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PLACENTAL MELATONIN AND ITS RECEPTORS : ROLE AND MECHANISM OF ACTION IN VILLOUS TROPHOBLAST SYNCYTIALISATION

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Baccalauréat en sciences pharmaceutiques

Mémoire présenté pour l'obtention du grade de
Maître ès sciences (M.Sc.)

Maîtrise en sciences expérimentales de la santé

Jury d'évaluation

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RÉSUMÉ

L'implication de la mélatonine dans le déroulement de la grossesse et le développement du fœtus a fait l'objet de plusieurs études. Notre laboratoire a montré que le trophoblaste villeux humain à terme, ainsi que les lignées cellulaires trophoblastiques (BeWo et JEG-3), expriment les récepteurs 1 et 2 (MT1, MT2) et les enzymes de synthèse (aralkylamine N-acétyltransférase (AANAT) et hydroxyindole O-méthyltransférase (HIOMT)) de la mélatonine. Cependant, le rôle et les mécanismes d'actions de la mélatonine et de ses récepteurs dans le placenta humain restent encore peu connus. Les objectifs spécifiques de la présente étude sont de déterminer le rôle de la mélatonine placentaire et ses récepteurs au cours de la syncytialisation des primocultures de trophoblaste villeux et déterminer les mécanismes qui régulent la production et la sécrétion de l'hormone gonadotrophine chorionique (β-hCG). Notre hypothèse de recherche est que (1) la mélatonine et ses récepteurs placentaires, jouent un rôle important dans la régulation de la syncytialisation de trophoblaste villeux et (2) réguler la sécrétion de la hCG via l'activation de la voie de signalisation PLC-PKC-MEK/ERK1/2. Les résultats montrent que le système mélatonine (MT1, MT2, AANAT et HIOMT) est exprimé dans les tissus placentaires à tous les trimestres de la grossesse. L'expression des enzymes AANAT et HIOMT est maximale au troisième trimestre de la grossesse. Le récepteur MT1 est significativement plus exprimé au premier trimestre par rapport au deuxième et troisième trimestre de la grossesse tandis que l'expression de MT2 ne varie pas. Au cours de la différenciation des primocultures de cytotrophoblaste villeux en syncytiotrophoblaste, on observe une augmentation et une diminution de l'expression (protéine et ARNm) des récepteurs MT1 et MT2, respectivement. La mélatonine (1mM) augmente la fusion du cytotrophoblaste villeux en syncytiotrophoblaste (21 % vs témoin) ainsi que la production de β-hCG (121% vs témoin). Dans les cellules BeWo, la mélatonine stimule la sécrétion basale, et inhibe la sécrétion stimulée par la forskoline, de β-hCG d'une manière dose dépendante. Ces effets sont médiés par la stimulation des voies PLC-β et ERK1/2. La mélatonine stimule la phosphorylation d'ERK1/2 après 5 min de culture (73% vs témoin), effet qui est renversé pas le luzindole (antagoniste MT1/MT2) mais non par 4-P-PDOT (antagoniste MT2). De plus, la stimulation d'ERK1/2 par la mélatonine est complètement renversée par les siRNA ciblant le récepteur MT1, tandis que ceux ciblant MT2 n'ont pas d'effet. L'ensemble de ces résultats suggère un rôle important de la mélatonine, et de ses récepteurs MT1, dans la régulation de la syncytialisation et la sécrétion de la β-hCG dans le trophoblaste villeux et, par conséquent, dans la grossesse et le bien-être fœtal.

Mots-clés: AANAT / HIOMT (ASMT) / le récepteur de la mélatonine MT1 / le récepteur de la mélatonine MT2 / syncytiotrophoblaste / cytotrophoblaste villeux / l'hormone gonadotrophine chorionique (β-hCG) / PLC-β /ERK 1/2 / luzindole/ 4-P-PDOT.

ABSTRACT

The role of melatonin in pregnancy well-being and fetal development was the subject of several studies. However, little is known about the function of melatonin and its receptors in human placenta. Our laboratory has demonstrated that human trophoblast at term and trophoblast-like cell lines express melatonin receptors (MT1, MT2 and ROR) and functional melatonin synthesizing enzymes (AANAT and HIOMT). In JEG-3 and BeWo choriocarcinoma cell lines, we have demonstrated that melatonin regulates the secretion of human chorionic gonadotropin (β-hCG) via an action mediated by MT1 and MT2 receptors. However, the role and mechanism of action of melatonin in villous trophoblast development and endocrine function is still obscure. This master's research aims to fill this gap. The specific objectives for the present proposal are to determine the role of placental melatonin and its receptors on the stages of villous trophoblast syncytialization and the mechanisms that regulate β-hCG production/secretion. Our research hypothesis is that (1) melatonin and its placental receptors, play an important role in the regulation of villous trophoblast syncytialization and (2) regulate β-hCG production via activating PLC-PKC signaling pathway with subsequent activation of MEK/ERK1/2. Our data show that melatonin system is expressed throughout pregnancy (from week 7 to term) in placental tissues. AANAT and HIOMT show maximal expression at the 3rd trimester of pregnancy. MT1 receptor expression is maximal at the 1st trimester compared to the 2nd and 3rd trimesters, while MT2 receptor expression does not change significantly during pregnancy. During the differentiation of villous cytotrophoblast into syncytiotrophoblast, we observed an increased and decreased expression (protein and RNA) of the MT1 and MT2 receptor respectively. Melatonin (1 mM) increased (21% vs control) the fusion of villous cytotrophoblast in syncytiotrophoblast accompanied by an increased production of human chorionic gonadotropin (β-hCG) (121% vs control) via an action mediated by MT1 and MT2 receptors. In BeWo cells, melatonin through a dose-dependent manner stimulates β-hCG basal secretion and inhibits forskolin-stimulated secretion of β-hCG. These effects of melatonin are mediated via the stimulation of PLC-β and ERK1/2 signaling pathways. Moreover, melatonin stimulates ERK1/2 phosphorylation after 5 min of culture (73% vs control), an effect that is reversed by luzindole (specific antagonist of MT1 and MT2), but not by 4-P-PDOT (specific MT2 antagonist). Furthermore, ERK1/2 stimulation by melatonin is completely reversed by small interfering RNA (siRNA) targeting the MT1 receptor, while MT2 targeting siRNA had no effect. Taken together, these results suggest an important role of melatonin and its MT1 receptor in the regulation of villous trophoblast syncytialization and secretion of β-hCG and, consequently, in

pregnancy and fetal well-being. **Keywords:** AANAT / HIOMT (ASMT) / MT1 melatonin receptor / MT2 melatonin receptor / Syncytiotrophoblast/ Villous cytotrophoblast/ Human chorionic gonadotropin (hCG)/ ERK 1/2 / PLC-β/ luzindole/ 4-P-PDOT.

ACKNOWLEDGMENT

First and foremost, I would like to express my deepest appreciation and my sincerest gratitude to my mentor, Dr. Cathy Vaillancourt, for her exceptional guidance, kindness, patience and providing me with an excellent atmosphere for doing research. Likewise, for patiently correcting my writing and financially supporting my research. I attribute the level of my Master's degree to her inspiration and effort. One simply could not wish for a better or nicer mentor.

Besides my mentor, I would like to thank my thesis juries: Dr. Denis Girard and Dr. Deborah Sloboda for their acceptance to revise my thesis.

A special thanks to all my laboratory colleagues for their assistance, upkeep and the exciting atmosphere of the laboratory, as well as nice times spent with them outside the lab.

I would also like to acknowledge my parents and my sister; they were always supporting me and motivating me with their best wishes.

Finally, I appreciate the financial support from Fondation Armand Frappier and Réseau Québécois en reproduction (RQR-CREATE) Program that sponsored the research discussed in this thesis.

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AANAT: Aralkylamine N-acetyltransferase

ASMT: Acetylserotonine - O – methytransferase

AFMK: N (1)-acetyl-N (2)-formyl-5-methoxykynuramine

AMK: N1-acetyl-5-methoxykynuramine

ATP: Adenosine triphosphate

AKAPs: A-kinase-anchoring proteins

AC: Adenylyl cyclase

ANOVA: Analysis of variance

BCL-2: B-Cell Lymphoma 2

BCA: Bicinchoninic acid

Caspases: Cysteine aspartase

CO_2: Carbon dioxide

CSF-1: Colony stimulating factor 1

CTB: Cytotrophoblast

Ca^{2+}: Calcium

cAMP: 3'-5'-cyclic adenosine monophosphate

cDNA: Complementary deoxyribonucleic acid

COX-2: Cyclo-oxygenase (also called inducible cyclooxygenase)

Cx 43: connexin 43

DMSO: Dimethylsulfoxide

DMEM-HG: Dulbecco's modified Eagle medium-High glucose

DNA: Deoxyribonucleic acid

ELISA: Enzyme linked immunosorbent assay

ERK 1/2: Extracellular signal regulated kinases

EVT: Extravillous trophoblasts

evCTB: Extravillous cytotrophoblast

EGF: Epidermal growth factor, epidermal growth factor

ETC: Electron transport chain

FBS: Fetal bovine serum

FSH: Follicle-stimulating hormone (follitropin)

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GAPDH: Glyceraldehyde -3- phosphate dehydrogenase

GC: Giant Cells

GIT: Gastrointestinal tract

GLUT1: Glucose transporter 1

GLUT3: Glucose transporter 3

GPx: Glutathion peroxidase

GPCR: G-protein coupled receptors

H/R: Hypoxia/reoxygenation

hCG: Hormone chorionic gonadotropin

hCG-H: Hyperglycosylated hCG

HDL: High-density lipoprotein

HIF-1: Hypoxia inducible factors 1

HIOMT: Hydroxyindole - O - methyltransferase

HLA: Human leukocyte antigen

hPL: Hormone placental lactogen

HPRT1: hypoxanthine phosphoribosyltransferase 1

IUGR: Intrauterine growth restriction

IVF: In vitro fertilization

IGF: Insulin-like growth factor

iNOS: Inducible nitric oxide synthase

JNK: C-Jun N-terminal kinase

MAPK: Mitogen-activated protein kinases

MMP: Matrix metalloproteinase

mRNA: Messenger ribonucleic acid

MT1: Melatonin receptor 1

MT2: Melatonin receptor 2

Na^+: Sodium

NF-kB: Nuclear factor-kappa B

PARP: Poly (ADP-ribose) polymerase

PDEs: Phosphodiesterases

pGH: Placental growth hormone

PI3K: Phosphatidylinositol - 3 - kinase

PKA: Protein kinase A

PKC: Protein kinase C

PLA_2: Phospholipase A_2

PLC-β: Phospholipase C-β

PS: Phosphatidylserines

PVDF: Polyvinylidene fluoride polyvinylidene difluoride

PTX: Pertussis toxin

LDL: Low-density lipoprotein

LH: Luteinizing hormone (lutropin)

LIF: Leukemia inhibitory factor

RLS: Rate limiting step

RNS: Reactive nitrogen species

ROR: Retinoic acid receptor related orphan

RZR: Retinoid Z receptor

ROS: Reactive oxygen species

RT-qPCR: Reverse transcription polymerase chain reaction

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

STB: Syncytiotrophoblast

SOD: Superoxide dismutase

siRNA: Small interfering RNA

SEM: Standard error of the mean

TGFα: Transforming growth factor alpha

TNF: Tumor necrosis factor alpha

TSH: Thyroid-Stimulating Hormone (thyrotropin)

VEGF: Vascular endothelial growth factor

vCTB: Villous Cytotrophoblast

CHAPTER 1: INTRODUCTION

Human placenta

1.1 Role of the placenta

The human placenta is a transitory organ that executes an imperative role in pregnancy well-being and fetal development. It provides a crossing point between the mother and baby where it facilitates oxygen and nutrients exchange as well as fetal waste and toxin elimination. The placenta also functions as an endocrine organ, and secretes specific peptide and steroid hormones throughout pregnancy (al-Lamki *et al.*, 1999, Aplin, 1991, Malassine, 2001, Morrish *et al.*, 1987, Teasdale *et al.*, 1985).It also protects the fetus from being rejected by maternal immune system, via contributing to maternal immune tolerance towards fetal antigens (Le Bouteiller, 2001).

A healthy and functional placenta is compulsory for appropriate fetal growth, and placental efficiency is conserved through the action of several hormones. During early pregnancy, trophoblast cells secrete human chorionic gonadotropin (hCG) into the maternal circulation that ensures continuous progesterone production by the corpus luteum. By the end of the 1st trimester, the yellow body degenerates and progesterone is then produced by the placenta. Human placental lactogen (hPL), a peptide hormone produced by the trophoblast cells, is released into maternal blood and stimulates lipolysis. The latter increases free fatty acids bioavailability which is required for progesterone production. In addition, hPL stimulates the expansion of maternal β-cells and consequently increases insulin production, thus it protects against the development of maternal gestational diabetes mellitus (Handschuh *et al.*, 2007a, Handschuh *et al.*, 2007b, Munro *et al.*, 1983, Newbern *et al.*, 2011).

The placenta permits maternal-fetal exchange and the transfer of various nutrients to the baby. Water, fatty acids, glucose, and amino acids are considered the main transferred nutrients (Fuglsang *et al.*, 2004, Graham *et al.*, 1992a, Marin *et al.*, 2003, Newbern *et al.*, 2011). Since glucose intake is very important and considered the main energy source, glucose transporters (GLUTs) are present in both placental and fetal cells. Precisely, GLUT1 transporter is present and numerous in the syncytiotrophoblasts (STB) while GLUT 3 is localized in fetal endothelial cells. Both transporters operate via facilitated diffusion (Smith *et al.*, 1992).

Transporters coupled to Na+ pumps and Na+ independent transporters facilitate amino acids transfer across the placenta. These transporters enable the influx of anionic, cationic and neutral amino acids (including L-tryptophan, the precursor of melatonin) across the STB (Avagliano *et al.*, 2012, Munro *et al.*, 1983, Smith *et al.*, 1992). Placental cholesterol is crucial as it serves as a precursor for estrogen production. The existence of lipid transporters across the placenta permits the transfer of maternal high-density lipoprotein (HDL) and low-density lipoprotein (LDL) to achieve this task (Woollett, 2011).

Placental dysfunctions through placental trauma or altered placental development result in pregnancy pathologies such as premature births, intrauterine growth restrictions (IUGR) and preeclampsia (Toal *et al.*, 2008). Collectively, this declares a healthy placental necessity for fetal growth, survival and pregnancy success.

1.2 Placental structure

The human placenta is of hemomonochorial structure that is characterized by an intensive extravillous trophoblast (EVT) invasion in the decidua and maternal myometrium. This type of placenta allows direct contact between maternal blood and trophoblasts and is capable of producing unique hCG, complicating the use of animal model (Crosley *et al.*, 2013, Malassine *et al.*, 2003).

1.2.1 Trophoblast differentiation

Villous cytotrophoblast (vCTB) are the stem cells of villous trophoblast. Throughout the syncytialization process, mononucleated vCTB fuse and differentiate into the multinucleated non-proliferative STB **(figure 1)** (Vaillancourt *et al.*, 2009a) .

STB performs villus cell external coating and accomplishes a principal role in maternal fetal exchange. STB comprises also several microvilli on its apical membrane that facilitates the exchange between the placental and maternal blood. Additionally, STB lacks human leukocyte antigen (HLA) class I resulting in proper maternal immune tolerance towards the placenta. The syncytialization process is not a common event that occurs only in the syncytiotrophoblast, osteoclasts (bone cell), male and female gametes and skeletal muscle cells (Graham *et al.*, 1992b, Kaufmann *et al.*, 2003, O. Nakamura, 2009, Teasdale *et al.*, 1985).

STB are the main endocrine cell of the placenta. These cells secrete pregnancy-related hormones like hCG, hPL, GH-v (placental growth hormone) and steroids (e.g. estrogens, progesterone). These hormones function in endocrine, paracrine and

autocrine fashion, each of which is essential for pregnancy well-being and fetal development (Orendi *et al.*, 2010).

EVT play a significant role in embryo implantation, placentation and in uterine spiral arteries remodeling. The EVT proliferates and invades the decidua and the myometrium to form the interstitial cytotrophoblast. Afterwards, some either migrate into maternal vessels acquiring endovascular trait, or form the giant cells in the decidua **(figure 2)** (Handschuh *et al.*, 2009, Handschuh *et al.*, 2007a, Ji *et al.*, 2013).

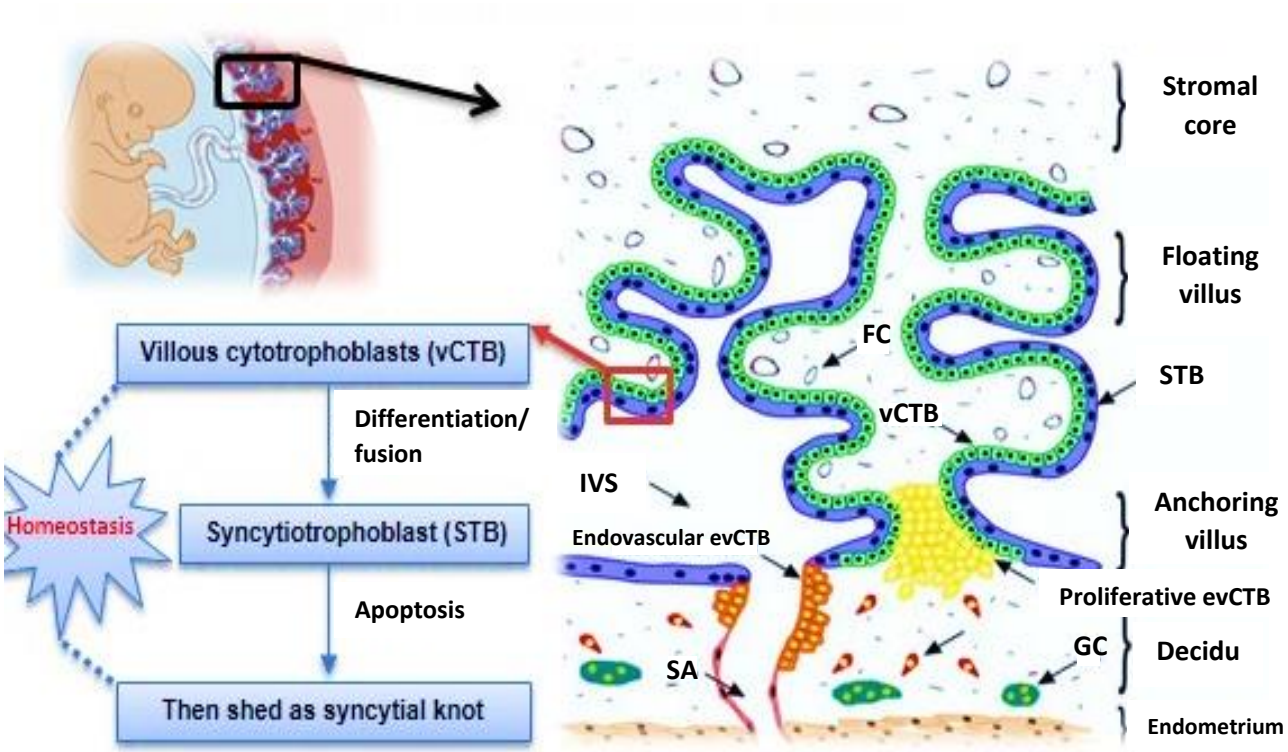

Figure 1 : Illustration of human villous trophoblast differentiation. Villous cytotrophoblast (mononucleated) aggregate and fuse into syncytiotrophoblast (multinucleated) in a highly organized process. SA: spiral artery; GC: giant cell; evCTB: extravillous cytotrophoblast; FC: fetal capillary STB: syncytiotrophoblast; vCTB: villous cytotrophoblast; IVS: intervillous space (Sagrillo-Fagundes *et al.*, 2014).

Maternal-fetal circulation is established by the end of the 1st trimester and the remodeling process of maternal spiral arteries is accomplished by the end of 2nd trimester. Endovascular ETVs are responsible for the invasion of the arteries, and substitute the endothelial cells. As a result, the small high resistance muscular arteries are converted into large low resistance fibrous vessels, which facilitate the entry of the blood into trophoblastic cavities (Cartwright *et al.*, 2010, Ji *et al.*, 2013, Kaufmann *et al.*, 2003). The giant cells (GC) are suggested to function as the STB, secreting both hPL and hCG hormones, emphasizing their role in pregnancy maintenance (al-Lamki *et al.*, 1999, Hu *et al.*, 2010, Simmons *et al.*, 2007).

1.3 Chorionic villi: villous trophoblast

After the adhesion of the blastocyst to the endometrium, the trophoblast begins to differentiate along two pathways: vCTB pathway and EVT pathway. During the 1st trimester of pregnancy, trophoblasts differentiate mainly towards the EVT path, allowing the invasion of the blastocyst into the uterus and the establishment of the utero-placental circulation. After the 1st trimester, vCTB pathway becomes the major one, a situation that demonstrates the adaptive and variable capacity of trophoblast differentiation during pregnancy **(figure 2)** (Graham *et al.*, 1992a, Ji *et al.*, 2013, Kaufmann *et al.*, 2003).

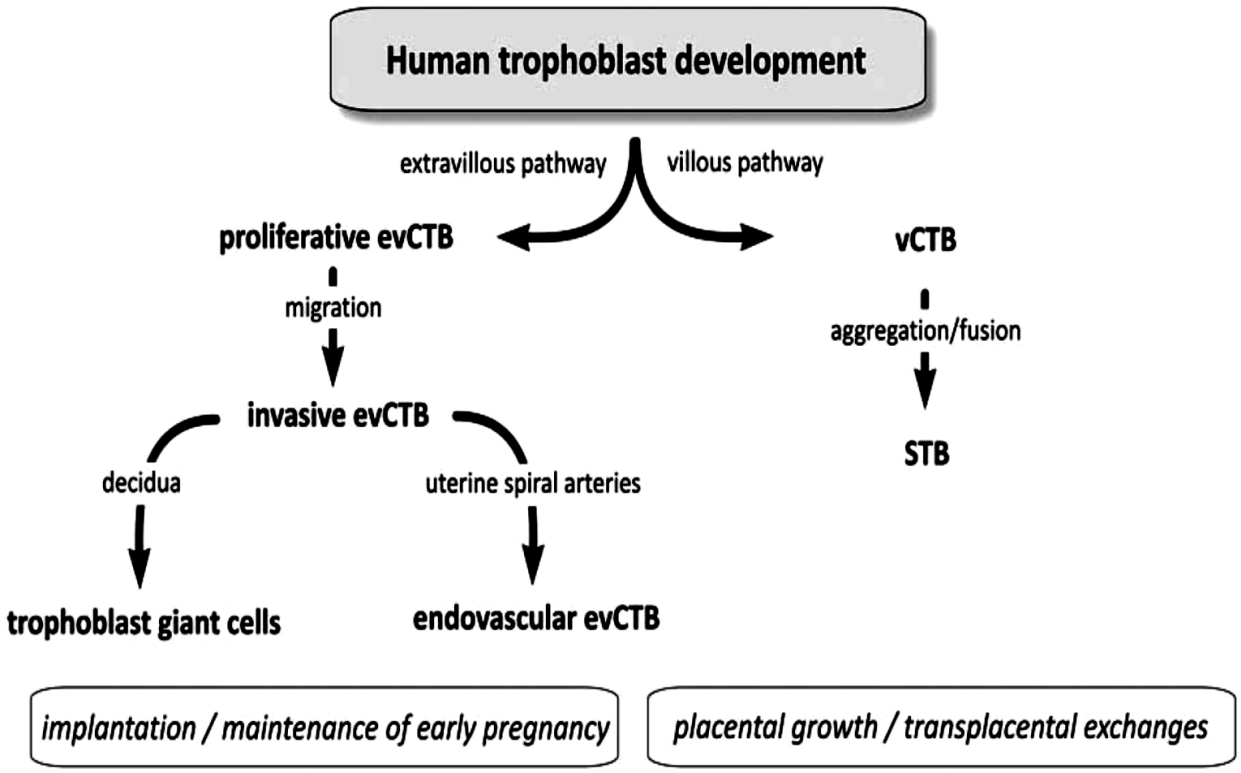

Figure 2 : Pathways of human trophoblast differentiation

Human trophoblast differentiates through either the villous or extravillous pathways. In the villous pathway, vCTB (mononuclear) merges and further differentiates to form the STB (multinucleated) which constitutes the outer layer of villus cells. Alternatively, in the extravillous pathway, evCTB proliferates and becomes invasive. Thereafter, some migrate to maternal vessels, and become a vascular cytotrophoblast (vascular evCTB) while other evCTB differentiate into multinucleated giant cells migrating in the decidua. vCTB: villous cytotrophoblast, STB: syncytiotrophoblast, evCTB: extravillous cytotrophoblast (Vaillancourt *et al.*, 2009a).

1.3.1 Syncytialisation

During the course of pregnancy vCTB differentiate and fuse to maintain STB hemostasis, and preserve its cellular constituents (proteins, nucleic acids, lipids, organelles, etc...) (Le Bellego *et al.*, 2009). STB undergoes apoptosis (both intrinsic and extrinsic) then expel apoptotic yields to the maternal circulation **(figure 3).** STB renewal process is complex and comprises a large number of factors such as hormones,

cytokines, growth factors (Ackerman *et al.*, 2012) or transcription factors (Heazell *et al.*, 2009, Huppertz *et al.*, 2011).

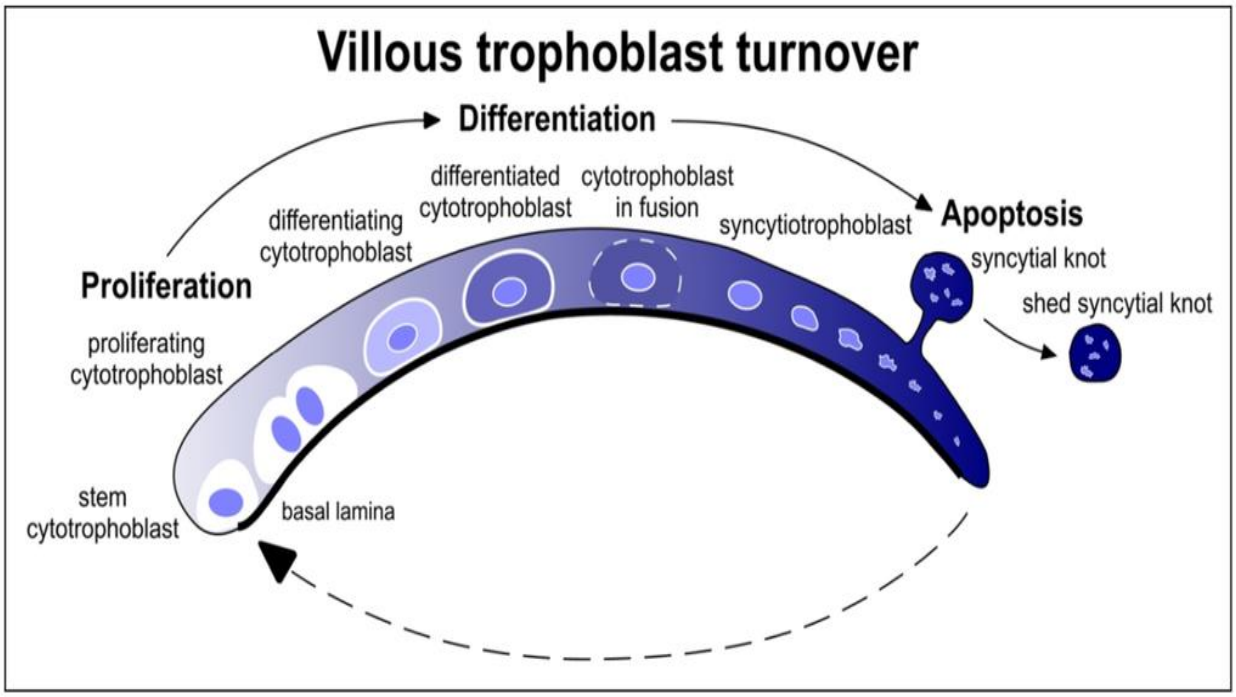

Figure 3 : Villous trophoblast turnover (homeostasis). Villous trophoblast is formed by proliferating stem cytotrophoblast that exit the cell cycle and differentiate in villous cytotrophoblast. Mononuclear villous cytotrophoblasts fuse and differentiate into a syncytium, the syncytiotrophoblast. Syncytiotrophoblast degeneration occurs through apoptosis to allow its regeneration and thus maintenance of villous trophoblast homeostasis (Lanoix *et al.*, 2012b).

The first evidence of trophoblast *in vivo* fusion into a syncytium was revealed upon using [^{3}H]-thymidine incorporation assays (Richart, 1961). Further studies on villous trophoblast differentiation were realized *in vitro* by isolating and purifying vCTB from human placenta, followed by harvesting them in primary culture (Kliman *et al.*, 1986). This technique employs Trypsin and DNase for performing enzymatic digestion then discontinuous Percoll gradient for VTB purification. Afterwards, freshly isolated

vCTB demonstrated a successful fusion and differentiation into functional STB (Benirschke *et al.*, 2000, Kliman *et al.*, 1986).

The differentiation process is characterized by morphological and biochemical parameters. Morphological differentiation is defined through the fusion of the mononucleated vCTB into a syncytium, while the biochemical differentiation is characterized by the production of specific hormones such as hCG, placental lactogen (hPL), placental growth hormone (PGH), neuropeptide Y and leptin. The hCG and hPL are the two hormones frequently examined for vCTB biochemical differentiation studies (Jacquemin *et al.*, 1996, Kliman *et al.*, 1986, Morrish *et al.*, 1987, Senaris *et al.*, 1997).

Complete morphological and biochemical differentiation of vCTB into STB - in culture- necessitates the presence of fetal serum (Bischof *et al.*, 2005, Morrish *et al.*, 1987). This process takes place after 48 hours of culture while both survival drop and increased apoptosis are observed after 96 hours of culture **(figure 4)**. Although the two apoptotic pathways (intrinsic and extrinsic) are involved in this process, the intrinsic pathway is predominantly found and is particularly up regulated during variable oxygen concentrations as well as hypoxia and oxidative stress in the placenta (Crocker *et al.*, 2001a, Heazell *et al.*, 2008, Hung *et al.*, 2010, Kar *et al.*, 2007, Lanoix *et al.*, 2013, Longtine *et al.*, 2012).

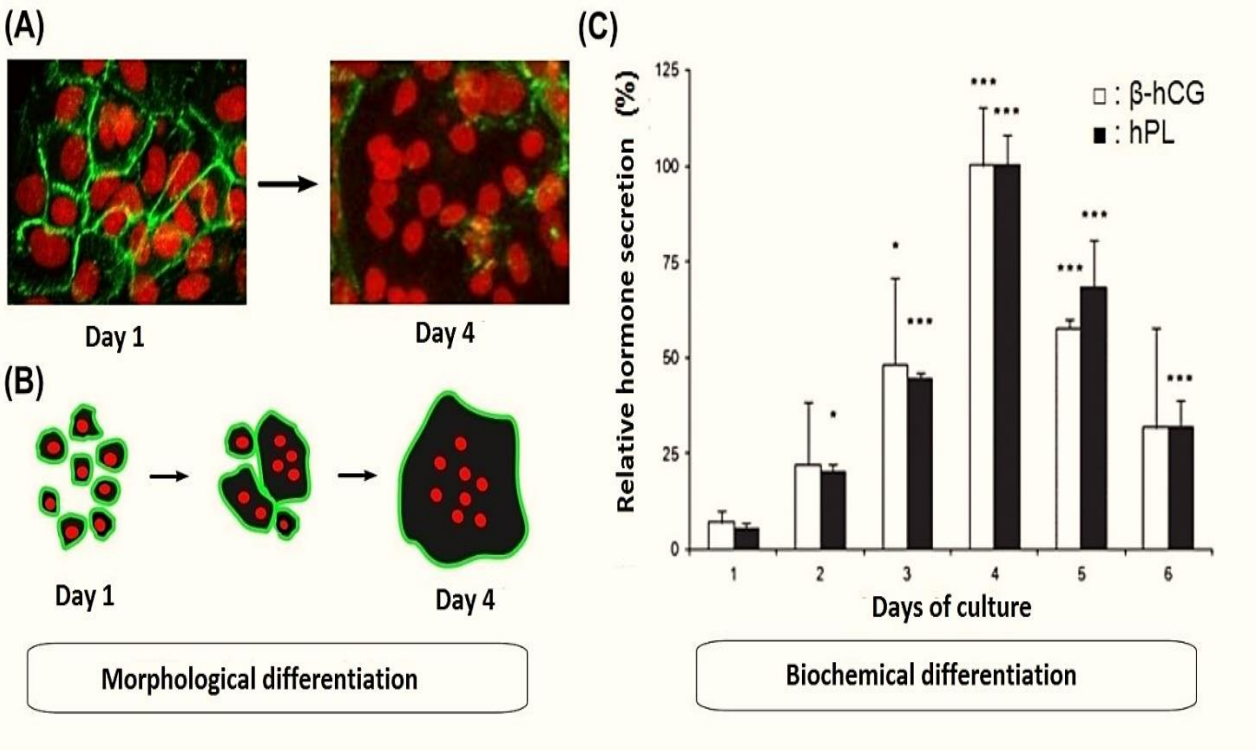

Figure 4 : Biochemical and morphological differentiation of villous trophoblast. (A) Morphological differentiation of primary cultures of villous trophoblast isolated from human placenta at term. Marking desmoplakin membrane and nuclei by confocal microscopy after one and four days of culture (400X). (B) Schematic of morphological differentiation in vitro. (C) Biochemical differentiation of primary cultures of villous trophoblast from human term placenta. Relative secretion of β-subunit of human chorionic gonadotropin (β-hCG) and placental lactogen (hPL) of a 6 day culture. The data represent the relative secretion ± SEM compared to the secretion at four days of culture (maximum secretion). * P <0.05; *** P <0.001 compared to the first day of culture (Vaillancourt *et al.*, 2009a).

Various obstetric complications, such as miscarriage, intra-uterine growth restriction or preterm delivery could be a result of altered villous trophoblast homeostasis (Evain-Brion, 2001, Jauniaux *et al.*, 2010, John *et al.*, 2012, Norwitz, 2006). For example, a lack of vCTB differentiation is involved in preeclampsia. This condition, affects between 2 to 7% of pregnant women and is characterized by maternal hypertension and proteinuria (Noris *et al.*, 2005). Although the pathophysiology of this complex disease is not yet fully understood, many factors involved in trophoblast

differentiation appear to be involved such as: vascular endothelial growth factor (VEGF), β-hCG, and caspase 8 (Ackerman *et al.*, 2012, Ji *et al.*, 2013).

The human placenta, as the other higher primates, is of hemomonochorial type. Since no type of - non primate- animal placenta is comparable to humans and none has the same type of trophoblast cells as the human placenta. Hence, animal models are not suitable for investigating human vCTB differentiation, thus emphasizing that primary cultures of vCTB isolated from human placentas as a model of choice to decrypt villous trophoblast differentiation (Benirschke *et al.*, 2000, Kliman *et al.*, 1986).

1.3.2 Factors regulating the syncytialization of villous trophoblast

Although the mechanisms involved in vCTB syncytialization are complex, some major mechanisms and factors that stimulate or inhibit the fusion process and biochemical differentiation were identified (e.g., growth factors, nucleic acid, kinases, hormones or retroviral particles) **(summarized in Table 1)** (Evain-Brion, 2001).

Table 1: Major factors modulating the syncytialization of vCTB in STB

Factor	Description	(-) (+)	Cell type	Action	Reference
hCG	Hormone	+	TT	Biochemical	(Shi *et al.*, 1993)
EGF	Growth factor	+	TT	Biochemical	(Morrish *et al.*, 1987)
VEGF	Growth factor	+	FT, TT	Biochemical	(Crocker *et al.*, 2001b)
CSF	Growth factor	+	TT	Biochemical	(Garcia-Lloret *et al.*, 1994)
TGF-α	Growth factor	+	TT	Biochemical	(Yang *et al.*, 2003)
ERK1/2	Protein kinase	+	TT	Biochemical and fusion	(Vaillancourt *et al.*, 2009a)
PKA	Protein kinase	+	B TT	Fusion Biochemical and fusion	(Keryer *et al.*, 1998, Knerr *et al.*, 2005, Knofler *et al.*, 1999)
p38	Protein kinase	+	TT	Biochemical and fusion	(Vaillancourt *et al.*, 2009a)
Syncytin 1	Membrane protein	+	TT, B	Fusion	(Frendo *et al.*, 2003)
Connexin 43	Membrane protein	+	TT	Fusion	(Frendo *et al.*, 2003)
Galectin 3	Membrane protein	+	B	Fusion	(Dalton *et al.*, 2007)
CD98	Transporter of amino acid	+	B	Fusion	(Kudo *et al.*, 2003)
Galectin 3	Membrane protein	+	B	Fusion	(Dalton *et al.*, 2007)
Calcium		+	TT, B	Fusion	(Alsat *et al.*, 1996)
TNF	Cytokine	-	TT	Biochemical	(Leisser *et al.*, 2006)
Hypoxia	Low oxygen tension	-	TT, B	Biochemical and fusion	(Alsat *et al.*, 1996)

Factors inhibiting the biochemical differentiation (hCG secretion) and fusion are expressed with (-); factors inducing the biochemical differentiation (hCG secretion) and fusion are expressed with (+) FT: First trimester; TT: Third trimester; B: BeWo cells. EGF: Epidermal growth factor, VEGF: vascular endothelial growth factor, CSF: colony stimulating factor, TGF-α: transforming growth factor alpha, ERK1/2: Extracellular signal regulated kinases, PKA: Protein kinase A, p38 mitogen-activated protein kinases, TNF: Tumor necrosis factor alpha. Adapted from (Gauster *et al.*, 2009)

1.4 Human chorionic gonadotropin (hCG) role and regulation of placentation

1.4.1 hCG structure

The hCG belongs to a family of glycoprotein hormones, which include luteinizing hormone (LH), thyrotropin (TSH) and follitropin (FSH). HCG consists of α-subunit and a β-subunit which are held together by non-covalent hydrophobic and ionic interactions **(Figure 5).** Secretion of hCG is constant all over the course of pregnancy, while it peaks at the 10th week of gestation. Conferring to sugar side chains, hCG can be present as a regular (hCG) or hyper glycosylated hCG (hCG-H) sharing a common hCG β-subunit amino acid sequence. In early pregnancy, hCG-H is the predominant form of hCG, and is secreted by the evCTB (Cole *et al.*, 2006, Sasaki *et al.*, 2008). hCG-H is a sugar variant of hCG and stimulates cytotrophoblasts invasion and implantation in an autocrine manner. Afterwards the regular hCG becomes the predominant form and is secreted by the differentiated and fused STB (Cole, 2010, Wide *et al.*, 1994).

Measurements of hCG is an indicator commonly used for pregnancy diagnosis. Recently, it has been shown that hCG-H levels quantification permits earlier pregnancy recognition than standard pregnancy tests, as its levels surge quickly during the early stages of pregnancy. Interestingly, ultrasensitive hCG-H assay permits pregnancy detection within 6 days in women undertaking *in vitro* fertilization (IVF) (Strom *et al.*, 2012). In contrast, inadequate levels of urinary hCG-H (less than 50% of total hCG) have been identified in a large proportion of early pregnancy losses in spontaneously conceived cycles (Sasaki *et al.*, 2008). Moreover, insufficient levels of hCG-H during the late stages of pregnancy are linked to preeclampsia development, suggesting that preeclampsia is associated with abnormal placentation and trophoblast invasion

(Bahado-Singh *et al.*, 2002, Uzan *et al.*, 2011). Symptomatic patients of ectopic pregnancy experience low circulating levels of another form of hCG, the disassociated β-subunit (Borrelli *et al.*, 2003). Conversely, down syndrome pregnancy are characterized with elevated hCG-H or hCG free β-subunit levels in maternal serum throughout the 1st trimester (Palomaki *et al.*, 2007).

Blood levels of hCG are elevated in patients with certain types of tumors. The β-subunit is highly secreted in case of choriocarcinoma as well as testicular and ovarian cancers. It's also higher in patients with germ cell tumors and hydatidiform mole formation. Accordingly, hCG blood levels can be used as a marker for these tumors, as a surveillance tool to monitor the efficiency of the treatment, and for post-treatment monitoring to avoid tumor relapse (Goyal *et al.*, 2014, Lempiainen *et al.*, 2012, Lempiainen *et al.*, 2014, van Cromvoirt *et al.*, 2014).

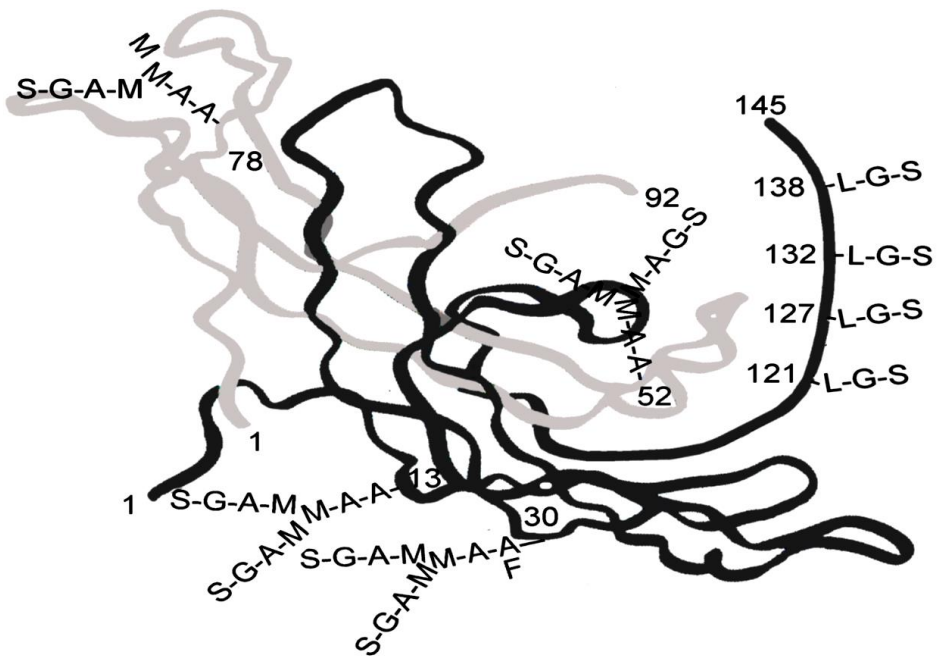

Figure 5 : The 3-D structure of hCG. L: N-acetylgalactosamine, A : N-acetylglucosamine, S: sialic acid or N-acetyl-neuraminic acid, G: galactose, M: mannose, F: fuctose (Cole, 2012).

1.4.2 hCG function

hCG plays a crucial role in pregnancy maintenance by preventing the regression of the corpus luteum. Accordingly, hCG sustains progesterone production by the corpus luteum during the early gestation. However, hCG levels drop during the late part of the first trimester and at that time the production of progesterone takes over by the placenta (Robert Hardin Melmed Shlomo Williams, 2011). hCG participates in the vital early pregnancy events such as the implantation and placentation. Its crucial tasks to pregnancy success is via enhancing the receptivity of the endometrium by promoting maternal immunotolerance of the fetus, remodulation of endometrial spiral artery, boosting natural killer population in the uterus and fostering the formation of new blood vessels (Cole, 2010, Licht *et al.*, 2007, Perrier d'Hauterive *et al.*, 2007).

hCG promotes the formation of new blood vessels (angiogenesis), thus increases the blood flow to the placenta and supports nutrient transfer to the fetus (Berndt *et al.*, 2009, Zygmunt *et al.*, 2002). It stimulates the growth and development of the umbilical cord and corresponding uterine growth to facilitate pregnancy progression and fetal development. It also maintains myometrial relaxation (Cole, 2010).

Stimulation of endometrial receptors by hCG result in various downstream molecules activation. For example, several growth factors are triggered such as transforming growth factor beta (TGF-β) and VEGF, which promote angiogenesis (Berndt *et al.*, 2009). Proteinases such as matrix metalloproteinase 9 (MMP-9) is stimulated and is involved in the regulation of tissues remodeling (Riley *et al.*, 2004). Cytokines as leukemia inhibitory factor (LIF) are also activated. However, the exact role of hCG in endometrial functions that is linked to implantation still remains a subject of

investigation (Gridelet *et al.*, 2013, Licht *et al.*, 2007, Perrier d'Hauterive *et al.*, 2007). It has been demonstrated that hCG/LH receptors are expressed in several fetal organs, but they are absent in the adult ones. Liver, kidneys, lung and spleen are among those fetal organs (Abdallah *et al.*, 2004, Goldsmith *et al.*, 1983, Rao *et al.*, 2007), this suggests a pivotal role of the hCG in enhancing their differentiation and development. The concentrations of hCG in the fetus are much lower than the levels of maternal circulation. Therefore, placental hCG is suggested to be leaked directly into maternal circulation rather than entering the circulation of the fetus (McGregor *et al.*, 1981).

1.4.3 Luteinizing hormone (LH)/hCG receptor

LH/hCG receptor is a member of G-protein coupled receptors family and is expressed in the testis, ovary and the placenta .The main pathway triggered upon its stimulation is cAMP/protein kinase A (PKA) pathway. hCG binding to its receptor activates adenylyl cyclase (AC), phospholipase C (PLC) and K^+ channels, which in turn control cellular cAMP, inositol triphosphates (IP3), Ca^{2+} and other secondary messengers. This process is spatiotemporally controlled (in space and time) by the compensating actions of ACs and phosphodiesterases (PDEs) inside the cell (**figure 6**) (Conti *et al.*, 1999, Houslay *et al.*, 2003, Lybaert *et al.*, 2013, J. L. Williams *et al.*, 2008).

Under the control of A-kinase-anchoring proteins (AKAPs), PDEs participate in the generation of cAMP localized pool (Dodge *et al.*, 2001, Tasken *et al.*, 2001) and both are present in the placenta. The AC is localized in the cytotrophoblast and syncytiotrophoblast while PDE in the syncytiotrophoblast (Bernatchez *et al.*, 2003, Matsubara *et al.*, 1987, Sato *et al.*, 1971, Stacey *et al.*, 1998). In trophoblasts, the increase in cAMP levels triggers new signal mediators expression. Consequently, this

results in an augmented secretion of hCG, hPL, PGH, gonadotropin-releasing hormone (GnRH), syncytin and aromatase initiating estrogen creation (Y. Chen *et al.*, 2013, Depoix *et al.*, 2011, Strauss *et al.*, 1992).Activation of cAMP/PKA signaling pathway stimulates both ERK1/2 and p38 MAPK pathways. Interestingly, this signaling pathway appears to stimulate β-hCG secretion during the syncytialization process in both BeWo and trophoblastic cells (Daoud *et al.*, 2005, Weedon-Fekjaer *et al.*, 2012).

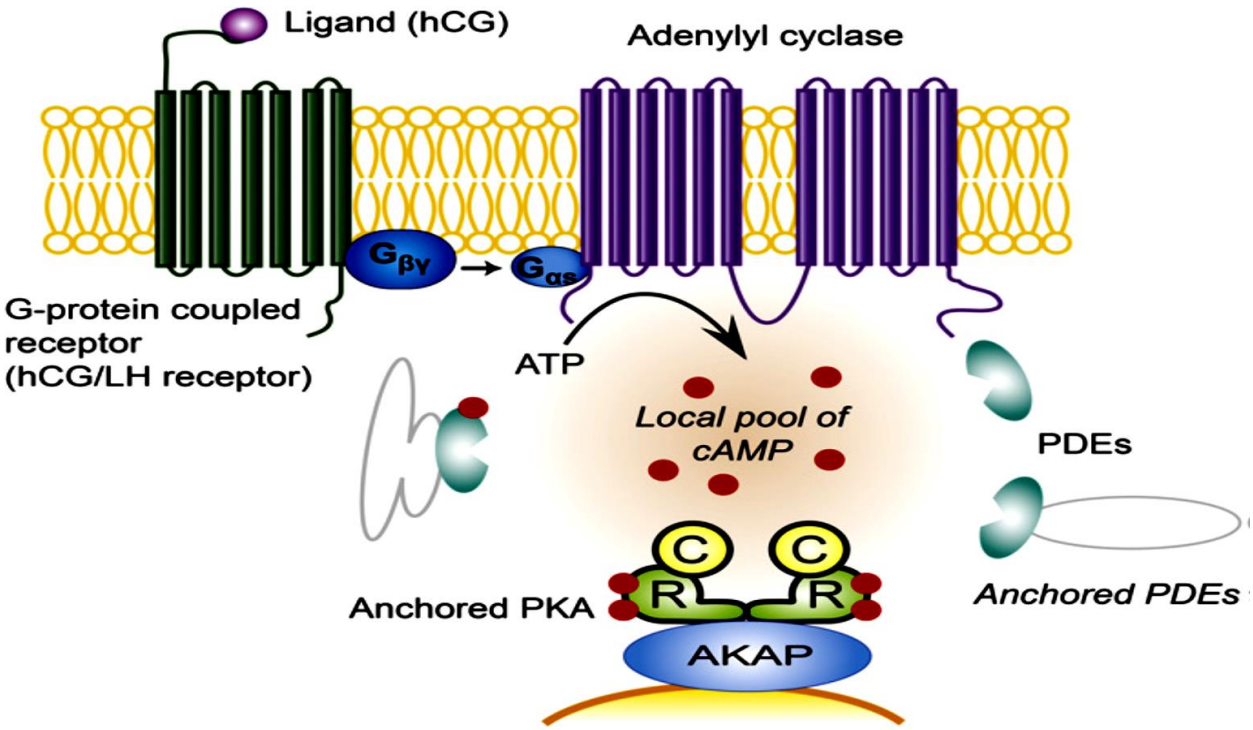

Figure 6 : hCG/LH receptor (G-protein coupled receptor) once stimulated by hCG results in AC activation and cAMP pool increase. Through the compensating actions of ACs and PDEs, AKAPs organize PKA anchoring in the area of cAMP pools, allowing for distinct control of its stimulation in space and time. AKAPs: A-kinase-anchoring proteins, AC: adenylyl cyclase, PKA: protein kinase A, PDEs: phosphodiesterases (Weedon-Fekjaer et al., 2012).

Melatonin

In the early days, melatonin was exclusively considered as the chief product of the pineal gland, that regulates the circadian and circannual cycle. However, further studies revealed that melatonin is also produced and present in various tissues and cells in the human body such as skin, retina, gastrointestinal tract (GIT), ovary, amniotic fluid and placenta (Bubenik, 2002, Lanoix *et al.*, 2008b, A. Slominski *et al.*, 2008, Tosini *et al.*, 1998, Zawilska *et al.*, 2009). Melatonin is known to protect cell, and its role in sustaining cell viability belongs to its receptor dependent and independent activities. Melatonin is a highly distributable molecule as it has both hydrophilic and lipophilic characteristics. This could explain the presence of melatonin in high levels at all subcellular components including the nucleus, the cytoplasm and the mitochondria. Melatonin can readily cross all morphological barriers including the placental and blood brain barrier (da Silva *et al.*, 2011, Reiter *et al.*, 2013a, R. M. Slominski *et al.*, 2012, Venegas *et al.*, 2012). Together, this highlights the significance of melatonin physicochemical properties in performing its protective action.

Melatonin is a potent antioxidant with higher activity compared to other antioxidants such as vitamin C and vitamin D (Milczarek *et al.*, 2010). When melatonin interacts with free radicals for their neutralization, a number of melatonin metabolites are produced such as N1-acetyl-5-methoxykynuramine (AMK). Interestingly, melatonin metabolites also have antioxidant properties and some of them maybe more active than melatonin itself (Galano *et al.*, 2013, Reiter *et al.*, 2013a). Melatonin has also an antioxidant action through receptor dependent activity. Specifically, the activation of

melatonin receptors MT1 and MT2 induce antioxidant enzymes expression and activity (Lanoix *et al.*, 2012b, Reiter *et al.*, 2014).

Melatonin has also anti-inflammatory properties as it modulates and lowers the expression and activity of the inducible form of nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), respectively (Carrillo-Vico *et al.*, 2005a, Carrillo-Vico *et al.*, 2005b). Inflammatory conditions with increased tumor necrosis factor (TNF) and nuclear factor kappa B (NFκB) levels reduce N-acetyl transferase arylalkylamine (AANAT) transcription, which is the rate limiting enzyme in melatonin synthesis (Cecon *et al.*, 2011, Fernandes *et al.*, 2006, Markus *et al.*, 2013). Alternatively, the anti-inflammatory properties of melatonin could inhibit NFκB activation (Negi *et al.*, 2011).

2.1 Melatonin synthesis

Synthesis of melatonin is carried out from the essential amino acid L-tryptophan. First, L-tryptophan is transformed into serotonin, then converted to N-acetyl-5-methoxytryptamine (melatonin) by the consecutive action of the two specific enzymes AANAT followed by hydroxyindole-O-methyltransferase (HIOMT or ASMT, acetylserotonine-O-methytransferase) that methylate the product of AANAT (**figure 7)** (Maronde *et al.*, 2011, A. Slominski *et al.*, 2008, Stehle *et al.*, 2011, Tan *et al.*, 2013, Wurtman *et al.*, 1968). Melatonin synthesizing enzymes are expressed in both the placenta and placental cell line as BeWo and JEG-3 (Lanoix *et al.*, 2008b).

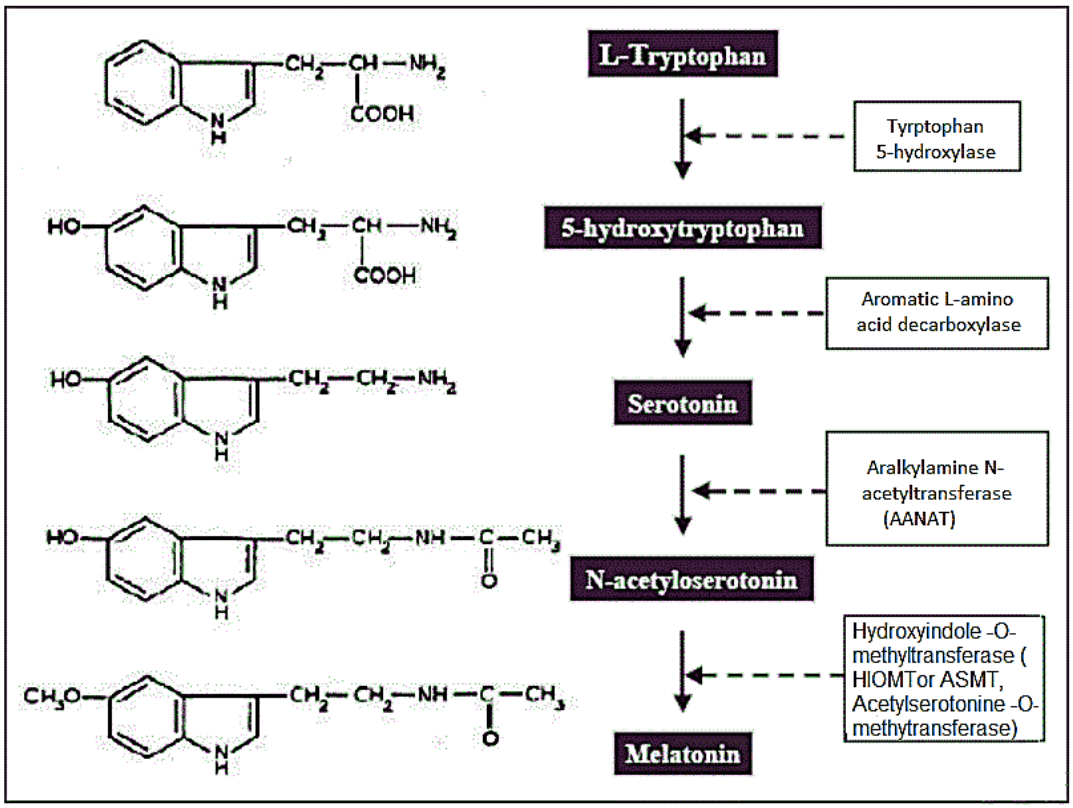

Figure 7 : Melatonin synthesis from L-tryptophan. L-tryprophan is converted into serotonin via the action of the two enzymes tryptophan 5-hydroxylase and aromatic L-amino acid decarboxylase, respectively. Afterwards, serotonin is transformed to melatonin via the consecutive action of arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT or ASMT) (Konturek et al., 2006).

2.2 Melatonin receptors

Melatonin has been identified to bind the two membrane receptors MT1 (Mel1a) and MT2 (Mel1b) and the nuclear receptor family RZR / ROR (retinoid orphan receptor and retinoid Z receptor) (Smirnov, 2001). Despite the affinity of ROR/RZR receptors for melatonin, there is still a controversy as to whether melatonin is their native ligand (Jetten, 2009, Luchetti *et al.*, 2010, Smirnov, 2001).

Melatonin MT1 and MT2 receptors are widely distributed and have been found in most human tissues. Melatonin via the activation of these receptors triggers different biological functions in various tissues (**Table 2**). Melatonin effects through the stimulation of its receptors could elicit the following actions: reducing the pressure of the cardiovascular system, stimulation of the antioxidant enzymes expression and activity, and the regulation of daily night-day cycle (Reiter, 1993, Rodriguez *et al.*, 2004, Sewerynek, 2002, Tomas-Zapico *et al.*, 2005).

Our group has demonstrated that melatonin receptors MT1, MT2 and RORα (retinoid orphan receptor alpha) are expressed in the villous trophoblast (vCTB and STB) as well as in JEG3 and BeWo cells (Lanoix *et al.*, 2008b).

Table 2 : Melatonin receptors role and distribution in human tissues

Table from (R. M. Slominski et al., 2012)

Tissues	Melatonin receptors expressed	Melatonin role
Suprachiasmatic nucleus (SCN)	MT2	Regulate circadian rhythm
Retina	MT2, RORα	Decrease dopamine release
Blood vessels	MT1, MT2	MT1: vasoconstrictor - MT2: vasodilator
Immune system	MT1, MT2, RORα	Inhibit leukotriene rolling Promote immune cell proliferation Stimulates IL2 and IL6 production
Reproductive system	MT1, MT2	Decreases GnRH, LH, FSH release
Pancreas	MT1, MT2, RORα	Decrease insulin release

Skin	MT1, MT2, RORα	Regulate hair growth, and functions of epidermis
Gastrointestinal tract	MT1, MT2	Decrease gastric contraction, peristalsis, and serotonin's actions Increases bicarbonate, amylase, and CCK release
Bone	MT1, RORα	Increases osteoblastic activity and decrease osteoclastic activity
Kidneys	MT1, MT2	Protects from inflammation, regulate glomerular filtration
Placenta	MT1, MT2, RORα	ROS scavenger, decrease apoptosis
Uterus	MT1, MT2	Myometrial contractility
Endometrium	MT1, MT2	Trophoblast invasion in early pregnancy

2.3. Melatonin MT1 and MT2 receptors

Melatonin MT1 and MT2 receptors are part of the GPCR family with seven trans-membrane (TM1-TM7) α-helices, 4 intracellular (I1-I4) and 4 extracellular (E1-E4) domains **(figure 8)** (Dubocovich *et al.*, 2005, R. M. Slominski *et al.*, 2012). MT1 receptor is a 350 amino acid protein while MT2 receptor is composed of 362 amino acids. MT1 and MT2 receptors are structurally similar with 60% homology in their amino acid sequence. Binding sites of both MT1 and MT2 receptors are located in the TM5 immediate vicinity and in the extracellular half of the trans-membrane domain. It has been observed that MT1 receptor possesses a relatively smaller binding site dimension compared to MT2 receptor. This is the principal structural difference that is anticipated to affect both binding selectivity and affinity. Residues at the C-terminal region of TM7 domain is recognized to be a G-protein binding site and also is involved in the interaction with other intracellular proteins. Cysteine residue, for example, appears to

mediate the inhibitory effect on AC. C-terminal cytoplasmic domains are also suggested to contain phosphorylation sites for PKA and PKC (Al-Ghoul *et al.*, 1998, Luchetti *et al.*, 2010).

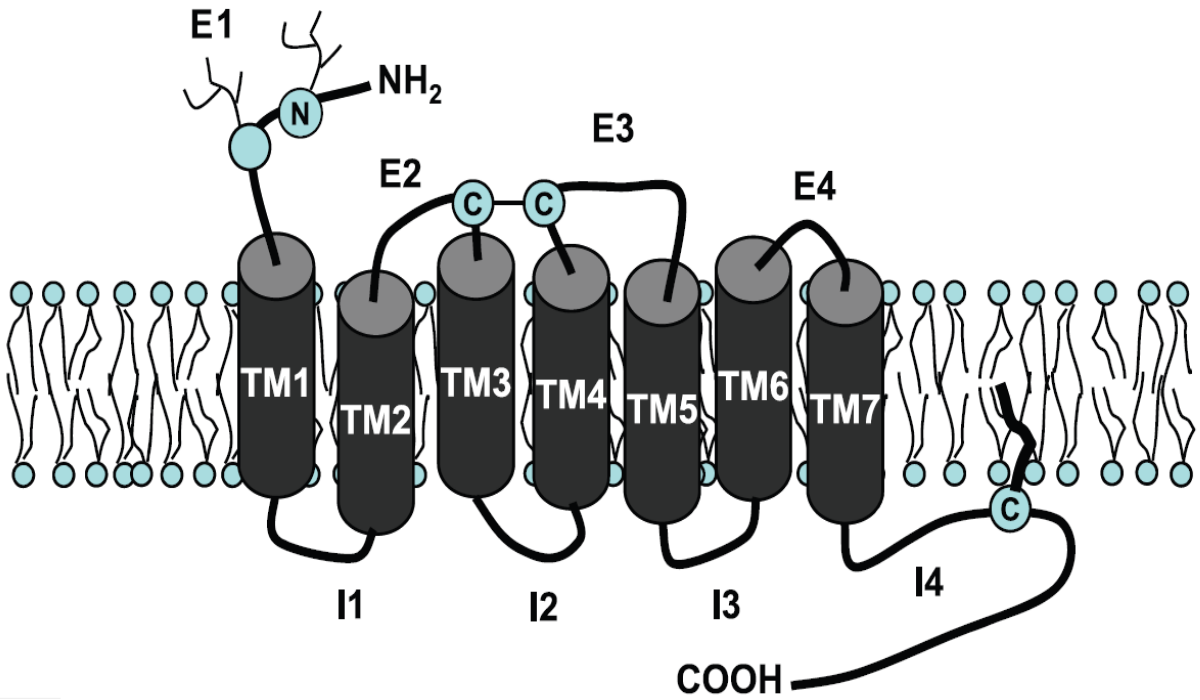

Figure 8 : Illustration of G-Protein coupled receptors (GPCRs). MT1 and MT2 melatonin receptors consist of seven hydrophobic trans-membrane domains of α-helical structure, joined by four intracellular and four extracellular loops (Luchetti *et al.*, 2010).

Activation of MT1 and MT2 is involved in the regulation of different signaling pathways depending on the tissues and cells (e.g. cAMP/PKA, PLC, PLA_2, ERK 1/2 and K^+ /Ca^{2+} channels) **(figure 9)** (Dubocovich *et al.*, 2005, Dubocovich *et al.*, 2003, Pandi-Perumal *et al.*, 2008). There are several commercially available compounds that act as either melatonin MT1/MT2 receptors agonists or antagonists **(Table 3).**

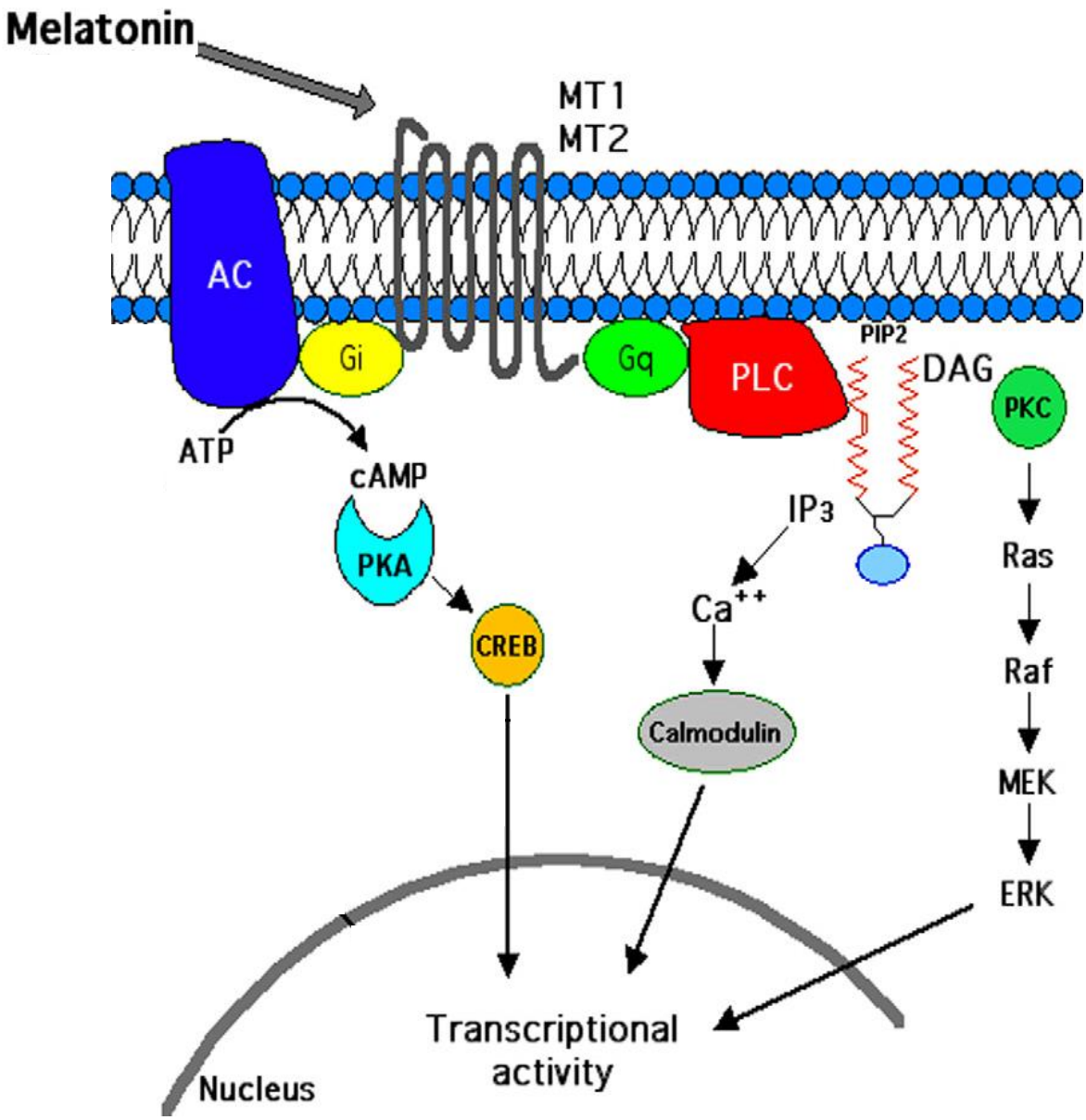

Figure 9 : Illustration of signaling pathways triggered by melatonin MT1/MT2 receptor. Stimulation of melatonin MT1 and MT2 receptors that activates pertussis toxin-insensitive G_q, triggers PLC activation. PLC hydrolyses PIP2 into DAG and IP3. DAG activates PKC, stimulating the Ras/Raf/MEK/ERK signaling pathway while IP3 stimulates Ca^{2+} signaling by calmodulin kinases (CaMK). Activation of pertussis toxin-sensitive G_i as a result of melatonin MT1 and MT2 receptors stimulation inhibits adenylyl cyclase (AC) activity. This inhibits cAMP/PKA pathway and lowers cAMP levels. PLC: phospholipase, PIP2: phosphatidylinositol 4, 5-bisphosphate, DAG: diacylglycerol, IP3: inositol 1, 4, 5- triphosphate, PKC: protein kinases type C, ERK1/2: Extracellular signal regulated kinases (R. M. Slominski *et al.*, 2012).

Table 3 : Melatonin MT1 and MT2 receptors agonists and antagonists

Drug	Action	Reference
	Melatonin receptors agonists	
TIK-301	Is a melatonergic agonist developed by Eli Lilly Co. (Indianapolis, IN, USA), It exhibits a high affinity towards MT1 and MT2 receptors. Its affinity for MT1 is similar to that of melatonin (pKi=10.09), but a little higher for MT2 (pKi=10.38).	(Hardeland, 2010, Rivara *et al.*, 2008)
Agomelatine	It is the naphtalenic analogue of MLT and is a potent MT1 (pKi=10.21) and MT2 (pKi=9.57) agonist. That is developed by Servier Laboratories, France	(de Bodinat *et al.*, 2010)
Ramelteon	In vitro binding studies revealed that ramelteon's affinity for MT1 (Ki=0.014 nM) and MT2 (Ki=0.112 nM) receptors is 3 to 16 times greater than for MLT. It is developed by Takeda Pharmaceutical Co, Japan	(Kato *et al.*, 2005)
Tasimelteon	Currently is at phase III clinical trial by Vanda Pharmaceuticals Inc (Washington, DC, USA). It has a slightly lower affinity to MT1 (pKi=9.45) and a moderately higher affinity to MT2 (pKi=9.80) than MLT.	(Hardeland, 2009, Ohta *et al.*, 2013, Rajaratnam *et al.*, 2009)
6-chloromelatonin	A potent agonist for melatonin MT1 (pKi= 9.10) and MT2 (pKi= 9.77) receptors	(Browning *et al.*, 2000)
	Melatonin receptors antagonists	
Luzindole	A competitive melatonin receptor antagonist, it has 11 to 25 fold higher affinity for the MT2 over the MT1 receptor	(Requintina et al., 2007)
4-P-PDOT	A melatonin receptor antagonist with more than 300 fold selectivity for the MT2 receptor compared to the MT1 receptor.	(Nonno *et al.*, 1999, Requintina *et al.*, 2007)

S22153	A specific antagonist of MT1 and MT2 melatonin receptors with high affinity MT1 (pKi= 8.7) and MT2 (pK i= 8.4).	(Li *et al.*, 2004, Weibel *et al.*, 1999)

MLT: melatonin

2.4 Melatonin and pregnancy

Maternal melatonin is able to reach the fetus and modulate its development. Melatonin is also produced by the reproductive system in the ovary, the oocyte and the placenta (Lanoix *et al.*, 2008b, Reiter *et al.*, 2014, Sakaguchi *et al.*, 2013, Tamura *et al.*, 2009).

Alterations in maternal melatonin levels have an impact on its levels in the fetal circulation and may affect gene expression in the fetal central nervous system (Novakova *et al.*, 2010, Okatani *et al.*, 1999, Reiter *et al.*, 2014). In rats, melatonin has been shown to protect the brain against oxidative stress, lipid peroxidation and DNA damage caused by ischemia and reperfusion during the embryonic period (Wakatsuki *et al.*, 1999). In pregnant rats, pinealectomy triggered maternal circadian cycle suppression where behavioral alterations in newborns have been observed, which was restored after its induction with melatonin (Bellavia *et al.*, 2006).

Studies suggest that births are more likely to occur during night-time under the influence of melatonin. The explanation of this phenomenon may be due to the interaction between melatonin and oxytocin, a hormone that modulates the uterine contractility. Both hormones activate the same signaling pathways in myometrial cells,

PLC and PKC pathways **(figure 11)** (Sharkey *et al.*, 2010, Sharkey *et al.*, 2009). The importance of this interaction is supported by studies that show that the oxytocin-induced contractility of myometrial cells is increased *in vitro* after being co-treated with melatonin. This phenomenon is not observed if the cells were treated only with oxytocin, suggesting that the melatonin sensitizes myometrial cells to the pro-contractile signaling of oxytocin (Reiter *et al.*, 2014, Sharkey *et al.*, 2010, Sharkey *et al.*, 2009).

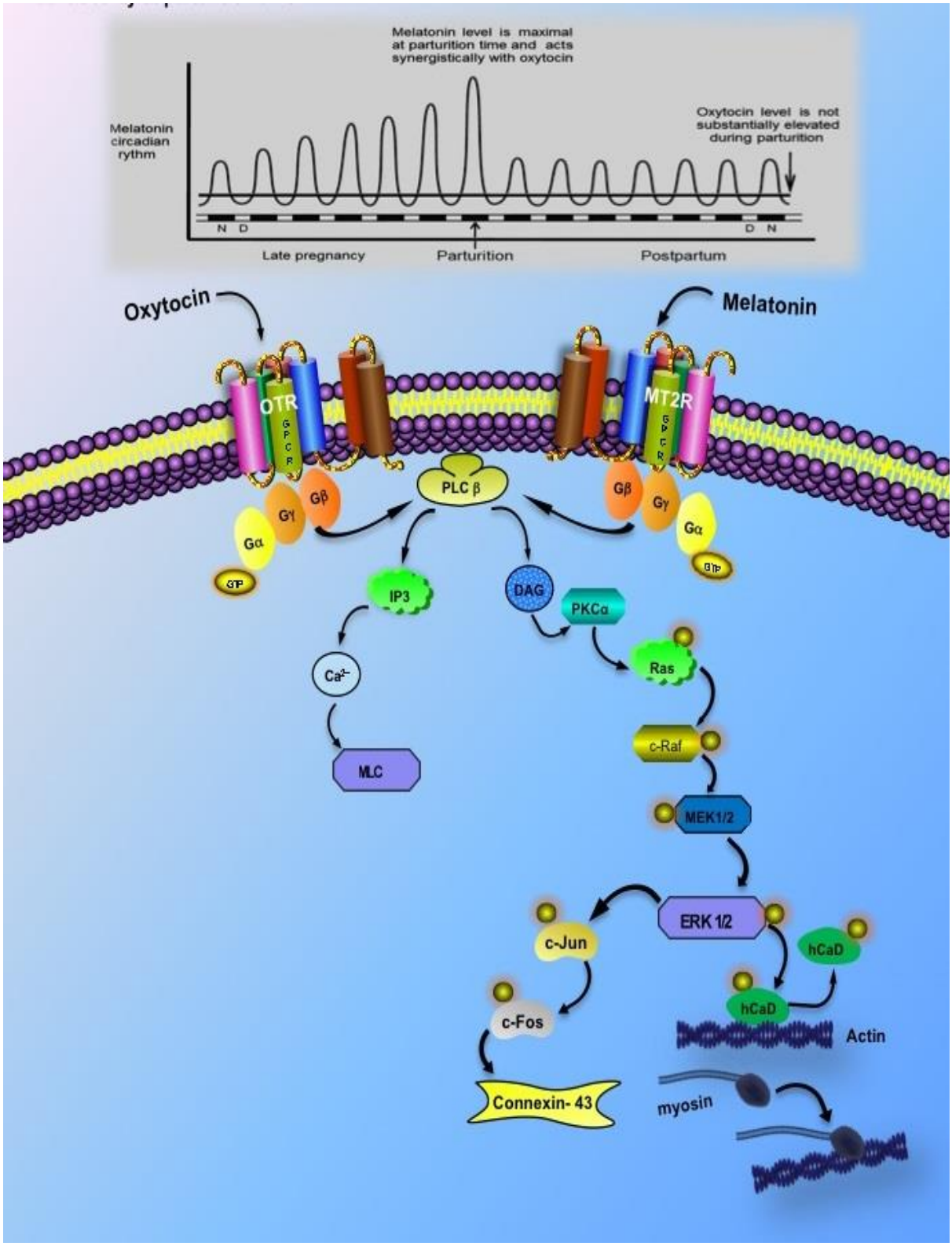

Figure 10: Suggested model of synergetic action of melatonin and oxytocin on night-time myometrial contractility at parturition time. Melatonin circadian rhythm and oxytocin level is described

through the last trimester of pregnancy, at parturition time and after delivery of human females. Melatonin and oxytocin stimulate respectively MT2 receptor (MT2R) and oxytocin (OTR) receptor to synergistically activate phospholipase (PLC)-β, which hydrolyses phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1, 4, 5- triphosphate (IP3). DAG activates protein kinases C (PKC)-α, activating the Ras/Raf/MAPK signaling pathway and leading to caldesmon (hCaD) phosphorylation. This increases actin availability for myosin binding and contractility induction. PLCβ stimulates both c-Jun and c-Fos, resulting in enhanced gap junctional communication via connexin 43 activation. Finally, IP3 stimulates Ca^{2+} release that activates myosin light-chain (MLC) and enhances myometrial contractility (Sagrillo-Fagundes *et al.*, 2014).

2.5 Melatonin and placenta

During the course of pregnancy, maternal circulating melatonin levels progressively increase with a maximum peak at the time of delivery. Pregnant women therefore have significantly higher plasma melatonin concentrations (day and night) compared to non-pregnant women. The placenta is believed to be the source of this increase as melatonin levels return to normal after the delivery of the placenta (Y. Nakamura *et al.*, 2001, Tamura *et al.*, 2008b, Vatish *et al.*, 2010).

It has been demonstrated that the mRNA of melatonin synthesizing enzymes (AANAT and HIOMT) is expressed in placental tissue from first trimester of pregnancy (Iwasaki *et al.*, 2005). Afterwards, our group confirmed the presence of melatonin synthesizing enzymes, and melatonin receptors (MT1, MT2 and RORα) mRNA and protein in normal human term placenta and in human placental choriocarcinoma cell lines. These data suggest that placental melatonin could be the source of the increased circulating melatonin levels observed in pregnant women (Lanoix *et al.*, 2008b).

Our group has shown that preeclampsia is associated with reduced activity and expression of AANAT, as well as the concentration of melatonin and its receptors (MT1

and MT2) in the placental tissue (Lanoix *et al.*, 2012a, Lanoix *et al.*, 2012b, Lanoix *et al.*, 2013). We have also previously shown that melatonin protects trophoblast cells from apoptosis and oxidative stress-induced by hypoxia-reoxygenation, *in vitro* **(figure 11)** (Lanoix *et al.*, 2013).

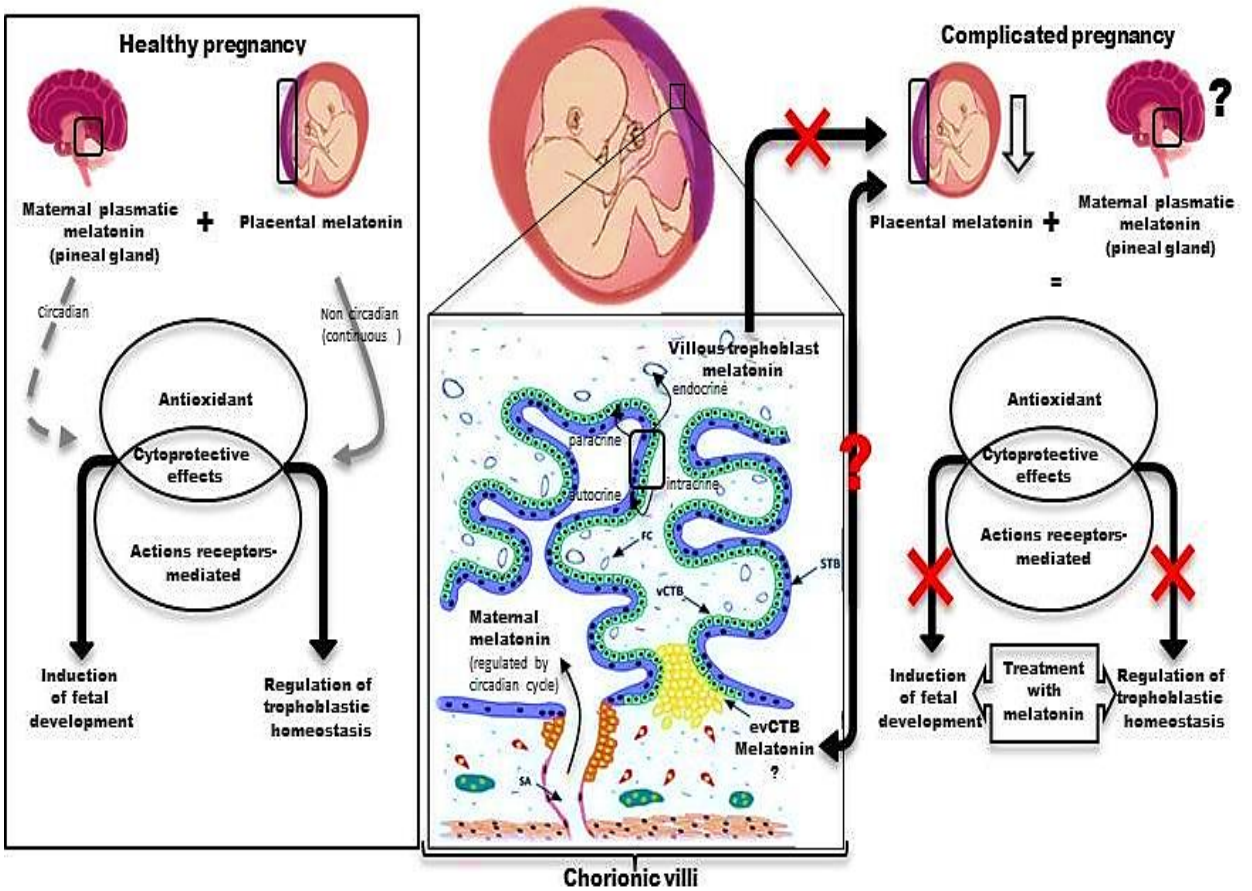

Figure 11: Potential role of melatonin in healthy and complicated pregnancies. Plasmatic melatonin, released in a circadian manner, reaches the intervillous space and accesses fetal capillaries (FC) through syncytiotrophoblast (STB) and villous cytotrophoblast (vCTB). In parallel, both STB and vCTB produce melatonin locally that can also reach fetal capillaries and maternal circulation. Both plasmatic and placental melatonin are related with maternal and fetal protective effects observed during pregnancy (Sagrillo-Fagundes *et al.*, 2014).

In summary, the presence of melatonin, its receptors and its synthesizing enzymes in the placental tissue implicate melatonin in maintaining the placenta during

pregnancy (Lanoix *et al.*, 2008b). The production of placental melatonin during pregnancy abnormalities is compromised. Based on this theory, recently, several studies have linked certain pregnancy diseases to alterations in its synthesis, in its receptors or its concentrations as in case of preeclampsia and intra uterine growth restriction (IUGR). Consequently, melatonin treatment is suggested to recover the absent protective effects of placental melatonin in those cases (Hobson *et al.*, 2013, Lanoix *et al.*, 2012a, Lanoix *et al.*, 2013, Y. Nakamura *et al.*, 2001, Reiter *et al.*, 2014).

2.6 Melatonin and hCG secretion

Activation of the PKA pathway induces both BeWo and trophoblast cell fusion and hCG secretion (Keryer *et al.*, 1998, Knerr *et al.*, 2005, Knofler *et al.*, 1999). Additionally, forskolin (inducer of AC), induces ERK1/2 and p38-MAPK phosphorylation in BeWo cells, and maximal stimulation is within 5 -10 minutes after forskolin treatment (Delidaki *et al.*, 2011). Our team has demonstrated that 6-chloromelatonin (a melatonin agonist) inhibited forskolin stimulated β-hCG secretion in a dose dependent manner (maximum effect at 10μM MLT), while it has no effect on basal β-hCG secretion in BeWo and JEG-3 cells (Lanoix *et al.*, 2006). Furthermore, BeWo and JEG3 pretreatment with pertussis toxin (PTX; inhibits the activation of Gi/o proteins) reversed the 6-chloromelatonin inhibitory effect on forskolin-stimulated β-hCG secretion. This reveals that 6-chloromelatonin inhibitory effect is mediated, in part, by a PTX-sensitive G protein, $G_{i/o}$. Indeed, activation of melatonin MT1 and MT2 receptors through $G_{i/o}$ pathway inhibits AC pathway and cAMP accumulation in various mammalian tissues, as reviewed in (Dubocovich *et al.*, 2005, Witt-Enderby *et al.*, 2003). On the other hand, the stimulatory effect of 6-chloromelatonin on basal β-hCG secretion in PTX-pretreated cells

could be due to $G_{q/11}$ signaling pathway activation. The $G_{q/11}$ pathway results in Ca^{2+} mobilization from intracellular stores and activation of PKC through PLC pathway (Feinman *et al.*, 1986, Lanoix *et al.*, 2006). The specific signaling pathways involved in melatonin effects on β-hCG production in normal trophoblasts as well as in choriocarcinoma cells remains to be explored.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

Our main research hypothesis is that melatonin and its placental receptors, play an important role in the regulation of villous trophoblast development and endocrine function. The objectives for the present project are to determine the role of melatonin and its placental MT1/MT2 receptors on villous trophoblast syncytialization and the mechanisms by which they regulate β-hCG secretion. The two specific objectives and hypotheses are:

Objective 1: Determine the effect of melatonin and its MT1 and MT2 receptors on villous trophoblast syncytialization (biochemical (β-hCG secretion) and fusion)**.** It has been demonstrated that melatonin has the ability to stimulate cellular differentiation of many cell types. Melatonin can stimulate the final stage of chick retinal cell differentiation, that is crucial for the development of several other cell types (Sampaio Lde *et al.*, 2010) and can decrease the apoptosis process in numerous cell types as $CD4^+$ T cells (Pedrosa *et al.*, 2010) and spermatozoa (Espino *et al.*, 2010). In neural cells, melatonin stimulates the differentiation of pluripotent P19 cells ($Oct4^+$ $Sosx2^+$) into neural stem cells ($Oct4^-$ $Sox2^+$) through MT1 receptor and ERK1/2 pathway activation (X. Chen *et al.*, 2014). Moreover, melatonin can stimulate human Saos2 osteoblast-like cell differentiation - a model of osteoblast - and consequently stimulates bone formation, through an action mediated by MT2 receptor and ERK1/2 signaling pathway activation (Matsumura *et al.*, 2014). Likewise, via receptor dependent and independent activities, melatonin triggers mesenchymal stem cells (MSCs) differentiation and survival (Luchetti *et al.*, 2014). **Hypothesis 1:** We propose that the differentiation of vCTB into STB is promoted by melatonin in a MT1 and MT2 receptor-dependent manner.

Objective 2: Determine the mechanisms by which melatonin and its MT1/MT2 receptors regulate β-hCG secretion. The MAPK signaling pathway plays a vital role in villous trophoblast morphological and biochemical differentiation. Dysregulation of this pathway could lead to the formation of aberrant STB and consequently lead to obstetric complications. Melatonin stimulates differentiation in various cell types via activating its MT1 and MT2 receptors with subsequent ERK1/2 signaling pathway stimulation. In addition, we observed earlier that melatonin stimulates β-hCG secretion in our *in vitro* models of human trophoblast; BeWo and JEG-3 cell lines (Lanoix *et al.*, 2006). **Hypothesis 2:** We propose that melatonin in a receptor-dependent manner stimulates β-hCG secretion throughout the activation of ERK1/2 and PLC signaling pathway.

CHAPTER 3

RESULTS

3.1: Placental melatonin system is present throughout pregnancy and regulates villous trophoblast differentiation

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Manuscript accepted for publication at the Journal of Pineal Research (under revision)

Contribution of the student

The student analyzed all the data, designed and conducted the protein expression of AANAT enzyme throughout pregnancy and wrote the manuscript.

De: onbehalfof+reiter+uthscsa.edu@manuscriptcentral.com
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À: Vaillancourt, Cathy
Objet: Journal of Pineal Research - Decision on Manuscript ID JPI-OM-11-14-0225

05-Dec-2014

Dear Prof. Vaillancourt:

Manuscript ID JPI-OM-11-14-0225 entitled "Placental melatonin system is present throughout pregnancy and regulates villous trophoblast differentiation" which you submitted to the Journal of Pineal Research, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter. **The reviewer(s) have recommended publication**, but also suggest some revisions to your manuscript. Therefore, I invite you to respond to the reviewer(s)' comments and revise your manuscript.

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Placental melatonin system is present throughout pregnancy and regulates villous trophoblast differentiation

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Short title: Placental melatonin system during pregnancy

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Keywords: AANAT / HIOMT (ASMT) / MT1 melatonin receptor / MT2 melatonin receptor / Syncytiotrophoblast/ Villous cytotrophoblast/ Human chorionic gonadotropin (hCG)

ABSTRACT

Melatonin is highly produced in the placenta where it protects against molecular damage and cellular dysfunction arising from hypoxia/re-oxygenation induced oxidative stress as observed in primary cultures of syncytiotrophoblast. However, little is known about melatonin and its receptor in the human placenta throughout pregnancy and their role in villous trophoblast development. The purpose of this study is to determine melatonin synthesizing enzymes; arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole O-methyltransferase (HIOMT), and melatonin receptor (MT1 and MT2) expression throughout pregnancy as well as the role of melatonin and its receptors in villous trophoblast syncytialisation. Our data show that the melatonin generating system is expressed throughout pregnancy (from week 7 to term) in placental tissues. AANAT and HIOMT show maximal expression at the 3rd trimester of pregnancy. MT1 receptor expression is maximal at the 1^{st} trimester compared to the 2^{nd} and 3^{rd} trimesters, while MT2 receptor expression does not change significantly during pregnancy. Moreover, during primary villous cytotrophoblast syncytialisation, MT1 receptor expression increases while MT2 receptor expression decreases. Treatment of primary villous cytotrophoblast with an increasing concentration of melatonin (10 pM -1 mM) increases the fusion index (syncytium formation; 21% augmentation at 1 mM melatonin (vs. vehicle)) and β-hCG secretion (121% augmentation at 1 mM melatonin vs. vehicle). This effect of melatonin appears to be mediated via its MT1 and MT2 receptors. In sum, melatonin synthetic machinery (synthetizing-enzymes and receptors) is expressed in human placenta throughout pregnancy and promotes syncytium formation, suggesting an essential role of this indolamine in placental function and pregnancy well-being.

INTRODUCTION

Human placenta is crucial for the growth and the development of the fetus as well as the adaptation of maternal physiology to pregnancy (Le Bouteiller, 2001, Longtine *et al.*, 2011). It mediates the exchange of nutrients and oxygen between the mother and the fetus, and eliminates waste products excreted by the fetus. The functional unit of the placenta is the chorionic villus that is formed by villous cytotrophoblast and is bathed in maternal blood (Aplin, 1991, Malassine, 2001, Rampersad *et al.*, 2011). Throughout the syncytialisation process, mononucleated villous cytotrophoblast cells fuse and differentiate into the multinucleated non-proliferative syncytiotrophoblast. Differentiation of villous cytotrophoblast is described by morphological and biochemical features. Morphological differentiation is characterized by the fusion of villous cytotrophoblast to syncytiotrophoblast. Biochemical differentiation is defined by the activation of genes expressed specifically in the syncytiotrophoblast, coding for secreted hormones such as human chorionic gonadotropin (hCG) (Handschuh *et al.*, 2007a, Jacquemin *et al.*, 1996, Kliman *et al.*, 1986, Morrish *et al.*, 1987, Mounier *et al.*, 2009, Newbern *et al.*, 2011). To maintain homeostasis, syncytiotrophoblast goes through apoptosis to be constantly renewed by the underlying villous cytotrophoblast. Syncytialization of villous cytotrophoblast *in vitro* is completed after approximately four days of culture (Castellucci *et al.*, 1990, Miller *et al.*, 2005, Vaillancourt *et al.*, 2009b). The disruption of syncytiotrophoblast homeostasis (via oxidative stress) contributes to various pregnancy complications such as intrauterine growth restriction and preeclampsia (Burton *et al.*, 2011, Ishihara *et al.*, 2002, Lanoix *et al.*, 2012a).

Melatonin was initially thought to be only produced by the pinealocytes under circadian rhythm control. However, it has been demonstrated that the gastrointestinal tract, skin, reproductive organs and placenta synthesize it as well (Bubenik, 2002, Hardeland *et al.*, 2006,

Lanoix *et al.*, 2008a, A. Slominski *et al.*, 2008, Tosini *et al.*, 1998, Zawilska *et al.*, 2009). Melatonin is synthesized from serotonin via two steps. The first step is by arylalkylamine N-acetyltransferase (AANAT) that acetylates serotonin to N-acetyl serotonin which is the rate limiting step. The second step is the methylation of N-acetyl serotonin and is via the enzyme hydroxyindole -O-methyltransferase (HIOMT- also called N-acetylserotonin O-methyltransferase (ASMT)) (Maronde *et al.*, 2011, Pandi-Perumal *et al.*, 2006, Wurtman *et al.*, 1968). Melatonin is a lipophilic hormone that allows its broad distribution in the body (Venegas *et al.*, 2012). This indolamine controls biological rhythms, has a strong antioxidant effect and possesses anti-inflammatory, cytoprotective and immunological properties [35] and plays a key role in sexual maturation and ovulation [36], as well as regulating cellular processes such as proliferation, differentiation, invasion, migration and apoptosis [37].

Melatonin mediates some of its effect through two G protein coupled receptors: MT1 (Mel1a) and MT2 (Mel1b) (Dubocovich *et al.*, 2005, Ekmekcioglu, 2006)-31]. Activation of MT1 and MT2, through PTX-sensitive G proteins (Gi/o) pathway, inhibits adenylyl cyclase pathway and blocks second messenger cAMP production. Both MT1 and MT2 receptors can bind and stimulate PTX-insensitive G proteins (Gq/11), which activates phospholipase C-β (PLC-β) (Dubocovich *et al.*, 2005, Dubocovich *et al.*, 2003, Lanoix *et al.*, 2006, Pandi-Perumal *et al.*, 2008).

We previously demonstrated the presence of melatonin receptors (MT1 and MT2) and functional melatonin synthesis enzymes (AANAT and HIOMT) in human primary term villous trophoblastic cells. We also demonstrated that melatonin modulates β-hCG secretion in placental cell lines (BeWo and JEG-3) through the stimulation of MT1 and/or MT2 (Lanoix *et al.*, 2008a, Lanoix *et al.*, 2006). This suggests that melatonin has an important role in human

placental function. However, the role of melatonin on β-hCG secretion in primacy trophoblast has never been studied. This study determined melatonin synthetizing-enzymes and receptor expression throughout pregnancy and defined their role in villous trophoblast syncytialisation (β-hCG secretion and fusion).

MATERIALS AND METHODS

Placental tissues

This study was approved by the ethical committee of Montreal University Health Centre (CHUM)-St-Luc hospital and Mother and Child University Hospital Center (CHU)-Sainte-Justine Hospital (Montreal, QC, Canada). Placental tissues were obtained from (CHU)-Sainte-Justine, where first (7-12 wk) and second (14-26 wk) trimesters of pregnancy were from therapeutic abortion. Third (27- 34 wk) and term (37- 41 wk) placental tissues were obtained immediately after spontaneous vaginal deliveries from uncomplicated pregnancies. Human placentas were immediately immersed in Dulbecco's minimal essential medium-high glucose (DMEM-HG; Sigma-Aldrich, Oakville, ON, Canada) containing a mixture of antibiotics (5 μg/ml amphotericin, 50 μg/ml gentamycin, 0.12 mg/ml penicillin), kept at 4°C and processed within 1h. Placental tissue samples were excised from randomly selected regions and were cut at 5-cm^2 sections, following the removal of the amnion, chorion and decidual layer and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Isolation and purification of term villous cytotrophoblast

Villous cytotrophoblasts from term placenta of uncomplicated pregnancy were obtained from (CHUM)-St-Luc. They were isolated, with minor modification as described previously (Lanoix *et al.*, 2008a), using the trypsin-DNase/Percoll method described by Kliman *et al.*

(Kliman *et al.*, 1986) and subsequently immunopurified according to Petroff *et al*. (Petroff *et al.*, 2006). Briefly, following the isolation, villous cytotrophoblast cells were suspended in fetal bovine serum (FBS; Gibco, Burlington, ON, Canada) and the percentage of viability and cell numbers were assessed by trypan blue exclusion. An average of 1.4 x 10^8 cells was obtained from 70 g of villous tissue. Cells were aliquoted in cryovials in freezing medium (10% DMSO in FBS) at a density of 1 x 10^7 cells/ml and stored at -80°C for 4-12 h. Then, cells were transferred to the vapor phase of a liquid nitrogen freezer and stored until analysis.

After thawing, villous mononuclear cytotrophoblasts were purified by immunomagnetic purification using autoMACS™, as previously described (Lanoix *et al.*, 2008a). Cells were selected negatively with mouse anti-human HLA-ABC (clone W6/32) antibody and autoMACS goat anti-mouse IgG microbeads. Typical yield after cryopreservation and immunopurification ranged from 75% to 85% of starting population. Cells were suspended in 37°C culture medium, consisting of DMEM-HG supplemented with 2mM L-glutamine, 25 mM HEPES, 10% FBS and 1x penicillin-streptomycin-neomycin (PNS) antibiotic mixture and seeded at a density of 4.5 x 10^6 cell/well in CellBind six-well microplate for protein extraction, RNA extraction and hormone assay. For immunofluorescence analysis, villous cytotrophoblast were seeded at a density of 6.4 × 10^5 cells/chamber in Permanox™ Lab-Tek 8 Chambers Slide System™ as previously (Lanoix *et al.*, 2008a).

mRNA expression analyses

Quantitative polymerase chain reaction (qPCR) analyses were performed as described by Lanoix, *et al.* (Lanoix *et al.*, 2012c). Briefly, total RNA from primary villous trophoblast and from placental tissues were extracted using Aurum Total mini kit (Bio-Rad, Mississauga, Canada) according to manufacturer's instructions. The Experion™ Automated Electrophoresis

Station (Bio-Rad) was used to determine RNA integrity. Complementary DNA (cDNA) was obtained from 0.5 - 1 µg RNA with the iScript cDNA synthesis kit following the manufacturer's instructions (BioRad) and stored at -20°C until further analysis. Specific primer for MT1, MT2, HIOMT, AANAT, peptidylprolyl isomerase A (PPIA), HPRT and TOP-1 were designed using Oligo 6 and software (Molecular Biology Insights, Cascade, USA), the specificity was determined with Primer-Blast program (http://www.ncbi.nlm.nih.gov/tools/primerblast/).

Validated primer sequences are shown in **Table 1**. PPIA, HPRT and TOP-1 were selected as reference genes (most stably expressed genes as validated by geNorm analysis) and as demonstrated previously (Lanoix *et al.*, 2012c). qPCR was conducted using the CFX-96 Real-Time PCR Detection System (BioRad). Amplification was performed with the SsoFast EvaGreen Supermix (BioRad) from 0.5 µl of RNA as described previously (Lanoix *et al.*, 2012c).

Proteins expression analyses

Whole cell lysates from primary villous trophoblast and from placental tissues were extracted as described previously (Lanoix *et al.*, 2008a). Protein concentration from each sample was determined using BCA protein assay reagents according to manufacturer's instructions (Pierce Biotechnology, Rockford, CA,). Protein expression was determined with semi-quantitative western blot experiments. 40 µg of proteins were resolved on 10% SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA). Specific protocols for various antibodies used are summarized in **Table 2.** Amido black was used to normalize the total protein expression because of its great stability, as we demonstrated earlier (Lanoix *et al.*, 2012d).

β-hCG secretion

For the evaluation of biochemical differentiation, the cell culture medium of primary villous trophoblast was collected every 24 h (from 24 h to 96 h of culture) and centrifuged, after which supernatants were stored at -80°C until assayed.

Cells were treated with increasing concentrations of melatonin (10 ρM, 1 nM, 100 nM, 10 μM or 1 mM) or vehicle (0.1 % DMSO) as well as with 1 mM melatonin, 1 mM melatonin combined with 1 mM luzindole (selective MT1 and MT2 antagonist from Tocris Bioscience, Minneapolis, USA), 1 mM luzindole or vehicle. Melatonin (100 % pure chromatographically) was a generous gift from Pr Russel J Reiter (University of Texas Health Science Center, San Antonio, USA).

The secretion of β-hCG was evaluated in culture medium in triplicate by enzyme-linked immunosorbent assay (ELISA) using EIA-1911 kits according to manufacturer's instruction (DRG International, Mountainside, USA). The Sensitivity of the kit is at 1.0: mIU/mL. Intra- and interassay coefficients of variation were 3.4 - 8.9% and 6.6 - 9.9% respectively. Results were normalized against protein content of whole cell lysate from corresponding culture well. Whole cell lysates were prepared as described above. Values are expressed as hCG secretion ± S.E.M.

Assessment of fusion

For the evaluation of morphological differentiation, primary villous trophoblast cells were treated with melatonin and luzindole as described above and stained for desmoplakin protein and nuclei as described previously (Lanoix *et al.*, 2008a) . Briefly, cells were fixed in methanol, blocked in 2% donkey serum and incubated with mouse anti-desmoplakin antibody (Sigma-Aldrich, Oakville, Canada), diluted to 1:800 in blocking buffer, overnight at 4˚C. Cells were then incubated with donkey anti-mouse IgG Alexa Fluor 488 antibody (1:1000; Invitrogen,

Burlington, Canada) for 1 h at room temperature in the dark. Nuclei were counterstained with propidium iodide (Invitrogen). Cells were mounted with ProLong Gold antifade reagent (Invitrogen) and analyzed at 30x using a Leica DMRE fluorescence microscope (Deerfield, IL, USA) and photograph with a Cook Sensicam High Resolution Performance digital CCD camera (Romulus, MI, USA). From a random point near the uppermost edge of the well, three random fields were selected for analysis. The results are expressed as the percentage of nuclei contained in multinuclear (≥ 3 nuclei) syncytia.

Statistical analysis

All data are represented as mean ± standard error of the mean (SEM), from at least three different placentas. Statistically significant differences were tested using Student's t-test, one-way ANOVA followed by Tukey's post hoc test, or two-way ANOVA followed by Bonferroni post hoc test. All statistical and graphical analyses, including concentration response analyses, were performed using Prism 5.0 (GraphPad, San Diego, CA) and a probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Figure 1 shows the mRNA and protein expression of MT1 and MT2 melatonin receptors between placentas from different trimesters of pregnancy. MT1 mRNA and protein expression are significantly higher in the 1st trimester than in the 2nd, 3rd and in term placentas (Fig. 1A/1C). On the contrary, MT2 mRNA and protein expression were not significantly altered among placentas from different trimesters of pregnancy (Fig. 1B/1D). Concerning mRNA and protein expression of AANAT, they are significantly different between the 2nd and 3rd as well as the 3rd and term, showing maximal expression at the 3rd trimester (Fig. 2A - 2C). Although there was no

significant difference observed in mRNA expression of HIOMT, it shows a similar pattern of expression as the AANAT with maximum value at the 3rd trimester (Fig. 2B). In sum, there is a variation in the expression of melatonin receptors during pregnancy and a higher expression of its synthesizing enzymes is during the 3rd trimester correlating with the observed high melatonin levels during late pregnancy.

During the *in vitro* differentiation of villous cytotrophoblast to syncytiotrophoblast, melatonin receptors are expressed differently. MT1 mRNA and protein are significantly more expressed at 96 h compared to 4 h (Fig 3A-B). In addition, MT2 mRNA and protein expression were more expressed at 4 h compared to 96 h (Fig. 3C-D). However, there is no significant difference observed in mRNA expression of melatonin enzymes, AANAT and HIOMT (Fig. 4). This indicates a different function of melatonin receptors during trophoblast differentiation.

Figure 5A shows that 1 mM of melatonin significantly increases β-hCG release during primary villous trophoblast differentiation in comparison to vehicle at 72 h and 96 h of culture. Interestingly, luzindole, a selective MT1 and MT2 melatonin receptor antagonist did not block the stimulatory effect of melatonin on β-hCG release but it has an additive effect and increased β-hCG secretion (Fig. 5B). Syncytial fusion was also significantly increased by 1 mM of melatonin at 96 h of culture (Fig 6A-B). Luzindole equally displays a stimulatory effect on syncytialisation when combined with melatonin (Fig 6A). Taken together, results of Fig. 5 and 6 indicate that melatonin significantly improves villous trophoblast biochemical and morphological differentiation.

DISCUSSION

This study assesses mRNA and protein expression of AANAT, mRNA expression of HIOMT and both mRNA and protein expression of MT1 and MT2 in human placenta during all trimesters of pregnancy. Both AANAT and HIOMT were highly expressed in the third trimester, which suggests a continuous production of placental melatonin with a peak in the late pregnancy. Other studies demonstrated consistent results with high melatonin levels in maternal serum throughout pregnancy, also reaching a peak at the end of pregnancy (Tamura *et al.*, 2008a, Tamura *et al.*, 2008b). Nakamura *et al*.(Y. Nakamura *et al.*, 2001) observed that melatonin levels return to normal after delivery. High levels of melatonin have been observed in human placenta even during daytime and lower levels were observed in abnormal pregnancies compared to normal (Tamura *et al.*, 2009, Tamura *et al.*, 2008a, Tamura *et al.*, 2008b, Vatish *et al.*, 2010). Taken together, this work confirms that melatonin is produced locally in the placenta at high levels and that the end of pregnancy the final rise may be a prerequisite to prepare for parturition. Parturition is characterized by sustained and spontaneous contractions of uterine smooth muscles. Childbirth is a light sensitive process with a peak of uterine contractions occurring at night in late pregnancy (Cagnacci *et al.*, 1998, Ducsay *et al.*, 1991, Glattre *et al.*, 1983). MT1 and MT2 have been detected in uterine myometrium (Schlabritz-Loutsevitch *et al.*, 2003). Experiments of melatonin administration to pinealectomized rats revealed that pineal melatonin controls the normal time of parturition (Reppert *et al.*, 1994, Takayama *et al.*, 2003). Moreover, oxytocin and melatonin utilize the same signaling pathway (phospholipase C (PLC) activation), and their receptors are up-regulated in human uterus (Sharkey *et al.*, 2010, Sharkey *et al.*, 2009). Interestingly, in case of low oxytocin levels, melatonin supplementation showed adequate full contraction of uterine smooth muscles *in vitro*. Thus, we suggest that placental melatonin might

be implicated in coordinating the process of human parturition via stimulating its receptors located in the myometrium. Alternatively, the pineal secretion of melatonin may coordinates a nocturnal delivery by ensuring maximum availability of melatonin (Reiter *et al.*, 2013b).

We observed that MT1 mRNA and protein are significantly more expressed in the first trimester compared to the 2^{nd} and 3^{rd} trimesters of pregnancy, while there is no change in the expression of MT2 receptor. This is consistent with Rafael et al (Sampaio *et al.*, 2012) findings, which established that MT1 is the initial receptor to be transcribed in mammalian embryo. This suggests a MT1 role in the increased embryo cleavage and blastocyst creation rates. However, further investigations are required for full understanding of its activity at this early embryonic period as well as embryonic development (Sampaio *et al.*, 2012).

Human trophoblast is a pseudo-tumorigenic tissue, requiring syncytiotrophoblast apoptosis to permit its renewal by villous cytotrophoblast. Caspase 8 is involved in the fusion of villous cytotrophoblast in syncytiotrophoblast and both chromatin condensation and DNA fragmentation were observed in syncytiotrophoblast (Sharp *et al.*, 2010). Altered regulation of syncytiotrophoblast apoptosis disrupts placental homeostasis, possibly leading to the development of complete molar pregnancy or choriocarcinoma (Burton *et al.*, 2011, Lanoix *et al.*, 2012a, Vaillancourt *et al.*, 2009b). Melatonin is a major ubiquitous regulator of apoptosis and a smart killer. In tumor cells, melatonin triggers intrinsic apoptosis pathways. Conversely, in non-tumor cells, it inhibits apoptosis and supports survival (Lanoix *et al.*, 2012b). We previously established that melatonin exerts anti-apoptotic activities in villous cytotrophoblast via modulating Bax/Bcl-2 pathway but stimulates mitochondrial membrane permeabilization and cellular apoptosis in BeWo choriocarcinoma cells (Lanoix *et al.*, 2012b, Sainz *et al.*, 2003).

MT1 and MT2 may play a different role in the villous trophoblast syncytialisation. In this study, we observed an increased expression of MT1 during the differentiation of villous cytotrophoblast in syncytiotrophoblast while the expression of MT2 decreases. Melatonin MT1 receptor is suggested to be involved in the regulation of apoptosis. Indeed, Winczyk *et al* (Winczyk *et al.*, 2002) observed that UCM386 (MT1 receptor agonist) exerts a pro-apoptotic and anti-proliferative effect as did melatonin in tumor cells. Similar action was also observed in ductal breast carcinoma and murine melanoma cells (Jablonska *et al.*, 2013, Jawed *et al.*, 2007). During human osteoblast differentiation and growth, Radio, et al. (Radio *et al.*, 2006) showed an important role of melatonin through MT2 melatonin receptor stimulation with subsequent activation of MEK/ERK 1/2 signaling pathway. Thus, in our study, increased expression of MT1 during syncytiotrophoblast formation is more likely linked to an apoptotic predominant action meanwhile high expression of MT2 in villous cytotrophoblast suggests an initial principal role in villous cytotrophoblast development and differentiation.

Syncytiotrophoblast is the main endocrine unit of the placenta. These cells secrete proteins and steroid hormones essential for pregnancy well-being and fetal development (Orendi *et al.*, 2010). β-hCG plays a key role in the differentiation and invasion of trophoblast cells, in pregnancy maintenance through preventing corpus luteum regression thus prevents progesterone drop, and as a marker of pregnancy from the 2nd week of pregnancy (Cole, 2010, Fujimoto *et al.*, 2002, Garrido-Gomez *et al.*, 2010, Gudermann *et al.*, 1992, Licht *et al.*, 2007). We previously showed that melatonin agonist modulates β-hCG secretion in placental cell lines (BeWo and JEG-3) through MT1 and/or MT2. Specifically, 6- chloromelatonin (melatonin receptor agonist) decreases β-hCG via activating Gi/o but increases β-hCG through stimulating PTX insensitive Gq (Lanoix *et al.*, 2006). In our study, melatonin (1mM) stimulated trophoblast differentiation

and increased the fusion of mononucleated villous cytotrophoblast into multinucleated syncytiotrophoblast, which was accompanied by an increased β-hCG production through an action mediated by MT1 and MT2. Luzindole, a selective MT1/MT2 antagonist, did not block the stimulatory effect of melatonin on hCG release. However, it also increased β-hCG secretion displaying an additive stimulatory effect. This phenomena could be explained by the reported cases of unexpected agonist action of luzindole in certain cell types, as well as its powerful antioxidant activity (Mathes *et al.,* 2008). β-hCG is recognized to be a key regulator of villous trophoblast differentiation. Consequently, melatonin is suggested to have a pivotal role in trophoblast differentiation as well as placental growth. Nevertheless, the specific signaling pathway involved in β-hCG secretion/production in both human trophoblast and BeWo cells still remains to be identified.

In this study we report, for the first time, that melatonin generating system is expressed in human placental tissues throughout pregnancy with higher expression of AANAT & HIOMT in the third trimester and greater expression of MT1 receptor in the first trimester. Moreover, during the differentiation of villous cytotrophoblast into syncytiotrophoblast, MT1 receptor expression is increased while MT2 is decreased, suggesting different roles of these receptors during trophoblast syncytialisation. Melatonin also plays an essential role in enhancing villous trophoblast differentiation and hCG secretion. Combined with our previous observations and this study the placenta seems to be the main origin of high melatonin levels in maternal blood throughout pregnancy, coordinating early pregnancy events along with guarding the feto-placental unit from oxidative stress until late pregnancy and finally it facilitates parturition. In summary, our data suggest an essential role of this indolamine in placental function and as a result, on pregnancy well-being and fetal development.

AUTHOR CONTRIBUTIONS

AS analyzed all the data, designed and conducted the protein expression of AANAT enzyme throughout pregnancy and wrote the manuscript. AAL and DL conducted and designed most of the experiment. LF and VB revised and critically analyzed the article and offered valuable suggestions. CV designed the research, analyzed data and revised the paper.

ACKNOWLEDGEMENTS

This work was supported financially by the discovery grant of the Natural Sciences and Engineering Research Council of Canada (NSERC) (no. 262011-2009) to CV and by studentship awards from the Fondation Armand Frappier and Réseau Québécois en reproduction (RQR)-NSERC-Collaborative Research and Training Experience Program (CREATE) Program to AS. AAL received a studentship from NSERC and Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), and DL received Fonds de la Recherche en Santé du Québec (FRSQ) doctoral scholarship. LSF from the Ministère de l'Éducation, du Loisir et du Sport (MELS - FRQNT).

CONFLICTS OF INTEREST

The authors confirm that there is no conflict of interest concerning the data elucidated in the manuscript.

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FIGURES LEGENDS

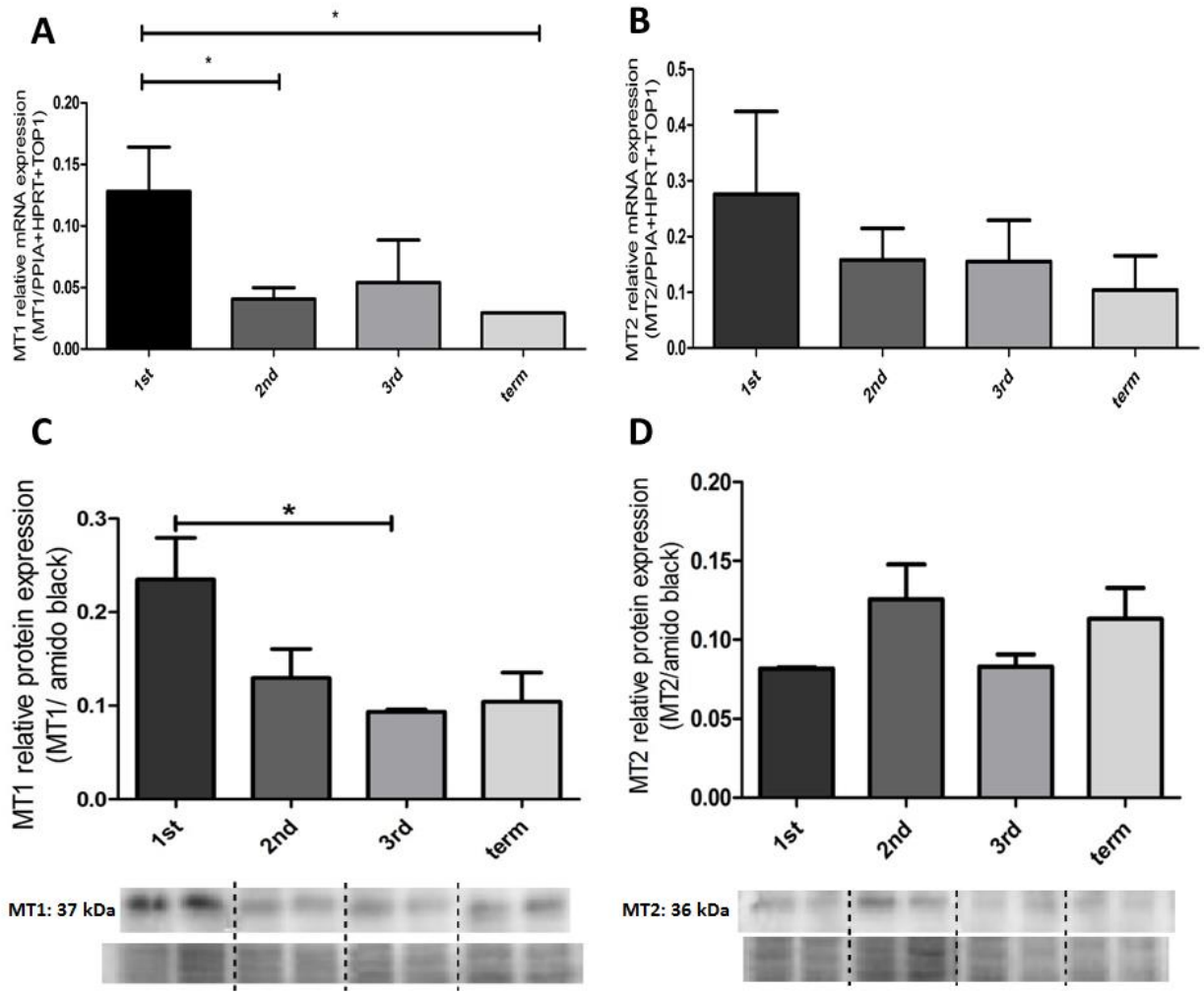


Fig. 1 Expression of melatonin receptors in human placenta at different trimesters of pregnancy. mRNA level of MT1 (**A**) and MT2 (**B**) melatonin receptors were analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of PPIA, HPRT1 and TOP1 was used to normalize gene expression. Protein expression of MT1 (**C**) and MT2 (**D**) was determined using semi-quantitative Western Blot. Amido black stain was used to normalize protein expression. Results shown are mean ± SEM. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test PPIA: peptidylprolyl isomerase A; HPRT1:

hypoxanthine phosphoribosyltransferase 1; TOP-1: topoisomerase-1. 1st trimester (7-12 weeks): n=3; 2nd trimester (14-26 weeks): n=6; 3rd trimester (27-34 weeks): n=6; term (37-41 weeks): n=3. * $P \leq 0.05$; *** $P \leq 0.001$.

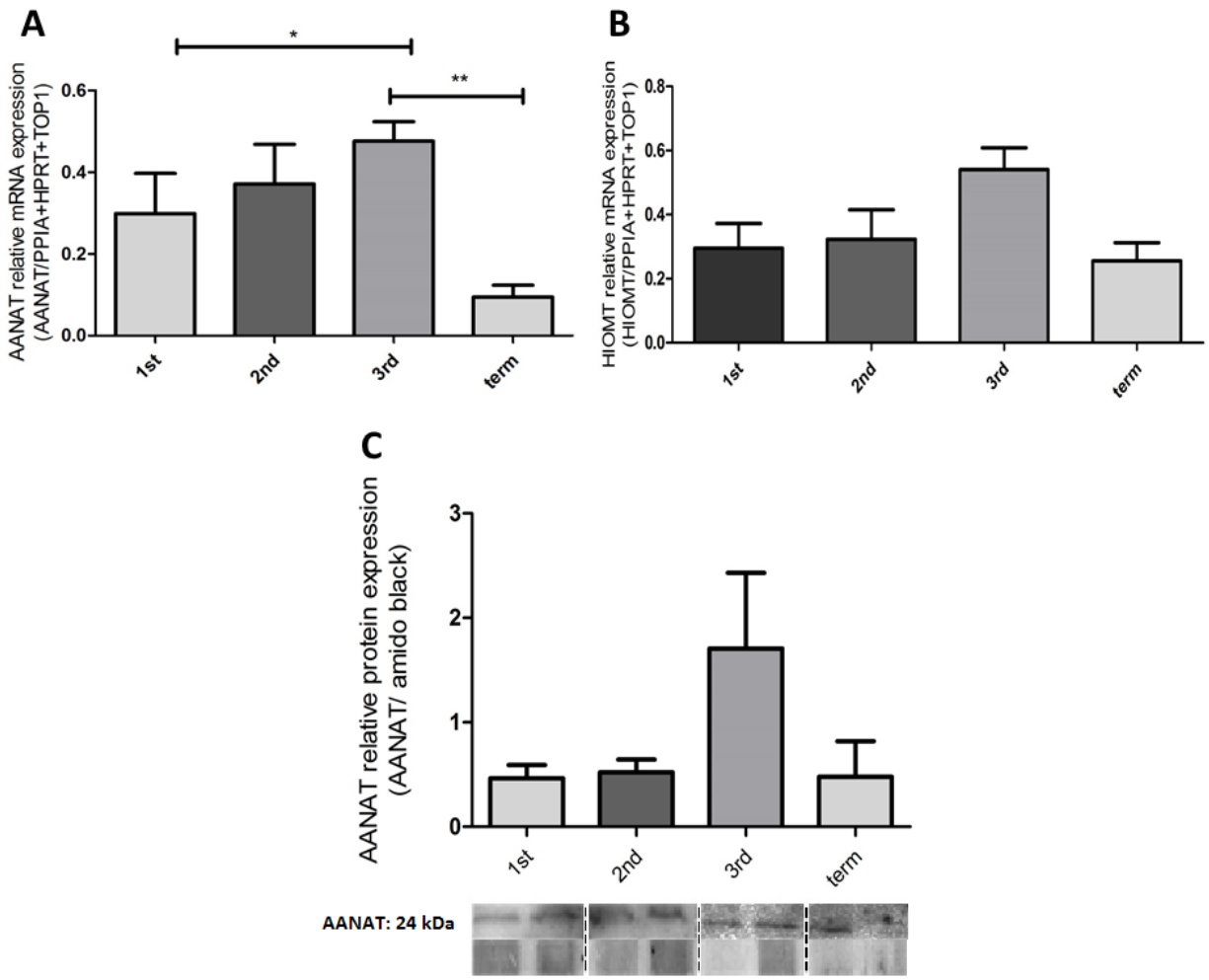


Fig 2 Expression of melatonin-synthesizing enzymes in human placenta at different trimesters of pregnancy. AANAT (**A**) and HIOMT (**B**) melatonin-synthesizing enzymes mRNA level was analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of PPIA, HPRT1 and TOP-1 was used to normalize gene expression. Protein expression of AANAT **(C)** was determined using semi-quantitative Western Blot. Amido black

stain was used to normalize protein expression. Values are represented as mean ± S.D. All data were analyzed by one-way ANOVA followed by Tukey's post hoc test. AANAT: aralkylamine N-acetyltransferase, HIOMT: hydroxyindole O-methyltransferase, PPIA: peptidylprolyl isomerase A, HPRT1: hypoxanthine phosphoribosyltransferase 1,TOP1: topoisomerase-1. 1st trimester (7-12 weeks): n=3; 2nd trimester (14-26 weeks): n=6; 3rd trimester (27-34weeks): n=6; term (37-41 weeks): n=3. * $P \leq 0.05$; *** $P \leq 0.001$.

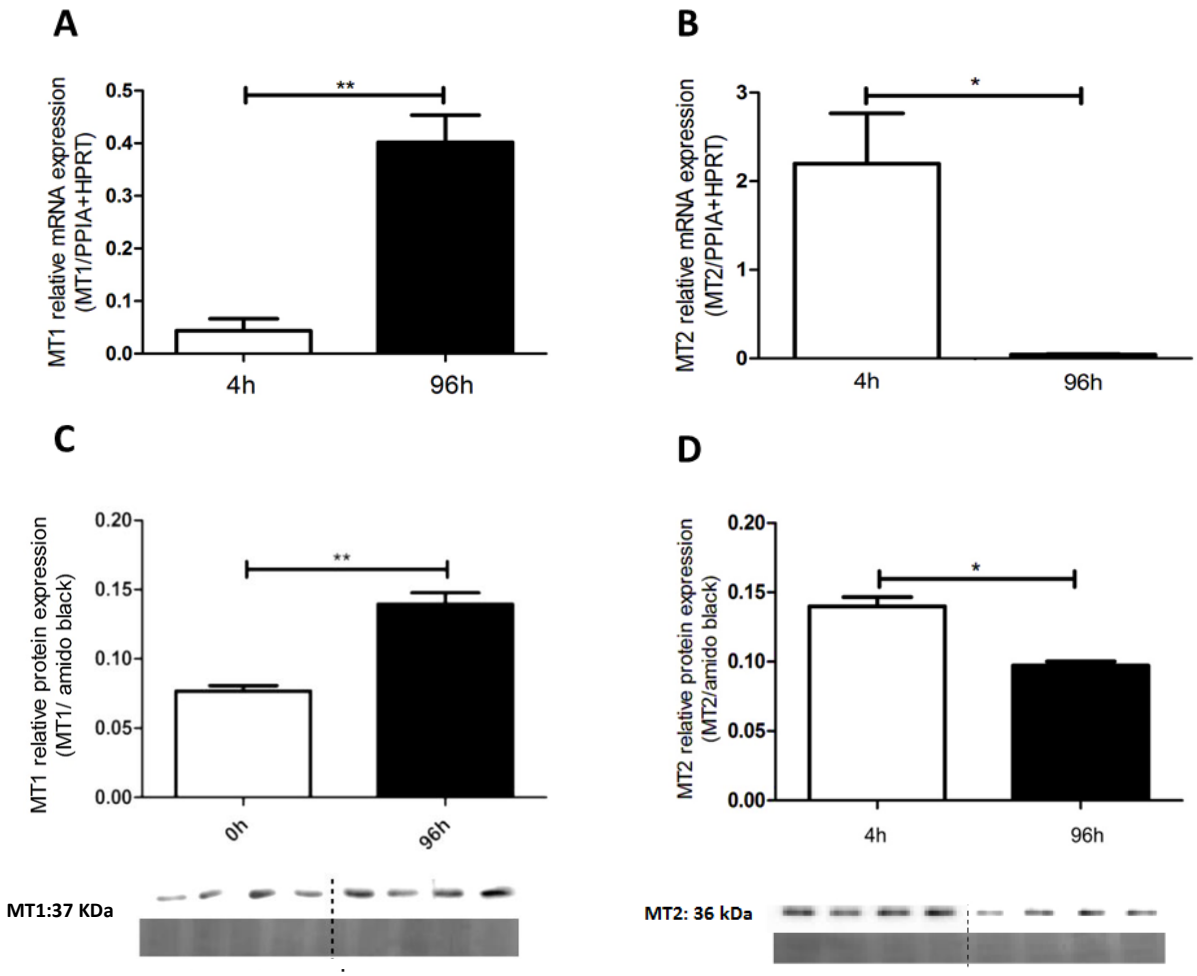


Fig 3 Expression of MT1 and MT2 melatonin receptors during villous trophoblast differentiation. Villous cytotrophoblasts were isolated and purified from normal human term

placentas. Melatonin receptors expression was determined at 4h (villous cytotrophoblast) and 96h (syncytiotrophoblast) of culture. MT1 (**A**) and MT2 (**B**) melatonin receptors mRNA was analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of PPIA and HPRT1 was used to normalize gene expression. Protein expression of MT1 (**C**) and MT2 (**D**) was determined using semi-quantitative Western Blot. Amido black stain was used to normalize protein expression. Values are represented as mean ± S.D. All data were analyzed by Student's t-test. PPIA: peptidylprolyl isomerase A, HPRT1: hypoxanthine phosphoribosyltransferase 1. 4h: n=3; 96h: n=3. * $P \leq 0.05$; *** $P \leq 0.001$.

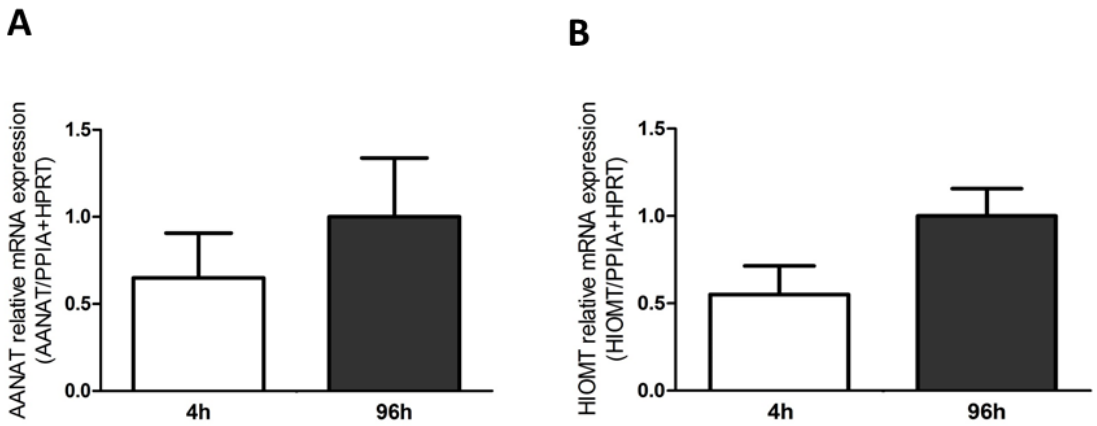


Fig. 4 Expression of AANAT and HIOMT melatonin-synthesizing enzymes during villous trophoblast differentiation. Villous cytotrophoblasts were isolated and purified from normal human term placentas. Melatonin synthesizing enzymes expression was determined at 4h (villous cytotrophoblast) and 96h (syncytiotrophoblast) of culture. AANAT (**A**) and HIOMT (**B**) mRNAs was determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The geometric average of PPIA and HPRT1 was used to normalize gene expression. Results shown are mean ± SEM. All data were analyzed by Student's t-test. AANAT: aralkylamine N-

acetyltransferase, HIOMT: hydroxyindole O-methyltransferase, PPIA: peptidylprolyl isomerase A, HPRT1: hypoxanthine phosphoribosyltransferase 1. 4h: n=3; 96h: n=3

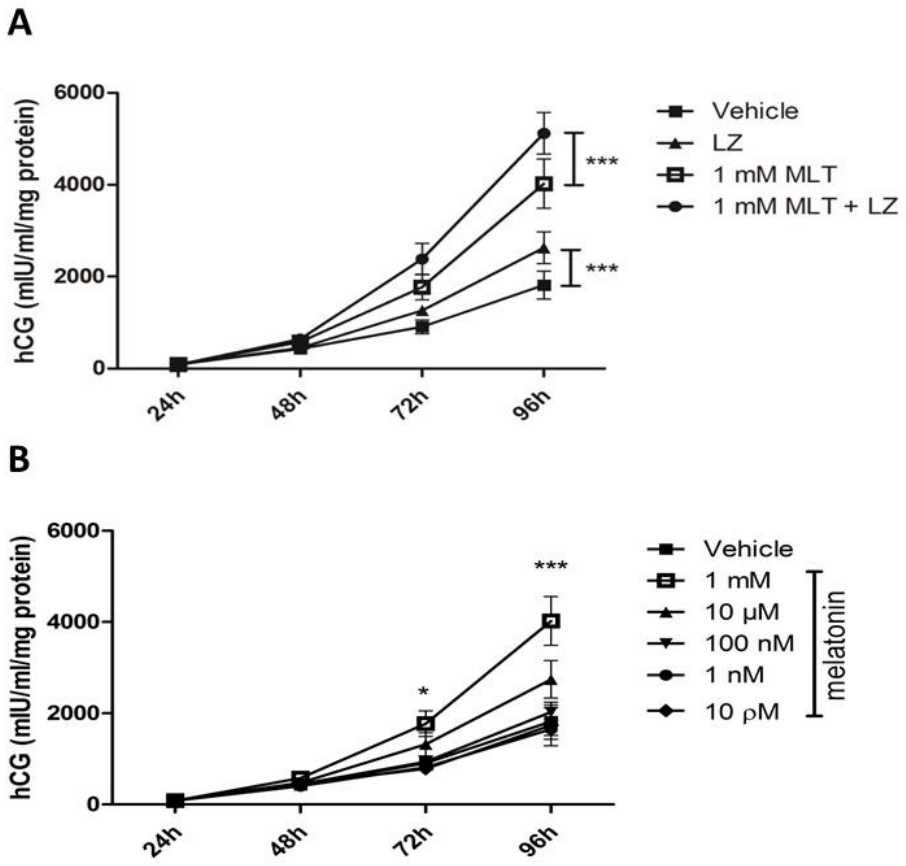


Fig. 5 Effect of melatonin and MT1/MT2 receptors antagonist luzindole on β-hCG release by syncytiotrophoblast isolated from human normal term placentas. (**A**) Cells were treated with increasing concentrations of melatonin or vehicle (0.1 % DMSO). (**B**) Cells were treated with 1 mM melatonin (MLT), 1 mM melatonin combined with 1 mM luzindole (a selective MT1 and MT2 receptor antagonist (MLT + LZ)), 1mM luzindole (LZ) or with vehicle. All treatments were replenished daily. Values are represented as mean ± S.D (n=3). All data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. Data have been normalized against the

protein content of whole cell lysate from corresponding culture well. *: $P < 0.05$; ***: $P < 0.001$. In **A**, the statistical difference is determined between vehicle and 1 mM melatonin.

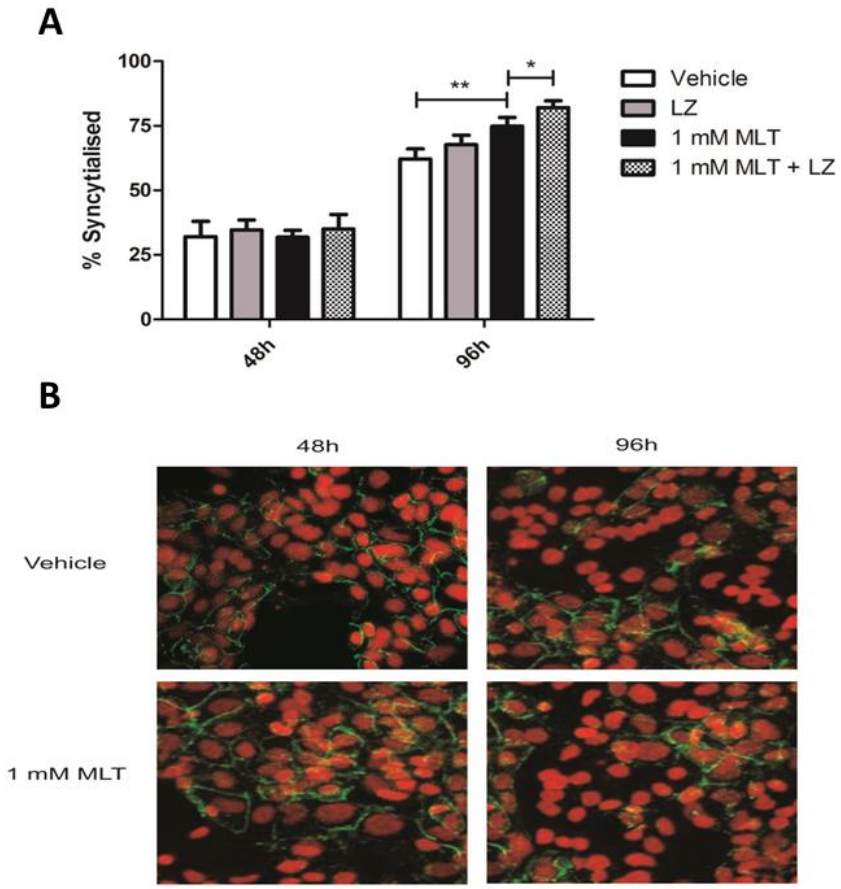


Fig. 6 Effect of melatonin and MT1/MT2 receptors antagonist luzindole on villous cytotrophoblast syncytialisation. Villous cytotrophoblast cells were isolated from human term placentas. (**A**) Level of syncytialisation at 48 h and 96 h of culture in the presence of 1 mM melatonin (MLT), 1 mM Luzindole (a selective MT1 and MT2 receptor antagonist (MLT + LZ)), 1mM luzindole (LZ) or vehicle (0.1 % DMSO). Values present mean ± S.D. (n=3). Data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. *: $P < 0.05$; **: $P < 0.01$. (**B**) Representative immunofluorescence staining of desmoplakin protein in untreated and in 1

mM melatonin treated primary villous trophoblasts, fixed in methanol, after 48 h and 96 h in` culture. Magnification, x30. All treatments were replenished daily.

Table 1 Genes and primer sequences used for RT-qPCR.

Gene	Accession number	Primer sequence	
		Sense	Antisense
AANAT	NMNM_001088	GCTGTCAGCGCCTTT	ACGCCCAAGACGGA
HPRT1	NM_ 000194	GACCAGTCAACAGGGGACATAA	AAGCTTGCGACCTTGACC
TOP-1	NM_ 003286	GGCAGAGTGAATCTAAGG	CTTAAAGGGTACAGGAATG
PPIA	NM_021130	GTTTGCAGACAAGGTCCCA	ACCCGTATGCTTTAGGATG
HIOMT	NM_004043	GGCGAGCGGCTACAGTT	ACAGGTCAAAGGCGGTCA
MT1	NM_005958	CCTGTCGGTGTATCGGAACA	TGACTTGGCAGTGCAGATAGC
MT2	NM_005959	TACGACCCACGCATCTATTCC	AGGTAGCAGAAGGACACGACA

AANAT: aralkylamine N-acetyltransferase; HIOMT: hydroxyindole O-methyltransferase; PPIA: peptidylprolyl isomerase A; TOP-1: topoisomerase1; HPRT1: hypoxanthine phosphoribosyltransferase 1.

Table 2 Antibodies used for western blot analysis.

Antibody	Dilution	Incubation	Source
Primary antibody			
anti-AANAT	1:10 000	O/N at RT	Millipore (AB5467)
anti-MT1	1:1000	O/N at 4ºC	Abnova (H00004543-A01)
Anti-MT2	1:1000	O/N at 4ºC	Novus Biologicals (NBP1-00922)
Secondary antibody			
anti-mouse-HRP	1:10 000	1 h at RT	Millipore (AP192P)
anti-rabbit-HRP	1:10 000	1 h at RT	Millipore (AP182P)

AANAT: aralkylamine N-acetyltransferase; HRP: horseradish peroxidase; O/N: overnight; RT: room temperature.

Supplemental data

Table 1: Characteristics of patients and placentas

Variables	Normal pregnancy (n =29)			
	1st trimester (n =5)	2nd trimester (n =14)	3rd trimester (n =6)	Term (n =4)
Maternal age (years)	16.3 ± 0.57	20.1 ± 7.70	29.7 ± 2.33	27.7 ± 11.4
Gestational age (years)	9.2 ± 1.64	18.07 ± 3.3	31 ± 3	39.5 ± 1.29
Mode of delivery (A/V/C)	A	A	V:C= 3:3	V:C= 2:2
Newborn sex (F:M)	---	---	3:3	3:1
Newborn weight (g)	---	---	1769.2 ± 584.4	3565 ± 422.6
Placenta weight (g)	---	---	252.8 ± 83.3	566.3 ± 122.4
Placental index	---	---	0.149 ± 0.054	0.158 ± 0.023

For the placenta from abortion, pathological analysis of the placenta indicated no abnormalities. A, abortion C, caesarean V, vaginal section; F, female; M, male.

Melatonin content assay

For the evaluation of melatonin content, primary villous trophoblasts were cultured in 96-well plate (125 000 cells/well). The cell culture medium was collected every 24 h (from 4 h to 96 h of culture) and centrifuged, after which supernatants were stored at -80°C until assayed.

The content of melatonin was evaluated in culture medium in duplicate by enzyme-linked immunosorbent assay (ELISA) using kits according to manufacturer's instruction (IBL- RE 54021, Hamburg, Germany). The detection range specified by the assay is 3.0 - 350 pg/mL. Values are expressed as melatonin secretion ± S.E.M.

Results

Figure 1 shows melatonin levels during trophoblast differentiation (from 4 h to 96 h of culture). Melatonin production (de novo) is similar during villous trophoblast differentiation.

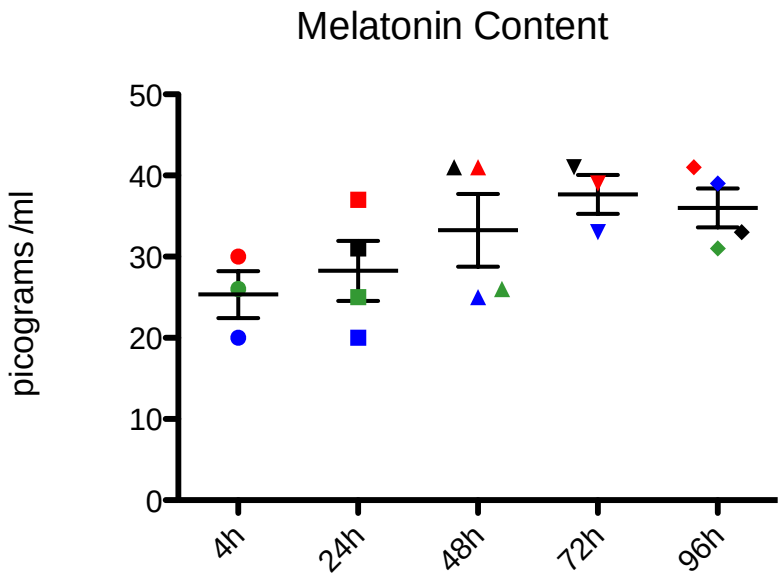


Figure 1 Melatonin levels during trophoblast differentiation (primary cell). Primary villous trophoblasts were cultured for 96 h, the cell culture medium was collected every 24 h (from 4 h to 96 h of culture). The content of melatonin was assessed in culture medium by ELISA as described in Materials and Methods. Results are represented as the mean ± SEM (n = 4 different placentas) and were analyzed with ANOVA paired-test, but no statistical difference was obtained.

3.2: Mechanisms by which melatonin and its receptors regulate β-hCG secretion

Materials and Methods:

Materials

Phospholipase C (PLC) inhibitor (U73122), MEK1/2 Inhibitor (U0126), 4P-PDOT (selective MT2 antagonist) and luzindole (MT1 and MT2 antagonist) were bought from Tocris Bioscience (Minneapolis, MN). Melatonin (M5250) and MEM/Ham's F-12K culture medium was purchased from Sigma-Aldrich (Oakville, ON, Canada). Fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT). TrypLE™ Enzyme was bought from Life technologies, Carlsbad, California. Cell-Bind T-75 flasks, 6 and 96 wells plates were purchased from Corning Life Science (Thermo Scientific, Canada). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL). Horseradish peroxidase (HRP)-conjugated secondary antibodies, and Re-Blot Plus Mild Antibody Stripping Solution were obtained from Millipore (Mississauga, ON, Canada). Lipofectamine ® RNAiMAX transfection reagent and Opti-MEM® reduced serum medium were purchased from (Invitrogen). Monoclonal antibody against phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204 - catalog # 4370S) was purchased from Cell signaling (Beverly, MA). Polyclonal antibody against ERK1/2 was purchased from Millipore, catalog # 06-182. MemCode™ Reversible Protein Stain Kit, Protease Inhibitor Cocktail and Phosphatase Halt Inhibitor Cocktail were from (Thermo Scientific, Canada). Trans-Blot® Turbo™ Mini PVDF membranes and Clarity™ Western ECL substrate was from Bio-Rad Laboratories (Mississauga, Canada). All other reagents and chemicals were purchased from Sigma Aldrich or Fisher Scientific.

BeWo cell culture

Human placental choriocarcinoma cell lines, BeWo, were acquired from the American Type Culture Collection (ATCC) (Rockville, MD). BeWo cells were cultured in MEM/Ham's F-12K culture medium with 10% FBS in Corning™ 75 cm^2 Cell Culture Flasks and kept in a humidified atmosphere of 5% CO_2 at 37ºC. Cells were split when they reached around 90% confluence, using TrypLE™.

Cell treatment

For β-hCG secretion analyses, BeWo cells were treated with an increasing concentrations of melatonin (1 nM, 10 µM and 1 mM) or vehicle (0.1 % DMSO) in the presence and/ or absence of forskolin (20 µM). In the presence of forskolin (20 µM), the effect of melatonin (10 µM) combined or not with Luzindole (10 µM), luzindole alone (10 µM) or vehicle has been analyzed. Similarly, in the presence of forskolin (20 µM), the effect of melatonin (10 µM) with or without 4P-PDOT (10 µM), 4P-PDOT alone (10 µM) or vehicle has also been analyzed. Cells were also treated with melatonin (10 µM) with or without of 1 h-pretreatment by PLC inhibitor (U73122 - 560 nm) or MEK 1/2 Inhibitor (U0126 – 25 µM) in the presence or absence of forskolin (20 µM).

To examine if melatonin stimulates ERK1/2, Bewo cells were cultured in a complete (10% FBS) medium for 24 h to allow cell attachment. Afterwards, cells were subjected to FBS deprivation (0% FBS) for 18 h, then were stimulated for 0, 5, 15, 30, and 60 min with 10 µM melatonin with or without 10 µM luzindole or 10 µM 4P-PDOT in culture medium containing 0.1% FBS. Stock solutions were diluted in dimethyl sulfoxide (DMSO). Further dilutions were made fresh on the day of experiment (with a final

concentration of DMSO at < 0.1%). The role of melatonin receptors was further explored using anti-sense technology with siRNA sequences for MT1 and MT2 receptors designed and synthesized by Qiagen according to their service guarantee 100% (HP Guaranteed siRNA). Briefly, after harvesting the cells in the plate in normal growth medium, the cells were transfected with siRNA using the Lipofectamine ® RNAiMAX transfection reagent according to manufacturer's instruction (Invitrogen).

Protein extraction and Western Blot analysis

Total protein was extracted as following: cells were washed two times with cold phosphate buffered saline (PBS) then harvested by scraping in 150 µL of cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 175 mM NaCl, pH 7.4) containing 1 µL Protease Inhibitor Cocktail and 1 µL Phosphatase Halt Inhibitor Cocktail. Lysates were mixed thoroughly by agitating for 30 min while kept on ice then centrifuged for 15 min at 14000 × g at 4 ºC. The supernatants were cautiously removed and stored at -80 ºC until analysis. BCA protein assay reagent along with the bovine serum albumin (BSA) as standard was used to measure the concentration of the protein in each sample. Assay procedures were done according to the manufacturer instructions and using spectrophotometric quantification (SpectraMax M5 with SoftMax Pro v5 software, Molecular Device, Sunnyvale, USA).

Proteins (40 µg) diluted in sodium dodecyl sulfate (SDS) sample buffer were heated for 7 min at 95 ºC for denaturation. Total proteins were then fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE - 4% stacking and 10% separating gels) then transferred to PVDF membranes. The membranes were blocked to avoid non-specific binding with Tris buffered saline solution (TBS) (20 mM Tris-HCl pH 7.4,

150 mM NaCl) containing 5% skim milk for 1 h at room temperature. Membranes were incubated with phosphospecific antibodies against p-ERK1/2 (1:1000 - overnight at 4ºC) diluted in TBS containing 5% BSA. Membranes were washed three times for 10 min with TBS containing 0.1% Tween (TBS-T) and then incubated with horseradish peroxidase conjugated anti-rabbit IgG secondary antibodies for 1 h at room temperature. Membranes were then washed 3 times for 10 min with TBS-T 0.1%. Bands were visualized with Clarity™ Western ECL substrate, scanned using ChemiDoc™ XRS+ System, and their intensities were quantified by densitometric analysis with Image Lab software (Bio-Rad Laboratories, Canada). The PVDF membranes were stripped with Re-Blot plus Mild solution at room temperature for 15 min, washed twice for 5 min with TBS then reprobed with antibodies against the non-phosphorylated proteins ERK1/2 (i.e. total protein) (1:1000 - overnight at 4ºC). For the normalization of total proteins transferred to PVDF, membranes were stained with MemCode™ Reversible Protein Stain Kit following the manufacturer protocol. Proteins expression was expressed as relative fold expression of MemCode band density.

β–hCG secretion

Bewo cells were cultured in 96-well plate (20,000 cells/well) for 24 h in complete culture medium then treated with increasing concentrations of melatonin, luzindole ,4-P-PDOT, with or without a pre-treatment of U0126 and U73122 for 1 h or vehicle (0.1 % DMSO) in the presence of 0.1% FBS. Culture media were collected every 24 h (from 24 h to 48 h of culture) centrifuged, and supernatants were stored at -20°C until assayed. The secretion of β-hCG was assessed in culture medium in triplicate by enzyme-linked

immunosorbent assay (ELISA) using DRG β-HCG ELISA Kit (EIA-1911) following the manufacturer's instruction (DRG International, Mountainside, New Jersey).

Statistical analysis

Statistically significant differences among treatments were tested using one-way ANOVA tracked by a Newman-Keul's post-hoc test. All statistical and graphical analyses, including concentration response analyses, were performed using Prism 5.0 (GraphPad, San Diego, CA) and a probability value of $P < 0.05$ was considered statistically significant.

RESULTS

Melatonin affects basal and stimulated β-hCG secretion in BeWo cells.

Figure 1 shows the effect of increasing concentrations (1 mM, 10 µM, 1 nM) of melatonin on β-hCG secretion in BeWo cells, in the presence or absence of forskolin (20 µM), which stimulates β-hCG secretion throughout the direct activation of AC pathway. Compared to control (DMSO), melatonin increases significantly basal β-hCG secretion at 1 mM (41% vs. control) **(Fig. 1A)** while it inhibits significantly forskolin-stimulated β-hCG secretion (22.8 % vs. control) **(Fig. 1B).**

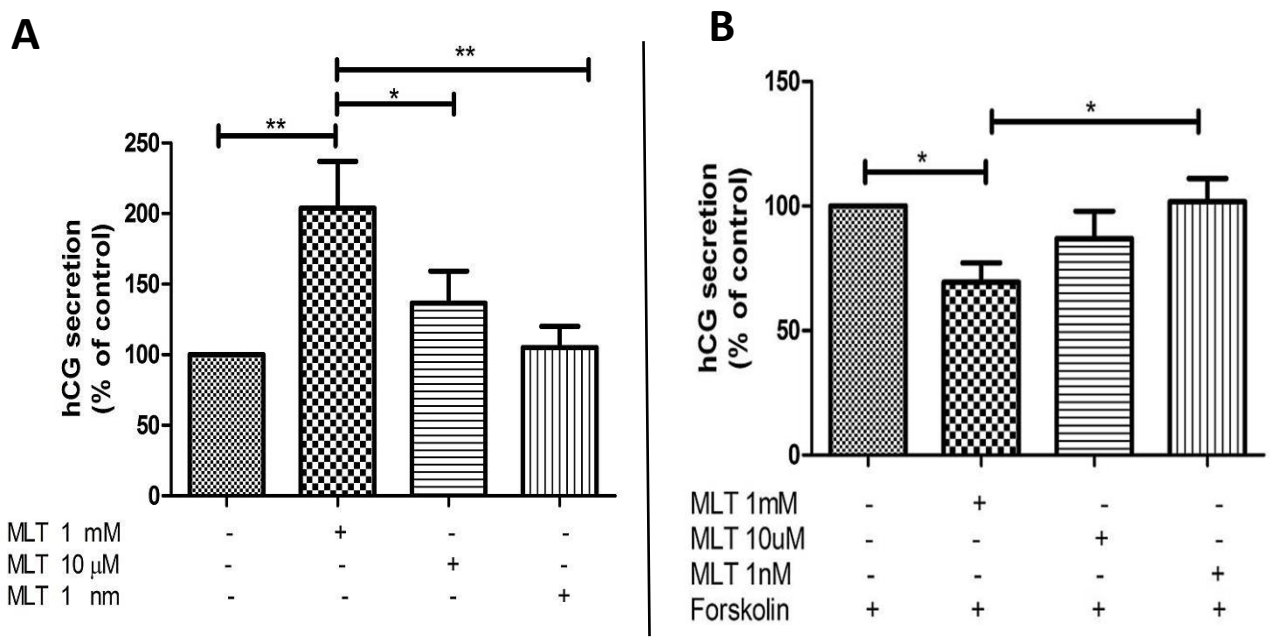


Figure 1 Melatonin effect on β-hCG secretion in BeWo cells. Cells were treated for 48 h with 1 mM, 10 µM or 1 nM of melatonin (MLT) or solvent alone (DMSO 0.1%, control) diluted in culture medium containing 0.1% FBS, in the absence **(A)** or presence **(B)** of forskolin (20 µM). The secretion of β-hCG was assessed in culture medium by ELISA as described in Materials and Methods. Results are represented as the mean ± SEM (n = at least 3 independent experiments (cell passage) and are presented as the percentage of either DMSO or forskolin; **P* < 0.05, ***P* < 0.01).

Effect of MT1 and MT2 receptors antagonists on melatonin regulation of β-hCG secretion in BeWo cells. **Figure 2** shows that treating the cells with luzindole (MT1/ MT2 antagonist) and melatonin together inhibits melatonin effect on β-hCG secretion, compared to luzindole alone **(Fig. 2A, B)**. Alternatively, treating the cells with 4P-PDOT (MT2 antagonist) and melatonin together does not inhibit completely melatonin effect on β-hCG secretion compared to 4P-PDOT **(Fig. 2C, D)**. Although the results were not statistically significant except in **(Fig. 2A**) they could indicate MT1 receptor involvement.

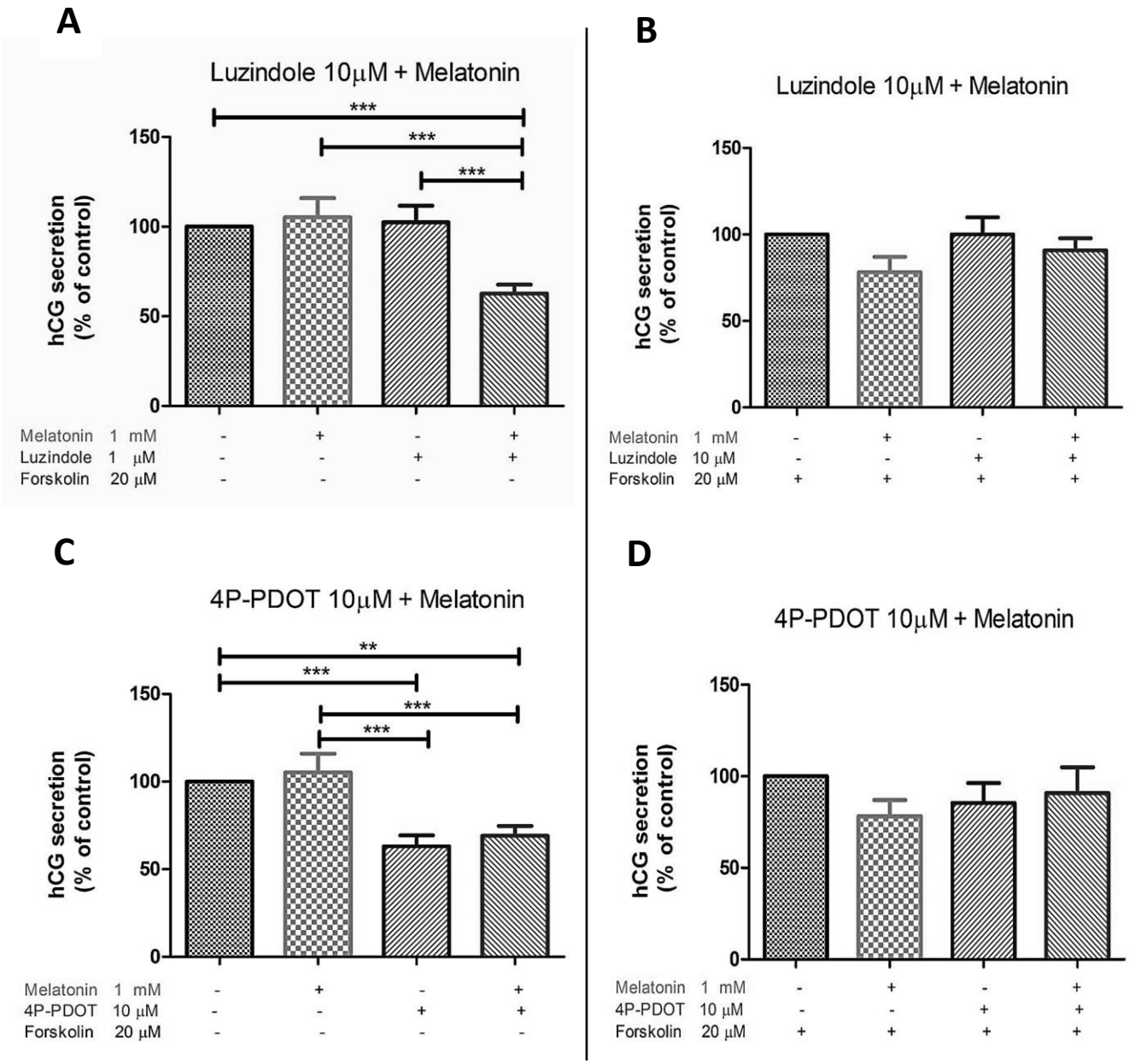


Figure 2 Effect of MT1 and MT2 melatonin receptors antagonists on β-hCG secretion in BeWo cells. Cells were treated for 48 h with 1 mM of melatonin (MLT) or solvent alone (DMSO 0.1%, control) with or without (A) MT1/MT2 antagonist (luzindole; 10 μM) or (B) MT2 antagonist (4P-PDOT; 10 μM), in the presence or absence of forskolin, as described in Methods. Secretion of β-hCG was assessed by ELISA as described in Materials and Methods. Results are represented as the mean ± SEM (n = at least 3 independent experiments and are presented as the percentage of either DMSO or forskolin; $^{**}P < 0.01$, $^{***}P < 0.001$).

Effect of PLC-β and ERK ½ inhibitors on melatonin regulation of β-hCG secretion in BeWo cells. A significant inhibition of β-hCG secretion was observed in the presence of (U73122 - 560 nM) and (U0126 - 25µM). This suggests the implication of the PLC-β and ERK1/2 signaling pathways in the stimulatory effect of 1mM of melatonin on basal β-hCG secretion.

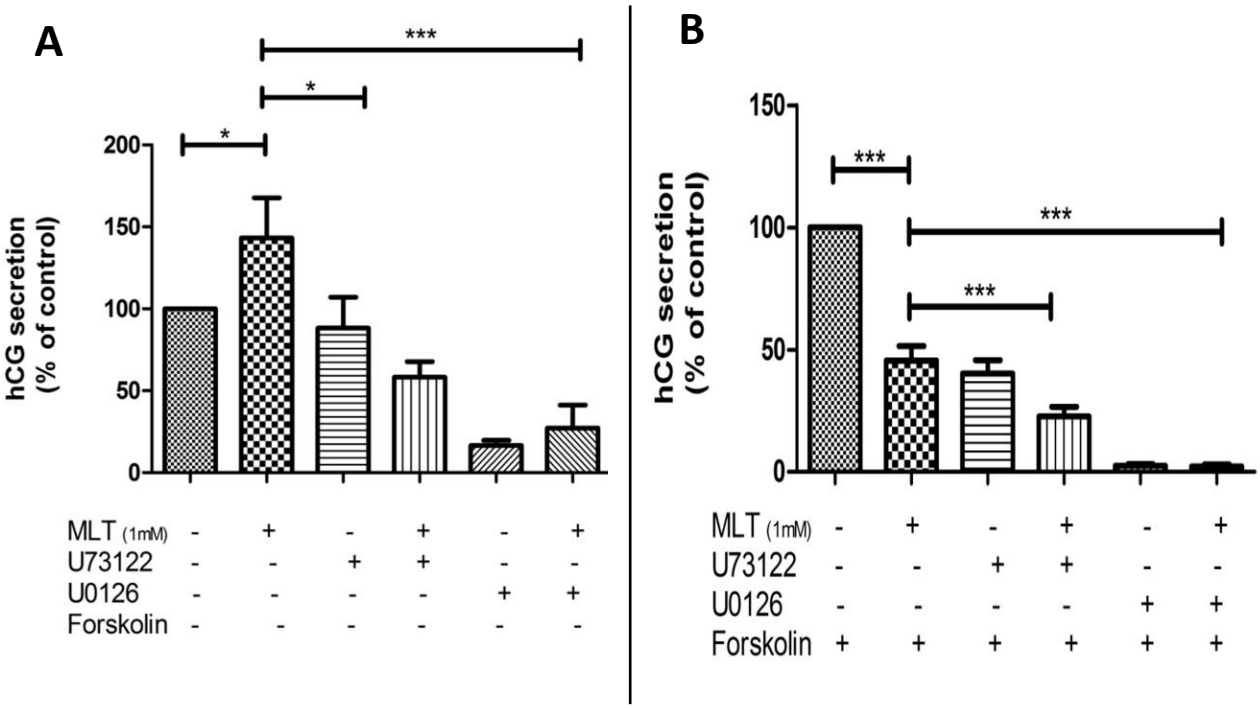
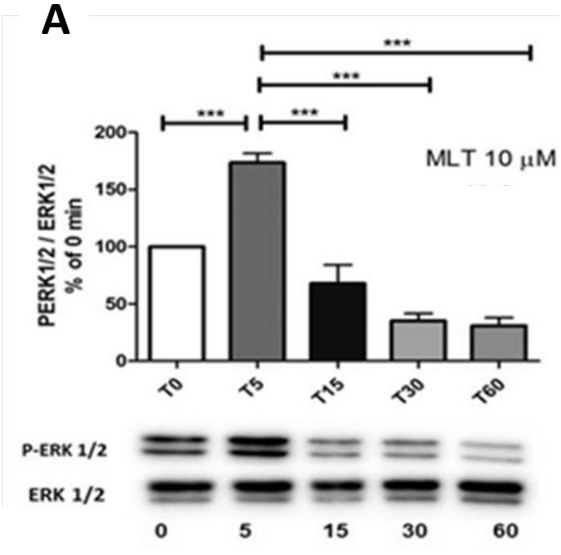
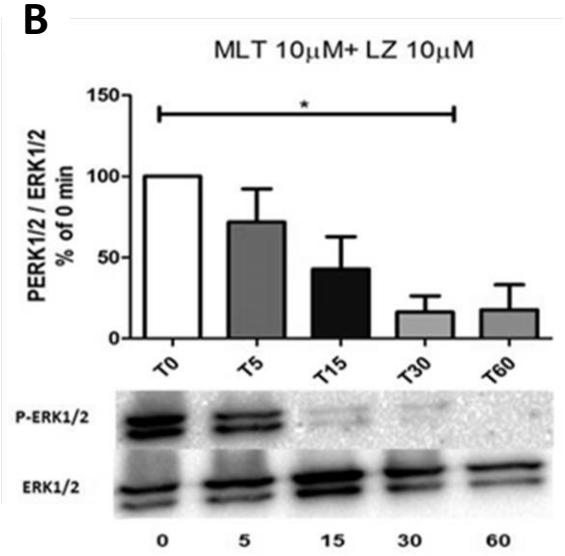


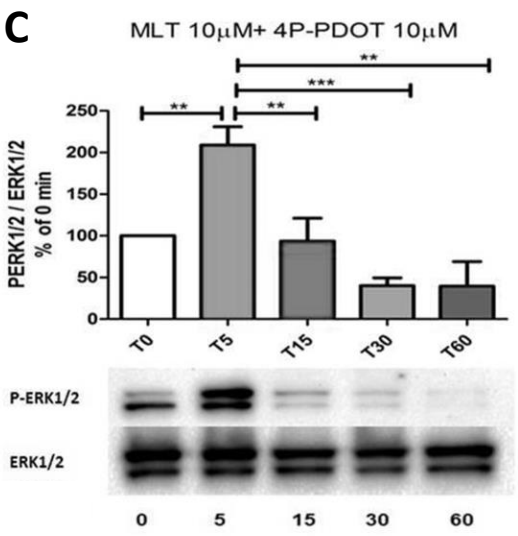
Figure 3 Effect of PLC and ERK½ inhibitors on β-hCG secretion in BeWo cells, in the presence and/ or absence of melatonin (MLT) and forskolin. Cells were pre-treated for 1 h with PLC-β Inhibitor (U73122 - 560 nm) or MAPK Inhibitor (U0126 - 25µM) or solvent alone (DMSO 0.1%, control) diluted in culture medium supplemented with 0.1% FBS. Then cells were treated for 48 h with 1mM MLT or solvent alone (DMSO 0.1%, control) diluted in 0.1% FBS culture medium, in the absence (A) or presence (B) of forskolin (20 µM). The secretion of β-hCG was assessed in culture medium by ELISA, as described in (Materials and Methods) using EIA-1911 kits. Results revealed are represented as the mean ± SEM (n = 2 independent experiments with at least 4 technical replicates and are presented as the percentage of either DMSO or forskolin; *$P < 0.05$, ***$P < 0.001$).

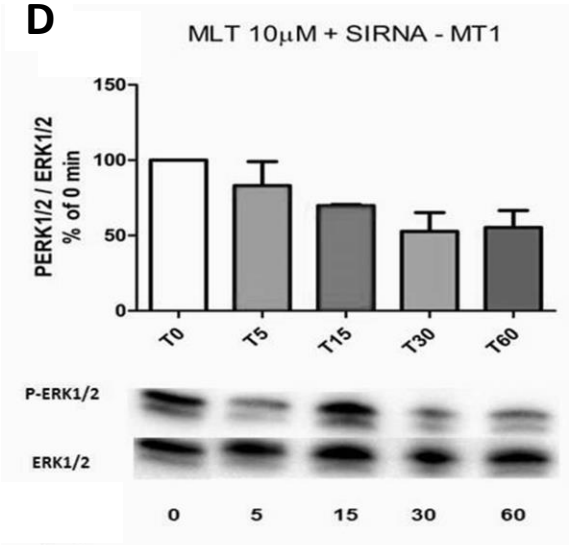
Effect of melatonin and its receptors on ERK1/2 phosphorylation in BeWo cells

Melatonin (10µM) increases significantly ERK1/2 phosphorylation at 5 min of culture (73% vs. control) **(Fig. 4A)**. To identify if melatonin stimulatory effect was receptor dependent, selective melatonin receptors antagonists and siRNA against MT1 and MT2 receptors were used. Luzindole (10 µM) inhibited melatonin-induced ERK1/2 activation **(Fig. 4B)**. On the other hand, 4P-PDOT (10 µM) cannot block melatonin stimulatory effect on ERK1/2 **(Fig. 4C)**. Specific siRNA towards MT1 receptor reversed melatonin stimulatory effect on ERK ½ protein phosphorylation **(Fig. 4D)**, while specific siRNA towards MT2 receptor has no effect **(Fig. 4E)**. Taken together, these results suggest an important role of melatonin and its MT1 receptor in the activation of ERK1/2 and consequently β-hCG secretion.


A
MLT 10 μM
PERK1/2 / ERK1/2
% of 0 min
T0
T5
T15
T30
T60
P-ERK 1/2
ERK 1/2
0
5
15
30
60


B
MLT 10μM+ LZ 10μM
PERK1/2 / ERK1/2
% of 0 min
T0
T5
T15
T30
T60
P-ERK1/2
ERK1/2
0
5
15
30
60


C
MLT 10μM+ 4P-PDOT 10μM
PERK1/2 / ERK1/2
% of 0 min
T0
T5
T15
T30
T60
P-ERK1/2
ERK1/2
0
5
15
30
60


D
MLT 10μM + SIRNA - MT1
PERK1/2 / ERK1/2
% of 0 min
T0
T5
T15
T30
T60
P-ERK1/2
ERK1/2
0
5
15
30
60

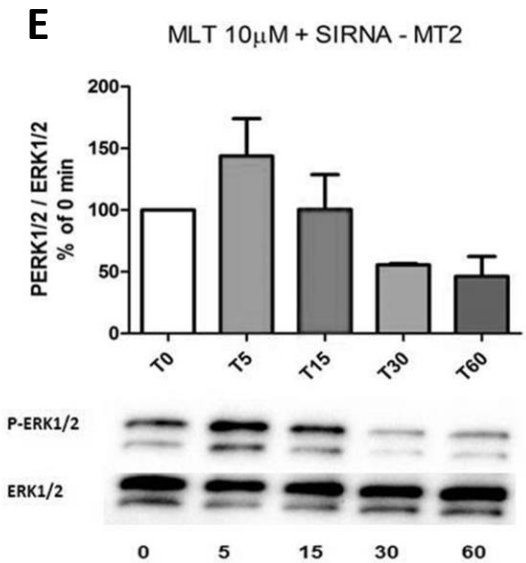

E
MLT 10μM + SIRNA - MT2
PERK1/2 / ERK1/2
% of 0 min
T0
T5
T15
T30
T60
P-ERK1/2
ERK1/2
0
5
15
30
60

Figure 4 Effect of melatonin and its receptors on ERK1/2 in BeWo cells. Cells were incubated with **(A)** 10 µM melatonin (MLT), **(B)** 10 µM MLT + 10 µM luzindole (LZ), **(C)** 10 µM MLT + 10 µM 4P-PDOT **(D)** 10 µM MLT + MT1 siRNA and **(E)** 10 µM MLT + MT2 siRNA for 0, 5, 15, 30 and 60 min. Protein lysates were fractionized on SDS-PAGE gel and incubated with phosphospecific antibody (p-ERK1/2), as defined in Materials and Methods section. Total ERK1/2 expression was analyzed by reprobing the membrane with specific antibody (total ERK1/2) after revealing the phospho-specific proteins. The upper section displays the densitometric analysis of band intensities stated as the ratio of p-ERK1/2 to ERK1/2, after normalization with memcode and as comparative to the level at 0 min (arbitrarily set at 100%). Densitometric data are the average of 3 independent experiments listed as mean± SD. Statistically significant variances vs. 0 min $*P < 0.05$, $**P < 0.01$, $***P < 0.001$) were defined by one-way ANOVA tracked by a Newman-Keul's post-hoc test.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

This project demonstrates that: 1) Melatonin MT1 and MT2 receptors and synthesizing enzymes are present throughout pregnancy; 2) The expression of melatonin synthesizing enzymes increases during pregnancy and peaks at term; 3) Melatonin increases villous trophoblast syncytialization (β-hCG secretion and fusion); 4) Melatonin MT1 and MT2 receptors are expressed differently during trophoblast syncytialisation, as the expression of MT1 increases while of MT2 decreases and; 5) Melatonin inhibits forskolin-stimulated whereas its increases basal β-hCG secretion and stimulates ERK1/2 signaling pathway. Activation of ERK1/2 by melatonin is mediated via MT1 receptor stimulation.

High melatonin levels observed during pregnancy appears to be of placental origin. This is evidenced by the fact that melatonin levels return to normal non-pregnant levels after delivery and that twin pregnancies showed higher melatonin levels compared to single pregnancies. Moreover, the content of melatonin in human placental tissues is 6 to 7-fold higher than in the plasma (Iwasaki *et al.*, 2005, Sagrillo-Fagundes *et al.*, 2014). In this project we show that melatonin synthetizing enzyme (AANAT and HIOMT) are expressed throughout pregnancy and that melatonin regulates pregnancy events as trophoblast syncytialization and β-hCG secretion. We observed also a maximal expression of AANAT and HIOMT in the 3rd trimester, suggesting a continuous melatonin production during pregnancy with a peak in the late pregnancy. Our findings are consistent with data published by others, demonstrating high melatonin levels in maternal serum during pregnancy, also reaching a peak by the end of pregnancy then returning to non-pregnant level after delivery (Tamura *et al.*, 2008a, Tamura *et al.*, 2008b). The increase in AANAT and HIOMT expression during pregnancy and their

peak at term suggest a role for placental melatonin in the preparation to parturition. Indeed, melatonin has been suggested to synchronize and facilitate parturition while alleviating the associated pain. MT1 and MT2 are expressed in uterine myometrium and pineal melatonin has been shown to control the normal time of parturition (Reppert *et al.*, 1994, Schlabritz-Loutsevitch *et al.*, 2003). Oxytocin and melatonin utilize the same signaling pathway to induce myometrium contractility (Sharkey *et al.*, 2009). Both activate PLC-β, which hydrolyses PIP2 into DAG and IP3. DAG activates PKCα, activating the Ras/Raf/MAPK signaling pathway and leading to hCaD phosphorylation. This increases actin availability for myosin binding and contractility induction. Interestingly, in case of low oxytocin levels, melatonin supplementation induced adequate full contraction of uterine smooth muscles *in vitro*. Moreover, melatonin via its MT1 and MT2 receptors have been observed to exert analgesic effect that would alleviate delivery associated pain (Srinivasan *et al.*, 2012). Further studies need to be done to determine if melatonin produced by the placenta plays a role in parturition.

The increased expression of MT1 receptors observed in STB compared to vCTB (i.e. during villous syncytialization) is consistent with the findings that melatonin stimulates ERK1/2 phosphorylation via MT1 receptor. As the STB are the main endocrine cells of the placenta that secrete β-hCG, we suggest that melatonin via MT1 receptor and ERK1/2 signaling pathway activation induces β-hCG secretion. Secretion of β-hCG is related to biochemical differentiation. Daoud *et al* (Daoud *et al.*, 2005) have demonstrated that ERK1/2 and p38 signaling pathways are essential to initiate trophoblast differentiation. Indeed they have showed that specific inhibitors for ERK1/2 and p38MAPK suppressed trophoblast differentiation. We demonstrate here that

melatonin increases trophoblast differentiation (fusion and β-hCG secretion) and that melatonin regulates β-hCG secretion via the activation of MT1 receptor coupled to PLC-ERK1/2 signaling pathway. Further studies are needed to determine if melatonin via its MT1 receptor also regulates trophoblast fusion and hCG secretion.

In agreement with our results, melatonin has been observed to stimulate pluripotent P19 (Oct4+ Sosx2+) cells differentiation into neural stem cells (Oct4- Sox2+). This effect was mediated by MT1 receptor and ERK1/2 pathway activation (X. Chen *et al.*, 2014). Melatonin also induces cellular differentiation of chinese hamster ovary (CHO) model, an effect triggered by MT1-receptor stimulation with subsequent MEK/ERK1/2 signaling pathway activation (Luchetti et al., 2009). We observed that MT1 receptor is significantly highly expressed in the first trimester compared to the 2nd and 3rd trimesters of pregnancy. This is consistent with other findings, which established that MT1 is the initial receptor to be expressed in mammalian embryo (Sampaio *et al*., 2012). This suggests a MT1 role in the increased embryo cleavage and blastocyst creation rates. Moreover, apoptosis is very common during embryogenesis and is necessary for shaping the embryo and sculpting the tissues (Haanen *et al.*, 1996, Penaloza *et al.*, 2006). Therefore, further investigations are required for full understanding of MT1 role and mechanism of action at this early embryonic period as well as during placental development.

Melatonin level is similar during primary villous trophoblastic cell differentiation. We suggest that melatonin could induce apoptosis in STB via MT1 receptors. However we could propose also that the melatonin uses its antioxidant activity, as well to balance such process and avoid the uncontrolled excessive STB death. Luzindole is also known

to exert an antioxidant activity that might overcome its antagonistic action in such situation. So luzindole antioxidant activity might be the reason for having an additive action rather than a blockage of melatonin effect. But further research is needed for full understanding of this phenomenon. On contrary to primary trophoblasts, Bewo cells treated with luzindole (MT1/ MT2 antagonist) inhibited melatonin stimulatory effect on hCG secretion. On the other hand, 4P-PDOT (MT2 antagonist) could not inhibit completely melatonin effect on hCG secretion, so we suggest that MT1 receptor could be involved in hCG secretion. However, further studies are required to confirm this role.

Similarly, it will be important to examine the expression of the genes implicated in trophoblast syncytialization, such as hCG gene, syncytin-1 and -2, mfsd2a, GCM1, CHD1, and connexin 43. MEK/ERK ½ pathway is known to be the upstream activator of PKCβ/Ras-1/Raf-1 signaling pathway, thus melatonin's effect on the phosphorylation of these proteins could be observed to identify if they are activated by melatonin in villous trophoblast. Alternatively, there are several mechanisms that potentiate trophoblast differentiation and will be of valuable interest to examine if melatonin exerts a role on them. For example, p38-MAPK signaling pathway is suggested to have a vital role in trophoblast differentiation. Src family kinase (SFK) is a member of the non-protein tyrosine kinases (PTK), they act as an upstream regulator for Ras-1/Raf-1 and both ERK1/2 and p38 signaling cascade, and thus they regulate trophoblast differentiation (Daoud *et al.*, 2005, Vaillancourt *et al.*, 2009a).

BeWo cells synthesize melatonin and express melatonin (MT1 and MT2) receptors (Aplin, 1991, Lanoix *et al.*, 2008b). It's used as an affordable and easy *in vitro* model to study the regulation of melatonin production and its function in human

trophoblast. In humans, studying the regulation of melatonin synthesizing enzymes is difficult, notably because of tissue availability restrictions also it is subjected to maternal status influence. However, it would be useful to use similar methods in primary cell line as in the BeWo line to look at the signaling pathways. Particularly, since we describe differences in melatonin's role in primary cells versus tumorigenic cell lines. Also as we demonstrated an opposite action of luzindole on hCG secretion-stimulated by melatonin. The differences in protein phosphorylation and glycosylation, which might occur between normal (placental tissue) and neoplastic cells (JEG-3 and BeWo), as the multiple bands observed with MT2 antibody could be attributed to such differences in protein post-translational modifications or receptor dimerization (Prada *et al.*, 2005).

In the primary cell line, but not in BeWo cells, we demonstrated that luzindole did not antagonize melatonin's effect on hCG but in fact it resulted in an additive effect. We could interpret this phenomenon as an unknown or yet to be identified an agonist action of luzindole in certain cell types. Several studies have identified a similar - unexpected - agonistic action of luzindole. For example, while studying the neuritogenic effect of melatonin on crustacean x-organ cells (Cary *et al.*, 2012), luzindole enhanced melatonin effect rather than blocking it. The authors provided the three hypotheses to interpret this unexpected effect: 1) Their cellular model (crustacean x-organ cells) may have an as-yet unidentified subtype of membrane-bound melatonin receptor for which luzindole acts as an agonist instead of an antagonist; 2) Luzindole may prevent the desensitization of MT1 receptors by melatonin or may enhance MT1 expression; 3). Luzindole may have a direct, non-receptor-mediated effect similar to melatonin's direct inhibition of calmodulin. This is because luzindole is structurally similar to melatonin (Dubocovich, 1988) and

therefore may also be a ligand to calmodulin. This remains to be studied in our trophoblastic cell model.

Luzindole also enhanced the anti-nociceptive effect of melatonin on the formalin-induced pain response in mice, rather than blocking it (Ray *et al.*, 2004). Kokkola et al observed that luzindole enhances MT1 receptor ligand binding and activation (Kokkola *et al.*, 2007). Melatonin via its MT1 receptor induces the cellular differentiation of Chinese hamster ovary. Luzindole either alone or in combination with melatonin produced similar effect as melatonin treatments. The authors suggested an agonist action of luzindole in their model system (Bondi *et al.*, 2008). Luzindole has been found to display a similar action of melatonin and protected HL-60 cells against heat shock-induced apoptosis. The authors proposed that Luzindole may act as a melatonin agonist in HL-60 cells (Cabrera *et al.*, 2003). Luzindole also behaves as a powerful radical scavenger as did melatonin, for example; luzindole inhibited the ABTS radical cation same as melatonin and more powerfully than did ascorbic acid (Mathes *et al.*, 2008). But this also remains to be studied in our trophoblastic cell model.

The increased expression of MT1 during STB formation might also be linked to the regulation of STB apoptosis. Both intrinsic and extrinsic apoptotic pathways have been found to be involved in STB apoptosis. Caspase 8 is known to play a chief role in placental cell differentiation, while caspases 10 and 14 involvement has also been expected (Black *et al.*, 2004, Fischer *et al.*, 2003, Huppertz *et al.*, 1999, White *et al.*, 2007). Trophoblast tissue is defined as a pseudo-tumorigenic and melatonin is known to be a major ubiquitous regulator of apoptosis and a smart killer. We previously showed that melatonin induces apoptosis in BeWo cell (cancer cell) while it inhibits apoptosis in

normal primary trophoblasts (non-tumor cell). Melatonin induces apoptosis through stimulating the loss of mitochondrial membrane potential. This action was suggested to be mediated by MT1 and/or MT2 receptors (Lanoix *et al.*, 2012b). UCM386 (MT1 receptor agonist) exerts a pro-apoptotic and anti-proliferative effect as did melatonin in tumor cells (Winczyk *et al.*, 2002). Similar action was also observed in ductal breast carcinoma and murine melanoma cells (Jablonska *et al.*, 2013, Jawed *et al.*, 2007). Therefore, further research is required to examine if melatonin MT1 receptor is involved is STB apoptosis, for example, activation of STB caspases (caspase 3, 9 and 8), PARP and ROCK-1, modulating Bax/Bcl-2 pathway as well as regulating DNA fragmentation and chromatin condensation.

Taken together, our previous work and the data of the present project help to better understand the role of melatonin and its receptors in placental health, and by consequence in pregnancy well-being and fetal development. Also, our data suggest a protective and therapeutic role of melatonin in pregnancy. Melatonin has been suggested for the potential treatment of a variety of disorders, including obstetric complications, depression, cancer, and neurodegenerative disease. Some authors suggested melatonin as a biomarker of preeclampsia and as a possible treatment for this disease and other obstetric pathologies associated with placental defect. In accord with this, earlier investigations by our group demonstrated alterations in melatonin system during pregnancies complicated by preeclampsia (Lanoix *et al.*, 2012a).Those alterations included down-regulation of melatonin receptors and synthesizing enzymes accompanied with reduced melatonin levels and increased oxidative stress in preeclamptic placentas. Melatonin is a powerful antioxidant; it has a greater effect than

vitamins C and E in many tissues, including placental tissue (Milczarek *et al.*, 2010, Reiter *et al.*, 2014). Our group has previously showed a protective effect of melatonin as it reverses the hypoxia-reoxygenation (H/R)-induced oxidative stress and apoptosis in human primary villous trophoblast *in vitro*, a model that mimics the H/R observed during preeclampsia (Lanoix *et al.*, 2013). It's noteworthy to indicate that oral melatonin is now undergoing a phase-1 clinical trial to evaluate its effectiveness as an official treatment for preeclampsia (Hobson *et al.*, 2013).

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ANNEXES

(A) Effect of PLC-β and ERK ½ inhibitors on melatonin regulation of β-hCG secretion in BeWo cells. A significant inhibition of β-hCG secretion was observed in the presence of (U73122 - 560 nM) and (U0126 - 25µM). This suggests the implication of the PLC-β and ERK1/2 signaling pathways in the stimulatory effect of 10 µM of melatonin on basal β-hCG secretion.

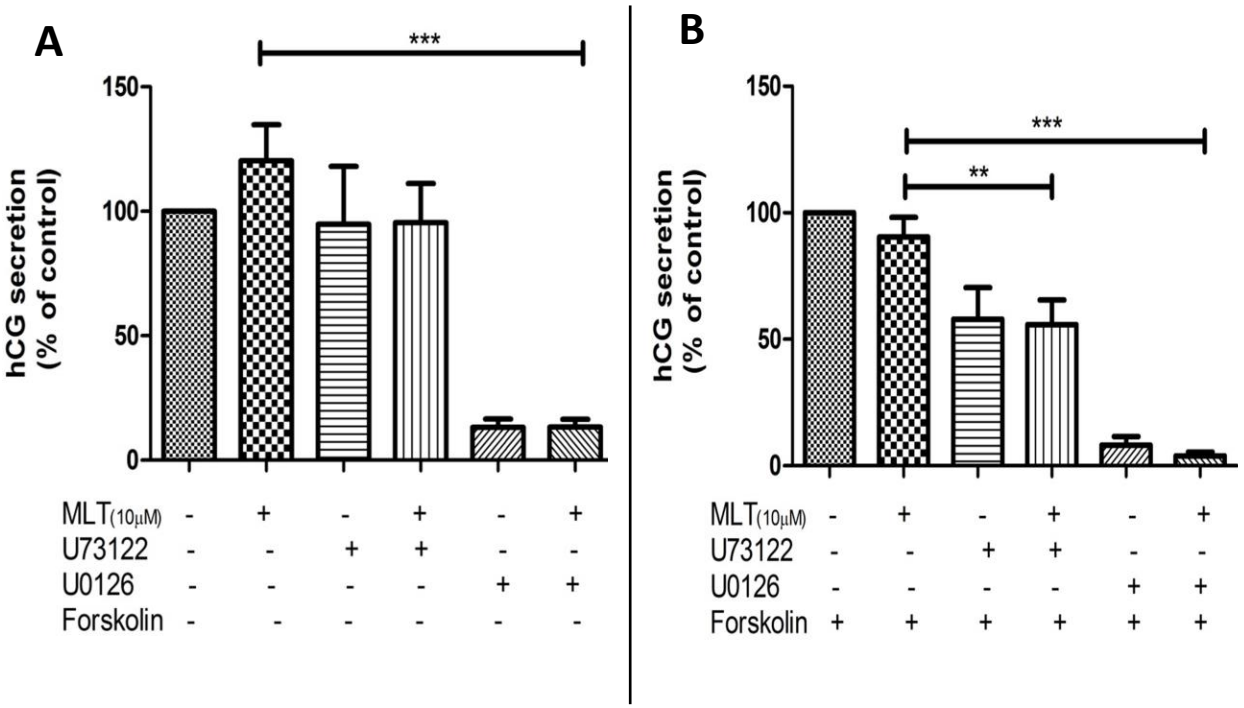

Figure 3 Effect of PLC and ERK½ inhibitors on β-hCG secretion in BeWo cells, in the presence and/ or absence of melatonin (MLT) and forskolin. Cells were pre-treated for 1 h with PLC-β Inhibitor (U73122 - 560 nm) or MAPK Inhibitor (U0126 - 25µM) or solvent alone (DMSO 0.1%, control) diluted in culture medium supplemented with 0.1% FBS. Then cells were treated for 48 h with 10 µM MLT or solvent alone (DMSO 0.1%, control) diluted in 0.1% FBS culture medium, in the absence (A) or presence (B) of forskolin (20 µM). The secretion of β-hCG was assessed in culture medium by ELISA, as described in (Materials and Methods) using EIA-1911 kits. Results revealed are represented as the mean ± SEM (n = 2 independent experiments with at least 4 technical replicates and are presented as the percentage of either DMSO or forskolin; $^{**}P < 0.01$, $^{***}P < 0.001$).

(B) Review article: Maternal and placental melatonin: actions and implication for successful pregnancies

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MINERVA GINECOL 2014; 66:251-66

Contribution of the student

Both the student and the other co-author contributed equally to this review

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MINERVA GINECOLOGICA

VOLUME 66 · N. 3 · GIUGNO 2014

EDIZIONI · MINERVA · MEDICA

PUBBLICAZIONE PERIODICA BIMESTRALE - POSTE ITALIANE S.P.A. - SPED. IN A. P. D.L. 353/2003 (CONV. IN L. 27/02/2004 N° 46) ART. 1, COMMA 1, DCB/CN - ISSN 0026-4784 TAXE PERÇUE

Maternal and placental melatonin: actions and implication for successful pregnancies

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Melatonin is one of the main sources of mitochondrial protection and its protective effects are equal or even better if compared with several consecrated antioxidants. Furthermore, the activation of specific melatonin receptors triggers several cellular pathways that improve the oxidoreduction and inflammatory cellular state. The discovery of the melatoninergic machinery in placental cells was the first step to understand the effects of this indoleamine during pregnancy. In critical points of pregnancy, melatonin has been pointed as a protagonist and its beneficial effects have been shown as essential for the control of trophoblastic function and development. On the contrary of the plasmatic melatonin (produced in pineal gland), placental melatonin does not vary according to the circadian cycle and acts as an autocrine, paracrine, intracrine, and endocrine hormone. The important effects of melatonin in placenta have been demonstrated in the physiopathology of pre-eclampsia with alterations in the levels of melatonin and in the expression of its receptors and synthetizing enzymes. Some authors suggested melatonin as a biomarker of pre-eclampsia and as a possible treatment for this disease and other obstetric pathologies associated with placental defect and increases in oxidative stress. This review will approach the beneficial effects of melatonin on placenta homeostasis and consequently on pregnancy and fetal health.

KEY WORDS: Melatonin - Placenta - Pre-eclampsia - Pregnancy.

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Melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule with wide protective effects that was thought to be exclusively produced in the pineal gland of superior animals.[1] However, it is well known now that its synthesis is virtually present in every cell of every organism, from bacteria throughout the animal and plant kingdoms.[2] This indoleamine is synthetized from the L-tryptophan pathway in a multistep process that has serotonin as an intermediate precursor. The serotonin is acetylated by the enzyme arylalkylamine *N*-acetyltransferase (AANAT) in *N*-acetylserotonin that is finally converted in melatonin by the enzyme serotonin *N*-acetyltransferase (ASMT, also known as hydroxyindole O-methyltransferase [HIOMT]). High concentrations of melatonin and the presence and activity of AANAT, considered the rate-limiting enzyme in melatonin synthesis production, in the mitochondrion assure that melatonin is produced at the cytoplasm and in the mitochondria.[3] Tan *et al.* (2013) suggested that this indoleamine was originally produced in the intermembrane space of the mitochondria and has migrated to the cytoplasm throughout the eukaryo-

tic evolution.[4] As mitochondria are the main cellular source of reactive species and consequently of oxidative stress, the massive presence of melatonin demonstrates its powerful antioxidant protection and is associated with the maintenance of the homeostasis in this organelle.[5] Besides the endogenous production of melatonin, it can also be obtained from exogenous sources. As melatonin is highly preserved in several species, it can be absorbed by the consumption of a variety of nuts, fruits, and vegetables.[6]

Melatonin produced by the pineal gland is delivered in the blood and cerebrospinal fluid and is responsible for the control of the circadian and circannual rhythms.[3] This photoneuroendocrine role of melatonin has been extensively studied and is related to variations on the reproductive competence. Equivalently to the universal phylogenic distribution of melatonin, its presence and production are attributed to several cellular types and organs, however only the pineal melatonin is available in the plasma; the synthesis in other organs is locally used with an autocrine and paracrine profile.[5] In other fluids than the blood and locally in organs where the production is well established, like intestine, skin and placenta, in general the concentration of melatonin exceeds the plasmatic levels. Conversely to the pineal production of melatonin, the production in these organs and fluids is not influenced by the variation of the diurnal length and photoperiod seasons.[7, 8] Besides the influence of melatonin to regulate the physiological alterations caused by the length of night's variations, it is also related to protective roles as free radicals scavenging, anti-inflammatory actions, improvement of immune system and an anti-cancer activity mediated by several cellular pathways.[2, 9]

Melatonin is highly soluble in lipophilic solutions and partially soluble in water, and this amphiphilic profile allows the prompt crossing of all physiological and cellular barriers. This characteristic allied to its intermediate size allows the presence of melatonin in all cells and its compartments.[9] The cytoprotective effect of melatonin goes beyond its direct antioxidant effect and is also related to the actions mediated by its receptors. The melatonin receptors type 1 and type 2 (MT1 and MT2, respectively) are members of the G protein-coupled receptors family (also known as seven-transmembrane domain receptors) and possess 60 % of homology in their sequence of amino acids.[3, 10] The effect of the interaction between melatonin and its receptors is variable regarding the tissue, the type of cell and the cellular phase. When MT1 and MT2 are activated, different isoforms of protein G lead to the activation of different signalling pathways (Table I).[3, 11-15] Melatonin is also able to interact with several members of the ROR/RZR (retinoid orphan receptor/retinoid Z receptor) nuclear receptors family. Regardless their affinity to melatonin, the controversial information that melatonin is their native ligand is still deliberated.[3] MT1 and MT2 are widely spread and are present in virtually all cells of the human body. MT1 is the main responsible of the regulation of the circadian cycle and is principally found in the central nervous system, whereas MT2 is related to the control of the body tem-

TABLE I.—*Cellular pathways modulated by melatonin and its receptors.*

Pathway	Effect of melatonin	Details	
Ca^{2+} channels	Inhibition	Inhibits the complex Ca^{2+}/calmodulin and stimulates the cytoskeleton polymerisation	3
K^{+} channels	Activation	Activated *via* PKA pathway	11
PLC	Activation	Activates ERK1/2	12
cAMP/PKA	Inhibition	Pathway inhibited via G_i	13
ERK1/2	Activation	Activated under oxidative stress	14
PLA2	Activation	Activated under oxidative stress	15

cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; PLC: phospholipase C; ERK1/2: extracellular-signal-regulated kinases; PLA2: phospholipase A2.

perature and is spread by the peripheral tissues.[3, 5] Several effects attributed to melatonin are related to its receptors, such as the stimulation of the expression of the antioxidant enzymes, regulation of the arterial tension and the inhibition of the expression of inflammatory factors.[16-18]

The increased interest and research on the cytoprotective action of melatonin are attributed to its unique characteristics. In a recent review, Galano *et al.* (2011) elegantly summarized the beneficial characteristics that qualify melatonin as an ideal radical scavenger molecule.[9] Melatonin has been proven to be versatile and is capable to mitigate several oxidants by mechanisms such as: Single electron transfer (SET: *Melatonin* $+ {}^{\bullet}R \rightarrow Melatonin^{\bullet+} + R^{-}$) and Radical adduct formation (RAF; *Melatonin* $+ {}^{\bullet}R \rightarrow$ *Melatonin - R*$^{\bullet}$). SET has been proposed as the main antioxidant mechanism of indole derivatives (*e.g.* tryptophan, melatonin, serotonin) against different oxidants.[19] This mechanism is characterized by the donation of an electron from melatonin to the radical and the consequent reduction of the reactivity of the latter and the formation of a less reactive radical from melatonin (expressed in the equation as Melatonin$^{\bullet+}$). The hydroxyl radical ($^{\bullet}$OH) is the most devastating free radical. It is normally produced intracellularly, but can also be generated by ultraviolet and ionizing radiation. The antioxidant activity of melatonin is effective against this radical by the RAF mechanism. Different techniques demonstrated that $^{\bullet}$OH reacts and is inactivated by the association with the carbon sites of melatonin. In this specific situation, the broad distribution of melatonin in different organelles, cells and body compartments is primordial to cut down the effect of $^{\bullet}$OH.[20, 21] The inactivation of $^{\bullet}$OH by melatonin generates a metabolite, cyclic-3-hydroxymelatonin (3-OHM), which has a weak reactivity and no cellular toxicity (in the RAF equation above, R$^{\bullet}$ represents $^{\bullet}$OH and 3-OHM is expressed as *Melatonin - R*$^{\bullet}$). The detection of this compound in the urine is a marker of the protection of melatonin against the $^{\bullet}$OH radical.[21] This metabolite of melatonin also acts as antioxidant, as well as other metabolites formed from the reaction between melatonin and reactive species, like N^2-acetyl N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) (Figure 1).

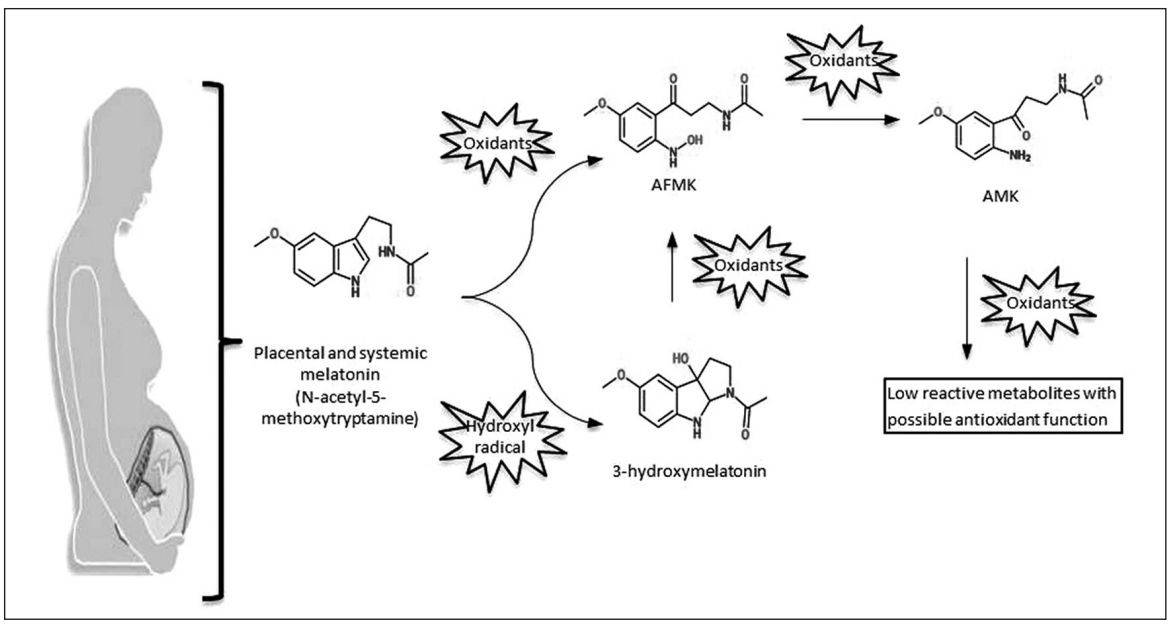

Figure 1.—Melatonin antioxidant cascade. Radical scavenging activity of melatonin that is found in several organs, among them the placenta. The melatonin metabolites have been shown as important antioxidant agents. AFMK: N^2-acetyl N^2-formyl-5-methoxykynuramine, AMK: N^1-acetyl-5-methoxykynuramine.

The importance of the metabolites of melatonin as antioxidants is already well established and there are evidences that AMK is even more potent than melatonin under specific situations.[9, 22] This unique feature distinguishes melatonin from several antioxidants and makes melatonin highly effective, even in low levels.[23]

Physiologically, the aerobic respiration generates reactive species of oxygen and nitrogen with an oxidant profile, called reactive oxygen species (ROS) and reactive nitrogen species (RNS). An unbalance between the levels of reactive species and antioxidants with an advantage for the formers is called oxidative stress. This situation is caused by an important reduction of antioxidants, increase of oxidants and the combination of both factors.[24] Mitochondria are the main cellular source of ROS and RNS, more specifically the complexes I and III of the electron transport chain (ETC). Even under normal conditions, 1-2% of the electrons are not completely reduced leading to the production of the anion radical superoxide ($O_2^{\bullet -}$). Subsequent reactions create several products, also with a reactive profile, such as hydrogen peroxide (H_2O_2), oxygen singlet ($^1O_2$) and the nitrous acid (HNO_2).[22, 24] Melatonin is able to improve the ETC efficiency, with a consequent reduction of free radicals generation. The wide localisation of melatonin, associated with its prolonged antioxidant activity and the stimulation of antioxidants enzymes expression are also sufficient to inactivate the free radicals effects and reduce the oxidative stress.[25]

Autophagy is the main cellular mechanism to eliminate cellular components (including mitochondria) that are dysfunctional or were affected by the oxidative stress with the aim to avoid the cellular death. This mechanism is constantly active and is one of the responsible to maintain the placental homeostasis, in spite of the constant alterations and stressors that affect this organ. The modulation of melatonin on the antioxidant enzymes of mitochondria and the direct effect of this indolamine as an oxidant scavenger demonstrate its mitochondrial protective effect. Recently, the relationship between the treatment with melatonin and the improvement of the homeostasis by the increase of autophagy and mitophagy (autophagy of mitochondria) was defined. However, Coto-Montes *et al.* demonstrated that, on time, there are no evidences to determine if the effect of melatonin on autophagy is related to its antioxidants effect or related to the action *via* its receptors.[26, 27]

The effect of melatonin on the inflammatory pathways is characterised by endocrine, autocrine, intracrine and paracrine responses. Markus *et al.* recently developed a hypothesis of the mutual control of melatonin and the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). Melatonin is considered a key factor of the immune system control and NF-κB can inhibit (in pinealocytes) or induce (in macrophages) the production of melatonin by the control of the enzyme AANAT.[18] Equivalently, tumor necrosis factor (TNF) also inhibits the production of melatonin by the suppression of AANAT and is inhibited under the treatment of melatonin.[28] Melatonin can inhibit, *via* its receptors and *via* free radical scavenging, the expression and activity of inflammatory mediators involved in the pathogenesis of several diseases of pregnancy, such as inducible nitric oxide synthase (iNOS), interleukin-2 (IL-2) and cyclooxigenase-2 (COX-2).[29, 30]

The "pleiotropic" effects of melatonin are extremely wide and include 1) mitochondrial homeostasis regulation; 2) induction of antioxidant enzymes; 3) inactivation of ROS and RNS; and 4) regulation of autophagy and inflammation. These actions are responsible for the cellular regulation and are extremely important for the cellular protection and also against challenging situations, commonly found during pregnancy.

Melatonin and pregnancy

Since the conception, the protective effects of melatonin are related to the improvement of the fertility and across the

pregnancy there is a continuous raise of plasmatic melatonin levels with the maximum during the parturition. During pregnancy, melatonin levels are higher during the nights than those measured in non-pregnant women. Interestingly, if compared to the non-pregnant women (whose melatonin is almost undetectable during the day), the diurnal levels of melatonin are high during pregnancy.[31] This variation of melatonin is hypothesised to be related with melatonin production by placental cells, which are also targets of this indoleamine. Despite of the site of melatonin production, it is essential for fetal development. The maternal variation of melatonin during pregnancy is also measured in fetal circulation and situations of circadian cycle alterations affect the expression of rhythmic genes in the fetus, which is reversed with daily melatonin injections.[32, 33]

Implantation

Local production of melatonin by human ovary was first established upon detecting its functional synthesizing enzymes (AANAT and ASMT) by Itoh *et al*.[34] Then the expression of melatonin receptors (MT1 and MT2) was revealed, being localized in human granulosa and luteal cells.[35] These findings explain – in part – the high melatonin level detected in ovarian follicular fluid compared to blood samples [7] giving rise to numerous research projects concerned with exploring the role of melatonin in ovarian functions.

The ovulation process is associated with inflammatory reactions.[36] Recruitment of macrophages, leucocytes and vascular endothelial cells in ovarian follicle results in elevated levels of inflammatory mediators and ROS.[37] Although ROS play a role in ovarian follicle rupture, elevated levels could result in diminished progesterone production by luteal cells and low oocyte quality due to oxidative injury.[38] Since melatonin is well-known to exert a powerful antioxidant effect through receptor-dependent and independent actions, high melatonin levels observed in preovulatory follicles compared to immature ones confirm its essential role in successful ovulation with high oocyte quality.[39]

In a clinical study with 25 infertile patients diagnosed with luteal phase defects (low serum progesterone level less than 10 ng/mL), melatonin administration to 14 patients (3 mg/day/oral) through luteal phase improved progesterone concentration in nine of them. On the other hand, the 11 non-treated patients experienced a non-significant increase in progesterone levels post-hCG injection. These results confirm ROS association with luteal phase defects and underline the antioxidant role of melatonin in protecting granulosa cells while enhancing progesterone production by the corpus luteum.[40]

Although assisted reproductive technologies (IVF-ET) have been significantly improved, poor oocyte quality remains the major challenge due to oxidative destruction.[41] Intriguingly, melatonin's powerful antioxidant effects through free radical scavenging, and increased antioxidant enzyme expression, augmented oocyte quality with extraordinary IVF-ET success rates.[17, 42] In 18 patients undertaking IVF-ET, melatonin administration (3 mg/tablet/day) at night from the 5th day of the previous menstrual cycle until the day of oocyte retrieval, triggered a higher intrafollicular melatonin level compared to controls. Remarkably, melatonin-treated patients experienced reduced intrafollicular concentrations of 8-hydroxy-2-deoxyguanosine (8-OHdG) and hexanoyl-lysine adduct (HEL), biomarkers of DNA damage and lipid damage, respectively.[43, 44]

To examine melatonin's usefulness on IVF-ET clinical outcome, 115 patients who failed to achieve pregnancy during the previous IVF-ET cycle and had a low fertilization rate (less than 50%) were recruited (Table II). Compared to previous cycle results, melatonin administration (3 mg/day) to 56 patients improved the fertilization rate with 11 patients achieving pregnancy. Conversely, for the 59 non-treated patients, no substantial change was observed in the fertilization rate and only six got pregnant.[43, 45]

TABLE II.—*Melatonin administration enhances in vitro fertilization pre-embryo transfer (IVF-ET) clinical outcome.*

	Melatonin administration 56 cycles	No melatonin administration 59 cycles
Fertilization rate in previous IVF-ET cycle	20.2±19%	20.9±16.5%
Fertilization rate	50±38%*	22.8±19%
Pregnanacy rate	11/56 (19.6%)	6/59 (10.2%)

* P<0.01 compared with the previous IVF-ET cycle (Mann-Whitney U-test)
Data from Tamura *et al.*[43]

These findings unravel a role for intrafollicular melatonin in reducing oxidative stress, protecting oocytes from injury and improving IVF-ET pregnancy success rates. Also demonstrate the effectiveness of oral melatonin supplementation during IVF-ET procedures, achieving enhanced oocyte quality in non-fertile women.

The aforementioned studies on oral melatonin administration and successful IVF-ET pregnancies support Tamura *et al.*[45] suggestion of plasmatic melatonin entry into ovarian follicular fluid. Subsequently, this suggests that *de novo* synthesized ovarian melatonin along with plasmatic melatonin might contribute to the high melatonin level observed in follicular fluid. One could propose that the activities of maternal melatonin could be associated with the reported high melatonin levels, which contribute to the high oocyte quality.

Parturition

Parturition or childbirth is the time of pregnancy culmination that involves spontaneous and vigorous myometrium contractions, resulting in cervical effacement and fetal descent. Published reports revealed parturition as a light sensitive event with an onset likely to occur at late night and early morning hours.[46] Although full explanation of this phenomenon is still ambiguous, some researchers expected that childbirth during darkness would guard both the mother and fetus from predators and maximize their survival, since labor process is known to be associated with maternal exhaustion and weakness.[47]

Irrespective of the role of oxytocin in parturition and its use for labor induction, its levels showed minimal increase during labor. Moreover, typical parturition occurred in the absence of posterior pituitary gland influence, after its removal.[48] Since parturition follows circadian rhythm as a nocturnal event, pineal gland melatonin was suspected to share a regulatory effect on this process. In this line of thought, Takayama *et al.*[49] examined the effect of melatonin injection on parturition timing in pinealectomized rats. This study revealed melatonin's importance in restoring normal parturition peak and maintaining photoperiodic rhythm synchronization. Similarly, a recent clinical study on pregnant women volunteers confirmed the role of night-time melatonin in augmenting uterine contraction. These women (at their >38 gestational week) were continually inspected for their uterine contractions from 7:00 pm till 7:00 am under dim light, and then they were eye-exposed to full spectrum light (10,000 lux) at 11:00 pm for 1 hour. Since this light spectrum is known to suppress nocturnal melatonin secretion,[50] uterine muscle contractions were shown to be impeded, which started following light exposure for the remainder of the night.[14] Owing to the widespread misuse of artificial light at night, this could explain the present-day drop in night-time melatonin along with nocturnal delivery.

Aside from the fact that uterine myometrium expresses both oxytocin and melatonin receptors,[51] melatonin and oxytocin activate the same signaling pathway of phospholipase C (PLC), protein kinase C (PKC) and myosin light chain kinase leading to uterine muscle contraction and offspring expulsion (Figure 2).[52, 53] While myometrial cells treated with low concentrations of oxytocin failed to show full contractions *in vitro,*

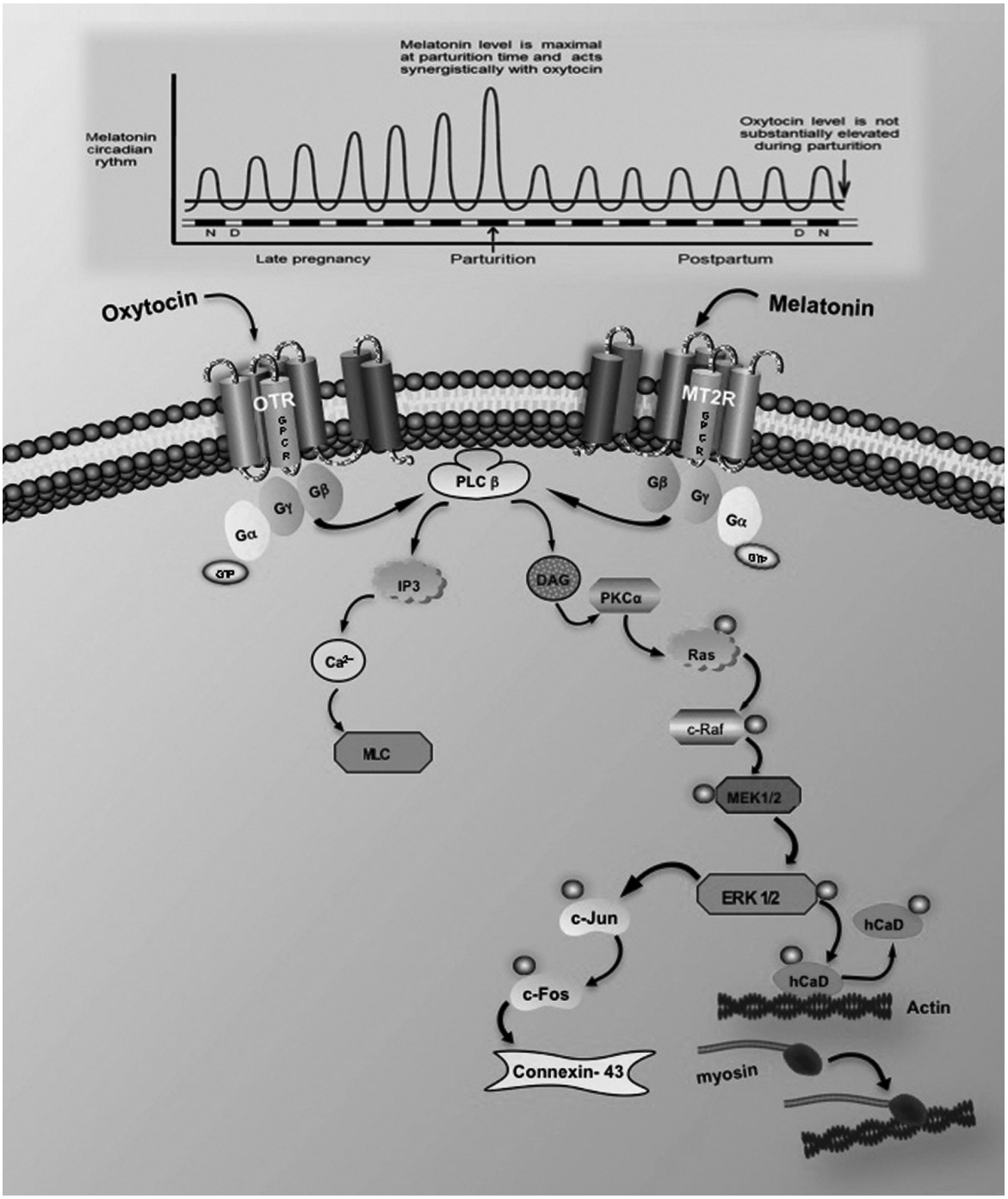

Figure 2.—Suggested model of synergetic action of melatonin and oxytocin on night-time myometrial contractility at parturition time. Melatonin circadian rhythm and oxytocin level is described through the last trimester of pregnancy, at parturition time and after delivery of human females. Melatonin and oxytocin stimulate respectively melatonin type 2 (MT2R) receptor and oxytocin (OTR) receptor to synergistically activate PLCβ, which hydrolyses PIP2 into DAG and IP3. DAG activates PKCα, activating the Ras/Raf/MAPK signaling pathway and leading to hCaD phosphorylation. This increases actin availability for myosin binding and contractility induction. Moreover, PLCβ stimulates both c-Jun and c-Fos, resulting in enhanced gap junctional communication *via* connexin 43 activation. Finally, IP3 stimulates $Ca2^{+}$ release that activates MLC and enhances myometrial contractility. PLC: phospholipase, PIP2: phosphatidylinositol 4, 5-bisphosphate, DAG: diacylglycerol, IP3: inositol 1, 4, 5- triphosphate, PKC: protein kinases type C, MAPK: mitogen-activated protein kinase, hCaD: caldesmon, MLC: myosin light-chain. Adapted from Reiter *et al.*[52]

melatonin supplementation (1 nmol/L) acted synergistically and sensitized uterine muscle to oxytocin resulting in their peak contraction. A possible explanation for the synergistic action of melatonin is the up-regulation of the activity and expression of connexin 43, a gap junction protein, consequently improving intracellular communication and myometrial contraction synchronization. Furthermore, laboring pregnant women showed corresponding upregulation of melatonin and oxytocin receptors compared to pregnant non-laboring ones.[14]

Interestingly, night-time circulating melatonin increases in late pregnancy reaching its peak by delivery time,[54] as shown by its levels in both maternal amniotic fluid [55] and urine [56] near term. Also nocturnal melatonin levels drops to normal (non-pregnant women level) instantly after delivery,[31, 57] assuming melatonin significance in determining parturition time through tracking light/dark cycle. Since placental melatonin levels have been shown to contribute to increased maternal blood levels of melatonin (*discussed in details in Melatonin and placenta section*) we could propose that it has a role in the parturition process due to its leakage into maternal blood or due to its paracrine activities on myometrial melatonin receptors.

In summary, the mentioned findings could explain the higher melatonin levels observed in late pregnancy as well as elevated night time delivery. It would be valuable to recommend melatonin supplementation at the onset of spontaneous labor, to surge uterine contractions and lessen its period as well as relieving delivery aches.[58]

Amnion and amniotic fluid

Amniotic sac is a transparent, thin and strong dual membrane bag inside which the fetus grows, the inner membrane is called amnion and includes both the amniotic fluid and the developing fetus, while the outer membrane is called chorion and forms part of the placenta. Together with the placenta and umbilical cord, amniotic sac is pushed out of the mother upon delivery.[59] In 1978, Mitchell *et al.*[60] detected high melatonin concentrations in the amniotic fluid during labor even greater than those observed in late pregnancy, questioning if melatonin might exert functional role during parturition. Interestingly, those findings match present-day data explaining melatonin activities during parturition accompanied by its high nocturnal level in late pregnancy, peaking by labor time.[61]

As melatonin is transferred easily and rapidly from maternal to fetal circulation,[62] Xu *et al.*[63] reported that maternal pretreatment with melatonin attenuated the lipopolysaccharides (LPS)-induced pro-inflammatory cytokine TNF presence in the amniotic fluid, emphasizing the anti-inflammatory action of melatonin. Moreover, melatonin receptor MT1 is expressed in the amniotic epithelial cells, which through melatonin stimulation increased cell proliferation. Interestingly, melatonin pre-treatment reversed amniotic epithelial cells demise after H_2O_2 exposure and promoted their survival, thus illustrating melatonin antioxidant activities in the amniotic epithelial cells.[64]

Because of the limited number and controversial findings of present-day studies on the involvement of melatonin in the amniotic fluid,[53, 65] extensive research is required to determine if melatonin is produced locally by amniotic membrane cells or due to its outflow from the placenta and maternal blood, or both. Moreover, it will be important to determine if amniotic fluid melatonin and amniotic sac melatonin receptors have implications in both normal pregnancy and various pregnancy complications.

Melatonin and placenta

Human placenta is a transitory organ that attaches the developing fetus to the wall of maternal uterus, where it plays a chief role in pregnancy well-being and fetal growth.[66] Human placenta mediates feto-maternal exchanges of nutrients, ions and oxygen as well as elimination of fetal waste products and metabolites. Furthermore, it functions

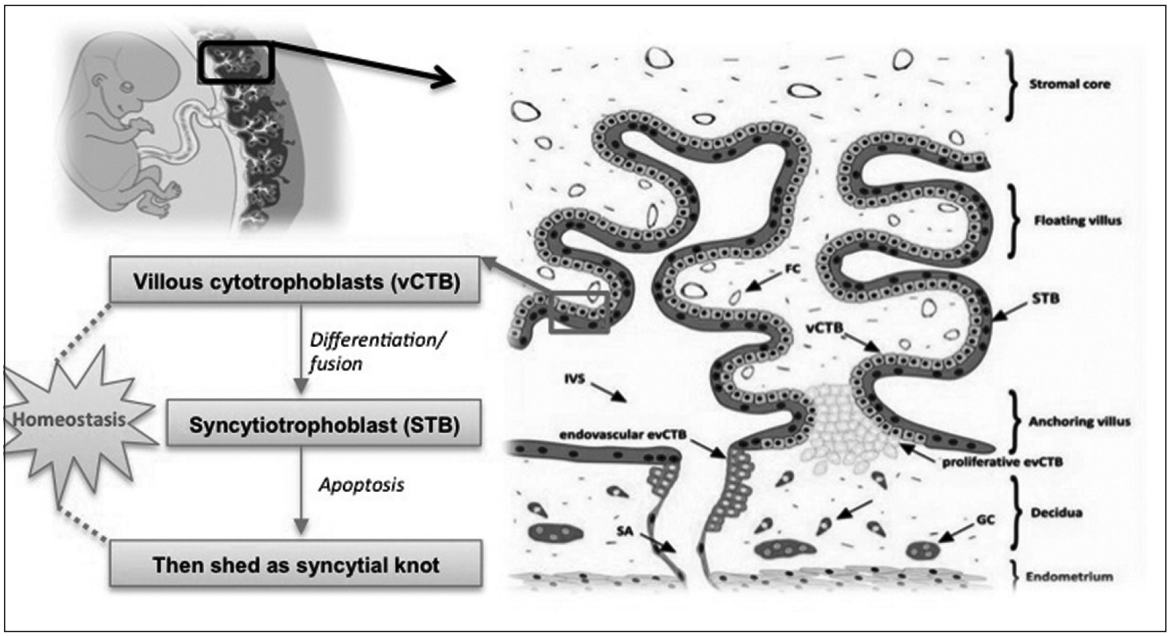

Figure 3.—Human villous trophoblast differentiation. Villous cytotrophoblast (mononucleated) aggregate and fuse into syncytiotrophoblast (multinucleated) in a highly organized process. SA: spiral artery; GC: giant cell; evCTB: extravillous cytotrophoblast; FC: fetal capillary STB: syncytiotrphoblast; vCTB: villous cytotrophoblast; IVS: intervillous space. From Zeldovich *et al.*[68] and Vaillancourt *et al.*[71]

as an endocrine organ producing specific steroid and peptide hormones, while protecting the fetus from being rejected by the maternal immune system through its immunomodulatory activities.[67]

The functional unit of the human placenta is the chorionic villi (Figure 3), formed of mononucleated villous cytotrophoblasts, which aggregate and fuse into multinucleated syncytiotrophoblast in a highly controlled process. Assuring the villous trophoblast homeostasis is vital to pregnancy success and fetal development (Figure 3). During the course of pregnancy, the syncytiotrophoblast undergo apoptosis to allow its continual regeneration by the underlying proliferative villous cytotrophoblasts.[68] Factors that disturb villous trophoblast homeostasis such as oxidative stress increase syncytiotrophoblast loss by apoptosis and trigger pregnancy complications such as pre-eclampsia and fetal growth restriction.[69-71]

In 2005, Iwasaki *et al.*[72] anticipated melatonin regulatory activities in human placenta upon identifying both melatonin synthesizing enzymes (AANAT and ASMT) and melatonin membrane receptors (MT1 and MT2) mRNA transcripts in first trimester placental tissues. Afterwards, our team conducted further extensive studies on placental melatonin system, where we demonstrated mRNA and protein expression of melatonin receptors (MT1, MT2 and RORα1) in normal human term placenta and in human placental choriocarcinoma cell lines (JEG-3 and BeWo).[8, 73] Subsequently, through immunohistochemistry, RT-PCR, Western Blot and LC-MS/MS techniques, we localized functional AANAT and ASMT as well as the melatonin receptors (MT1, MT2 and RORα1) on both villous cytotrophoblast and syncytiotrophoblast of normal human term placenta.[8] Our findings were consistent with the observations of elevated extra-pineal melatonin production during pregnancy, as detected in maternal blood, which peaks by term and does not follow the circadian rhythm. This also matches propositions about the placental origin of melatonin during pregnancy. Since the level of melatonin returns to normal after delivery and is higher in twin pregnancies compared to single pregnancies,[72] this supports the idea that placental melatonin contrib-

utes to the high maternal blood level during pregnancy. As demonstrated by Nakazawa *et al.*, the melatonin content in human and rat placental tissue is high even during day hours. Even more, melatonin content in human placental tissues is 6 to 7-fold higher than in the plasma.[74]

A recent study by our team showed a protective effect of melatonin on villous cytotrophoblasts and syncytiotrophoblast in a situation that mimics the pre-eclampsia and generates high levels of oxidative stress, the exposition to an environment of hypoxia/reoxygenation (H/R).[75] Exposition of primary syncytiotrophoblast cell to H/R induced the activation of the apoptosis by the mitochondrial pathway, which was completely reversed upon treatment with 1mM of melatonin. In this model, melatonin treatment significantly increases the levels and activity of antioxidant enzymes (superoxide dismutase and glutathione peroxidase), reduces both pro-apoptotic factors (caspases 9 and 3) and the proteins of Bcl2 with a consequent protection of the mitochondrial integrity. Under oxidative stress, the raise of the permeabilization of the mitochondrial membrane releases activating factors of the intrinsic apoptotic pathway. This mechanism is regulated by members of B-Cell Lymphoma 2 (Bcl2) family, which are responsible for the formation of pores in the outer membrane of mitochondria and the liberation of pro-apoptotic factors (*e.g.*, cytochrome-c). These factors activate the apoptotic pathway with the cleavage of pro-caspase 9 and pro-caspase 3, and the consequent condensation of the chromatin and fragmentation of the DNA.[75, 76]

Modulation of antioxidants and oxidants levels and the consequent oxidative stress during pregnancy leads to a proper implantation and placental development.[69] For example, mild levels of oxidative stress regulate the syncytialization of the villous trophoblasts; however super-expression of the antioxidant enzyme superoxide dismutase impairs the switch from villous cytotrophoblasts to syncytiotrophoblast.[77] The increase of oxidative stress during pregnancy is related to the tension of oxygen, significantly increased in the uterine arteries in comparison with the intervillous space. This is essential for the placental exchanges and is a key-factor for the migration and invasion of the extravillous cytotrophoblasts and its differentiation to endovascular extravillous cytotrophoblasts. The optimal invasion of the extravillous cytotrophoblasts leads to the rise of the oxygen tension from <20 mmHg (first trimester) to 45-55 mmHg (second and third trimester), essential for the proper fetal development.[69] Improper extravillous cytotrophoblast invasion in the maternal decidua will alter the increase of oxygen tension, leading to complicated pregnancies, such as pre-eclampsia.[69]

Owing to our curiosity in identifying melatonin functions in placental physiology, we were the first to demonstrate that melatonin has a dual effect on apoptosis in tumor cells versus normal trophoblast cells. Melatonin induces apoptosis in BeWo choriocarcinoma cell line (tumor villous trophoblast) cells while inhibiting apoptosis and promoting survival in normal villous trophoblast. Melatonin prevents tumor cell proliferation through pro-apoptotic pathways activation and reactive oxygen species induction, *via* receptor dependent and independent activities, resulting in mitochondrial membrane permeabilization and cell death. By contrast, melatonin promotes non-tumor cell survival through its antioxidant properties and pro-apoptotic pathways inhibition, which occurs *via* a MT1/MT2 receptor pathway that inhibits Bax/Bcl-2 intrinsic apoptosis.[78] Nevertheless, further research is needed to unravel the mechanisms by which melatonin and its receptors preserve and regulate trophoblast homeostasis.

In sum, melatonin is *de novo* synthesized and its receptors are expressed in human placental trophoblastic cell, providing a unique model for the study of the mechanisms and signaling pathways involved in the protective actions of melatonin. Also, placental melatonin is strongly associated with the improvement of placental functions and development and with the protection

from oxidative stress, and is pointed as a potential watchdog against H/R-induced alterations in villous trophoblast homeostasis.

Pregnancy complications: melatonin and placenta

The comparison between the average weight of placentas and pineal glands indicates that both enzymes responsible for the production of melatonin, AANAT and ASMT, have their activity at least 2-fold higher in villous cytotrophoblast and in the syncytiotrophoblast compared to the pineal production. The concentrations of melatonin is 225-fold higher in placental cells.[8] These data show the massive level of melatonin in placenta, however its functionality remains partially understood. One role that has been characterized is its cytoprotective effect on trophoblast cells exposed to H/R.[75] During the first trimester of normal pregnancies, the invasion performed by extravillous trophoblasts is responsible for the improvement of the oxygen availability in the intervillous space, however, in preeclamptic placentas the lack of extravillous trophoblast invasion compromise the oxygen availability and the feto-placental homeostasis. Roberts and Hubel [79] distinguished pre-eclampsia in two phases. The first phase is defined as failure of vessel remodelling. The second phase is characterized by clinical signs and by the uncontrolled enhancement of placental oxidative stress, inflammation and erroneous expression of placental factors. These issues arise from the incomplete arterial remodelling leading to an insufficient or, more frequently, to intermittent blood supply, properly reproduced *in vitro* by a H/R exposure. As described previously, melatonin is effective to avoid the apoptosis and oxidative effects caused by H/R by the modulation of several steps of intrinsic apoptosis pathway and storage of oxidative status.[22, 77]

Aside the protection provided by melatonin to villous cytotrophoblast and syncytiotrophoblast in an environment that simulate pre-eclampsia, our group observed that preeclamptic pregnancies demonstrate decreased placental melatonin levels associated with lower expression of placental melatonin receptors, lower expression and activity of AANAT and lower activity of ASMT.[80] To define if the decrease of melatonin production was due to a lack of a precursor or an effect on its synthesizing enzymes, the level of serotonin was assessed and, on the contrary of melatonin, it was increased in pre-eclamptic placentas, which suggest the direct effect of pre-eclampsia on the melatoninergic synthesis machinery. This corroborates the results of Nakamura *et al.* (2001), which show decreased plasmatic melatonin levels in subjects with pre-eclampsia if compared with normal matched pregnant women and suggests that the decrease observed is related to placental production.[31] Together, these data suggest that *de novo* synthesized placental melatonin contributes to high maternal blood levels observed during pregnancy, and that this *de novo* synthesized melatonin exerts paracrine, autocrine, and intracrine activities in placental function. Finally, the variation of placental and plasmatic levels of melatonin of preeclamptic patients leaded us to suggest plasmatic melatonin as a biomarker of pre-eclampsia. Yamamoto *et al.* (2013) investigated the effect of four hypertensive diseases during pregnancy on placental melatoninergic system: chronic hypertension, chronic hypertension associated with pre-eclampsia, gestational hypertension and pre-eclampsia.[81] In this study, all four alterations were responsible for the increase of expression of receptor MT1. All three hypertensions increased the expression of tryptophan hydroxylase (TH), but pre-eclamptic placentas demonstrated reduced expression of this enzyme whose role is to initiate the melatonin synthesis pathway by the addition of an OH^- to tryptophan. Despite the analysis of placental MT1 and TH, neither plasmatic nor placental melatonin was analyzed.

Hyperglycemia associated with vascular alterations during pregnancy characterize gestational diabetes mellitus (GDM), and affects between 8% and 17% of pregnancies.[82] This disease stimulates the placental

oxidative stress and affects the feto-placental vascular exchange.[83, 84] Recently, the risk of GDM has been positively associated with polymorphic expressions of the melatonin MT2 receptor. The first study that found this association was performed in a population of Korean ethnic origin. A group of almost one thousand GDM patients without previous diabetes mellitus were selected and two single-nucleotide polymorphisms (SNPs) were identified in MT2 gene and were strongly associated with GDM.[85] Other studies followed this pattern and evaluated the relationship of GDM and SNPs in MT2 gene in different populations. Regardless the population, MT2 gene polymorphisms were strongly associated with GDM.[83] To date, the melatonin effect on GDM is not well described, however the melatonin cytoprotective effect would possibly prevent the alterations caused by GDM.[86]

Appropriate placental development is necessary for the fetal growth and development. Pregnancies with insufficient subsistence usually are related to obstetrics complications, such as fetal growth restriction (FGR). The fetal morbidity associated with this condition is caused by the altered placental development and compromised umbilical blood flow and is extremely high and harmful. The main consequences of FGR are perinatal death, preterm births, low weight at the partum, and retardation in the physical growth and brain development.[87, 88] As the pathophysiology of FGR is related with placental inflammation, hypoxemia and rise of placental oxidative damage, melatonin has been tested in experimental models to alleviate the prenatal effects of FGR. Lemley *et al*.[89] developed an experimental study to evaluate the effects of melatonin on pregnant ewes compromised by FGR. This model was based on the nutriment restriction and demonstrated that oral melatonin was able to increase the uterine blood flow and the fetuses ponderal mass. Based on the placental responsiveness to melatonin treatment, the same group demonstrated that chronic uterine injection of melatonin improves the feto-placental blood flow in pregnant ewes.[90] In accordance with these results, a recent study that mimics FGR demonstrated that injection of melatonin during pregnancy reduces fetal oxidative stress levels, improves neurodevelopment, and enhances cognitive capacity of the lambs.[91]

The disproportion between oxidants and antioxidants in preeclamptic pregnancies leaded to the evaluation of antioxidants as a treatment to this disease. Vitamin C and E were tested as a possible treatment for pre-eclampsia in a vast clinical trial.[92] Despite of theoretical and previous assumptions, the results of the clinical trial demonstrate no beneficial effect of the treatment and even showed decreased birth weight of babies from women treated with vitamin C and E. Based on these results, Milczarek, *et al.* demonstrated that the association of both vitamins C and E with melatonin is extremely effective in the protection of placental mitochondria against the oxidative stress and suggested the combination of this three antioxidants as a possible treatment for pre-eclampsia (Figure 4).[23]

Regardless the concentration and the type of treatment with melatonin, its toxicity is very low. Assays with increased doses of melatonin under chronic and acute treatment have demonstrated that several experimental models are not affected by this indolamine.[93, 94] Moreover, oral treatment with melatonin has generated neither important side effects nor toxic effects.[95]

The toxicity of melatonin was already tested in a variety of experiments that include even experimental models of pregnancy. Oral melatonin was not related to any kind of toxicity during pregnancy of rats and ewes.[94, 96] This fact, in association with the recent demonstrations of its effectiveness against placental abnormalities, resulted in establishing clinical trials that try to correlate the effect of melatonin against alterations as pre-eclampsia and FGR. Hobson, *et al.* initiated a clinical trial (phase I) to evaluate the effect of 30 mg/day of oral melatonin in patients with pre-eclampsia.[97] The length of the treatment begins with the recruitment and is kept until the birth. Several markers of eligible women enrolled in this study are constantly determined, such

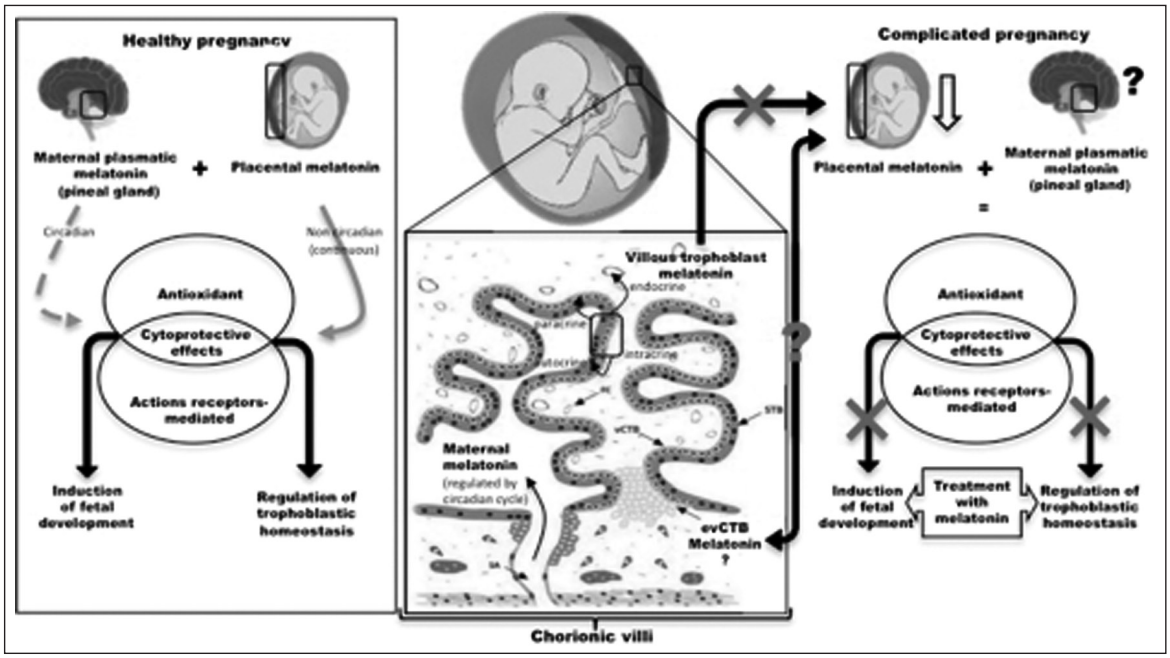

Figure 4.—Potential role of melatonin in healthy and complicated pregnancies. Plasmatic melatonin, released in a circadian manner, reaches the intervillous space and accesses fetal capillaries (FC) through syncytiotrophoblast (STB) and villous cytotrophoblast (vCTB). In parallel, both STB and vCTB produce melatonin locally that can also reach fetal capillaries and maternal circulation. Both plasmatic and placental melatonin are related with maternal and fetal protective effects observed during pregnancy. Although extravillous cytotrophoblast (evCTB) are responsible for the placental implantation in the beginning of pregnancy and may have a relationship with complicated pregnancies, melatonin production in those cells is still unknown. Furthermore, since placental melatonin production during pregnancy abnormalities is compromised, melatonin treatment is suggested to recover the absent protective effects of placental melatonin in those cases.

as plasmatic oxidative stress, markers of pre-eclampsia, markers of maternal morbidity and ultrasound. The same research group that related the treatment of melatonin and the improvement of cognitive capacity of lambs is developing a clinical trial with the aim of determining the effect melatonin treatment on patients with FGR.[98] Similarly to the other clinical trial, this study is a single-arm, open-label study, however the daily treatment in this case is 8 mg/day. Placental, maternal and fetal oxidative stress will be assessed as well as maternal and fetal markers that indicate the possible protection of melatonin against FGR.

Conclusions and future perspectives

Both placental and plasmatic melatonin work synergistically and, despite of the source, this indoleamine has effects that go beyond the endocrine pattern. Maternal melatonin modulates fetal circadian cycle and its development, whereas placental melatonin regulates trophoblast homeostasis and thus placental function and development in a paracrine, autocrine and intracrine manner. Accordingly, despite of its origin, melatonin is a key-factor for successful pregnancy and fetal development.

Research using trophoblast cells is a novel approach to discover the mechanisms by which melatonin plays a pro-survival and cytoprotective role crucial for placental health and, by consequence, for pregnancy well-being and fetal development. Therefore, further researches are needed to better understand the role of the plasmatic and placental melatonin as well as its receptors in normal and abnormal pregnancies. Hence, this will open the way for the development of new preventive and therapeutic approaches in this area.

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Funding.—This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grant (no. 262011-2009) to CV as well as by studentship awards to LSF from the *Ministère de l'Éducation, du Loisir et du Sport* (MELS) *du Québec - Fonds de recherche du Québec - Nature et technologies* (FRQ-NT) and to AS from the *Fondation Armand Frappier* and *Réseau Québécois en reproduction* (RQR)-NSERC-Collaborative Research and Training Experience Program (CREATE) Program.

Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.