ATC CA
Syncytin-2		CCA AAT TCC CTC CTC TCC TC CGG GTG TTA GTT TGC TTG GT
hCGα	Ex2/3	TCT GGT CAC ATT GTC GGT GT TTC CTG TAG CGT GCA TTC TG
hCGβ	Ex2/3	GTG TGC ATC ACC GTC AAC AC GGT AGT TGC ACA CCA CCT GA
GATA2	Ex4/5	CTC ATC AAG CCC AAG CGA AG ACA ATT TGC ACA ACA GGT GCC
GCM1	Ex3/4	GGC GCA AGA TCT ACC TGA GA CAC AGT TGG GAC AGC GTT T

Quantitative ChIP. Cells were collected and protein-DNA cross-linking was achieved by treating the cells with 1% formaldehyde (Sigma) for 10 minutes at room temperature with gentle rocking. 125 mM Glycine was added to quench the reaction. Chromatin immunoprecipitation was completed following a previously established protocol. Immunoprecipitation with different antibodies: Gata2 (abcam), H3K9me (abcam), H3K9me2 (abcam), RNA Pol II (Covance) and Mouse IgG (BD Bioscience) was used as a negative control for antibody binding. After immunoprecipitation, chromatins were reverse cross-linked, digested with proteinase K (Sigma) and purified. Amplification by real-time PCR was used to quantify precipitated DNA. A standard curve was set using input DNA samples at concentrations of ⅕, 1/25, 1/125 and 1/625.

ChIP Primer	Location	Forward Sequence Reverse Sequence
Syncytin-1	-1kb	GGA GAC TTG TGG CTG TCA GA AGC ACT GGC CCT TCA AGT AA

Syncytin-2	-1kb	CAA CAC AAA TAG ATT TCA AAA GCA TGT AAA TCA AAT CAC ACA ACA TGC
hCGα	-1kb	GCC AAC ATG GTG AAA CTC CT AGC TGG GAC TAC AGG TGT GC
hCGβ	-1kb	CAC ATG TGG TTT TGA AGG TTG CAT CAT GAT TAC CCT CAA GTT CC
GATA2	-500bp	CTG ATT GTA TGG CCA TGT GTG GCG AGG ACA TCC TCA GAC AG
GCM1	-1kb	AAG GCC TGG CAG ACT ACC TA AGA TTG CGT CAC TGC AGT CA
GCM1	-500bp	AGG ATG TTG GCT ATC CAC TGT C TCT CTC TCA TGT GTG CAA GAG A

Western blot analysis. Whole-cell lysates were extracted with a lysis buffer. Electrophoresis was performed using a 10-12% polyacrylamide gel in Tris-glycine running buffer (pH 8.3). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Blocking was performed with 5% nonfat dry milk (Difco) in 20 mM Tris-HCl, pH 7.6 (Tris-buffered saline with Tween [TBS-T]). Primary antibody incubation, washing, secondary antibody incubation and development of chemiluminescent signals were done following a previously published protocol. The HRP substrate used was Luminata crescendo (Millipore).

Immunofluorescence. Procedure was adapted from a previously published protocol. Cells were washed with PBS and fixed on glass coverslips with 4% paraformaldehyde (Sigma) in PBS. Cells were permeabilized in 0.25% Triton X-100 in PBS and blocked with 10% FBS in PBS with 1% Triton X-100. Primary antibody incubation was completed in 1 hour, followed by washing with 0.05% Triton X-100 in PBS and then secondary antibody (conjugated with Alexa fluor 488 [green]) incubation for 30 minutes while shielded from light. Next, the cells were counterstained

with DAPI (Invitrogen) and observed under a fluorescence microscope (Leica). All incubations took place at room temperature.

shRNA knockdown via lentiviral transfection. Short hairpin RNAs (shRNAs) targeting human LSD1/Kdm1a (Sigma). Lentiviral supernatants were prepared in HEK293T cells following a previously described procedure. Five different shRNA constructs were used. shRNA construct 068 was the most effective for the knockdown of LSD1. Lentiviral particles were transduced in BeWo cells and selected with puromycin in BeWo culture condition. Experimental analyses were performed after 3-4 days of culture.

Immunohistochemistry. Human placental FFPE sections were deparaffinized and rehydrated using Histoclear (National Diagnostics) followed by decreasing ethanol gradient and distilled water. Antigen retrieval was done using Reveal Decloaker in the Decloaking chamber (Biocare Medical) at 90℃ for 15 minutes. Peroxidase blocking was done using 3% hydrogen peroxide for 10 minutes at room temperature, followed by blocking for nonspecific binding with 10% Normal Goat Serum (Invitrogen) for 30 minutes. Primary antibody and secondary antibody incubations were done for 30 minutes using antibody dilution buffer (1% BSA, 0.3% Tween 20 in PBS (pH 8.0)) for secondary antibody (biotinylated anti-rabbit or mouse IgG (H+L) [Vector] with 0.05% Normal human serum). Tissues were incubated with HRP-streptavidin (Life Technologies) for 10 minutes, followed by DAB chromogen solution (Dako) for color change and Mayer's Hematoxylin solution (Sigma) for 5 minutes. Hematoxylin color change was achieved using warm, running tap water for approximately 2 minutes. Dehydration of the tissue was achieved using distilled water, increasing ethanol gradient and Xylene. Stained tissues were imaged using a bright field microscope (Nikon).

Chapter III: Discussion and Future Perspectives

Epigenetic modifier LSD1 plays a major role in the regulation of human trophoblast syncytialization. Expression of the retroviral proteins Syncytin-1 and Syncytin-2 initiates cell fusion, or syncytialization, in cell cycle-terminated cytotrophoblasts [16, 31]. In addition to activation of Protein Kinase A (PKA), this cellular signal is critical to the proper development of the human placenta and, ultimately, growth and survival of the fetus. Alterations in the epigenome appear to be sufficient in shutting down transcription and further signaling in this developmental process.

BeWo cells, in lieu of primary cytotrophoblasts, exhibit nuclear expression of LSD1 and mimic trophoblast syncytialization. 8Br-cAMP treatment in BeWo cells causes an increase in cytosolic cAMP concentration, triggering the activation of PKA [29]. Based on our findings, PKA activation appears to be the initial signal which changes the cellular environment, leading to the signaling cascade that initiates syncytialization. The amount of LSD1 is not affected by the 8Br-cAMP treatment, but its histone modification activity seems to respond to the activation of PKA. For our purposes, it looks as if hCGα and hCGβ are expressed during syncytialization. When LSD1 is significantly reduced or inhibited, the expression of these syncytialization-associated genes is greatly impacted, which offers the possibility that LSD1 is involved in the initiation of syncytialization.

The importance of LSD1 becomes clear when it is knocked down in BeWo cells. *Syncytin* genes are unable to activate without a sufficient level of LSD1 present. Hormone secretion that is essential to the pregnancy microenvironment also does not occur properly in this condition. Treating the LSD1-KD cells with 8Br-cAMP reveals the necessity of LSD1 in this signaling pathway where its demethylase function is pertinent. Seemingly, *Syncytin-1* transcription is prevented in an LSD1-deficient environment because RNA Polymerase II is not

recruited at the Syncytin-1 locus in these conditions. To further assess the importance of LSD1, it is necessary to understand its histone demethylation properties.

Histone demethylation is a key post-translational modification that is a crucial step in our proposed signaling mechanism. Many histone demethylases have been reported and shown to be instrumental in altering gene expression without changing the genome [25]. In relation to altering expression of syncytialization-associated genes, LSD1 appears to be associated with repressive chromatin, specifically histone H3 lysine 9-methylation. 8Br-cAMP treatment results in a decrease in the level of H3K9 methylation at syncytialization-associated gene loci, suggesting that LSD1 is removing the methyl groups in response to PKA activation, which then leads to the expression of Syncytin proteins to initiate syncytialization. When LSD1-KD cells are treated with 8Br-cAMP in an attempt to force syncytialization, methylation increases which results in repression of the genes necessary for syncytialization. As the number of methyl groups at one site increases, so does the level of repression [25]. Our findings have definitively shown that an increase in H3K9-methylation causes suppression of Syncytin-1, Syncytin-2, hCGα and hCGβ in the absence of LSD1. Thus, H3K9 demethylation by LSD1 results in an entirely different gene expression profile compared to one which lacks LSD1, suggesting that epigenetic modification provides a significant means of regulation in this context.

Furthermore, small molecule inhibition of LSD1 demonstrates similar results to the knockdown experiment. Primary cytotrophoblasts express LSD1, as determined visually by staining placenta tissues and quantitatively when cultured *in vitro*. Loss of LSD1 activity prevents syncytialization in primary cells, as observed by loss of Syncytin-1, hCGα and hCGβ expression. As seen in the BeWo cells, levels of mono- and dimethylation at hCGα and hCGβ increase significantly where LSD1 activity is inhibited in cytotrophoblasts, which is consistent

with our finding that LSD1 is important for allowing expression of syncytialization-associated genes.

In continuing the mechanistic investigation of human trophoblast syncytialization, we have found that transcription factor *GATA2* is involved. Previous studies have shown the importance of GATA2 in early trophoblast differentiation [26-28], which gives rise to the question of whether it is also a regulator of trophoblast syncytialization. Based on our data, there appears to be a mutual relationship between *LSD1* and *GATA2*. *GATA2* is recruited at Syncytin-1 and Syncytin-2 upon induction of syncytialization, suggesting that initiation of syncytialization may be GATA2-dependent. Consistent with our findings thus far, the loss of *GATA2* recruitment at Syncytin-1 and Syncytin-2 where LSD1 is knocked down implies a relationship between GATA2 and the LSD1-regulation of Syncytin expression. Further, the increase in methylation at GATA2 when LSD1-KD samples are treated with 8Br-cAMP supports the idea that the repressive effect on GATA2 is specific to its role in trophoblast syncytialization. GATA inhibition leads to a significant down-regulation of *LSD1* and *Syncytin-1*, also leading to the conclusion that this process is GATA2-dependent. Syncytin-2 may be regulated by an additional transcription factor or mechanism since the data consistently shows overexpression of Syncytin-2 following GATA inhibition.

In summary, the data we present brings to light, for the first time, an epigenetic signaling pathway for initiation of human trophoblast syncytialization. In response to the change in cellular environment caused by the activation of PKA, *GATA2* is recruited at the Syncytin-1 and Syncytin-2 loci. Simultaneously, LSD1 is activated and demethylates H3K9 due to the requirement of Syncytin-1 and Syncytin-2 mediated cell-cell fusion. This histone modification is the key step in this pathway where these essential genes are able to activate and initiate the

syncytialization process (Figure 8). Epigenetic regulation has a significant impact on which genes are expressed or silenced and how cellular signals are interpreted. Histone demethylases, along with many other epigenetic factors, provide a method for approaching pathological pregnancies and other pathological conditions caused by particular gene expression patterns without altering the genome [19, 23]. For example, this method offers an alternative to gene therapy. Prevention of detrimental post-translational modifications or enhancement of beneficial changes allows the genome to remain intact while epigenetic manipulations take place, one implication being that histone demethylases present a possible platform for drug targeting.

It is important to continue investigation of signaling pathways which control trophoblast differentiation for a more complete understanding of this developmental process. The advancement of early detection technology is also necessary. The detrimental effects of pathological conditions such as PE, IUGR and HELLP can be severe or even fatal, and some of these effects last throughout the entire life of the mother and the child after birth [33]. Detecting these complications earlier in the pregnancy may greatly reduce the percentage of failed pregnancies [34]. In addition to further mechanistic studies, the development of drugs to target epigenetic factors would cause the particular gene to be switched on or off to improve the condition [19, 23]. Personalized medicine is another possible treatment option [19], though much further research is required before implementing these therapies.

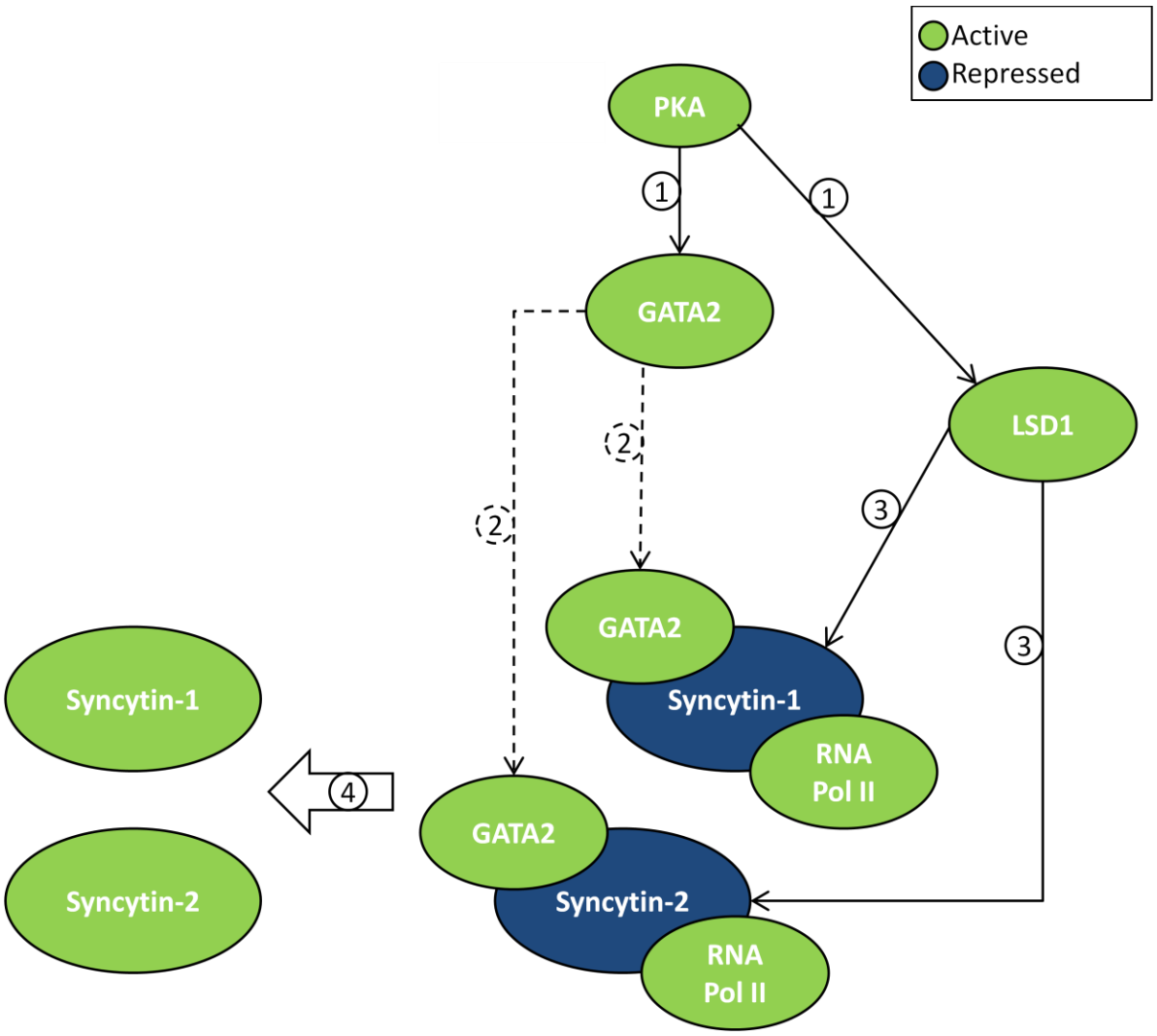

Figure 8. Summary: LSD1 regulates trophoblast syncytialization via a GATA2-dependent pathway.

8Br-cAMP treated BeWo cell condition is representative of the CTB to SynTB transition.

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