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Role of decorin at the fetal-maternal interface

Chidambra D. Halari, *The University of Western Ontario*

Supervisor: Lala, Peeyush. K, *The University of Western Ontario*
Co-Supervisor: Renaud, Stephen J., *The University of Western Ontario*
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Abstract

The human placenta is an invasive tumor-like structure, this invasion being physiological. A subset of placental trophoblast called extra-villous trophoblast invades the uterine decidua and remodels uterine arteries into low-resistance, high-flow tubes to permit adequate flow of maternal blood to nourish the fetus. A poor extra-villous trophoblast invasion and uterine arterial remodeling can lead to fetal growth restriction and a serious pregnancy-associated maternal disease preeclampsia. Decorin, a leucine-rich proteoglycan produced by uterine decidual cells restrains multiple trophoblast functions: self-renewal and differentiation of trophoblast stem cells, migration, invasion, proliferation and endovascular differentiation. Additionally, decidual overproduction of decorin was associated with preeclampsia, and increased decorin levels in the maternal plasma during the second trimester could predict preeclampsia.

I discovered that decorin plays a critical role in maturation of human endometrial stromal cells into decidual cells. Using CRISPR-Cas9 approach for knocking out decorin, I showed that decorin-depleted human endometrial stromal cells failed to mature, as revealed by fibroblastic morphology and reduced production of decidual cell maturation markers, insulin like growth factor protein-1 and prolactin. I also found that interleukin 1 beta produced by trophoblast, macrophage and endometrial glands is the main stimulus for decorin production in the decidua by recruiting a transcription factor, nuclear factor kappa B. Additionally, I discovered a new microRNA-mediated mechanism of decorin action on trophoblast. Two microRNAs (let 7c-5p and 512-3p), induced by decorin restrained multiple trophoblast functions known to be compromised in preeclampsia. They were elevated in preeclampsia-associated placentas. Collectively, my research is fundamental to understanding the pathogenesis of preeclampsia. This knowledge can be exploited to improve maternal and fetal health. For example, decorin induced microRNAs may be used as a biomarker for early diagnosis and intervention of preeclampsia. Furthermore, non-toxic molecules that can reduce decorin production by decidual cells may have therapeutic potential.

Keywords

Pregnancy, Decorin, Interleukin 1beta, Preeclampsia, MicroRNA, Placenta, Decidualization, Trophoblast, NF-κB, Fetal growth restriction

Summary for Lay Audience

During pregnancy, the mother's uterus undergoes a process called decidualization in which uterine cells transform into specialized cells called decidual cells. This process is essential for implantation of embryo and development of the placenta. The placenta is the physical connection between a mother and her baby and must invade the mother's uterine blood vessels to draw nutrients for the baby. This placental invasion is exquisitely controlled by many locally-derived molecules to maintain the health of the mother and the developing baby. If this invasion is not adequate, it can result in poor blood flow to the baby, leading to fetal growth restriction and also a serious maternal disease called preeclampsia. Fetal growth restriction has a life-long impact on the baby's health with an increased risk of developing diabetes and heart disease. Preeclampsia can be life-threatening to the mother, because of high blood pressure, kidney damage and chance of stroke. It can only be cured by delivering the placenta and the baby. Many uterine molecules control placental invasion to maintain a healthy balance including a small protein, decorin which is made by decidual cells. Decorin controls placental invasion and its over-production is associated with preeclampsia. But its role in decidual cell development is not known. Therefore, I explore this role of decorin and found that decorin is produced during decidualization and is necessary for the decidual cell maturation. Moreover, I found that another protein Interleukin 1beta is the key stimulus for decorin production. Additionally, I found out that decorin action on placental cells could be due to small RNAs called microRNAs which can be biomarkers for preeclampsia. Collectively, these findings can help us improve our understanding of early diagnosis and management of preeclampsia.

Co-Authorship Statement

Some parts of Chapter 1 are adapted from Halari CD, Zheng M, Lala PK. Roles of Two Small Leucine-Rich Proteoglycans Decorin and Biglycan in Pregnancy and Pregnancy-Associated Diseases. Int J Mol Sci. 2021 Sep 30;22(19):10584. Figures were reproduced for this thesis with permission from International Journal of Molecular Sciences.

Chapter 2 is adapted from Halari CD, Nandi P, Jeyarajah MJ, Renaud SJ, Lala PK. Decorin production by the human decidua: role in decidual cell maturation. Mol Hum Reprod. 2020 Oct 1;26(10):784-796. Text and figures were reproduced for this thesis with permission from the Molecular Human Reproduction (Appendix B). CD Halari designed the study, conducted all the experiments, analyzed the data and wrote the manuscript. P Nandi worked on the preliminary data for this study. M Jeyarajah helped with shRNA-mediated DCN knockdown. SJ Renaud obtained the approval from human ethics review board for placenta sample collection and provided critical feedback and revised the manuscript. PK Lala conceived and directed the study and revised the manuscript to completion.

Chapter 3 is adapted from Halari CD, Renaud SJ, Lala PK. Molecular mechanisms in IL-1β-mediated decorin production by decidual cells. Mol Hum Reprod. 2021 Nov 27;27(12):gaab068. Text and figures were reproduced for this thesis with permission from the Molecular Human Reproduction (Appendix B). CD Halari designed the study, conducted all the experiments, analyzed the data and wrote the manuscript. SJ Renaud provided critical feedback and revised the manuscript. PK Lala conceived and directed the study and revised the manuscript to completion.

Chapter 4 is entitled Roles of Decorin-induced microRNAs 512-3p and let-7c-5p in Trophoblast Functions Disrupted in Preeclampsia. CD Halari designed all experiments, analyzed the data and wrote the chapter. P Nandi worked on the preliminary data for this study. J Sidhu and M Sbirnac provided some preliminary data. M. Zhang performed experiments pertaining to figure 8. SJ Renaud provided some critical feedback on this chapter. PK Lala conceived, directed the study and provided critical feedback on the chapter at all levels of completion.

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List of Abbreviations

8-br-cAMP	8-bromo-cyclic adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
CTB	Cytotrophoblast
DSCs	Decidual stromal cells
DCN	Decorin
EGF	Epidermal growth factor
E	Estrogen
ER	Estrogen receptor
EVT	Extravillous trophoblast
FGR	Fetal growth restriction
hPMC	highly proliferative mesenchymal cells
hCG	human chorionic gonadotrophin
hTS	Human trophoblast stem
ICM	Inner cell mass
IL	Interleukin
LRR	Leucine rich repeats
miRNA	MicroRNA
PE	Preeclampsia

P	Progesterone
PR	Progesterone receptor
PGE2	Prostaglandin E2
PKA	Protein kinase A
qRT-PCR	quantitative RT-PCR
STB	Syncytiotrophoblast
TE	Trophectoderm
TS	Trophoblast stem

Chapter 1

1 INTRODUCTION

1.1 The Placenta

1.1.1 Human placental development

The placenta in eutherian mammals is a unique exchange organ that develops between the mother and the fetus and is essential for successful pregnancy and fetal health. It serves multiple functions essential for fetal growth and survival, including provision of O_2, nutrients and hormones, removal of CO_2 and other waste products, and immune-protection from maternal immune attack. It invades the uterine endometrium to variable degrees to allow a transfer of nutrients from the mother to the fetus. Depending on the degree of invasion it is classified as (a)“epithelio-chorial” (e.g., in ruminants) in which the chorionic trophoblast does not breech the endometrial epithelium; (b)“endothelio-chorial” (in carnivores), in which the trophoblast invades the endometrial stroma to reach the maternal endothelium; (c)“hemo-chorial” (in rodents, and most primates, including humans) in which the trophoblast invades the endometrial arteries to derive oxygen and nutrients from the maternal arterial blood (reviewed by Halari, Zheng and Lala, 2021). Within the hemo-chorial class, the placenta in most primates including the human is the most invasive tumor-like structure which invades the uterine endometrium, endometrial glands and the vasculature (arteries, veins and lymphatics). Extensive development of new lymphatic vessels (lymphangiogenesis) has been demonstrated in the pregnant uterus (Red-Horse, 2008) and trophoblast invasion of the lymphatics and endometrial glands has been noted during the second week of gestation (Moser et al., 2018; Huppertz et al., 2019).

Placenta develops from the extraembryonic epithelial tissue layer called the trophectoderm (TE), which in humans is formed 5 days post conception in the pre-implantation embryo (termed a blastocyst). At this stage cells in the blastocyst are segregated into two lineages: the inner cell mass (ICM) and the TE. The polar TE, that is adjacent with the underlying ICM attaches to the uterine surface epithelium of the endometrium (Fig. 1-1) and lays the foundation for the beginning of placenta formation. Although the earliest stages of

implantation have not been directly visualized in humans, our knowledge of the first weeks of placental development is based on the early pregnant hysterectomy specimens (Centre of Trophoblast Research, Cambridge), Carnegie (Human Developmental Anatomy Center, Washington DC) collections and higher primate studies (Hertig et al., 1956; Hamilton and Boyd, 1960; Carter et al., 2015; Ojosnegros et al., 2022). These studies suggest that at around 7-8 day post conception, TE fuses to form a primary syncytium, which represents the first invasive placental cells. (Boss et al., 2018). The placenta then develops rapidly during the first weeks of gestation dynamically changing its structure and function (Burton and Fowden, 2015).

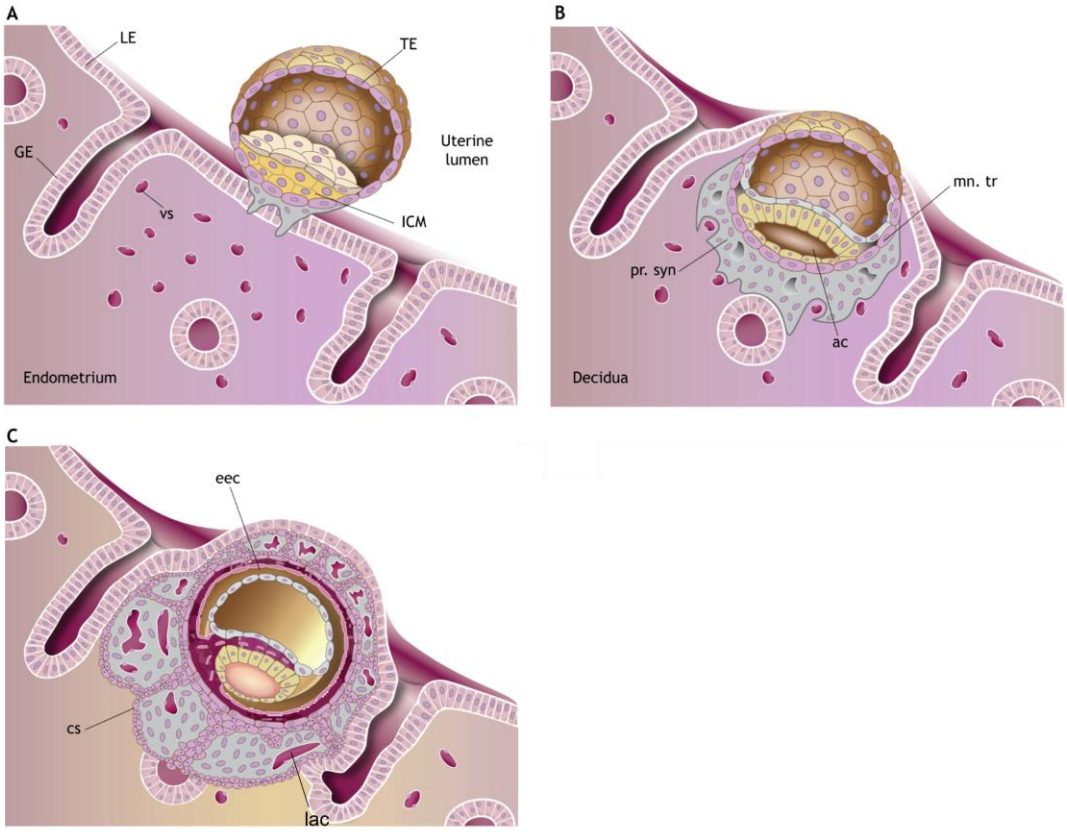

Figure 1-1: Human placental development.

Schematic representation of early stages in placenta formation following implantation. (A,B) The pre-lacunar stages. (C) The primary villous stage. cs, cytotrophoblastic shell; eec, extra-embryonic coelom; GE, glandular epithelium; ICM, inner cell mass; lac, lacunae; LE, luminal epithelium; mn. tr, mononuclear trophoblast; pr. syn, primary syncytium; TE, trophectoderm; vs, blood vessels. (Adapted and modified from Turco and Moffett, 2019).

The primary syncytium invades through the uterine surface epithelium into the underlying endometrium and contributes to the transformation of the endometrium into a specialized tissue known as the decidua (Fig. 1-1) (Schlafke and Enders, 1975). Fluid-filled spaces (lacunae) then appear within the syncytial mass that enlarge and merge, partitioning it into a system of trabeculae. The syncytium also erodes into decidual glands, allowing secretions to bathe the syncytial mass (Hertig et al., 1956).

The TE generates another trophoblast lineage called mononuclear cytotrophoblasts (CTB) which are beneath the primary syncytium and initially not in direct contact with maternal tissue. During primary syncytium expansion, CTB rapidly expands by continuous proliferation and cell fusion to form projections that will push through the primary syncytium to form primary villi with a CTB core and an outer layer of syncytiotrophoblast (STB). The STB is primarily engaged in absorptive, exchange and endocrine functions and secretes critical pregnancy hormones into the maternal circulation, such as human chorionic gonadotrophin and placental lactogen (Evain-Brion et al., 2003; Aplin, 2010). The villous trees are formed by further proliferation and branching, and the lacunae become the intervillous space. CTB eventually penetrate through the primary syncytium and merge laterally to surround the conceptus in a continuous CTB shell between the villi and the decidua. The blastocyst is now covered by three layers: the inner chorionic plate in contact with the original cavity; the villi separated by the intervillous space; and the CTB shell in contact with the decidua.

Soon afterwards, extraembryonic mesenchymal cells migrate through the villous core to form secondary villi. By day 18-post conception, fetal blood vessels develop from the mesenchymal cells in the villus core, when the villi are called tertiary villi. The villi continue to grow rapidly by progressive branching from the chorionic plate to form a system of villous trees. Where the CTB shell is in contact with the decidua (the maternal-fetal interface), CTB cells migrate as cell columns to invade into decidua as extravillous trophoblast (EVT) and invade uterine decidua & spiral arteries (Lala and Hamilton, 1996; Knöfler and Pollheimer 2013). Some of the EVT cells undergo endothelial-like (endovascular) differentiation to invade and remodel distal segments of the arteries into

low-resistance tubes that allow steady flow of maternal blood for fetal nourishment (Kaufmann et al., 2003; Cartwright et al., 2010) (Fig. 1-2).

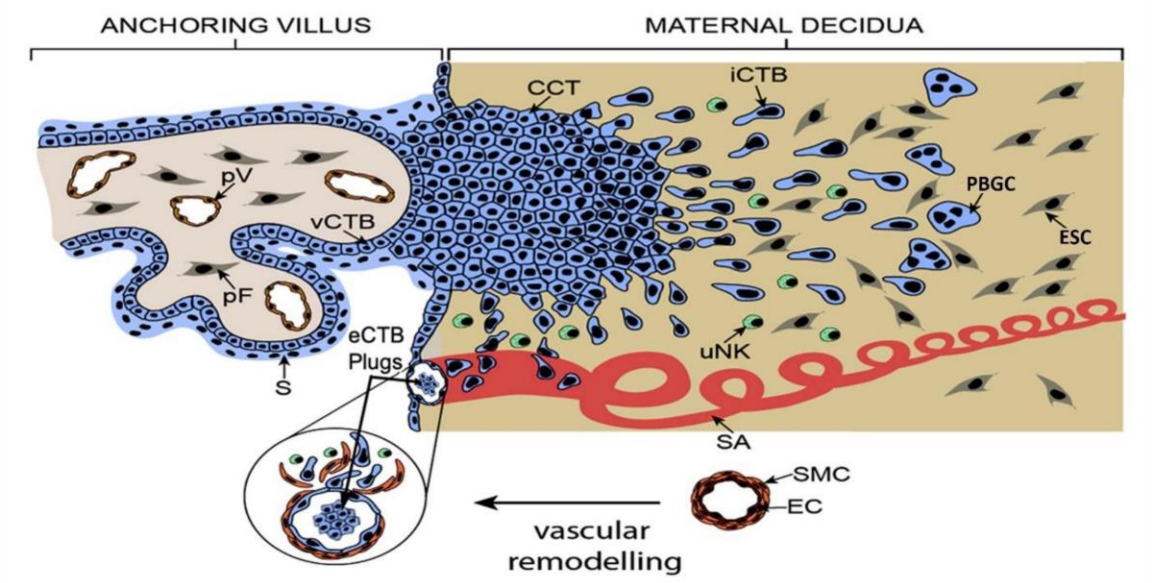

Figure 1-2: Cell columns migrating out of the CTB layer form the anchoring villi invading the decidua.

The cells give rise to different invasive extravillous trophoblast (EVT) subtypes**.** Interstitial cytotrophoblasts (iCTBs) migrate into the decidual stroma and differentiate into placental bed giant cells (PBGCs). Endovascular trophoblasts migrate into spiral arteries and contribute to uterine natural killer (uNK) cell-initiated remodeling of spiral arteries (SA). Bulmer et al. (2020) recently challenged the long-held view that endovascular trophoblasts (eCTBs) replace the endothelium of spiral arteries during arterial remodeling. By immunostaining a large number of chorionic villus biopsy samples, they found that, while eCTB plugs appear within the arterial lumen, arteries were found with either missing or intact endothelium, but never lined by eCTBs. CCT, cell column trophoblast; ESC, endometrial stromal cell; eCTB, endovascular cytotrophoblast; PBGC, placental bed giant cell; iCTB, interstitial cytotrophoblast; pF, placental fibroblast; pV, placental vessel; SA, spiral artery; uNK, uterine NK cell; vCTB, villous cytotrophoblast. Reproduced with permission from Halari, Zheng and Lala, 2021.

Human Trophoblast stem cells: Possible sources of human trophoblast stem (hTS) cells, which are the multipotent cells capable of giving rise to all trophoblast lineages, have remained controversial for a long time. Trophoblast stem (TS) cell lines derived from mouse blastocyst have been instrumental in defining the molecular mechanism in differentiation pathways (Tanaka et al., 1998, Rossant, 2007). The stem cells were categorized into embryonic stem like and more committed TS-like cells with distinct embryonic stem and TS cell markers. Until recently, it has not been possible to establish hTS cell lines from the preimplantation blastocyst, and the source of stem cells that sustains trophoblast growth, renewal and differentiation in the human placenta remained poorly characterized. Genbacev et al (2011) reported that chorionic mesenchyme serves as a niche for hTS cells. They isolated cells expressing pluripotency markers from first and second trimester chorion, and grew them in the presence of FGF and inhibitors of the activin/nodal pathway to obtain self-replicating cells, which gave rise to both STB and CTB with invasive phenotype simulating EVT. It is likely that chorionic mesenchyme is a source of pluripotent stem cells, which were induced to form trophoblast *in vitro*. Similarly human embryonic stem cells lines have also been successfully manipulated *in vitro* with bone morphogenetic protein (BMP) 4 treatment to obtain cells with STB and EVT phenotype (Xu et al., 2002, Golos et al., 2013, Hori et al., 2016). Recently, Okae et al (2018) succeeded in the establishment of hTS cell lines both from the pre-implantation blastocyst and from primary trophoblast cells isolated from first trimester chorionic villi. They show that activation of Wingless/integrated (Wnt) and Epidermal growth factor (EGF), as well as inactivation of Transforming Growth Factor (TGF)β and Rho/Rho-associated coiled-coil containing protein kinase pathways could sustain the stem cell phenotype in blastocyst-derived and first trimester villus- derived hTS cells. These hTS cells exhibited similar transcriptomes and methylomes indicating similar genetic and epigenetic profiles. Under specific culture conditions the hTS cells could differentiate into both syncytial and EVT pathways. Recently our lab identified hTS cells in early first trimester (7-9 weeks gestation) villus CTB cells from the self-renewal capacity of single cells to form spheroids on ultra-low attachment plates for multiple generations (Nandi et al., 2018), showing that they contained 0.1-0.4% hTS cells, very similar to the report by Okae et al (2018). Nandi et al (2018) also utilized the immortalized first trimester human trophoblast cell line HTR-

8/SVneo developed by our lab three decades ago (Graham et al., 1993) to show that this cell line had an incidence of 13-15% hTS cells. In both cases, the stem cells had markers of both Embyonic stem and TS cell phenotypes. The TS cells isolates from villi could differentiate into both syncytial (STB) and EVT pathways, whereas TS cells in HTR-8/SVneo cells primarily differentiated into EVT pathway and had restricted capacity to give rise to STB. The CTB-like cells in the BeWo choriocarcinoma cell line, on the other hand, is more restricted for differentiation into STB pathway. Despite the limitations of using cell lines, these findings suggest a hierarchy of self-renewing hTS cells which give rise to progenitors of EVT or STB pathways having limited self-renewal capacities. The hTS cells in HTR-8/SVneo cell line may be more committed to EVT pathway, whereas BeWo cell line may be more committed to syncytial pathway. This concept of stem cell hierarchy is supported by the transcriptomic profile of cells derived from trophoblast organoids produced with primary trophoblast cells (Haider et al., 2018). The incidence of hTS cells almost becomes non-detectable in chorionic villi older than 9 weeks (Okae et al., 2018, Nandi et al., 2018). However, for the first time, very recently Wang et al (2022) succeeded in generating hTS cell from term human placentas. They demonstrated that the induction efficiency of hTS from CTB is determined by functional antagonism between the stemness regulator ΔNp63α and the placental transcription factor Glial Cells Missing Transcription Factor 1 (GCM1). They used a cocktail of EGF/CASVY to activate ΔNp63α, thereby partially inhibiting GCM1 activity and reverting term CTB into hTS cells. By applying hypoxia, they could further reduce GCM1 activity and more efficiently induce term CTB into hTS cells. This study presents a major milestone in TS cell research.

1.1.2 Decidualization

The inner lining of the uterus known as “endometrium” is a complex, dynamic, heterogeneous tissue made up of multiple cell types. It is the one organ that changes its size every month during female’s fertile years (Evans et al., 2016). Upon conception, this endometrium undergoes functional and morphological changes to form the specialized endometrial lining into which the blastocyst implants and, called “decidua”. This process is called “decidualization”. The name “decidua” derives from its transient nature of being

shed into the uterine lumen like the deciduous leaves that are shed in autumn. Decidua serves as a critical organ in performing the endocrine and immunological functions. Upon implantation of the blastocyst, three distinct decidual regions are formed in the uterus, each serving its own separate function. The region where placenta develops is called decidua basalis. Decidua capsularis overlies the embryo and chorion, whereas decidua basalis lies between the chorion and the uterine wall and is the region where placenta develops (Fig. 1-3). Decidua parietalis is the remaining decidua covering the sides of the uterus not occupied by the embryo. Decidua capsularis and parietalis eventually come in contact by mid pregnancy, fuse and obliterate the uterine cavity.

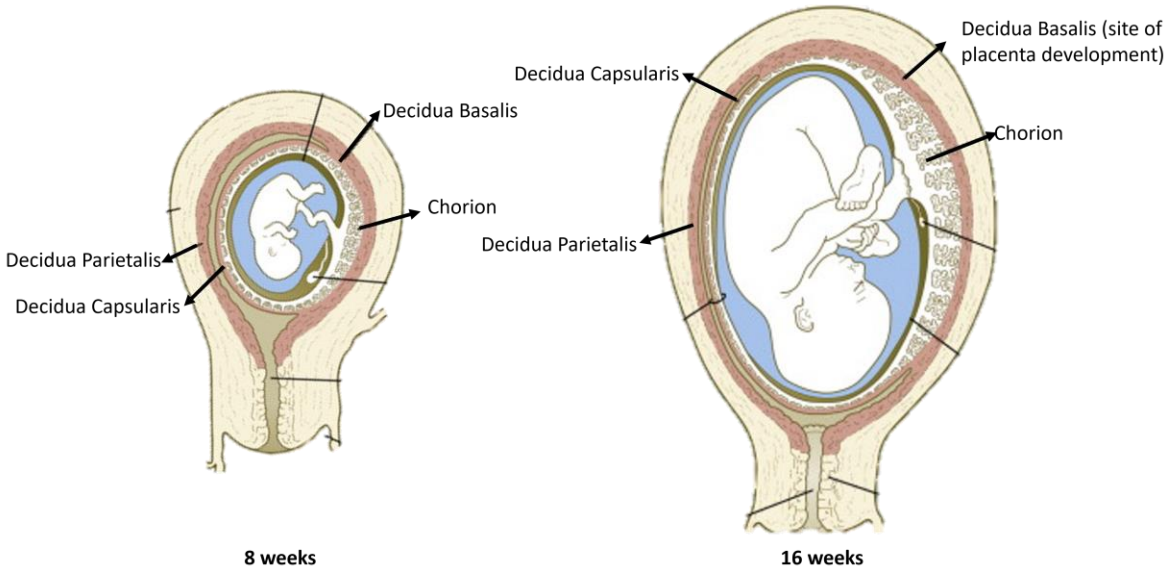

Figure 1-3: Anatomic arrangement of the decidua

8 and 16 week gestation uterus showing decidua basalis, the region where placenta develops along with decidua capsularis and parietalis Modified and adapted from Ng et al 2020.

1.1.2.1 The process of decidualization

Decidualization is an important step for uterine receptivity which ensures successful implantation and placentation. Following implantation, proper decidualization of the endometrium plays a key role in the development of the placenta and is likely to involve all the major cellular elements of the endometrium: glands, vessels, stromal cells and immune cells. Importantly, these changes include the differentiation of endometrial stromal fibroblast-like cells (ESCs) into decidual stromal cells (DSCs). It is the ability of ESCs to differentiate into this alternative cell state that appears to be the key element in decidual transformation. DSCs are not simply modified ESCs; they are a distinct cell type resulting from terminal differentiation and the genetic reprogramming of ESCs. This reprogramming includes the downregulation of genes involved in the pro-inflammatory response and in resisting tissue invasion along with increased expression of genes that promote cellular proliferation, immune tolerance, and tissue invasion. DSCs make up 10–30% of decidual cells in the first trimester and up to 60–70% of cells in term decidua. DSCs behave as quiescent cells during their late stage of decidualization associated with increase in cellular death through apoptotic pathways (Rytkonen et al., 2019). Thus, there should be a supplementary pool of cells that continuously replenishes DSCs throughout pregnancy. Evidence from several studies of decidualization conducted with uterine stromal fibroblasts cultures isolated from human first trimester, term and peri-partum decidua, supports the theory that decidual fibroblasts must be functioning as a pool of DSC precursors during pregnancy (Richards et al., 1995; Gargett et al., 2016; Huang et al., 2021).

During early weeks of human pregnancy, fetal development depends upon secretions from endometrial glands as the source of histotrophic nutrition, since endovascular trophoblast plugs only allow seepage of maternal blood into the intervillous space (Burton et al., 2002, 2007). ESCs produce a number of growth factors that stimulate glandular development and secretions. As they decidualize, the DSCs starts to secrete basement membrane proteins, fibronectin, and laminin (Aplin et al., 1988) and decorin (DCN) (Xu et al., 2002) which provides a framework for the EVT to move through in a highly controlled manner. But there is still a big question mark on what specific factors of receptive endometrium are crucial to support the developing placenta (Koot et al., 2016; Young, 2017). Since defective

decidualization has been linked to pregnancy disorders and failure of implantation, there is a growing need to answer this question and conduct more research in this field (Conrad et al., 2017; Garrido-Gomez et al., 2017).

1.1.2.2 Physiology of decidualization

In most mammals that exhibit decidualization, the decidual reaction that transforms the endometrium into decidua is initiated by the arrival of the blastocyst. Humans are one of the few mammalian viviparous species in which decidualization is initiated independent of the presence of a blastocyst and minor cyclic decidualization is observed in stromal cells in the mid-to late secretory phase of the menstrual cycle, transforming the stromal cells into larger, rounded decidual cells (Maruyama and Yoshimura, 2008). Ultrastructural studies of human decidual cells indicate their characteristics as: enlarged and rounded nuclei, increased numbers of nucleoli, dense membrane-bound secretory granules, cytoplasmic accumulation of glycogen and lipid droplets, and the expansion of the rough endoplasmic reticulum and Golgi complex (Gellersen and Brosens, 2014). As a result of this differentiation, decidual cells acquire unique biochemical and cellular properties that enable them to support embryo implantation. This process, preceding implantation, is exclusively observed in mammals with menstruation and deep, hemo-chorial placentation, such as humans and old-world primates (Ramsey et al., 1976; Emera et al., 2012). The steroid hormones Estradiol and Progesterone are critical for the regulation of the cyclical events in human endometrium (Young, 2013). Extensive proliferation of both epithelial and stromal cells occurs in response to rising estradiol levels during the first half of the menstrual cycle, or proliferative phase. During the second half of the cycle, termed secretory phase, progesterone action dominates in the endometrium where it induces differentiation of stromal cells (Critchley et al., 1998). Estradiol plays an important role in the expression of the progesterone receptor, which permits the endometrium to respond to progesterone in the secretory phase (Cole, 2010; Koos, 2011; Hamilton et al., 2014). In the event of no blastocyst implantation, progesterone levels drop and continued decidual formation is prevented, while the transformed endometrial stroma cells are lost due to shedding of the upper layer of the decidualized endometrium (i.e., menstruation). However,

if there is implantation of the blastocyst, the placenta will secrete human chorionic gonadotropin which leads to sustained progesterone production and full development of the decidua (Cole, 2010).

Progesterone acts by binding and activating the nuclear progesterone receptor, PR, which is highly expressed in the stromal cells during the secretory phase and in pregnancy (Telgmann and Gellersen, 1998). Activation of PR alters the expression of numerous genes that are play a role in preparation for pregnancy. Decidua-derived factors promote immune-protection of the allogeneic conceptus and prevent over-invasion of the uterus by the placental trophoblast. For example, Prostaglandin E2 (PGE2) was identified as a key local immunosuppressor molecule produced by decidual cells. PGE2 suppress activation of decidual natural killer cells with potential anti-trophoblast killer function (Parhar and Lala, 1988; Parhar et al., 1989) by blocking the production of interleukin (IL)2 and development of IL2 receptors on lymphocytes. Murine pregnancy could be interrupted by activation of antigen-non-specific killer cells in the endometrium following treatment with the prostaglandin inhibitor indomethacin, high dose IL-2 or a combination of the two (Lala, 1990). Additionally, two decidua-derived molecules TGF-β (Graham and Lala, 1991, 1992, Lala et al., 1998) and DCN (Nandi et al., 2015) were shown by our lab as key factors controlling trophoblast invasion of the uterus.

1.1.2.3 Origin of decidual cells

The origin of ESC that serve as precursors of DSC in the pregnant uterus remains controversial. Some three decades ago Lysiak and Lala (1992) from this laboratory, using a beta-globin transgene marker, demonstrated that some or most decidual cell precursors in the mouse pregnant uteri are derived from progenitors in the bone marrow. Bone marrow from CD1 mice bearing a thousand copies of the beta-globin gene was injected into the amniotic cavity of 17-day-old embryos of wild type CD1 mice. The pregnant females were allowed to deliver normally, and the female offspring raised to puberty were mated with CD1 males and then killed on day 12 of gestation. The extent of donor cell reconstitution (chimerism) was evaluated in sections of the host spleens, uteri, and other organs was

evaluated by *in situ* hybridization with a biotinylated cDNA probe specific for the beta-globin genes. The chimerism in decidual cells as well as uterine natural killer cells was similar to that in the spleen, indicating that bone marrow contained the ultimate precursor of decidual cell progenitors that may have migrated to the uterus at any time during ontogeny or pregnancy. However, whether these cells were pleuripotent hemopoietic stem cells or a distinct population of bone marrow mesenchymal cells remained unknown. More recently, Tal et al (2019) confirmed these findings using a different mouse model and showed that the precursors are non-hemopoietic mesenchymal stem cells. They used Homeoboxa11 (HOXA-11) deficient mice having endometrial stromal-specific defects precluding decidualization and successful pregnancy. Hoxa11 expression in the bone marrow was restricted to non-hematopoietic cells. BM transplants from wild type to Hoxa 11 null mice resulted in uterine stromal cell expansion, gland formation, and successful decidualization. Moreover, in Hoxa11 +/- mice, which have increased pregnancy losses, bone marrow transplant from WT donors led to rescue of pregnancy loss and normalized uterine expression of multiple decidualization-related genes. Collectively, these reports reveal that adult BM serves as a major source of decidual cell progenitors during pregnancy.

During the window of implantation, the mid-luteal human endometrium has a discrete population of highly proliferative mesenchymal cells (Diniz-da-Costa et al., 2021). These cells had high colony forming ability *in vitro*. Using single-cell transcriptomics, the authors demonstrated that highly proliferative mesenchymal cells (hPMC) express genes involved in chemotaxis and vascular transmigration. Although distinct from resident endometrial stromal cells, hPMC also expressed genes encoding pivotal decidual transcription factors and markers, most prominently prolactin. The hPMCs were enriched around spiral arterioles, scattered throughout the stroma, and occasionally present in glandular and luminal epithelium. The clonogenic cells in culture expressed a gene signature partially conserved in hPMC. They found that recurrent pregnancy loss was associated with hPMC depletion. These midluteal hPMCs represent novel decidual precursors that are likely derived from the bone marrow as described earlier.

1.1.2.4 Morphological changes in decidualized endometrial stromal cells

During decidualization, ESCs undergo remarkable morphological changes, in which elongated, spindle-shaped endometrial stromal fibroblasts transform into secretory, epithelioid-like decidual cells (Gellersen et al., 2007; Maruyama and Yoshimura, 2008; Yu et al., 2016). (Fig. 1-4)

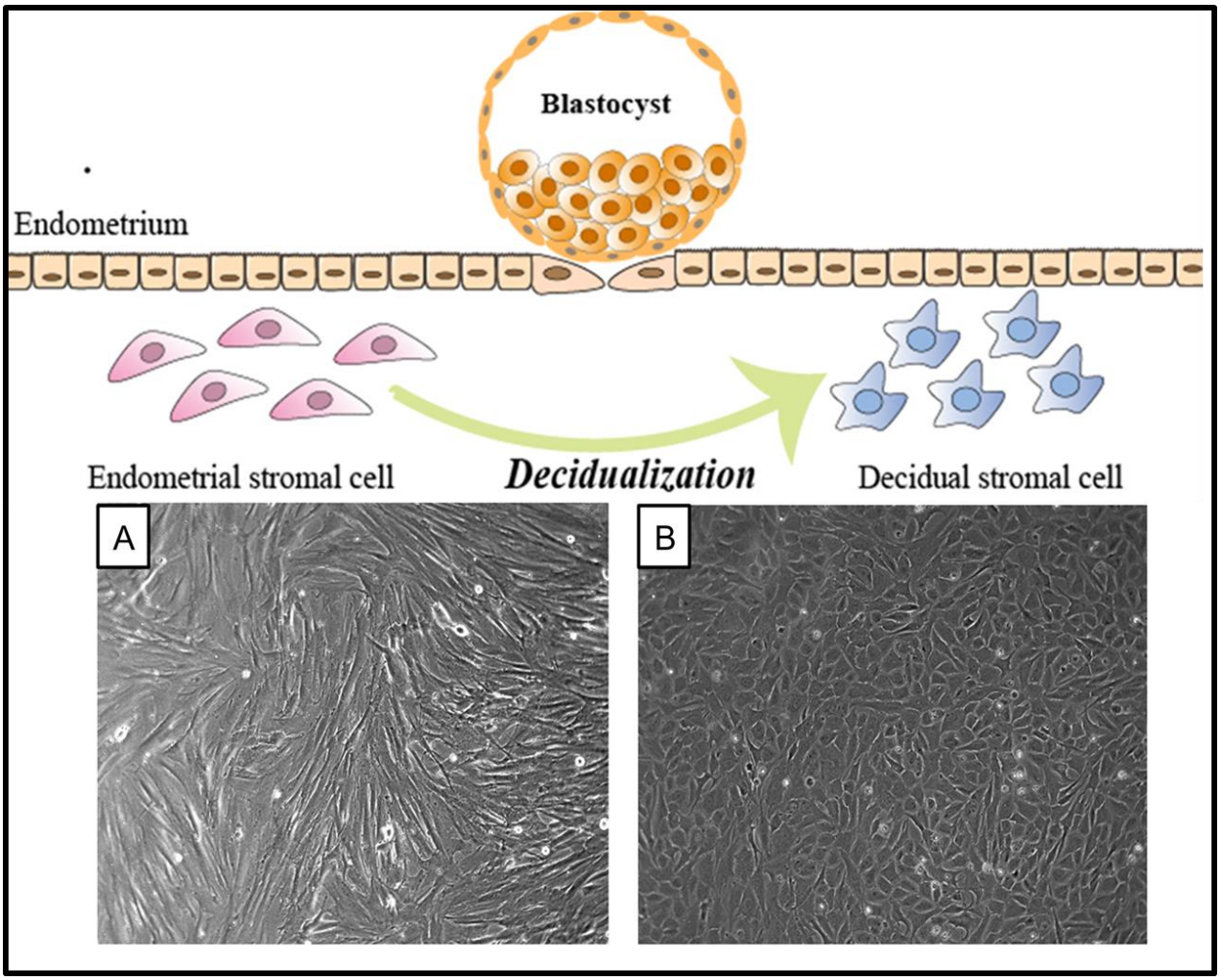

Figure 1-4: Diagram showing changes in cellular morphology of human endometrial stromal cells following implantation of blastocyst.

Immortalized human endometrial stromal cells prior to (Panel A) and following *in vitro* decidualization (Panel B) showing change from fibroblastic to epithelioid appearance. Modified and adapted from Owusu-Akyaw et al., 2019 and Xu et al., 2021.

These morphologic changes have been extensively characterized in various studies including Halari et al., 2020. Before ovulation starts (endometrial proliferative phase), ESCs have very little cytoplasm, and are elongated with indented nuclei (Gellersen and Brosens, 2014). As the endometrial stromal cells transition from fibroblastic to polygonal like, they show multiple intracellular changes such as, increase in glycogen and lipid, expansion of rough endoplasmic reticulum, and cytoskeletal reorganization (actin microfilaments) (Aplin et al., 1988; Abrahamsohn and Zorn, 1993; Gellersen et al., 2007). As they approach the decidualized stage, cytoplasm shows significant expansion, nuclei become more rounded in comparison to previous elongated shape and develop more nucleoli, and show increase in intracellular phagosomes and lysosomes (Lawn et al., 1971; Cornillie et al., 1985). *In vitro* studies using different combinations of hormones such as (estradiol, medroxy progesterone acetate) have been used to promote the remarkable decidualization of ESC, but it is clear that increased intracellular cyclic adenosine monophosphate (cAMP) is essential for this process no matter what hormones are used.

1.1.2.5 Regulation of uterine decidualization

There are many similarities in the factors required for decidualization in mice and humans (reviewed by Ramathal et al., 2010). The steroid hormones estrogen (E) and progesterone (P) play a pivotal role in directing early uterine events during pregnancy in both species. The PR knockout mouse uterus cannot support embryo implantation and are also non-receptive to an artificial deciduogenic stimulus, such as the intrauterine injection of oil. Under chronic E stimulation, increased hyperplasia and an inflammatory response is observed, due to unopposed action of E in the uterus. On the other hand, estrogen receptor (ER)α-null mice have impaired uterine growth and fail to prepare for blastocyst attachment. The ablation of uterus specific ERα in PR-positive uterus in the mutant animals exhibited a failure in the decidual response to an artificial stimulus indicating an important role for ERα in decidualization. Both murine and human decidualization requires ERα and PR proteins to promote uterine stromal differentiation. In the post-implantation mouse uterus, during the next 3 days of gestation, decidual cells surrounding the site of embryo attachment proliferate and differentiate extensively, eventually becoming larger, often with

bi-nucleated or polyploid status (Ansell et al., 1974). Polyploid cells eventually die by apoptosis. By the end of the invasive period of the trophoblast (day 10.5), the murine decidua has regressed totally. Polyploidy has also been observed in *in vitro* decidualized human ESCs (Halari et al., 2020; Chapter 2).

Several growth factors, cytokines and protein hormones are recognized as crucial markers for the initiation and maintenance of decidualization. Differentiating ESCs carry a molecular signature of mesenchymal–epithelial transition as they are reprogrammed to become DSCs with widespread changes in gene expression, Examples include EGF, IGF, ECM proteins (laminin, type IV collagen, fibronectin, heparin sulfate), and an increase in the production of secretory proteins including prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1), two established markers of decidualization. (Telgmann and Gellersen ,1998). In 2011, Garrido-Gomez et al. conducted a proteome and secretome screening study using decidualized ESCs, and reported that there are several decidualization markers besides PRL and IGFBP1 that might be involved in promoting angiogenesis (a critical component of decidualization during early pregnancy), including platelet/endothelial cell adhesion molecule-1 (PECAM-1) and myeloid progenitor inhibitory factor-1. Another study conducted using primary ESCs reported that a coordinated and synchronized changes in the secretome of 23 secreted factors were associated with successful implantation, including IL-13, IL-6, CCL3, chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL8 (IL-8) whereas, failed implantation was reported in the group with disordered secretome profile in comparison to the pregnant group (Peter et al., 2017).

Progesterone along with various progesterone regulated proteins forms a complex network for decidualization. They include Indian hedgehog (IHH) (Lee et al., 2006), Bone morphogenetic protein (BMP2) (Lee et al., 2007), nuclear receptor subfamily 2 group F member 2 (NR2F2/COUP-TFII) (Kurihara et al., 2007), Wingless-type MMTV integration site family (WNT) 4 (Franco et al., 2011), heart and neural crest derivatives expressed transcript 2 (HAND2) (Li et al., 2011), Forkhead box O1 (FOXO1) (Vasquez, 2015), CCAAT/enhancer-binding protein beta (CEBPB) (Wang et al., 2012), and homeobox A10 (HOXA10) (Godbole et al., 2010), mammalian target of rapamycin complex 1 (MTORC1)

(Baek et al., 2018), and the tumor necrosis factor alpha-nuclear factor kappa-light-chain-enhancer of TNFα/NFκβ pathway in activated B cells (Sugino et al., 2004). NODAL-signaling pathway inhibitor left-right determination factor-2 is another factor profusely secreted by decidualized cells (Tang et al., 2005).

Expression of local factors such as relaxins and prostaglandins stimulate elevated levels of cAMP in ESCs (Gellersen and Brosens, 2014). This cAMP activity leads to the activation of protein kinase A (PKA) that crucial for decidualization (Cheng et al 2008). PR is the dominant member of the 3-ketosteroid nuclear receptor family that responds to progesterone and cAMP/PKA signaling during decidualization (Gellersen and Brosens, 2014; Wu et al., 2018). Studies using PKA inhibitors have shown downregulated expression of genes associated with decidualization in cultured human ESCs (Brar et al., 1997; Matsuoka et al., 2010). Activation of protein kinase A through cAMP signalling sensitizes ESCs to progesterone (Gellersen and Brosens, 2003), but this mechanism is not yet fully understood. Decidualization can be induced *in vitro* in cultured ESCs within 3 days by factors that increase intracellular cAMP concentrations (Brosens et al., 1999). These factors include prostaglandin E2, relaxin, corticotrophin- releasing hormone, gonadotrophins or cAMP analogues such 8-bromo-cAMP (Nemansky et al., 1998). Treatment with 8-Br-cAMP alone increases decidual markers expression, which is further elevated when cells are exposed to both 8-Br-cAMP and the synthetic progesterone, progestin.

It appears that cAMP elevation and progestin are the minimal signals for decidualization. This hypothesis was tested by Stadtmauer and Wagner (2022) who performed a genome-wide gene expression analysis in human ESCs decidualized with PGE2 and progestin. In comparison to cAMP induced decidualization, PGE2 mediated decidualization revealed shared activation of core decidual genes and decreased induction of senescence-associated genes. Single-cell transcriptomics of PGE2-mediated decidualization revealed a distinct, early-activated state transitioning to a differentiated decidual state. PGE2-mediated decidualization was found to depend upon progestin-dependent induction of PGE2 receptor 2 subtype (EP2), leading to PKA activation. Progesterone-dependent induction of EP2 is

absent in opossum, a placental mammal incapable of decidualization. Thus, EP2 activation is a prime requirement for decidualization.

Another factor that can affect decidualization is reactive oxygen species. During the decidualization process, changes in oxygen tension due to arterial remodeling is observed and this can generate reactive oxygen species. DSCs have the capacity to resist a certain range of cellular oxidative stress signals. Many molecular mechanisms play a role in this, including the inhibition of stress pathways such as c-Jun N-terminal kinase (Leitao et al., 2010), reduced inositol trisphosphate signaling (Muter et al., 2016), microRNA-mediated gene silencing resistance (Shah et al., 2013), and the increase of free radical scavengers (Kajihara et al., 2006).

Involvement of microRNA (miRNA) in epigenetic regulation of decidualization is another growing topic. Using the miRNA profiling of primary ESCs before and after *in vitro* decidualization, Estella et al. (2012) reported increased expression of 26 miRNAs and decreased expression of miR-96, miR-135b, miR-181, and miR-183. Furthermore, introducing miR-96 and miR-135b in decidualizing ESCs, resulted in the expression of FOXO1 and HOXA10 and decrease in IGFBP1 secretion by decidual cells (Estella et al., 2012). Another study demonstrated an upregulation of the miR-200 family during *in vitro* decidualization, correlated with the downregulation of IHH signaling (Jimenez et al., 2016). Several other studies have shown the functional importance of miRNAs in decidualization such as increased miR-181a expression promotes hESC decidualization by inhibiting transcription factor KLF12 (Zhang et al., 2015), miR-542-3p decreases decidualization by down-regulating key decidual genes such as IGFBP1, PRL, integrin-linked kinase and TGF-β1(Tochigi et al., 2017, Qu et al., 2021) and miR-194-3p reduces decidualization by repressing PR (Pei et al., 2018).

Overall, decidualization is a complex process regulated by a variety of extrinsic and intrinsic factors that converge on activation of PR and cAMP. Since proper decidualization is beneficial for healthy pregnancy, disruptions in the regulatory mechanisms controlling decidualization can compromise extra cellular matrix (ECM) remodeling, which involves

synthesis of collagens and non-collagenous molecules such as DCN, a leucine-rich proteoglycan.

1.1.3 Decorin

DCN is a well-characterized member of the widely distributed small leucine rich protein family, ubiquitously expressed in the ECM of most tissues (Iozzo, 1999). Its so named because of its capacity to bind and 'decorate' collagen fibrils. (Hocking et al., 1998; Vogel et al., 1984; Scott et al., 1988). It is mainly known for its vital functions as a powerful antifibrotic, anti-inflammation, antioxidant, and antiangiogenic molecule (Iozzo et al., 1999; Salomaki et al., 2008; Neill et al., 2012; Järveläinen et al., 2015). DCN was the first proteoglycan that was associated with regulating the cell cycle by inhibiting TGFβ signaling (Yamaguchi et al., 1990).

DCN is a small leucine-rich proteoglycan containing a 40 kDa backbone protein with 4 domains (Iozzo, 1998; Lala and Nandi, 2016). Domain I in the nascent DCN molecule is ultimately cleaved off and absent from the mature protein, domain II is covalently linked with a glycosaminoglycan (chondroitin or dermatan sulphate) chain, domain III has 10-12 leucine-rich repeats (LRR), whereas domain IV has two cysteine residues and forms a large loop. (Fig. 1-5).

DCN is produced by several stromal cell classes including dermal fibroblasts, chondrocytes, chorionic villus mesenchymal cells of the placenta and DSCs (Lysiak et al., 1995). DCN can function by binding to ECM molecules e.g., collagen (Danielson et al., 1997), fibronectin, thrombospondin (Winnemoller et al., 1992) or growth factors, e.g., TGFβ (Iozzo, 1998) and IGF-I (Winnemoller et al., 1992), Toll-like receptors (TLR)-2 or TLR4 (Schönherr et al., 2005) or as an antagonistic ligand for some tyrosine kinase receptors (TKRs) EGFR (Frey et al., 2013; Santra et al., 2002; Zhu et al., 2005), IGFR-1 (Schönherr et al., 2005; Iozzo et al., 2011), MET (Goldoni et al., 2009) and VEGFR-2 (Khan et al., 2011). VEGFR-2 binding site of DCN was shown to be confined to a 12 amino acid span of the LRR5 in domain III (Khan et al., 2011). This binding blocked VEGFR-2 dependent migration and endovascular differentiation of the EVT (Lala et al., 2012), events

that allow EVT to replace uterine arterial endothelium in the process of arterial remodeling during normal pregnancy. This process is known to be compromised in preeclampsia (PE) and a subset of fetal growth restriction (FGR) (Lala and Nandi, 2016).

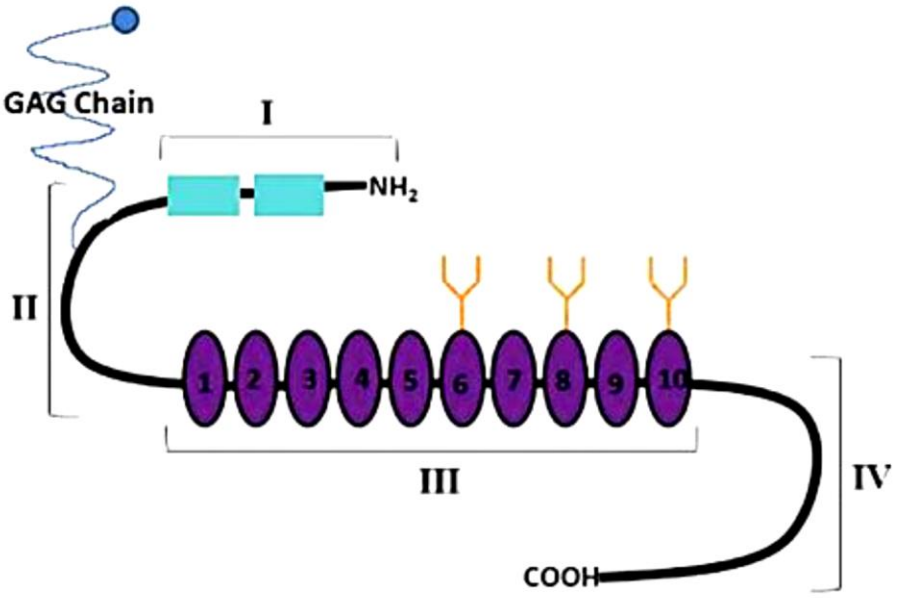

Figure 1-5: DCN structure

40 kDa core protein and a glycosaminoglycan (GAG) chain that is tissue specific. The core protein is composed of four domains: domain I contains a signal peptide and propeptide; domain II contains four evenly spaced cysteine residues and the GAG attachment site; domain III contains ten leucine rich repeats and domain IV contains a relatively large loop with two cysteine residues. (Reproduced with permission from Nandi et al., 2016)

The most vital function of DCN in collagen fibrillogenesis was first shown in DCN null mice. These mice displayed poor collagen assembly and skin fragility with significant reduction in tensile strength (Danielson et al., 1997). Additionally, decidualized endometrium from DCN null mice showed collagen fibrils with larger diameter and abnormal outline (uncontrolled lateral fibril assembly), in comparison to the wild type mice (Sanches et al., 2010). In human, DCN was found to be produced by DSCs, colocalized with TGFβ in the ECM throughout human gestation (Lysiak et al., 1995). An increase in DCN mRNA with upregulation of human *PRL* and its receptor (*PRLR*) in human decidua was reported (Brar et al., 2001; Eyal et al., 2007). Other DCN functions include: myogenesis by binding to and sequestering myostatin, a myogenic inhibitor (Kishioka et al., 2008); restraint of angiogenesis (Järveläinen et al., 2015) by binding to thrombospondin (Davies et al., 2001) or VEGFR-2 (Khan et al., 2011; Lala et al., 2012; Lala and Nandi, 2016). Furthermore, DCN overproduction by the decidua was shown to be causally associated with PE, by compromising trophoblast invasion, endovascular differentiation and uterine arterial remodeling (Lala et al.,2012; Siddiqui et al., 2016). Lastly, DCN plays a role in restraining human TS cell self-renewal and differentiation capacity into STB and EVT (Nandi et al., 2018).

In summary, we found that decidua-derived DCN plays a paracrine role in controlling multiple trophoblast functions which are dysregulated in PE. However, no information exists as to whether DCN has any autocrine role in decidual development.

1.1.4 Preeclampsia

Poor EVT invasion and arterial remodeling resulting in a hypo-perfused placenta have been linked with maternal PE and and a subset of FGR (Meekins et al., 1994; Pijnenborg, 1996; Lala and Chakraborty, 2003; Kaufmann et al., 2003; Cartwright et al., 2010; Redman and Sargent, 2005; Burton et al., 2009; Cartwright et al., 2010). Fig. 1-6

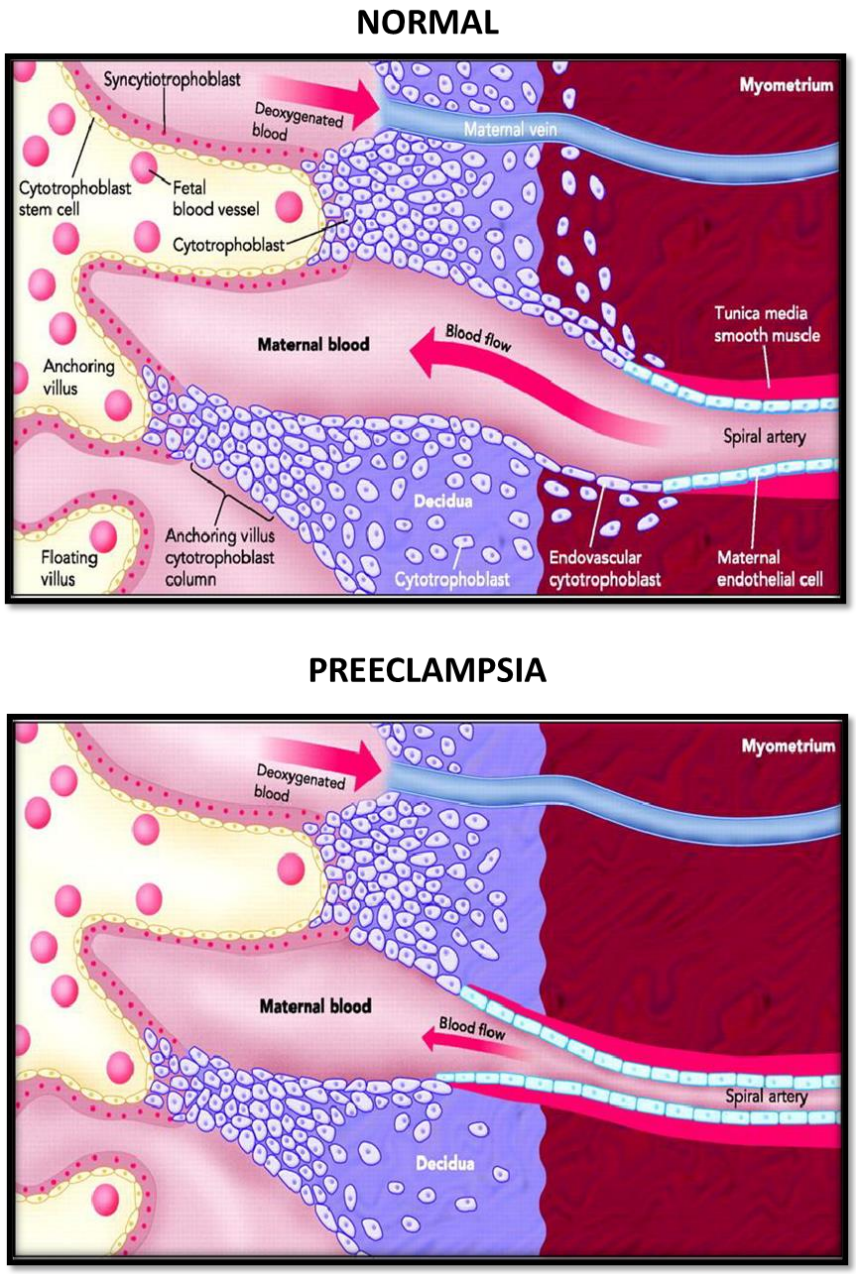

Figure 1-6: An illustration comparing normal and PE placentation.

Top: during normal placentation, invasive EVTs invade the decidua (coloured blue) up to the inner myometrium (red) and remodel maternal spiral arteries, transforming them to low-resistance tubes that allow steady flow of maternal blood for fetal nourishment.

Bottom: in PE, poor formation and invasiveness of EVTs results in shallow invasion of spiral arteries. (Modified and Adapted from Lam et al., 2005).

PE is a pregnancy related multisystem progressive disorder affecting around 5-7% of pregnancies worldwide (Umesawa and Kobashi, 2017). It is diagnosed clinically by sudden-onset hypertension in an otherwise normotensive mother, often accompanied by at least one other symptom such as new-onset proteinuria, end organ dysfunction or headaches at 20 weeks or later in gestation (ACOG, 2020). A revised definition excludes proteinuria as an essential feature (ACOG, 2013, incorporated by SOGC) (Magee et al., 2014). If left untreated, PE-afflicted mothers can develop eclampsia (or epilepsy-like seizures) that can be fatal. PE affects 5% to 7% of all pregnancies worldwide every year but in North America, it is a leading cause of maternal mortality, morbidity and premature deliveries (Hogan et al., 2010, Wanderer et al., 2013, Rana et al., 2019). There is no known cure for PE except delivering the fetus (and therefore removing the placenta). PE has a multi-factorial etiology, culminating in a hypo-perfused placenta that releases toxic products into the maternal circulation and damages maternal vascular endothelium in multiple organs at a later stage (Roberts and Bell., 2013). PE progression can be broadly classified into 2 subtypes: early-onset (before 34 weeks; placental) and late-onset (34 weeks or later, maternal) PE. Both appear to have distinct etiology and phenotypes. Recent reports (Wójtowicz et al., 2019, Wadhwani et al., 2020) suggest that placental PE is more severe than maternal PE. This may be because, in contrast to placental preeclmapsia, there is no increase in STB microparticles in maternal PE (Goswami et al., 2006). Placental PE is associated with incomplete EVT invasion and uterine arterial remodeling, thereby causing resistance to blood flow in the uterine arteries (Sugimoto et al., 2003; Eremina et al., 2003; Eremina et al., 2008). Maternal PE arises when the placenta releases toxic lipid peroxidases and inflammatory chemokines into maternal circulation, usually due to underlying maternal conditions (Roberts and Lain, 2002). Once in the circulation, these factors promote widespread vasoconstriction and endothelial damage that restrict blood flow to essential organs. Several risk factors are associated with PE that apply to both early and late onset types. These include past history of PE, previous chronic hypertension, pregestational diabetes, chronic renal disease, high pre pregnancy body mass index (Duckitt and Harrington, 2005), short stature, multifetal gestation, nutritional deficiencies (Schmidt et al., 2008), heredity, autoimmune disorders (systemic lupus erythematosus and antiphospholipid antibody syndrome), hydatidiform mole and fetal macrosomia (Hong and

Ruiz-Beltran, 2007; Walker and Chesnut, 2010; Powers et al., 2010; Jardim et al., 2015), nulliparity, advanced maternal age and use of assisted reproductive technology. Though our understanding of PE has improved in recent years, the development and interpretation of clinical tests remain difficult for PE. Impaired spiral artery remodeling leads to oxygen deprivation, or placental ischemia. By hitherto unknown mechanisms, this is followed an imbalance between angiogenic and anti-angiogenic factors in the placenta (Maynard and Karumanchi, 2011). There is evidence the pathogenesis of PE relates to an imbalance in angiogenic and antiangiogenic factors produced by the placenta, which can cause blood vessel instability in the mother (Maynard and Karumanchi, 2011).

Angiogenic imbalance is considered as one of the causes in the pathogenesis of PE. Angiogenic proteins such as VEGF, placental growth factor (PIGF), β-fibroblast growth factor (β-FGF), TGF-β, and angiopoietins are known to influence vasculogenesis and angiogenesis. The binding of these angiogenic proteins with their receptors Flt-1/ VEGFR-1, VEGFR-2, Tie-1, and Tie-2 are found to be essential for normal vascular development. VEGF is a cytokine produced by CTB during pregnancy and any disruption in the expression or function of VEGF, its receptor and isoforms have shown to an affect endothelium. Free bioactive VEGF levels is found to be decreased significantly in PE (Buhimschi et al., 2005; Aggarwal et al., 2006), whereas sFlt-1 that binds with free VEGF, is found to be highly elevated in preeclamptic subjects (Maynard et al., 2003). Several studies have confirmed the altered VEGF signaling in PE (Maynard et al., 2003; Ahmad and Ahmed, 2004; Stepan, 2009). Soluble endoglin (s-endoglin), another anti-angiogenic molecule released by the placenta (Oggè et al., 2010) is elevated in the maternal blood and considered a biomarker for predicting PE.

PlGF is another potent angiogenic protein used as a biomarker for PE. PlGF is mainly secreted by STB of the placenta, which is also required for vasculogenesis throughout embryonic development during normal pregnancy. Decreased levels of PlGF have been observed in PE mothers. In fact, lower level of PlGF is noticed quite early in women who are destined to develop PE (Levine et al., 2004; Thadhani et al., 2004; Shibata et al., 2005). sFlt-1 binds to both VEGF and PlGF in PE and prevents their binding to endogenous

receptors (Kendall and Thomas, 1993). Therefore, higher levels of sFlt-1 and lower levels of PLGF are observed in PE.

It has been suggested that the anti-angiogenic molecules such as sFlt-1 produced by the PE-associated placenta is compensatory defense mechanism against over-production of VEGF, rather that causative of PE (Fan et al., 2014). These authors showed that in women with PE, sFlt-1 is upregulated in placental trophoblasts, while VEGF is upregulated in adjacent maternal decidual cells. Endometrium-specific *VEGF* overexpression in mice by lentiviral gene delivery induced placental sFlt-1 production and elevated sFlt-1 levels in maternal serum. This led to pregnancy losses, placental vascular defects, and PE-like symptoms, including hypertension, proteinuria, and glomerular endotheliosis in the mother. Knockdown of placental *sFlt-1* with a trophoblast-specific transgene caused placental vascular changes that were consistent with excess VEGF activity. Furthermore, *sFlt-1* knockdown in VEGF-overexpressing animals enhanced symptoms produced by VEGF overexpression alone. These findings by Fan et al (2014) indicate that sFlt-1 plays an essential role in maintaining vascular integrity in the placenta by sequestering excess maternal VEGF and suggest that a local increase in VEGF can trigger placental overexpression of sFlt-1, potentially contributing to the development of PE and other pregnancy complications. These findings speak against sFlt-1 removal by plasmapheresis in PE subjects as a suggested therapy.

1.1.5 Fetal Growth Restriction

Placental maldevelopment can also lead to FGR, where fetus fails to attain the growth potential as determined by the genetic makeup. FGR is defined clinically as < 10th percentile for gestational age-related fetal weight or abdominal circumference <10th percentile documented by ultrasound. When this information is unavailable, the documented birthweight, combined with the gestational age is used to determine FGR postnatally. FGR occurs in about 10-12 % births in North America and at a much higher rate in poorer countries (Lee et al., 2017). It is associated with increased risks of coronary heart disease and related disorders such as stroke, hypertension and type 2 diabetes in

adulthood (Barker, 2006). FGR is also a multifactorial disease, a subset sharing the same placental pathology (hypo-invasive trophoblast) as in early-onset PE. While a variable incidence of FGR (15-30%) has been reported in PE subjects, it is more common in early-onset PE. Weiler, Tong, & Palmer (2011) reported that FGR is associated with 40% of women displaying early-onset PE, however this association did not alter the severity of PE symptoms. It has been suggested that compromised uterine arterial remodeling is the primary cause of idiopathic FGR. Huppertz (2011) challenged this notion and suggested that both poor villous and EVT development can lead to FGR. In cases with FGR EVT fails to adequately transform uterine spiral arteries. However, in FGR cases abnormal development of villous CTB may have an impact on fetal nutrition without the induction of an inflammatory response of the mother. It is still unclear why the villous trophoblast fails to achieve an adequate turnover both in PE and in FGR. Based on the information above, it is evident that further research into pathophysiology, new biomarkers for early diagnosis, prevention and intervention of PE/ FGR are highly relevant to reducing their impacts on maternal and child health.

1.2 ROLES OF CYTOKINES IN PREGNANCY

Successful implantation as well as the establishment and progression of pregnancy require highly coordinated molecular communications between the mother and fetus. During pregnancy, there is extensive cross talk mediated by hormones, cytokines, growth factors, proteins, lipids, ions and the extracellular matrix between uterine cells (uterine epithelium, DSCs, immune cells) and the placenta (Moffett and Loke, 2006). The maternal immune system is a critical participant in the establishment and maintenance of pregnancy (Munoz-Suano et al., 2011). Pregnancy is a process of physiological inflammation in which a balanced action of pro- and anti-inflammatory mediators is needed to establish, maintain and successfully complete the entire process. In this context we can divide pregnancy into three distinct immunological phases according to its regulation by cytokines with different profiles (Mor and Koga, 2008).

Initially, a pro-inflammatory environment dominates in the endometrium during the time of embryo implantation (referred to here as the first immune phase) (Dimitriadis et al., 2005) (Robertson et al., 2010). During the implantation window, a receptive endometrium is dependent on multiple cytokines. Mice lacking LIF are unable to support embryo implantation (Cheng et al., 2017). Evidence from infertile women supports the notion that abnormal levels of LIF, or the related cytokine IL-6 in the endometrium may underlie some forms of human infertility (Sharkey, 1998). The endometrial side strictly regulates cytokine production, which starts with the decidualization process that leads to uterine tissue differentiation, recruitment of uterine natural killer (NK) cells and macrophages to aid inflammation and to facilitate invasion of trophoblast into the uterine endothelium and maternal blood vessels (Zhou et al., 2003; Salamonsen et al., 2007). Vascularization in the decidua is also regulated by cytokines such as PlGF, vascular endothelial growth factor (VEGF) and the IL-12/IL-15/IL-18 system. (Torry et al., 2004; Králícková et al., 2005). Leukocyte activation in the decidua further contributes to the pro-inflammatory environment generating an array of regulatory cytokines needed for implantation (Alamonsen et al., 2007).

During the first immune phase, the blastocyst invades and implants itself into the endometrial lining of the uterus damaging the mother's endometrial tissue. This process leads to infiltration of various invading cells and the need to repair the damaged uterine epithelium by the mother's body. IL-6, EGF and basic fibroblast growth factor (bFGF) produced by the blastocyst promotes the production of multiple cytokines by the endometrium. Members of the gp130 cytokine family, IL-11 and LIF, the transforming growth factor beta superfamily, the colony-stimulating factors, and the IL-1 and IL-15 systems are crucial molecules for a successful implantation (Guzeloglu-Kayisli et al., 2009). Thus, during the first phase, both implantation and placentation are pro-inflammatory processes involving various growth factors and pro-inflammatory cytokines.

During the second phase, a shift from pro to anti-inflammatory mediators (cytokines and growth factors) is established. A vast array of cytokines are present in gestational tissues and studies in cytokine-null mutant mice show that several cytokines influence the course of pregnancy and the health of the offspring after birth. (Robertson et al., 1994; Ingman

and Jones, 2008). For most of pregnancy an anti-inflammatory environment predominates, sustaining the regulatory T cells that are a hallmark of pregnancy tolerance (Aluvihara et al., 2004; Guerin et al., 2009). The fetus grows rapidly establishing a symbiotic relationship with the mother and by now, the fetus, the placenta and the mother are adapted to each other. The immunological process moves towards an anti-inflammatory state where Th1 cytokines shift to Th2 cytokines via natural killer cell regulation and lymphokine activated cells (Raghupathy, 1997). Cytokines such as IL-3, IL-4, IL-5, IL-10 are observed at the fetomaternal interface which minimize Th1-type responses. (Carp, 2004) The trophoblast and decidual tissue contribute towards the Th2 cytokines (Chaouat et al., 1999). These cytokines not only establish immune tolerance but also promote the mechanical stretch in the uterine wall which leads to TGF-β3 production which further accelerates uterine growth (Shynlova et al., 2007; Orsi and Tribe 2008). During this growth, uterine endometrium produces IL-1α, IL-1β, IL-6 and IL-8 for the sustenance of the anti-inflammatory state (Orsi and Tribe, 2008).

The final phase marks the completion of fetal development and the onset of parturition. Now, the mother is getting ready to deliver the baby. The exact mechanisms regulating the onset and progression of labor are not fully understood but it is suggested to be an inflammatory state triggered by cytokines and prostaglandins (Romero et al., 2006). Reduction in maternal progesterone leads to increase in surfactant protein A which activates fetal amniotic macrophages. These macrophages migrate towards the uterine wall to release IL-1β triggering inflammatory prostaglandin production resulting in uterine contractility (Condon et al., 2004) IL-1β, IL-6 and IL-8 are also produced by myometrial leukocyte invasion (Orsi and Tribe, 2008). The chorioamniotic membrane-mediated cytokine production increases matrix metalloproteinase (MMP) levels to mediate extracellular matrix remodeling and weakening leading to rupture and dissociation of the fetal membranes (Cockle et al., 2007). During this phase, cervical fibroblasts upregulate IL-6, IL- 8, MMP-1 and MMP-3 and inhibition of their tissue inhibitors (tissue inhibitors of metalloprotease, TIMPs) helps to in remodel the cervix. Thus, the infiltration of immune cells into the myometrial tissue stimulates an inflammatory process that promotes contraction of the uterus, delivery of the baby and expulsion (simulating “rejection”) of the placenta (Romero et al., 2006).

1.2.1 IL-1beta (IL-1β)

The IL-1 system is a key regulator of inflammatory response between the maternal endometrium and the embryo and hypothesized to play a significant role in pregnancy (Krussel et al., 2003; Paulesu et al., 2008). Among others, IL-6, IL-12, IL-15, IL-18 and IL-1βare shown to play role during pregnancy (Yokhimoto et al., 1998; Nakanishi et al., 2001; Ashkar et al., 2003; Griffith et al., 2017), and IL-1α and IL-1β are most studied in relation to pregnancy and parturition (Ishiguro et al., 2016).

1.2.1.1 IL-1β mediated signal induction

IL-1β activity is regulated by Interleukin-1 receptor antagonist (IL-1RA), which has been implicated in pregnancy and labour induction (Dimitriadis et al., 2005). Both, IL-1β and IL-1RA interact with IL-1 receptor type 1 (IL-R1), which can initiate signal transduction pathways and stimulate cellular responses. IL-1β and IL-1RA also bind to a decoy receptor, IL-1 receptor type 2 (IL-R2), which dampens cellular responses to high concentrations of IL-1β. IL1β as a cytokine is capable of a wide spectrum of effects on numerous cell types such as T cells, tissue macrophages, blood monocytes and dendritic cells. (Bankers-Fulbright et al., 1996; Kim et al., 2012; Schober et al., 2012). At the feto-maternal interface it is expressed by human EVTs, macrophages, and decidual stromal fibroblasts (Kauma et al., 1990). IL-1RA inhibits IL1β activity by binding to its receptor with high affinity (Hannum et al., 1990). A soluble form of the IL-1 receptor accessory protein (AcP, sIL-1RAcP) enhances IL-1β binding to IL-1R2 by around 100 folds. Nevertheless, this does not affect the low-binding affinity of IL1A, thereby decreasing IL-1β binding to IL-1R1 and reducing inflammation (Gabay et al., 2010). (Fig. 1-7)

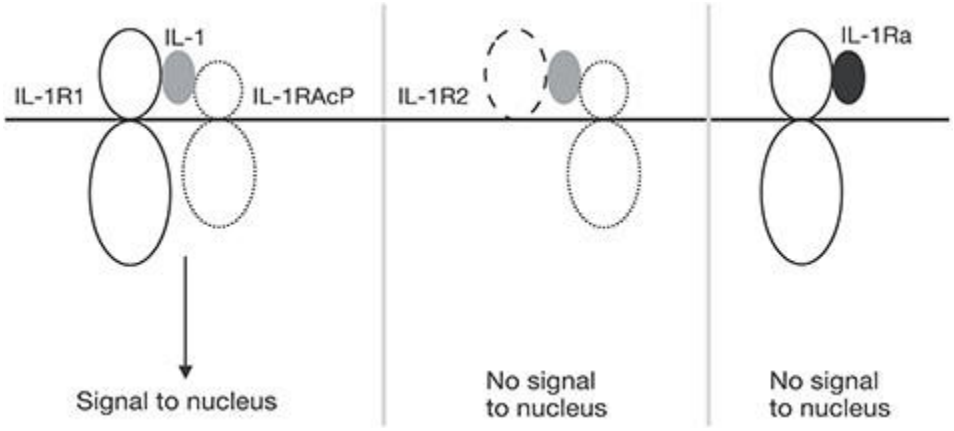

Figure 1-7: Regulation of IL-1 activity by IL-1 receptor.

The binding of IL-1 induces a conformational change in the extracellular component of IL-1R1, enabling its interaction with IL-1RAcP, which is required for intracellular signaling. IL-1R2 might only act as a decoy receptor. Binding of active IL-1 to cell surface receptors can be inhibited by IL-1Ra. IL, interleukin; IL-1R1, IL-1 receptor type I; IL-1R2, IL-1 receptor type II; IL-1RAcP, IL-1 receptor accessory protein; IL-1Ra, IL-1 receptor antagonist. (Modified and adapted from Gabay et al., 2010)

Once IL-1β binds to IL-1R1, IL-1RAcP is recruited to form a heterodimeric receptor. The cytoplasmic domains on IL-1R1 and IL-1RAcP come to lie in close proximity to each other, which facilitates recruitment of MyD88. MyD88 stimulates the phosphorylation of IL-1 receptor–associated kinases IRK-4, IRK-2, and IRK-1 (Martin and Wesche, 2002). Next, Tumor necrosis factor receptor–associated factor (TRAF-6) is recruited to this complex. Phosphorylated IRK-1 and TRAF-6 migrate to the membrane and associate with TAK1 (TGF-β-activated kinase1), TAK1-binding protein TAB1, and TAB2. The complex of TAK1, TAB1, TAB2, and TRAF-6 migrates to the cytosol, where TAK1 is phosphorylated following the ubiquitination of TRAF-6. Phosphorylated TAK1 activates IKKβ, and phosphorylated IKKβ in turn phosphorylates IκB. Phosphorylated IκB degrades, releasing NF-κB, which enters the nucleus. In addition to the phosphorylation of IKKβ, TAK1 also phosphorylates and activates mitogen-activated protein kinase kinases 3, 4 and 6 (MEK3, MEK4 and MEK6). The three MEKs phosphorylate and activate the MAPKs p38, ERK and JNK that activates another transcription factor Activator protein 1 (AP-1) (O'Neill, 2002; Dunne and O’Neill, 2003) (Fig. 1-9).

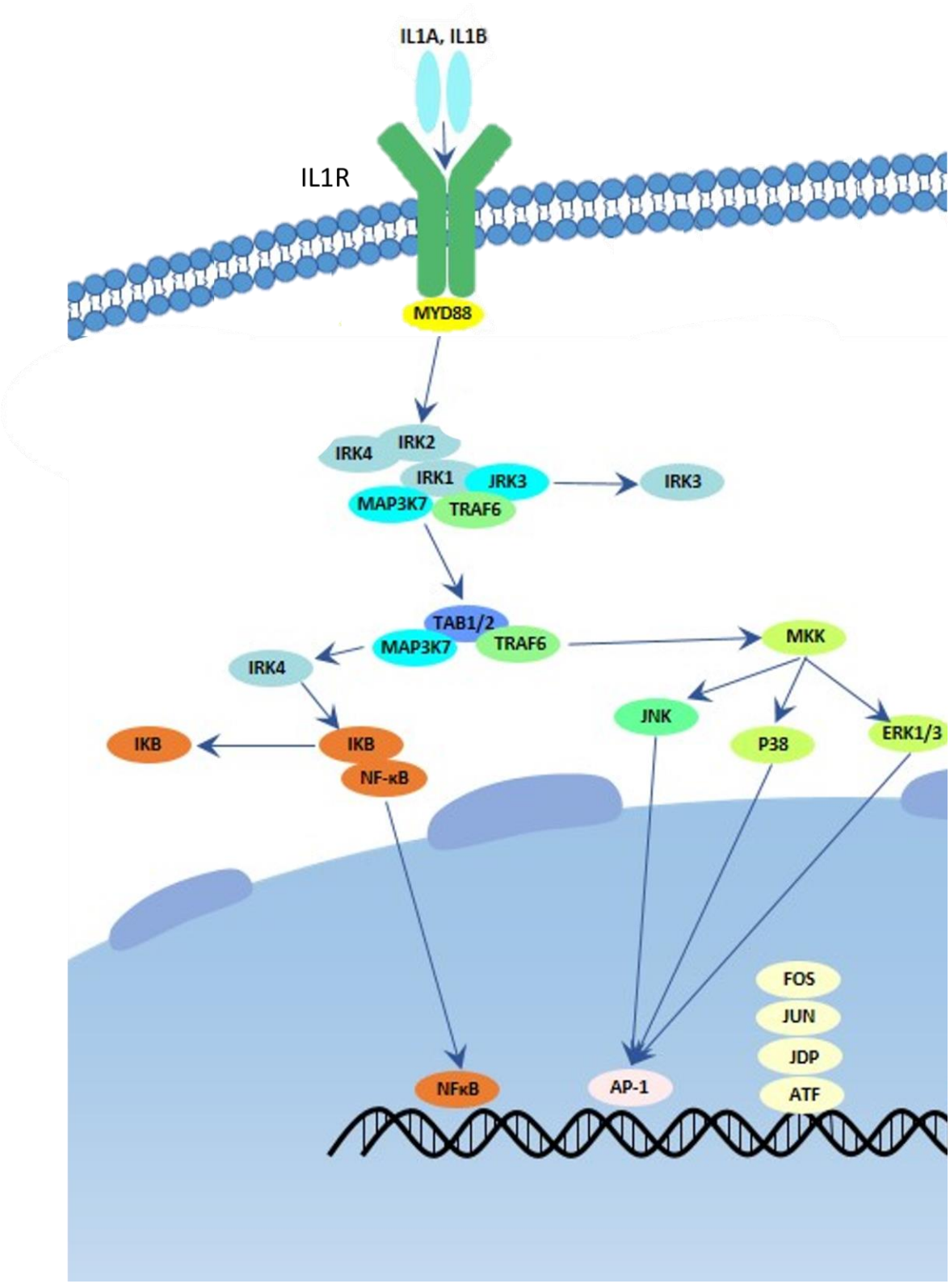

Figure 1-8: IL-1β signaling pathway

The signal initiates when cytokines (IL-1α or IL-1β) interact with by IL-1R1 and IL-1RAcP, forming a heterodimeric signaling receptor complex. This creates a scaffold for the association of MyD88 causing a downstream signalling and leading to activation of AP-1 and NF-κBtranscription factors. IRK, IL-1 receptor–associated kinase; TRAF, Tumor necrosis factor receptor–associated factor; TAB, TGF-β-activated kinase1 binding protein; IKB, IkappaB kinase; MAP3K7, Mitogen-activated protein kinase kinase kinase 7; MKK, MAPK kinases; ERK, Extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases; AP-1, Activator protein 1; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells. (Modified from a figure published by cusabio (www.cusabio.com)

1.2.1.2 Roles of IL-1β in pregnancy

IL-1β and IL-1RA seem to play important roles in implantation and development of placenta. Also, it appears the IL-1 system mediates the immunological tolerance required for the blastocyst implantation into the maternal uterus (Herrler et al., 2003). CTBs express both IL-1β and IL-1R. IL1β secreted by CTB regulates its invasion *in vitro* through its effects on MMP-9 (Fisher and Damsky, 1993). In addition, IL1β stimulates proMMP-3 expression in primate endometrial stromal fibroblasts acting through the MAP kinase pathway (Rawdanowicz et al., 2004). Using a primary trophoblast model, Prutsch et al (2012) showed that IL-1β induced motility in first trimester EVT cells in a dose-dependent manner and it could be inhibited by IL-1RA. Also, IL-1β induces the expression of well-known implantation marker, β3 integrin, in the endometrium (Gonzalez et al., 2004).

IL-1 is also known to influence levels of human chorionic gonadotrophin (hCG), a key placental peptide hormone that sustains early pregnancy. An *in vitro* study conducted by Nilkaeo and Bhuvanath (2006), using primary human first trimester trophoblast, showed that these cells release hCG on exposure to recombinant human IL-1. In addition, exposing endometrial stromal cells to IL1β was shown to induce IGFBP1 (key decidual marker) expression in the presence of steroid hormones (Strakova et al., 2000), suggesting a possible involvement in decidualization.

Another role of IL-1β is in the initiation of parturition, as it mediates the inflammatory state needed for induction of spontaneous labor (Mittal et al., 2010). IL-1β has been shown in the chorioamniotic membranes of mothers undergoing spontaneous labor (Romero et al 2018). It has been suggested that there is a shift of inflammatory markers before labor in which IL-1β increases and IL-1RA decreases. The ratio of IL-1RA /IL-1β is lower in the decidua of women who underwent spontaneous labor in comparison to women not undergoing labor (Ammälä et al., 1997). Similarly, Heng et al (2014) reported that levels of IL-1β and IL-1RA , along with IL-1α in the cervicovaginal fluid can predict onset of labor with 86% sensitivity and 92% specificity.

Overall, it is evident that IL-1 system cytokines, in particular IL-1 β plays a significant role in pregnancy. Present research investigates a hitherto un-explored role of IL-1 β in human decidualization.

1.3 MICRORNAs

miRNA are small noncoding (21–25 nucleotide), endogenous, single-stranded RNAs that regulate gene expression at the level of post-transcription; preferentially by binding to the untranslated region (3′ UTR) of a target gene (Krek et al., 2005). Since the discovery of the first miRNA in 1993, about 2,500 miRNAs have been identified and many of them have been functionally validated in humans (Baek et al., 2008; Friedlander et al., 2014). miRNAs are partially complementary to one or more messenger RNA (mRNA) molecules (Bartel, 2004). One miRNA can bind to multiple gene targets and result in the translational suppression of numerous genes. They are known to regulate the expression of 30% of all human genes (Baek et al., 2008) and are key regulators of fundamental cellular processes such as cell differentiation, proliferation, migration (Zhang, 2008). Not surprisingly miRNAs also contribute to the conception and maintenance of pregnancy (Robertson et al., 2017) by regulating key processes such as inflammation, immune tolerance, angiogenesis, and apoptosis (Veit and Chies, 2009; Lycoudi et al., 2015; Santa et al., 2015). Aberrant expression of miRNAs is found in several pregnancy-related disorders such as PE, intrauterine growth restriction, and preterm birth (Cai et al., 2017).

1.3.1 MicroRNA biogenesis and function

Biogenesis of miRNA is a multi-step process (Fig. 1-9) that consists of both nuclear and cytoplasmic phases. It begins with RNA polymerase-II dependent transcription of long primary transcripts (pri-miRNA) from the genes encoding miRNAs. Pri-miRNAs (100-140 nucleotides) fold into distinctive hairpin loop precursors (Denli et al., 2004). In mammals, the microprocessor complex composed of nuclear RNase III enzyme, Drosha, and a double stranded RNA binding protein, DGCR8 cuts the pri-miRNA leaving behind about 70

nucleotide hairpin sequence called the precursor miRNA or (pre-miRNA) within the nucleus. The pre-miRNAs are then exported from the nucleus into the cytoplasm by a protein called exportin 5 and a nuclear protein Ran-GTP complex. The pre-miRNAs are further processed by another RNase III enzyme, Dicer, and double-stranded RNA-binding protein TRBP into a miRNA duplex without the loop (Hutvagner et al., 2001). This miRNA duplex then associates with an Argonaute family protein and undergoes the coordinated process of RNA-induced silencing complex (RISC) assembly (Meister et al., 2004).

Argonaute (AGO) proteins are the core components of the RISC complex, and in humans there are four main types of AGO (AGO1 to AGO4) proteins have been identified. Finally, the miRNA duplex is unwound; one strand functions as the mature miRNA, and the other is often degraded. The miRNA RISC complex binds to specific sites in the 3'UTR of target mRNAs and promotes translational inhibition and mRNA degradation (Wu et al., 2006). In general, perfectly complementary mRNA targets are cleaved while partially complementary targets are silenced through translational repression, mRNA deadenylation and degradation (Lee et al., 1993). The cellular concentration of a miRNA is expected to correlate with its repressive activity. This is supported by several observations of overexpression of miRNAs leading to dose-dependent decreases in the levels of the target mRNAs (Shu et al., 2012; Wang et al., 2017). The importance of miRNA biogenesis machinery is highlighted by the observation that the lack of even a single protein in this pathway causes profound defects in different organs.

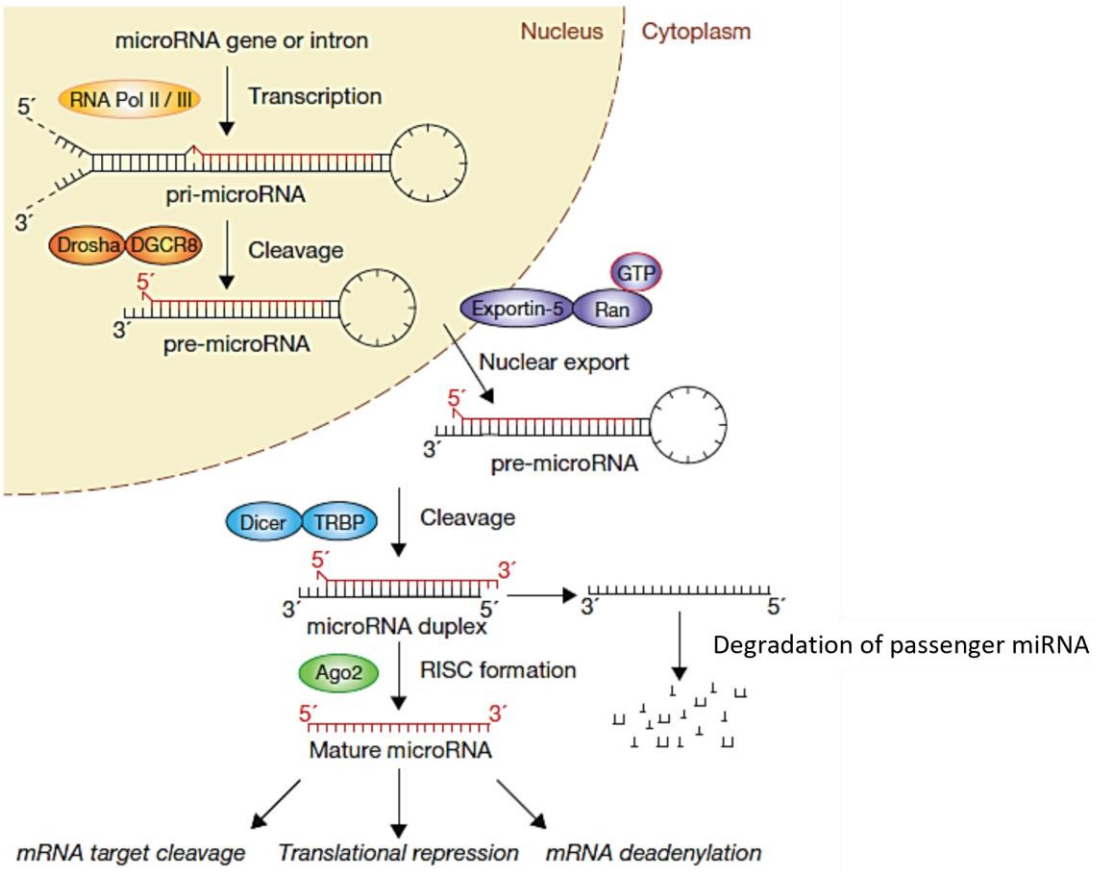

Figure 1-9: Schematic representation of canonical pathway of microRNA processing

This pathway includes the production of the pri-miRNA by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5. In the cytoplasm, the RNase Dicer and TRBP cleaves the pre miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with AGO proteins into the RISC, where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is degraded. (Modified and adapted from Winter et al., 2009)

1.3.2 Placenta-associated microRNAs

MicroRNAs (miRNAs) are abundantly expressed in the placenta, often, in a spatio-temporal manner. This suggests that some miRNAs may play a role in controlling pregnancy-associated genes and chorio-decidual interactions. Interestingly, some miRNAs are only expressed in the placenta (Baek et al., 2008) and are differentially expressed at different stages of pregnancy. These placenta-specific miRNAs are largely clustered in three groups such as chromosome 14 miRNA cluster (C14MC), C19MC, and miR-371-373 cluster (Miura et al., 2010; Morales-Priesto et al., 2012).

The C19MC is the largest cluster of miRNA's in the human genome, containing 46 highly homologous miRNA genes (Bortolin-Cavaille et al., 2009). The miRNAs in this cluster are almost exclusively expressed in the placenta but also in some fetal tissues, testis as well as some tumor cells (Bentwich et al., 2005; Zhang et al., 2008; Setty et al., 2020) (Luo et al., 2009; Donker et al., 2012). At early stages of pregnancy, tumor-suppressor, anti-apoptotic, and angiogenic miRNAs are upregulated in the placenta; in contrast, miRNAs involved in cell differentiation, tumor progression, and immune regulation are highly expressed at later stages of pregnancy. Although the complete range of the biological functions of C19MCs are still unknown, a recent study by Mouillet et al. (2020) found that a member of C19MC, miR-519d-3p, promoted trophoblast cell proliferation and decreased cell migration function. Other members of C19MC miRNAs, such as miR-519, miR-517a, and miR-517c, also exhibit tumor-suppressive activity via triggering cell senescence (Marasa et al., 2010) or inhibiting cell proliferation (Liu et al., 2013). It was reported that miR-517a-3p, miR-519a-3p, and miR-520c-3p are also abundantly expressed in placenta-derived mesenchymal stromal cells (Flor et al., 2012), indicating that their placenta-specific functions are not just restricted to the trophoblast. Expression of miRNAs from these clusters changes throughout pregnancy and differs among placentas from patients with pre-term labor compared to normal term pregnancies (Mayor-Lynn et al., 2011). In addition, some of these placenta-specific miRNAs have been detected in the maternal circulation throughout gestation with a significant decrease after delivery (Miura et al., 2010; Kotlabova et al., 2011). MiRNA's also have differential expression patterns between normal and PE pregnancies (Gao et al., 2018). These observations, in addition to recent

discoveries surrounding miRNA regulation of EVT invasion and migration, suggest that miRNAs play key roles in the process of PE development, and that maternal circulating miRNAs may have the potential to be novel diagnostic agents for pregnancy disorders and potential therapeutic targets (Gao et al., 2018) (Skalis et al., 2019).

1.3.3 Let-7c-5p

MiRNA let-7c-5p is a highly conserved member of the let-7 family. It is located on human chromosome 21q21.1 within the LINC00478 gene (http://www.mirbase.org). The miRNA *let-7* family was one of the earliest identified human miRNAs and they participate in various biological processes such as cell proliferation, differentiation, apoptosis, hormone secretion, metabolism, immune regulation and tumorigenesis. Let-7c-5p is a tumor suppressor in various cancers such as prostate, colorectal adenocarcinoma and acute myeloid leukemia, in which it acts by repressing tumor cell migration, invasion and proliferation (Hertel et al., 2012; Nadiminty et al., 2012). Let-7c-5p targets many genes in the Wnt signalling pathway, a pathway known to be important for embryogenesis and placentation (Hosseini et al., 2018). Some of the genes targeted by let-7c include TLR4 (encodes a receptor involved in recognition of bacteria and inflammatory signaling), low-density lipoprotein receptor 1 (LOX-1), Bcl-xl (encodes an anti-apoptotic protein), and AGO1, which encodes a protein involved in miRNA biogenesis (Tokyol et al., 2008; Du et al., 2010; Chen et al., 2013). Bcl-xl expression is suppressed by let-7c leading to increased apoptosis of endothelial cells (Qin et al., 2012). Maternal plasma from early pregnancy loss subjects revealed an upregulation of let-7c (Hosseini et al., 2018). Therefore, increased levels of let-7c may result in increased apoptosis thereby adversely affecting embryonic and placental development (Qin et al., 2012).

1.3.4 miR-512-3p

The gene coding for miR-512-3p is located on chromosome 19, where many other placenta-specific miRNA genes involved in PE and FGR are located (Luo et al., 2009).

The C19MC is the largest miRNA cluster ever reported (Prieto et al., 2011). One intriguing aspect of these placental-specific miRNAs is their ability to be secreted in maternal circulation during gestation. miR-512-3p has been reported to act as a tumor suppressor in hepatocellular carcinoma (Chen et al., 2010) and lung adenocarcinoma (Zhu et al., 2015). Multiple studies have reported upregulation of miR-512-3p in PE patients (Martinez-Fierro et al., 2018; Wang et al., 2012). In a study by Luo et al., normal expression patterns of miR-512-3p demonstrated placenta-restricted expression of this miRNA compared to 9 other organs (brain, liver, skeletal muscle, small intestine, testis, ovary, lung, heart, and kidney) (Luo et al., 2009). Therefore, altered levels of miR-512-3p in maternal blood may serve as a diagnostic biomarker for pregnancy disorders such as PE (Luo et al., 2009). This miRNA also confers resistance against viruses that cause perinatal infection (Bayer et al., 2018). This antiviral mechanism is derived by the induction of autophagy in virus-infected non-placental recipient cells, by incorporating trophoblast derived exosomes containing packaged miRNAs, thereby attenuating viral replication (Delorme-Axford et al., 2013).

While miR-512-3p was shown to be increased in PE-associated placentas, no information is available about whether it is induced in the trophoblast. These questions are addressed in the present research.

1.4 Rationale and Study aims

DCN was reported during decidualization of human endometrial stromal cells *in vitro* (Gellersen and Brosens, 2003; Gellersen and Brosens, 2007). However, the kinetics of DCN production during decidualization *in vitro* or post-implantation decidua *in vivo* remains undetermined. Our lab has extensively examined the paracrine role of decidua derived DCN on human trophoblast functions, a possible autocrine role of DCN in decidual cell development has never been tested.

IL-1β has been implicated in pro-inflammatory responses of the maternal endometrium to the embryo to induce immunotolerance. Considering IL-1β and DCN are increased during

PE, it raises the possibility of a causal link between the two in PE. This possibility remains to be tested.

DCN controls EVT cell differentiation from stem cells (Nandi et al., 2018); and proliferation, migration, invasion (Xu et al., 2002; Iacob et al., 2008) and endovascular differentiation (Lala et al., 2012), events needed for uterine spiral artery remodeling (Merline et al., 2011; Nandi et al., 2016). While DCN was shown to control inflammation and tumor growth by upregulating miR-21 which targets Programmed cell death 4 (PDCD 4) (Merline et al., 2011), possible roles of DCN-regulated miRNAs in the above named DCN actions on the trophoblast remain to be identified. This identification is important in understanding putative epigenetic mechanisms in DCN action in the pathogenesis of PE.

The overall hypothesis is that DCN plays an essential role in decidual cell differentiation and trophoblast functions by multiple mechanisms.

The specific objectives of this project were:

1. To explore the autocrine roles of DCN in decidual cell development.
2. Define molecular mechanism(s) in IL-1β mediated DCN production by the decidua.
3. Identify the roles of DCN-regulated miRNAs in EVT cell functions and their roles in PE.

My research will help to identify mechanisms of DCN action in pregnancy. Since DCN production by the decidua is increased in serious pregnancy diseases such as PE and FGR, the goal of this research is to ultimately provide a foundation in which better diagnosis and possibly intervention of these diseases can be achieved.

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Chapter 2

2 Decorin Production by the Human Decidua: Role in Decidual Cell Maturation.

Decidualization involves the proliferation and differentiation of fibroblast-like endometrial stromal cells into epithelioid-shaped and secretory ‘decidual’ cells in response to steroid hormones. Human decidual cells produce insulin-like growth factor-binding protein-1 and prolactin (PRL), two well-recognized markers of decidual cell maturation and a proteoglycan decorin (DCN). We reported that DCN restrains the human trophoblast renewal, migration, invasion and endovascular differentiation needed for uterine arterial remodeling during normal pregnancy. DCN overproduction by the decidua is associated with a hypo-invasive placenta and a serious pregnancy disorder, pre-eclampsia (PE). Furthermore, elevated maternal plasma DCN levels during the second trimester is a predictive biomarker of PE. While these paracrine roles of decidua-derived DCN on trophoblast physiology and pathology have been well-defined, it remains unknown whether DCN plays any autocrine role in decidual cell development. The objectives of this study were to examine: the kinetics of DCN production during decidualization of human endometrial stromal cells; gestational age-related changes in DCN production by the first trimester decidua; and a possible autocrine role of DCN on decidual cell maturation. We found that DCN production is enhanced during decidualization of both primary and immortalized human endometrial stromal cells *in vitro* and during early gestation in decidual samples tested ex vivo, and that it is important for endometrial stromal cell maturation into a decidual phenotype. Decorin-depleted human endometrial stromal cells exposed to decidualizing stimuli failed to mature fully, as evidenced by fibroblastoid morphology, reduced insulin-like growth factor-binding protein-1 and PRL expression, and reduction in cellular ploidy. We identified heart and neural crest derivatives-expressed protein 2, and progesterone receptor as potential downstream mediators of DCN effects.

2.1 Introduction

Successful implantation and placentation depend on a receptive uterine endometrium. This receptivity is ensured by extensive tissue remodeling of the endometrium, which includes transformation of fibroblast-like endometrial stromal cells (ESC) into highly specialized, epithelioid-shaped ‘decidual’ cells having unique biochemical and secretory properties, a process known as decidualization (Gellersen et al., 2007; Okada et al., 2018). This process provides a nutritive and immunoprivileged matrix essential for embryonic and placental development. Defective decidualization is associated with a variety of human pregnancy disorders such as miscarriage, fetal growth restriction (FGR) and pre-eclampsia (PE) (Cha et al., 2012; Rabaglino et al., 2015; Garrido-Gomez et al., 2017).

In humans, decidualization is independent of the presence of a blastocyst and minor cyclic decidualization is observed in ESCs in the mid-to-late secretory phase of the menstrual cycle (Maruyama and Yoshimura, 2008; Ramathal et al., 2010; Gellersen and Brosens, 2014). The steroid hormones estradiol and progesterone are critical for the regulation of the cyclical events in human endometrium (Gellersen and Brosens, 2014). Extensive proliferation of both epithelial and stromal cells occurs in response to rising estradiol levels during the first phase of the menstrual cycle or the proliferative phase. During the second phase of the cycle, termed the secretory phase, progesterone action dominates in the endometrium where it induces differentiation of stromal cells. Estradiol plays an important role in the induction of progesterone receptor (PGR), which permits the endometrium to respond to progesterone in the secretory phase. In the absence of blastocyst implantation, progesterone levels drop, the decidual response ceases and the transformed ESCs are shed during menstruation. However, if implantation occurs, placental trophoblast cells secrete hCG leading to sustained progesterone production by ovarian granulosa cells and full development of the decidua (Cole, 2010).

Several growth factors, cytokines, hormones, proteins and proteoglycans are recognized as markers for the initiation and maintenance of decidualization. Examples include epidermal growth factor, insulin-like growth factor (IGF), extracellular matrix (ECM) molecules and two secreted proteins: IGF-binding protein (IGFBP)1 and prolactin (PRL) (Bell, 1991; Telgmann and Gellersen, 1998; Gellersen and Brosens, 2003). IGFBP1 and PRL are both

well-recognized as markers for decidual cell maturation. Furthermore, during this differentiation process, there is an increase in cellular ploidy resulting from endomitosis without cytokinesis (Sroga et al., 2012).

Decorin (DCN) is a transforming growth factor (TGF)-β-binding, small leucine-rich proteoglycan (SLRP), produced by many stromal cells in the body including dermal fibroblasts and chondrocytes (Iozzo, 1998; Neill et al., 2012). It is also produced by fetal mesenchymal cells of the placenta and decidual cells (Lysiak et al., 1995). Decidua-derived TGF-β provides a key control mechanism for trophoblast invasion (Graham and Lala, 1991). We found that DCN is co-localized with TGF-β in the human decidua at various gestational ages (Lysiak et al., 1995), possibly serving as a storage device for TGF-β in the decidual ECM in an inactive form, until activated by the trophoblast-derived protease cascade at the invasion front. However, decidua-derived DCN, on its own, was shown to restrain human trophoblast proliferation, migration and invasiveness, independent of TGF-β (Xu et al., 2002) by binding to multiple tyrosine kinase receptors including vascular endothelial growth factor receptor 2 (VEGFR2) (Iacob et al., 2008; Khan et al., 2011). The VEGFR2-binding site was confined to a 12 amino acid span of the leucine rich repeat 5 domain of the DCN protein, overlapping with the VEGF-binding site (Khan et al., 2011). This binding provided a mechanism for DCN to restrain VEGF-dependent endovascular differentiation of the trophoblast, a process essential for uterine arterial remodeling (Lala et al., 2012). We noted that DCN over production by the decidua leading to poor trophoblast invasion and uterine angiogenesis was a hallmark of PE-associated placentas and that elevated maternal plasma DCN level during the second trimester is a predictive biomarker for PE and a subset of FGR (Siddiqui et al., 2016; Nandi et al., 2016). Finally, DCN was shown to restrain trophoblast stem cell renewal and differentiation (Nandi et al., 2018). In light of multiple DCN functions during normal and pathological pregnancy, DCN production by human ESCs (HESCs) during decidualization *in vitro* and pregnancy-associated decidua *in vivo* needs to be characterized further.

Decidualization of ESCs can be achieved *in vitro* in the presence of factors stimulating cAMP, and further enhanced with progesterone (Brosens et al., 1999; Gellersen and Brosens, 2003), leading to an upregulation of many genes including DCN (Gellersen et al.,

2007; Okada et al., 2018). However, the temporal kinetics of DCN production during decidualization of ESCs *in vitro* or during early gestation *in vivo* remains undefined. While the paracrine roles of decidua-derived DCN on trophoblast physiology and pathology have been well-defined, as outlined above, it is currently unknown whether DCN plays an autocrine role in decidual cell development or maturation. The present study was therefore designed with three objectives: first, to examine the kinetics of DCN production *in vitro* during decidualization of HESCs; second, to examine the gestational age-related changes in DCN production by the pregnancy-associated decidua during the first trimester (7–11 weeks); and third, to examine the possible autocrine role of DCN on decidual cell development or maturation *in vitro*. Results reveal that DCN production is enhanced during decidualization of both primary and immortalized HESCs *in vitro* and during early gestation in decidual samples tested ex vivo, and that it is required for ESC maturation to a decidual phenotype. We also identified PGR and heart and neural crest derivatives-expressed protein 2 (HAND2), a transcription factor required for decidualization, as candidate downstream mediators of DCN effects. Together, our results identify a novel autocrine role of DCN during decidualization.

2.2 Material and Methods

2.2.1 Decidual samples

Decidual samples from healthy pregnant subjects undergoing elective termination of pregnancy at the London Health Sciences Center (Westminster campus), during the first trimester of pregnancy (between 7 and 11 weeks of gestation, estimated on the basis of the first date of the last menstrual period and ultrasound validation) were collected following approval by the University of Western Ontario Human Ethics Review Board and informed written consent by the subjects. They were processed for isolation of ESCs or for tissue RNA isolation and histology.

2.2.2 Isolation of primary HESC

Primary HESCs (pHESCs) were isolated from 9- to 11-week-old decidual tissues with a procedure modified from Perdu et al. (2016). Each decidual tissue sample (approximately 50–100 mg) without visible contamination by chorionic villi or fetal membranes was rinsed with ice-cold Ca2+ and Mg2+-inclusive Hank's balanced salt solution (HBSS; Gibco, Grand island, NY, USA) on a sterile stainless-steel sieve three times, followed by three washes with Ca2+ and Mg2+-free HBSS (Gibco). The washed tissue was incubated at room temperature for 1.5 h in wash buffer 1 (Ca2+ and Mg2+-free HBSS, 2 mM sodium bicarbonate; Sigma, St. Louis, MO, USA, gentamicin; Gibco, and Amphotericin B; Gibco). The tissue was then rinsed with PBS (Gibco) on a sterile stainless-steel mesh strainer and minced with sterile fine scissors in a petri dish. Minced tissue was placed in a 500 ml conical flask and incubated for 15 min at 37°C in 50 ml HBSS containing gentamicin, amphotericin B and 0.02% trypsin (Gibco). Then an equal amount of wash buffer 2 (PBS + 2% fetal bovine serum, FBS; Gibco) was added to neutralize the trypsin. The tissue fragments were then placed in a 500 ml conical flask and covered with 50 ml digestion solution (approximately 50 ml digestion solution for 10 mg of tissue) and incubated for 1.5 h at 37°C with intermittent agitation. Digestion solution was prepared using 1 × collagenase/hyaluronidase (StemCell Technologies, Vancouver, BC, Canada), 1 mg/ml DNase I (Sigma) and penicillin/streptomycin (P/S, Gibco) in 1:1 DMEM/F12 medium (Gibco). After digestion, the contents were filtered in a sterile stainless-steel fine mesh strainer to discard the undigested tissue and recover the dispersed cell suspension. The suspension was centrifuged (695 g, 24°C, 5 min), washed twice with wash buffer 2 and subjected to lysis of erythrocytes using ammonium chloride (StemCell Technologies) for 10 min on ice. This was followed by centrifugation (conditions same as above), washing the pellet and filtering through a 70 μm nylon mesh (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain a single-cell suspension. The cell suspension was layered on top of histopaque (approximately 2 ml cell suspension on 4 ml of histopaque; Sigma) in 15 ml tubes and centrifuged in a swinging bucket rotor at 700 g for 20 min at room temperature. After centrifugation, the translucent upper layer was carefully aspirated and discarded with a Pasteur pipette to within 1 cm of the opaque interface containing decidual cells. The opaque interface was carefully transferred with a Pasteur pipette into a clean conical

centrifuge tube, washed and centrifuged again. Isolated cells were seeded in phenol red-free DMEM/F12 medium supplemented with 10% heat-inactivated FBS, 1% ITS+ Premix (Corning, Corning, NY, USA) and 1% P/S and cultivated at 37°C, 5% CO2 and 95% humidity. Purity of pHESC was routinely examined by immunofluorescence using antibodies against Vimentin (see Immunofluorescence section). The pHESCs were maintained in hormone-free medium containing basal DMEM/F12, 2% charcoal-stripped FBS (Life Technologies, Carlsbad, CA, USA), and 1% P/S for a minimum of 2 weeks before use in experiments.

2.2.3 Cells and culture

Telomerase-immortalized HESCs (THESCs) were purchased from American Type Culture Collection (CRL-4003) and cultivated as instructed by the supplier. Briefly, cells were cultured in phenol red-free DMEM/F12 medium supplemented with 10% heat-inactivated FBS, 1% ITS+ Premix and 1% P/S. Cells were passaged via light trypsinization prior to reaching confluency and were maintained at 37°C in an atmosphere consisting of 5% CO2 for no more than 20 sequential passages.

HTR8/SVneo (referred to as HTR-8 cells) is an SV40Tag immortalized first trimester human trophoblast cell line developed previously in our laboratory (Graham et al., 1993). The cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 1% P/S and were maintained at 37°C. Cells were used within 2 weeks of passage both as monolayers and spheroids, as detailed later.

Human embryonic kidney (HEK)-293T cells (used to produce lentivirus) obtained from American Type Culture Collection (CRL-3216) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 1% P/S, and maintained at 37°C, 5% CO2. Cells were passaged via light trypsinization prior to reaching confluency.

2.2.4 *In vitro* decidualization

THESC and isolated pHESC (between passages two and five) were grown to confluence (maximum time to confluence 6 days) with control or decidualizing media. The control medium consisted of basal DMEM/F12 supplemented with 2% charcoal-stripped FBS and 1% P/S. The decidualization medium included control medium plus 250 nM medroxyprogesterone-acetate (a synthetic progesterone derivative; United States Pharmacopeia) and 0.5 mM 8-bromo-cAMP (a stable, cell permeable analog of cAMP; Sigma), henceforth called as decidualizing agents. Cells were left to grow until harvested at various time points. Supernatants were collected, and immediately frozen and stored at −80°C for further analyses. Morphological changes in cells were monitored daily with a phase contrast optics and images were captured using a Leica inverted microscope (Leica Microsystems, Wetzlar, Germany).

2.2.5 RNA extraction and RT-PCR

RNA was extracted from pHESCs, THESCs and freshly isolated first trimester decidual tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using a BioTek Epoch Microplate Spectrophotometer. Per sample, 1 μg of RNA was subjected to cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA). The resulting cDNA was diluted 3-fold and then subjected to quantitative RT-PCR (qRT-PCR). qRT-PCR was run on a LightCycler (Bio-Rad, Hercules, CA, USA) using custom designed primers (Table 2-1) and SYBR Green PCR mix (Quanta Bioscience, Beverly, MA, USA). Cycling conditions involved an initial holding step (95°C for 10 min), followed by 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 1 min) and a dissociation phase. Relative mRNA expression in treated samples compared to control samples was calculated with the ΔΔCt method using GAPDH as reference RNA for pHESC and THESC cells, and the geometric means of four constitutively expressed genes GAPDH, 18S Ribosomal N1 (RNA18SN1), eukaryotic elongation factor 2 (EEF2), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) as reference RNA for decidual tissue samples.

Table 2- 1: Primers used for qRT-PCR

Gene	Forward primer	Reverse primer
DCN	TGTTGTTCGATTATGGGTGCT	GGCTGTACTGAAGACAGAAAGTG
IGFBP1	GATGTCAGAGGTCCCCGTTG	TAGGTACTGATGGCGTCCCA
PRL	GGGGTTCATTACCAAGGCCA	TGGATAGGATAGCCTCCGGG
HAND2	ATGAGTCTGGTAGGTGGTTTTCC	CATACTCGGGGCTGTAGGACA
FOXO1	AAGAGCGTGCCCTACTTCAA	TTCCTTCATTCTGCACACGA
PGR	AAATCTACAACCCGAGGCGG	CGGCTCCTTTATCTCCCGAC
BCHE	CTGGCTCGGGTTGAAAGAGT	CCAAGGCCTCACGGTAGTTT
ROR1	AGTTCGCCATTCCTTCCCTG	CTTCTATTTACCGCACGCTGG
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
EEF2	AGGCGTAGAACCGACCTTTG	GACAGCGAGGACAAGGACAA
RNA18SN1	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA
YWHAZ	ATGCAACCAACACATCCTATC	GCATTATTAGCGTGCTGTCTT

2.2.6 Enzyme immunoassays

Secreted DCN (diluted 1:100) and IGFBP1 (diluted 1:10) proteins were measured in cell-free culture supernatants of THESC or pHESC using a human ELISA kit according to the manufacturer's instructions (DCN; DY143, IGFBP1; DY871, R&D Systems, Minneapolis, MN, USA) and normalized to cell numbers plated for culture. A standard curve was generated using absorbance value plotted against defined concentrations of recombinant DCN and IGFBP1. Conditioned media was diluted in assay buffer to ensure absorbance values fell within the linear range of the standard curve.

2.2.7 Immunofluorescence

pHESC and THESC cells plated on cover slips were cultured. At the indicated times, culture medium was removed and 500 µl of 4% paraformaldehyde was added to the cells for 30 min. Paraformaldehyde was removed, and cells were washed with PBS. Next, 0.5% Triton X-100 in PBS was added to permeabilize cells for 10 min followed by washing with PBS and blocking with 500 µl PBS containing 8% bovine serum albumin (BSA) and 0.01% Tween-20 for 30 min. After blocking, cells were incubated overnight at 4°C with primary antibody (Vimentin, 1 µg/ml, Cat no. MA5-11883, Invitrogen, Carlsbad, CA, USA) or HAND2 (5 µg/ml, Cat no. AF3876, R&D Systems) diluted in 8% BSA-PBS. A non-specific mouse IgG served as the negative control (2 µg/ml, cat # 31903, Invitrogen). The next day, coverslips were washed twice with PBS and an Alexafluor 488 conjugated anti-mouse secondary antibody (Biotium, Hayward, CA, USA; 1:10 000 dilution) was added. Following a 1 h incubation at room temperature, secondary antibody was removed and cells were washed with PBS. For actin filaments staining, cells were stained with phalloidin (1:20 dilution, Cat no. 8878, Cell signaling, Danvers, MA, USA) for 15 min followed by washing with PBS. Coverslips were then placed onto microscope slides precoated with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and slides were dried horizontally at 4°C overnight. The next day, clear nail polish was used to seal the edges of the coverslip, and samples

were stored at 4°C until imaged using a confocal microscope (Zeiss, Oberkochen, Germany).

2.2.8 Lentivirus generation and shRNA-mediated DCN knockdown

To knockdown DCN expression, a cocktail of two short hairpin (sh)RNA constructs encoded within PLKO.1 vectors (shRNA1—TRCN0000058553 and shRNA2—TRCN0000058554) were obtained from Sigma. A control PLKO.1 shRNA vector containing an shRNA that does not target any known mammalian transcript was obtained from Addgene (plasmid 1864) (Watertown, MA, USA). Since THESCs were immortalized based on resistance to puromycin, the puromycin cassette encoded within the PLKO.1 vectors was replaced with a cassette encoding blasticidin resistance. To generate shRNA-encapsulated lentiviruses, HEK-293T cells were transfected using Lipofectamine 2000 (Life Technologies) with the shRNA-containing vectors and third-generation lentiviral packaging plasmids, as described previously (Jeyarajah et al., 2019). Lentivirus-containing culture supernatants were collected every 24 h for a total of 48 h. Lentivirus was stored at −80°C until use. To transduce THESCs, cells were exposed to viral particles for 48 h in the presence of 8 μg/ml hexadimethrine bromide (Sigma). After 48 h incubation, transduced cells were selected with blasticidin (2 μg/ml). Efficiency of selection was monitored by exposing untransduced THESCs to blasticidin.

2.2.9 CRISPR-mediated generation of DCN-mutant cells

For clustered regularly interspaced short palindromic repeats (CRISPR) mediated generation of DCN-mutant cells, guide RNA targeting DCN (target sequence 5′GGCTCGAAGTCGCGGTCATC-3′, as reported by Daquinag et al., 2017) was first designed and incorporated into lentiCRISPRv2 plasmid (Addgene 98293, Watertown, MA, USA). Control cells were transduced with lentiCRISPRv2 vector without DCN guide RNA. Lentivirus was generated as stated above, and THESCs were transduced with viral supernatant for 48 h. Transduced cells were selected with blasticidin and clonal selection

was utilized to isolate multiple clones. DNA was isolated from individual clones (Geneaid DNA isolation kit, Cat no. GEC150, New Taipei city, Taiwan) and DNA sequencing was conducted at the Robarts Research Institute DNA Sequencing Facility (London, ON, Canada) to identify clones possessing a mutation in the DCN gene. Confirmation of reduced DCN protein secretion in DCN-mutant cells was performed by ELISA.

2.2.10 Analysis of cellular ploidy

Polyploidy was shown to be another maturation marker for decidual cells (Sroga et al., 2012). Cellular ploidy level, as shown by the distribution of DNA contents, was analyzed with a FACS analyzer (BD FACS Celesta), using propidium iodide staining of fixed cells (Darzynkiewicz and Huang, 2004). The levels were compared between control and DCN knockout (KO) THESC subjected to decidualizing stimuli. Relative incidence of cells with enlarged nuclei (30 μm or more in diameter) plus binucleate cells in phalloidin and DAPI stained preparations also provided a measure of polyploidy in control versus DCN KO cells. This method was shown to correlate with the measurement of cellular DNA content (Ma et al., 2011).

2.2.11 Whole transcript expression arrays and pathway analysis

Comprehensive gene expression analysis was performed using the Clariom S GeneChip Assay, human (ThermoFisher, Waltham, MA, USA). Control and DCN-mutant cells (one independent clone for each assay) decidualized for 6 days were subjected to this analysis. RNA was extracted as described above, and 100 ng of RNA was used for the reaction. The assay was conducted at Hamilton Health Sciences Corporation (Hamilton, ON, Canada). The RNA integrity was checked by bioanalyzer and the batch effect was controlled by Affymetrix® Transcriptome Analysis Console software 4.0 (ThermoFisher). The batch effects module offers two functions: source of variation analysis and batch effect removal. Samples were normalized using the SST-RMA (Signal Space Transformation-Robust Multiarray Analysis) data normalization algorithm to reduce background. In preliminary

experiments, we also did a Clariom S GeneChip Assay with non-decidualized control and DCN depleted cells to evaluate whether the ESC gene signature for decidualization markers was affected by DCN depletion in undifferentiated THESC.

2.2.12 *In vitro* spheroid invasion assay

Spheroid attachment and invasion has been used as an *in vitro* model for studies of trophoblast adhesive interactions on different endometrial epithelial cell lines (Weimar et al., 2013). Here, we used trophoblst sheroids to study invasion of endometrial stromal cells. HTR-8 trophoblast cells were grown as spheroids on ultra-low attachment plates using the protocol reported by Nandi et al. (2018). Spheroids were allowed to grow for 2 weeks and transferred onto a 90% confluent monolayer of decidualized control THESC or DCN KO THESC. Phase-contrast images were captured at 24 and 48 h (using Leica Microsystems) to visualize spheroid attachment and trophoblast invasion by sprouting from the spheroids.

2.2.13 Cell migration analysis by wound-healing assay

As reported earlier (Jeyarajah et al., 2019), HTR-8 cells were seeded at ~70–80% confluence into a 6-well plate and cultured to 100% confluence. Cells were then treated with mitomycin C (250 nM, Sigma Aldrich, cat no. M4287) for 1 h to prevent proliferation. Multiple wounds were then created on the cell monolayer by scratching using 200 µl pipette tips. The wounded monolayers of HTR-8 cells were cultured in media collected from control THESC or in media collected from DCN KO THESC at 6 days of decidualization treatment. The wound area was recorded using light microscopy (Leica Microsystems) at 0 and 24 h. Migration was recorded as the percentage wound closure at 24 h. To calculate the area of the wound, images were imported into ImageJ (version 1.5.3), where cell frontiers bordering the wound were traced. The percentage of wound closure was determined using the following equation: $[(A0 - A24)/A0] \times 100\%$, where A0 represents the initial area of the wound at 0 h and A24 represents the area of the wound after incubating for 24 h.

2.2.14 Statistical analysis

For most experiments, between three and seven biological replicates (a minimum of three) were used. SE of the mean was calculated to determine the level of uncertainty. GraphPad Prism software version 6 (San Diego, CA, USA), unequal variances Student's t-test or ANOVA, followed by Tukey–Kramer post hoc comparisons were used to calculate significant differences between samples. A P-value of 0.05 or less was considered to be statistically significant.

2.3 Results

2.3.1 DCN production during decidualization of pHESCs

pHESCs isolated from first trimester decidua were cultured until they reached confluency. Before treating with decidualizing agents, immunostaining for vimentin (mesenchymal cell marker, absent in trophoblast and glandular epithelial cells) confirmed cell purity. More than 98% of cells were vimentin positive (Fig. 2-1A). The cells were cultured for 6 days with medium changed every 2 days with or without decidualizing agents. The experimental group received media with decidualizing agents. The control group was exposed to media without decidualizing agents. Cells of both groups exhibited significant expression of both *IGFBP1* and *PRL* mRNA from Day 1 of decidualization treatment. This was not surprising since these cells, although fibroblast-like in morphology, were isolated from first trimester pregnant (decidualized) endometrial tissue. The mRNA expression levels of both molecules increased further in the experimental group reaching a peak on Day 6 when *IGFBP1* increased 50-fold (Fig. 1B) and *PRL* 150-fold (Fig. 2-1C). Secreted IGFBP1 protein increased 20-fold (Fig. 1D). A significant increase in DCN mRNA expression (5-fold, Fig. 2-1E) and secreted protein levels in cell-free supernatants (ELISA) (2-fold, Fig. 2-1F) was also noted in the experimental group on Day 6.

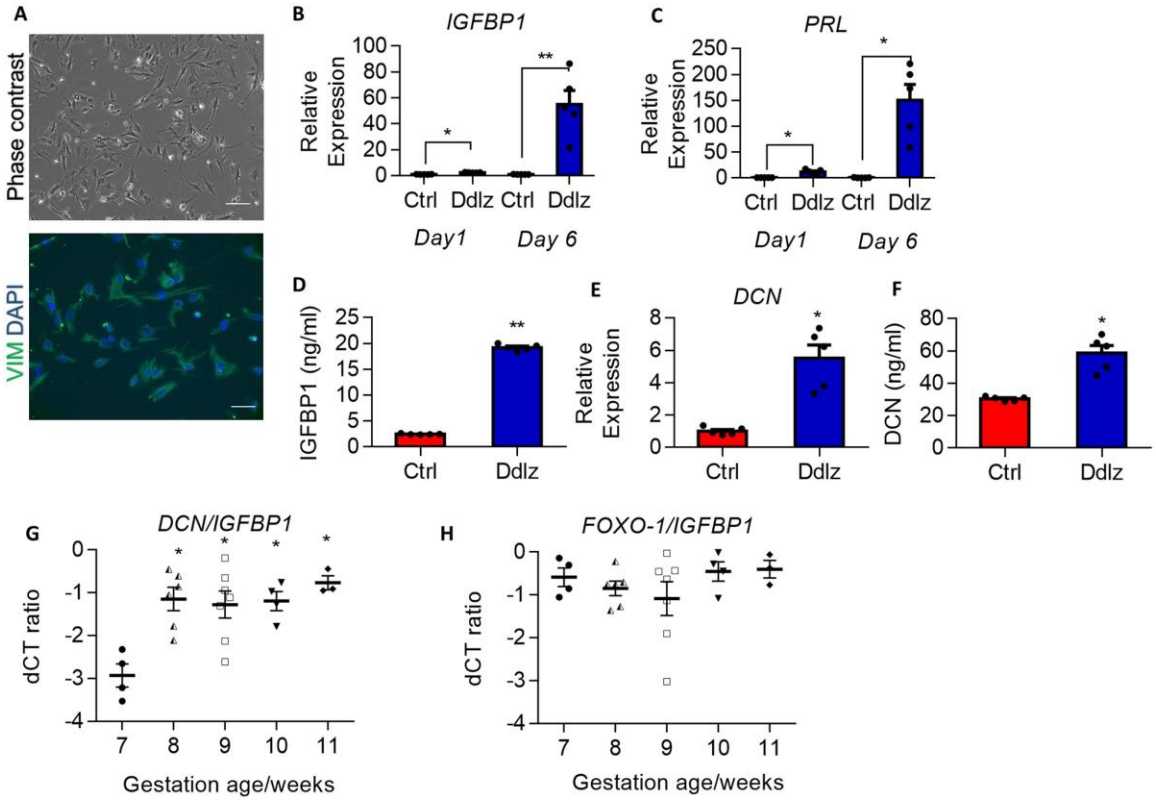

Figure 2-1: DCN production in decidualized pHESCs and human decidua.

Light micrograph and fluorescent image of pHESCs after isolation showing immunofluorescence for Vimentin (VIM, green) and DAPI staining for nuclei (blue). Magnification bar: 10 μm. Data for IGFBP1 mRNA (B) and PRL mRNA (C) measured at Days 1 and 6 with qRT-PCR and secreted IGFBP1 protein (D), measured with ELISA (Day 6) after treating pHESC with decidualizing agents (Ddlz), compared to control (Ctrl) cells in the absence of decidualizing agents. (E) DCN mRNA expression and secreted DCN protein concentration (F) at 6 days in decidualized (Ddlz) cells compared to controls (Ctrl). In B, C, E: mRNA expression levels were normalized to GAPDH. Statistical analyses were performed with Student's t-test (n = 5) ± SE. (*P < 0.05, **P < 0.005). (G) DCN and (H) FOXO-1 mRNA expression relative to IGFBP1 mRNA (used as a decidual maturation marker). The data represent dCT ratios of DCN/IGFBP1 or FOXO-1/IGFBP1 for decidua

isolated between 7 and 11 weeks of gestation from different mothers. Expression levels were normalized to the geometric means of 4 housekeeping genes-GAPDH, RNA18SN1, EEF2 and YWHAZ. Data are expressed as means (n = 3–7) ± SE. (*P < 0.05). Statistical analyses were performed with one-way ANOVA followed by Tukey–Kramer post hoc test. DAPI, 4′,6-diamidino-2-phenylindole; DCN, decorin; EEF2, eukaryotic elongation factor 2; FOXO-1, forkhead box protein O1; IGFBP1, insulin-like growth factor binding protein 1; pHESC, primary human endometrial stromal cells; PRL, prolactin; RNA18SN1, 18S Ribosomal N1; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

2.3.2 DCN production by the early gestational decidua *ex vivo*

We had earlier examined the localization of DCN protein in various cell types and the ECM in the decidua and chorionic villi Lysiak et al., 1995). However, gestational age-related changes in DCN production by the first trimester decidua *in situ* has not been previously quantified. Hence, we measured levels of DCN mRNA in freshly isolated samples of 7–11 weeks decidua ($n = 24$). Expression levels (relative to geometric means of GAPDH, RNA18SN1, EEF2 and YWHAZ) at different gestational ages presented in Fig. 2-S1A shows a rise between 7 and 8 weeks, followed by no change between 8 and 11 weeks. However, these data do not represent DCN expression by decidual cells alone, since decidua is a heterogeneous tissue inclusive of stromal cells at various stages of maturation and leukocytes such as uterine natural killer cells. Hence, we used IGFBP1 mRNA as a maturation marker of decidual cells. Gestation age-related changes in the expression of IGFBP1 mRNA in the decidual tissues are shown in Fig. 2-S1B. These data were used to derive the ratio of DCN/IGFBP1 mRNA as a measure of DCN expression relative to decidual cell maturation. A cluster plot of this ratio (shown in Fig. 2-1G) at gestational ages from 7 to 11 weeks reveals that the level of DCN production by decidual cells significantly increases at 8 weeks ($P < 0.05$) and remains stable thereafter up to 11 weeks gestation. FOXO1 is a key transcription factor needed for many decidualization-associated genes (Vasquez et al., 2015; Okada et al., 2018). FOXO1 expression relative to IGFBP1 expression remained unchanged between 7 and 11 weeks (Fig. 2-1H).

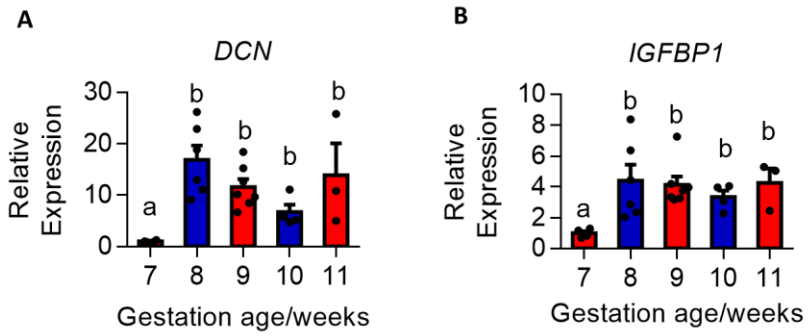

Figure 2-S 1: qRT-PCR data showing DCN expression

(A) and IGFBP1 expression (B) relative to GAPDH in decidual tissues at different gestational ages. Both DCN and IGFBP1 exhibit similar gestation age-dependent changes. Values 8 to 11 weeks were significantly higher (*$p<0.05$) than the value at 7 weeks in both for DCN and IGFBP1, as indicated by letters a and b.

2.3.3 DCN production in decidualized THESCs

To confirm earlier reports that THESCs represent a reliable model for decidualization *in vitro* (Gellersen and Brosens, 2003; Gellersen et al., 2007), cells were cultured for 6 days in the presence or absence of decidualizing agents (in the same manner as presented for pHESC). As shown in Fig. 2-2, decidualization was evident in the experimental group by morphological changes from spindle to epithelioid shapes in immunofluorescent images of phalloidin-stained cells (Fig. 2-2A) and increased expression of decidualization markers IGFBP1 and PRL (mRNA level of IGFBP1: 50-fold increase and PRL: 20-fold increase) measured with qRT-PCR and secreted IGFBP1 protein measured with ELISA (20-fold increase, Fig. 2-2B). Expression of DCN mRNA (Fig. 2-2C) was very similar to pHESC (Fig. 2-1E) after 6 days of decidualization. There was no significant difference in DCN mRNA expression normalized to GAPDH between non-decidualized THESC and pHESC (Fig. 2-2D). The time course of IGFBP1, PRL and DCN mRNA expression by THESC is presented in Fig. 2-S2. While THESC in the control group produced DCN during the culture period, a significant upregulation of DCN levels during decidualization reveals that DCN production is stimulated by decidual stimuli, as was shown with pHESC.

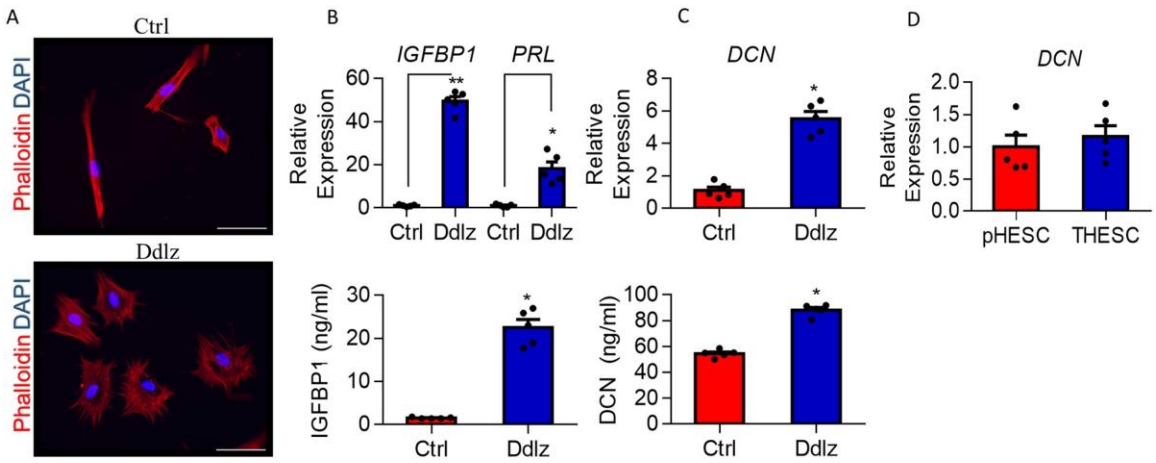

Figure 2-2: DCN production in decidualized THESCs.

A) Morphology of THESC (telomerase-immortalized endometrial stromal cell line): non-decidualized (Ctrl, fibroblast-like spindle-shaped), and decidualized for 6 days (Ddlz, polygonal shaped), stained for phalloidin (red), and DAPI (blue). Magnification bar: 10 µm. (B) qRT-PCR data showing expression of decidual markers: IGFBP1 and PRL mRNA (top) along with secreted IGFBP1 protein (ELISA, bottom) in THESCs in Ddlz cells compared to Ctrl cells at 6 days (cultured respectively with or without the decidualizing agents). (C) DCN mRNA expression (top) and secreted DCN protein (ELISA, bottom), after 6 days of culture with or without decidualizing agents. (D) qRT-PCR data showing no significant difference in DCN mRNA expression normalized to GAPDH between THESC and pHESC. (n = 5 ± SE); (*P < 0.05; **P < 0.005). Statistical analyses were performed with Student's t-test.

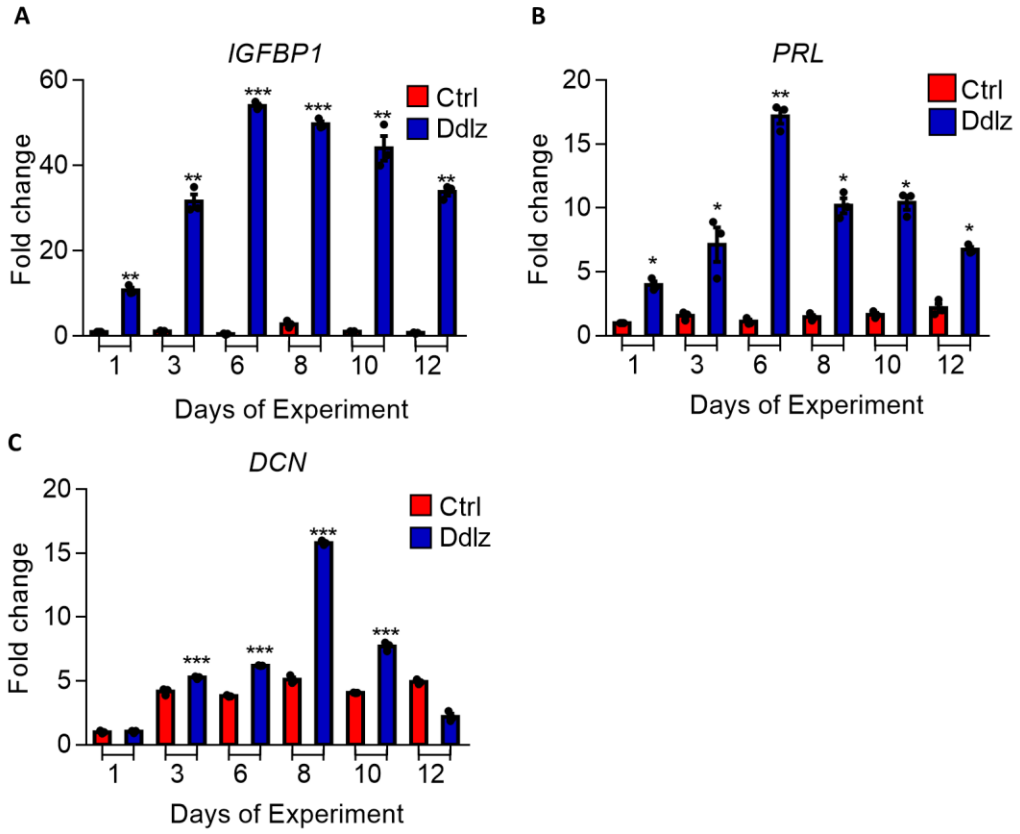

Figure 2-S 2: Temporal kinetics of changes in IGFBP1, PRL and DCN expression in THESC in the presence or absence of decidualizing agents.

qRT-PCR values for mRNA, normalized to GAPDH, relative to day 1 values of untreated control cells presented for IGFBP1 (A), PRL (B) and DCN (C) production by THESC during decidualization in vitro. (Control: Ctrl; Decidualized: Ddlz). N=3 ± SE (*p<0.05, **p<0.01, ***p<0.001)

2.3.4 DCN knockdown in THESCs impedes decidualization

In order to understand the role of DCN in decidualization, we used two distinct approaches to disrupt DCN expression in ESCs: shRNA and CRISPR-Cas9. We were unsuccessful in transfecting pHESCs efficiently, hence all subsequent experiments were conducted using THESCs. Knocking down DCN by 60% (Fig. 2-3A), associated with reduced secretion of DCN protein (by 70%, Fig. 2-3B), in THESC using shRNA significantly impeded the process of decidualization after exposure to decidualizing agents. This was evidenced by: poor morphological transformation of spindle-shaped stromal cells into epithelioid-shaped decidual cells (Fig. 2-3C); reduced expression of decidual maturation markers: IGFBP1 (4-fold decrease) and PRL (2-fold decrease), as assessed by qRT-PCR; and decreased IGFBP1 secretion (3-fold), as measured by ELISA (Fig. 2-3D–F). These findings for the first time suggest an autocrine role of DCN in decidual cell maturation.

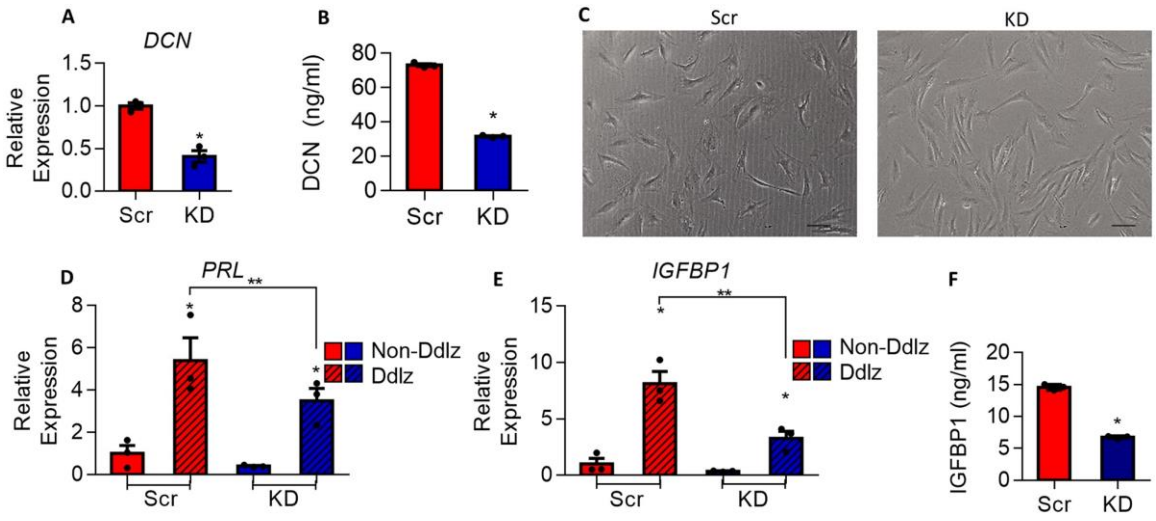

Figure 2-3: Effect of DCN knockdown on decidualization in THESCs.

(A) qRT-PCR for DCN mRNA and (B) ELISA for secreted DCN protein showing significant DCN knockdown in THESCs transfected with DCN-specific shRNA (KD) compared to THESCs transfected with scrambled shRNA (Scr). (C) Morphology (phase-contrast) of Scr versus DCN KD THESCs after treatment with decidualizing agents for 6 days. Magnification bar: 10 μm. (D) PRL and (E) IGFBP1 expression in Scr and KD cells in the absence (non-Ddlz) and presence of decidualizing agents (Ddlz). GAPDH was used as a reference gene. (F) IGFBP1 secretion (determined by ELISA) in Scr and KD cells cultured with decidualizing agents for 6 days. (n = 3 ± SE). (*P < 0.05; **P < 0.005). Statistical analyses were performed with Student's t-test.

To further substantiate our findings, we used CRISPR-Cas9 to generate THESCs possessing a mutation within the gene encoding DCN. THESCs were transduced with guide RNA plasmid targeting nucleotide sequences of the second exon of the DCN gene (Fig. 2-4A). Following selection, we identified a clonal population of cells possessing a 252 bp deletion including exon 2 of the DCN gene. Compared to control cells, these DCN-mutant cells exhibited a 95% reduction in DCN protein secretion, measured with ELISA (Fig. 2-4B). Since DCN production was almost completely abrogated in these cells, they will henceforth be referred to as DCN KO cells. To determine the efficiency of decidualization in control and DCN KO cells, cells were cultured with or without decidualizing agents, as established earlier. The time course of IGFBP1 (mRNA and protein) and PRL (mRNA) production by DCN KO cells upon decidualization is presented in Fig. 2-S3. No DCN mRNA or secreted DCN protein was detectable in DCN KO cells on any day. We found that DCN KO cells did not attain the decidual morphological phenotype (epitheloid shape) after 6 days of decidualization treatment (Fig. 2-4C) and showed a profound reduction in decidual markers IGFBP1 (7-fold) and PRL (8-fold) (Fig. 2-4D), which were more pronounced than noted with DCN knocked down (KD) cells. Furthermore, a reduction in cellular ploidy, another maturation marker was observed in DCN KO cells from cellular morphometry and flow cytometry analysis of DNA content. Fluorescence (DAPI and phalloidin) microscopic analysis for cellular polyploidy showed that control cells contained more polyploid cells than DCN KO cells, as judged by the presence of cells with large (30 μm or more in diameter) nuclei and bi-nucleated cells (Fig. 2-4E). Flow cytometry displayed a shift in the distribution of cellular DNA contents in DCN KO THESCs cells (from high to low N) compared to empty vector control cells (Fig. 2-S4). These results suggest a possible role of DCN in maintaining decidual cell polyploidy.

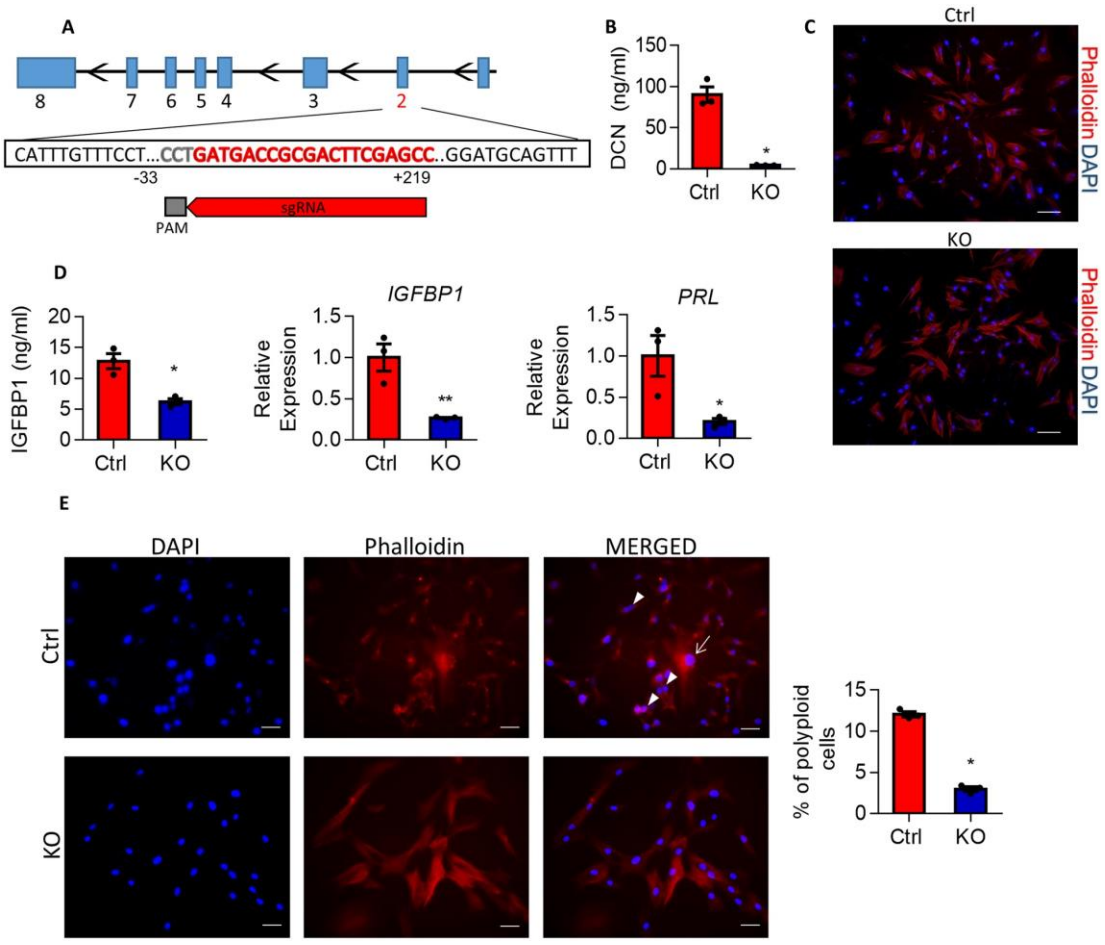

Figure 2-4: CRISPR-Cas9 mediated disruption of the DCN gene in THESCs.

(A) Schema showing the location of the guide RNA (sgRNA) sequence in relation to the DCN gene. (B) Levels of secreted DCN in THESCs in control (Ctrl, transduced with vector lacking in DCN guide RNA) and DCN-Knock out (KO) cells. (C) Morphology of Ctrl and DCN KO cells treated with decidualizing agents for 6 days, stained for phalloidin (red), and DAPI (blue). Magnification bar: 10 µm. (D) IGFBP1 (secreted protein and mRNA) and PRL (mRNA) in DCN KO cells compared to Ctrl cells treated with decidualizing agents. mRNA expression normalized to GAPDH. (n = 3 ± SE). (*P < 0.05; **P < 0.005). (E) Immunofluorescence images of Ctrl and DCN KO THESC. Cells show cytoplasmic Phalloidin (red) and nuclear DAPI (blue). Ctrl shows a higher incidence of binucleate cells (shown by arrow heads) plus cells with large (30 µm or more in diameter) nuclei (shown by arrows) than seen in DCN KO cells. Magnification bar: 100 µm. Bars on the right

represent mean incidences derived from counting single slide each from 3 Ctrl and 3 DCN KO clones ± SE. *$p < 0.01$. Statistical analyses were performed with Student's t-test.

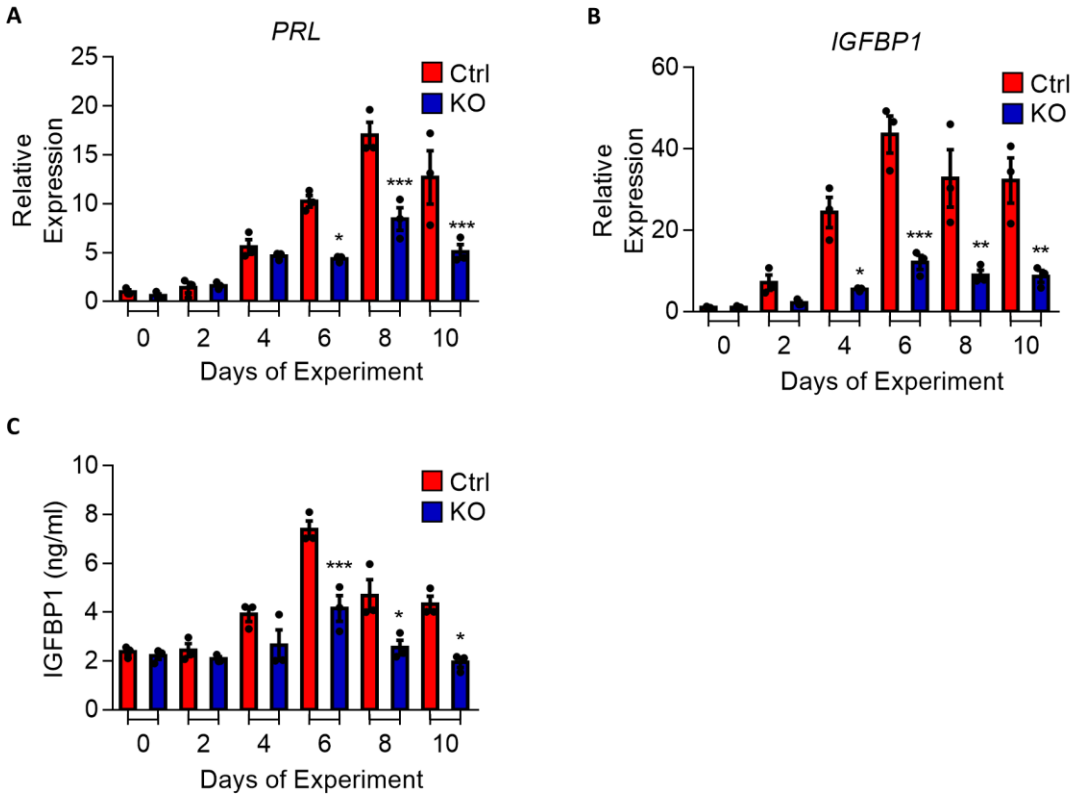

Figure 2-S 3: Temporal kinetics of changes in PRL (mRNA) and IGFBP1 (mRNA and protein) expression in Control (Ctrl) vs DCN Knockout (KO) THESC in the presence of decidualizing agents.

qRT-PCR values for PRL (A) and IGFBP1 mRNA (B) and IGFBP1 secreted protein (C), normalized to GAPDH, relative to day 0 values of treated Ctrl cells during decidualization in vitro. (Control: Ctrl; DCN knockout: KO). N=3 ± SE (*p<0.05, **p<0.01, ***p<0.001)

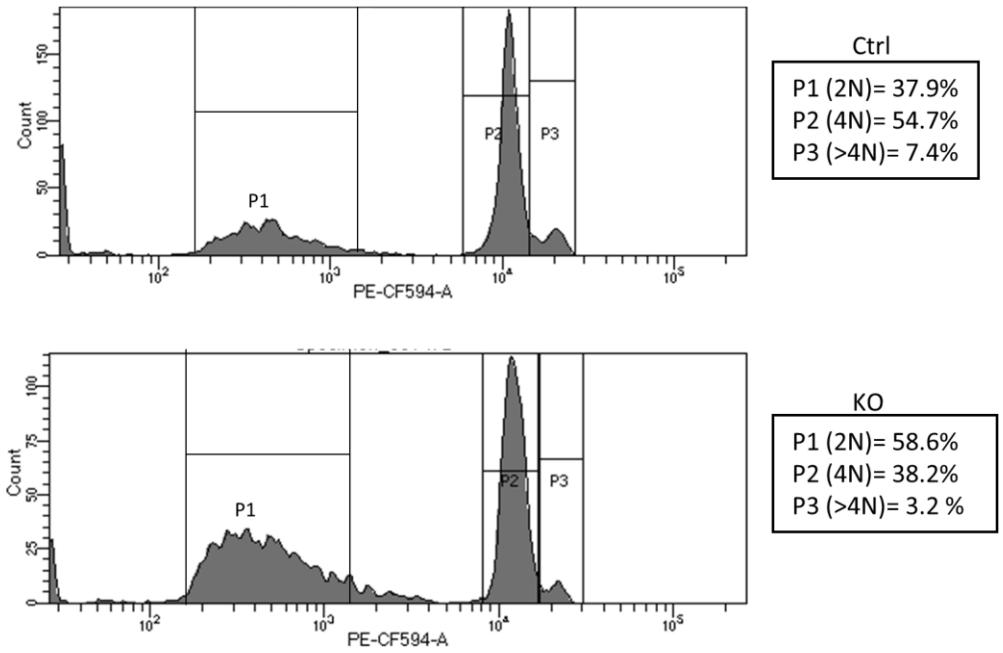

Figure 2-S 4: Flow cytometry

Representative data from one experiment on flow cytometry of distribution of DNA contents in Ctrl vs DCN KO cells. Data show a progressive shift in the distribution DNA contents from high to low N values (> 4N>4N>2N) in DCN KO cells, compared to Ctrl cells.

2.3.5 Paracrine effects of DCN depletion in decidualized THESC on trophoblast migration and invasion

We have earlier shown than decidua-derived DCN restrains trophoblast migration and invasion of the decidua (Xu et al., 2002) by binding to multiple tyrosine kinase receptors. Here we compared the effects of cell-free supernatants from decidualized DCN KO versus control THESC on trophoblast migration in a wound-healing assay. Results presented in Fig. 2-S5A show that DCN depletion in THESC promoted trophoblast migration. Adhesive interactions of trophoblast cells with the endometrium are essential for embryo implantation in the uterus. We have reported that human trophoblast stem cell renewal and differentiation into syncytial and invasive (extravillous) pathways can be studied with primary and HTR-8 trophoblast spheroids developing from single cells (Nandi et al., 2018). Here, we show that trophoblast sprouting and invasion of THESC monolayers from HTR-8 spheroids were stimulated by DCN depletion in HESC (Fig. 2-S5B).

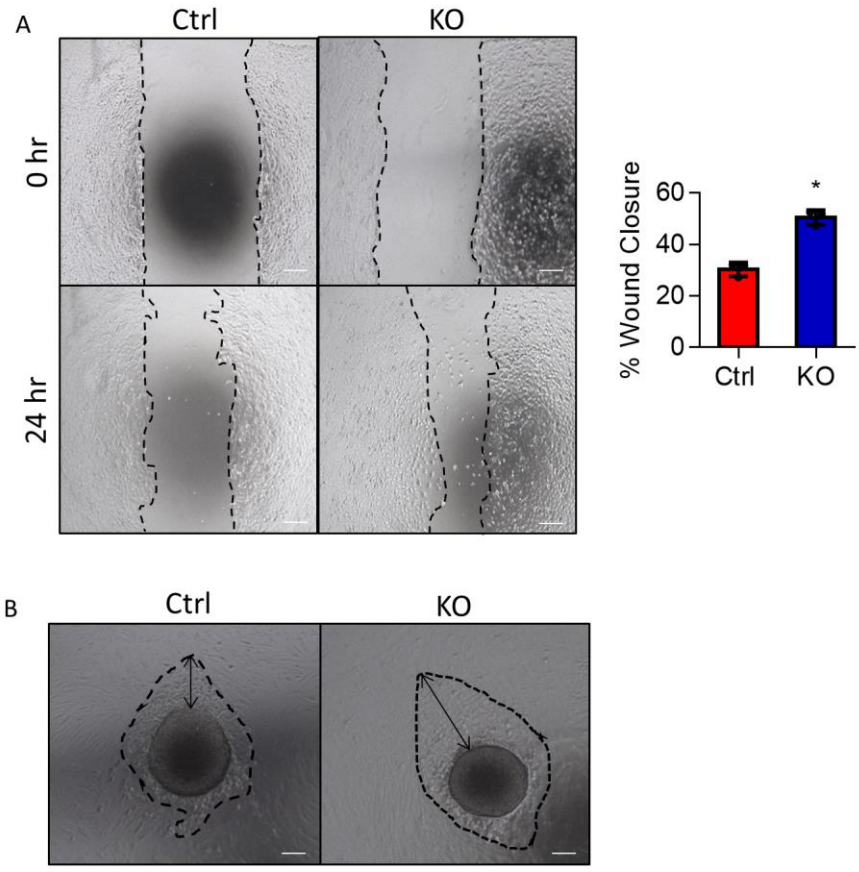

Figure 2-S 5: Paracrine effects of DCN depletion in THESC on trophoblast migration and invasion.

A. Migration (wound Healing) assay showing the scratch gap (black dotted line) in HTR-8 cells at time 0hr and 24hr following treatments with control THESC media (Ctrl) and DCN KO THESC media (KO) collected at 48hr of treatment with decidualizing agents. Cellular migration, shown as percent wound closure area, was more pronounced (*$p<0.05$) in HTR-8 cells cultured with DCN KO THESC media than with Ctrl media. N=3 ± SE

B. Invasion assay showing attachment and invasion of trophoblast cells after 48hr of co-culturing trophoblast spheroids on monolayers of Ctrl or DCN KO THESCs. Arrow shows the level of trophoblast sprouting and invasion to be higher on DCN KO THESC monolayer than on the Ctrl monolayer. Magnification bar: 200µm

2.3.6 Reduced expression of genes associated with decidualization in DCN KO cells

We performed whole transcript expression analysis using the Clariom S assay of control versus DCN KO THESCs after treatment with decidualizing agents for 6 days. Overall, from a total of 21 448 transcripts examined, 880 differentially expressed genes (2-fold expression change) were evident in DCN KO cells; 556 genes were downregulated and 324 were upregulated (Fig. 2-S6A). To validate results obtained from the array, qRT-PCR was conducted using five other clones derived from DCN KO cells. We detected decreased expression of IGFBP1, PRL, HAND2 and PGR, and increased expression of butyrylcholinesterase (BCHE) and receptor tyrosine kinase like orphan receptor 1 (ROR1) in DCN KO cells following decidualization treatment, with fold changes consistent with results obtained using microarray (Fig. 2-S6B–H). Changes in these genes were also validated in a second array with another DCN KO clone (not shown). These genes did not show any significant difference between non-decidualized control and DCN KO THESC (Figs 2-S3: IGFBP1 and PRL; and 2-S7: HAND2, PGR and FOXO1). In support of downregulation of HAND2 mRNA, immunoflourescence analysis showed near absence of nuclear HAND2 protein in DCN KO cells in comparison to control cells (Fig. 2-5A). While IGFBP1 and PRL are decidual maturation markers, HAND2 and PGR are obligatory for decidualization of HESCs.

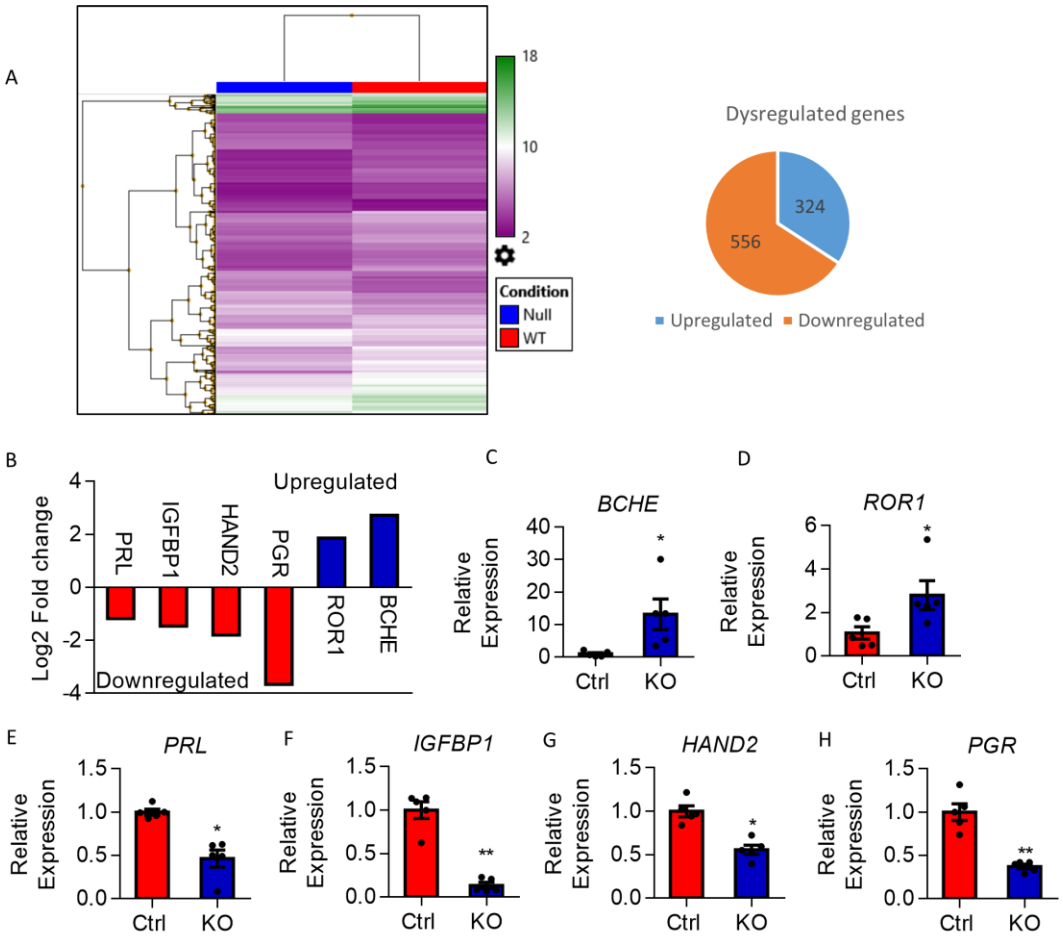

Figure 2-S 6: Differential gene expression between control and DCN KO THESCs exposed to decidualizing agents.

(A) Heatmap of 880 differentially-expressed genes in control THESC (Ctrl) and DCN KO cells. Pie chart showing the number of up-regulated and down-regulated genes in DCN KO cells is shown on the right. (B) Microarray data of selected up-regulated and down-regulated genes (shown as log2 fold change). (C-H) qRT-PCR validation of selected up-regulated and down-regulated genes in Ctrl and DCN KO cells. (N=5 ± SE). (*p<0.05, **p<0.005).

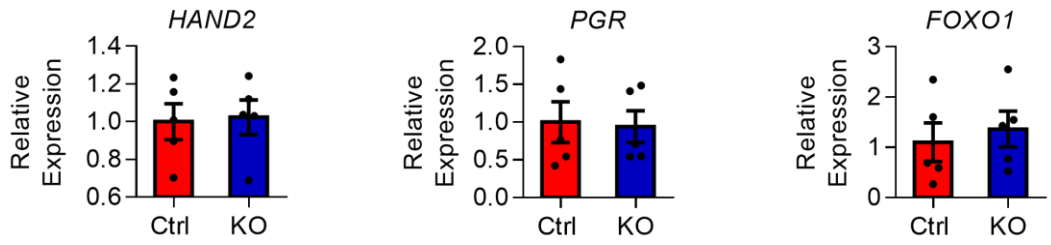

Figure 2-S 7: Differential gene expression between non-decidualized control and DCN KO THESCs .

qRT-PCR of some selected genes (HAND2, PGR, FOXO1) in non-decidualized Ctrl and DCN KO cells. (N=5 ± SE). No significant difference was observed in these genes.

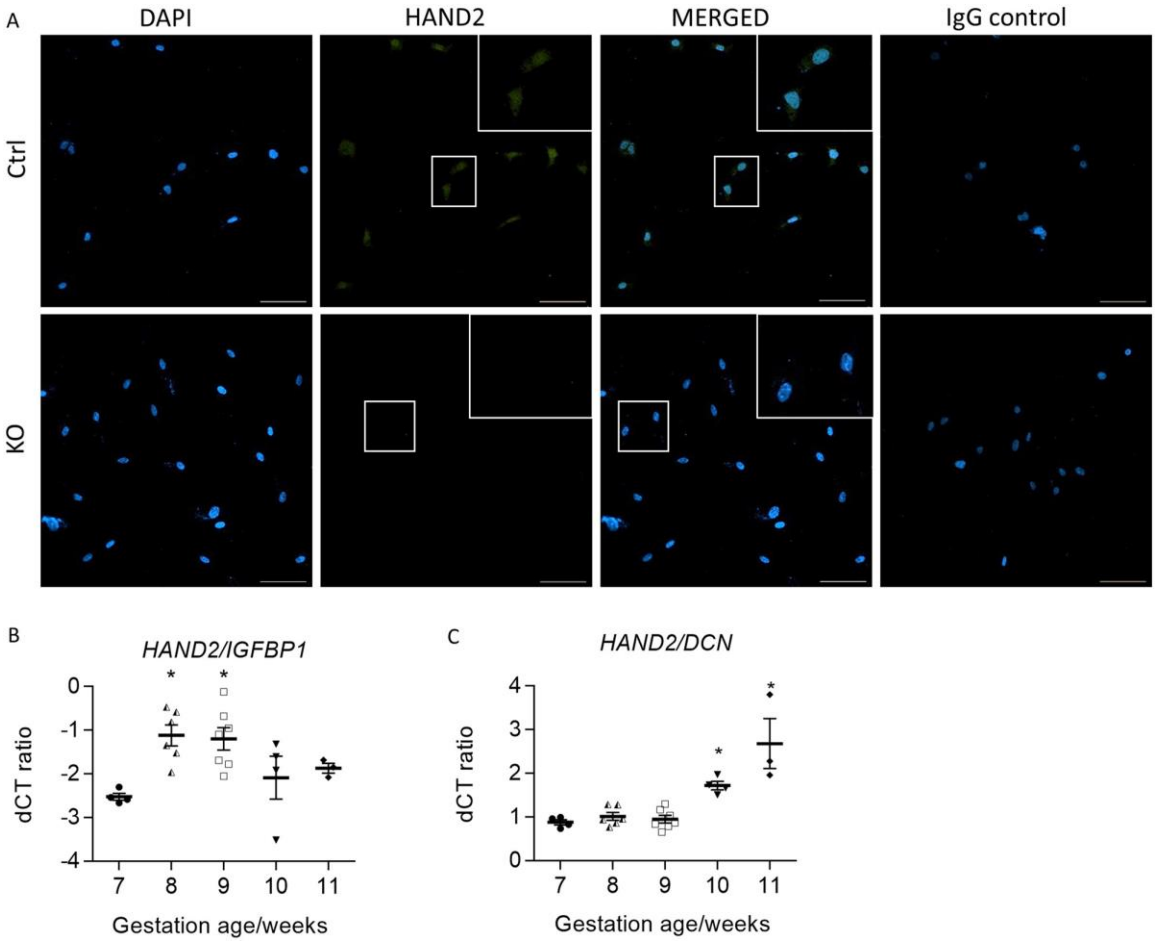

Figure 2-5: HAND2 expression in THESC and decidual tissues.

(A) Immunofluorescent images of Ctrl and DCN KO THESC (including magnified insets) showing nuclear Heart And Neural Crest Derivatives Expressed 2 (HAND2) in Green and DAPI (Blue). KO cells exhibit very little staining for HAND2 protein. IgG controls shown on the right panel confirm antibody specificity. (B) HAND2 expression relative to IGFBP1 expression in first trimester decidua at various gestational ages. Expression levels were normalized to the geometric means of 4 housekeeping genes-GAPDH, RNA18SN1, EEF2 and YWHAZ. HAND2 mRNA expression relative to IGFBP1 mRNA (used as a decidual maturation marker). The data represent dCT ratios of HAND2/IGFBP1 for decidua isolated between 7 and 11 weeks of gestation from different mothers showing a significant increase from Week 7 to Weeks 8 and 9. (C) HAND2 mRNA expression relative to DCN mRNA. The ratios reveal significant increase from Week 7 to Weeks 10 and 11. n = 3–7 ± SE. (*P

< 0.05). Statistical analyses were performed with one-way ANOVA followed by Tukey–Kramer post hoc test.

Next, we performed gene ontology analysis and KEGG analysis (data not shown) to identify pathways that were significantly altered in the DCN KO cells. Not surprisingly, the cells showed an upregulation of pathways associated with tumorigenesis, proliferation, migration and angiogenesis, since DCN is a well-known tumor-suppressor molecule (Neill et al., 2012, 2015) and an inhibitor of trophoblast proliferation, migration and angiogenesis (Lala and Nandi, 2016). A downregulation of the cAMP signaling pathway in DCN KO cells is consistent with the well-known role of cAMP in decidualization. An upregulation of VEGF signaling was expected from our demonstration that DCN is a negative regulatory ligand for VEGFR2 to antagonize VEGF signaling (Khan et al., 2011). An upregulation of TGF-β receptor signaling pathway can be explained by an increased TGF-β activity in DCN KO cells, since DCN can bind to and inactivate TGF-β (Lysiak et al., 1995). Our findings of a down regulation of HAND2 in DCN KO cells shown with Clariom microarray above was confirmed with qRT-PCR (Fig. 2-6G) and immunoflourescence (Fig. 5A), indicating that the DCN-mediated regulation of IGFBP1 noted earlier may be HAND2-dependent. This notion is consistent with the report that knocking down HAND2 in HESC resulted in downregulated IGFBP1 (Huyen and Bany, 2011). Our gene expression analysis with non decidualized control and DCN depleted THESC reveal that the ESC gene signature for decidualization markers, including HAND2, was unaffected by DCN depletion without decidualizing stimuli (data not shown).

2.3.7 HAND2 expression in early gestational decidua

HAND2 is one of the transcription factors upregulated in HESC during the process of decidualization (Gellersen and Brosens, 2003; Okada et al., 2018) and is considered as obligatory for decidual cell development (Huyen and Bany, 2011; Vasquez et al., 2015). Since HAND2 expression in early gestation human decidua has not been reported before, we examined gestation age-related changes in HAND2 expression levels in decidual tissues retrieved at 7–11 weeks of gestation. We found that HAND2 expression in decidual tissues relative to IGFBP1 (Fig. 2-5B) exhibited a significant increase from Week 7 to Weeks 8 and 9, very similar to that of DCN expression relative to IGFBP1 (Fig. 2-1G). This

similarity is further demonstrated by plotting HAND2 expression relative to DCN expression (Fig. 2-5C), which increased from Week 7 to Weeks 10 and 11.

2.4 Discussion

DCN is a small leucine rich proteoglycan produced by many stromal cells in the body (Iozzo, 1998; Neill et al., 2012) including the placenta and the decidua (Lysiak et al., 1995). Our laboratory has uncovered multiple paracrine roles of DCN in trophoblast functions, in health and in disease (Xu et al., 2002; Iacob et al., 2008; Khan et al., 2011; Lala et al., 2012; Siddiqui et al., 2016; Nandi et al., 2018). The present study confirms previous reports of an upregulation of DCN production during decidualization of HESCs *in vitro* (Gellersen et al., 2007; Okada et al., 2018). We have shown here that primary as well as immortalized ESCs exhibit a similar kinetics of increase in DCN production. Furthermore, using a large sample size (n = 24) of early gestation (7–11 weeks) decidua, we have shown that DCN production relative to decidual maturation is a very early event in human gestation. This is not surprising in view of the multiple roles of decidua-derived DCN in placental development including trophoblast proliferation, differentiation, migration, invasion and uterine angiogenesis. Here, for the first time, DCN was shown to exert an autocrine role in decidual cell maturation.

The process of decidualization of the endometrium is required for successful implantation, placentation, and embryo development (Okada et al., 2018). This process involves proliferation of ESCs (the decidual cell progenitors) followed by their differentiation into mature decidual cells, evidenced by morphological transformation from a fibroblastic to epithelioid morphology. While morphological features of decidual cell maturation have been described at the light and electron microscope levels (Lawn et al., 1971; Wewer et al., 1985), molecular markers of maturation, such as IGFBP1 and PRL, are considered as additional quantifiable parameters (Bell, 1991; Gellersen and Brosens, 2003). An increase in cellular ploidy is another maturation-associated event (Sroga et al., 2012). In the present

study, using both DCN KD and DCN KO ESCs exposed to decidualizing stimuli, we demonstrate for the first time that decidua-derived DCN plays an autocrine role in decidual cell maturation as evidenced by poor morphological transformation, a reduction in IGFBP1 and PRL expression, and reduction in cellular ploidy. We also demonstrate the functional consequences of DCN depletion in HESC on trophoblast biology. We confirm the paracrine roles of DCN in restraining trophoblast migration and invasion by using decidualized control and DCN KO THESC. Whether DCN depletion in decidualizing uterine stromal cells compromises attachment and invasion by human IVF-derived embryos remains unknown. The presently used HTR cell line has its limitations as an *in vitro* model for placentation, which cannot replace the human blastocyst.

It is interesting to note that DCN-null mice can conceive and produce viable offspring but suffer from skin fragility owing to disorganized collagen fibrils in the dermis (Danielson et al., 1997). This is due to the obligatory requirement of DCN in collagen fibrinogenesis in the dermis. However, DCN-null mice show an upregulation of another class 1 SLRP protein called biglycan, which compensates for the loss of some DCN functions (Zhang et al., 2009). In the uterus, DCN is necessary for myometrial contraction in a DCN gene dose-dependent manner, while biglycan exhibits partial compensation for the loss of DCN (Wu et al., 2012). For these reasons, we checked the expression of the gene encoding biglycan (by qRT-PCR) in DCN KO THESC and found no difference in the expression level from that in control cells (data not shown), indicating that biglycan does not compensate for DCN by increased expression in DCN-depleted cells.

While ESCs serve as the local progenitors for decidual cells, studies in chimeric mice using a β globin transgene as a donor bone marrow cell marker (Lysiak and Lala, 1992) or HOXA-11 null mice deficient in ESC (Tal et al., 2019) suggest that there is a variable recruitment of bone marrow-derived progenitors in ESC expansion during decidualization in pregnancy. Cell renewal in the decidua has been well studied in rodents, indicating regional differences at different gestational ages (Mikhailov, 2003). While this information

is lacking in the human, decidual tissue at the implantation site (decidua basalis) continues to grow along with the placenta indicating that cell proliferation and/or survival continues at the placental bed during the period of placental growth. We suggest that the maturation promoting action of DCN is important *in vivo* during the growth phase of decidua basalis.

Microarray analysis of DCN KO THESC cells subjected to decidualization identified both PGR and HAND2 as downstream candidates involved in DCN-mediated decidual maturation. We validated the downregulation of both genes in DCN KO cells by qRT-PCR. PGR is a nuclear receptor essential for progesterone-mediated HESC differentiation into decidual cells by utilizing multiple transcriptional pathways (Mazur et al., 2015). Unsurprisingly, both HAND2 and DCN were among some 695 genes identified by these authors bound by the PGR (using chromatin immunoprecipitation-sequencing) and differentially expressed upon decidualization with or without PGR silencing. Our findings suggest a positive feedback regulation of PGR and HAND2 by DCN for decidual cell development.

HAND2 and FOXO1 are key transcription factors upregulated during decidualization of ESC (Okada, 2018), and considered as obligatory for decidual cell development (Huyen and Bany, 2011; Vasquez et al., 2015). The antiproliferative action of progesterone on endometrial epithelium is also HAND2-dependent (Li et al., 2011). HAND2 is expressed in the pre-implantation and post-implantation decidua in mice suggesting that an implanting embryo is not obligatory for its expression. Moreover, knocking down HAND2 in both murine and human ESC impeded decidualization (Huyen and Bany, 2011): these authors also demonstrated down-regulation of both FOXO1 and IGFBP1 in HAND2-knockdown HESCs, indicating that FOXO1 is downstream of HAND2 in decidual response, and IGFBP1 may be directly regulated by HAND2, FOXO1, or both. A schematic of the role of DCN in decidual cell development is presented in Fig. 6. We identified that IGFBP1 contains multiple putative HAND2-binding sites (CAGATG/CATCTG) less than 150 base-pairs upstream of the transcription start site.

HAND2 binding at these sites remains to be validated with chromatin immunoprecipitation. Our present data combined with the above evidence strongly suggests that DCN-mediated decidual cell maturation is dependent on HAND2.

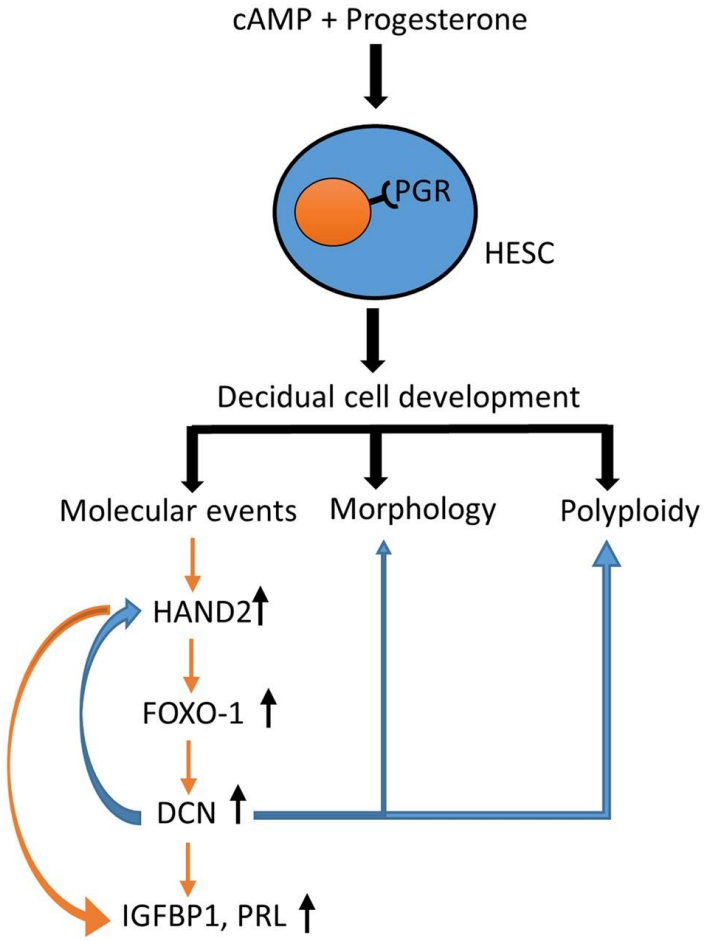

Figure 2-6: Schema of the role of DCN in decidual cell development.

Proposed schema is based on previously reported data combined with the present data. Decidualizing stimuli (cAMP stimulators and MPA) act on HESCs to make them proliferate and differentiate into decidual cells as evidenced by molecular, morphological and ploidy changes. HAND2 is a highly conserved transcription factor shown to upregulate FOXO-1 (Huyen and Bany, 2011) which is known to upregulate DCN (Gellersen et al., 2007). HAND2 upregulates IGFBP1 (Huyen and Bany, 2011), whereas DCN can upregulate HAND2 (present data). DCN regulation of HAND2 may be indirect, mediated by other molecules, likely providing positive feedback for decidual cell maturation. DCN regulation of PGR is likely mediated by HAND2. cAMP, cyclic adenosine monophosphate; MPA, medroxy progesterone acetate; PGR, progesterone receptor.

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Chapter 3

3 Molecular mechanisms in IL-1β-mediated decorin production by decidual cells

Decorin, a small leucine-rich proteoglycan produced by decidual cells restrains trophoblast differentiation, migration and invasiveness of extra-villous trophoblast cells. Decidual overproduction of decorin is associated with preeclampsia, and elevated decorin levels in maternal plasma are a predictive biomarker of preeclampsia. Furthermore, decorin plays an autocrine role in maturation of human endometrial stromal cells into decidual cells. Thus, a balanced decorin production by the decidua is critical for healthy pregnancy. However, the molecular mechanisms regulating decorin production by the decidua are unclear. Interleukin-1 beta is an inflammation-associated multi-functional cytokine, and is reported to induce decidualization in primates. Hence, the present study was designed: (i) to test if exogenous Interleukin-1 beta stimulated decorin production by human endometrial stromal cells; and if so, (ii) to identify the cellular source of Interleukin-1 beta in first trimester decidual tissue; (iii) to identify the downstream molecular partners in Interleukin-1 beta mediated decorin production by human endometrial stromal cells. Results revealed that (i) amongst multiple pro-inflammatory cytokines tested, Interleukin-1 beta alone stimulated decorin production by these cells; (ii) both macrophages and decidual cells in first trimester decidua produced Interleukin-1 beta; (iii) Interleukin-1 beta mediated decorin production was dependent on Interleukin-1 receptor activation, followed by activation and nuclear translocation of nuclear factor kappa B and its binding to the decorin promoter. These results reveal that Interleukin-1 beta plays a novel role in inducing decorin production by human endometrial stromal cells by activating nuclear factor kappa B.

3.1 Introduction

Decidua-derived decorin (DCN) is an important molecule exerting multiple paracrine effects on trophoblast functions, and an autocrine role in decidual cell maturation, which are all essential for maintenance of pregnancy. The present study was designed to test if exogenous interleukin (IL)-1β induced decorin production by human endometrial stromal

cells (HESC), and if so, define the cellular source of IL-1β in pregnancy associated decidua and understand the molecular mechanisms in DCN induction by IL-1β in HESC during the process of decidualization.

DCN is a leucine-rich proteoglycan produced by many stromal cells in the body including dermal fibroblasts, chondrocytes (Neill et al., 2012), mesenchymal cells of the placenta and decidual cells (Lysiak et al., 1995). A major function of DCN is collagen fibrillogenesis, so that DCN null mice suffer from fragile skin resulting from disorganized collagen fibrils in the dermis (Danielson et al., 1997). DCN can bind to and inactivate transforming growth factor beta (TGFβ) (Neill et al., 2012), a key molecule controlling trophoblast invasion of the decidua (Graham and Lala, 1992). Our studies revealed that DCN is colocalized with TGFβ in the decidual extracellular matrix indicating that DCN is a storage device for TGFβ keeping it inactive. TGFβ bound to DCN is likely activated by the protease cascade at the trophoblast invasion front to prevent placental over-invasion of the uterine endometrium (Lysiak et al., 1995). Subsequently, we found that DCN can restrain proliferative, migratory and invasive functions of trophoblast in a TGFβ-independent manner (Xu et al., 2002). DCN binding to multiple tyrosine kinase receptors on trophoblast, such as epidermal growth factor receptor, insulin-like growth factor receptor-1 and vascular endothelial growth factor receptor-2 (VEGFR2), was found to differentially mediate these trophoblast functions (Iacob et al., 2008; Khan et al., 2011). The VEGR2-binding site of DCN was mapped to a 12-amino acid span of the leucine-rich repeat 5 domain of the DCN protein core (Khan et al., 2011). This binding controlled VEGF-dependent endovascular differentiation of the trophoblast, during the process of remodeling of the uterine arteries (Lala et al., 2012; Lala and Nandi, 2016). DCN also restrained renewal and differentiation of ‘stem’ trophoblast cells into extra-villous trophoblast (EVT) and syncytiotrophoblast (Nandi et al., 2018). We found that decidual overproduction of DCN is associated with preeclampsia (PE), a trophoblast hypo-invasive disease, and elevated DCN level in the maternal plasma is a predictive biomarker of PE during the second trimester of gestation (Nandi et al., 2016; Siddiqui et al., 2016). Recently, we found that DCN production by human decidual tissue *in vivo* rises during early gestation and DCN plays an autocrine role promoting maturation of HESC into decidual cells (Halari et al., 2020). Thus, a balanced production of DCN by the decidua is critical for healthy

pregnancy; low production of DCN leads to a decidual maturation defect, and high production predisposes to PE. However, what regulates decidual DCN production remains unknown. Interestingly, IL-1β was reported to stimulate insulin-like growth factor-binding protein 1 (IGFBP1) production and decidualization in the primate endometrial stromal cells (ESC), suggesting a possible role of IL-1β in DCN production (Strakova et al., 2000).

IL-1 cytokine family members are key regulators of physiological inflammatory responses of the maternal endometrium to the embryo (Paulesu et al., 2008). Studies in mice-lacking individual cytokines show that many of them including IL-1β are essential for successful pregnancy (Ingman and Jones, 2008). IL-1β is expressed by various cells at the feto-maternal surface, including human EVT cells, macrophages and fibroblasts of the decidual stroma (Kauma et al., 1990). Trophoblast-derived IL-1β was shown to promote its own invasiveness in an autocrine manner by activating matrix metallopeptidase 9 (MMP-9) (Librach et al., 1991). Exogenous IL-1β promoted trophoblast migration and invasion by regulating the uPA/PAI-1 pathway (Prutsch et al., 2012). In addition, IL-1β exhibited multiple effects on human or primate ESC: (i) proMMP-3 expression following activation of the mitogen-activated protein kinase (MAPK) pathway (Rawdanowicz et al., 1994); (ii) expression of IGFBP1 (a key decidual marker) in the presence of steroid hormones (Strakova et al., 2000) suggesting a role of IL-1β in decidualization.

Effects of IL-1β on target cells are mediated by the type 1 IL-1 receptor (IL-1R1) (Kuno and Matsushima, 1994; Dinarello, 1998), which then complexes with the IL-1 receptor accessory protein (IL-1RAcP) and activates multiple downstream signaling networks including P38 MAPK, extracellular signal-regulated kinase (ERK) and nuclear factor kappa B (NF-κB) pathways, depending on the cell type (Bankers-Fulbright et al., 1996; Srinivasan et al., 2004). Functions of IL-1β can be blocked by an IL-1 receptor antagonist (IL-1RA) that prevents binding of IL-1α/β to IL1R. Furthermore, The soluble form of IL-1 receptor accessory protein (IL-1RAcP) enhances the ability of soluble type IL-R2 receptor to inhibit IL-1 action, thus reducing inflammation (Smith et al., 2003).

Based on the above information, the objective of our study was to test if exogenous IL-1β stimulated DCN production by HESC. Additionally, we sought to identify the cellular source of IL-1β in first trimester decidual tissue and determine the downstream molecular partners in IL-1β-mediated DCN production by HESC. Results revealed that (i) amongst multiple pro-inflammatory cytokines tested, IL-1β alone stimulated DCN production by the HESC; (ii) both macrophages and decidual cells in first trimester decidual tissue produced IL-1β; and (iii) IL-1β-mediated DCN production was dependent on IL-1R1 activation, followed by activation and nuclear translocation of NF-κB, and NF-κB binding to the DCN promoter.

3.2 Materials and methods

3.2.1 Decidual samples

Decidual samples were collected as mentioned previously (Halari et al., 2020). Briefly, decidual samples from healthy pregnant subjects undergoing elective termination of pregnancy at the London Health Sciences Centre (Westminster campus), during the first trimester of pregnancy (between 7 and 11 weeks of gestation, estimated on the basis of the first date of the last menstrual period and ultrasound validation) were collected following approval by the University of Western Ontario Human Ethics Review Board and informed written consent by the subjects. They were processed for extraction of primary (p) HESC, immunostaining, *in situ* hybridization and isolation of RNA.

3.2.2 Cells and cell culture

Telomerase-immortalized HESC (THESC) were purchased from American Type Culture Collection (CRL-4003) and cultivated as instructed by the supplier. Briefly, cells were cultured in phenol red-free DMEM/F12 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), and 100 µg/ml penicillin/streptomycin (P/S; Gibco). Cells were passaged after light trypsinization prior to reaching confluency and were maintained at 37°C in an atmosphere consisting of 5% CO2 for no more than 20

sequential passages. The cells were plated with fresh medium with or without the cytokines: IL-1β (10 ng/ml; Cat no. 200-01B, Peprotech, USA), IL-12 (10 ng/ml; Cat no. 200-12, Peprotech), IL-15 (10 ng/ml; Cat no. 200-15, Peprotech), IL-18 (50 ng/ml; Cat no. 592102, Biolegend, USA), IL-6 (10 ng/ml; Cat no. 206-IL-010, R&D Systems, USA), IL-1RA (100–300 ng/ml; Cat no. CLCYT203, Cedarlane, USA) or inhibitors of IL-1β pathway (P38-MAPK: 10 μM SB203580, NFκB: 10 μM parthenolide and mitogen activated protein kinase kinase (MEK)/Extracellular signal regulated protein kinase (ERK)-MAPK: 10 μM U0128; Sigma, USA) dissolved in dimethyl sulfoxide (DMSO) for different time points. Control cells were treated with equal concentration of DMSO. Optimal concentrations of cytokines used in cultures were derived from previously published information (Yoshimoto et al., 1998; Nakanishi et al., 2001; Ashkar et al., 2003; Sharpe-Timms et al., 2010). Primary (p)HESC was isolated from first trimester decidua using a protocol reported by us (Halari et al., 2020). In brief, single cell suspensions of decidual cells were freed of leukocytes by immuno-magnetic exclusion and maintained for 2 weeks in hormone-free medium when the cells reverted to stromal cell phenotype.

3.2.3 *In vitro* decidualization

THESC were grown with control or decidualizing media for 6 days. The control medium consisted of basal DMEM/F12 supplemented with 2% charcoal-stripped FBS and 1% P/S. The decidualization medium included control medium plus 250 nM medroxyprogesterone-acetate (a synthetic progesterone derivative; USP, USA) and 0.5 mM 8-bromo-cAMP (a stable, cell permeable analog of cAMP; Sigma).

3.2.4 RNA extraction and reverse transcription PCR

RNA was extracted from THESC and freshly isolated first trimester decidual tissues using Ribozol (VWR International, Canada), according to the manufacturer's instructions, and converted into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). cDNA was diluted 3-fold, then subjected to quantitative PCR run on a

CFX96 real time PCR detection system (Bio-Rad Laboratories, USA) using custom designed primers (Table 3-I) and SYBR Green PCR mix (Quanta Bioscience, USA). Cycling conditions involved an initial holding step (95°C for 10 min), followed by 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 1 min) and a dissociation phase. Relative mRNA expression in treated samples compared with control samples was calculated with the ΔΔCt method using the geometric means of GAPDH and RNA, 18S Ribosomal N1 (RNA18SN1) as reference RNA for THESC cells and the geometric means of four constitutively expressed genes (GAPDH, RNA18SN1, eukaryotic translation elongation factor 2 (EEF2), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ)) as reference RNA for decidual tissue samples.

Table 3- 1: Primer sequence used for qRT-PCR

Gene	Forward primer	Reverse primer
IGFBP1	GATGTCAGAGGTCCCCGTTG	TAGGTACTGATGGCGTCCCA
PRL	GGGGTTCATTACCAAGGCCA	TGGATAGGATAGCCTCCGGG
IL1B	TGAGCTCGCCAGTGAAATGA	AACACGCAGGACAGGTACAG
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
EEF2	AGGCGTAGAACCGACCTTTG	GACAGCGAGGACAAGGACAA
RNA18SN1	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA
YWHAZ	ATGCAACCAACACATCCTATC	GCATTATTAGCGTGCTGTCTT

Primers for quantitative real-time polymerase chain reaction (qRT-PCR): IGFBP1, insulin-like growth factor-binding protein 1; PRL, prolactin; IL1B, interleukin 1 beta; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EEF2, eukaryotic translation elongation factor 2; RNA18SN1, 18S ribosomal N1; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

3.2.5 ELISA

Secreted DCN protein was measured in cell-free culture supernatants of THESC or pHESC using a human DCN ELISA kit according to the manufacturer's instructions (DCN; DY143, R&D Systems) and normalized to cell numbers plated for culture. A standard curve was generated using absorbance values plotted against defined concentrations of recombinant DCN. Conditioned media were diluted (1:50) in assay buffer to ensure absorbance values fell within the linear range of the standard curve.

3.2.6 Immunohistochemistry

Paraffin sections of 9-week-old human decidual tissues were deparaffinized and rehydrated in a graded series of ethanol washes, then subjected to heat-mediated antigen retrieval by immersing in citrate buffer for 20 min at 95°C. Sections were then treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidases, permeabilized using 0.3% Triton-X (Bioshop, Canada), and blocked with 10% normal goat serum (ThermoFisher Scientific, USA). Sections were then probed with antibodies specific for CD68 (1:400, Cat no. 76437, Cell Signaling Technology, USA), IL-1β (1:200, Cat no. AF-201, R&D Systems), Cytokeratin 7 (1:300, Cat no. MA5-11986, Invitrogen, USA) or IGFBP1 (1:400, Cat no. 31025, Cell Signaling Technology) overnight and then incubated with a species-specific biotinylated secondary antibody, followed by Vectastain peroxidase (Vector Laboratories, USA). Sections were then treated with an aminoethyl carbazole chromogen solution (ThermoFisher Scientific), nuclei counterstained with hematoxylin, and sections mounted using Fluoromount-G (Southern Biotech, USA) and imaged using a Nikon DS-Qi2 microscope.

3.2.7 Immunocytochemistry

Cells were plated on cover slips and then placed into culture. At the indicated times, culture medium was removed and fixed by immersing in 4% paraformaldehyde for 30 min.

Paraformaldehyde was then removed, and cells were washed with phosphate buffered saline (PBS). Next, 0.5% Triton X-100 in PBS was added to permeabilize cells, followed by blocking with PBS containing 8% bovine serum albumin (BSA) and 0.01% Tween-20 for 30 min. After blocking, cells were incubated overnight at 4°C with Phalloidin (1:20 dilution, Cat no. 8878, Cell Signaling Technology) or with primary antibodies (IL-1β, 1:200, Cat no. AF-201, R&D Systems; IL-1R1, 1:100, Cat no. AF-269, R&D Systems or p65, 1:200, Cat no. 8242, Cell Signaling Technology) diluted in 8% BSA–PBS. A non-specific species-appropriate IgG served as the negative control. Following a 1 h incubation with species-appropriate fluorescence-conjugated antibodies, cells were washed, coverslips were placed onto microscope slides precoated with vectashield mounting medium containing DAPI (Vector Laboratories) and then slides were dried horizontally at 4°C overnight. The next day, clear nail polish was used to seal the edges of the coverslip, and samples were stored at 4°C until imaged using a confocal microscope (Zeiss, Germany).

3.2.8 Western blotting

Whole cell lysates were prepared by immersing in radioimmunoprecipitation assay lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)) supplemented with protease inhibitor cocktail (Sigma). A modified bicinchoninic acid assay (Bio-Rad Laboratories) was used to measure protein concentrations. Approximately 30 μg of cell lysate was mixed with 4× reducing loading buffer (0.25 M Tris, 8% SDS, 30% glycerol, 0.02% bromophenol blue, 0.3 M dithiothreitol), boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes and probed using antibodies for phospho-p65 (1:500, Cat no. 3033T, Cell Signaling Technology) or β-actin (1:1000, Cat no. 47778, Santa Cruz Biotechnology, USA). Membranes were then incubated for 1 h with species-appropriate secondary antibodies, and signals detected using an LI-COR Odyssey imaging system (LI-COR Biosciences, USA).

3.2.9 Luciferase assay

A luciferase reporter construct was used to assess NF-κB activation. THESC were seeded into a 96-well plate at 15 000 cells/well. Following 24 h, the cells were transfected with 100 ng of pNL3.2.NF-κB-RE (Promega, USA) using Lipofectamine 2000 (ThermoFisher Scientific). The pNL3.2.NF-κB-RE plasmid contains five copies of an NF-κB response element that drives transcription of NanoLuc luciferase in the presence of active NF-κB. Following 24 h the cells were treated with or without IL-1β for different duration. Lipopolysaccharide (1 µg/ml for 1 h, Cat no. L3024, Sigma) was used as a positive control. The cells were harvested, lysed with 5× passive lysis buffer, and luciferase activity read using a luminometer (Perkin Elmer, USA) according to the manufacturer's instructions (Promega).

3.2.10 *In situ* hybridization

Insitu hybridization was conducted using RNAScope, as per the manufacturer's instructions (Advanced Cell Diagnostics, USA). Briefly, paraffin embedded human decidual sections (at 9 weeks of gestation) were hydrated through a graded series of ethanol washes, subjected to peroxidase and protease treatment, and hybridized with probes specific to human IL1B (Cat no. 310361, Advanced Cell Diagnostics). Additional sections treated with probes specific for human PPIB (Cat no. 313901, Advanced Cell Diagnostics) and bacterial dapB (Cat no. 310043, Advanced Cell Diagnostics) served as positive and negative controls, respectively. Sections were then subjected to a series of amplification steps, treated with 3,3′-diaminobenzidine chromogen solution, and nuclei were counterstained with hematoxylin. Sections were dehydrated using increasing concentrations of ethanol, cleared using xylene, mounted with Cytoseal (ThermoFisher Scientific), and imaged using a Nikon DS-Qi2 microscope.

3.2.11 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (Jeyarajah et al., 2020). Briefly, THESC were treated with IL-1β for 30 min, fixed for 10 min with 0.7% formaldehyde, lysed, and sonicated using a Bioruptor (Diagenode, USA) to prepare DNA fragments of less than 400 base pair (bp). One percent of sonicated nuclear lysate was removed to serve as an input control. Half of the remaining lysate was incubated with NF-κB p65 antibody (3 μg, Cat no. 8242, Cell Signaling Technology), the other half was incubated with a negative control rabbit IgG (3 μg, 2729, Cell Signaling Technology) overnight at 4°C. Immunoprecipitated chromatin fragments were then captured using protein G-conjugated Sepharose beads (Sigma). Following capture, DNA fragments were eluted and assessed by quantitative PCR. Values were normalized relative to input. We found two potential binding sites upstream of DCN (TGGACTTTT: −1279 and GGGTCTTC: −9501). Primers were designed specific for these regions of DNA that span the consensus NF-κB p65 DNA-binding motif (shown in Table 3-2). A proprietary human I-Kappa-B-Alpha (IKBA) promoter primer pair (SimpleChIP, Cat no. 5552, Cell Signaling Technology) was used as a positive control for NF-κB p65 DNA binding (Nowak et al., 2005; Cookson et al., 2015).

Table 3- 2: List of ChIP primers used for flanking putative NF-κB binding sites upstream of *DCN*.

Binding site	Forward Primer	Reverse Primer
TGGACTTTT:-1279	TGCCATATAAGGAGAGTTCAGAAAG	ATTGTCCCTGGACATTGGTG
GGGGTCTTC:-9501	TGATCTCATAAGGTTGGTGAACT	CAGGTGTATAGATATTTGGAAGGTTTC

ChIP, chromatin immunoprecipitation; NF-Kb, nuclear factor kappa B; DCN, decorin.

3.2.12 Statistical analysis

For most experiments, three to five biological replicates (a minimum of three) were used. SD was calculated to determine the level of uncertainty. Using GraphPad Prism software version 6, an unequal variances t-test or analysis of variance, followed by Tukey–Kramer post hoc comparisons was used to calculate significant differences between samples. A P-value of 0.05 or less was considered to be statistically significant.

3.3 Results

3.3.1 IL-1β stimulates DCN production

Many inflammatory cytokines are present in gestational tissues and studies in mutant mice show that some of them influence the course of pregnancy and the health of offspring (Ingman and Jones, 2008). Hence, we explored the effects of several ILs, known to play a role during pregnancy, on DCN production by HESC. THESC were cultured in serum free media with IL-6, IL-12, IL-15, IL-18 and IL-1β (10–50 ng/ml) for 24 h. Amongst these cytokines, IL-1β alone stimulated DCN production by the THESC (Fig. 3-1A and B). We found that neither pHESC nor THESC displayed any change in IL1B mRNA after decidualization *in vitro* (Fig. 3-1C and D), indicating that the effects of IL-1β on DCN production by HESC within the decidua are likely paracrine in nature. Since we had shown that DCN production increases during decidualization *in vitro* (Halari et al., 2020), we examined the mRNA expression of decidual markers (IGFBP1 and prolactin; PRL) after treating the cells with exogenous IL-1β for 24 h. We found no change in the expression of either marker (Fig. 3-1E and F) or in the morphology of cells after 24 h treatment (Fig. 1G). They maintained a fibroblastic morphology, rather than attaining a polygonal morphology typical of mature decidual cells. Cells treated with decidualizing agents (Ddlz) were used as a positive control for decidual markers. These findings confirmed that DCN upregulation after IL-1β treatment is not an indirect effect of decidualization by IL-1β.

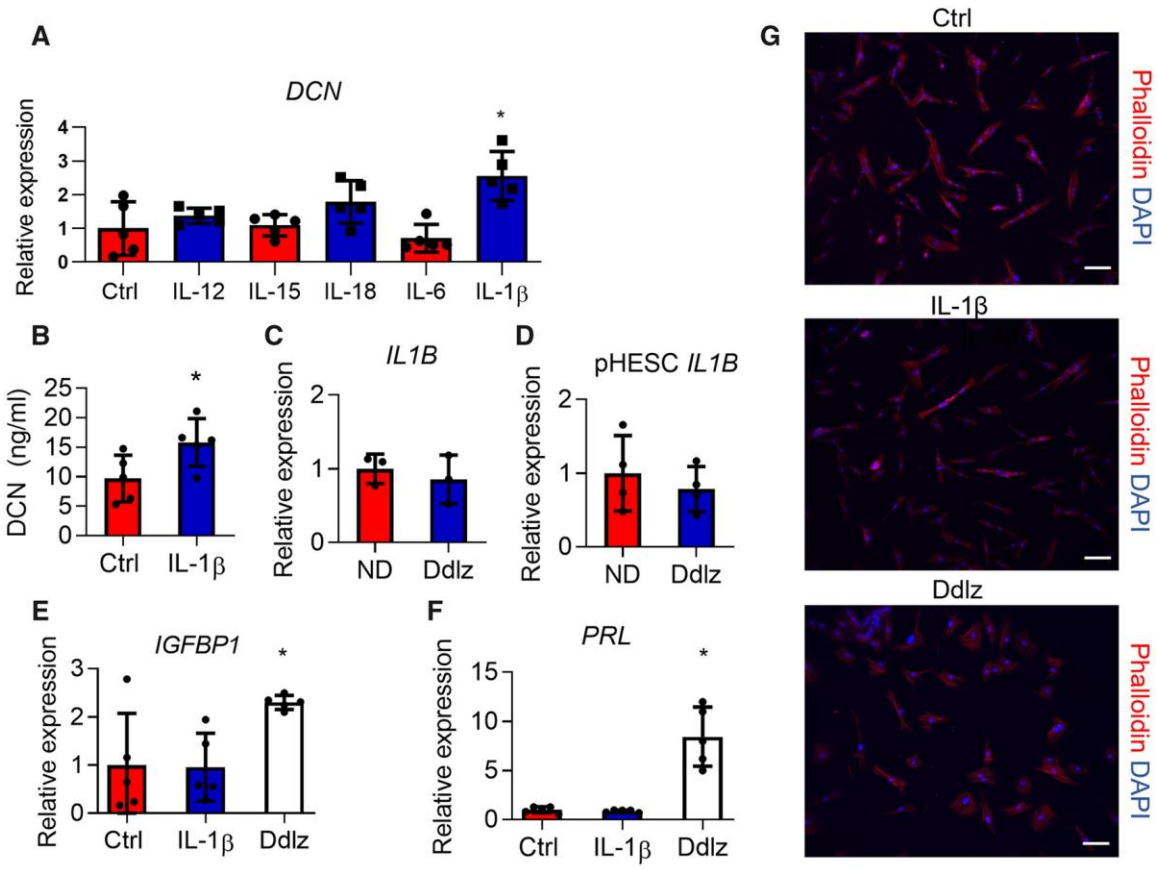

Figure 3-1: Interleukin-1 (IL-1)β stimulates decorin (DCN) expression in telomerse-immortilized human endometrial stromal cells (THESC).

(A) Effect of various cytokines on DCN expression in THESC in comparison to control (Ctrl) cells not exposed to cytokines. (B) IL-1β effect on DCN secretion as determined by ELISA. (C) IL1B expression in THESC and primary human endometrial stromal cells (pHESC) (D) subjected to decidualization *in vitro* using decidualizing agents for 6 days (decidualized, Ddlz) compared with non-decidualized controls (ND). Data for Insulin-like growth factor-binding protein (IGFBP)1 mRNA (E) and prolactin (PRL) mRNA (F) after treating THESC with exogenous IL-1β and decidualizing agents as positive control (Ddlz) for 24 h. (G) Morphology of Ctrl cells, IL-1β treated THESCs and decidualized cells (Ddlz) stained for phalloidin (red) and DAPI (blue). Data are expressed as means (n = 5 for (A), (B), (E), (F)), (n = 3 for (C)), (n = 4 for (D)) ±SD (*P < 0.05). Statistical analysis in (A) was performed using one-way ANOVA followed by Tukey–Kramer post hoc test. In (B–F), statistical analysis was performed using Student's t-test. Scale bars: 10 μm.

3.3.2 Identifying the cellular source of IL-1β in human decidual tissues

In order to identify the cellular source of IL-1β, we first conducted immunohistochemical examination of IL-1β and CD68 (macrophage marker) individually in first trimester decidual tissues (8, 9, 10 and 11 weeks) in semi-serial sections (shown for 9 weeks in Fig. 3-2). We found that macrophages were positive for IL-1β. Moreover, cytokeratin 7-positive endometrial glandular epithelia were also positive for IL-1β, indicating that these cells secrete IL-1β.

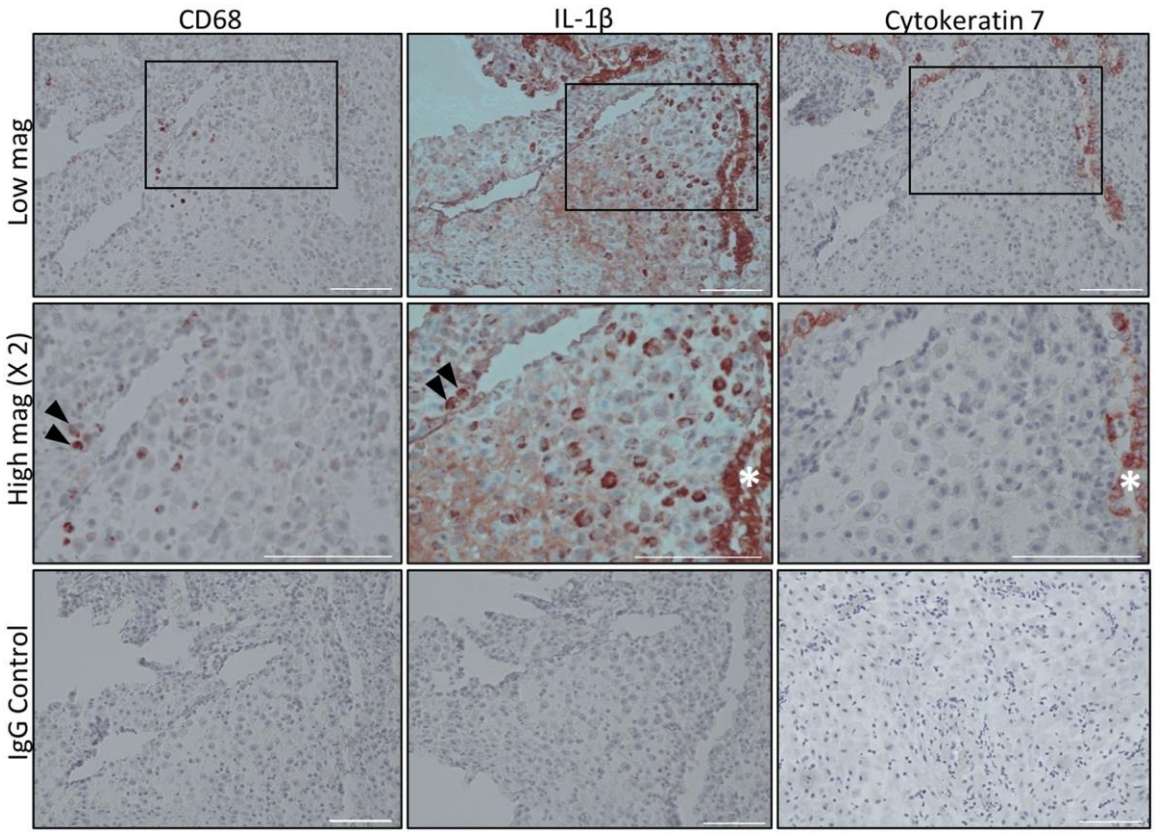

Figure 3-2: IL-1β is detectable in macrophages and decidual glands in first trimester decidual tissue.

Immunohistochemistry of paraffin-embedded first trimester decidual tissue sections (9 weeks) using primary antibodies specific for CD68 (macrophage marker), IL-1β, and cytokeratin 7 (demarcates glandular epithelia indicated with asterisk). Non-specific IgG (negative controls) used at the same concentration as corresponding primary antibodies are shown in the bottom panels. Arrowheads indicate cells that are CD68 and IL-1β positive. Scale bars: 100 μm.

To determine localization of IL1B mRNA in human decidua, we conducted *in situ* hybridization on human decidual tissue sections at 9 weeks of gestation (Fig. 3-3A and D). In addition, semi-serial sections were immunostained for CD68 (macrophage marker; Fig. 3-3B and E) and IGFBP1 protein (decidual cell marker; Fig. 3-3C and F) to identify macrophages and decidual cells, respectively. Expression of IL1B was detected in both IGFBP1 positive and CD68 positive cells within the decidua. Since we could not conduct dual immunostaining for these markers in the same section, we could not measure the incidence of double-positive cells. We suggest that decidual cells *in vivo* are either induced to express IL1B by other unknown mediators or binding IL-1β protein secreted by other non-decidual cells including decidual glands. Computation of the incidence of IL-1β+, CD68+ and IGFBP1+ cells (Fig. 3-3I) suggested that many cells were lacking any of the markers, and these cells likely belonged to stromal cells or other uterine immune cells

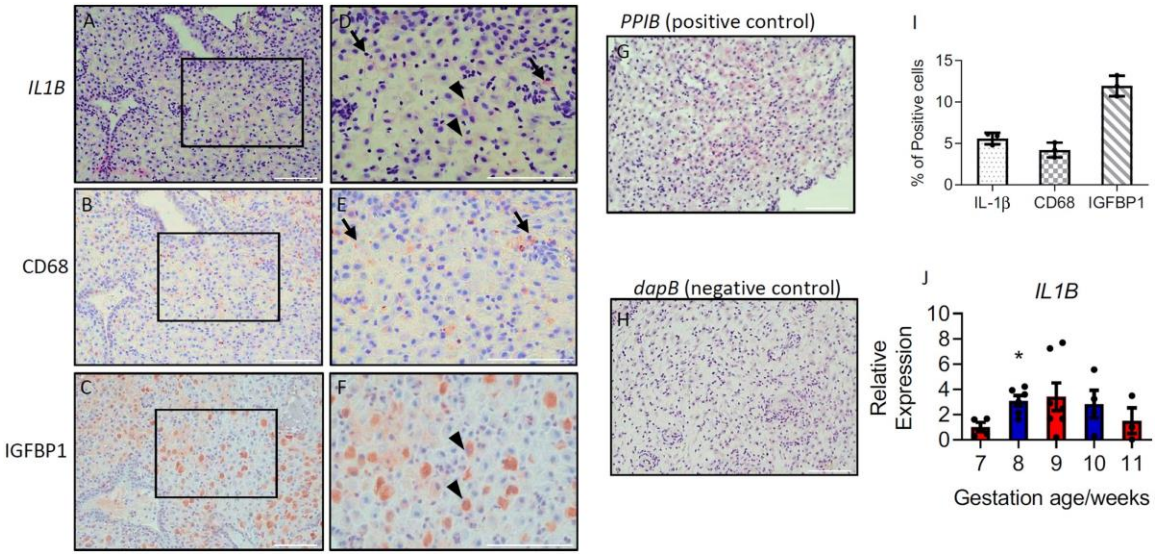

Figure 3-3: IL-1β is expressed in the decidua during early pregnancy.

(A) *In situ* hybridization of IL1B (red) in 9-week decidual tissue. Immunohistochemistry of CD68 (B) and IGFBP1 (C) using semi-serial sections of 9-week decidual tissue showing location of macrophages and decidual stromal cells, respectively. Please note that IL1B is expressed in cells corresponding to both macrophages and decidual stromal cells. Boxed inserts in (A–C) are shown as higher magnification images in (D–F). (G) Hybridization using a positive control probe (PPIB) and (H) negative control probe (dapB). (I) Incidence of cells expressing IL1B mRNA, CD68 protein and IGFBP1 protein in 9-week decidual tissue (J) IL1B expression relative to the geometric means of four housekeeping genes: GAPDH, RNA18SN1, EEF2 and YWHAZ in decidual tissues at 7–11 weeks. Data are expressed as means (of N = 3–6) ±SD (*P < 0.05). Statistical analysis was performed with one-way ANOVA followed by Tukey–Kramer post hoc test. Scale bars: 100 μm. Arrows in (D) and (E) show IL1B positive cells which are CD68 positive. Arrow heads in (D) and (F) show IL1B positive cells that are IGFBP1 positive.

We also measured gestational age-related changes in IL1B production by first trimester decidua *in vivo*, which to the best of our knowledge has not been previously reported. We quantified levels of IL1B mRNA in freshly isolated samples of 7–11 weeks decidua (n = 24). Expression levels (relative to the geometric means of GAPDH, RNA18SN1, EEF2 and YWHAZ) at different gestational ages (Fig. 3-3J) show a rise between 7 and 8 weeks, followed by no change between 8 and 11 weeks. This trend is very similar to the kinetics of DCN production in the decidua reported earlier (Halari et al., 2020).

3.3.3 Role of IL-1R in IL-1β induced DCN production by HESC

Since IL-1β action is mediated by IL-1R, we used immunofluorescence to confirm that both pHESC and THESC express IL-1R1 protein (Fig. 3-4A). Next, we tested if blocking IL-1R1 in THESC inhibits IL-1β-induced DCN production. Specifically, we measured DCN production (secreted protein) by THESC in the presence of IL-1RA at blocking concentrations (100–300 ng/ml). This antagonist binds to IL-1 receptors in competition with IL-1, but does not elicit an intracellular response from this binding. Cells were primed with IL-1RA for 1 h, followed by IL-1β (10 ng/ml) treatment for 24 h. IL-1RA alone (at 200 ng/ml) did not alter DCN production, but IL-1RA blocked IL-1β-induced DCN production at all doses tested (Fig. 3-4B). These findings confirm that IL-1β mediated upregulation of DCN in THESC is mediated through IL-1R1.

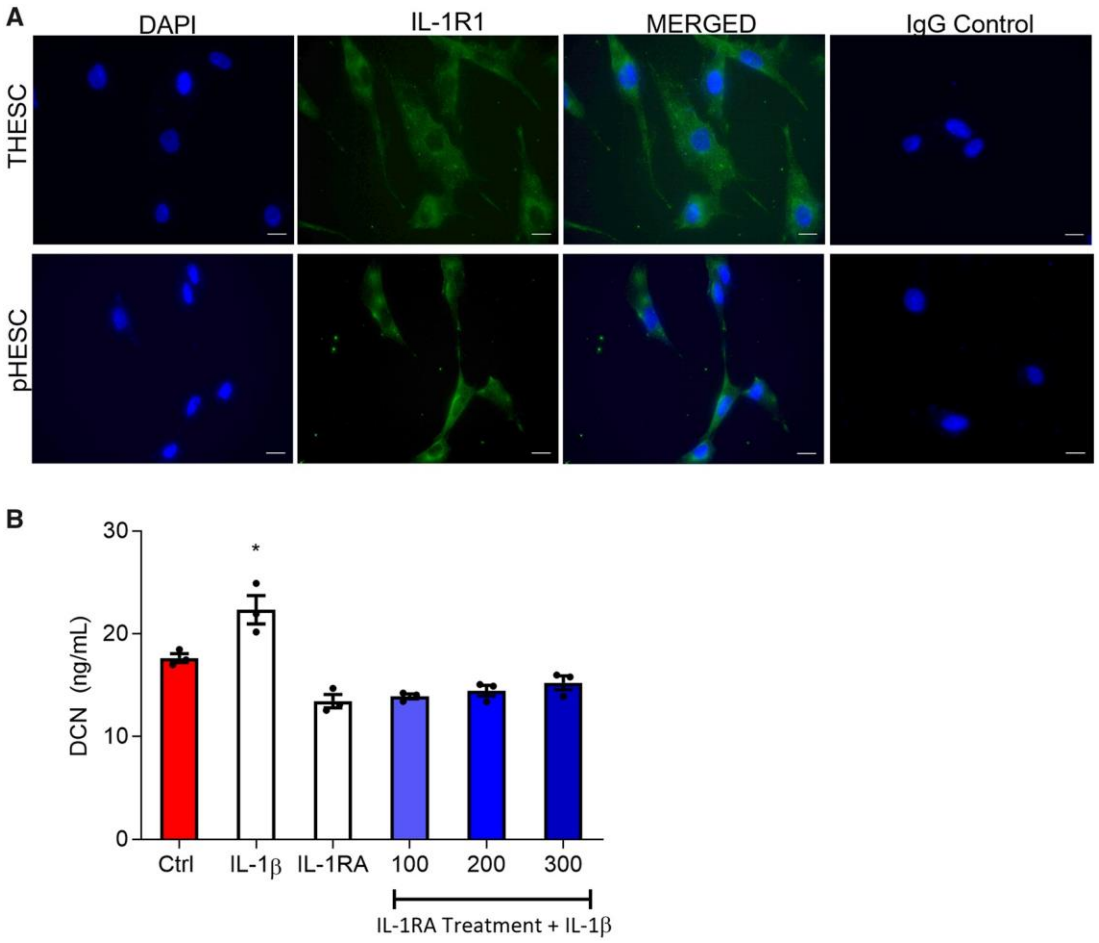

Figure 3-4: IL-1R is expressed by PHESC and THESC and mediates IL-1β-induced DCN production.

(A) PHESC and THESC immunostaining with IL-1R1 (green). Nuclei were counterstained with DAPI (blue). (B) DCN secretion following exposure of THESCs to IL-1RA (100–300 ng/ml) for 1 h, followed by treatment with IL-1β for 24 h. Data represent means ± SD, N = 3 (*P < 0.05). Statistical analyses were performed with one-way ANOVA followed by Tukey–Kramer post hoc test. Scale bars: 10 μm.

3.3.4 IL-1β-induced DCN production is dependent on NF-κB pathway

Next, we measured IL-1β-induced DCN production by HESC in the presence/absence of inhibitors of well-known IL-1β signaling pathways (P38, ERK and NF-κB). THESC were cultured with these inhibitors in serum free media for 1 h and then media were replaced with fresh media containing IL-1β for 24 h. DCN (secreted protein) was measured. DCN protein was significantly reduced after exposure of THESC to NF-κB inhibitor alone for 1 h followed by IL-1β treatment for 24 h, when compared with cells pretreated with vehicle (Fig. 3-5A). Inhibition of P38-MAPK or ERK had no effect on IL-1β-induced DCN production. Hence, subsequent experiments focused on the NF-κB pathway. Phosphorylation of NF-κB protein p65 was rapidly increased in THESC exposed to IL-1β, confirming that IL-1β activates NF-κB in these cells (Fig. 3-5C; Figs 3-S1 and S2). This was further validated by immunostaining for p65 and noting its transient translocation to nuclei stained with DAPI (Fig. 3-5D). To determine if IL-1β promotes NF-κB transcriptional activity, we used a construct containing a promoter with NF-κB binding sites upstream of a luciferase reporter gene. This construct was then transfected into THESC and cells were treated with IL-1β for up to 4.5 h. The levels of luminescence increased by more than 2-fold within 2 h after treatment with IL-1β (Fig. 3-5E). Lipopolysaccharide was used as positive control for NF-κB activation.

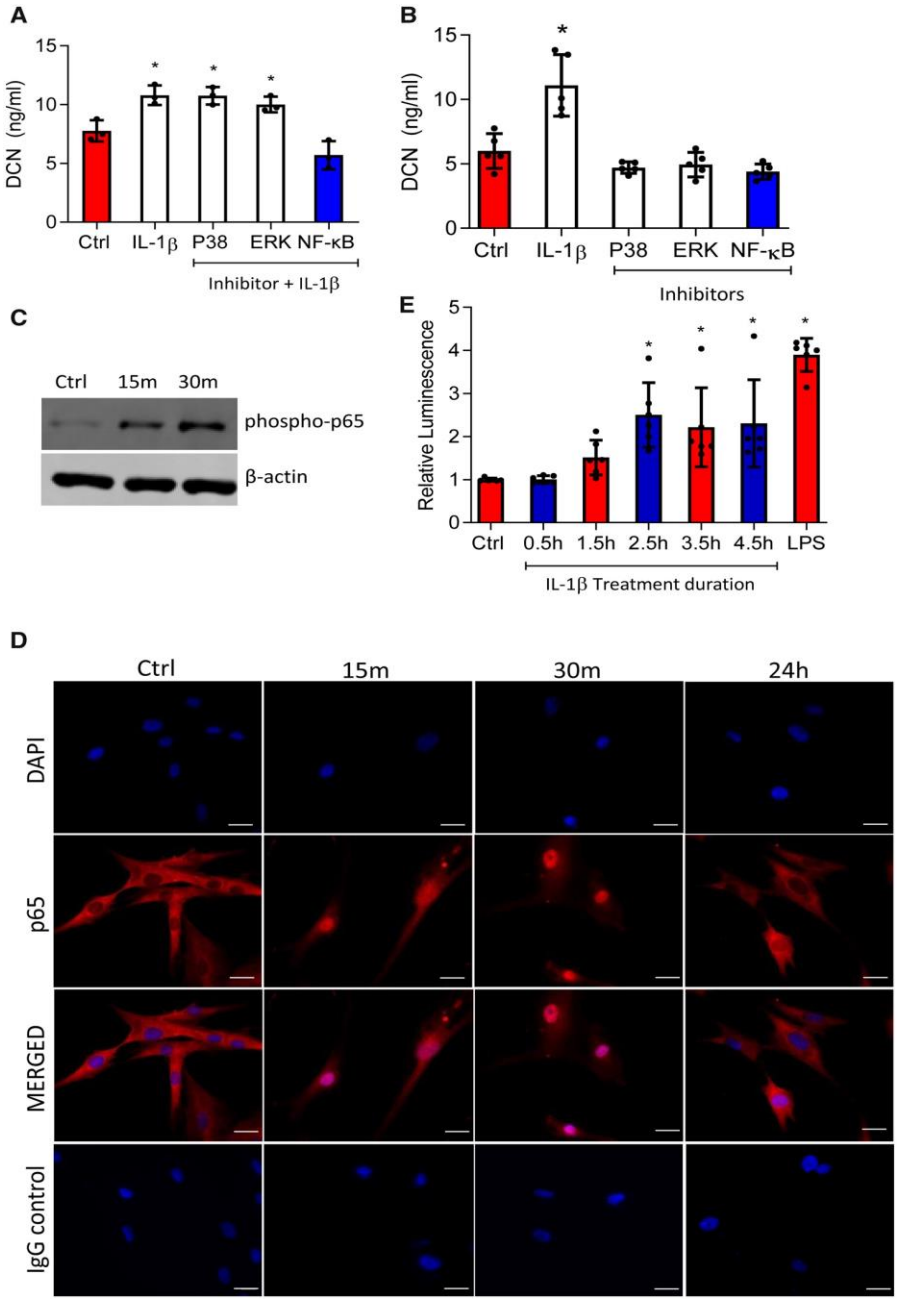

Figure 3-5: Activation of Nuclear Factor Kappa B (NF-κB) promotes DCN production following exposure of THESCs to IL-1β.

(A) DCN secretion by THESCs exposed to IL-1β following pretreatment with pharmacological inhibitors of NF-κB, P38-Map kinase (MAPK) or MAPK kinase (MAPKK or MEK)/extracellular signal-regulated kinase (ERK). (B) DCN secretion by THESCs exposed to pharmacological inhibitors alone. Control (Ctrl) cells were exposed

to DMSO only. (C) Western blot showing phospho-p65 NF-κB protein after 15 min (15 m) or 30 min (30 m) IL-1β treatment compared with THESCs not exposed to IL-1β (Ctrl). (D) Immunofluorescence of p65 NF-κB (red) following IL-1β treatment for 15 min (15 m), 30 min (30 m) or 24 h. Note that rapid and transient nuclear translocation of p65 NF-κB following exposure to IL-1β. Nuclei were counterstained blue using DAPI. (E) Relative luciferase activity in THESC transfected with NF-κB-Luc and treated with IL-1β for up to 4.5 h. Lipopolysaccharide (LPS) was used as a positive control for NF-κB activation. Data are presented as means ± SD, N = 3–5 (*$P < 0.05$). Statistical analyses were performed with one-way ANOVA followed by Tukey–Kramer post hoc test. Scale bars: 10 μm.

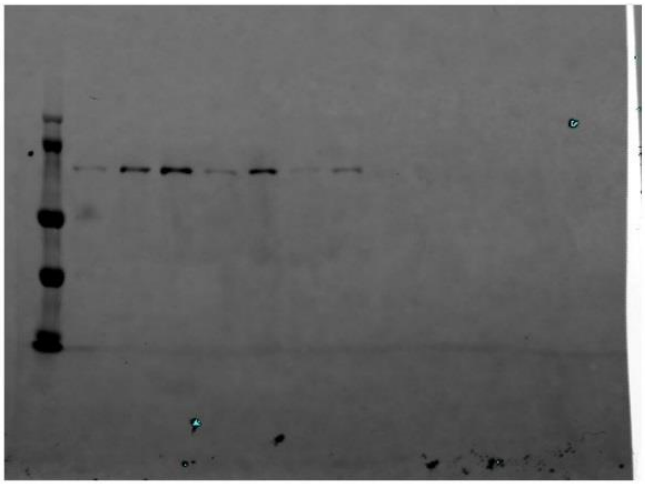

Figure 3-S 1: Uncropped western blot for p-P65 corresponding to fig 3-5 C showing ladder on the left side.

First, second and third band on the right corresponds to Ctrl, 15minutes and 30minutes of IL-1β treatment respectively.

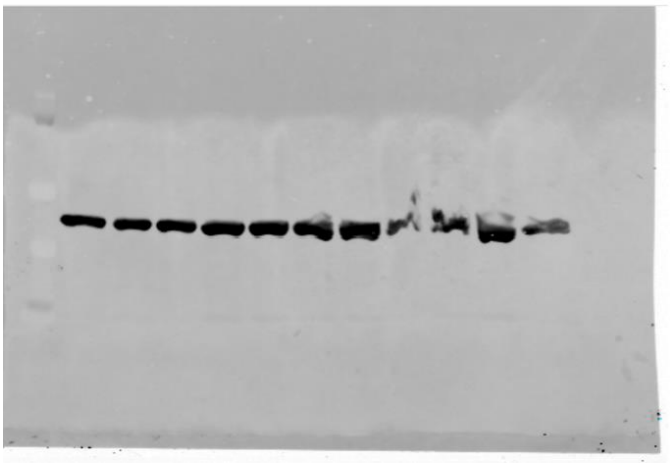

Figure 3-S 2: Uncropped western blot for β actin corresponding to fig 3-5 C showing ladder on the left side.

3.3.5 NF-κB binds upstream of DCN

NF-κB binds to DNA at the consensus sequence 'GGGRNYYYCC'. Through in silico analysis of the promoter region of DCN, we found two potential NF-κB binding sites: TGGACTTTT and GGGGTCTTC, which are 1279 and 9501 bp upstream of the DCN transcription start site, respectively (Fig. 3-6A). We performed ChIP to determine whether NF-κB binds to these sites after THESC are exposed to IL-1β for 30 min. IKBA has multiple well-characterized NF-κB DNA-binding sites (Nowak et al., 2005; Cookson et al., 2015) and we used commercially available IKBA ChIP primers that bind to one of these sites, as a positive control. As expected, NF-κB displayed robust binding upstream of IKBA and also bound strongly to the putative binding site 1279 bp upstream of DCN, but not at the site 9501 bp upstream of DCN (Fig. 3-6B and C; Figs 3-S3 and S4). Therefore, NF-κB binding 1279 bp upstream of DCN likely contributes to IL-1β-mediated stimulation of DCN production.

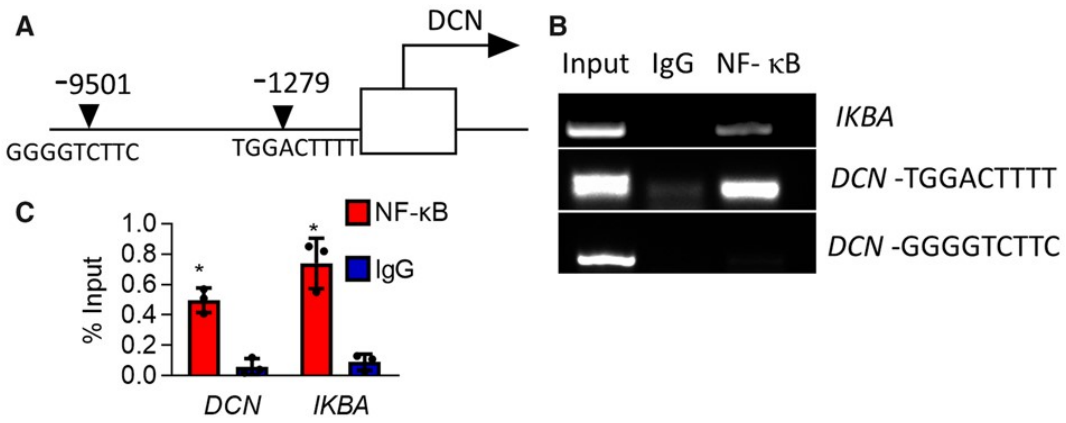

Figure 3-6: NF-κB binds upstream of the DCN gene.

Chromatin immunoprecipitation (ChIP) was performed using NF-κB or IgG (negative control) antibodies in lysates of THESC treated with IL-1β. (**A**) Schematic showing two potential NF-κB-binding sites upstream of the DCN transcription start site. (**B**) Determination of NF-κB or IgG-binding upstream of I-kappa-B-alpha (*IKBA*) (positive control) and *DCN* at two binding sites (DCN–TGGACTTTT and DCN–GGGGTCTTC). Input indicates total DNA. (**C**) Quantitative PCR showing enrichment of NF-κB or IgG using primers flanking regions upstream of *IKBA* and *DCN* (TGGACTTTT), presented as a percentage of Input DNA. No binding was observed at the GGGGTCTTC sequence 9501 bp upstream of DCN (data not shown). Statistical analysis was performed using Student's *t*-test. Data represent means ± SD, N = 3, (*$P < 0.05$).

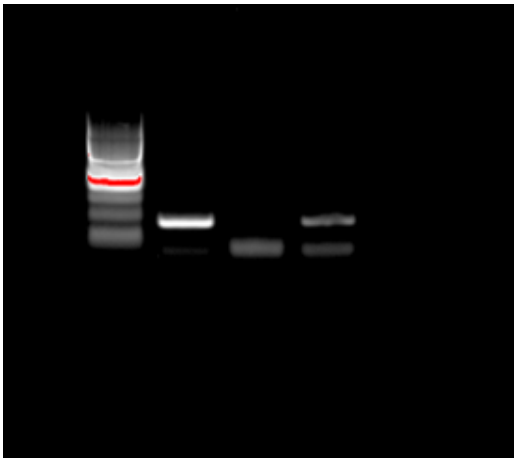

Figure 3-S 3: Uncropped IKBA ChIP gel corresponding to fig 3-6 B showing ladder on the left side and Input, IgG and NF-κB band on the right.

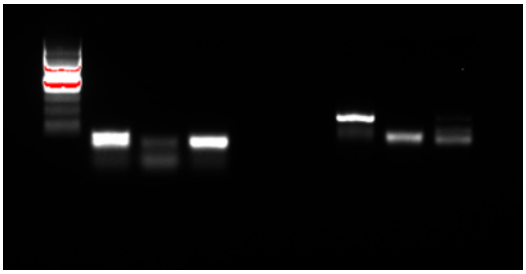

Figure 3-S 4: Uncropped DCN ChIP gel corresponding to fig 3-6 B showing ladder on the left side and Input, IgG and NF-κB for DCN at 2 binding site (TGGACTTTT on the left) and GGGGTCTTC on the right).

3.4 Discussion

The present study revealed that treating with exogenous IL-1β alone does not induce decidualization in THESC, but does so in the additional presence of decidualizing agents as early as 24 h. Studies in primates suggest a role of IL-1β in decidualization (Strakova et al., 2000). These authors found that IL-β response was hormone-dependent. However, if IL-1β and dbcAMP were added together, IGFBP-1 expression was inhibited. Frank et al. (1995) suggested that decidual IL-1β inhibits PRL and IGFBP-1 expression through an autocrine/paracrine mechanism. Mizuno et al. (1999) showed that exogenous IL-1β inhibits cAMP induced decidualization in HESC. Taken together, these findings suggest that decidualization is a complex process involving a molecular network of positive and negative regulators (Gellersen and Brosens, 2014).

We have shown that decidua-derived DCN plays an important role in successful pregnancy by virtue of its paracrine effects in controlling trophoblast proliferation, differentiation, and invasion of the decidua and uterine spiral arteries and autocrine effects in maturation of HESC into decidual cells. Furthermore, decidual overproduction of DCN is associated with PE. Thus, DCN production by the decidua must be exquisitely controlled to maintain feto-maternal health. The present study revealed that IL-1β is a key mediator of DCN production by the decidua and that the cellular sources of IL-1β in first trimester decidual tissue are macrophages and decidual cells. The extent of contribution of either cell class remains undetermined. We further demonstrated that IL-1β mediated DCN production is dependent on IL-1R1 activation followed by NF-κB activation and binding of NF-κB to the DCN promoter. Our findings of the stimulatory role of IL-1β in DCN production by the decidua suggest that increased IL-1β levels reported in PE subjects (Kalinderis et al., 2011) may contribute to DCN overproduction by PE-associated decidua (Siddiqui et al., 2016). Interestingly, another pro-inflammatory cytokine IL-6 has been reported to be elevated in the sera of PE subjects (Kalinderis et al., 2011) raising the possibility that IL-6 may upregulate DCN or IL-1β or both in HESC. However, we excluded the role of IL-6 in promoting DCN production (Fig. 1) or IL-1β production (data not shown) by HESC. Thus, the role(s) of IL-6 in PE are likely independent of DCN and IL-1β. Since both IL-1β and

DCN are upregulated in PE, our findings of molecular pathways of DCN upregulation in HESC may potentially be exploited in the future for prevention or intervention of PE.

We have confirmed that both pHESC and THESC express IL-1R1 (the signaling receptor for both IL-1α and IL-1β) and that IL-1β-mediated DCN production is IL-1R1 dependent. Downstream signaling of IL-1β is usually mediated by several pathways including P38-MAPK, ERK and NF-κB (Bankers-Fulbright et al., 1996; Srinivasan et al., 2004). In the case of DCN upregulation by IL-1β we have excluded the roles of P38-MAPK and ERK, and shown that NF-κB plays an obligatory role. We found two potential NF-κB-binding sites upstream of the DCN transcription start site, and our results with ChIP assay confirmed strong NF-κB binding at one of these sites.

In this study, we have focused on the molecular mechanisms in IL-1β-mediated DCN production by the HESC. Many inflammation-associated cytokines and other stimuli besides IL-1β can exert their actions via NF-κB activation, and it is possible that these other factors may similarly promote DCN expression. On the other hand, NF-κB activity can also induce some cytokines such as IL-1β, IL-6, IL-12 and TNF-α, to perpetuate the inflammatory response (Liu et al., 2017). While we have excluded the roles of some cytokines such as IL-6 and IL-12 in DCN induction in the HESC, roles of others such as IL-11 and TNF-α remains to be tested. IL-11 was shown to be one of the cytokines needed for decidualization and its deficiency is associated with infertility (Karpovich et al., 2005).

In summary, this is the first study reporting IL-1β-mediated DCN upregulation in HESC, via IL-1R1/NF-κB pathway, unveiling a novel mechanism in DCN production by HESC. DCN exerts multiple essential roles in pregnancy including an autocrine role in decidual cell maturation from HESC (Halari et al., 2020). IL-1R-null mutation in mice caused reduced litter size in females but had no effect on male fertility (Abbondanzo et al., 1996). These mice exhibited no implantation defect, indicating that impaired fertility in these mice may be due to a decidual maturation defect resulting from local DCN deficit. Given the integral role of DCN in regulating decidualization and placentation, our data implicating IL-1β as a key regulator of DCN production provides a potential link between inflammation and increased DCN production in various pregnancy pathologies.

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Chapter 4

4 Roles of Decorin-induced microRNAs 512-3p and let-7c-5p in Trophoblast Functions Disrupted in Preeclampsia

4.1 Introduction

Preeclampsia (PE) is a serious pregnancy specific-disorder that affects around 5% of pregnancies worldwide and is characterized by new onset hypertension and (typically) proteinuria after 20 weeks of gestation (Gao et al., 2018). If left untreated, PE may lead to multisystem organ damage such as renal failure, pancreatitis and hemolytic anaemia (Sheikh et al., 2016). PE ise one of the leading causes of maternal and perinatal morbidity and mortality (Duley, 2009). It has long been recognized that pathogenesis of PE lies within the placenta because removal of the placenta eradicates the clinical manifestations of PE (Zhu et al., 2009; Chen et al., 2013).

During normal placental development, trophoblast stem cells contained within the cytotrophoblast (CTB) layer of the chorionic villi differentiate into two subpopulations: syncytiotrophoblast (STB) and extra-villous trophoblast (EVT). STB arises by cell fusion and EVT as migratory cell columns. EVT cells proliferate at the villus base and invade uterine decidua and spiral arteries (Lala et al., 1996; Knöfler et al., 2013). Some of the EVT cells undergo endothelial-like (endovascular) differentiation to invade and remodel distal segments of the arteries into low-resistance tubes that allow steady flow of maternal blood for fetal nourishment (Kaufmann et al., 2003; Cartwright et al., 2010). While both EVT and STB differentiation pathways are dysregulated in PE, poor EVT invasion is thought to be the root cause of various pathological manifestations such as defective uterine arterial remodeling and resulting in a hypo-perfused placenta (Meekins et al., 1994, Lala et al., 2003, Kaufmann et al., 2003, Burton et al., 2009, Cartwright et al., 2010). A hypoxic placenta releases toxic lipid peroxides and inflammatory chemokines into maternal circulation, leading to maternal vascular damage in multiple organs (Roberts and Lain, 2002). Currently, there is no known cure for PE except delivering the fetus and the placenta (Backes et al., 2011). However, premature delivery increases perinatal morbidity and mortality rates (Backes et al., 2011). As such, further research to understand the

mechanisms underlying PE and to identify the novel biomarkers for its early detection is urgently needed.

Decorin (DCN), a leucine rich-proteoglycan produced by various mesenchymal cells including decidual stromal cells, is overexpressed by the PE-associated decidua, as shown by *in situ* hybridization for mRNA and immuno-localization of the protein (Siddiqui et al., 2016). Additionally, plasma DCN levels in second trimester patients are elevated in PE compared to control (non-PE) patients matched for body-mass index (Siddiqui et al., 2016). DCN controls EVT cell proliferation, migration, invasion (Xu et al., 2002; Iacob et al., 2008) and endovascular differentiation (Lala et al., 2012), events needed for uterine spiral artery remodeling (Kaufmann, 2003; Lala and Nandi, 2016). Furthermore, DCN is a key regulator of human trophoblast stem cell self-renewal and differentiation (Nandi et al., 2018) andplays an autocrine role in decidual cell differentiation from human endometrial stromal cells (HESC) (Halari et al., 2020, Chapter 2). These findings reveal that a balanced DCN production by the decidua is essential for healthy pregnancy. Decreased DCN production may result in decidual maturation defects, whereas increased levels may result in compromised trophoblast functions associated with PE.

MicroRNAs (miRNAs) are single-stranded non-coding RNAs composed of approximately 17-25 nucleotides that typically bind to the 3′ untranslated region of target mRNAs to facilitate degradation (Dexheimer and Cochella, 2020). The human placenta expresses numerous miRNAs, many of which are exclusively expressed by trophoblasts and are not expressed by other normal human tissues (Baek, 2008). These placenta-specific miRNAs are largely clustered in three groups: chromosome 14 miRNA cluster (C14MC), chromosome 19 miRNA cluster (C19MC), and miR-371-373 cluster (Miura et al., 2010; Morales-Prieto et al., 2012). Expression of miRNAs from these clusters changes throughout pregnancy and differs among placentas from patients with preterm labor compared to those in normal term pregnancies (Mayor-Lynn et al., 2011). In addition, some of these placenta-specific miRNAs have been detected in the maternal circulation throughout gestation with significant decrease after delivery (Miura et al., 2010; Kotlabova et al., 2011). Many microRNAs (miRNAs) are dysregulated in PE-associated placentas (Ishibashi et al., 2012; Hong et al., 2014; Xu et al., 2021) and some may appear in the

maternal blood as PE biomarkers (Munaut et al., 2016; Jelena et al., 2020; Xu et al., 2021). These observations, in addition to recent discoveries surrounding miRNA regulation of EVT invasion and migration, suggest that miRNAs play key roles in the process of PE development (Fu et al., 2013; Gao et al., 2018). Furthermore, maternal circulating miRNAs may have the potential to be novel diagnostic agents for pregnancy disorders (Xu et al., 2021). MiR-512-3p is part of the C19MC (Prieto et al., 2011) and multiple studies have reported upregulation of miR-512-3p in PE patients (Martinez-Fierro et al., 2018; Wang et al., 2012; Martinez-Fierro et al., 2021). A recent study showed elevated level of this miR-512-3p at 20 weeks of gestation in the serum of women who later developed severe PE (Martinez-Fierro et al., 2021). Another miRNA, let-7c-5p is also upregulated in PE (Caldeira-Dias et al., 2018) but little is known about the contribution of this miRNA to the pathogenesis of PE.

The objective of the present study was to determine whether exposure of EVTs to DCN affects expression of specific miRNAs, and to test the role of these miRNAs in altering EVT functions. Our results identified two DCN-upregulated miRNAs let-7c-5p and miR-512-3p, which were also upregulated in PE. Both miRNAs were shown to be elevated in ectopic DCN-over-expressing or exogenous DCN-treated first trimester human trophoblast cell line HTR-8/SVneo. They variably compromised trophoblast cell migration, invasion and VEGF-dependent endovascular differentiation. Finally, *PPP3R1,* a known target of miR-512-3p, was paradoxically elevated in miR-512-3p overexpressing trophoblast cells and PE-associated placentas. *PPP3R1* elevation appears to be intermediated by a transcription factor USF2. No target could be validated for miR-let-7c-5p.

4.2 Material and Methods

4.2.1 Cell line and culture

HTR-8/SVneo, originally derived from immortalization of EVTs derived from explant outgrowths (Graham et al., 1993), and commonly used as a model of invasive EVTs, were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μM streptomycin. Cells were passaged via light trypsinization prior to

reaching confluency and were maintained at 37ºC in an atmosphere consisting of 5% CO2 for no more than twenty sequential passages.

4.2.2 Placenta sample collection

Flash-frozen placenta samples collected from normotensive and preeclampsia were obtained from the Research Centre for Women's and Children's Health Biobank (RCWIH, Mount Sinai Hospital, Toronto, Canada, http://biobank.lunenfeld.ca). All samples were collected from caesarean section deliveries with informed consent, and were approved by the Mount Sinai Hospital and University of Western Ontario research ethics boards. The details of the subjects including the gestational age of the pregnancies and parameters used to define preeclampsia have been reported by Jeyarajah et al., 2019.

4.2.3 Transfection with miRNA-mimics and inhibitors

Prior to transfection, HTR-8/SVneo cells were plated in a 12-well plate and grown to 70-80% confluency. Following the removal of culture media, the cells were incubated with 800 μL/well Opti-MEM media (supplemented with 7.5% FBS) for 1 h. Cells were then treated with Lipofectamine 2000 RNAiMax (Invitrogen,2.5 μL Lipofectamine/100 μL of Opti-MEM media) and oligonucleotides (2 μg/well) and incubated for 4 h. Subsequently, cells were washed two times with RPMI-1640 complete media and incubated overnight. Successful transfection was verified by determining miRNA levels in cells 24 h after exposure to lipofectamine. MiRNA overexpression and knockdown were achieved via transfection with miRIDIAN miR-512-3p mimic (Dharmacon, cat no. C-300769-03-0005), miR-512-3p Inhibitor (Dharmacon, cat no. IH-300769-05-0005), miRNA let-7c-5p mimic (Dharmacon, cat no. C-300477-03-0005) and miRNA let-7c-5p hairpin inhibitor (Dharmacon, cat no. IH-300477-05-0005). Controls for overexpression and knockdown experiments consisted of transfection with miRIDIAN™ Dharmacon mimic (Dharmacon, cat no. CN-001000-01-05) and inhibitor (Dharmacon, catno. IN-001005-01-05),

respectively. The sequences of these control oligonucleotides do not target any known mammalian transcript.

4.2.4 SiRNA-mediated PPP3R1 knockdown

Transient transfection of PPP3R1 siRNA oligomers (Thermofisher, cat no. AM16708) and negative control (Thermofisher, cat no AM4641) was carried out using lipofectamine 2000 RNAiMax (Invitrogen, Thermo Fisher). All plasmid transfections (1 μg) were carried out similar to the protocol described above in miRNA transfection. Successful transfection was verified by determining *PPP3R1* mRNA levels in cells 24 h after transfection using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Experiments with PPP3R1 knockdown cells were conducted within 3 days of transfection.

4.2.5 MiRNA extraction from HTR-8/SVneo cells

MiRNA was extracted from HTR-8/SVneo cells using the miRNeasy Kit (Qiagen) according to the manufacturer's instructions (Qiagen, 2020). Briefly, HTR-8/SVneo cells were lysed using 700 μL of TRIzol (Thermofisher) and collected in 1.5 mL Eppendorf tubes. The resulting lysate was treated with 140 μL of chloroform, agitated for 15 seconds, and centrifuged at 12,000 rpm for 15 min (4°C). The upper aqueous phase was then transferred to a new collection tube and mixed in a 1:1 ratio with 70% ethanol solution. The resulting mixture was then put through the RNeasy minielute spin column (Qiagen, cat no. 1026497) in increments of 700 μL and centrifuged at 10,000 rpm for 1 minute (25°C). Flowthrough was collected, mixed with 0.65 volume of 100% ethanol, and passed through the miRNeasy column at 10,000 rpm for 1 minute (25°C) (Qiagen, cat no. 74104) to isolate miRNA. Once done, the resulting flowthrough was discarded and the isolated miRNA was washed in two steps: 500 μL of RPE buffer (Qiagen) was added, the column was centrifuged at 10,000 rpm for 1 min (25°C) and flow through was discarded; 500 μL of 80% ethanol was added, the column was centrifuged at 10,000 rpm for 1 minu (25°C), and flowthrough was discarded. The column was then centrifuged at 10,000 rpm for 5

minutes (25°C) to dry. Lastly, the column was transferred to a new 1.5 mL Eppendorf collection tube, 12 µL of RNase-free water (Qiagen) was added, and the new tube was centrifuged at 10,000 rpm for 2 minu (25°C). Following purification, the sample concentration and 260/230-absorbance purity ratio were quantified using the Epoch/Take Multi-Volume Spectrophotometer System (BioTek Instruments, Inc.).

4.2.6 MiRNA extraction from placental tissue samples

MiRNA was extracted from placental tissue samples using the miRNeasy Kit (Qiagen) according to manufacturer's instructions (Qiagen). Briefly, a small amount of flash-frozen tissue was placed in a 15 mL collection tube with 700 µl of TRIzol® (Invitrogen Life Technologies) and homogenized for 40 seconds using the Omni TH Tissue Homogenizer (Omni International, Inc.). The tube was then left to sit at room temperature for 5 min to promote nucleoprotein complex dissociation. After 5 min, 140 µl chloroform was added to the tube and the isolation proceeded with the same protocol as the HTR-8/SVneo cellular miRNA extraction mentioned above.

4.2.7 cDNA synthesis from miRNA

Purified miRNA was utilized for cDNA synthesis using the qScript miRNA cDNA Synthesis Kit (Quantabio) according to manufacturer's instructions (Quantabio, 2020). Briefly, 2 µL of Poly(A) Tailing Buffer (5X), 1 µL of Poly(A) Polymerase, and 7 µL of 500 ng/µL purified miRNA solution were added to a PCR tube to give a final volume of 10 µL. The resulting mixture was then incubated in the C1000 thermal cycler (Bio-Rad) at 37°C for 60 min followed by incubation at 70°C for 5 min. Once finished incubating, 9 µL of miRNA cDNA Reaction Mix and 1 µL of qScript Reverse Transcriptase were added to the PCR tube to give a final volume of 20 µL. The resulting mixture was then placed back in the Thermal Cycler at 42°C for 20 min, followed by incubation at 85°C for 5 min. The resulting cDNA was stored at –20°C prior to qRT-PCR analyses.

4.2.8 Reverse transcription-quantitative polymerase chain reaction

qRT-PCR was performed using the qScript miRNA cDNA Synthesis Kit (Quanta bio, cat no. 95107-025) according to manufacturer's instructions. Briefly, 2 µL of 500 ng/µL cDNA sample (diluted 1:10) was added to PCR tubes containing 18 µL of master mix (10 µL PerfeCTa SYBR Green SuperMix, 0.4 µL Custom miRNA Assay Primer (2 µM), 0.4 µL PerfeCTa Universal PCR Primer (10 µM), 7.2 µL Nuclease-Free Water). Samples were then run through the Rotor-Gene 3000 (Corbett Research) thermal cycler using custom designed primers (Table 4-1, 4-2). Cycling conditions involved initial holding step (95°C for 13 min), followed by 45 cycles of a two-step PCR (95°C for 15 s and 60°C for 60 s) and a dissociation phase. Fold-changes in miRNA and mRNA expression in treated samples compared to control samples were calculated using the 2-ΔΔCt method. *RNU6* was used as a reference miRNA and the geometric mean of *GAPDH* and 18S rRNA (*RNA18SN1*) as reference RNA for HTR-8/SVneo and placental tissue samples.

Table 4- 1: mRNA primer sequences used for qRT-PCR

Gene	Forward primer	Reverse primer
PPP3R1	GAGGGCGTCTCTCAGTTCAG	GCTGGACGTCTTGAGCAGAT
USF2	AATGGAGGACAGACAGGAACAC	CTCCTTTACTCGCTCCCGTC
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
RNA18SN1	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA

Primers for quantitative real-time polymerase chain reaction (qRT-PCR): PPP3R1, Protein Phosphatase 3 Regulatory Subunit B, Alpha; USF2, Upstream Transcription Factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RNA18SN1, 18S ribosomal N1.

Table 4- 2: miRNA primer sequences used for qRT-PCR

MiRNA	Forward primer
miR-512-3p	5’GTGCTGTCATAGCTGAGGTCAA3’
miR-512-5p	5’CTCAGCCTTGAGGGCACTTT3’
miR-195-3p	5’GGCTGTGCTGCTCCAAAA3’
miR-195-5p	5’GCTAGCAGCACAGAAATATTGG3’
miR-18b-3p	5’GCCCTAAATGCCCCTTCTAAA3’
miR-18b-5p	5’GGTGCATCTAGTGCAGTTAGAAAA3’
miR-363-3p	5’CAATTGCACGGTATCCATCTG3’
miR-363-5p	5’GGGTGGATCACGATGCAAT3’
miR-374a-3p	5’CGCGCTTATCAGATTGTATTGT3’
miR-374c-5p	5’CATAATACAACCTGCTAAGTGCTAAAA3’
miR-155-3p	5’GCTCCTACATATTAGCATTAACAAAAA3’
miR-155-5p	5’TGCTAATCGTGATAGGGGTAAA3’
let-7c-5p	5’-CCGAGCTGAGGTAGTAGGTTGTATG-3’
U6	5’GCAAATTCGTGAAGCGTTCC3’

4.2.9 Wound healing assay

To measure migration, we used wound-healing assay. Cells were treated with mitomycin C (500 ng/mL, Sigma, cat: M4287) for 1 h to block cell proliferation, scratched multiple times (8 linear scratches each, vertically and horizontally) in the absence or presence of exogenous DCN (250nM, a concentration previously shown to have the highest anti-migratory effect, Sigma, cat: D8428) for 24 h. The wound area was recorded using light microscopy (Leica Microsystems) at 0 and 24 h. Migration was recorded as the percentage wound closure at 24 h. To calculate the area of the wound, images were imported into ImageJ (version 1.5.3), where cell frontiers bordering the wound were traced. The percentage of wound closure was determined using the following equation: [(A0 – A24)/A0] × 100%, where A0 represents the initial area of the wound at 0 h and A24 represents the area of the wound after incubating for 24 h.

4.2.10 Transwell migration and invasion assays

A transwell migration assay was performed using a 24 well plate and transwell inserts (Corning, CLS3464) containing microporous (8 μm pores) membranes. 40,000 cells were resuspended in serum free media, placed on top of each transwell, and allowed to migrate through the microporous membrane for 24 h towards the complete FBS-containing media. After 24 h, non-migratory cells on top of the membrane were removed using a cotton bud and membranes were stained with hematoxylin and eosin. Once dried, membranes were imaged at 40x total magnification using the Leica Inverted Light Microscope and cells were counted using ImageJ software. For the invasion assay, transwells were coated with a thin layer of matrigel (BD Biosciences, 400 μg/ml diluted in serum free RPMI-1640 medium) for 4 h before performing the assay same as above.

4.2.11 Spheroid invasion assay

This assay allows one to quantify the invasion of HTR-8/SVneo cells into Matrigel at various time points (Siddiqui et al., 2016). Spheroids were formed using 24-well

AggreWell 800 plates (Stemcell Technologies) containing 800 µm microwells according to manufacturer's protocol (Stemcell Technologies, 2017). Briefly, AggreWell 800 plate wells were pre-treated with 500 µL of Anti-Adherence Rinsing Solution (Stemcell Technologies, cat no. 07010) and then the plate was centrifuged at 1300 × g for 5 min. Following microscope verification that no bubbles remained, the Anti-Adherence Rinsing Solution was aspirated from the wells. Each well was then rinsed with 2 mL of basal RPMI-1640 media prior to addition of 1 mL of complete RPMI-1640 media. Next, 900,000 HTR-8/SVneo cells suspended in 1 mL complete media were plated per well and the AggreWell plate was centrifuged at 100 × g for 3 min. This resulted in approximately 3,000 HTR-8/SVneo cells captured in each microwell. The plate was then incubated at 37°C and 5% CO_2 for 24 h. After 24 h, spheroids were collected using a P1000 pipette and passed through a 37 µm reversible strainer into a 15 mL conical tube. Next, 1 mL of basal RPMI-1640 media was dispensed across the surface of the well to dislodge any remaining spheroids, then media was collected and passed again over the strainer. This washing step was repeated three times prior to inverting the strainer, placing it over a well in a 6-well plate and rinsing with 4 mL of complete RPMI-1640 media. The spheroids were then collected individually using a P200 pipette and 2 spheroids were plated per well in a 12-well plate pre-coated with 200 µL of 8 mg/mL growth factor-reduced Matrigel. The plated spheroids were then incubated at 37°C with 5% CO_2 for 48 h. Images were taken every 24 h under 100 × magnification using the Leica Inverted Light Microscope. Spheroid area and percent invasion (measured as the area of sprouts invading Matrigel) relative to the spheroid area was quantified using ImageJ software.

4.2.12 5-Ethynyl-2 Deoxyuridine (EdU) proliferation assay

Cellular proliferation was measured using EdU (5-ethynyl-2 deoxyuridine) incorporation. Briefly, glass coverslips, which had been sterilized via treatment with 70% ethanol solution for 15 min, were placed at the bottom of each well in a 12 well plate. Next, 50,000 cells suspended in 1 mL of RPMI-1640 complete media were plated into each well and incubated overnight. The following day the medium was changed with fresh media

containing 1 μL/mL EdU. The cells were then incubated at 37°C with 5% CO_2 for 72 h and media was replaced with fresh EdU supplemented media every 24 h. After 3 days, cells were fixed using 4% paraformaldehyde solution and stained using Hoechst 33342 dye and the Click-iT EdU Cell Proliferation Kit for Alexa Fluor 488 dye (ThermoFisher Scientific, cat no. C10337) according to manufacturer's protocol. The cells were then imaged using fluorescence microscopy (Hoechst 33342 excitation/emission: 350/461 nm; Alexa Fluor 488 excitation/emission: 495/519 nm) and percent EdU-positive cells in each representative image were quantified using ImageJ software.

4.2.13 Endothelial-like tube formation assay

Tube formation capabilities of HTR-8/SVneo cells were measured using an endothelial-like tube formation assay. Accelerated tube formation, conducted in the presence of VEGF-A is a measure of endovascular differentiation (Lala et al., 2012). Briefly, 20,000 HTR-8/SVneo cells were diluted in RPMI-1640 complete media supplemented with 30ng/ml $VEGF_{121}$ (Sigma, cat no.H9041), plated on 100 μL of 8 mg/mL Growth Factor Reduced Matrigel (Corning, cat no. 354230) in a 24 well plate, and incubated at 37°C with 5% CO_2 overnight. Cells were then imaged at 24 h under 100x total magnification using the Leica Inverted Light Microscope. Tube length and branch points were quantified using ImageJ software.

4.2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism Software version 8. Student's t-test was used to measure differences between two means. All figures present mean data with error bars extending to ± the standard error of the mean (SEM). Significance is represented by: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$.

4.3 Results

4.3.1 DCN upregulates expression of miRNAs let-7c-5p and miR-512-3p

Trophoblast cells do not produce DCN. To make trophoblast cell exposed to DCN continuously, my lab generated DCN overexpressing HTR-8/SVneo cell line (WT-HTR-DCN), which steadily produces substantial amounts of DCN (confirmed by qPCR, ELISA). We conducted a differential gene/miRNA micro-array analysis using WT-HTR-DCN and the control (mock-transfected) cell line. Of the large number of DCN-dysregulated miRNAs, we selected some which showed fold change > or < 1.5 and were reported to be dysregulated in PE (Table 4-3).

Table 4- 3: Selected miRNAs from our microarray data along with their literature reference.

↑ Indicates upregulation and ↓ indicates downregulation

MiRNA	Microarray data	Literature Refs
miR-512 (Wang et al., 2012)	↑	↑
miR-195 (Chen and Wang 2013)	↑	↑
miR-18b (Wang et al., 2017)	↑	↓
miR-363 (Zhang et al., 2015)	↓	↓
miR-374 (Li et al., 2013)	↓	↓
miR-155 (Gan et al., 2017)	↓	↑
let-7c-5p (Timofeeva et al., 2018)	↑	↑

To determine whether DCN altered expression of these miRNAs in trophoblasts using a different approach, HTR-8/SVneo cells were treated with 250 nM DCN for 24 h, and expression of select miRNAs was evaluated using qRT-PCR. This concentration was based on a pilot experiment conducted by Nandi et al., 2018 showing a maximal inhibitory effect of DCN was observed between 200 nM and 300 nM. We found that only two miRNAs out of 13 tested were significantly upregulated ($p<0.05$, Fig. 4-1).

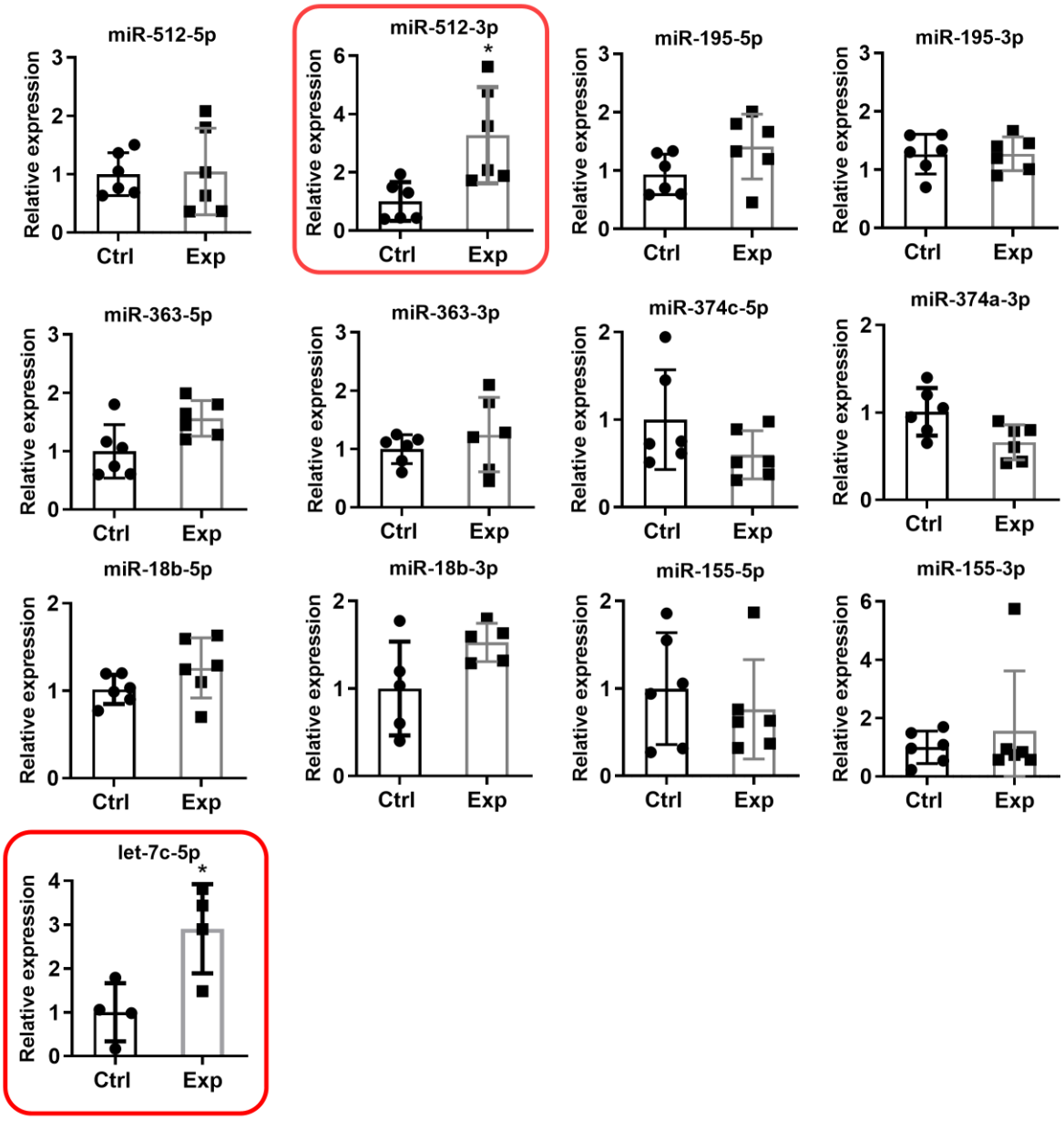

Figure 4-1: Determination of miRNA expression in HTR-8/SVneo cells in the presence or absence of exogenous DCN.

HTR-8/SVneo cells were exposed to vehicle (Ctrl) or 250 nM DCN (Exp) for 24 h, and then qRT-PCR was used to measure expression of select miRNAs."

Only miR-512-3p and let-7c-5p were significantly upregulated. Ctrl: cells not exposed to DCN. Exp: cells exposed to DCN. Time frame used for experiment: 24hrs. (N=4-6, *= $P<0.05$).

4.3.2 MiRNAs let-7c-5p and miR-512-3p are overexpressed in PE placentas

To validate literature reports of increased let-7c-5p and miR-512-3p expression in PE, miRNA was extracted from gestational age matched placental tissues derived from healthy normotensive (control) pregnancies and PE complicated pregnancies. Purified miRNA was then used to synthesize cDNA for qRT-PCR analysis of miRNA expression level. Both let-7c-5p and miR-512-3p expression levels in PE placentas were found to be significantly higher than in control placentas ($*p \leq 0.05$, n=5 biological replicates, (Fig. 4-2)

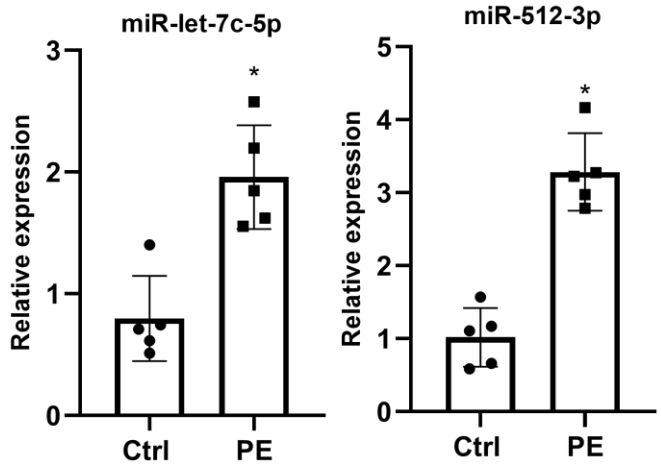

Figure 4-2: Increased expression of let-7c-5p and miR-512-3p in PE placentas.

Relative expression of let-7c-5p and miR-512-3p based on qRT-PCR analyses of placenta tissue samples derived from healthy normotensive (control) and preeclamptic (PE) pregnancies. Analysis performed using Student's t-test. Error bars extending to ± SEM. (*$p \leq 0.05$, n=5 replicates).

4.3.3 Overexpression of miR-512-3p resulted in inhibition of trophoblast migration, invasion, proliferation and tube-like formation

To investigate the role of miR-512-3p on various EVT functions, a miR-512-3p mimic was transfected into HTR-8/SVneo cells. Compared to mock-transfected cells, HTR-8/SVneo cells transfected with the miR-512-3p mimic had a 50-fold increased expression of miR-512-3p (Fig. 4-3A, n=4, $p \leq 0.05$). Cells with increased miR-512-3p had reduced capacity to migrate through a transwell membrane compared to control cells (Fig 4-3B, n=4, $p < 0.05$), showing that miR-512-3p has an inhibitory effect on trophoblast motility. To study the role of miR-512-3p on EVT invasion, a 3D spheroid invasion assay was done. Quantification of percent invasion (based on area of sprouts) relative to spheroid area revealed a significant decrease in the invasion capacity of HTR-8/SVneo cells over-expressing miR-512-3p at 24 h (Fig 4-3C, $p \leq 0.001$, n=4 replicates).

Cells transfected with the miR-512-3p mimic also showed decreased numbers of proliferating EVT cells in comparison to the mock-transfected control cells, as indicated by reduced EdU fluorescence signals (Fig 4-3D, $p \leq 0.0001$, n=4 replicates). To investigate the role of miR-512-3p on EVT endovascular differentiation, an endothelial-like tube formation assay was performed. Extensive tube formation was observed under the control conditions; however, this was drastically disrupted in cells over-expressing miR-512-3p, including a significant decrease in both the total tube length and the number of branch points between miR-512-3p overexpression and control treatments (Fig 4-3E, $p \leq 0.01$, n=3

replicates). Collectively, these results indicate that miR-512-3p compromised proliferative, migratory, invasive and tubulogenesis functions of EVT cells.

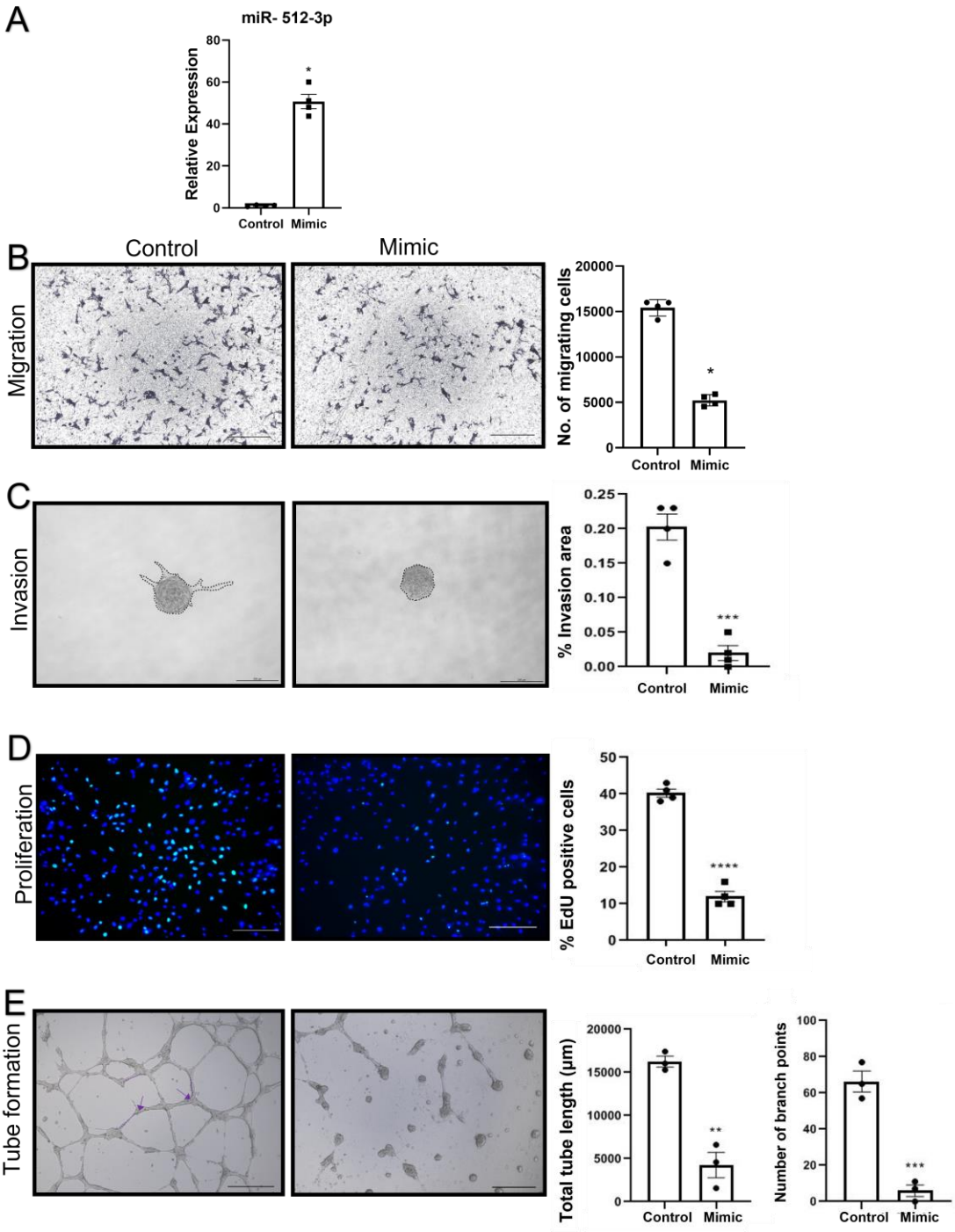

Figure 4-3: miR-512-3p overexpression decreased all HTR-8/SVneo trophoblast functions.

(A) Expression of miR-512-3p in mock-transfected HTR-8/SVneo cells (Control) or following transfection with miR-512-3p (Mimic). Results were analyzed using Student's

t-test (*p<0.05, n=3 replicates). **(B) Migration** measured using transwell assay. Representative images at 20x of membranes after 24hrs with migrated cells stained dark purple (noted here as dark spots) for both control and miRNA overexpressing cells. The assay was quantified by calculating number of cells migrating through the membrane post 24hrs of starting the assay. Error bars extending to ± SEM. (*p ≤ 0.05, n=4 replicates). **(C) Invasion** measured using spheroid invasion assays. Representative images of HTR-8/SVneo cells at 100x total magnification (200 µm scale bar) following 24 h of growth on GFR Matrigel.Invasion quantified by measuring percent invasion area (μm^2) under control and mimic conditions at 24 h. Analysis performed using Student's t-test. Error bars extending to ± SEM. (***p ≤ 0.001, n=3 replicates). (D) **Proliferation** measured using the 5-Ethynyl-2 Deoxyuridine (EdU) assay. Representative images of HTR-8/SVneo cells at 100x total magnification (200 µm scale bar) following 72 h of EdU treatment. Nuclei stained with Hoechst (blue) and proliferating cells with incorporated EdU stained with Alexa Fluor™ 488 dye (green). Proliferation quantified by counting percent EdU-positive cells under control and mimic treated conditions. Analysis performed using Student's t-test. Error bars extending to ± SEM. (****p ≤ 0.0001, n=4 replicates). (E) **Endovascular differentiation** measured using endothelial-like tube formation assays. Representative images of HTR-8/SVneo cells at 100x total magnification (200 µm scale bar) following 24 h of VEGF treatment and growth on GFR Matrigel.Tube formation quantified by measuring total tube length (µm) (shown by dotted line) and total number of branch points (shown by arrows) under control and mimic treated conditions. Analysis performed using Student's t-test. Error bars extending to ± SEM. (**p ≤ 0.01, (***p ≤ 0.001, n=3 replicates).

4.3.4 Knockdown of miR-512-3p resulted in increase in proliferation

Knockdown of miR-512-3p was performed by transfecting HTR-8/SVneo cells with a specific miR-512-3p antisense olignonucleotide (inhibitor). Using this strategy, we achieved approximately 50% knockdown of miR-512-3p (Fig 4-4A, n=4, $p<0.05$). Migration, invasion, and tube formation did not show any significant change between the control and knockdown cells (Fig 4-4B, C, E, n=3-4). However, cells with reduced miR-512-3p expression showed significant increase in proliferation as determined by the number of cells that incorporated EdU (Fig 4-4D, n=4, $p<0.01$). Collectively, these findings of an absence of signicicant changes in most EVT functions by miRNA knockdown may have resulted from the possibility that miR-512-3p is a DCN-inducible miRNA, expressed at relatively low levels in native EVT cells; or a more efficient knockdown was needed to achieve the expected results.

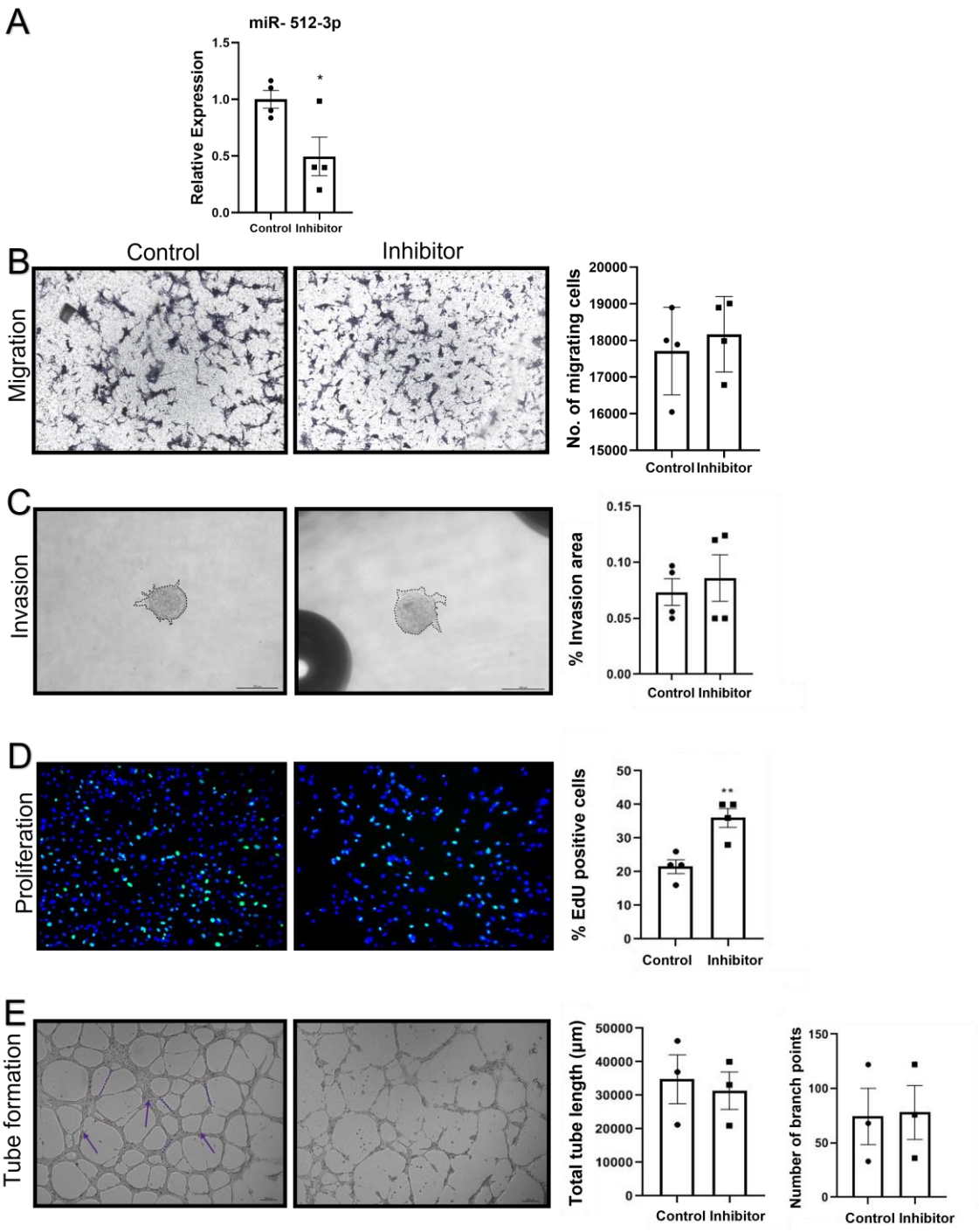

Figure 4-4: miR-512-3p knockdown increased trophoblast proliferation.

(A) Expression of miR-512-3p in mock-transfected HTR-8/SVneo cells (Control) or following transfection with miR-512-3p antisense olignonucleotide (Inhibitor). Results were analyzed using Student's t-test (*$p<0.05$, n=3 replicates). (B-E) All assays were conducted and quantified as indicated in figure legend 4-3. Migration (B), invasion (C) and Tube formation (E) did not show any significant difference between control and miR-512-3p inhibitor treated cells. (n=3-4) (D) Proliferation quantified by counting percent EdU-positive cells showed significant increase in inhibitor treated conditions vs control. (**$p \leq 0.01$, n=4). All analysis were performed using Student's t-test. Error bars extending to ± SEM.

4.3.5 Over-expression of let-7c-5p decreased invasion and disrupted endothelial-like tube formation ability of trophoblast cells

To investigate the role of miR-let-7c-5p on EVT functions, transfection mediated over-expression of miR-let-7c-5p in HTR-8/SVneo cells was performed, which resulted in a 170 fold increase in let-7c-5p compared to mock-transfected cells (Fig. 4-5A, $n=4$, $p<0.05$). We observed a significant decrease in spheroid invasion assay (more than 90% in comparison to control) (Fig 4-5C, $p<0.01$, $n=4$). We also noticed significant reduction in tube formation in the overexpressing cells measured via total tube length (Fig 4-5E, $p<0.01$) and total branch points ($p<0.05$, $n=3$). Migration and proliferation did not show any difference between the control and overexpressing cells (Fig 4-5B, D).

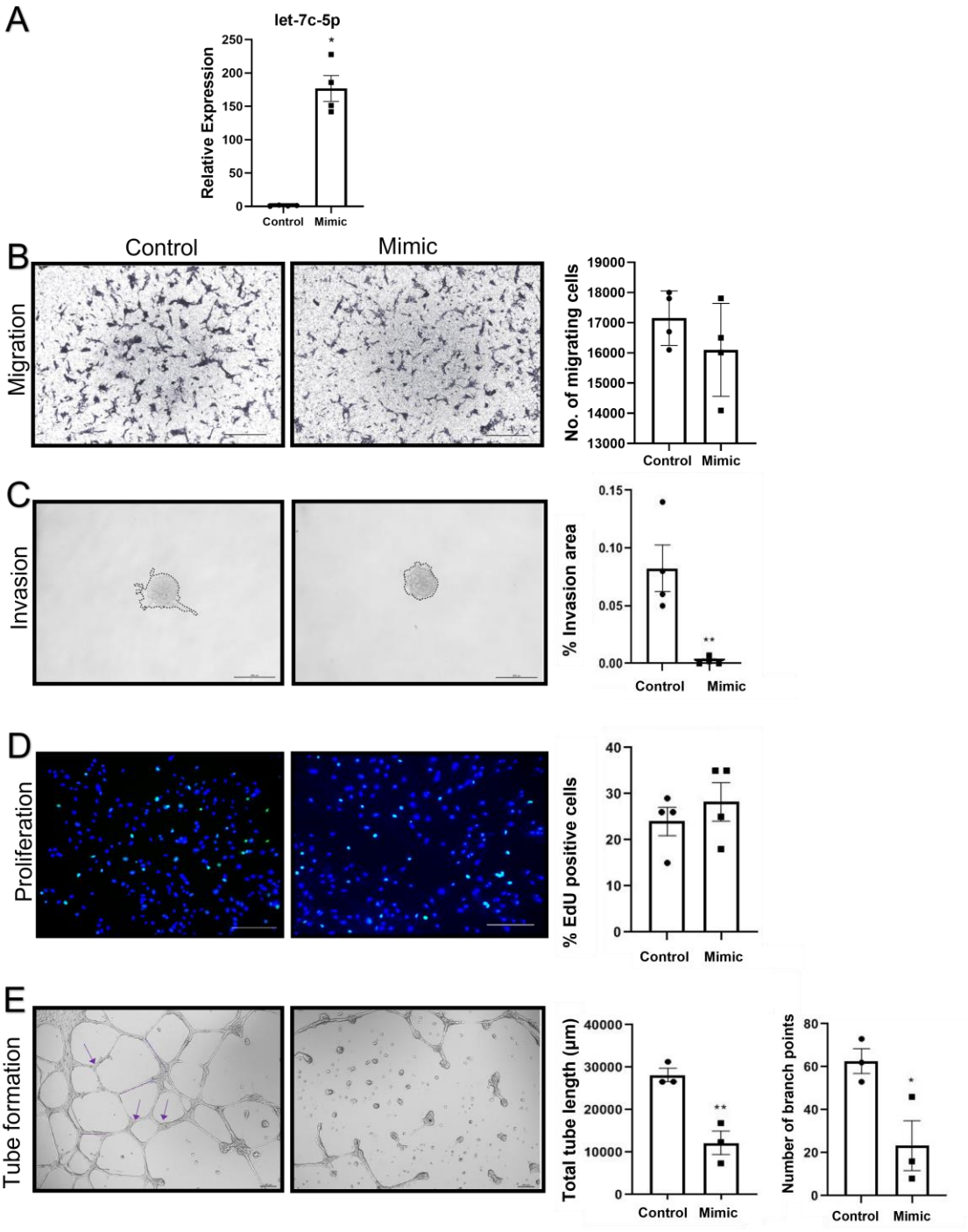

Figure 4-5: let-7c-5p overexpression decreased invasion and disrupted endothelial-like tube formation ability of HTR-8/SVneo trophoblast cells

(A) let-7c-5p overexpression (let-7c-5p Mimic;) confirmation by qRT-PCR analyzed using Student's t-test (*$p<0.05$, n=4 replicates). (B-E) All assays were conducted and quantified as indicated in figure legend 4-3. Migration (B) and proliferation (D) did not show any significant difference between control and let-7c-5p Mimic treated cells. (n=4) (C) Invasion quantified by measuring percent invasion area (μm^2) showed significant decrease (>90%) in mimic treated cells vs control. (**$p \leq 0.01$, n=4). (E) Tube formation ability was significantly reduction in let-7c-5p mimic treated cells in comparison to control, measured by total tube length (**$p<0.01$) and total number of branch points (*$p<0.05$, (n=3). All analysis were performed using Student's t-test. Error bars extending to ± SEM.

4.3.6 Knockdown of let-7c-5p did not affect trophoblast motility, invasion, proliferation or tube forming ability

To determine the effect of reduced let-7c-5p on trophoblast functions, HTR-8/SVneo cells were transfected with specific let-7c-5p antisense oligonucleotide (inhibitor) or control. Compared to mock transfected cells, HTR-8/SVneo cells transfected with the miR-let-7c-5p inhibitor had almost 90% reduced expression of miR-let-7c-5p (Fig. 4-6A, $p<0.05$, $n=4$). None of the functional assays on quantification showed significant change between the control and let-7c-5p inhibitor cells (Fig. 4-6B-E, n=3-4).

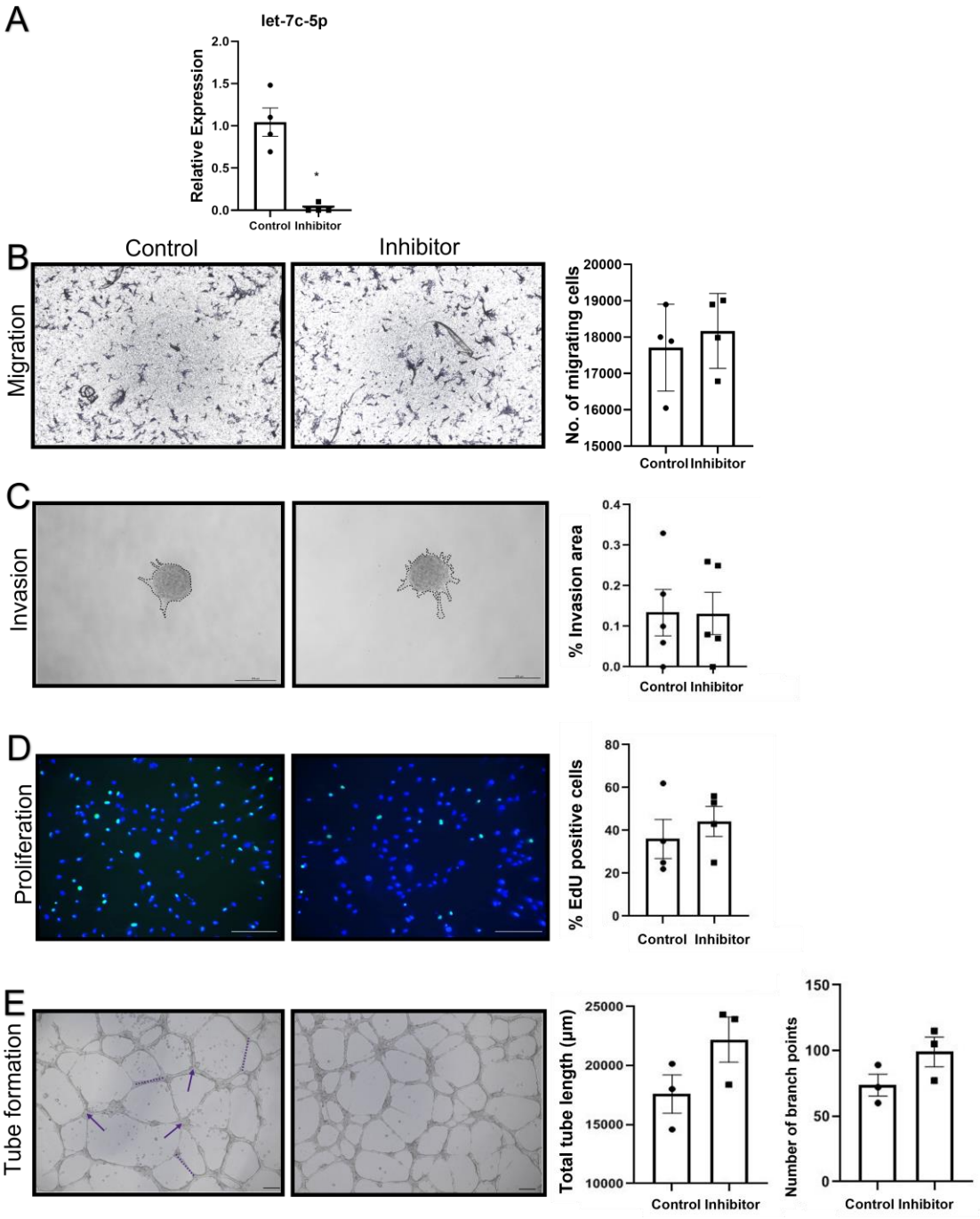

Figure 4-6: let-7c-5p knockdown resulted in no significant changed in HTR-8/SVneo trophoblast functions.

(A) let-7c-5p knockdown (let-7c-5p Inhibitor) confirmation by qRT-PCR analyzed using Student's t-test (*$p<0.05$, n=4 replicates). (B-E) All assays were conducted and quantified as indicated in figure legend 4-3. Migration (B), Invasion (C), proliferation (D) and tube formation (E) did not show any significant difference between control and let-7c-5p inhibitor treated cells (n=3-4). All analysis were performed using Student's t-test. Error bars extending to ± SEM.

Collectively, these findings of an absence of significant changes in most EVT functions by miRNA knockdown may have resulted from the possibility that let-7c-5p is also a DCN-inducible miRNA, expressed at relatively low levels in native EVT cells

4.3.7 Knocking down PPP3R1, a known target of miR-512-3p, increases migration and invasion of trophoblast cells

PPP3R1 is a known target for miR-512-3p (Kurashina et al., 2014). The authors showed a decrease in PPP3R1 in miR-512-3p overexpressing BeWo choriocarcinoma cells. Paradoxically, our qRT-PCR data consistently showed an increase in *PPP3R1* mRNA in miR-512-3p overexpressing cells (Fig 4-7A; $p<0.05$, n=7). We proceeded with knocking down PPP3R1 in HTR-8/SVneo cells (60% knockdown) and performed migration and invasion assays. There was a 50% increase in percent wound closure after 24 h in PPP3R1-knockdown cells in comparison to control cells ($p<0.05$, n=3). Invasion assay was performed by placing cells into transwells coated with a thin layer of Matrigel, and determining how many cells invaded through the Matrigel after 48 h. There was a significant increase in the number of cells invading through the membrane in PPP3R1-knockdown cells in comparison to control (Fig 4-7B, $p<0.05$, n=3). Therefore, PPP3R1 function in EVT cells was consistent with the upregulation of this gene by miR-512-3p overexpression. This finding called for investigating the possibility of an intermediary miR-512-3p binding molecule which targets PPP3R1.

4.3.8 PPP3R1 expression is upregulated in PE and after exogenous DCN.

To the best of my knowledge, there is no information on the levels of *PPP3R1* in placentas from PE. Therefore, we decided to measure *PPP3R1* transcript levels using gestation age-matched control and PE placental tissues and found a significant increase of *PPP3R1* mRNA expression in PE-associated placentae (Fig 4-7C, $p<0.05$, n=5). Furthermore, since exposure of HTR-8/SVneo to DCN increased levels of miR-512-3p, and miR-512-3p overexpression increased *PPP3R1* expression, we also checked the expression level of

PPP3R1 after treatment with exogenous DCN for 24 h. We found a significant increase in *PPP3R1* mRNA after DCN treatment (Fig 4-7 D, $p<0.05$, $n=4$).

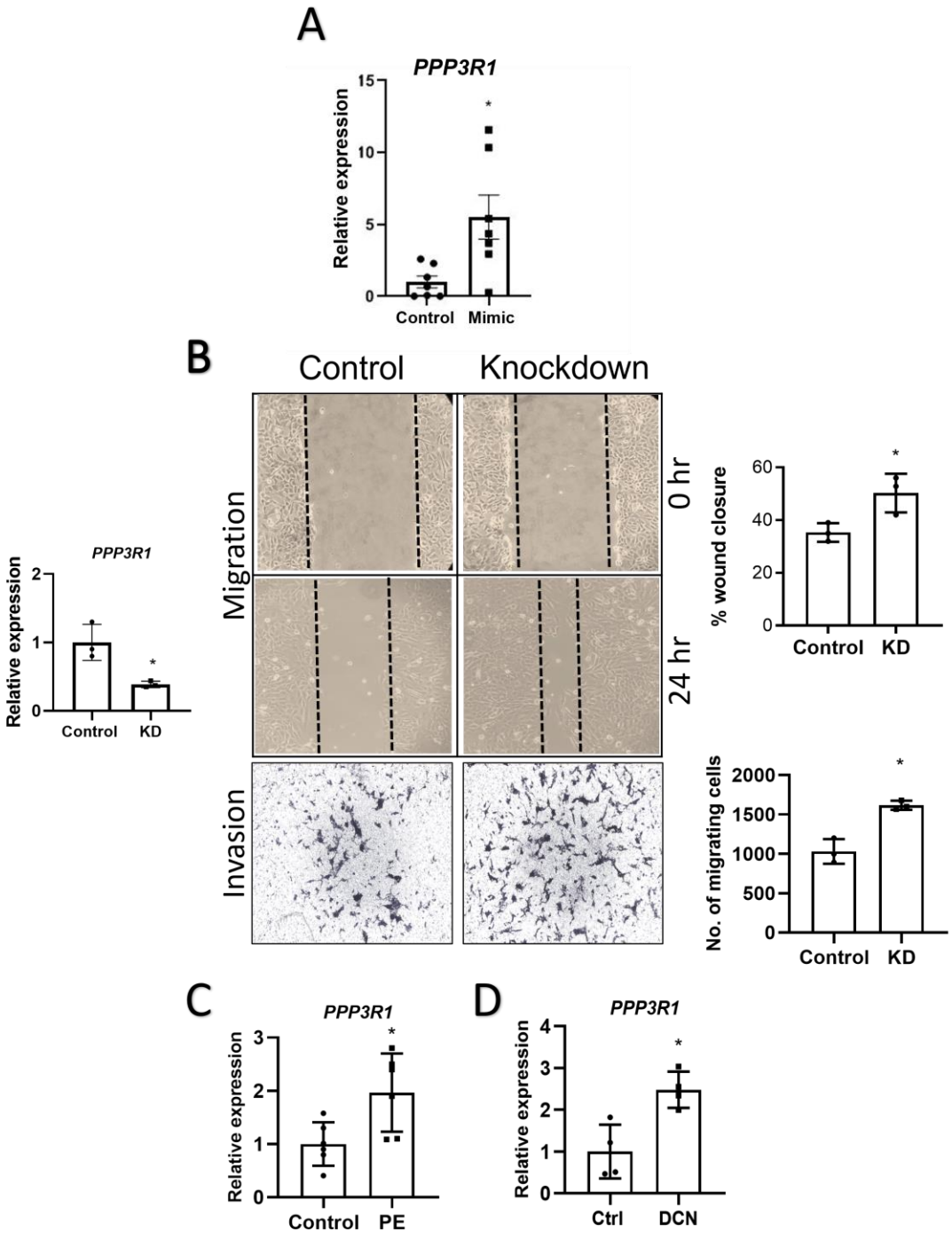

Figure 4-7: PPP3R1 knockdown increases migration and invasion of HTR-8/SVneo trophoblast cells

(A) miR-512-3p overexpression (miR-512-3p mimic) resulted in significant increase in *PPP3R1* confirmed by qRT-PCR (*p<0.05, n=7 replicates). (B) qRT-PCR showing knocking down *PPP3R1* in HTR-8/SVneo cells (60% knockdown, *p<0.05) and the resulting effect on migration and I nvasion assays. Migration assay performed using scratch assay. Images taken at 0 hr and 24 hr (20x), measured by the percentage of wound closure at 24 hr showing significant increase in wound closure in PPP3R1 knockdown cells. Invasion assay images shown for control and knockdown cells taken at 48 hmeasured by calculating the number of cells migrating through the Matrigel covered membrane. (*p<0.05, n=3). (C) qRT-PCR showing significant increase of *PPP3R1* mRNA expression in placentae from PE (*p<0.05, n=5) and (D) after exogenous DCN treatment (*p<0.05, n=4). All analysis were performed using Student's t-test. Error bars extending to ± SEM.

4.3.9 PPP3R1 upregulation by miR-512-3p is intermediated by the transcription factor USF2

Elevated expression of PPP3R1 in miR-512-3p overexpressing cells indicated that this miRNA might be targeting negative regulators of PPP3R1. To identify these transcription factors, we used Enrichr, a tool that consists of both a validated user-submitted gene list and a search engine for transcription factors. By comparing miRNA target genes from TargetScan (https://www.targetscan.org/vert_80/), we identified PPP3R1 regulatory transcription factors. Of these transcription factors, we identified USF2 as the only negatively regulated transcription factor of PPP3R1. We validated this prediction by comparing *USF2* mRNA expression in control and miRNA-512-3p over-expressing trophoblast cells. There was a robust downregulation of *USF2* with a concomitant upregulation of PPP3R1 in miR-512-3p over-expressing trophoblast cells (Fig 4-8A). Similarly exogenous DCN treatment (250 ng/ml for 24 h) also downregulated *USF2*, although this did not reach statistical significance (Fig 4-8B, p=0.065). Interestingly, PPP3R1 was found to be a direct target of USF2.
(https://maayanlab.cloud/Harmonizome/gene_set/USF2/ENCODE+Transcription+Factor+Targets)

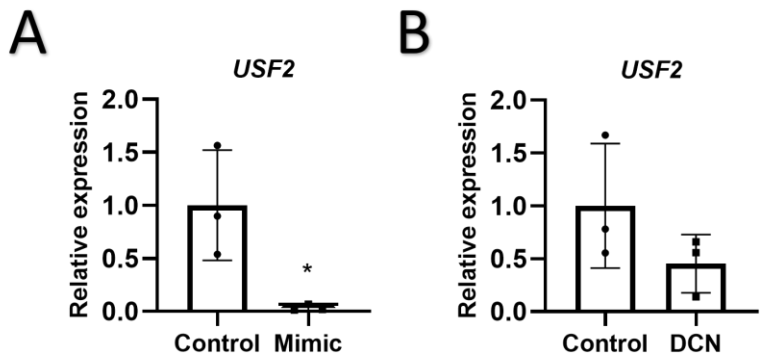

Figure 4-8: USF2 transcription factor may act as an intermediary for PPP3R1 upregulation by miR-512-3p in HTR-8/Svneo trophoblast cells

qRT-PCR showing *USF2* upregulation in overexpressing miR-512-3p cells (A) and in exogenous DCN treated cells for 24 h. (B) analyzed using Student's t-test (*$p<0.05$, n=3).

4.4 Discussion

PE is a life-threatening maternal pregnancy complication arising from abnormal placentation (Roberts and Lain, 2002). Poor placentation and uterine invasion by the trophoblast in PE severely reduce oxygen supply to the placenta and developing fetus, resulting in placental hypoxia and subsequent secretion of toxic factors into maternal circulation (Roberts and Lain, 2002). Recently, the proteoglycan DCN was shown to be overproduced by the decidua associated with PE and elevated in the plasma of PE patients (Siddiqui et al., 2016). DCN has been shown to facilitate development of placental hypoxia by impairing trophoblast proliferation, migration, invasion, and endovascular differentiation (Xu et al., 2002; Iacob et al., 2008; Khan et al., 2011; Lala et al., 2012). While these observations have been attributed to DCN interaction with several receptors, including EGFR, IGFR2, VEGFR2 (Iacob et al., 2008, Khan et al., 2011), the role of miRNAs in DCN actions on EVT cells remains unknown. With the observation that several miRNAs are dysregulated in PE pregnancies, the contribution of miRNAs to PE development has been gathering much attention in recent years (Gao et al., 2018, Xu et al., 2021).

The current study aimed to identify DCN dysregulated miRNAs that could play a role in DCN mediated compromise in trophoblast functions associated with PE. We found multiple miRNAs that were altered in the miRNA microarray analysis with DCN-overexpressing trophoblast and selected those that were reported to be dysregulated in PE. Upon validation of selected miRNAs, we identified two miRNAs, let-7c-5p and miR-512-3p that showed significant upregulation in DCN-treated HTR-8/SVneo cells as well as an ability to alter EVT cell functions, known to be compromised in PE

Prior to studying miRNA effects on EVT functions, we analyzed the expression of let-7c-5p and miR-512-3p miRNA molecules in gestation age matched PE vs control placental tissues. We found that both miRNAs were significantly upregulated in placentas derived from PE pregnancies compared to normotensive controls. This was consistent with previous studies that reported upregulated expression of let-7c-5p and miR-512-3p in PE patients (Timofeeva et al., 2018; Wang et al., 2012).

The current study is first report on the roles of miR-512-3p and let-7c-5p on trophoblast functions such as migration, invasion, proliferation and tube formation (endovascular differentiation). We found that all these functions were variably downregulated by both miRNAs, more notably by miR-512-3p. To ascertain that the transwell migration or invasion assay was not influenced by cell proliferation, we used mitomycin C treated cells in our assay. Similarly, to ascertain that spheroid invasion assay was not influenced by proliferation, we quantified the average spheroid area between 24 and 48 h for both control and over-expression conditions and did not find any significant change in spheroid area. Endothelial-like tube formation gave the same results both measured as total tube length and branching points. Although the effects of miR-512-3p on EVT endovascular differentiation have not been previously reported, these results are consistent with studies reporting that other members of the C19MC cluster, such as miR-515-5p, may play inhibitory roles in trophoblast differentiation processes (Zhang et al., 2016).

Knocking down miR-512-3p did not show any significant change in migration, invasion or tube formation. This may reflect relatively low expression of this miRNA in trophoblast cells in the absence of DCN. However, we observed a significant increase in trophoblast proliferation on miR-512-3p downregulated cells, indicating its dominant anti-proliferative role relative to other effects on the trophoblast.

Let-7c-5p was found to compromise multiple trophoblast functions variably. Overexpressing let-7c-5p significantly compromised invasion and tube formation, but did not show any significant effect on migration and proliferation of EVT cells. Our findings are consistent with several studies reporting that let-7c-5p plays an inhibitory role in mediating invasion capacities of lung, liver, and colorectal cancer cells (Han et al., 2012; Li et al., 2021; Zhao et al., 2014). Let-7c-5p was reported to exert anti-proliferative effects on erythroleukemia cells (Mortazavi and Sharifi, 2018). Let-7a-5p, another member of the let-7 family was found to have anti-apoptotic effects on trophoblast cells (Zha et al., 2020).

Overexpressing let-7c-5p was found to substantially disrupt tube formation by EVT cells, suggesting that let-7c-5p inhibits EVT endovascular differentiation. Our results are consistent with previous studies that have observed let-7c-5p to be downregulated during

flow-stimulated angiogenesis, suggesting it may play an inhibitory role in angiogenic processes (Henn et al., 2019).

Even though altering levels of miR-512-3p showed more profound and consistent effects on trophoblast functions compared to miR-let-7c-5p, we searched for potential targets of both miRNAs both miRNAs using literature search and targetscan (https://www.targetscan.org/vert_80/). We narrowed down the targets of both miRNAs by selecting those that are known to play a role in pregnancy or placenta development. We made a list of 34 targets combined for both miRNAs and tried to validate them by qRT-PCR of control versus respective miRNA overexpressing cells. We could not validate any target for miR-let-7c-5p but we validated a target (PPP3R1) for miR-512-3p. *PPP3R1* is a gene coding for calcineurin-B, and a known target of miR-512-3p (Kurashina et al, 2014). Calcineurin-B pathway has been reported to cause renal podocyte injury in PE (Yu et al., 2018).

Traditionally, miRNAs negatively regulate gene expression by repressing translation or directing sequence- specific degradation of target mRNAs (He and Hannon, 2004). But contrary to the traditional view, we consistently found an increase of PPP3R1 in cells overexpressing miR-512-3p. Indeed, there is an increasing number of recent reports suggesting that miRNAs can also induce or promote the expression of target genes (Place et al., 2008, Rusk et al., 2008). For example, Huang et al (2012) reported miR-744 and miR-1186 induced transcriptional activation by targeting promoter of Cyclin B1 gene. Another study by Xiao et al., (2017) showed that in HEK293T cell line, miR-24-1 overexpression increased histone 3 lysine 27 acetylation by targeting enhancers.

Nevertheless, since *PPP3R1* was increased in HTR-8/SVneo cells overexpressing miR-512-3p, the role of PPP3R1 on trophoblast functions was tested. We found a significant increase in both functions in PPP3R1-knockdown cells in comparison to control cells. This suggested that PPP3R1 is an anti-migratory and anti-invasive molecule which could contribute to the anti-migratory actions observed in HTR-8/SVneo cells overexpressing miR-512-3p.

We attempted to address how miR-512-3p increased the expression of its target gene *PPP3R1*. Using bioinformatics, we compared miR-512-3p target genes and PPP3R1 regulatory transcription factors to search for potential intermediaries. The analysis revealed that miR-512-3p is a putative negative regulator of USF2, a transcription factor which represses expression of PPP3R1. Decreased expression of *USF2* and increased expression of *PPP3R1* in miR-512-3p overexpressing cells indicate that PPP3R1 upregulation may result from an intermediary transcription factor USF2. These findings reveal a novel mechanism in miR-512-3p action. A schema is shown is fig 4-9.

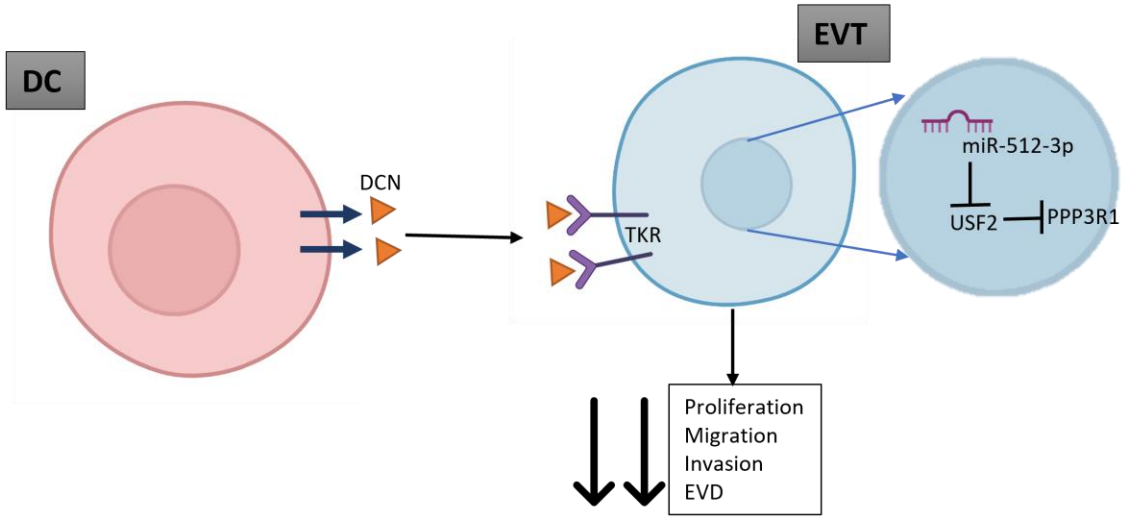

Figure 4-9: A schematic indicating the possible role of DCN mediated downregulation of PPP3R1.

DCN released by decidual cells (DC) binds to multiple tyrosine kinase receptors (TKR) on extravillous trophoblasts (EVT) and induces expression of miR-512-3p (binding partner unknown). MiR-512-3p downregulates *USF2*, which increases expression of *PPP3R1* and results in decreased EVT proliferation, migration, and endovascular differentiation (EVD).

In summary, the present study offers new mechanistic insights into the roles of miR-512-3p and let-7c-5p miRNAs in PE development, while also highlightings possible novel miRNA-mediated mechanisms in DCN inhibitory actions on EVT cells. Future studies will explore a direct relationship between DCN over-expression and DCN-induced miRNA over-expression in the same PE subjects. Current findings, together with future studies, may facilitate development of novel miRNA plasma biomarkers and treatments that will improve early diagnosis and management of pregnancies affected by PE.

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Chapter 5

5 Discussion

5.1 Significance of Research

Decorin (DCN) is a leucine-rich proteoglycan made at the fetal-maternal interface by decidual cells and fetal mesenchymal cells. My lab has been a pioneer in uncovering the paracrine roles of DCN on the human trophoblast in maintaining a normal utero-placental homeostatsis and their alterations in preeclampsia (PE)/ fetal growth restriction (FGR). DCN, by binding to multiple tyrosine kinase receptors on the trophoblast, restrains placental over-invasion of the uterus and its arteries, whereas over-production of DCN by decidual cells is causally associated with PE. Furthermore, elevated blood DCN level in the mother during the second trimester in pregnancy can predict PE, before clinical diagnosis. My project aimed to answer three major questions on DCN in human pregnancy: (1) Does DCN have an autocrine role in decidual cell development or maturation (Chapter 2); (2) When and how is DCN produced by the decidua during pregnancy? (Chapter 3); (3) Does DCN induce any microRNAs (miRNAs) in the trophoblast that may compromise trophoblast functions known to be associated with PE? (Chapter 4).

The overall goal was to elucidate the roles of DCN in decidual cell development in normal pregnancy and identify molecular mechanisms leading to alterations in DCN production that may lead to PE and FGR. This research is fundamental to understanding the origin and PE and FGR. The knowledge can be exploited to improve maternal and fetal health.

I hypothesized that DCN is crucial for decidual cell development, that DCN is produced in early gestational decidua, this production induced by some pregnancy-associated pro-inflammatory cytokine (such as IL-1beta; IL-1β) and that it induces miRNAs that compromise trophoblast functions in PE.

The main findings supporting my hypothesis are that:

(1) DCN plays an autocrine role in decidual maturation and is a crucial regulator of the decidualization process.

(2) IL-1β produced by cells at the fetal-maternal interface promotes DCN production by human endometrial stromal cells (HESC), via IL-1R1/NF-κB pathway, unveiling a novel mechanism in DCN production by HESC.

(3) DCN-induced miRNAs miR-512-3p and let-7c-5p in the trophoblast present as novel meditators of DCN effects that compromise trophoblast functions in PE. miR-512-3p action was mediated by a paradoxical upregulation of PPP3R1 (calcineurin B subunit), possibly due to an intermediary transcription factor USF2 downregulated by this miRNA.

5.1.1 Decorin plays an autocrine role in decidual maturation and is a crucial regulator of decidualization process.

While a minor decidualization of the human endometrium occurs during the post-ovulatory (mid-late secretory) phase of the menstrual cycle (Gellersen et al 2007), it is enhanced and sustained after successful implantation. This process can be faithfully mimicked *in vitro* by treating HESC with 250nM medroxyprogesterone-acetate (MPA), and 0.5mM 8-bromo-cAMP (a stable, cell permeable analog of cAMP). In chapter 2, my results using these treatments on a telomerase- immortalized (T)-HESC linereveal that the peak decidualization, characterized by epitheloid morphology and insulin like growth factor 1 (IGFBP1) and prolactin (PRL) production (markers of decidualization) occurs on day 6. Similar results were also noted with primary (P)-HESC that I derived from the first trimester decidua using my own protocol. The decidual response in both cases was associated with a nearly parallel rise in DCN production (mRNA measured with RT-qPCR and secreted protein measured with ELISA).

To examine the effects of DCN depletion on Decidualization of THESC, we knocked down the expression of DCN in THESC with a cocktail of shRNAs (resulting in 65-70% knockdown) and exposed them to decidualizing agents. Knocking down DCN significantly blocked their transformation into decidual cells, as indicated by retention of fibroblast-like morphology and a reduction in the synthesis of decidual markers IGFBP1 and PRL, compared to control shRNA transfected cells. To validate these findings further, we generated DCN-knock out (DCN-KO) THESCs by CRISPR/Cas9 double nickase

approach. Guide RNA targeting DCN was designed and incorporated into lentiCRISPRv2 plasmid. DCN-KO cells exposed to decidualizing agents exhibited poor decidual differentiation as revealed by fibroblast-like morphology, as well as profoundly reduced synthesis of IGFBP1 and PRL. These findings with DCN-KD/KO cells revealed for the first time an autocrine role of DCN in sustaining decidual cell differentiation. Whether decidual cell development is compromised in DCN-null mice remains unclear. These mice are fertile, but they display abnormal collagen fibrillogenesis in their endometrium (Sanches et al., 2010). They exhibit partial compensation of DCN functions by upregulating biglycan protein (Calmus et al., 2011; Wu et al., 2012). However, we found no change in biglycan expression in our DCN-KO HESC, indicating no role of biglycan in regulating IGFBP1 and PRL synthesis.

I also examined the kinetics of DCN production by the decidua *in vivo* (tested *ex-vivo*) during the first trimester of gestation. Currently, there is no information on DCN production *in vivo* at early stages in human pregnancy. We obtained 28 samples of first trimester decidua (at different gestational ages; week 7 to 14) from elective termination of pregnancy in subjects with no known history of recurrent pregnancy loss. Decidual tissues, carefully separated from fetal membranes and chorionic villi, were freed of blood by repeated washes with sterile DMEM. A part of the fresh tissue was subjected to qRT-PCR for measuring mRNA levels of DCN, IGFBP1 and PRL. We also measured the expression of FOXO-1 and HAND2, two key transcription factors involved in upregulation of many decidual response genes (Labied et al., 2006; Kim et al., 2005, Huyen and Bany 2011; Vasquez et al., 2015). We found that HAND2 expression in decidual tissues relative to IGFBP1 showed a significant increase from Week 7 to Weeks 8 and 9, very similar to that of DCN expression relative to IGFBP1.

To examine the effects of DCN depletion on changes in transciptomes of THESC upon decidualization, we performed whole transcript expression analysis using Clariom S assay of control versus DCN KO THESCs after treatment with DM and found that HAND2, transcription factor known to be upregulated during decidualization, was significantly decreased. I identified that IGFBP1 contains multiple putative HAND2-binding sites (CAGATG/CATCTG) less than 150 base-pairs upstream of the transcription start site. In

future experiments, HAND2 binding at these sites can be validated with chromatin immunoprecipitation. Present data combined with the above evidence strongly suggests that DCN-mediated decidual cell maturation is dependent on HAND2. This notion is consistent with the report by Huyen and Bany (2011) that knocking down HAND2 in HESC downregulated IGFBP1. These authors also demonstrated downregulation of both FOXO1 and IGFBP1 in HAND2-knocked down HESCs, indicating that FOXO1 is downstream of HAND2 in decidual response, and IGFBP1 may be directly regulated by HAND2, FOXO1, or both.

Findings from this chapter provide evidence that DCN plays a critical autocrine role in decidual cell development during pregnancy.

5.1.2 IL-1β-mediated DCN upregulation in HESC, via IL-1R1/NF-κB pathway

IL-1β promotes IGFBP1 production in primate decidua (Strakova et al., 2000). Furthermore, elevated serum IL-1β levels are evident in PE subjects (Kalinderis et al., 2011), raising the possibility of a causal link between elevated IL-1β and DCN production in PE. In chapter 3, I analyzed the effect of multiple cytokines on DCN production and showed that DCN can be induced in HESC by IL-1β. Therefore, we hypothesized that IL-1β is the key cytokine needed for DCN production by decidual cells. We found that neither PHESC nor THESC before or after decidualization *in vitro* produced any measurable IL-1β at the mRNA or secreted protein levels, indicating that the effects of IL-1β on DCN production by HESC within the decidua are likely paracrine in nature and DCN production due to IL-1β is independent of decidualization

We also identified the cellular source of IL-1β in the decidua by immunostaining and *in situ* hybridization and found that both decidual cells and macrophages were positive for IL-1β. Macrophages as well as trophoblast cells are already known to express IL-1β at the feto-maternal interface (Kauma et al., 1990). We suggest that decidual cells *in vivo* are either induced to express IL-1β by other unknown mediators or binding IL-1βprotein secreted by other cells. We also showed that IL-1β mediated DCN production was dependent on IL-1R

activation, followed by activation and nuclear translocation of NF-κB and its binding to the DCN promoter. These results reveal that IL-1β plays a novel role in inducing DCN production by human endometrial stromal cells by activating NF-κB. Findings from this chapter provide new insights into the role of IL-1β in DCN production in the decidua.

5.1.3 Novel roles of miRNAs 512-3p and let-c-7-5p in mediating DCN effects on EVT cell functions

In chapter 4, I aimed to answer the question whether DCN exerts its effects on the trophoblast, at least in part, by inducing miRNAs that compromised trophoblast motility, proliferation, and endovascular differentiation. Using the miRNA microarray data obtained from an ectopic DCN-overexpressing HTR-8/SVneo trophoblast cell line (HTR-DCN) and the wild type control cell line, we identified a large number of DCN-regulated miRNAs. We selected those that were validated by qRT-PCR using both HTR-DCN and exogenous DCN-treated wild-type HTR-8/SVneo cells. From these results, we selected two miRNAs (Let 7c-5p and 512-3p), which were upregulated by DCN and also increased in PE placentas. We overexpressed and knocked-down these two miRNAs in HTR-8/SVneo cells and performed EVT functional assays: migration, invasion, proliferation and endovascular differentiation (VEGF-dependent tube formation on Matrigel). While both miRNAs exerted variable inhibitory effects on all four trophoblast functions, miR-512-3p had more consistent and robust effects, leading us to investigate putative target gene(s). Unexpectedly, we found that PPP3R1 (calcineurin B subunit), a previously identified target of miR-512-3p, was upregulated by miR-512-3p mimic. Consistent with this finding, like miR-512-3p, *PPP3R1* was also elevated in PE placentas. In recent years several laboratories have reported target upregulation by miRNAs by binding to reporter and enhancer sites (Place et al., 2008; Rusk et al., 2008). In the present case the target upregulation was likely intermediated by downregulation of a transcription factor *USF2*, which targets PPP3R1. Our findings represent a novel mechanism of miR-512-3p action.

5.2 Future directions

Following the results observed in chapter 2, we predicted multiple HAND2-binding sites (CAGATG/CATCTG) less than 150 base-pairs upstream of the gene encoding IGFBP1. It would be interesting to validate this and conduct chromatin immunopreciptation assays for this binding in decidualized HESC cells, following the method reported by Vasquez YM et al for binding of PGR to IRF4 (Vasquez et al., 2015). In the case of negative binding, we shall purify and sequence the HAND2- bound DNA (ChIP-Seq, Illumina) to identify other HAND2-binding partner(s) that may bind to *IGFBP1*.

We demonstrated that DCN-KO cells have a decidualization defect and a reduced expression of HAND2 and PGR (Halari et al., 2020). Future studies are aimed to test whether over-expressing HAND2 or PGR in DCN-KO ESCs, when exposed to decidual stimuli, restores DC maturation as indicated by morphology, IGFBP1 and PRL production. We shall expose the transfected cells to decidual stimuli (8Br-cAMP and medroxy progesterone acetate) for 6 days and compare the degree of decidualization by morphology (epithelioid phenotype), the level of IGFBP1 and PRL production at the mRNA and secreted protein levels for a comparison with decidualized un-tarnsfected cells.

Additionally, future studies are warranted to conduct *in situ* hybridization of the decidual tissues collected a 7 to14 weeks gestation for relative expression of IGFBP1, PRL, DCN, FOXO-1 and HAND2 mRNAs in decidual cells, which has never been done before at the cellular level. These future experiments combined with my results from chapter 2 will increase our understanding of the mechanism behind the autocrine role DCN in decidual cell development.

In chapter 3, while we identified IL-1β as the best candidate inducer of DCN upregulation in HESC, we cannot exclude additional candidate(s) which may act in parallel or proximal to IL-1β *in vivo*. Since DCN is upregulated *in vivo* between 7-8 weeks (Halari et al., 2020) differential gene /protein arrays, using decidual tissues between these gestational ages may aid their identification. Also, my data reveals that THESCs express IL-1R and IL-1β induced DCN production is significantly blocked by an IL-1R antagonist**.** For future experiments, we shall produce *IL-1R*-knockout THESC using CRISPR/Cas9 approach as reported by us for knocking

out the *DCN* gene. (Halari et al., 2020) We shall measure DCN production by at least 3 clones of IL-1β treated *IL-1R*-Knockout versus control cells. This genetic approach will strengthen our data. We also intend to conduct RNA sequencing in the above cells to identify changes in genes and pathways, as reported by us for DCN-Knockout cells (Halari et al., 2020). This approach being unbiased, should be highly informative on any intermediary genes and pathways in IL-1β mediated DCN production by HESC. Since, we found an NF-κB binding site upstream of the DCN gene, we could also use CRISPR/Cas9 to mutate these binding sites in order to determine their importance for driving *DCN* transcription

In chapter 4, we used HTRSV/neo cells which are a valuable tool for screening DCN-induced miRNAs. Considering the limitations that immortalized cell line does not completely mimic primary cells. Future studies using human trophoblast stem cell line and cells isolated from chorionic villus sprouting, should provide this valuable information. Chorionic villus sprouting is an ex-vivo assay that closely mimics events *in vivo*. Therefore, we shall use intact undisrupted first trimester chorionic villi in explant cultures on Matrigel (Danielson et al., 1997; Luo et al., 2012; Genbacev et al., 1992; Nicola et al., 2005; Nicola et al., 2008), that allow EVT sprouting, shown by us to be stimulated by prostaglandin E2 (Nicola et al., 2005; Nicola et al., 2008) and inhibited by DCN (Iacob et al., 2008), and also used to study miRNA effects (Luo et al., 2012; Farrokhnia et al., 2014). Explants will be cultured on growth factor reduced Matrigel for 24-96 h to examine the effects of miRNAs found to regulate EVT migration. For overexpression, pre-miR mimetics will be transfected into villus explants with an Amaxa Nucleofector, as reported (Nicola et al., 2008).

Overexpression will be confirmed in some explants by qPCR using specific primers. For each miRNA, the effect of the three pre-miR sequences on EVT outgrowth will be compared with that of un-transfected and empty vector (mock)-transfected controls. IGF-I (10 nM, a trophoblast mitogen) will be used as a positive control. The degree of villus sprouting is a combined effect of proliferation, migration and invasion. Proliferation will also be directly measured with EdU uptake at 48 h. Tissues will be fixed and processed for immunohistochemistry using anti-EdU antibody (Sigma).

Another important question that we would like to answer in future studies is how DCN induces the mentioned miRNAs in the trophoblast. We propose that this might be due to DCN binding to one or more of TKRs (IGFR, EGFR1 and VEGFR-2) (Iacob et al., 2008; Khan et al., 2011; Lala et al., 2012) on trophoblast. In order to validate this, we will use currently available peptides shown to block multiple TKRs (Hojjat-Farsangi, 2014).

Lastly, we shall test whether DCN-upregulated miRNAs shown to be upregulated in PE placentas are elevated in the plasma of PE subjects exhibiting elevated DCN.

5.3 Conclusion

This research combines mechanisms in autocrine roles of DCN in decidual development, mechanisms in DCN production by the decidua and miRNA-mediated paracrine roles of DCN in regulating trophoblast functions (a schematic is presented in Fig. 5-1). This figure summarizes my research findings in the figure legend.

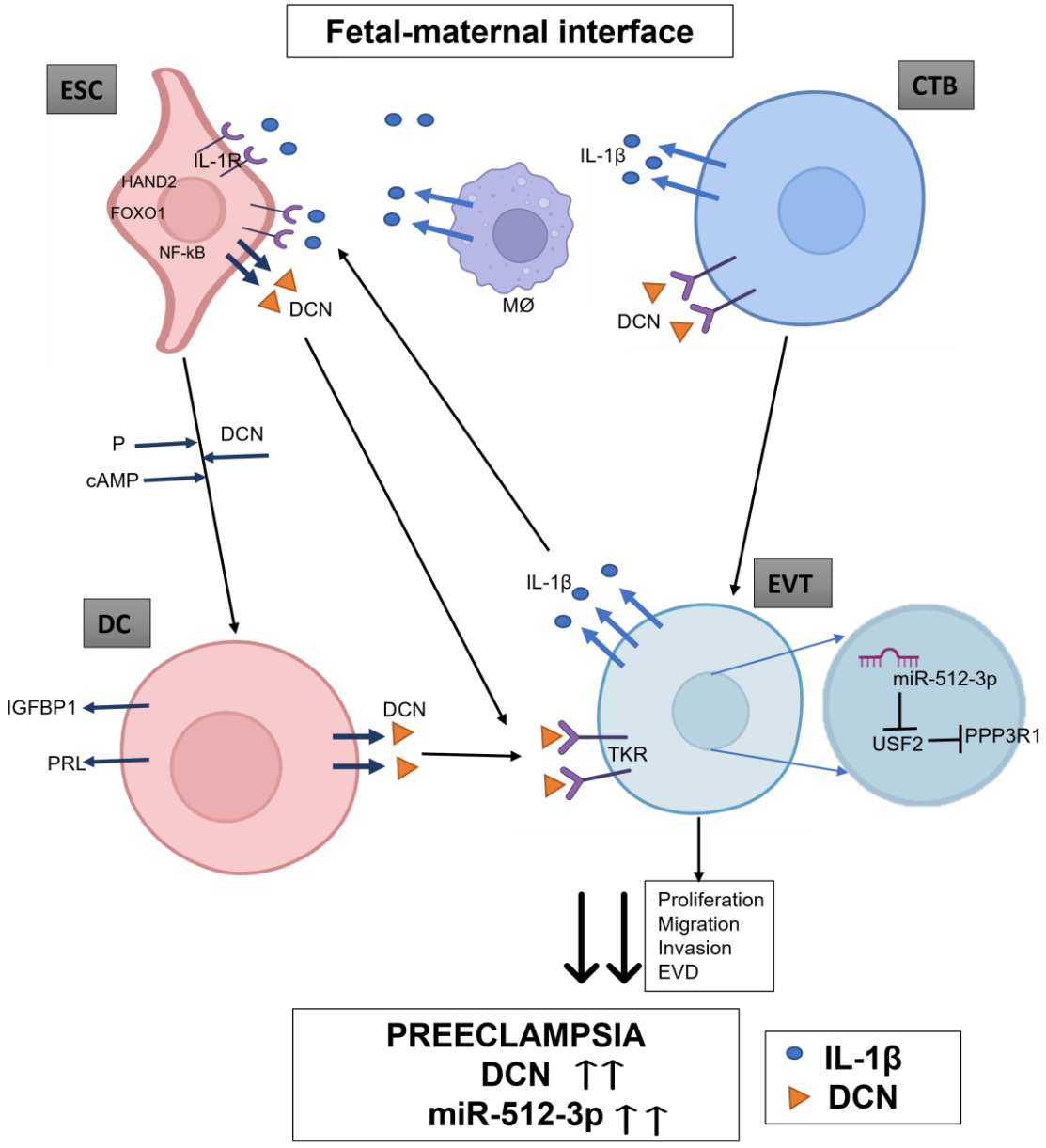

Figure 5-1: DCN at the fetal-maternal interface.

Endometrial stromal cells (ESC) and decidual cells (DC) produce DCN. DCN plays an autocrine stimulus for decidual cell maturation in the presence deciduogenic stimuli progesterone (P) and cAMP. DCN-mediated maturation process involves recruitment of HAND2→FOXO-1→ Progesterone receptor (PR). DCN production is promoted by exogenous IL-1β produced by all trophoblast subsets and macrophages (MØ). IL-1β binds to IL-1 receptor (IL-1R) to recruit NF-κB. DCN plays a paracrine role in restraining cytotrophoblast (CTB) differentiation along extravillous trophoblast (EVT) pathways.

DCN also inhibits EVT proliferation, migration and endovascular differentiation (EVD) by binding to multiple tyrosine kinase receptors (TKR), as well as inducing miR-512-3p (binding partner unknown). miR-512-3p action is mediated by upregulation of its target *PPP3R1* owing to its binding to and negative regulation of an intermediary transcription factor *USF2*. DCN overproduction by the PE decidua may be causally related to miR-512-3P upregulation in PE.

Since DCN overproduction is implicated in the pathogenesis of PE, this research is fundamental to understanding the origin of PE. It should identify molecular mechanisms in DCN regulation of decidual development, their alterations in PE and FGR and help developing highly predictive biomarkers for these maladies. It can be exploited to improve early diagnosis and management of PE.

5.4 References

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Appendix A

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Delegated Initial Approval Notice

Principal Investigator: Dr. Stephen Renaud
Department & Institution: Schulich School of Medicine and Dentistry\Anatomy & Cell Biology,Western University

Review Type: Delegated
HSREB File Number: 108948
Study Title: Mechanisms controlling trophoblast differentiation
Sponsor: Natural Sciences and Engineering Research Council

HSREB Initial Approval Date: March 10, 2017
HSREB Expiry Date: March 10, 2018

Documents Approved and/or Received for Information:

Document Name	Comments	Version Date
Revised Western University Protocol	Received February 28,	
Other	References	
Revised Letter of Information & Consent	Elective Termination of Pregnancy	2017/03/13
Revised Letter of Information & Consent	C-section	2017/03/13

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

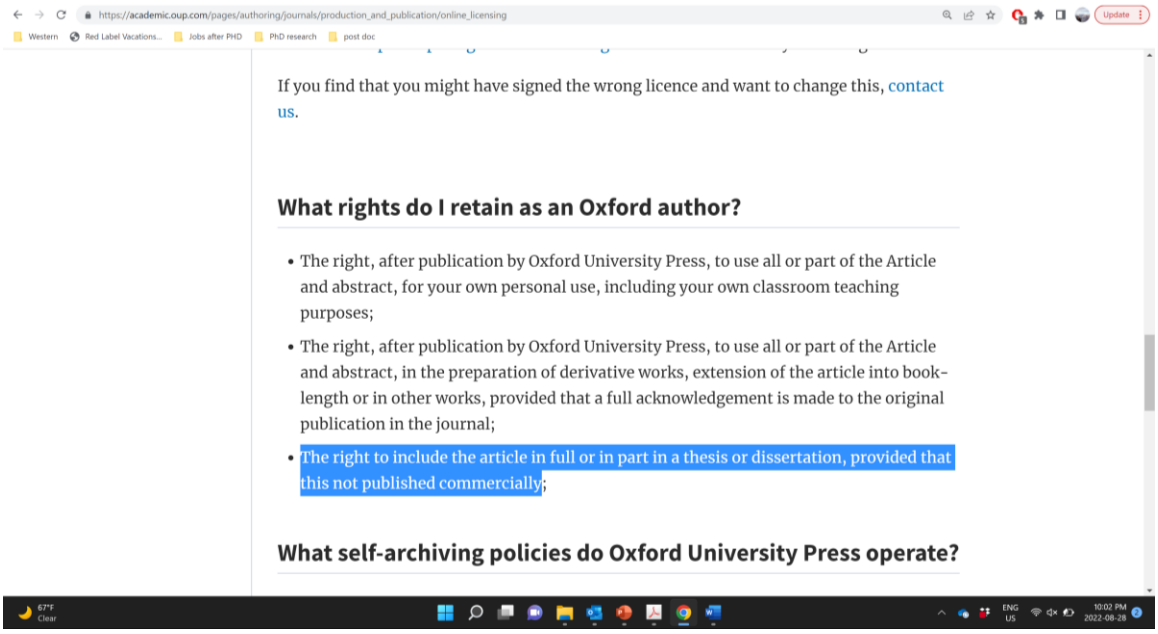
The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[redacted]
Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair
EO: Erika Basile ___ Nicole Kaniki ___ Grace Kelly ___ Katelyn Harris___ Nicola Morphet ___ Karen Gopaul

Western University, Research, Support Services Bldg., Rm. 5150
London, ON, Canada N6G 1G9 t. 519.661.3036 f. 519.850.2466 www.uwo.ca/research/ethics

Appendix B

- Molecular Human Reproduction Journal gives authors " **The right to include the article in full or in part in a thesis or dissertation provided that this is not published commercially".** **(https://academic.oup.com/pages/authoring/journals/production_and_publication/online_licensing)**
- Below is the screenshot from the website



Curriculum Vitae

Academic Training & Degrees

- PhD in Anatomy and Cell Biology, 2017 (In progress)
  University of Western Ontario, London, Canada
  Supervisors: Dr. Peeyush Lala (Chief Supervisor) Dr. Stephen Renaud (Co-Supervisor)
- Certificate in University Teaching and Learning, 2018-2021 (In progress)
  University of Western Ontario, London, Canada
- Certificate, Western Graduate Management Consulting Association, 2019 (Completed)
  Mini- MBA Seminar Series in Business & Consulting, Ontario, Canada
- Certificate, Global Clinical Scholars Research Training Program, 2015-2016 (Completed)
  Harvard Medical School, Boston, U.S.A.
- MSc, Clinical Embryology, 2012-2013 (Completed)
  University of Oxford, Oxford, U.K.
- Undergraduate M.B.B.S (M.D. Equivalent), 2006-2011 (Completed)
  Krishna Institute of Medical Sciences University, Maharashtra, India.

Scholarships and Awards

- Children's Health Research Institute Trainee Award, 2020-21
  Lawson Health Research, University of Western Ontario
- Anatomy and Cell Biology Travel Award, April 2020
  University of Western Ontario, London
- 3 Minute Thesis Competition, 3rd Price, 2020
  University of Western Ontario, London
- Elsevier Trophoblast Research New Investigator Award, judged as among top 10 poster, 2019
  International Federation of Placenta Associations, Argentina
- Children's Health Research Institute Trainee Award, 2019-20
  Lawson Health Research, University of Western Ontario
- Interdisciplinary Development Initiative (IDI) in Stem Cells and Regenerative Medicine training award, 2019-20
  University of Western Ontario
- Mitacs Globalink Research Award, 2018 (collaboration with Dr. Nihar Nayak, Wayne State Univ)
- Children's Health Research Institute -Internal Research Fund (CHRI-IRF), 2017
  Lawson Health Research, University of Western Ontario
- Partial HMS Scholarship, 2015
  GCSRT Program, Harvard Medical School, Boston, U.S.A.

Journal Publications

1. Halari CD, Zheng M, Lala PK. Roles of Two Small Leucine-Rich Proteoglycans Decorin and Biglycan in Pregnancy and Pregnancy-Associated Diseases. Int J Mol Sci. 2021 Sep 30;22(19):10584.
2. Halari CD, Renaud SJ, Lala PK. Molecular mechanisms in IL-1β-mediated decorin production by decidual cells. Mol Hum Reprod. 2021 Nov 27;27(12):gaab068.
3. Nandi P, Jang CE, Biggar K, Halari CD, Jansson T, Gupta MB. Mechanistic Target of Rapamycin Complex 1 Signaling Links Hypoxia to Increased IGFBP-1 Phosphorylation in Primary Human Decidualized Endometrial Stromal Cells. Biomolecules. 2021 Sep 18;11(9):1382
4. Halari CD, Nandi P, Jeyarajah MJ, Renaud SJ, Lala PK. Decorin production by the human decidua: role in decidual cell maturation. Molecular Human Reproduction. 2020 Oct;26(10):784-796.
5. Lala PK, Nandi P, Hadi A, Halari C. A crossroad between placental and tumor biology: What have we learnt? Placenta. 2021 Dec;116:12-30.